

# Genomic Adaptations to the Loss of a Conserved Bacterial DNA Methyltransferase

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**ABSTRACT** CcrM is an orphan DNA methyltransferase nearly universally conserved in a vast group of *Alphaproteobacteria*. In *Caulobacter crescentus*, it controls the expression of key genes involved in the regulation of the cell cycle and cell division. Here, we demonstrate, using an experimental evolution approach, that *C. crescentus* can significantly compensate, through easily accessible genetic changes like point mutations, the severe loss in fitness due to the absence of CcrM, quickly improving its growth rate and cell morphology in rich medium. By analyzing the compensatory mutations genome-wide in 12 clones sampled from independent  $\Delta ccrM$  populations evolved for ~300 generations, we demonstrated that each of the twelve clones carried at least one mutation that potentially stimulated *ftsZ* expression, suggesting that the low intracellular levels of FtsZ are the major burden of  $\Delta ccrM$  mutants. In addition, we demonstrate that the phosphoenolpyruvate-carbohydrate phosphotransfer system (PTS) actually modulates *ftsZ* and *mipZ* transcription, uncovering a previously unsuspected link between metabolic regulation and cell division in *Alphaproteobacteria*. We present evidence that point mutations found in genes encoding proteins of the PTS provide the strongest fitness advantage to  $\Delta ccrM$  cells cultivated in rich medium despite being disadvantageous in minimal medium. This environmental sign epistasis might prevent such mutations from getting fixed under changing natural conditions, adding a plausible explanation for the broad conservation of CcrM.

**IMPORTANCE** In bacteria, DNA methylation has a variety of functions, including the control of DNA replication and/or gene expression. The cell cycle-regulated DNA methyltransferase CcrM modulates the transcription of many genes and is critical for fitness in *Caulobacter crescentus*. Here, we used an original experimental evolution approach to determine which of its many targets make CcrM so important physiologically. We show that populations lacking CcrM evolve quickly, accumulating an excess of mutations affecting, directly or indirectly, the expression of the *ftsZ* cell division gene. This finding suggests that the most critical function of CcrM in *C. crescentus* is to promote cell division by enhancing FtsZ intracellular levels. During this work, we also discovered an unexpected link between metabolic regulation and cell division that might extend to other *Alphaproteobacteria*.

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Homologs of the solitary DNA adenine methyltransferase CcrM are almost universally conserved in the ancient and ecologically very diverse clade of non-*Rickettsiales* *Alphaproteobacteria* (1). This extensive conservation supports the idea that CcrM homologs have been essential for fitness (2, 3) in these *Alphaproteobacteria* over hundreds of millions of years of evolution and suggests that virtually no strain or species in which the *ccrM* gene was inactivated by accident would have been able to compete efficiently with its *ccrM*-encoding relatives. Accordingly, CcrM homologs show major signs of phylogenetic persistence, being very conserved in sequence, highly expressed in tested models, and located in the vicinity of other essential or persistent genes (1, 4–7). Moreover, the nearly universal conservation of CcrM suggests that the function of DNA methylation by CcrM in *Alphaproteobacteria* is difficult to compensate genetically under common evolutionary scenarios in natural environments. It has indeed been shown that essential genes whose function can be replaced through easily accessible genetic changes, like a small number of

single nucleotide substitutions or a change in expression of a paralogous gene, tend to be less conserved than genes whose function cannot be so easily compensated (8, 9). Our objective was to shed some light on the striking persistence of CcrM DNA methyltransferases by using experimental evolution, whole-genome sequencing and classical genetics to study the compensability of the CcrM homolog in the alphaproteobacterial model *Caulobacter crescentus*.

CcrM plays a central role in the regulation of the cell cycle of *Caulobacter crescentus*, methylating the adenine in nearly all GANTC sequences along the chromosome in a cell cycle-dependent way (5, 10, 11). Since CcrM is active during only a short time window before cell division and after the replication of the chromosome, the methylation state of GANTC motifs alternates periodically during the cell cycle between a fully methylated and a hemimethylated form (after the replication of each GANTC motif until the predivisional stage, when CcrM is expressed again) (11, 12). The methylation state of GANTC motifs influences

among others the transcription rate of the genes coding for two global regulators of the cell cycle: DnaA, which initiates DNA replication and regulates the transcription of about 40 genes, and CtrA, which represses DNA replication and regulates the transcription of about 95 genes (13–16). In addition, the activity of a third key regulator of the cell cycle, the transcription factor GcrA, seems to be influenced by the methylation state of GANTC motifs located at or close to its binding sites on the promoter of at least some of its many target genes (17, 18). On its own, a regulatory cascade connecting DnaA, GcrA, and CtrA might control the cell cycle-dependent transcription of nearly 200 genes (13). An estimate based on a transcriptomic study suggests that the transcription of at least 40 genes could be influenced directly by GANTC methylation; among them, essential functions related to cell division and DNA metabolism are overrepresented (1). In particular, the transcription of *mipZ* and *ftsZ*, whose products are involved in the early steps of the divisome assembly, is strongly activated by the methylation of GANTC motifs located in their promoter region (19). Overall, these results indicate that CcrM is an important element in the architecture of the network regulating the cell cycle and, especially, in the control of cell division in *C. crescentus*.

Despite its key regulatory role, the *ccrM* gene is not essential for viability in *C. crescentus*:  $\Delta ccrM$  mutants can easily be constructed and cultured in minimal medium (M2G [M2 minimal salts plus 0.2% glucose]), where they have a moderate growth disadvantage relative to wild-type cells (19). However, when they are transferred to rich medium (peptone yeast extract [PYE]),  $\Delta ccrM$  mutants lose the capacity to divide, forming long filaments that quickly end up lysing. Complementation with an increased expression of the FtsZ protein can partially restore cell division and viability of  $\Delta ccrM$  mutants, suggesting that low levels of FtsZ are responsible for the most severe phenotypes of the mutant or that other missing functions can be compensated through higher expression of FtsZ. Overall, it appears that, in the absence of CcrM, *C. crescentus* cannot cope with elevated growth rates under conditions where organic matter (amino acids) is abundant, where it probably fails to coordinate growth and cell division (19). Such a limitation would be extremely disadvantageous in a natural environment where changes in nutrient abundance and type might be quick and radical (20). Naturally arising  $\Delta ccrM$  mutants could simply not be able to survive sudden “feast” conditions, missing most opportunities to increase in population size. This might apply to other *Alphaproteobacteria* as well, given that the *ccrM* gene seems to be essential in rich medium in several of them (4, 21, 22).

As the starting point of our research, we asked whether and to what extent the fitness disadvantage associated with the disruption of *ccrM* could be compensated through mutations in *C. crescentus* cells cultivated under very selective conditions (rich medium). We took advantage of the fact that CcrM is not essential in minimal medium and that cell death is not immediate upon transfer to rich medium (19). Indeed, for potential compensatory mutations to appear and spread in the population, cells have to be able to survive and, ideally, divide, even slowly, in the restrictive conditions. Since fitter mutants appeared quickly and reproducibly in our experimental setup, we prolonged the experiment for a total of 300 generations to get a more extensive view of the mutational landscape of evolving  $\Delta ccrM$  mutants. Through the analysis of the genetic basis of the adaptation, we were able to learn more about the fitness determinants of *C. crescentus* and to get a better

understanding of CcrM interactions within the complex regulatory network that underlies the bacterial cell cycle.

