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Summary

Bacterial cell proliferation is highly efficient, both because bacteria grow fast and multiply with a low failure rate. This efficiency is underpinned by the robustness of the cell cycle and its synchronization with cell growth and cytokinesis. Recent advances in bacterial cell biology brought about by single cell physiology in microfluidic chambers suggest a series of simple phenomenological models at the cellular scale, coupling cell size and growth with the cell cycle. We contrast the apparent simplicity of these mechanisms based on the addition of a constant size between cell cycle events (e.g., two consecutive initiation of DNA replication or cell division) with the complexity of the underlying regulatory networks. Beyond the paradigm of cell cycle checkpoints, the coordination between the DNA and division cycles and cell growth is largely mediated by a wealth of other mechanisms. We propose our perspective on these mechanisms, through the prism of the known crosstalk between DNA replication and segregation, cell division and cell growth or size. We argue that the precise knowledge of these molecular mechanisms is critical to integrate the diverse layers of controls at different time and space scales into synthetic and verifiable models.

One-sentence summary

The efficiency of the bacterial cell cycle relies on its robustness, speed and low failure rate. Such qualities are based on a tight coordination between cell cycle events, cytokinesis and cell growth. Here we review the recent advances mediated by single cell microscopy in

regards with the molecular data accumulated over the past century. At the cellular scale simple principles emerge from a complex regulatory network at the molecular level. Bridging our understanding over the two levels of organization will require integrative approaches and promise to unveil new governing principles underlying bacterial cell proliferation.

Bacteria are very efficient at proliferating

The mechanisms underlying the capacity of a cell to self-replicate are primordial for life. The core mechanisms are highly conserved throughout evolution. Bacteria have evolved over billions of years into very efficient and highly integrated proliferative agents. As single cell organisms, they rely on cell duplication to proliferate. The population growth rate is often argued to be the parameter under selective pressure through evolution. The efficiency of proliferation may be just as important. Bacteria typically multiply by binary fission and cannot increase the number of offspring per generation beyond 2. However, they have acquired mechanisms to maintain an average number of descendants close to the maximal value of 2, even under non-optimal growth conditions. Maintaining cell growth during e.g., the repair of DNA damage, allows buffering a temporary delay in the completion of DNA replication, and sustain the same population growth rate as unstressed cells (Darmon et al., 2014). Cultivation of clonal populations of millions of cells in the laboratory highlights the faithfulness and the efficiency of the cell duplication process. An estimation of the death rate of *E. coli* cells under constant growth conditions (microfluidic system) shows that *E. coli* cells are able to proceed through all the cellular duplication process and give birth to two viable daughter cells with more than 99.95% efficiency (1 death in 2000 exponentially growing cells) (Stewart et al., 2005). The bacterial cell cycle is thus highly efficient and robust, requiring an effective coordination of the different cell cycle events, between each other and with cell growth.

Early evidence of coupling between the cell cycle and growth

In bacteria as in every living cells, two main cycles have to be coordinated: the DNA cycle – DNA replication and segregation – and the division cycle. Both cycles must also be coupled to cell growth. These connections are fundamental because they define the time and size scales relevant to a cell. Cytological observations in the first half of the 20th century already pointed to such coupling. Gerhard Piekarski and Bern Stille successfully used Feulgen's staining method to describe the dynamics of what was then called "nucleoid" (term coined by G. Piekarski (Piekarski, 1937)) along the life-cycle of a bacterial cell. The stained "thymonic material", or DNA, in multiple Gram positive and negative bacteria formed nucleus-like

structures, hence the term nucleoid. The nucleoid was shown to be duplicated and segregated to future daughter cells, or spores (Moore, 1941; Piekarski, 1937; Stille, 1937). Somehow, the nucleoid dynamics was coupled to cell growth and division to maintain a stable number of nucleoids per cell.

