Identification of cyanobacterial cell division genes by comparative and mutational analyses

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Summary
We performed comparative and mutational analyses to define more comprehensively the repertoire of genes involved in cyanobacterial cell division. GenesftsE, ftsI, ftsO, ftsW, and (previously recognized)ftsZ, minC, minD, minE and sulA were identified as homologues of cell division genes of Gram-negative and Gram-positive bacteria. Transposon mutagenesis of Synechococcus elongatus PCC 7942 identified five additional genes, cdv1, cdv2, cdv3, ftn6 and cikA, involved in cell division. cdv1 encodes a presumptive periplasmic peptidyl-prolyl cis-trans isomerase. cdv2 has similarity to ylmF which, like divIVA, lies within the Gram-positive bacterial ylm gene cluster whose members have functions associated with division. Conservation of other ylm genes in cyanobacteria suggests that cyanobacteria and Gram-positive bacteria share specific division proteins. Two ylm homologues are also found in algal and plant genomes. cdv3 has low but significant similarity to divIVA, suggesting that minE and cdv3 both mediate division-site determination in cyanobacteria. In contrast, Gram-positive bacteria lack minE, and (Gram-negative) proteobacteria lack divIVA. ftn6, of unknown function, and the circadian input kinase, cikA, are specific to cyanobacteria. In S. elongatus, unlike in other bacteria, FtsZ rings are formed at sites occupied by nucleoids. Thus, the division machinery of cyanobacteria differs in its composition and regulation from that of Gram-negative and Gram-positive bacteria.

Introduction
Cyanobacteria are the most ancient organisms that perform oxygen-producing photosynthesis. Plastids evolved from an endosymbiont of cyanobacterial ancestry (reviewed in Gray, 1999; Cavalier-Smith, 2000; McFadden, 2001) and multiply by division as do cyanobacteria and other bacteria. Several studies have shown that plastid division is performed by a combination of both cyanobacterial-derived and eukaryote-specific systems (reviewed in Miyagishima et al., 2003; Osteryoung and Nunnari, 2003).

Over the past decade, the molecular mechanisms responsible for bacterial division have been extensively elucidated. Several proteins involved in bacterial cell division were identified mainly from Escherichia coli (Gram-negative bacterium; proteobacterium)fts mutants, which have temperature-sensitive cytokinesis defects and, as a result, elongate to form filaments (reviewed in Errington et al., 2003). The protein FtsZ, which is ubiquitous in bacteria and archaea and is also found in plastids and some mitochondria (summarized in Gilson and Beech, 2001), assembles into a ring structure (the Z ring) at the site of division before other known proteins of the division complex assemble (Errington et al., 2003). In E. coli, once a Z ring is formed at the division site, FtsA and ZipA bind directly to FtsZ. After that, the remaining proteins, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsW, FtsI and FtsN, are recruited to the division site to activate septation (Errington et al., 2003; Schmidt et al., 2004). Placement of the Z ring in E. coli is governed by the minB operon, which encodes three gene products: MinC, MinD and MinE (de Boer et al., 1989). MinC inhibits division by suppressing formation of the Z ring; this activity is dependent on the membrane-associated protein MinD, which is thought to form a heterodimer with MinC. MinE prevents MinCD from acting at the cell centre, thereby allowing Z ring assembly only at that position (de Boer et al., 1989; Errington et al., 2003). Recent time-lapse observations revealed that MinCD oscillates from pole to pole and that this oscillation depends on MinE, which also oscillates (summarized in Errington et al., 2003). Another protein known to regulate Z ring formation is SulA, which inhibits Z ring formation. Transcription of sulA is induced by the SOS response, and the corresponding protein inhibits FtsZ polymerization, delaying cell division until DNA damage is repaired (Bi and Lutkenhaus, 1993; Mukherjee et al., 1998). Cell division has also been extensively studied in Bacillus subtilis and differs from that in E. coli, especially with regard to positioning of the Z ring. B. subtilis and several other Gram-positive bacteria lack MinE, and their MinCD complex does...
not oscillate. Instead, another protein, DivIVA, regulates the positioning of the MinCD complex; DivIVA recruits MinCD to the cell poles, thereby allowing a Z ring to form only at the mid-cell position (Errington et al., 2003).

Although less well studied, cyanobacterial orthologues of ftsZ (Doherty and Adams, 1995; Zhang et al., 1995), minC, minD, minE (Mazouni et al., 2004) and sulA (Raynaud et al., 2004) also function in cell division. As in other bacteria, cyanobacterial FtsZ localizes to a septal ring (Mazouni et al., 2004), and overexpression inhibits cell division and produces filamenous cells (Mori and Johnson, 2001). In plants, nuclear orthologues of ftsZ (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Strepp et al., 1998), minD (Colletti et al., 2000), minE (Itoh et al., 2001) and sulA (Maple et al., 2004; Raynaud et al., 2004) also function in plastid division. Although these results indicate that the cell division mechanisms are partially conserved in diverse bacteria as well as in plastids, some of the cell division genes identified in other bacteria are apparently missing from cyanobacterial genomes (Rothfield et al., 1999), and cyanobacteria and plastids have some unique division genes. For example, the orthologous cell and plastid division genes ftn2 and ARC6, respectively (Koksharova and Wolk, 2002; Vitha et al., 2003), are confined in their distributions to cyanobacteria and plants. Both proteins localize to their respective division sites where they may interact with and regulate the activity of FtsZ (Vitha et al., 2003; Mazouni et al., 2004). Further, cyanobacteria are photoautotrophic. Their cell cycles and ftsZ expression patterns, where studied, are influenced by light or circadian rhythms (Mori et al., 1996; Holtzendorff et al., 2001; Mori and Johnson, 2001; Asato, 2003). These facts suggest that, just as there are differences between division proteins and processes in E. coli and B. subtilis, there are unique molecular mechanisms that regulate cyanobacterial cell division. Identification of the cell division genes of cyanobacteria is needed both for understanding the evolutionary and mechanistic diversity among bacterial cell division systems and as a basis for understanding the evolution of chloroplast division.

Although the genetic basis of cell division has been studied much less in cyanobacteria than in heterotrophic bacteria, the generation of filamentous mutants by insertional inactivation in Synechococcus elongatus PCC 7942 has led to the identification of three cell division loci: flm (Dolganov and Grossman, 1993), ftn2 and ftn6 (Koksharova and Wolk, 2002). Therefore, analysis of such mutants appears to be an effective strategy for investigating cyanobacterial cell division. In addition, the availability of the complete genomic sequences of several cyanobacteria facilitates comparative analysis.

To initiate a comprehensive analysis of cyanobacterial cell division, we first searched cyanobacterial genomes for cell division genes previously identified in E. coli and B. subtilis and found nine related genes, ftsE, ftsI, ftsQ, ftsW, ftsZ, minC, minD, minE and sulA. As noted above, orthology of cyanobacterial division genes to the last five of these had previously been reported. By screening for filamentous mutants of Synechocystis PCC 7942, we then identified an additional five genes, cdv1, cdv2, cdv3, ftn6 and cikA. These include cyanobacteria-specific genes and genes shared with Gram-positive bacteria. Finally, we searched in cyanobacterial genomes for genes encoded in the cell division gene cluster of Gram-positive bacteria and identified three additional candidate cyanobacterial cell division genes related to ylmE, ylmG and ylmH, the latter two of which are also present in plant and algal nuclear genomes.