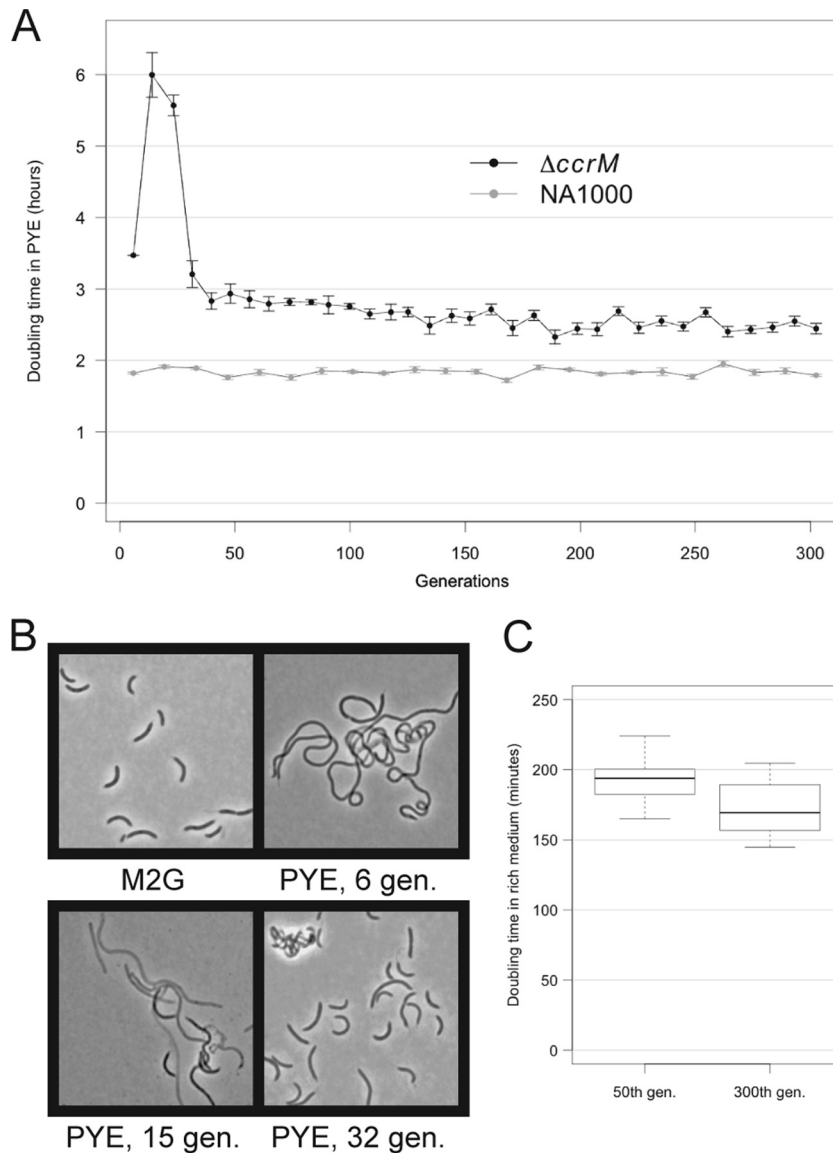
## RESULTS

**Phenotypic adaptation of  $\Delta ccrM$  populations growing in rich medium.** We let 12 independent populations of a *C. crescentus* strain lacking CcrM (NA1000  $\Delta ccrM$ ) evolve in rich medium (PYE) at 28°C for about 300 doublings in optical density (corresponding to doublings in cell mass; referred to here as “generations”), rediluting the cultures every day and collecting/freezing samples every 50 generations. We started the cultures from three independently obtained  $\Delta ccrM$  clones; the evolving populations were numbered 1.1 to 1.4 (ancestral strain, JC1149), 2.1 to 2.4 (ancestral strain, JC1150), and 3.1 to 3.4 (ancestral strain, JC1151). As controls, 10 independent populations of wild-type NA1000 *C. crescentus* were cultured under the same conditions for the same number of generations. During the course of the experiment, we monitored the growth rate and the morphology of the cells in the evolving populations. In each of the 12  $\Delta ccrM$  populations, but in none of the NA1000 populations, we observed significant changes in growth and morphology over the 300 generations. In particular, we distinguished two phases during the evolution of the  $\Delta ccrM$  populations: a phase of rapid adaptation, between the start of the experiment and the ~40th generation, involving rapid changes in cell shape and growth rates, and a phase of slower evolution, between the ~40th and the 300th generation (Fig. 1A).

Right after the transfer from minimal medium (M2G) to rich medium at the beginning of the experiment, the *C. crescentus* cells lacking CcrM started to elongate and to form filaments more than 10 times longer than the wild-type cells, as previously reported (19) (Fig. 1B). The filamentous cells eventually started to lyse: the populations sampled at generations ~15 and ~23 were composed of more than 50% “ghost” cells either lacking DNA (no 4',6-diamidino-2-phenylindole [DAPI] coloration) or permeable to the dead stain propidium iodide (data not shown), which is a known characteristic of the  $\Delta ccrM$  strain cultivated in rich medium (19); by that time, most live cells were filamentous or very elongated (Fig. 1B). However, between generations 30 and 40, the average length of the live cells decreased suddenly in all the populations simultaneously, while the frequency of dying and highly filamentous cells dropped (Fig. 1B). The morphological evolution correlated with changes in growth rate: while the average doubling time remained between 3 and 4 h during the 5 to 7 first generations in rich medium, it increased up to more than 6 h during the next 20 generations, as dead cells were accumulating in the populations (Fig. 1A; also, see Fig. S1A in the supplemental material). After that, the doubling time dropped back to about 3 h, as the average cell size and the frequency of dead cells decreased.

The changes observed during the rapid evolution phase could have been caused either by a physiological adaptation (epigenetic or regulatory) or by a change in the genetic composition of the population (selective sweep). To test this second alternative, we isolated colonies at generation 50 on rich medium agar plates, grew them in minimal medium and then transferred them back to rich medium. No obvious decrease in growth rate was observed upon transfer (data not shown), indicating that the adaptation of the cells to rich medium was stable and, thus, most likely had a genetic rather than epigenetic or regulatory basis.

