The environmental Alphaproteobacterium Caulobacter crescentus is a classical model to study the regulation of the bacterial cell cycle. It divides asymmetrically, giving a stalked cell that immediately enters S phase and a swarmer cell that stays in the G1 phase until it differentiates into a stalked cell. Its genome consists in a single circular chromosome whose replication is tightly regulated so that it happens only in stalked cells and only once per cell cycle. Imbalances in chromosomal copy numbers are the most often highly deleterious, if not lethal. This review highlights recent discoveries on pathways that control chromosome replication when Caulobacter is exposed to optimal or less optimal growth conditions. Most of these pathways target two proteins that bind directly onto the chromosomal origin: the highly conserved DnaA initiator of DNA replication and the CtrA response regulator that is found in most Alphaproteobacteria. The concerted inactivation and proteolysis of CtrA during the swarmer-to-stalked cell transition license cells to enter S phase, while a replisome-associated Regulated Inactivation and proteolysis of DnaA (RIDA) process ensures that initiation starts only once per cell cycle. When Caulobacter is stressed, it turns on control systems that delay the G1-to-S phase transition or the elongation of DNA replication, most probably increasing its fitness and adaptation capacities.

**Introduction**

DNA replication is controlled with exquisite precision in all cell types to ensure that each daughter cell inherits one copy of complete chromosome(s) following each division event. Control mechanisms largely focus on the initiation step of the replication process, when the replisome is assembled onto DNA. In nearly all bacteria, the highly conserved initiator of chromosome replication is DnaA [1,2]. It typically binds to several DnaA boxes located on the chromosomal origin and oligomerizes to open the DNA double helix at an AT-rich region within the origin. It also interacts with helicases to load them onto the DNA. Subsequently, the replicative DNA polymerase and its β-sliding clamp (DnaN) are recruited onto leading and lagging strands to initiate bi-directional and processive DNA replication. The proper timing of chromosome replication is highly dependent on controlling the levels, the activity and the availability of DnaA in bacteria. The analysis of a variety of bacteria now reveals to which extent control mechanisms are conserved, or not, in different bacterial classes or species [3,4]. It also shows that bacteria often control the DNA replication process to modulate their proliferation in response to environmental cues directly connected to their biological niche, through the regulation of DnaA or of other more specific regulators.

Caulobacter crescentus (henceforth Caulobacter) is an aquatic Alphaproteobacterium that emerged as a powerful model system to study the regulation of the bacterial cell cycle. This bacterium divides asymmetrically at the end of each cell cycle giving daughter cells with distinct developmental and replicative fates (Figure 1) [5]. The first one is a replication incompetent (G1-phase) swarmer cell, while the second one is a replication competent (S-phase) stalked cell. To initiate the replication of its unique circular chromosome, the swarmer cell must first differentiate into a stalked cell. This relatively slow growing bacterium, compared with the most studied Escherichia coli model system, never displays...
more than two replication forks at work within the same cell and over-initiation events are severely deleterious [6]. It is relatively easy to isolate nearly pure populations of swarmer cells from mixed populations of *Caulobacter* cells, facilitating studies on the regulation of the timing of DNA replication during the bacterial cell cycle.

This review focuses on recently discovered mechanisms that control the replication of the chromosome of *Caulobacter* cultivated in favorable or less-favorable growth conditions, with particular emphasis on the regulation of the DnaA initiator and of the CtrA inhibitor of chromosome replication that are both highly conserved in *Alphaproteobacteria*.

The origin of replication of the *Caulobacter crescentus* chromosome

The origin of replication of the *Caulobacter* chromosome (*Cori*) was mapped years ago at a chromosomal locus located close to the *hemE* and overlapping the CCNA_00001 (*duf299*) open reading frames (ORF) (Figure 2) [6–8]. It carries DNA motifs with affinity for several known regulators of the *Caulobacter* cell cycle, including the initiator DnaA, the response regulator CtrA, the nucleoid associated proteins IHF (Integration host Factor) and GapR, and the DNA methyltransferase (MTase) CcrM [6,7,9]. As assumed in nearly all bacterial species, DnaA and several of the DnaA boxes found on the origin are indispensable for the initiation of chromosome replication in *Caulobacter* [7,10]. DnaA binds to seven DnaA boxes on the *Cori*; five of these are low-affinity W-boxes and two are moderate-affinity G-boxes (Figure 2) [7]. Although one of the W-box overlaps with a DNA motif that can be methylated by the DNA MTase CcrM (Figure 2), it appears that methylation of the *Cori* by CcrM is not required for DNA replication in *Caulobacter*, since the chromosome content of ΔccrM cells is identical with that of wild-type cells [11,12]. Also, consistent with this finding, a homolog of the