Results

Identification of cell division genes in cyanobacteria by comparative analyses

Molecular genetic studies in E. coli and B. subtilis have identified 13 proteins that localize at the division site, and other proteins that regulate Z ring formation (Errington et al., 2003; Schmidt et al., 2004). ftsZ is the most conserved gene and is found in almost all prokaryotic taxa. However, the wide phylogenetic distribution of ftsZ is not shared by the other division genes of E. coli or B. subtilis (Rothfield et al., 1999). Previous comparative analyses have shown that the genome of Synechocystis sp. PCC 6803 has orthologues of minC, minD, minE, ftsZ, ftsI and ftsW (Rothfield et al., 1999). Some bacterial division genes were not included in these analyses, however, and several other cyanobacterial genomes and some plastid and nuclear genomes of algae and plants have as been sequenced completely. Therefore, before screening for new genes, we conducted new BLAST searches of cyanobacterial and photosynthetic eukaryotic genomes to clarify how many cell division genes identified in E. coli and B. subtilis are conserved in cyanobacteria or plastids.

Many divergent bacteria have a chromosomal region, the dcw cluster (division and cell wall), containing many genes whose products are involved in cell division and cell wall synthesis (Ayala et al., 1994; Vicente et al., 1998). Usually, ftsL, ftsI, ftsW, ftsQ, ftsA and ftsZ are included in this region along with other genes (Ayala et al., 1994; Vicente et al., 1998). Although ftsI, ftsW, ftsQ and ftsZ were found in all sequenced cyanobacterial genomes (Table 1 and Tables S1 and S2), they are spread throughout the genome and no conservation was found in the order of neighbouring genes other than in the clustering of ftsQ and ftsZ. In the dcw clusters of E. coli and B. subtilis, ftsQ, ftsA and ftsZ are aligned in this order (Ayala et al., 1994; Vicente et al., 1998). Although ftsQ and ftsZ...
Table 1. Distribution, among the genomes of cyanobacteria and plants, as determined by searches based on sequence similarity, of cell division genes identified in *E. coli* or *B. subtilis*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genes whose products localize at the septum in <em>E. coli</em> and/or <em>B. subtilis</em></th>
<th>Genes whose products regulate FtsZ ring formation or positioning in <em>E. coli</em> and/or <em>B. subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>ftsE</td>
<td>ftsI</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>ftsE</td>
<td>ftsI</td>
</tr>
</tbody>
</table>

| Cyanobacteria | | |
| G. violaceus PCC7421 | gil0649 | gil0929 | – | gil0299 | gil3581 | gil0298 |
| A. sp. PCC 7120 | alr1706 | alr0718 | all7666 | all3857 | all0154 | all3858 |
| T. elongatus BP-1 | tli0453 | tli2074 | – | tli0136 | tli0440 | tli2382 |
| S. sp. PCC 6803 | sll0828 | sll1633 | – | sll1632 | sll1267 | sll1633 |
| S. sp. WH 8102 | SYNW1760 | SYNW1643 | SYNW0475 | SYNW1644 | | |
| S. sp. PCC 7942 | oft2266 | oft853 | oft2355 | oft854 | | |
| P. marinus MED4 | PMM0518 | PMM1308 | PMM1458 | PMM1309 | | |
| P. marinus MIT9313 | PMT1249 | PMT0320 | PMT1475 | PMT0319 | | |
| P. marinus SS120 | Pro0041 | – | Pro1382 | Pro1611 | Pro1383 | |

| Plastid genomes | | |
| M. viride | – | AAF43792 | – | – |
| N. olivacea | – | AAD54780 | – | – | AAD54904 |
| C. paradoxa | – | – | – | – | P48280 |
| G. theta | – | – | – | – | – |
| C. vulgaris | – | – | – | – | – |
| Others | – | – | – | – | – |

| Nuclear genomes | | |
| C. merolae | – | – | – | – | CMS004C |
| A. thaliana | – | – | – | – | At2g36250 |

| *E. coli* | minC | minD | minE | SulA | yfhF |
| *B. subtilis* | minC | minD | – | – | – |

| Cyanobacteria | | |
| A. sp. PCC 7120 | alr3455 | alr3456 | asr3457 | alr2390 | |
| T. elongatus BP-1 | tlr2016 | tlr2017 | tlr2018 | tlr0465 | |
| S. sp. PCC 6803 | sll0288 | sll0289 | sll0546 | sll1223 | |
| S. sp. WH 8102 | SYNW1970 | SYNW1971 | SYNW1972 | SYNW2218 | |
| S. sp. PCC 7942 | oft1975 | oft213 | oft214 | oft633 | |
| P. marinus MED4 | PMM0322 | PMM0321 | PMM0320 | PMM0120 | |
| P. marinus MIT9313 | PMT1652 | PMT1653 | PMT1654 | PMT1973 | |
| P. marinus SS120 | Pro0364 | Pro0363 | Pro0362 | Pro0140 | |

| Plastid genomes | | |
| L. viride | – | AAF43874 | – | – |
| N. olivacea | – | AAD54488 | – | – | AAD54904 |
| C. paradoxa | – | – | – | – | – |
| G. theta | – | – | – | – | – |
| C. vulgaris | – | O78436 | O78435 | – | – |
| Others | – | – | – | – | – |

| Nuclear genomes | | |
| C. merolae | – | – | – | – | – |
| A. thaliana | – | At5g24020 | At1g69390 | At2g21280 | – |

d. Another gene was identified (Pro0518).
e. Another gene was identified (Pro1735).
f. Lemieux et al. (2000).
g. Turmel et al. (1999).
h. Another gene was identified (CMO089C) (Matsuzaki et al., 2004; Miyagishima et al., 2004).
i. Two other genes were identified (At3g52750, At3g55280) (Osteryoung et al., 1998).
j. Similarity between cyanobacterial and *E. coli* SulA proteins is very low (Raynaud et al., 2004).
k. Relationship between yfhF and cell division has not been examined. YfhF has significant similarity to cyanobacterial SulA.
l. Two other genes were identified (ai12275 and ai12233).
m. Another gene was identified (tr0963).
n. Raynaud et al. (2004).o. Douglas and Penny (1999).p. Wakasugi et al. (1997).q. Colletti et al. (2000).r. Itoh et al. (2001).orf ID or GenBank accession numbers are shown and dash indicates not identified. Only genes for which orthologues were identified in cyanobacteria are listed. No orthologues of the *E. coli* or *B. subtilis* genes ftsA, ftsL, yabQ, ftsN, zipA, ezrA (Errington et al., 2003), zapA (Gueiros-Filho and Losick, 2002) and ftsX (Schmidt et al., 2004) were found.
are also adjacent in most cyanobacterial genomes, \( ftsA \) is missing. \( ftsK \) was only found on one of the six plasmids of *Anabaena* sp. PCC 7120. Also missing were genes encoding FtsL, YabQ and ZapA. These proteins are poorly conserved, however, and we cannot rule out the possibility that distant homologues may exist in cyanobacteria. In total, nine of 18 cell division genes found in *E. coli* and *B. subtilis* (\( ftsE, ftsI, ftsQ, ftsW, ftsZ, minC, minD, minE \) and \( sulA \)) are well conserved in cyanobacterial genomes and readily identifiable by BLAST (Table 1 and Tables S1 and S2).