A further slower decrease in doubling time was observed after



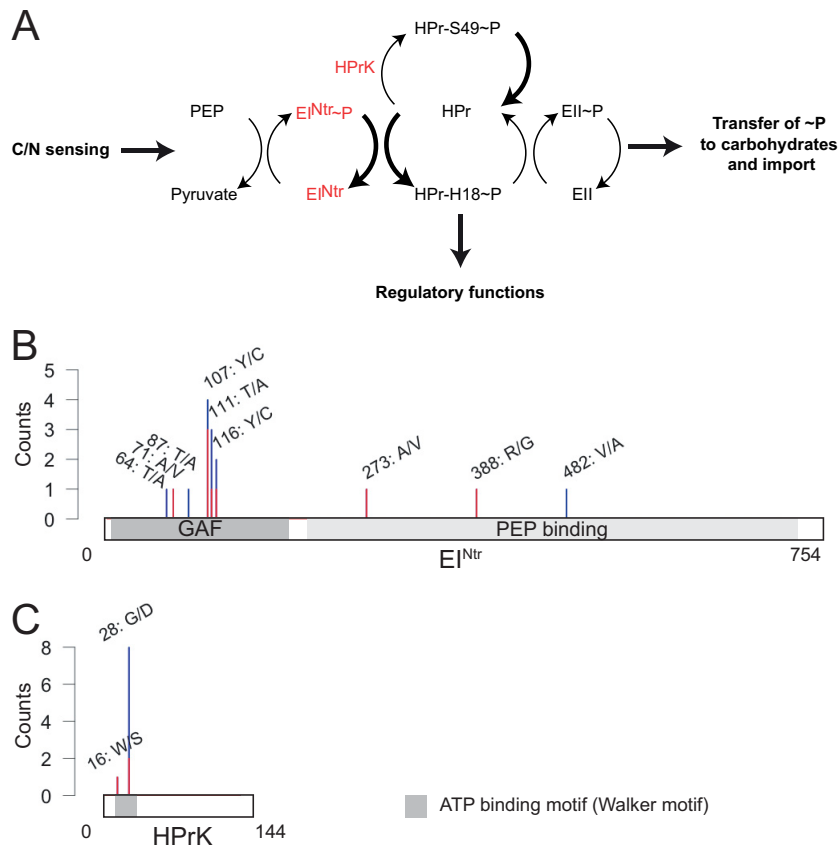
**FIG 1** Changes in growth rate and morphology during the evolution of  $\Delta ccrM$  populations in rich medium. Twelve independent  $\Delta ccrM$  populations and 10 independent wild-type (NA1000) populations were transferred from minimal medium (M2G) to rich medium (PYE) at an  $OD_{660}$  of 0.01 at the beginning of the experiment. Each second day for the first 40 doublings and each day for the following doublings, the culture was rediluted to an  $OD_{660}$  of 0.001. (A) Changes in average overnight generation time during the course of the experiment for the 12  $\Delta ccrM$  populations and for the 10 NA1000 control populations. The overnight generation time was measured, taking the initial and final  $OD_{660}$  and time interval into account. The error bars show standard deviations from the means for the 10 or 12 populations of each starting genotype. (B) Morphology of representative cells in a  $\Delta ccrM$  population during the initial phase of evolution: before transfer to rich medium (M2G) and 6, 15, and 32 generations (gen.) after transfer. After 6 generations in rich medium, cells formed 10- to 40- $\mu$ m-long filaments that did not show signs of lysis; after 15 generations, more than 50% of the filamentous cells seemed to lyse (light grey cells); after 32 generations, viable shorter cells invaded the population. (C) Between generation 50 and generation 300, the clones sampled from the  $\Delta ccrM$  populations gained in fitness (growth rate). Two clones were isolated from each of the 12  $\Delta ccrM$  populations at generation 50 and generation 300 and grown independently in rich medium. The doubling times were measured in exponential phase. The thick line represents the median, the limits of the box show the 25th and 75th quartiles, and the whiskers show the 5th and 95th quantiles.

generation 40 for most evolving  $\Delta ccrM$  populations on the basis of their generation time (see Fig. S1A and B in the supplemental material). To confirm this result, we calculated the generation time in rich medium by a more traditional method using pure cultures of clones isolated from the 12  $\Delta ccrM$  evolving populations at generations 50 and 300. The median difference in generation time between the first and the second set of clones was 24 min and statistically significant (Student's *t* test *P* value, <0.01)

(Fig. 1C). We could not demonstrate significant differences in cell morphology or size between two sets of clones sampled from the 12 evolving populations at the 50th and 300th generations by either microscopy or flow cytometry (data not shown). The existence of consistent and stable differences in growth rate between the two sets of clones suggested that the adaptation during the second phase of evolution might also have a genetic origin.

We concluded that, over time, the growth rate and morphol-





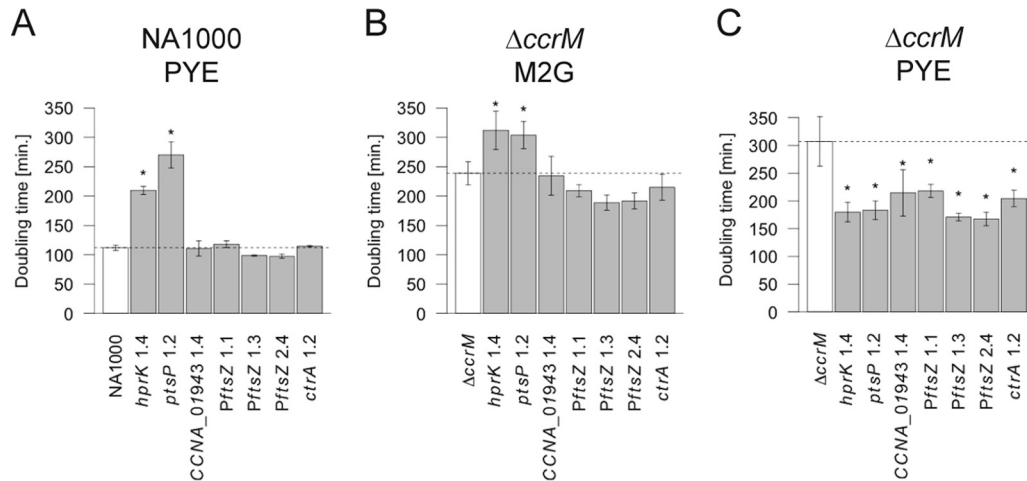
**FIG 3** Point mutations in genes encoding components of the phosphoenolpyruvate-carbohydrate phosphotransfer system (PTS) are the most frequent in evolved  $\Delta ccrM$  clones. (A) A simplified model for the PTS in *C. crescentus* based on homology searches (24, 47) and on the experimental work done on the *Rhizobiales* members *Brucella melitensis* and *Sinorhizobium meliloti* (36, 41, 48). The PTS allows a phosphate group to be transferred from phosphoenolpyruvate (PEP) to specific carbohydrates via several protein intermediates—EI<sup>Ntr</sup>, HPr, and EII—to regulate their import into the cytoplasm. EI<sup>Ntr</sup> and HPr may have additional regulatory functions via unidentified factors in *Alphaproteobacteria*. (B) Amino acid changes (counts) caused by point mutations found in the coding sequence of *ptsP* (EI<sup>Ntr</sup>) in 12 clones isolated at generation 300 (blue, whole-genome sequencing) or at generation 50 (red, targeted PCR and Sanger sequencing). (C) Amino acid changes (counts) caused by point mutations found in the coding sequence of *hprK* (HPrK) in 12 clones isolated at generation 300 (blue, whole-genome sequencing) or at generation 50 (red, targeted PCR and Sanger sequencing).

uted in a total of 25 functional regions (coding sequences or intergenic regions). The recurrence of almost 40% of the point mutations (Fig. 2; Table 1), their concentration in a limited number of functional regions and the biased  $K_a/K_s$  and  $K_i/K_s$  ratios strongly suggest that the fixation of the point mutations in the different populations was promoted primarily by selection rather than drift.

**Point mutations in PTS or PTS-associated genes.** All 14 evolved  $\Delta ccrM$  strains that we sequenced contained at least one point mutation affecting the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS), whereas none of the 10 NA1000 strains evolved in rich medium for 300 generations presented mutations in the same regions (see Table S1 in the supplemental material). The PTS is a pathway that mediates the transfer of a phosphate group from phosphoenolpyruvate to a sugar via several protein intermediates (Fig. 3A); it often controls the import of carbohydrates into the cytoplasm and regulates the metabolism in response to nutrient availability (23). In the genomes of the evolved  $\Delta ccrM$  strains, point mutations were found in sequences encoding either EI<sup>Ntr</sup>, which is often the only EI enzyme in *Alphaproteobacteria* (24), or the HPr serine kinase HPrK (Fig. 3B and C). One particular mutation was found in 6 different

strains (Table 1): it changed into an aspartate a very conserved glycine 28 residue in the ATP-binding motif of the HprK serine kinase (encoded by *hprK*, also called *CCNA\_00239*) (Fig. 3C). All the strains carrying the *hprK* mutation also carried one of two mutations in the *CCNA\_01943* gene, which changed an arginine into a histidine or a cysteine (Table 1). The *CCNA\_01943* gene codes for a protein of unknown function that is highly conserved among *Alphaproteobacteria* (including members of divergent orders, like *Rickettsiales*) and beyond. The co-occurrence of *hprK* and *CCNA\_01943* mutations suggests that there is a genetic interaction between the two proteins. The 8 strains that did not carry the *hprK* mutation harbored at least one mutation in the gene coding for the PTS EI<sup>Ntr</sup> (*ptsP*, also called *CCNA\_00892*) (Fig. 3B; Table 1). In 7 of these strains, the mutation affected different residues in the N-terminal regulatory GAF domain of the protein.