One of the most influential result in bacterial cell physiology came 2 decades later with the discovery by Schaechter and colleagues that the mass (or size) of individual cells in a growing population is tightly coupled to the growth rate of the population and can be describe by an exponential relationship (Schaechter et al., 1958). This simple law (*i.e.,* an equation describing empirical data) has been a central element in bacterial cell biology over the next 60 years. Importantly, the composition of the medium is irrelevant to predict cell mass, as long as we know which growth rate is achieved. Moreover, in a given growth medium, a change of growth rate by a temperature shift does not alter cell mass. Growth rate is simply a measure of the "pattern of biochemical activities imposed by the medium" that sets the average cell size (Schaechter et al., 1958). This strong correlation between cell mass/size and growth rate reflects *i)* a sharp coupling between growth rate and division rate and *ii)* a predictable change in this coupling according to the growth rate.

Cell size results from the balance between growth and division rates, and is widely used as a proxy for cell mass since cell density does not change significantly along the cell cycle under steady state growth (Martínez-Salas et al., 1981). The tight coupling between cell size and cell growth has therefore strong implications on cell cycle progression. Within the next decade, measurement of the timing of the replication and post-replication periods (C and D periods, respectively, in the bacterial nomenclature) showed that, for fast growth conditions (>0.8-1 doubling/hour), these periods were nearly constant, irrespective of the doubling time, at C = 40min and D = 20min (Cooper and Helmstetter, 1968). The definition of the C and D periods, as well as the B period, extending from cell birth to the initiation of DNA replication, formalized the current bacterial cell cycle model, which is therefore centrally defined by the DNA replication period C. Note that the BCD model is not fully equivalent to the eukaryotic G1/S/G2/M cell cycle model. In bacteria, DNA segregation occurs concomitantly with DNA replication and each locus segregates shortly after its replication (on average 10-20 min (Lesterlin et al., 2012; Wang et al., 2008)) while in eukaryotic cells, DNA synthesis (S phase) is independent from chromosome segregation, which occurs during Mitosis (M phase).

The first prominent model of the coupling between cell growth and the cell cycle.

Combining population average data from Schaechter and colleagues on *Salmonella typhimurium* (Schaechter et al., 1958) and Cooper and Helmstetter on *E. coli* B/r (Cooper

and Helmstetter, 1968), William Donachie proposed that the mass versus growth rate relationship discovered by Schaechter Maaløe and Kjeldgaard (the SMK law) results from initiation of DNA replication at multiples of a critical cell mass (Donachie, 1968). This model assumes that the initiation of DNA replication, occurring at multiples of a fixed cell mass, is the molecular event determining the timing of all other cell cycle events, including cytokinesis.

William Donachie's insight was that the correlation between cell size and growth rate – or maybe more appropriately growth medium richness – finds a quantitative interpretation in the way cells progress through the cell cycle based on the SMK law. He proposed that for fast growth conditions, the population average cell size can be expressed as :

$$S = \frac{S_i}{N_i} 2^{(C+D)/T} = \frac{S_i}{N_i} e^{\lambda(C+D)} \quad (1)$$

where S_i/N_i is the ratio of population averages of size over the number of origin of replication at the time of initiation of DNA replication, C and D are the durations of the C and D periods, T the population doubling time and λ the growth rate. The major assumptions of this model are that the C+D period and the ratio S_i/N_i, later dubbed 'unit cell', are constant across growth conditions. Stated differently: "If cells have a constant C and D, and if the initiation mass is a constant, the mass per cell will be an exponential function when plotted against growth rate" (Cooper, 1997).

This interpretation of the coupling between mass (or size), growth and the cell cycle based on population averages suggests a cell size homeostasis model where a critical size triggers the initiation of DNA replication (sizer model), which is followed by cell division after a constant period C+D. This is the sizer model applied to cell mass at the initiation of DNA replication, instead of cell size at division as it was initially envisioned (Koch and Schaechter, 1962). However, both the SMK growth law and the assumptions of the sizer models have been challenged.