Among these nine genes, \( ftsZ, minD, minE \) and \( sulA \) are also found in plant and algal genomes (Osteryoung and Vierling, 1995; Osteryoung et al., 1998; Strepp et al., 1998; Colletti et al., 2000; Itoh et al., 2001; Maple et al., 2004; Raynaud et al., 2004), and \( ftsI \) and \( ftsW \) are found in the plastid genomes of a few green algae (Turmel et al., 1999; Lemieux et al., 2000). \( ftsE \) and \( ftsQ \) were not found in eukaryotic organisms. Consistent with the endosymbiotic theory, none of the genes missing from cyanobacterial genomes was found in either the plastid or nuclear genomes of plants or algae.

**Identification of chromosomal loci involved in cell division using transposon mutagenesis**

In order to identify other genes involved in cyanobacterial cell division, we mutagenized *S. elongatus* PCC 7942 by the introduction of pRL692, which carries a derivative of transposon Tn5 (Tn5-692; Koksharova and Wolk, 2002) and confers resistance to erythromycin, spectinomycin and streptomycin. Among approximately 50,000 colonies screened on plates containing erythromycin and spectinomycin, 350 exhibited a spreading morphology (Dolganov and Grossman, 1993; Koksharova and Wolk, 2002) and contained filamentous mutants (Fig. 1). To identify the transposon insertion sites, the right border of the transposon and flanking genomic region in 292 of these mutants were amplified by inverse polymerase chain reaction (PCR) and sequenced, and 254 sites of insertion were assigned in the genome.

Seven loci were sites of insertion three times or more, strongly suggesting they were linked to the phenotypes (Fig. 2). \( orfs \) are numbered according to the draft annotation of the complete genome; Version 01dec03; http://genome.ornl.gov/microbial/syn_PCC7942/01dec03/syn_PCC7942.html). These included \( ftn2 \) (orf2009) and \( minE \) (orf214), whose roles in cyanobacterial cell division have been recently investigated (Koksharova and Wolk, 2002; Mazouni et al., 2004); \( fim3 \) region \( orf3 \) (orf1974) and \( ft6 \) (orf1703), previously identified as possible cell division loci (Dolganov and Grossman, 1993; Koksharova and Wolk, 2002); and three genes, \( orf322, orf375 \) and \( orf1938 \) (Fig. 2; described below), not previously associated with cell division in cyanobacteria. \( orf375 \) encodes CikA, a regulator of the *S. elongatus* circadian clock (Schmitz et al., 2000; Mutsuda et al., 2003). An insertion was also found in the 5′-untranslated region of \( ftsZ \) and coding region of \( minE \). The \( minE \) mutant was completely segregated whereas the \( ftsZ \) mutant was not fully segregated. Scale bar = 5 mm (A–C) and 10 μm (D and E).

**Fig. 1.** Isolation of filamentous mutants of *S. elongatus* PCC 7942 by random insertion of transposon Tn5-692. A–C. Spreading colonies on plates containing spectinomycin and erythromycin. D and E. Filamentous mutants from spreading colonies. Tn5 was inserted in the 5′-untranslated region of \( ftsZ \) (D) and coding region of \( minE \) (E). The \( minE \) mutant was completely segregated whereas the \( ftsZ \) mutant was not fully segregated.

**minE behaves differently in S. elongatus PCC 7942 and Synechocystis sp. PCC 6803**

Because cyanobacteria have multiple genomes (Binder and Chisholm, 1990), PCR was used to determine whether the mutations in four of the \( minE \) mutant strains were completely or incompletely segregated after transfer of the original spreading colonies to fresh plates.
Although incompletely segregated in three of the strains, the mutation was fully segregated in one strain in which the transposon was inserted near the 5' end of the coding region. This finding indicates that *S. elongatus* cells lacking MinE are viable. The phenotypes of the *S. elongatus minE* mutants (Fig. 1) resemble those of *E. coli minE* mutants (de Boer et al., 1989), suggesting that MinE functions similarly in these two rod-shaped bacteria in regulating Z ring placement. In contrast, disruption of *minE* in *Synechocystis* sp. PCC 6803, which is spherical, produced no obvious defect in cell division (Mazouni et al., 2004).

cikA, *ftn6*, and three new genes, *cdv1, cdv2* and *cdv3*, are involved in cyanobacterial cell division

An earlier study showed that insertional inactivation of *flm3* region *orf2* (*orf1973; Fig. 2) resulted in filamentation in *S. elongatus* PCC 7942 (Dolganov and Grossman, 1993). Our screen identified seven transposon insertions in *flm3* region *orf3* (*orf1974; Fig. 2), which is the adjacent downstream gene, but only one in *flm3* region *orf2* (Fig. 2). These results suggested the possibility that the cell division defects in the *orf1973* and possibly other transposon insertion mutants could be attributable to polar effects on the downstream genes. To test this, we disrupted *orf322, orf375* (cikA), *orf1703* (*ftn6*), *orf1938*, and their corresponding adjacent downstream genes (Fig. 2) by homologous recombination using the omega cassette (Prentki and Krisch, 1984). We also disrupted *orf1974*, but as its downstream *orf* is on the opposite strand and would not show a polar effect, that gene was not disrupted.

The growth rates of all of the disruption mutants on agar or in liquid medium appeared to differ little from that of the wild type. The *orf323, orf1703, orf1704* and *orf1939* mutants were completely segregated, whereas segregation of the *orf322, orf375, orf376, orf1938* and *orf1974* mutations was incomplete even after five serial transfers of single colonies to new plates (Fig. 2). We conclude that complete inactivation of the latter five *orfs* is probably lethal. All spectinomycin-resistant transformants formed by disruption of *orf322, orf375, orf1703, orf1938* and
orf1974 exhibited a spreading morphology (Fig. 2). Cells of these mutants were longer than those of the wild type and were heterogeneous in size, indicating that they were defective in cell division (Fig. 3). In contrast, disruption of corresponding downstream genes (orf323, orf376, orf1704, orf1939) yielded no spreading colonies (Fig. 2) and the cells showed no defects in cell division (Fig. 3). These results suggested that orf322, orf375 (cikA), orf1703 (ftn6) and orf1938, and not their downstream genes, are responsible for the cell division phenotypes. Although our screen identified one insertion of the transposon in orf1973 (ftm3 region orf2), we cannot determine at present whether this gene and the downstream orf1939 (ftm3 region orf3) are both involved in cell division or whether the cell division defect in the orf1973 mutant is attributable to a polar effect. Also, because complete segregation of orf376 could not be achieved, we cannot at present completely rule out that orf376 affects cell division. However, as no spreading colony morphology was observed even after five serial transfers, and no insertion of the transposon was detected in orf376, orf375 and not orf376 is likely to be the division-associated gene.

Based on the above results, we designate orf322, orf1938 and orf1974 as cdv1, cdv2 and cdv3 (cell division) respectively. The above gene disruption experiments also indicate that ftn6, rather than its downstream genes, and cikA are involved in cell division.