The fact that the two clones isolated from two independent populations at generation 50 already carried a mutation in genes encoding a PTS component suggested that the initial increase in fitness of the  $\Delta ccrM$  strain cultivated in PYE was specifically linked to PTS mutations (Table 1). To confirm this, we sequenced the *hprK* and *ptsP* loci in 12 clones isolated from the 12 evolving  $\Delta ccrM$  populations at generation 50. We found that all but one

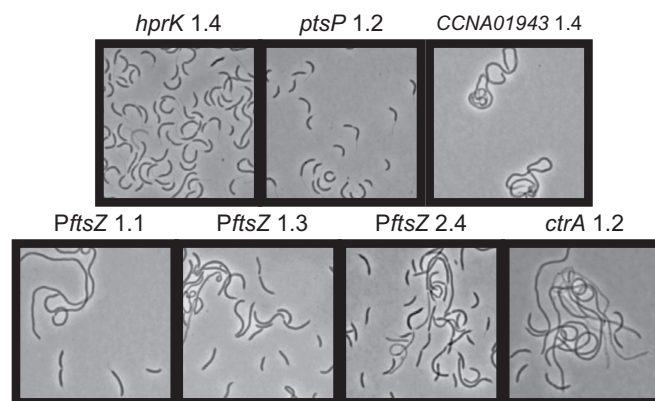


**FIG 4** Growth rates of NA1000 and  $\Delta ccrM$  strains carrying a single point mutation in *hprK*, *ptsP*, *CCNA\_01943*, the promoter region of *ftsZ* ( $P_{ftsZ}$ ) (three different mutations), or *ctrA*. (A) Average growth rate in exponential phase of NA1000 mutants (*hprK* 1.4, JC1323; *ptsP* 1.2, JC1336; *CCNA\_01943* 1.4, JC1327;  $P_{ftsZ}$  1.1, JC1325;  $P_{ftsZ}$  1.3, JC1328;  $P_{ftsZ}$  2.4, JC1338; *ctrA* 1.2, JC1343) and control strains (NA1000, JC1322) in rich medium based on three replicates. (B) Average growth rate in exponential phase of  $\Delta ccrM$  mutant (*hprK* 1.4, JC1339; *ptsP* 1.2, JC1340; *CCNA\_01943* 1.4, JC1344;  $P_{ftsZ}$  1.1, JC1345;  $P_{ftsZ}$  1.3, JC1341;  $P_{ftsZ}$  2.4, JC1342; *ctrA* 1.2, JC1346) and control ( $\Delta ccrM$ , JC1347) strains in minimal medium based on three replicates. (C) Average growth rate of  $\Delta ccrM$  mutant and control strains during the 23 generations after transfer from minimal medium to rich medium based on two replicates. Error bars show standard deviations from the means; the dotted line represents the average for the control strain.

(2.1) had a nonsynonymous mutation in the *ptsP* gene (8 strains) or the *hprK* gene (3 strains) (see Table S2 in the supplemental material). None of these strains carried a mutation in the *CCNA\_01943* gene, suggesting that mutations in this gene appear secondarily in strains already carrying a mutation in the *hprK* gene (see Table S2 in the supplemental material).

To characterize the effect of point mutations affecting the PTS in the absence of other mutations, we constructed wild-type and  $\Delta ccrM$  strains carrying the individual point mutations found in the evolved strains 1.2 (*ptsP* 1.2, an A-to-G change at position 970633 [970633 A/G]) and 1.4 (*hprK* 1.4, 254890 G/A). The growth of wild-type strains carrying these point mutations was heavily impaired in rich medium: the doubling times of the mutants were 270 and 210 min, respectively, compared to about 110 min for the control isogenic strains under the same conditions (Fig. 4A). In a  $\Delta ccrM$  genetic background, in contrast, the point

mutations affecting the PTS were very advantageous in rich medium, as these strains had growth rates on average 1.7 times higher than that of the control  $\Delta ccrM$  strain; in minimal medium, the same mutations were only slightly disadvantageous (300 min of doubling time compared to 250 min for the control) (Fig. 4B and C). Phase-contrast microscopy confirmed that the morphology of the  $\Delta ccrM$  strains carrying the PTS mutations did not obviously change upon transfer from minimal to rich medium (Fig. 5; also, see Fig. S2 in the supplemental material), while control  $\Delta ccrM$  cells became highly filamentous and lysed (Fig. 1). All together, these results indicate that either mutation is sufficient to compensate for the most deleterious phenotypes of the  $\Delta ccrM$  strain in rich medium. Interestingly, one additional characteristic of these  $\Delta ccrM$  cells carrying a mutation in PTS-encoding genes cultivated in both rich and minimal media was a long-stalk phenotype (Fig. 5; also, see Fig. S2 in the supplemental material) similar to the



**FIG 5** Phase-contrast micrographs of  $\Delta ccrM$  strains with single point mutations 7 generations after transfer from minimal to rich medium. Cells from strains JC1323, JC1325, JC1327, JC1328, JC1336, JC1338, and JC1343 are shown. Compare these images with the micrographs of a  $\Delta ccrM$  population after ~6 generations in rich medium (PYE) (Fig. 1B). The morphology of the same mutants in minimal medium (M2G) and at later generations in rich medium is presented in Fig. S2 in the supplemental material.

one seen in wild-type *C. crescentus* under phosphate limitation (25, 26); this suggests a possible cross talk between the PTS and phosphate sensing in *C. crescentus*.

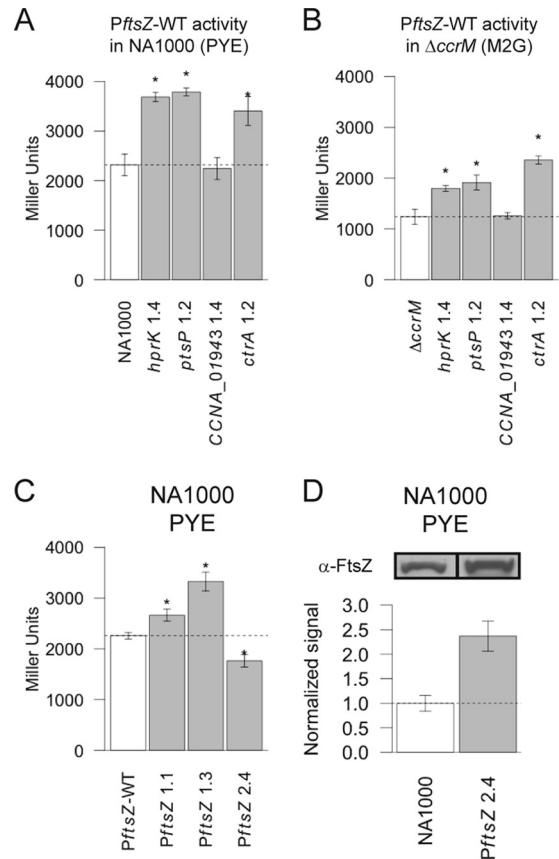
Since we had previously shown that the main phenotypes of the  $\Delta ccrM$  strain could be complemented by enhanced *ftsZ* transcription (19), we conjectured that the PTS mutations that we identified might increase the intracellular concentration of FtsZ and thereby promote cell division (Fig. 5). To determine whether the transcription of *ftsZ* might be promoted by the point mutations affecting the PTS, we quantified the activity of the *ftsZ* promoter in the wild-type and  $\Delta ccrM$  strains carrying the *hprK* 1.4 or *ptsP* 1.2 point mutation. We found that, in both PTS mutants, the *ftsZ* promoter was ~1.6 times more active than in the wild-type strain, suggesting that the PTS actually regulates *ftsZ* transcription in *C. crescentus* (Fig. 6A). In a  $\Delta ccrM$  background, the effect was similar: *ftsZ* transcription increased ~1.5 times in both PTS mutants, thereby compensating for 50% of the decrease in expression of *ftsZ* due to the absence of CcrM (Fig. 6A and B). This suggests that part of the fitness advantage that the  $\Delta ccrM$  PTS mutants have over their  $\Delta ccrM$  ancestors could be a consequence of partially restored levels of FtsZ.