Debated assumptions

The interpretation of the relationship between cell growth and cell cycle encapsulated in Equation (1) does not capture the increase in C and D period durations in slow growth conditions. In fact, the C and D periods have been shown to not be as constant as initially proposed and they tend to increase with the generation time (Allman et al., 1991; Bipatnath et al., 1998; Michelsen et al., 2003; Skarstad et al., 1983; Stokke et al., 2012; Woldringh, 1976). Moreover, the fundamental hypothesis of invariance of the unit cell S_i/N_i has been repeatedly challenged. On the one hand, some studies substantiated the model by showing

a narrower variation of cell size at the onset of DNA replication as compared to cell age (Boye et al., 1996; Koppes et al., 1980), thereby identifying the initiation of DNA replication as the most likely cell cycle event coupled to cell mass or size. The constancy of the initiation mass also received strong experimental support (Herrick et al., 1996; Hill et al., 2012; Si et al., 2017; Wallden et al., 2016) under the assumption that the C+D period is constant (see also (Cooper, 1997, 2006) for a review of the arguments in favor of this model). On the other hand, some studies challenged the invariance of the initiation mass (Churchward et al., 1981; Wold et al., 1994), and other results are difficult to reconcile with the simplest versions of the model of constant mass at initiation. For instance, the number of origin of replication in the cell cannot play a role in size sensing as *E. coli* cells can maintain multiple copies of mini-chromosomes (plasmids with the chromosomal origin of replication as only origin of replication) (Leonard and Helmstetter, 1986; Messer et al., 1978), or even live with multiple copies of the origin of replication on the chromosome (Løbner-Olesen and von Freiesleben, 1996; Wang et al., 2011).

Cell size results from the balance between growth and cell division rates

Zheng and colleagues proposed a more comprehensive interpretation of the empirical relationship between cell growth and the cell cycle. This growth law, unlike the SMK law, also describes the dependence of cell size/mass on growth rate and the duration of C and D periods under slow growth conditions (Zheng et al., 2020). The growth law becomes:

$$S = S_0\lambda(C+D) = S_0\log(2)\,(C+D)/T \qquad (2)$$

where symbols have the same meaning as in Equation (1) and S_0 is a 'fundamental' cell size (or mass) unit without explicit meaning so far. The model from Zheng and colleagues (Equation (2)) is incompatible with the one presented in Equation (1). In this framework, the initiation mass is dependent on growth rate. Despite the small degree of variation – 20-50% (Wold et al., 1994; Zheng et al., 2020) – and the experimental difficulty of measuring the initiation mass, this result is probably the best circumstantial evidence in favor of the model defined in Equation (2). Importantly, while Donachie's model assume constant C+D period, this model is based on the observation that the duration of the C+D period is inversely proportional to the growth rate, with $C+D = 0.3\lambda^{-1} + 0.99$ (Zheng et al., 2020). This expectation is in line with the proportional relationship identified between the C period at slow growth rates and the generation time T (Kubitschek and Newman, 1978), hence a reciprocal relationship between the C period and the growth rate λ. The precise nature of the dependence of the C+D period on the growth rate may require further consensus as others have identified a power law relationship $C+D = 0.3\lambda^{-0.84} + 0.7$ (Wallden et al., 2016). Note that we modified the time unit in the latter expression, from minutes to hours, to match the

former relationship from Zheng and colleagues. The exponent of -0.84 is slightly different from the value of -1 implied in the reciprocal relationship, but both expressions are in broad agreement. The exact value of the exponent change the respective impacts of the growth rate and the C+D period on cell size or mass, but does not alter the model presented in Equation(2). The value close to one suggests a balanced importance of both parameters throughout the broad range of growth conditions tested. Importantly, the relationship between the duration of the C+D period and the growth rate predicts a small change in C+D period (~15% decrease) as the growth rate increases from 1 to 3 doublings/hour. This limited increase, together with the experimental difficulties to measuring C and D periods, may explain why the C+D period has been considered to be invariant for fast growth rates.

Cell biology at the single cell level: new perspectives on the correlation between cell size and growth rate

How cells know and control how big they are? The answer to this cell size homeostasis question is inherently linked to the coordination of cell growth with the cell cycle. This old question remains unanswered, but has been intensely investigated over decades. And although we do not have definitive answers yet, tremendous progress has been made over the last decade. In the following paragraphs we review the different models of cell size homeostasis, how they emerged and why they may not be fully satisfactory.