Properties of the ftn6, cikA, cdv1, cdv2 and cdv3 gene products

ftn6, cikA, cdv1, cdv2 and cdv3 are conserved in other cyanobacteria (Table 2 and Tables S3 and S4). cikA and ftn6 are specific to cyanobacteria whereas cdv1, cdv2 and cdv3 are conserved in other bacterial taxa. All five gene products are predicted to be soluble proteins (http://sosui.proteome.bio.tuat.ac.jp/sosui)frame0.html).

ftn6 was identified as a possible cell division gene in a previous study (Koksharova and Wolk, 2002). The gene predicts an unknown protein of 228 amino acids with no detectable conserved domains. cikA encodes a phytochrome-related protein that bears similarity to histidine protein kinases of bacterial two-component signal transduction systems (Schmitz et al., 2000; Mutsuda et al., 2003). The protein is a sensory component in the input pathway of the circadian clock in S. elongatus PCC 7942, but a role in cell division has not been reported.

cdv1 encodes a 243-amino-acid protein with strong similarity to a group of peptidyl-prolyl cis-trans isomerases (PPIase), which function in protein folding and cell signaling (Fulgości et al., 1998). The protein has a predicted signal peptide (SIGNALP; http://www.cbs.dtu.dk/services/SIGNALP/) and has been detected in the periplasmic proteome in Synechocystis sp. PCC 6803 (Fulgości et al., 2000). Although a protein similar to CdV1 is encoded in plant nuclear genomes (Fulgości et al., 1998), this plant protein is more closely related to another cyanobacterial PPIase. Therefore, the plant homologue and CdV1 are probably not orthologous.

cdv2 and cdv3 encode proteins of 191 and 152 amino acids respectively. These proteins were placed within COG1799 (uncharacterized protein conserved in bacteria) and COG3599 (cell division initiation), respectively, in the database of Clusters of Orthologous Groups of proteins (COGs; http://www.ncbi.nlm.nih.gov/COG; Tatusov et al., 2000). CdV2 has strong similarity to YlmF, a protein of unknown function that was shown recently to be involved in cell division in Streptococcus pneumoniae (Fadda et al., 2003). A putative orthologue of CdV2 is also encoded in the plastid (cyanelle) genome of Cyanophora paradoxa (Table 2 and Tables S3 and S4). COG3599 contains DivIVA, which is involved in septum site determination in Gram-positive bacteria (Cha and Stewart, 1997; Edwards and Errington, 1997; Marston et al., 1998). In the alignment of proteins in COG3599, the CdV3 orthologues from Synechocystis sp. PCC 6803 and Anabaena sp. PCC 7120 have weak but significant similarity to DivIVA, especially in their N-terminal 100 amino acids (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=COG3599). In addition, as in DivIVA (Stahlberg et al., 2004), all but about 50 amino acids at the N-terminus are predicted to form a coiled coil by MULTICOIL (http://theory.lcs.mit.edu/~elwolf/multicoil.html). Although a BLAST search did not show similarity between CdV3 and B. subtilis DivIVA, a BLAST search using Bacillus cereus ZK DivIVA (GenBank Accession No. YP_085237; word size was set to 2 and without low complexity filter) showed weak similarity to the CdV3 orthologue of Thermosynechococcus elongatus BP-1 (II1166, expectation was 8.2e-2 and identity was 26%).

YlmE, CdV2 (YlmF), YlmG and YlmH are conserved in cyanobacteria and orthologues of YlmG and YlmH are present in plant and algal genomes

CdV2 (YlmF) and CdV3 (DivIVA-like protein) are well conserved in both Gram-positive bacteria and cyanobacteria, whereas these two genes are not found in E. coli or other Gram-negative bacteria (proteobacteria) (Massidda et al., 1998; Table 2 and Tables S3 and S4). These findings suggest that the cell division system in cyanobacteria has specific components in common with that in Gram-positive bacteria, even though cyanobacteria have an outer membrane characteristic of Gram-negative bacteria (reviewed in Hoiczyk and Hansel, 2000). Interestingly, in Gram-positive bacteria, YlmF and DivIVA are encoded in a gene cluster, downstream of ftsZ (Massidda et al., 1998). This cluster contains ylmE, ylmF, ylmG, ylmH and divIVA. Of
Fig. 3. Morphology of gene disruptants of orf322 (cdv1), cikA, orf323 (cdv2), orf324 (cdv3) and their adjacent downstream genes. A and B. Wild type and orf2 (orf2009) mutant isolated previously (Koksharova and Wolk, 2002) for comparison. C–K. The omega cassette, which confers resistance to streptomycin and spectinomycin, was inserted into orf322 (cdv1) (C), orf323 (D), orf325 (cikA) (E), orf326 (F), orf327 (ftn6) (G), orf328 (ftn6) (H), orf329 (cdv2) (I), orf330 (orf1938) and orf331 (cdv3) (K) to inactivate each gene. Cells were observed under Nomarski optics. Scale bar = 10 μm.

L. Distribution of cell lengths in cultures of wild-type (WT) and mutant cells. Lengths of 100 cells for each strain were measured at logarithmic growth phase and are shown in the histograms. The average cell length is shown in each graph with the standard deviation.

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Table 2. Distribution, in other taxa, of cell division genes identified or predicted in *S. elongatus* PCC 7942 by transposon mutagenesis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Identified cell division genes</th>
<th>Candidate cell division genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>ftn2</em></td>
<td><em>ftn6</em></td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sp. PCC 7942</em></td>
<td>orf2009</td>
<td>orf1704</td>
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<td><em>G. violaceus</em> PCC 7421</td>
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<td><em>P. marinus</em> SS120</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>–</td>
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<td><em>B. subtilis</em></td>
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<td>Plastid genomes</td>
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<tr>
<td><em>C. paradoxa</em></td>
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<tr>
<td><em>G. theta</em></td>
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<tr>
<td><em>C. merolae</em></td>
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<tr>
<td><em>P. purpurea</em></td>
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<td>Others</td>
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<td>Nuclear genomes</td>
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<tr>
<td><em>C. merolae</em></td>
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<tr>
<td><em>A. thaliana</em></td>
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<td><em>P. purpurea</em></td>
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<td>a. <em>P. purpurea</em>, <em>Porphyra purpurea</em>. Other abbreviations are the same as Table 1.</td>
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<td>b. Koksharova and Wolk (2002).</td>
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<td>c. Another gene was identified in each strain (orf1861, gsr2010, asl0940, trl1577, ssi0353, SYNW0335, PMM0061, PMT1589, Pro0075).</td>
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<td>d. Two other genes were identified (glr2610, glr0973).</td>
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<td>e. Mazouni et al. (2004).</td>
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<td>f. Although CMS406C, At1g11930 and At4g26860 have similarity to <em>ylmE</em>, they are not closely similar to cyanobacterial proteins.</td>
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<td>g. Another gene was identified (CMT057C).</td>
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<td>h. At5g42480 is <em>ARC6</em> (Vitha et al., 2003). Another gene was identified (At3g19180).</td>
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<tr>
<td>i. Three other genes were identified (At5g36120, At4g27990, At3g07430).</td>
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<td>orf ID or GenBank accession numbers are shown; dash indicates not identified.</td>
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</table>
these genes, only ylmE and ylmG are found in E. coli and other proteobacterial genomes (Fadda et al., 2003; Table 2 and Tables S3 and S4). Recently, inactivation of ylmE, ylmF, ylmG and ylmH was shown to cause morphological and/or division abnormalities in Streptococcus (Fadda et al., 2003). What roles their products may play in cell division is unknown, although YlmE has an N-terminal domain of alanine racemase, which is involved in cell wall synthesis (Shaw et al., 1997), YlmG is clustered in the YGGT family of membrane proteins (function unknown), and YlmH contains an S4 domain in its C-terminus, which probably mediates binding to RNA (Davies et al., 1998). These findings led us to search for other members of this cluster in cyanobacteria and in photosynthetic eukaryotes.