We also reconstructed the *CCNA\_01943* (2086092 C/T) mutation, which is systematically associated with *hprK* mutations, in wild-type and  $\Delta ccrM$  backgrounds and did not observe major effects on growth rate or *ftsZ* expression (Fig. 4A and B, 5, and 6A; also, see Fig. S2 in the supplemental material). The only significant difference relative to the control strain was found upon transfer of the  $\Delta ccrM$  *CCNA\_01943* single mutant to rich medium: cells grew slightly faster than  $\Delta ccrM$  cells (Fig. 4C). These results suggest that *CCNA\_01943* point mutations do not promote *ftsZ* transcription or cell division *per se*; they might rather modulate potential negative effects that the mutations found in *hprK* have on growth, consistent with their late appearance during our evolution experiment.

Taking all these data together, we concluded that mutants carrying specific point mutations in *hprK* or *ptsP* quickly invaded the ancestral  $\Delta ccrM$  populations cultivated in rich medium. We show that, on their own, these mutations are sufficient to suppress cell filamentation and lysis and to give a strong selective advantage to their carriers during adaptation to rich medium. Finally, we provide evidence that this selective advantage could be a consequence of enhanced transcription of the *ftsZ* gene, uncovering an interesting connection between the PTS and cell division.

**Point mutations affecting cell cycle regulators and cell division.** Among the 28 mutations that did not affect the PTS or *CCNA\_01943*, 6 (21%) were connected with the control of the cell cycle and, especially, of cell division (Table 1).

A point mutation was found in the promoter region of the *ftsZ* gene ( $P_{ftsZ}$ ) in three different strains (Table 1), while none of the 10 wild-type control strains sampled from populations that evolved under the same conditions carried any mutation in this genetic context (see Table S1 in the supplemental material). The fact that 3 out of 8 point mutations found in intergenic regions were located in a narrow 100-bp window suggests that this region is a strong determinant of the adaptation of  $\Delta ccrM$  to rich medium. To determine whether any point mutation was present in  $P_{ftsZ}$  after the first selection sweep, we analyzed the *ftsZ* promoter region in one clone isolated from each of the 12 populations sampled at the 50th generation. No point mutation was detected in this region in any of the strains, suggesting that the mutations



**FIG 6** Effect of selected point mutations on *ftsZ* transcription and/or on the intracellular levels of the FtsZ protein. (A) Average activity of the wild-type *ftsZ* promoter in selected NA1000 mutant strains (NA1000, JC1353; *hprK* 1.4, JC1354; *ptsP* 1.2, JC1369; *CCNA\_01943* 1.4, JC1368; *ctrA* 1.2, JC1370) as measured by  $\beta$ -galactosidase assays using cells cultivated in rich medium (PYE). (B) Average activity of the wild-type *ftsZ* promoter in selected  $\Delta ccrM$  mutant strains ( $\Delta ccrM$ , JC1371; *hprK* 1.4, JC1372; *ptsP* 1.2, JC1386; *CCNA\_01943* 1.4, JC1387; *ctrA* 1.2, JC1388) as measured by  $\beta$ -galactosidase assays using cells cultivated in minimal medium (M2G). (C) Effect of the three different point mutations found in the *ftsZ* promoter region of evolved  $\Delta ccrM$  strains on *ftsZ* transcription in NA1000, as measured by  $\beta$ -galactosidase assays using cells cultivated in PYE (NA1000, JC1353;  $P_{ftsZ}$  1.1, JC1383;  $P_{ftsZ}$  1.3, JC1384;  $P_{ftsZ}$  2.4, JC1385). (D) Relative quantification of FtsZ levels, from immunoblots, in an NA1000 derivative and in an isogenic strain carrying the 2.4 point mutation in the *ftsZ* promoter region (strains JC1347 and JC1342). In panels A through C, the averages are based on 3 or 4 replicates and error bars indicate standard deviations from the means; the dotted line represents the average for the control strain. In panel D, the averages are based on duplicates, and error bars indicate standard deviations from the means.

located in  $P_{ftsZ}$  became predominant in the population only during the phase of slower evolution, after the 50th generation (see Table S2 in the supplemental material).

One of the  $P_{ftsZ}$  mutations (2773108 G/A, strain 2.4) was located 3 bp upstream of the translational start codon of *ftsZ*. The two other mutations (2773188 T/C, strain 1.1; 2773191 C/T, strain 1.3) were located 2 and 5 nucleotides upstream of the transcription start site of *ftsZ* (27). To evaluate the effect of the  $P_{ftsZ}$  point mutations on growth and morphology, we constructed wild-type and  $\Delta ccrM$  strains carrying one of each of the three  $P_{ftsZ}$  mutations. While no significant effect on growth was observed in the wild-type genetic background (Fig. 4A), we found that each

$\Delta ccrM$  derivative had a higher growth rate in minimal medium than either the parental  $\Delta ccrM$  strain or the  $\Delta ccrM$  strains carrying the mutations affecting the PTS (Fig. 4B). In rich medium, the growth rates of the  $\Delta ccrM$  derivatives were much higher than that of the  $\Delta ccrM$  strain and similar to or slightly higher than those of the  $\Delta ccrM$  PTS mutants (Fig. 4C). However, the  $P_{ftsZ}$  point mutations had a much milder suppressive effect than PTS mutations on  $\Delta ccrM$  cell morphology: the  $P_{ftsZ}$  mutant cells were on average longer than the PTS mutant ones, and the frequency of filamentous cells was higher in the population (Fig. 5; also, see Fig. S2 in the supplemental material).

Considering the position of the point mutations found in  $P_{ftsZ}$ , we hypothesized that these could lead to increased  $ftsZ$  expression. We constructed transcriptional reporters to compare the activity of the three mutant promoters with the wild-type promoter using  $\beta$ -galactosidase assays. We found that the mutated  $ftsZ$  promoters found in the evolved  $\Delta ccrM$  strains 1.1 and 1.3 were 1.2 and 1.5 times more active than the wild-type promoter (Fig. 6C). In contrast, the mutated  $ftsZ$  promoter found in the evolved  $\Delta ccrM$  strain 2.4 was less active than the wild type (Fig. 6C). Since the point mutation present in the latter promoter region was located close to the translational start site, we conjectured that translation efficiency might be enhanced rather than transcription. We therefore evaluated the impact of the  $P_{ftsZ}$  2.4 mutation on the intracellular levels of the FtsZ protein using immunoblots; we found that FtsZ levels were 2.4 times higher in the mutant strain than in the wild-type strain, suggesting that the  $P_{ftsZ}$  2.4 mutation enhances  $ftsZ$  translation (Fig. 6D). In conclusion, all three point mutations found in the  $ftsZ$  promoter region in our evolved  $\Delta ccrM$  strains enhance  $ftsZ$  expression, either by increasing transcription or by a posttranscriptional mechanism.