It was clear from the beginning that the growth laws, derived from population averages, do not necessarily describe the behavior of single cells and do not constrain the list of possible cell size homeostasis mechanisms. Under *steady state conditions*, every cell cycle event will indeed occur on *average* at the same average cell size or age, and will be followed *on average* by other cell cycle events after a constant time period, irrespective of the underlying cell size homeostasis mechanism (see (Boye and Nordström, 2003)). Therefore, it is of paramount importance to take advantage of the noisy nature of cellular physiology to explore how cells respond to the small perturbations they experience at each generation.

Clues from cell size distributions and correlations

The underlying mechanisms coupling cell growth with cell size and the cell cycle induce specific patterns in the distribution or correlation between cell size, growth or cell cycle parameters. In the following paragraphs, we present examples illustrating how statistical features (*i.e.*, (i) the degree of variability of cell size at the initiation of DNA replication, (ii) the correlation between cell lengths at birth and division and (iii) the skewness of the interdivision time distribution) can help falsify or support a specific family of model of cell size homeostasis and coupling between cell size and the cell cycle.

Single cell information was collected in many studies, often by quantitative analyses of light or electronic microscopy images. Identifying the cell cycle stage associated with the smallest cell size variability would strongly suggest that cell size or growth is coordinated with the cell cycle at this specific stage. As a corollary, the relatively high variability of cell size at the time of initiation of DNA replication questioned the sizer model from Cooper and Helmstetter (Cooper and Helmstetter, 1968) and Donachie (Donachie, 1968) (see for a comprehensive discussion on the topic (Koch, 1977)).

Correlations between cell size or age distributions at various cell cycle stages are highly predictive of the mode of size control at work. Simple snapshots of synchronized or even asynchronous populations provide us with cell size distributions. Cell size distributions allow for the estimation of the degree of correlation between the inception and termination of cell cycle periods, respective to cell age or size. For instance, the correlation between cell length at birth and division was estimated to be as high as 0.55 (Koppes et al., 1980). This result is incompatible with the absence of correlation predicted by a sizer model as in this family of models (Cooper and Helmstetter, 1968; Donachie, 1968; Koch and Schaechter, 1962) cell division is triggered irrespectively of cell size at birth.

Quantitative single cell tracking was achieved as early as 1932 (Rahn, 1932), unveiling the variability in generation times of bacterial cells growing in the same growth conditions. Complementary studies confirmed this variability and revealed the continuous nature of the single cell growth rate (*i.e.*, no cell cycle arrest) (Powell, 1956; Schaechter et al., 1962). Voorn and Koppes realized that the different cell size control mechanisms implied very different levels of skewness of the interdivision time distribution and used the experimental estimation of the skewness to falsify the sizer model as proposed by (Cooper and Helmstetter, 1968; Donachie, 1968; Schaechter et al., 1962), and favor an incremental, or adder model where cells would grow on average by the same amount, irrespectively of cell size at birth (Voorn and Koppes, 1997)

More recently, Ariel Amir developed a mathematical framework able to capture the mode of size control in one parameter (Amir, 2014). This versatile model allowed for an objective comparison between modes of control. Both correlations (length at birth versus division and length at birth versus interdivision time), and the skewness of the interdivision time distribution was used to estimate that the most likely range of value for this control parameter correspond to the incremental model.

Direct experimental observation of the incremental/adder phenomenon

Inspection of the relation between cell cycle, cell size and the individual growth rate of the cells using microfluidic devices revealed that cells grow indeed on average by a constant amount before dividing irrespective of their size at birth. As a result, cells shorter than average will tend to be relatively longer, while cells longer than average tend to be relatively shorter and cell size converges toward the average added size between divisions (Campos et al., 2014; Taheri-Araghi et al., 2015). The name of 'adder' designing the incremental model was coined (Taheri-Araghi et al., 2015) to match other generic names for cell size control mechanisms sizer and timer. The adder behavior seem to be conserved and was characterized in a diverse set of organisms (Campos et al., 2014; Deforet et al., 2015; Fievet et al., 2015; Taheri-Araghi et al., 2015; Willis and Huang, 2017), including yeast cells (Soifer et al., 2016), and even in mammalian cells (Cadart et al., 2018). Given the diversity of concerned organisms, this degree of conservation suggests that the adder phenomenon is an emergent cellular property rather than based on a molecular mechanism sensing added size.