Like cdv2 (ylmF) and cdv3 (divIVA-like gene), ylmE, ylmG and ylmH are conserved in cyanobacterial genomes (Table 2 and Tables S3 and S4). In addition, ylmE and cdv2 (ylmF) are juxtaposed in several cyanobacterial genomes, showing another similarity between cyanobacteria and Gram-positive bacteria. ylmE could not be identified in any eukaryote and ylmF was detected only in C. paradoxa. However, genes encoding proteins related to YlmG and YlmH were found in the Cyanidioschyzon merolae and Arabidopsis nuclear genomes (Table 2 and Supplementary material, Tables S3 and S4). In both organisms, the YlmG- (C. merolae, CMC030C, CMT057C; Arabidopsis, At1g53120, At5g21920, At3g07430) and YlmH-like proteins (C. merolae, CMK194C; Arabidopsis, At1g53120) are closely related to those of cyanobacteria.

In addition, these eukaryotic proteins have N-terminal extensions, and TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicted that some are localized in plastids (prediction scores: 0.957, At5g36120; 0.834, At5g21920; 0.719, At3g07430; and 0.550; CMK194C), suggesting that they may function in these organelles.

**Cell length in the gene disruptants**

Targeted disruption of ftu6, cikA, cdv1, cdv2 and cdv3 (divIVA-like gene) produced cells longer than those of wild type. Cell lengths of the disruptants were measured during exponential growth phase. ftu2 and ftu6 mutant cells were more than 10 times longer than wild-type cells (Fig. 3). In contrast, cdv1, cdv2, cdv3 and cikA mutant cells were two to three times longer than wild-type cells, but these four mutant populations also contained cells more than five times longer than those in the wild-type (Fig. 3). Stationary-phase cultures of the cikA mutant contained filamentous cells longer than those in exponential phase (data not shown). All six mutants exhibited greater heterogeneity in cell length than wild-type cells (Fig. 3). Most of the original transposon insertion mutants showed similar phenotypes (data not shown).

**FtsZ localization and nucleoid distribution in the gene disruptants**

Z ring formation is the first known step in bacterial septation (Errington et al., 2003; Schmidt et al., 2004). Therefore, to gain insight into the roles of Ftn6, CikA, CdV1, CdV2 and CdV3 in cyanobacterial cell division, we examined the localization of FtsZ in the corresponding disruption mutants. Exponential phase cultures (OD<sub>730</sub> = 0.1–0.2) were fixed, and the localization of FtsZ was examined by immunofluorescence microscopy using anti-<i>Anabaena</i> FtsZ antibodies, which have been shown to react with FtsZ specifically (Kuhn et al., 2000). The antibodies clearly labelled the Z ring at mid-cell in wild type (Fig. 4).

Z rings were also observed in cdv1, cdv2 and cdv3 mutants, although their frequency (number of rings per unit cell length) was reduced by up to 50% compared with that in wild type (Fig. 4). The Z rings in these mutants were often not positioned at the mid-cell, but quantitative analyses showed that the frequency of ring positioning was still higher around the mid-cell position than at other positions in cdv1 and cdv2 mutants. In the cdv2 mutant, half of the rings that were not positioned at mid-cell were observed in cells having two or three Z rings, in which the one-third or one-fourth positions may represent potential division sites. In contrast to cdv1 and cdv2 mutants, in the cdv3 mutant the Z rings were positioned nearly randomly throughout the cell (Fig. 4K). This observation suggests that, at least in the cdv3 mutant, the mechanism responsible for positioning of the Z ring is disturbed.

Z rings were not observed in <i>ftu2</i>, <i>ftu6</i> and <i>cikA</i> mutants (Fig. 4), suggesting that cells of these mutants were elongated because of defects in Z ring formation. Immunoblot analysis showed little difference in FtsZ protein levels between the mutant and wild-type strains (Fig. 4L), indicating this phenotype was not a consequence of reduced FtsZ levels. Because the growth rates of these three mutants differed little from that of wild-type cells and the lengths of the cells changed little during exponential phase, these cells must divide. In fact, constriction was observed by Nomarski optics or autofluorescence in <i>ftu2</i>, <i>ftu6</i> and <i>cikA</i> mutants. At the constricted sites in <i>ftu2</i>, <i>ftu6</i> and <i>cikA</i> mutants, there were no condensed Z rings but we observed slight and diffuse localization of FtsZ proteins (Fig. 4F, H and J). These observations suggest that <i>ftu2</i>, <i>ftu6</i> and <i>cikA</i> mutants may be defective in recruitment of FtsZ to the division site or in subsequent assembly of the Z ring.

In <i>E. coli</i> and <i>B. subtilis</i>, Z ring positioning is controlled not only by the Min system, but also by the location of the nucleoid. In these species, the Z ring cannot form at the mid-cell until chromosome partitioning creates a nucleoid-free gap at that site (Margolin, 2001). To investigate
Identification of cyanobacterial cell division genes

Fig. 4. Localization and levels of FtsZ in the gene disruptants.
A–J. Immunofluorescence images showing localization of FtsZ in wild type (A), ftn2 mutant (Koksharova and Wolk, 2002) (E and F), and gene disruptants of cdv1 (B), cdv2 (C), cdv3 (D), ftn6 (G and H) and cikA (I and J). F, H and J are magnified images of division sites. Cells from growing cultures (OD730 = 0.1–0.2) were fixed and FtsZ was detected by indirect immunofluorescence microscopy using anti-Anabaena FtsZ antibodies (Kuhn et al., 2000). Green fluorescence shows localization of FtsZ and red is autofluorescence of chlorophyll. Scale bar = 10 μm (I) for A–E, G and I, and 2 μm (J) for F, H and J.

K. Analyses of FtsZ ring position and FtsZ ring frequency in wild-type and mutant cells. Micrographs were obtained of 268 wild-type cells, 132 cdv1 mutant cells, 122 cdv2 mutant cells and 118 cdv3 mutant cells. For each cell, the cell length, as well as the distance of the ring to the most proximal cell pole, was determined. The plane midway between the both poles was defined as the mid-cell position. The relative distance of the Z ring from the mid-cell position was calculated, where the mid-cell and pole positions were defined as 0 and 0.5, respectively, for each cell. The frequency of ring positions is shown by the histograms. The black and the white sections of the bar show rings in cells with one Z ring and cells with multiple rings respectively. Also shown in the graphs are the mean distances from mid-cell and standard deviation (upper number) and the frequency of FtsZ rings (lower number; total number of rings divided by total cell length). Total cell length was 1057 μm (wild type), 1181 μm (cdv1 mutant), 1149 μm (cdv2 mutant) and 1595 μm (cdv3 mutant) respectively.

L. Levels of FtsZ in the gene disruptants. Total proteins extracted from growing cultures (OD730 = 0.1–0.2) were separated and FtsZ was detected with anti-Anabaena FtsZ antibodies (Kuhn et al., 2000). Equal amounts of total protein were loaded in each lane and the equality of loading was confirmed by Coomassie brilliant blue staining after SDS-PAGE (data not shown).