Two other point mutations were found in the sequence encoding the cell cycle regulator CtrA, which represses  $ftsZ$  transcription (27). One of them (*ctrA*3.2; 8 D/G) affects the predicted active site (“acidic pocket”) of the response regulator (28). The other one (*ctrA* 1.2; 209 H/R) is located in the C-terminal part of the protein very close to a determinant of CtrA degradation (29). These two mutations could potentially lower the repressing activity of CtrA on the  $ftsZ$  promoter by affecting either the phosphorylation state or the proteolysis of CtrA. Another point mutation (*divL*2.4) was found in the sequence encoding the DivL histidine kinase. DivL affects the phosphorylation and the degradation of CtrA, and although this mutation (392 G/S) should not affect the phosphorylation of DivL at Y550, it might disturb its regulation or possible phosphorylation-independent functions and modulate CtrA activity (30–32).

We reconstructed the *ctrA* 1.2 mutation in the wild-type and  $\Delta ccrM$  genetic backgrounds, to test whether it had an effect on growth or  $ftsZ$  transcription on its own. We found that the *ctrA* 1.2 mutation had a negligible effect on growth in the wild-type strain cultivated in rich medium or in the  $\Delta ccrM$  strain cultivated in minimal medium (Fig. 4A and B). Upon transfer to rich medium, in contrast, the  $\Delta ccrM$  *ctrA* 1.2 mutant grew much faster than the  $\Delta ccrM$  strain (Fig. 4C); in contrast with the  $\Delta ccrM$  control (Fig. 1B), shorter cells were present in the  $\Delta ccrM$  *ctrA* 1.2 mutant population, although the morphology of a significant proportion of the cells was still filamentous (Fig. 5; also, see Fig. S2 in the supplemental material). These observations indicated that this mutation enhances growth rate but that it is not sufficient to improve cell division as much as PTS mutations. To test whether

it might derepress  $ftsZ$  transcription in cells cultivated in rich medium, we compared the transcriptional activity of the  $ftsZ$  promoter in a wild-type strain carrying or lacking the *ctrA* 1.2 mutation. We observed a significant increase (1.6-fold) in  $ftsZ$  transcription in the presence of the *ctrA* 1.2 mutation (Fig. 6A); in a  $\Delta ccrM$  background, the effect was even stronger (1.9-fold increase) (Fig. 6B). We therefore suppose that the aberrant morphology of many cells seen in the  $\Delta ccrM$  background despite the presence of the *ctrA* 1.2 mutation might be due to a pleiotropic effect associated with a dysfunctional CtrA protein (28), rather than to a strong deficiency in  $ftsZ$  expression.

The occurrence of three independent mutations in the  $ftsZ$  promoter region supports our previous hypothesis that a low FtsZ concentration is a major burden on the fitness of the  $\Delta ccrM$  strain cultivated in rich medium (19). The presence of three additional mutations in sequences encoding two essential regulators of the cell cycle, CtrA and DivL, more generally confirms the genetic connection between DNA methylation by CcrM and the cell cycle control circuit (1).

**Other point mutations of interest.** In addition to the point mutations affecting PTS and PTS-associated genes or converging on the regulation of  $ftsZ$  expression, 28 additional mutations were found in the evolved  $\Delta ccrM$  strains (Table 1). Some of them were recurrent, like a frameshift mutation found in four strains in the *CCNA\_00608* gene (predicted to code for a polygalacturonase [pectin hydrolase]), the same 84 V/G substitution found in three strains in the *CCNA\_03537* gene (encoding a glyoxalase family protein), and two different point mutations found in *CCNA\_00999* (encoding a conserved hypothetical protein). Several independent nonsynonymous mutations could affect membrane receptors, transporters, or permeases (in the *CCNA\_00451*, *CCNA\_03524* or *CCNA\_01257* coding sequences or in the intergenic region between *CCNA\_02570* and *CCNA\_02571*). Some of these mutations might modulate adverse effects that PTS mutations have on growth rate and thus enhance the fitness of the strains rather than having a direct impact on the  $\Delta ccrM$  phenotype.

**Other mutations.** A few larger mutational events specific to the evolved  $\Delta ccrM$  strains were identified during the analysis. A deletion of nearly 50 kbp encompassing 56 predicted coding sequences (bp 2932736 to 2982516 in the genome) was detected as missing coverage in 11 out of the 12 sequenced strains collected after 300 generations but not in the wild-type or in the ancestral  $\Delta ccrM$  strains (see Fig. S3A in the supplemental material). The presence of the deletion was confirmed by PCR using primers annealing on the flanking regions of the deletion. Two 100% identical sequences of 927 bp coding for a transposase (*CCNA\_02772* and *CCNA\_02828*) belonging to an *IS511* transposable element (33, 34) were found on each side of the deleted region, suggesting that the ~50 kbp was excised through homologous recombination between these two sequences. The deletion was not present in any of 10 wild-type strains that evolved for the same number of generations under the same conditions, indicating that this deletion is not an adaptation to long-term culture in rich medium but might confer a specific advantage in the  $\Delta ccrM$  or  $\Delta ccrM$ /PTS background. Surprisingly, this large deletion was already present in 3 out of 12  $\Delta ccrM$  clones sampled after the 50th generation (see Fig. S4B in the supplemental material). This early presence suggests that the advantage provided by this mutation is strong enough for its carriers to quickly outcompete  $\Delta ccrM$ /PTS mu-



tants. Another possibility is that homologous recombination is more efficient in  $\Delta ccrM$  cells than wild-type cells, promoting the excision of this region.

One of four IS511 insertion sequences present in the *C. crescentus* genome (33) had been transposed in two of the  $\Delta ccrM$  evolved strains sampled at the 300th generation to position 1054365 (*CCNA\_00974*) in population 1.3 and to position 1111235 (*CCNA\_01023*) in population 3.1. No point mutation was found in these two genes in any of the sequenced strains, suggesting that the insertion position might be neutral from a selective viewpoint. It is possible that IS511 transposition frequency could be enhanced in the  $\Delta ccrM$  strain, considering that three transposases were found to be overexpressed 2-fold or more in that strain (1).

## DISCUSSION

We previously showed that DNA methylation by CcrM is critical for the viability of *C. crescentus* in rich medium and for the regulation of its cell cycle (1, 19). In this study, using an original experimental evolution approach, we demonstrated that the most important function of CcrM is to stimulate *ftsZ* transcription and found an unexpected connection between the regulation of cell division and the central metabolism (through the PTS).

**The lack of FtsZ is a major fitness burden for  $\Delta ccrM$  cells.** Twelve independent  $\Delta ccrM$  populations were cultivated for ~300 generations in rich medium. All ancestral  $\Delta ccrM$  populations were quickly invaded by mutants with improved cell division, able to partially compensate for the loss of fitness due to the absence of CcrM (Fig. 1). Whole-genome sequencing revealed that each evolved strain carried two to seven different mutations in addition to the  $\Delta ccrM$  deletion (Table 1) and that two thirds of these mutations (32/55) clustered in only eight chromosomal regions (Fig. 2). More detailed analysis of several of these mutations demonstrated that nearly 40% (21/55) of all detected point mutations could be connected with the regulation of *ftsZ* expression and that each of the twelve evolved strains carried at least one mutation promoting *ftsZ* transcription (Fig. 6; Table 1).

Three of these mutations were located upstream of the *ftsZ* coding sequence (Fig. 7) and were sufficient to strongly ameliorate the growth and division of  $\Delta ccrM$  cells in rich medium (resulting in a doubling time nearly two times shorter) (Fig. 4). Since these mutations promote *ftsZ* transcription or translation *in cis* (Fig. 6), they are unlikely to affect cellular factors other than FtsZ. Consistently, we previously showed that an artificial induction of *ftsZ* transcription was sufficient to restore the viability of  $\Delta ccrM$  cells cultivated in rich medium (19). Taken together, these findings demonstrate that the lack of FtsZ is a major burden slowing the growth of  $\Delta ccrM$  cells.