The adder phenomenon does not specify a family of conserved mechanisms and could arise from a diversity of control mechanisms. In fact, an adder between division events was rapidly shown to be compatible with a replication centric model reminiscent of the sizer model at the initiation of DNA replication: an adder between replication initiation events define cell size and is coupled to the division cycle through a constant C+D period (Ho and Amir, 2015; Taheri-Araghi, 2015). The added size per division cycle is proportional to the number of origins of replication in the cell. Others proposed that the very same old sizer model could recapitulate the adder behavior between divisions if the C+D period is not constant and depends on growth rate (Wallden et al., 2016). The interdivision adder was shown to be also compatible with a division centric model where cell division is the limiting process through the accumulation of a cell envelope precursor necessary to build the new poles, provided that the rates of cell surface area and cell volume are proportional (Harris and Theriot, 2016).

Ojkic and colleagues observed a remarkable scaling of cell surface area and volume (Ojkic et al., 2019):

$$S = \gamma V^{\frac{2}{3}} \quad (3),$$

where S represents the cell surface area, V cell volume, and γ a constant pre-factor dependent on cell shape. Equation (3) captures the tight control of the cell aspect ratio (cell length over width) (Zaritsky, 2015; Zaritsky and Pritchard, 1973). This scaling relationship is proposed to emerge from cell shape homeostasis at the single cell level. Regardless of the

growth conditions and the achieved growth rate, cells tend to add on average the same added length when normalized by cell width (constant $\Delta L/width$) (Ojkic et al., 2019). This generalization of the adder phenomenon is consistent with the work from Harris and Theriot and with the cell division being the rate limiting process.

From a single limiting process to a concerted control

All the models presented in the previous section were based on a *single* rate limiting process that was size-dependent (*i.e.*, the division or replication process is sensitive to cell size, not age). However, the division rate of *E. coli* cells was shown to be both size and age-dependent: the division rate of a young cell remains lower than an older cell with the same size. These results called for the notion of concerted control (Osella et al., 2014) with two interdependent, but different controlling elements, or triggers.

Exploring cell size homeostasis beyond the interdivision time, by segmenting the cell cycle into multiple periods, further substantiated this notion of concerted control. In *E. coli*, cell size compensation occurs during the B and D, but not C period of the cell cycle under relatively slow growth conditions (no overlapping cycles) (Adiciptaningrum et al., 2015). This study echoes earlier findings showing that restricting DNA replication through thymine limitations resulted in longer C period and shorter D period (Meacock and Pritchard, 1975), and highlights at least 2 important points: (i) The durations of the B and D periods can be modulated by both size and growth rate; (ii) The D period can be modulated, suggesting that cell division can be licensed by an event other than DNA replication initiation.

Concurrent and parallel processes: a matter of correlations and variances

The problems of the coupling between the division and DNA cycles and of the varying C+D (or D only) period called for more complex models than a single adder driving a rate limiting process. Single cell tracking of the replication process revealed that the adder phenomenon could be observed between consecutive replication initiation in *Mycobacterium smegmatis* and *E. coli* (Logsdon et al., 2017; Witz et al., 2019). In both cases, the comparison between observed and simulated variances and correlations between variables led the authors to propose a second adder running from the initiation of DNA replication to the following cell division to couple division and DNA cycles in order to recapitulate the experimental observations (Figure 1A). Both models posit a size dependent trigger for the initiation of DNA replication, while DNA replication triggers cell division through an independent process (see (Koch, 1977) for early support for this family of models).

