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The architecture and conservation pattern of whole cell control circuitry

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Abstract

The control circuitry that directs and paces *Caulobacter* cell cycle progression involves the entire cell operating as an integrated system. This control circuitry monitors the environment and the internal state of the cell, including the cell topology, as it orchestrates orderly activation of cell cycle subsystems and *Caulobacter's* asymmetric cell division. The proteins of the *Caulobacter* cell cycle control system and its internal organization are co-conserved across many alpha-proteobacteria species, but there are great differences in the regulatory apparatus' functionality and peripheral connectivity to other cellular subsystems from species to species. This pattern is similar to that observed for the "kernels" of the regulatory networks that regulate development of metazoan body plans. The *Caulobacter* cell cycle control system has been exquisitely optimized as a total system for robust operation in the face of internal stochastic noise and environmental uncertainty. When sufficient details accumulate, as for *Caulobacter* cell cycle regulation, the system design has been found to be eminently rational and indeed consistent with good design practices for human-designed asynchronous control systems.

While there are alternatives for characterizing the functional organization of the bacterial cell, we focus here on the control circuitry that drives and coordinates progression of the *Caulobacter crescentus* cell cycle and the developmental and adaptive responses that encapsulate the organism's fitness strategy for its targeted environmental niche (Figure 1). Another common alternative for viewing the cells' functional organization is to focus entirely on the metabolic network. Metabolic network models have been constructed building on pioneering research on *E. coli* and yeast metabolism[1]. The resulting models are captured in sophisticated databases[2] and the ever more complex metabolic cycle charts found in every laboratory. A third alternative, more theoretical and perhaps too abstract[3], is to focus on the general structure of interaction networks within the cell[4]. The cell cycle control system is in a sense the "brain" of the cell where diverse sensor inputs are integrated into the moment to moment coordination of the cell's progress toward its premier objective: growth and cell division.

Initial concepts that led to recognition of genetic regulatory networks was articulated in Jacob and Monod's prescient explanation of regulation of *E. coli's lac* operon[5]. This initial recognition that groups of genes are linked both within operons and in regulatory feedback

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loops has evolved, and we now recognize that cell regulation is a phenomenon that involves the entire cell operating as an integrated system. The control circuitry that directs and paces cell cycle progression in *Caulobacter* is powerful example of such an integrated system.

Caulobacter is a gram negative α-proteobacterium that is adapted to a specific environmental niche: survival in clear lakes and streams where nutrient sources are generally highly dispersed and often low for extended periods. Intricate details of the operations of this tiny organism have emerged from many "reductionist" research approaches, and we now understand that the organism is an integrated, highly organized machine that is rigorously controlled by a relatively simple (compared to the human-engineered electronic devices that surround us) biochemically-based control logic[6; 7; 8; 9].

Figure 1 depicts a hierarchical, signaling and control oriented perspective on the organization of cellular function. The network logic organizes and paces progression of the subsystems that implement the cell cycle. Various sensors monitor the environment and lead the network logic to slow or stop cell cycle progression in the face of environmental challenges. The "housekeeping" metabolic and synthetic functions provide the energy and the structural resources needed for cell growth, chromosome replication, polar development, and cell division. The housekeeping functions are coupled bidirectionally to the cell cycle control system. However, they can adapt, somewhat independently of the cell cycle control logic, to changing composition and levels of the available nutrient sources.

The *Caulobacter* cell cycle control system

Caulobacter divides asymmetrically to produce two different progeny, a swarmer cell and a stalked cell, each with distinct morphological features and regulatory programs. The swarmer cell has a period of motility prior to differentiating into a stalked cell identical to its sibling (Fig. 2A). The cell cycles of both daughter cell types are driven by a cyclical genetic circuit involving five master regulator proteins (Fig. 2B)[10; 11; 12; 13]. Two of these proteins, DnaA and the CtrA response regulator, have central and multi-faceted roles in cell cycle control, and they are subject to multiple levels of regulation. DnaA is a dual function protein that serves as an initiator of chromosome replication and as a transcription factor controlling the expression of approximately 40 genes, including the GcrA global transcription factor that, in turn, initiates transcription of *ctrA*[11]. CtrA, in its phosphorylated form, binds to and silences the origin of replication and directly controls the transcription of about 95 cell cycle-regulated genes. In the swarmer cell, DnaA is present in low amounts and is inactive, while CtrA~P binds to and represses the origin of DNA replication[14; 15]. Clearance of CtrA and accumulation of active DnaA is necessary for DNA replication initiation following the swarmer to stalk (Sw→St) cell transition (Fig 1A).