Three other mutations were identified in the *ctrA* and *divL* genes (Table 1), encoding the CtrA master regulator and the DivL regulator of CtrA phosphorylation and degradation (Fig. 7) (30). Consistent with the fact that CtrA is a direct inhibitor of *ftsZ* transcription (27), we demonstrated that one of these mutations (*ctrA* 1.2) promoted *ftsZ* transcription (Fig. 6) and the growth of  $\Delta ccrM$  cells cultivated in rich medium (Fig. 4).

Finally, at least one mutation in the *hprK* or *ptsP* genes, encoding PTS components, was identified on the chromosome of each of the twelve strains evolved for ~300 generations (Table 1; Fig. 7). We showed that cells carrying one of these mutations were already frequent in 11  $\Delta ccrM$  populations after only ~50 generations (Ta-

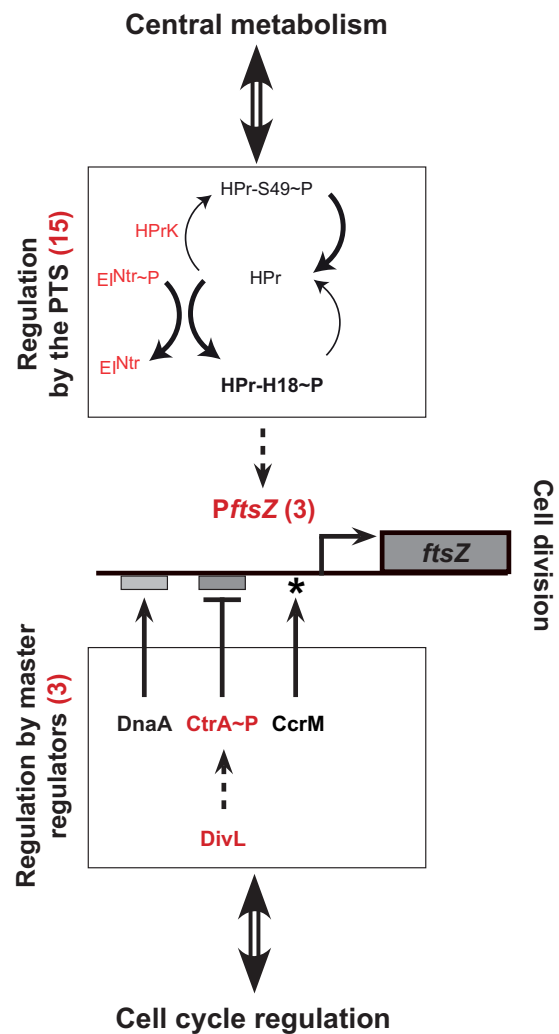


FIG 7 Model for the network connecting *ftsZ* transcription with cell cycle progression (through master cell cycle regulators) and central metabolism (through PTS proteins). About 40% (21/55) of the point mutations identified in the 14 evolved  $\Delta ccrM$  strains converge on promoting *ftsZ* expression. The red numbers in parentheses are the actual mutation counts identified in the 14 evolved  $\Delta ccrM$  strains. Fifteen mutations were found in sequences encoding the Elntr and HprK PTS proteins; these mutations probably promote the accumulation of Hpr-H18~P (bold arrows), leading to an activation of the transcription of *ftsZ* through unknown intermediates. Three mutations were found in the *ftsZ* promoter region (two close to the transcription start site and one near the ribosome binding site). Two mutations were found in the sequence encoding CtrA, a repressor of *ftsZ* transcription, and one in the sequence encoding DivL, which activates the phosphorylation and the activation of CtrA. Dashed arrows indicate potentially indirect effects.

ble 1; also, see Table S2 in the supplemental material) and that mutations in *hprK* or *ptsP* improved the division of  $\Delta ccrM$  cells cultivated in rich medium very efficiently compared to other mutations (Fig. 5; also, see Fig. S2 in the supplemental material). Altogether, these findings indicate that PTS mutations might provide the easiest and most efficient way to improve the fitness of  $\Delta ccrM$  cells cultivated in rich medium. Supporting our proposal that the lack of FtsZ is one of the main factors limiting the growth of  $\Delta ccrM$  cells, we showed that mutations in *hprK* or *ptsP* alone are sufficient to increase *ftsZ* transcription (Fig. 6).

**Control of *ftsZ* transcription by the PTS.** The components of the PTS have not been characterized so far in *C. crescentus*, but their orthologs in other *Alphaproteobacteria* have been studied (23, 35). The *C. crescentus* HprK protein is the homolog of the *Sinorhizobium meliloti* HprK serine kinase, which phosphorylates the Ser53 residue of the *S. meliloti* HPr protein (36). By analogy, it is very likely that *C. crescentus* HprK phosphorylates the conserved Ser49 residue of the *C. crescentus* HPr (Fig. 3A). The mutation that we identified six times in *hprK* (Table 1) and that stimulates *ftsZ* transcription (Fig. 6) changed the conserved Gly28 residue of HprK into an aspartate. This residue is located in the putative ATP-binding motif of the kinase (Fig. 3C), suggesting that the mutation leads to a loss of function that blocks the phosphorylation of the conserved Ser49 residue of HPr by HprK (Fig. 7). This mutation may at the same time promote the phosphorylation of the His18 residue of HPr, leading to an accumulation of HPr with His18 phosphorylated (HPr-H18~P) (36). Similarly, the *C. crescentus* EI<sup>Ntr</sup> protein (encoded by *ptsP*) is the homolog of the *S. meliloti* EI<sup>Ntr</sup> protein (Fig. 3A). This protein phosphorylates the His22 residue of the *S. meliloti* HPr protein when its activity is not inhibited by glutamine binding to its GAF domain (36, 37). By analogy, it is likely that the five different mutations that we identified in the GAF domain of the *C. crescentus* EI<sup>Ntr</sup> protein (Fig. 3B), including the one that was confirmed to stimulate *ftsZ* transcription (*ptsP* 1.2) (Fig. 6), promote the phosphorylation of the conserved His18 residue of the *C. crescentus* HPr by the mutant EI<sup>Ntr</sup>, leading again to an increase in HPr-H18~P levels (Fig. 7). Taken together, our results suggest that *ftsZ* transcription might be directly or indirectly affected by the phosphorylation state of the HPr protein, with HPr-H18~P appearing to be, potentially, the most efficient at stimulating *ftsZ* transcription (Fig. 7). HPr has no obvious DNA binding domain, but it was shown that it binds to transcription factors or to anti-sigma factors in other bacterial species and that it sometimes phosphorylates transcriptional regulators, to control gene expression indirectly in a manner that is often dependent on its phosphorylation state (23, 38, 39). None of these known HPr-interacting proteins (CcpA, BglG, Rsd, etc.) seems to be conserved in the *C. crescentus* proteome, suggesting that the *C. crescentus* HPr might regulate *ftsZ* transcription through novel intermediates.