---

*Figure 1. Graphical representation of the *Caulobacter* cell cycle and of the abundancy of the DnaA and CtrA main regulators of the initiation of chromosomal replication. In swarmer cells, the initiation of chromosomal replication is inhibited by high levels of CtrA∼P (red color) binding to the chromosomal origin of replication (*Cori*, blue circle). During the swarmer-to-stalked cell transition, CtrA∼P is proteolyzed at the cell pole where the stalk will be built. Coincidentally, DnaA is synthesized and binds to ATP. DnaA-ATP (green color) binds to the *Cori* and is active to initiate replication. As soon as DNA replication has started, HdaA is recruited to the replisome and activated to stimulate the conversion of DnaA-ATP into DnaA-ADP (blue color) and DnaA proteolysis. This Regulated Inactivation of DnaA (RIDA) process prevents re-initiation during the same cell cycle. In early predivisional cells, CtrA is re-synthesized and phosphorylated, but gets efficiently dephosphorylated and proteolyzed in the swarmer cell compartment of late pre-divisional cells to inhibit the initiation of DNA replication in the swarmer progeny. Although this was not directly demonstrated, CtrA∼P may form a gradient in pre-divisional cells, being the most abundant at the flagellated cell pole even before cell compartmentalization, due to polarized upstream regulators of CtrA phosphorylation and degradation [83].*
methylated methylation-dependent DNA binding protein SeqA, which controls replication initiation in E. coli [3], cannot be found in the Caulobacter proteome. The relatively low affinity of DnaA for the Cori DnaA boxes might be connected with the existence of CtrA, which inhibits the initiation, supposedly through a direct competition with DnaA when binding to the Cori. Indeed, five CtrA binding sites are found on the Cori and one of them overlaps one of the two moderate-affinity DnaA G-boxes (Figure 2). The ctrA gene is essential for the survival of Caulobacter and partial loss of CtrA function leads to premature initiation of DNA replication in swarmer cells [13,14]. Notably, an IHF binding site overlaps one of the CtrA binding sites (Figure 2) [15], indicating that IHF may promote the disassembly of CtrA on the Cori during the swarmer-to-stalked cell transition [16]. Targeted mutagenesis experiments eliminating the CtrA binding sites on the Cori, however, showed that the inhibition of DNA replication by CtrA is dispensable when Caulobacter is cultivated in minimal medium [17]. In addition to DnaA, CtrA and IHF, the GapR protein shows affinity for Cori [9]. GapR is a newly discovered and conserved nucleoid associated protein that appears as critical for chromosome replication and growth in Caulobacter [9,18,19], most probably by promoting the ability of topoisomerases to relax positive supercoils that accumulate ahead of the replication fork [20]. Considering that GapR is more abundant in stalked than in swarmer cells [9], it might also promote the binding of DnaA to the Cori, although this was not tested directly. Finally, the presence of two ORFs [21] and of a gene transcribed into a small non-coding RNA [22,23] in the Cori region (Figure 2) suggests that the transcription of these elements could potentially interfere with or promote the opening of the DNA double helix for initiation. Consistent with this possibility, early findings showed that transcription from one of the hemE promoters is required for chromosome replication in Caulobacter [24].