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whether the cell division defects in the ftn2, ftn6, cikA, cdv1, cdv2 and cdv3 mutants could result from impaired nucleoid segregation or morphology, nucleoid distribution in the mutant strains was investigated by DAPI (4',6-diamidino-2-phenylindole) staining.

Nucleoid regions in both wild-type and mutant cells often appeared as clusters of small particles (Figs 5 and 6), perhaps reflecting the distribution of the multiple genomes in *S. elongatus*. During exponential phase, all three mutants had elongated nucleoid regions compared with those in wild type. However, the nucleoids appeared to be evenly spaced throughout the cells and none of the mutant cells was anucleate (Fig. 5). These data suggest that the replication and distribution of nucleoids occur normally in the mutants. However, the number of large gaps between nucleoids per micrometre of cell length (arrowheads in Fig. 5) was lower in the mutants than in the wild-type strain. In dividing wild-type cells, such gaps were observed at the site of constriction (arrowheads in Fig. 5A).

To ascertain the relationship between the positioning of Z rings and large nucleoid-free gaps in the cdv1, cdv2 mutants, the timing of nucleoid segregation and FtsZ ring formation was investigated in wild-type and mutant cells. Immunofluorescence and DAPI-staining images showing localization of FtsZ (A, C, E, G, I, K, M, O, Q and S) and distribution of nucleoids (B, D, F, H, J, L, N, P, R and T) in wild type (A–H), cdv1 mutant (I–L), cdv2 mutant (M–P) and cdv3 mutant (Q–T). Paired photographs (A and B, C and D, E and F, G and H, I and J, K and L, M and N, O and P, Q and R, S and T, and U and V) show images of identical cells. Arrowheads indicate large nucleoid-free gaps. Scale bar = 10 µm.
and cdv3 mutants, we compared the localization of nucleoids and Z rings in these mutants and in wild type. All the wild-type cells without a Z ring (Fig. 6A and B), or in which the Z ring had formed but with no evident constriction (Fig. 6C and D), lacked large nucleoid-free gaps; such gaps (arrowheads, Fig. 6F and H) were detected only in those wild-type cells having slight or deep constrictions. In the cdv1, cdv2 and cdv3 mutants, Z rings also formed before visible constriction of the cells (Fig. 6I, M and Q), and large nucleoid-free gaps were detected only at already constricted sites (compare Fig. 6J, L, N, P, R and T). To further investigate the relative timing of Z ring formation and nucleoid separation, we treated wild-type cells with cephalaxin, which specifically inhibits Fts1 (Spratt, 1977; Botta and Park, 1981). In E. coli, cephalaxin treatment interferes with Z ring constriction, producing filamentous cells that divide only occasionally. In regions of these cells lacking constrictions, gaps between nucleoids can still be observed (Pogliano et al., 1997). In contrast, although cell division was also largely inhibited in cephalaxin-treated S. elongatus cells (Fig. 6U), large nucleoid-free gaps were only observed at the occasional constriction sites (Fig. 6V). Together with the FtsZ localization studies described above, these observations provide evidence that, unlike in E. coli and B. subtilis in which separation of the nucleoids at the division site is a prerequisite for Z ring formation according to the nucleoid occlusion model (Margolin, 2001), Z ring formation in S. elongatus can occur in the plane occupied by a nucleoid, suggesting that nucleoid occlusion does not regulate Z ring assembly in cyanobacteria. These results further indicate that nucleoid occlusion probably does not account for the irregular positioning of the Z rings in the cdv1, cdv2 and cdv3 mutants.

Discussion

By comparative and mutational analysis, we have expanded the repertoire of genes known or predicted to govern cell division in S. elongatus PCC 7942. The list now includes ft2, ft6 and cikA, which are unique to cyanobacteria, and ftsE,ftsI,ftsK,ftsQ,ftsW,ftsZ, minC,minD,minE,SulA,cdv1,cdv2 and cdv3, which are shared with other bacteria (Tables 1 and 2). In addition, we have determined that cyanobacteria, which are Gram-negative in morphology (Hoiiczky and Hansel, 2000), have several cell division genes in common with Gram-positive as well as Gram-negative bacteria. Finally, we have found that Z ring formation does not require the absence of nucleoids at the plane of assembly in S. elongatus as it does in E. coli and B. subtilis. These findings indicate that the composition of the division machinery in cyanobacteria and the mechanisms that regulate its assembly are distinctive.

Formation of well defined Z rings as observed by immunofluorescence microscopy could not be detected in the ft2, ft6 or cikA mutants despite the presence of normal FtsZ protein levels. Therefore, these three genes may mediate aspects of Z ring assembly in cyanobacteria. Because we did not obtain transposon insertions in the ftsZ coding region in S. elongatus, nor has ftsZ been successfully mutated in either Anabaena sp. PCC 7120 or Synechocystis sp. PCC 6803 (Zhang et al., 1995; Mazouni et al., 2004), we suggest that the ftsZ gene in cyanobacteria, as in other bacteria, is indispensable. Therefore, unless FtsZ function in cyanobacterial cell division differs significantly from that in other bacteria, it seems likely that some degree of Z ring assembly must have occurred in the ft2, ft6 or cikA mutants, although at a lower frequency and probably more transiently and in less condensed form than in the wild-type strain.

The similarity in growth rates but differences in mean cell lengths among the ft2, ft6 and cikA mutants indicates that cell division, and presumably at least partial Z ring assembly, occurred more frequently per micrometre of cell length in the shorter cikA mutant cells than in the much longer ft2 and ft6 mutant cells. The reasons for these differences are currently unclear because we do not yet understand how these genes function in cell division. However, Ft2 as well as the closely related plastid division protein ARC6, both of which are localized at their respective division sites, probably interacts directly with the division machinery, potentially with FtsZ itself, such that loss of this gene severely perturbs the division process (Koksharova and Wolk, 2002; Vitha et al., 2003; Mazouni et al., 2004). The same may be true of Ft6 because the phenotypes of the ft6 and ft2 mutants are similar, although at present we have little on which to base speculations about ft6 function.

In contrast, the role of CikA seems more likely to be regulatory. CikA is a phytochrome-related histidine kinase that is proposed to modulate the behaviour of the circadian clock in S. elongatus PCC 7942 by relaying information on light, temperature and clock status to the central timing mechanism (Schmitz et al., 2000; Mutsuda et al., 2003). In addition, the timing of cell division in S. elongatus, but not the rates of DNA synthesis or cell growth, has been shown to be subject to circadian regulation (Mori et al., 1996; Asato, 2003; Johnson, 2004). Because cikA mutants exhibit altered phasing of several endogenous rhythms (Schmitz et al., 2000) as well as a reduced frequency of cell divisions, it is possible that cell septation, perhaps triggered by Z ring formation, is regulated by the circadian clock. As cell division of Synechococcus sp. PCC 7942 also requires light (Herdman and Carr, 1971; Marino and Asato, 1986; Asato, 2003), CikA could also
be a photoreceptor (Schmitz et al., 2000; Mutsuda et al., 2003) that imparts light status information to a system that regulates cell division more directly than the circadian clock; loss of light sensory input from CikA could consequently reduce the frequency of cell division. Analysis of other clock mutants, including kaiA, kaiB and kaiC mutants (Ishiura et al., 1998), will be important for understanding the relationship between the circadian clock, Z ring formation and cell division in *S. elongatus*.