The exquisite integration of regulatory events that propel the forward progression of the *Caulobacter* cell cycle is well illustrated by the link between chromosome replication and the temporal control of DnaA accumulation. DnaA begins to accumulate at the Sw→St transition to its peak concentration in the early stalked cell, commensurate with replication initiation. The transcription of the *dnaA* gene occurs preferentially when its promoter is in the fully methylated state[16]. Upon initiation of replication of the fully methylated chromosome, passage of the replication fork through the *dnaA* gene located near the origin, yields two hemimethylated copies of *dnaA*, resulting in a drop in *dnaA* transcription[16]. The hemi-methylated DNA is only re-methylated upon production of the CcrM DNA methyltransferase near the time of completion of chromosome replication. Thus, *dnaA* transcription is inhibited after passage of the replication fork, which occurs only once per cell cycle. In addition, DnaA activity is modulated by the HdaA protein[17] via the RIDA mechanism that has been well characterized in *E. coli*.

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After the completion of chromosome segregation and decatenation in the predivisional cell, the cytoplasm is divided by fission of the inner membrane into two compartments inside the contiguous outer membrane, well before completion of cell division (Fig. 2A)[18; 19]. This cytoplasmic compartmentalization event triggers divergent genetic programs in the nascent stalked and swarmer cell compartments, so that a new swarmer cell regulatory control network begins in the swarmer compartment at that moment[18; 20]. The distinct genetic program followed by each nascent sibling after compartmentalization is determined by two component signal transduction proteins and ATP-dependent proteases that are differentially localized to the cell poles at the time of compartmentalization[20; 21; 22; 23; 24]. Dynamic subcellular localization of regulatory proteins, as in regulation of *Caulobacter* cell cycle progression and in *Bacillus* sporulation, is an integral component of bacterial regulatory systems[25]. There are strong parallels between the cellular strategy for asymmetric cell division in bacterial and eukaryotic cells[7].

Figure three shows two sub-networks, the cyclical genetic circuit and the network of dynamically-localized phospho-signaling proteins that create the overall regulatory network that drives cell cycle progression, establishment of asymmetry, and creation of the two distinct daughter cells. These two sub-networks play complementary roles in the operation of the cell cycle. The phospho-signaling network depends on polar localization and the phosphorylation state of the CckA histidine kinase, which in turn determines the activation and stability of CtrA through the two phospho-cascades involving the ChpT phosphotransferase[23] and the CpdR phosphorylation state-dependent ClpXP localization[26]. The phospho-signaling networks act to achieve differential regulation of the presence and phosphorylation state of CtrA in the nascent swarmer and stalked cell compartments and also the morphological differentiation that distinguishes the two daughter cells. Among the functions of the cyclical genetic circuit (Fig 2B) is the tight control of chromosome replication through the opposing actions of DnaA and CtrA to assure (i) that initiation of replication only occurs when the cell state is such that completion of replication is assured and (ii) that there is only one round of replication per cell cycle. Another essential function of the genetic circuit, driving and coordinating cell cycle progression, is accomplished by means of the successive synthesis and removal of the cell cycle master transcriptional regulators, DnaA, GcrA, SciP, and CtrA (Fig. 2A).