**CtrA restricts the initiation of chromosome replication to stalked cells**

CtrA is a conserved response regulator, which is found in most Alphaproteobacteria [25]. It needs to be phosphorylated to be active and to inhibit the initiation of DNA replication [13]. Logically, very strict regulatory mechanisms control the levels of active CtrA-P (Figures 1 and 3A), to ensure that chromosome replication can still start once per cell cycle and specifically during the swarmer-to-stalked cell transition of Caulobacter or in the stalked progeny following cell division. CtrA-P is very abundant in swarmer cells, where it binds to the Cori with high affinity to maintain cells in G1 phase, and in pre-divisinal cells, where it plays essential roles in the regulation of gene expression (Figure 1) [26,27]. Following cytoplasmic compartmentalization in late pre-divisional cell, the levels of CtrA become highly dissimilar: the flagellated compartment of the cell contains ~22,000 molecules of CtrA [28], while the stalked compartment contains undetectable levels of CtrA. These strong temporal and spatial variations are mostly dependent on the activity of the essential CckA histidine kinase/phosphatase, which is at the top of a phasorelay controlling CtrA phosphorylation and proteolysis (Figure 3A) [5,6]. Indeed, although ctrA transcription varies significantly during the cell cycle, due to tight control by the GcrA epigenetic regulator [29,30], by the CtrA-associated SciP regulator [31,32] and by self-regulation [33], this temporal regulation is not required for the control of the initiation of replication since the constitutive transcription of ctrA does not lead to replication defects [26]. Still, sufficient transcription of ctrA
Figure 3. Graphical representation of the regulatory circuits controlling CtrA and DnaA activity in Caulobacter cells cultivated in optimal (no background color) or stressful (brown background color) growth conditions. Part 1 of 2

(A) Model for the regulatory network controlling the levels and the activity of CtrA. During the swarmer-to-stalked cell transition, the activity of CckA switches from a kinase (CckAKIN; red arrows) to a phosphatase (CckAPHOS; green arrows), reducing the levels of ChpT–P. In turn, CtrA–P can no more accumulate and gets actively degraded by a CpdR- and PopA/RcdA-dependent ClpXP proteolysis complex, triggering the initiation of DNA replication by DnaA-ATP.
DnaA controls the frequency of the initiation of chromosome replication

Control of the abundancy of active DnaA ensures that replication can start once, but only once, per cell cycle [6,50]. Levels must be high enough in stalked cells, but shut down right after replication initiation to prevent over-initiation events in stalked and early pre-divisional cells, when CtrA–P levels are minimal.

A first level of regulation is through the control of dnaA transcription that peaks right before the initiation of chromosome replication during the swarmer-to-stalked cell transition [6,51]. Mechanisms controlling dnaA transcription are still unclear although the analysis of dnaA promoter elements indicates that the efficiency and the timing of dnaA transcription may be controlled by a transcriptional activator binding close to a GAGTC motif upstream of the −35 region [52,53]. A second level of regulation is through the inhibition of dnaA translation by a long untranslated region (5′UTR) upstream of the translational start codon (Figure 3B), but this mechanism does not appear to control the timing of dnaA expression [53]. Importantly, the transcriptional and post-transcriptional controls of DnaA synthesis play only a marginal role in controlling when, and at which frequency, DNA replication can initiate during the Caulobacter cell cycle, since the artificial and constitutive expression of dnaA does not lead to replication defects in Caulobacter. Instead, these mechanisms may be more useful to control DnaA levels in response to environmental signals, rather than to cell cycle cues.

The most important mechanism controlling the frequency of replication initiation is the so-called Regulated Inactivation and proteolysis of DnaA (RIDA) process that appears as essential for the survival of Caulobacter [14,54,55]. This
process restricts the levels of active DnaA by stimulating its ATPase activity, leading to the concerted inactivation and degradation of DnaA (Figure 3B) [6,50]. There are now clear indications that DnaA needs to be associated with ATP to initiate DNA replication [14,54,55], as it is also the case in many other bacterial species [3]. The RIDA process takes place right after the initiation of DNA replication, through a stimulation of the ATPase activity of DnaA by the HdaA protein, preventing lethal over-initiation events. The current model is that HdaA interacts with the β-sliding clamp of the replisome once it is loaded onto the DNA at the onset of DNA replication and that this interaction switches on the activity of HdaA and the subsequent RIDA process [54,56]. In addition, the conversion of DnaA-ATP into DnaA-ADP during the RIDA process leads to a significant destabilization of DnaA at the onset of DNA replication during the swarmer-to-stalked cell transition [57,58]. Two different ATP-dependent proteases can recognize DnaA in Caulobacter (Figure 3B). The first one is ClpAP, which appears to destabilize DnaA independently of the nucleotide bound to it and preferentially under stress conditions, since the ClpS adaptor inhibits this pathway in exponentially growing cells [59]. The second one is Lon [60] and seems to have a preference for DnaA-ADP rather than DnaA-ATP [59], suggesting that it preferentially degrades DnaA during the S phase of the cell cycle when the RIDA process is active. Thus, Lon probably contributes to controlling the intracellular levels of active DnaA as a function of the cell cycle to prevent over-initiation events. As described below, it also plays a key role in adjusting DnaA levels and regulating DNA replication in response to stresses.