**Genes common to cyanobacteria, Gram-positive bacteria and Gram-negative bacteria**

*ftsE, ftsI, ftsQ, ftsW, ftsZ, minC and minD* (Errington et al., 2003) are found in both *E. coli* and *B. subtilis*, and are well conserved in cyanobacteria. *cdv1*, identified as a potential cell division gene in this study, is also well conserved in other bacterial phyla including *E. coli and B. subtilis*. *cdv1* encodes a PPIase previously shown to be present in the periplasm of *Synechocystis* sp. PCC 6803 (Fulda et al., 2000). PPIase is an enzyme that accelerates protein folding by catalysing the cis–trans isomerization of proline imidic peptide bonds in oligopeptides (Fischer and Schmid, 1990). Therefore, CdV1 may regulate the involvement of some protein in septum formation. As the function of this type of PPIase has not been studied in bacteria, further study, especially identification of its ligand, is needed to clarify its relationship to bacterial cell division.

**Similarity between cell division systems in cyanobacteria and Gram-positive bacteria**

Positioning of the MinCD complex, which inhibits Z ring assembly near the cell poles, is regulated by MinE in Gram-negative bacteria and by DivIVA in Gram-positive bacteria. These two proteins have distinct modes of action (Errington et al., 2003). Our study showed that, unlike in Gram-positive and Gram-negative bacteria, both MinE and the DivIVA-like protein CdV3 participate in cell division in cyanobacteria. Cyanobacterial *minE* is well conserved with its Gram-negative counterparts, and, as in *E. coli* (de Boer et al., 1989), all the *minE* mutants identified in our screen produced very long filamentous cells. CdV3 and DivIVA are less well conserved, but are structurally similar based on protein size, the position and length of coiled-coil structure, and the placement of both in COG3599. In addition, the *cdv3* mutants divided asymmetrically as does a *B. subtilis* divIVA mutant (Cha and Stewart, 1997; Errington et al., 2003). In *B. subtilis*, the phenotype of the divIVA mutant can be suppressed by disruption of *minD*, or *minC* and *minD*, suggesting that DivIVA regulates the MinCD complex (Cha and Stewart, 1997; Edwards and Errington, 1997). Similar experiments in cyanobacteria will be needed to define further the similarity between CdV3 and DivIVA. Although MinC and MinD were shown to be involved in cyanobacterial division (Mazouni et al., 2004), it is still unknown whether they oscillate as in *E. coli* (Errington et al., 2003), localize at the cell poles as in *B. subtilis* (Errington et al., 2003) or perhaps neither. Study of the localization of the MinCD complex, MinE and CdV3 in cyanobacteria should provide significant insight into how these two different systems cooperate in cyanobacteria.

Notably, certain (Gram-positive) clostridia also appear to possess both *minE* and *divIVA* (Stragier, 2000), suggesting that this newly identified system is shared by some Gram-positive bacteria and cyanobacteria. Perhaps most Gram-positive bacteria have lost MinE and proteobacteria have lost DivIVA while their common ancestor, like cyanobacteria, had both. Although MinC, MinD, MinE and CdV3 are encoded in all cyanobacterial genomes examined, orthologues of CdV3 and MinC have not been identified in algal and plant genomes.

Conservation of CdV2 (YlmF) in Gram-positive bacteria and its apparent absence from proteobacteria show an additional similarity in the cell division systems of Gram-positive bacteria and cyanobacteria. These observations, together with the identification of CdV3, led to the recognition that *ylmE*, *ylmG* and *ylmH* may play a role in cell division in cyanobacteria, and that *ylmG* and *ylmH* may play a role in plastid division in algae and plants (Table 3). Despite their involvement in cell division in *Streptococcus* (Fadda et al., 2003), their functions in cyanobacteria await study. Analysis of the possible role of the eukaryotic homologues of *ylmG* and *ylmH* in plastid division is in progress.

**Division mutants not identified by transposon mutagenesis**

Although our screen identified mutations in various genes in filamentous mutants, we found no transposon insertions in several cyanobacterial genes whose homologues are known to mediate cell division in other bacteria, including *ftsE, ftsI, ftsQ, sulA, ylmE, ylmG and ylmH*. Therefore, other cell division genes are probably still unidentified in cyanobacteria. Because we found many insertions in some genes (Fig. 2), inactivation of other genes may have been lethal even when segregation was incomplete. In particular, disruption of *ftsZ* in cyanobacteria could not be achieved (Zhang et al., 1995; Mazouni et al., 2004). Although we found one transposon in the 5′-untranslated region of *ftsZ*, the culture contained many cells like wild type and no significant decrease of FtsZ level was detected in the culture by immunoblotting (data not shown).

Whereas inactivation of *sulA* (Raynaud et al., 2004) or ARTEMIS (Fulgosi et al., 2002) in *Synechocystis* sp. PCC
Nucleoid separation and FtsZ ring formation in S. elongatus

The observation that defects in nucleoid separation at the division site prevented Z ring assembly in E. coli prompted a model in which Z ring positioning is controlled independently by the Min system, which restricts Z ring assembly to the mid-cell, and by nucleoid occlusion, which suppresses Z ring assembly at sites occupied by the nucleoid (Margolin, 2001). The latter mechanism, which functions in both E. coli and B. subtilis, is thought to couple cytoskeleton to chromosome replication as nucleoid partitioning and Z ring formation cannot occur until after the chromosome has replicated (Margolin, 2001). In contrast, we observed the formation of Z rings at presumptive division sites despite the presence of nucleoids at those sites as indicated by DAPI staining. Consistent with this observation, DNA content and cell division are not tightly coupled in S. elongatus and other Synechococcus species (Binder and Chisholm, 1990; Mori et al., 1996; Asato, 2003), and nucleoid staining and electron microscopy in some cyanobacterial species indicate that septation can be initiated before the movement of nucleoids away from the division site (Beck, 1963; Porta et al., 2000). These data are consistent with the hypothesis that nucleoid occlusion does not regulate Z ring formation in cyanobacteria. This hypothesis makes sense for two reasons: (1) the presence of multiple genome equivalents in cyanobacteria (Binder and Chisholm, 1990) might obviate the need for tight coupling between DNA replication and cell division and (2) the cell periphery, where the Z ring presumably assembles, and the nucleoids are not closely juxtaposed as they are in non-photosynthetic bacteria, but are separated by the thylakoid membranes; therefore, it might be predicted that the presence or absence of nucleoids at the division site would not directly regulate Z ring assembly in cyanobacteria. Related considerations also apply to the regulation of Z ring assembly in chloroplasts.

Although cyanobacteria have an outer membrane as do Gram-negative bacteria, molecular phylogenetic studies group cyanobacteria apart from other Gram-negative bacteria (proteobacteria) (Woese et al., 1990; Doolittle, 1999; Gupta and Griffiths, 2002). Further study of cyanobacterial division will no doubt reveal additional similarities and differences among bacterial division systems and provide new insights into the evolution of chloroplast division.
Experimental procedures

Database search

DNA and protein sequence databases were accessed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and from the Kazusa DNA Research Institute of Japan (http://www.kazusa.or.jp/cyano/). The draft genome sequence data for *S. elongatus* PCC 7942 were obtained from the Department of Energy Joint Genome Institute (Version 01dec03; http://www.jgi.doe.gov/JGI_microbial/html/index.html). Protein motifs were searched by Pfam (http://www.sanger.ac.uk/Software/Pfam/).