A critical element in the control of the sequential expression of large groups of genes as the cell cycle progresses is not only their activation, but also their subsequent repression. CtrA activates many chemotaxis, pili, and flagellar genes in the late predivisional cell, and many of these are then repressed in the daughter swarmer cell, even in the presence of CtrA. How does the cell accomplish this critical modulation of gene expression? To control the timing of CtrA function the cell regulates CtrA activity via phosphorylation[27], control of timing of CtrA accumulation by inhibiting *ctrA* transcription via a methylation ratchet mechanism[16] described above for the temporal control of *dnaA* transcription, and by temporally regulating proteolysis of the CtrA substrate by the ClpXP protease[26; 28; 29]. Yet another level of control has recently been discovered: the transcription of *sciP*, encoding an essential helix-turn-helix transcription factor, is activated when CtrA~P accumulates in the predivisional cell (Fig. 2B)[13; 30]. The SciP protein then accumulates specifically in the daughter swarmer cell where it represses the transcription of at least 58 genes that were activated by CtrA as well as the transcription of *ctrA*. The promoters of each of the downstream genes contain both SciP and CtrA binding motifs[13] so that CtrA, SciP, and each of these downstream co-regulated genes are organized as a type 1 incoherent feedforward motif[31; 32], where CtrA activates transcription of *sciP* and the gene is then repressed after a delay while SciP accumulates[13; 30]. The role of these SciP regulatory pathways within the cell cycle control circuit is to make the level of both CtrA and the downstream co-regulated gene products much less sensitive to the cell growth rate. In *Caulobacter* and other alpha-proteobacteria,

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sciP and its homologs are located on the chromosome in close proximity to flagellar genes, suggesting that the SciP-related feedback pathways are particularly important in multiple species in control of the stoichiometry of flagellar, and probably chemotaxis, proteins.

Conservation and repurposing of the *Caulobacter* cell cycle control system among the α-proteobacteria

All the proteins of *Caulobacter* cell cycle regulatory system are widely co-conserved across the alpha-proteobacteria, but the ultimate function of this regulatory system varies widely in different species. In thirty alpha-proteobacteria genomes available in 2009, the CcrM, CckA, ChpT, CtrA, DnaA, ClpP and ClpX components of the core cell cycle engine are conserved[33]. Of these, 15 have also conserved the genes required for *Caulobacter* asymmetry and polar differentiation, including the polar-localized two component signal transduction proteins DivJ, DivK, PleC, CpdR, and the RcdA localization factor[33]. However, the individual species built around this conserved genetic network have enormous differences in fitness strategies and ecological niches. For example, *Agrobacterium tumefaciens* is a plant pathogen, *Brucella abortus* is an animal pathogen, and *Misorhizobium meliloti* is a soil bacterium that invades, and becomes a symbiont in, plant root nodules that fix nitrogen. The specific coupling between the protein components of the cell cycle control network and the downstream read-out of the circuit differs from species to species. The pattern is that the internal functionality of the network circuitry is conserved, but the coupling at the "edges" of the regulatory apparatus to the proteins controlling specific cellular functions differ widely among species. In *Rhodobacter capsulatus*, the *Caulobacter* CtrA and the CtrA binding site motif are completely conserved, but the set of genes CtrA regulates is quite different[34]. In particular, *R. capsulatus* CtrA is not involved in cell cycle regulation, and it is not essential. The evolutionary transcriptional rewiring of *R. capsulatus* has been achieved primarily by replacing CtrA binding sites with motifs for alternative transcription factors in some downstream genes. The proteins transcriptionally controlled by CtrA in *Brucella abortus* also differ from those in *Caulobacter*, but in *Brucella*, CtrA is both essential and involved in cell cycle regulation[35]. The evolution of CtrA from an ancient common ancestor has involved divergence of its function within different classes of alpha-proteobacteria. In one class, as in *Caulobacter*, CtrA is essential and central to cell cycle regulation, while in others as in *Rhodobacter*, it is not essential and not involved in cell cycle regulation though important for other cellular functions[34]. Throughout, interestingly, the role of CtrA in regulation of motility has been maintained.

In six alpha-proteobacteria, where CtrA plays a central role, the main differences between orthologs of proteins controlling CtrA activity in each of the species were in the N-terminal extremity of regulatory histidine kinases, where sensor domains are usually located[36]. Again this is consistent with maintenance of the CckA-associated regulatory apparatus for CtrA, as in *Caulobacter*, but with divergence of the connectivity of the apparatus to the remainder of the cellular control system.