### Control of chromosome replication during non-optimal growth conditions

Bacteria must coordinate the replication of their genome with their growth rate. This is all the more important for bacteria that live in oligotrophic environments, like Caulobacter, and that are thus frequently exposed to nutrient limitations and environmental stresses.

When exposed to nutrient limitations, Caulobacter turns on a so-called stringent response that targets DnaA and CtrA to slow down or arrest the replication of its chromosome. This response is based on the production of guanosine tetra- or penta-phosphate ((p)ppGpp) by the SpoT enzyme [61], in response to carbon or nitrogen starvation [62] or in response to fatty acid depletion (Figure 3A) [63]. Recent findings also showed that SpoT is specifically activated by elements of the nitrogen-related phosphotransferase system (PTS Ntr) in response to low glutamine levels [64,65]. The (p)ppGpp alarmone then appears to inhibit DnaA synthesis (Figure 3B) and CtrA degradation (Figure 3A), leading to a severe inhibition of the initiation of chromosome replication [66]. The stabilization of CtrA by (p)ppGpp appears to be dependent on the up-regulation of the conserved MopJ regulator, which targets the DivL-dependent pathway controlling CckA activity (Figure 3A), although this may not be the only pathway involved [67]. The complex regulatory system controlling DnaA levels in response to nutrient limitations appears to include an inhibition of dnaA translation (Figure 3B), leading to a rapid clearance of DnaA by the Lon protease [68]. Interestingly, this translational inhibition also takes place in starved ΔspoT mutant cells, indicating that other unknown (p)ppGpp-independent pathways are also involved in the regulation of DNA replication in response to low nutrient levels [68].

When exposed to DNA/protein damaging conditions, Caulobacter also stops or slows down the replication of its chromosome. Two such regulatory pathways have now been identified (Figure 3B). The first one is based on the detection of unfolded or damaged proteins by the DnaK chaperone, which leads to a stabilization of the σ32 heat-shock factor and the consequent activation of lon transcription and Lon-mediated degradation of DnaA [60]. Thus, proteotoxic and severe heat-shock stresses block the initiation of DNA replication by DnaA [60,69]. The second one is based on the stabilization of the SocB toxin in response to DNA damage. In turn, SocB can bind to the β-clamp of the DNA polymerase to inhibit the elongation of DNA replication, most probably through a disassembly of the replicative DNA polymerase complex [70].

Surprisingly, Caulobacter adopts a rather opposite strategy when facing stress conditions affecting the integrity of its membrane, such as exposure to ethanol or high salt concentrations in its environment or exposure to mild heat-shocks. Indeed, these stresses promote the phosphatase activity of CckA in a DivL- and c-di-GMP-independent manner, leading a rapid decrease in the levels of active CtrA/P [71]. As a consequence, fewer stressed cells are in G1 phase and cells appear longer with an abnormally high DNA content compared with non-stressed cells. Future work should aim at understanding why the inhibition of CtrA activity provides an advantage to cells facing membrane stresses, while it appears to be deleterious to cells facing nutritional stresses.
Perspectives