Culture conditions

*Synechococcus elongatus* PCC 7942 and its derivatives were grown in BG-11 medium (Allen, 1968) in 25 or 125 ml Erlenmeyer flasks or on Petri dishes containing 1.2% agarose at 30°C in continuous light (c. 140 μE m⁻² s⁻¹) on a rotary shaker. Growth of cells in liquid cultures was measured by determining OD₇₃₀. For inhibition of FtsI, cephalexin (100 μg ml⁻¹) was added to liquid cultures at an early phase of growth (OD₇₃₀ = 0.1); cells were observed 72 h after addition of the drug.

**Transposon mutagenesis**

Tn5-692 was transferred to *S. elongatus* PCC 7942 cells by conjugation with *E. coli* strain HB101 bearing pRL443 (conjugal plasmid), pRL528 (helper plasmid) and pRL692 (containing Tn5-692) as described (Elhai and Wolk, 1988a; Koksharova and Wolk, 2002). Filters bearing exconjugants were incubated for 72 h at 30°C (light intensity, c. 60 μE m⁻² s⁻¹) before transfer to medium containing erythromycin and spectinomycin (10 μg ml⁻¹ each). Spreading colonies identified between 15 and 30 days after the initiation of selection were streaked to new plates. Isolated colonies were used for further experiments.

**Determination, by inverse PCR, of the sequences flanking the transposon in the transformants**

A single colony was grown in liquid culture containing erythromycin and spectinomycin (2 μg ml⁻¹ each) and the genomic DNA was extracted from 5 to 10 ml of culture (OD₇₃₀ > 0.5) using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The DNA was digested with TaqI or Hhal for 2 h; Hhal, when used, was heat-inactivated at 65°C for 15 min. The digested DNA preparations were ligated overnight at 16°C with T4 DNA ligase (Invitrogen). The genomic regions were amplified and sequenced with primers (Q. Fan and C.P. Wolk, unpubl.) based on the sequence of Tn5-692 (GenBank Accession No. AF424805). The primer sequences and reaction protocol are to be published elsewhere but are available upon request. Segregation of mutations was assessed by PCR using primers (Table 3) up- and downstream from the insertion. Segregation of mutations was assessed by PCR using primers (Table 3) up- and downstream from the insertion.

**Targeted gene disruption**

To inactivate a gene, the relevant genomic region was amplified with primers (Table 3) by PfuTurbo DNA polymerase (Stratagene, La Jolla, CA). Each amplified DNA was cloned into the vector pGEM-T (Promega, Madison, WI, USA), and the insert was sequenced to identify error-free clones. The omega cassette (the omega interposon; Prentki and Krisch, 1984; cut from pRL453 and pRL463; Elhai and Wolk, 1988b), which confers resistance to erythromycin, streptomycin and spectinomycin, was inserted into the target gene at a unique restriction site (Table 3). The orientation of the omega cassette inserted to each *orf* was determined by digestion with a combination of SpeI, which cuts once in pGEM-T, and SpI, which cuts once in the omega cassette and once in pGEM-T. Constructs in which the antibiotic resistance gene of the omega cassette was inserted in the same orientation as the gene were used for gene disruption, except for *orf1939*, into which the omega cassette was inserted in the opposite orientation. Gene disruptants were generated by transformation of wild-type cells with these constructs and selected on BG-11 plates containing spectinomycin (10 μg ml⁻¹). The single colonies were streaked on new plates five times. Segregation of the mutations was confirmed by PCR using the primers listed in Table 3.

**Microscopy**

All observations by microscopy were carried out using cells grown in liquid. *S. elongatus* PCC 7942 cells were collected by centrifugation at 5000 g for 10 min and then observed with Nomarski optics (BH2; Olympus America, Melville, NY).

For observation of nucleoids, cells were fixed at room temperature for 30 min with 1% glutaraldehyde dissolved in phosphate-buffered saline (PBS) and then stained with DAPI at a concentration of 1 μg ml⁻¹.

FtsZ localization and nucleoids were observed together by staining cells first with anti-FtsZ antibodies for indirect immunofluorescence microscopy and then with DAPI. Cells were fixed in 3% (w/v) paraformaldehyde dissolved in 50 mM PIPES-KOH, pH 6.8, 10 mM EGTA, 5 mM MgSO₄ for 30 min at room temperature and washed twice with PBS. After treatment with 0.05% Triton X-100 in PBS for 15 min, the samples were permeabilized for 30 min at 37°C with 0.2 mg ml⁻¹ lysozyme dissolved in Tris-HCl, pH 7.5, 10 mM EDTA, and then washed twice with PBS. After blocking with 5% bovine serum albumin in PBS (blocking buffer) for 30 min, cells were labelled at 30°C for 2 h with anti-Anabaena FtsZ antibodies (Kuhn et al., 2000) diluted 1:500 in blocking buffer. Cells were then washed twice with blocking buffer, and incubated with Oregon green-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR; 1:300 dilution) for 1 h at 30°C. Cells were then washed twice with PBS, stained with DAPI at a concentration of 1 μg ml⁻¹ and observed by fluorescence microscopy (DMR A2; Leica Microsystems, Wetzlar, Germany).

**Immunoblotting**

Pellets of cells dissolved in Laemmli sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol,
and 0.01% bromphenol blue) were incubated at 95°C for 5 min. The samples were centrifuged at 15 000 g for 10 min and the supernatant solutions were subjected to electrophoresis and immunoblotting. Immunoblot analyses were performed as previously described (Stokes et al., 2000) using 10% acrylamide gels. The primary antibody (anti-Anabaena FtsZ) and secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody; Pierce, Rockford, IL) were diluted 1:2000 and 1:20 000 respectively.

Acknowledgements

We are deeply indebted to the US Department of Energy Joint Genome Institute and Dr Susan Golden (Texas A&M University) for permission to use the S. elongatus PCC 7942 sequences before publication. We are also grateful to Drs I. Kuhn and C.-C. Zhang (Université Louis Pasteur de Strasbourg) for their generous gift of the anti-Anabaena FtsZ antibodies. We also thank Drs Q. Fan and S. Lachno-Yessef for their technical instruction in mutagenesis and inverse PCR, Brad Olson for helpful discussions, Mary Fantacone for editorial help and our reviewers for excellent suggestions. This study was supported by a research fellowship from the Japanese Society for the Promotion of Science for Young Scientists (No. 7498 to S.M.), and by grants from the US Department of Energy (DOE-FG02-91ER20021 to C.P.W.) and the National Science Foundation (MCB-0313520 to K.W.O.).

Supplementary material

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Table S1. E-values of similarities between proteins encoded by E. coli or B. subtilis and related cyanobacterial or eukaryotic genes.

Table S2. Per cent identity between proteins encoded by E. coli or B. subtilis and related cyanobacterial or eukaryotic genes.

Table S3. Expect-value of similarities between proteins encoded by the cell division genes identified or predicted in S. elongatus PCC 7942 and those in other species.

Table S4. Identities between proteins encoded by cell division genes identified or predicted in S. elongatus PCC 7942 and those in other species.

References


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