This mode of conservation and evolution of function of the *Caulobacter* cell cycle control network with peripheral plasticity in connectivity is similar to the evolutionary pattern observed in the “kernels” of gene regulatory networks that regulate metazoan body plan development[37]. These kernels are positioned high in complicated hierarchical regulatory networks, and they are resistant to evolution, but changes at the periphery and in the subordinate subroutines that encode specific functionality, produce speciation[37].

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The overall cell cycle control system design is evolutionarily selected for robustness

Robustness is the ability to maintain performance in the face of perturbations and uncertainty[38]. Analysis of robustness is commonly focused on robust performance of subsystems, e.g. gene expression networks[39; 40; 41], chemotaxis control[42; 43] or circadian rhythms[44; 45].

The effects of the stochasticity in gene expression due to fluctuations in transcription and translation rates has been extensively studied[41; 46], but the aggregate temporal effects of stochastic variations in other processes are also important. For example, replication of each arm of the *Caulobacter* chromosome involves 2 million successive nucleotide addition reactions occurring over about an hour. Fluctuations in availability of substrates and pauses to correct errors or for other reasons lead to significant variance in timing in progression of replication and in the time for completion. In *Caulobacter*, CtrA is cleared from the cell early in the stalked cell phase of the cell cycle. Since CtrA is a master transcriptional regulator that directly controls about 95 genes, including *ftsZ* and the *ftsAQ* operon that encodes components of division apparatus that initiates cytokinesis[47], the timing of CtrA's reintroduction to the cell relative to the progression of chromosome replication is important. As referred to above, a mechanism involving the methylation state of the *ctrA* promoter region provides a ratchet-like mechanism that favors *ctrA* expression only after the replication fork has passed through the *ctrA* gene[16]. A variation of this same mechanism is operative in the timing of *dnaA* transcription[16]. In contrast to *dnaA*, however, the *ctrA* promoter is preferentially activated when it is in the hemi-methylated state, so that initial *ctrA* expression is delayed until the replication fork passes. *ctrA* has a second promoter (P2) that is auto-activated by CtrA~P. The first CtrA molecules expressed from P1 are quickly phosphorylated so that they activate the strong *ctrA* P2 promoters on both daughter chromosomes, and the CtrA level in the cell rises quickly to activate or repress downstream genes. In this way, the timing of synthesis of DnaA and CtrA is coordinated with the variable rate of cell cycle progression. This ratchet mechanism and other mechanisms are coupled to the cyclical genetic circuit that drives progression of the cell cycle (Fig. 2B) to create a closed-loop cell cycle control system that is hierarchical, distributed, asynchronous, and parallel[6]. Detailed analysis of the logical design of this circuit using tools and techniques developed for design of asynchronous electric circuits has shown that the *Caulobacter* cell cycle control circuit has features evolved by selection to assure that timing failures do not occur even in the face of extreme excursions in reaction rates[6].

Genetic circuit design features that prevent timing glitches are not restricted to cell cycle control. The *Caulobacter* polar flagellum is a complex machine that is constructed largely autonomously after CtrA-directed initiation of the transcriptional cascade of flagellar genes (~50 genes) late in the cell cycle. The flagellar genes are organized hierarchically and transcribed sequentially, with the order of assembly of the gene products into the growing flagellum corresponding to the order of their transcription[48]. The CtrA global regulator activates transcription of class II flagellar genes[14; 49]. Transcription of the class IV flagellar genes follows transcription of the class III genes, which follow transcription of the class II genes[50; 51]. Two checkpoints operate to ensure the ordered transcription of the genes encoding flagellum components and both checkpoints are dependent on assembly status as a reference. Only after the assembly of an early flagella component, the MS ring within the inner membrane (class II), is complete, can the transcription of the Class III and IV flagella genes commence[52]. The class III genes encode the components of the flagellar hook and basal body components. Only after the hook and basal body is fully assembled, can expression of the filament flagellin genes (Class IV) proceed[53]. This structure of the transcriptional cascade and the internal assembly checkpoints assure proper ordering of

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construction of components of this machine (e.g., the base, the rotor, the hook, and the filament) independent of timing variations in synthesis of the parts.