This review aimed at summarizing the current view on the control of DNA replication in *Caulobacter*. Most, although not all, of the pathways regulating chromosome replication target the DnaA initiator or the CtrA response regulator. In fast growing conditions, the tight regulation of their activity is mostly dependent on the RIDA process and on the activity of the CckA kinase/phosphatase. A common point between the RIDA process and the CckA phosphatase is that they promote the inactivation and the proteolysis of their targets (Figure 3). Interestingly, the (p)pGpp alarmone affects CtrA and DnaA to adjust the length of the G1 phase under fast-growing conditions [72], or to block cells in the G1 phase under stressful conditions [64,65,67]. DnaA synthesis also appears to be regulated by (p)pGpp and growth conditions [66,68], but mechanisms involved in these control systems are still ill-defined. Interestingly, experimental and computational evidences suggest that up to 10 small non-coding RNAs (sncRNAs) might target the dnaA and hdaA messenger RNAs (Figure 3B) [22,23,73]. Considering that the expression of several of these is cell cycle-regulated or regulated in response to stresses [22,23,74,75], and that the Hfq RNA chaperone is required for metabolic homeostasis [76], it is tempting to predict that translational regulation by sncRNAs might also play an important role in fine-tuning the levels and the activity of DnaA (Figure 3B). Other unknown pathways are probably involved in adjusting DNA replication with cell growth, most probably by connecting the central metabolism with DNA replication. A recent study, for example, raised the possibility that a member of the Lrp family of transcription factors, named PutR, might connect proline intracellular levels with the elongation of DNA replication and cell division in *Caulobacter* [77].

It is noteworthy that DnaA and CtrA are also important transcription factors [27,78]. The impact of the RIDA process on the control of DnaA-regulated genes has not been investigated in detail, but preliminary findings suggest that the nucleotide binding to DnaA might also influence the transcription of a subset of these genes and chromosome segregation [55,79]. This might be used by *Caulobacter* to coordinate DNA replication with other processes required for cell cycle progression.

Finally, it is also interesting to mention that while the regulation of DnaA appears to be a widespread mechanism used by most bacteria to control chromosome replication, the involvement of CtrA in replication control is restricted to only a subset of them. Indeed, although CtrA is well conserved in Alphaproteobacteria, its regulon appears to evolve rapidly [80–82]. Then, determining whether DNA replication is under negative control in other Alphaproteobacteria and understanding the associated mechanisms could provide interesting new information on the evolution of complex networks controlling chromosome replication in bacteria.

Funding

The Swiss National Science Foundation provided financial support (SNSF fellowships CRSII3_160703 and 31003A_173075).

Acknowledgements

We thank Giulia Cheloni for the critical reading of this manuscript. We apologize to authors not cited in this short mini-review, which mostly focuses on articles published over the last five years.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References

1. Katayama, T., Ozaki, S., Keyamura, K. and Fujimitsu, K. (2010) Regulation of the replication cycle: conserved and diverse regulatory systems for DnaA and oriC. Nat. Rev. Microbiol. 8, 163–170. https://doi.org/10.1038/nrmicro2314
2. Katayama, T., Kasho, K. and Kawakami, H. (2017) The DnaA cycle in Escherichia coli: activation, function and inactivation of the initiator protein. Front. Microbiol. 8, 2496. https://doi.org/10.3389/fmicb.2017.02496
3. Skarstad, K. and Katayama, T. (2013) Regulating DNA replication in bacteria. Cold Spring Harb. Perspect. Biol. 5, a012922. https://doi.org/10.1101/cshperspect.a012922
4. Heinrich, K., Leslie, D.J. and Jonas, K. (2015) Modulation of bacterial proliferation as a survival strategy. Adv. Appl. Microbiol. 92, 127–171. https://doi.org/10.1016/bs.aambs.2015.02.004
5. Collier, J. (2016) Cell cycle control in Alphaproteobacteria. Curr. Opin. Microbiol. 30, 107–113. https://doi.org/10.1016/j.mib.2016.01.010
6. Collier, J. (2012) Regulation of chromosomal replication in Caulobacter crescentus. Plasmid 67, 76–87. https://doi.org/10.1016/j.plasmid.2011.12.007
7. Taylor, J.A., Ouimet, M.C., Wargachuk, R. and Marczenki, G.T. et al. (2011) The *Caulobacter crescentus* chromosome replication origin evolved two classes of weak DnaA binding sites. Mol. Microbiol. 82, 312–326. https://doi.org/10.1111/j.1365-2958.2011.07785.x
194
70 Aakre, C.D., Phung, T.N., Huang, D. and Laub, M.T. (2013) A bacterial toxin inhibits DNA replication elongation through a direct interaction with the beta sliding clamp. Mol. Cell 52, 617–628 https://doi.org/10.1016/j.molcel.2013.10.014