It was previously shown that *ftsZ* transcription is inhibited or activated by the direct binding of CtrA or DnaA, respectively, to the *ftsZ* promoter (Fig. 7) (15, 27, 40), raising the possibility that the PTS might affect the regulation of the *ftsZ* promoter by these regulators. To test this hypothesis, we measured the effect of the *ptsP* 1.2 mutation on the activity of mutant *ftsZ* promoters that cannot bind to DnaA or CtrA. We found that these DnaA- or CtrA-independent mutant *ftsZ* promoters were still more active in the *ptsP* 1.2 mutant than in the wild-type strain (see Fig. S4 in the supplemental material), suggesting that the binding of DnaA or CtrA to the *ftsZ* promoter is not required for the activation of *ftsZ* transcription by PTS components (Fig. 7). We also found that the integrity of the GANTC motif methylated by CcrM in the *ftsZ* promoter (19) is unnecessary (see Fig. S4 in the supplemental material), consistent with our previous finding that PTS mutations promote the activity of the methylated (in wild-type cells) or nonmethylated (in  $\Delta ccrM$  cells) *ftsZ* promoter (Fig. 6). Taken together, our observations suggest that two independent modules might regulate *ftsZ* transcription (Fig. 7). One module would include the DnaA, CtrA, and CcrM master cell cycle regulators,

which bind or methylate the *ftsZ* promoter and which coordinate cell division with other events of the cell cycle, such as DNA replication (15, 19, 27). The other module would be connected with the activity of PTS components, maybe to coordinate cell division with central metabolism. Understanding how PTS components such as HPr influence *ftsZ* transcription in a manner that seems independent of known direct regulators of *ftsZ* transcription will require further investigations.

**The PTS might connect cell division with central metabolism.** PTS play a great variety of biological roles in bacteria, including detecting available nutrients or metabolites and controlling central metabolism, chemotaxis, or virulence (for example, see reference 23). Still, despite sparse and very indirect evidence in *Escherichia coli* (24), no clear connection between the PTS and cell division in bacteria had been described.

Canonical PTS from *Enterobacteriaceae*, *Vibrionales*, and *Firmicutes* regulate the assimilation of alternative carbon sources through the uptake and phosphorylation of carbohydrates (23, 35). The presence or absence of specific sugars is translated into relative levels of phosphorylated versus nonphosphorylated forms of enzyme II (EII) permeases and HPr, causing diverse effects, including carbon catabolite repression. As many *Alphaproteobacteria* lack EII permeases, it has been proposed that *Alphaproteobacteria* could sense carbon and nitrogen availability through their PTS (24, 35). Consistent with this hypothesis, the PTS of *Brucella melitensis* has been connected with central metabolism through interactions of EI<sup>Ntr</sup> and EI<sub>IA</sub> with the  $\alpha$ -ketoglutarate dehydrogenase and its substrates (41); in *S. meliloti*, EI<sup>Ntr</sup> binds to glutamine to control its activity in response to carbon and nitrogen levels in the cell (37). *S. meliloti* *hprK* mutants grow slowly on all types of carbon sources, suggesting that the PTS is connected with central metabolic pathways in this bacterium (36). Considering that wild-type *C. crescentus* strains carrying *hprK* and *ptsP* 1.2 mutations showed severe growth defects in rich medium (Fig. 4A), it is tempting to assume that the *C. crescentus* PTS may communicate the metabolic state of the cell to the *ftsZ* promoter to coordinate cell division with cell growth (Fig. 7).

Interestingly, we observed that the activity of a second promoter controlling the transcription of another important cell division gene, *mipZ*, was also stimulated in *hprK* and *ptsP* 1.2 mutants (see Fig. S5 in the supplemental material). This gene encodes the MipZ protein, which controls the subcellular localization of the FtsZ ring before cell division in *C. crescentus* (42). It is also regulated by the DnaA, CtrA, and CcrM regulators, like *ftsZ* (15, 16, 19). The coregulation of *ftsZ* and *mipZ* by a PTS-dependent mechanism might explain why  $\Delta ccrM$  mutants carrying PTS mutations divided more efficiently than  $\Delta ccrM$  mutants carrying suppressor mutations in the *ftsZ* promoter (Fig. 5). This finding suggests that master regulators of the *C. crescentus* cell cycle and the PTS share common targets to coordinate cell division with other cell cycle events (through master regulators) and with the central metabolism of the cell (through the PTS) (Fig. 7). This study also illustrates the power of genetic screens using CcrM-deficient strains to identify novel pathways controlling the cell cycles of *Alphaproteobacteria*.

**Concluding remarks on the conservation of CcrM.** During the evolution experiment described in this study, *C. crescentus*  $\Delta ccrM$  cells quickly gained fitness through point mutations, suggesting that the *ccrM* gene might be lost if compensating PTS mutations arise during natural evolution. This appears to be in

contradiction with the high degree of conservation of the *ccrM* gene in *Alphaproteobacteria* (1). However, PTS mutations improved the fitness of  $\Delta$ *ccrM* cells in rich medium, but not in minimal medium (Fig. 4), providing an example of environmental sign epistasis (43). Since most *Alphaproteobacteria* are frequently exposed to changing environments in their natural habitats, including nutrient-poor environments, PTS mutations that could have compensated for or allowed the loss of *ccrM* would be strongly counterselected. Furthermore, even in rich medium,  $\Delta$ *ccrM* cells with PTS mutations, although fitter than  $\Delta$ *ccrM* cells (Fig. 4C), were still much less fit and competitive than wild-type *C. crescentus* cells. Taken together, these observations add a possible explanation for the phylogenetic conservation of CcrM in so many *Alphaproteobacteria*.

## MATERIALS AND METHODS

**Culture conditions and strains.** *C. crescentus* strains were cultivated in peptone yeast extract (PYE) rich medium or in M2 minimal salts plus 0.2% glucose (M2G) minimal medium at 28°C (44), except when indicated otherwise. Agar (1.5%) was added to make solid media (M2GA or PYEA). Strains used in this study are listed and described in Table S4 in the supplemental material.

**Experimental evolution.** Overnight cultures in M2G of three independently obtained  $\Delta$ *ccrM* strains (JC1149, JC1150, and JC1151 [19]) were diluted in 3 ml of PYE (rich medium) to an optical density at 660 nm ( $OD_{660}$ ) of 0.01, each in four replicates numbered 1.1 to 1.4 (ancestral culture, JC1149), 2.1 to 2.4 (ancestral culture, JC1150) and 3.1 to 3.4 (ancestral culture, JC1151). After that, for a total of 300 doublings in  $OD_{660}$  (37 days), the 12 populations were rediluted to an  $OD_{660}$  of 0.001 in 3 ml of PYE as soon as the cultures reached an  $OD_{660}$  of 0.5 to 1.0 (late exponential phase). The doubling times of each population were estimated on the basis of the initial and final  $OD_{660}$  and the time interval at every dilution. Aliquots of each population were frozen every ~50 doublings. As a control, a similar experiment was carried out with 10 wild-type NA1000 populations; we used a total volume of PYE of 5 ml and daily dilutions to an  $OD_{660}$  of 0.0001 for these populations.

**Whole-genome sequencing and assembly.** One clone was reisolated from the 12  $\Delta$ *ccrM* populations sampled at generation 300 and from 2 populations (1.2 and 3.3) sampled at generation 50 for sequencing. As controls, one colony from our NA1000 *C. crescentus* strain and one colony from each of the three  $\Delta$ *ccrM* strains JC1149, JC1150, and JC1151 were also included. Illumina libraries of 400 to 500 bp were constructed from the extracted genomic DNA using barcoded adaptors optimized for high GC content and sequenced (single reads) on a HiSeq 2500 system in a single lane (18-plex sequencing). The filtering, alignment of the reads to the reference NA1000 genome (NC\_011916), point mutations and new junction (rearrangements) calling was done using breseq 0.22 software (45, 46). The mean coverage depth was >180 for all strains.

Other methods, including those used to calculate generation times and to construct strains and transcriptional reporters, are described in Text S1 in the supplemental material.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00952-15/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.  
Figure S1, PDF file, 0.3 MB.  
Figure S2, PDF file, 0.9 MB.  
Figure S3, PDF file, 0.1 MB.  
Figure S4, PDF file, 0.03 MB.  
Figure S5, PDF file, 0.02 MB.  
Table S1, PDF file, 0.02 MB.  
Table S2, PDF file, 0.02 MB.  
Table S3, PDF file, 0.1 MB.  
Table S4, PDF file, 0.1 MB.

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