In summary, the overall design of the *Caulobacter* cell-cycle circuitry has been optimized by evolutionary selection to operate over a wide range of nutrient conditions and to be resistant to stochastic variations in the time for completion of various subsystem operations or signaling pathways. Robustness of the cell cycle is not just a matter of using particular small regulatory motifs preferentially in the parts of circuitry, but it is an integral property of the overall circuit design. (The overall design of metabolic networks has also been shown to be optimized for robustness and evolvability[54].) The perception that cellular regulation is extraordinarily complex is primarily a consequence of the experimental difficulty of reverse engineering the circuitry and thus the paucity of details on the regulatory structure of most cells. However, when sufficient details accumulate, as for *Caulobacter* cell cycle regulation, the system design is found to be eminently rational and indeed consistent with good design practices for human-designed asynchronous control systems. However, the observed conservation of the internal regulatory circuitry of the *Caulobacter* cell cycle control system across the alpha-proteobacteria, coupled with great plasticity in the regulatory apparatus' functionality and external connectivity, emphasizes that understanding a bacterial species requires a cell wide investigation of the organization of its regulatory logic.

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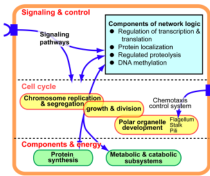

Figure 1. Hierarchical organization of the cell

The cell's signaling and the control subsystem interface interfaces with the environment by means of sensory modules largely located on the cell surface. The genetic network logic responses to signals received from the environment and from internal cell status sensors to adapt the cell to current conditions. A major function of the top-level control is to assure that the operations involved in the cell cycle occur in the proper temporal order. In *Caulobacter* this involves a genetic regulatory circuit with five master regulators organized as a cyclical genetic circuit (Fig. 2B) and an associated phospho-signaling network (Fig. 3). The phospho-signaling network monitors the state of progression of the cell cycle and plays an essential role in accomplishment of asymmetric cell division. The cell cycle control system is tightly integrated with the mechanisms that implement the cell cycle. The control system manages the time and place of the initiation of chromosome replication and cytokinesis as well as the development of polar organelles appropriate to the cell type and stage in the cell cycle. Underlying all these operations are the mechanisms for production of protein and structural components and energy production. The metabolic and catabolic subsystems provide the energy and the molecular raw materials for protein synthesis cell wall construction and other operations of the cell.

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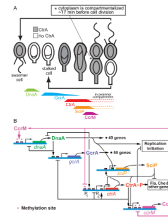

Figure 2.

(A) *Caulobacter* cell cycle. Shading shows temporal and spatial localization pattern of CtrA. Dynamic protein concentrations are indicated below for DnaA, GcrA, CtrA, SciP, and CcrM. Diagrams inside the cell show progression of chromosome replication. (B) Five genes organized in a cyclical genetic circuit provide the core engine that drives the *Caulobacter* cell cycle[10; 16].

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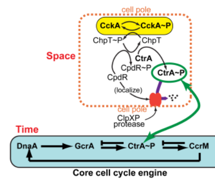

Figure 3.

Polar localized phospho-signaling proteins central to asymmetric cell division are coupled to the core cell cycle engine. The primary function of the cyclical genetic circuit comprising the cell cycle engine is to activate the subsystems that implement the cell cycle in the proper order and to drive the cell cycle forward. The phosphosignaling circuitry senses the cell topology and is tightly coupled to the progression of the engine through control of the stability and phosphorylation state of CtrA. Localization of DivL and CckA leads to phosphorylation of the phospho-signaling protein, ChpT, that phosphorylates CtrA and prevents CtrA proteolysis by simultaneously phosphorylating CpdR. Subsequent interruption of this cascade at the instant of the cell compartmentalization leads to rapid dephosphorylation and proteolysis of CtrA to enable initiation of chromosome replication.