71 Heinrich, K., Sobetzko, P. and Jonas, K. (2016) A kinase-phosphatase switch transduces environmental information into a bacterial cell cycle circuit. PLoS Genet. 12, e1006522 https://doi.org/10.1371/journal.pgen.1006522

72 Boutte, C.C., Henry, J.T. and Crosson, S. (2012) Pyppp and polyphosphate modulate cell cycle progression in Caulobacter crescentus. J. Bacteriol. 194, 28–35 https://doi.org/10.1128/JB.05932-11

73 Beroual, W., Brilli, M. and Biondi, E.G. (2018) Non-coding RNAs potentially controlling cell cycle in the model Caulobacter crescentus: a bioinformatic approach. Front. Genet. 9, 164 https://doi.org/10.3389/fgene.2018.00164

74 Landt, S.G., Abeliuk, E., McGrath, P.T., Lesley, J.A., McAdams, H.H. and Shapiro, L. (2008) Small non-coding RNAs in Caulobacter crescentus. Mol. Microbiol. 68, 600–614 https://doi.org/10.1111/j.1365-2958.2008.06172.x

75 Lasker, K., Schrader, J.M., Men, Y., Marshik, T., Dill, D.L., McAdams, H.H. et al. (2016) Caulobrowser: a systems biology resource for Caulobacter crescentus. Nucleic Acids Res. 44, D640–D645 https://doi.org/10.1093/nar/gkv1050

76 Irnov, I., Wang, Z., Jannetty, N.D., Bustamante, J.A., Rhee, K.Y., Jacobs-Wagner, C. et al., (2017) Crosstalk between the tricarboxylic acid cycle and peptidoglycan synthesis in Caulobacter crescentus through the homeostatic control of alpha-ketoglutarate. PLoS Genet. 13, e1006978 https://doi.org/10.1371/journal.pgen.1006978

77 Mouammine, A., Eich, K., Frandi, A. and Collier, J. (2018) Control of proline utilization by the Lrp-like regulator PuR in Caulobacter crescentus. Sci. Rep. 8, 14677 https://doi.org/10.1038/s41598-018-32660-3

78 Hottes, A.K., Shapiro, L. and McAdams, H.H. (2005) DnaA coordinates replication initiation and cell cycle transcription in Caulobacter crescentus. Mol. Microbiol. 58, 1340–1353 https://doi.org/10.1111/j.1365-2958.2005.04912.x

79 Mera, P.E., Kalogeraki, V.S. and Shapiro, L. (2014) Replication initiator dnaA binds at the Caulobacter centromere and enables chromosome segregation. Proc. Natl Acad. Sci. U.S.A. 111, 16100–5 https://doi.org/10.1073/pnas.1418989111

80 Pini, F., De Nisco, N.J., Ferri, L., Penterman, J., Fioravanti, A., Brilli, M. et al. (2015) Cell cycle control by the master regulator CtrA in Sinorhizobium meliloti. PLoS Genet. 11, e1005232 https://doi.org/10.1371/journal.pgen.1005232

81 Figueroa-Cuilan, W., Daniel, J.J., Howell, M., Sulaiman, A. and Brown, P.J.B. (2016) Mini-Tn7 insertion in an artificial attTn7 site enables depletion of the essential master regulator ctrA in the phytopathogen agrobacterium tumefaciens. Appl. Environ. Microbiol. 82, 5015–5025 https://doi.org/10.1128/AEM.01392-16

82 Francis, N., Ponzín, K., Fioravanti, A., Vassen, V., Willemart, K., Ong, T.A.P. et al., (2017) CtrA controls cell division and outer membrane composition of the pathogen Brucella abortus. Mol. Microbiol. 103, 780–797 https://doi.org/10.1111/mmi.13589

83 Trokes, C.G. and Laub, M.T. (2012) Polarity and cell fate asymmetry in Caulobacter crescentus. Curr. Opin. Microbiol. 15, 744–750 https://doi.org/10.1016/j.mib.2012.10.011