A fundamentally different perspective came from Lagomarsino's group who proposed that any of the two cycles could be rate limiting for triggering cell division at each division cycle

(Micali et al., 2018a, 2018b) (Figure 1B). In this model, the division and the replication cycles are coupled to cell size via near-adders running between consecutive replication initiation or cell division events. The two cycles are coupled through the duration of the C+D period. In this AND gate, the slowest process between divisome assembly and DNA replication plus segregation period sets division size (Figure 1B). As a result, the added size during the C+D period negatively correlates with cell size at the initiation of DNA replication, but to a milder degree than if DNA replication was never limiting. In addition, the C+D period should depend on growth rate: if the division process sets the division size though an adder, cells growing faster will grow by the added size sooner, thereby shortening the D period whenever the division process is limiting. This property of the model describes the relationship between C+D duration and growth rate measured at the single cell level (Adiciptaningrum et al., 2015; Wallden et al., 2016). Note that this concurrent processes model may be parametrized to avoid any correlation between the size at replication initiation and the added size during the C+D period and match the prediction of the double adder proposed by Witz and colleagues (Witz et al., 2019).

Interestingly, Si and coworkers experimentally altered independently the duration of the division and replication processes, effectively making each of the two processes the limiting process in independent experiments (Si et al., 2019). They observed that altering the adder behavior between division events had no impact on the adder between interdivision events. They also observed that altering the inter-initiation adder did not affect the inter-division adder. These results led the author to conclude that the DNA and division cycles are not coupled to cell size through the same cell cycle event (Figure 1C). In fact, they propose that cell size is set by the replication process (added size proportional to the number of origin of replication), while the control of the division process ensures cell size homeostasis. In light of the concurrent processes model (Micali et al., 2018a), one would expect a strong negative correlation between the added size during the C+D period and the size at the initiation of DNA replication, indicative of cell division being always the limiting process. Note that the double adder model proposed in (Si et al., 2019) is compatible with earlier data (Cooper and Helmstetter, 1968) to the extent that the C+D period is not constant and is predicted to negatively correlate with growth rate. This negative correlation explained by Micali's and Si's models are consistent with the observed relationship between these two parameters at the single cell level (Adiciptaningrum et al., 2015; Wallden et al., 2016) and links the single cell behavior to the population average data described by the model presented in Equation (2). The clear difference of dependence of C and D periods on growth rate (Adiciptaningrum et al., 2015) suggests that the two periods must be separated to establish a meaningful relationship with growth rate.

About correlations and variances

The differences between the models lie in their ability to capture correlations between parameters across the population or over generations. The characterization of cell to cell variability in single-cell studies enable the estimation of these correlations and test or falsify the different models. Early studies focused on specific correlation coefficients or degree of variation to build and choose the model best describing the data, or falsify other models (e.g., the correlation between size at birth and division or the skewness of the interdivision time distribution (Amir, 2014; Voorn and Koppes, 1997)). Logsdon and colleagues used stochastic simulations of different models to estimate 13 parameters (coefficients of correlation and variation). Witz et al., proposed a more systematic method based on the generalized variance to identify the set of most independent variables to identify the model that describes best the data (Witz et al., 2019). In fact, all studies dealing with single cell variability were built on the premise that the minimal set of independent variables providing maximal information about the system would define the natural variables for cell size homeostasis.

Grilli and colleagues proposed a more formal and general approach, based on the linear response theory framework, and built a general and versatile model capturing all considered models through a limited set of parameters (Grilli et al., 2017, 2018). This approach also allows for the exploration of the importance of the coupling parameters and the sensitivity to noise under different parametrization of the general model corresponding to the different modes of control envisioned. This formalism is largely inaccessible to biologists but opens potential fruitful collaboration between theorists and experimentalists.

It remains to be seen how all these models are sensitive to the segmentation of the cell cycle in specific steps. The end of DNA replication, or late DNA segregation steps have the potential to be major cell cycle transitions as mentioned earlier (Adiciptaningrum et al., 2015; Meacock and Pritchard, 1975) (see also (Huls et al., 2018)). How would models evolve if new cell cycle periods were included in the measurements (e.g., the time from DNA replication initiation to the inception of cell constriction (U period))? Would we identify new natural variables? The present models suggest that it might be useful to further investigate the coupling of the late cell cycle periods with cell size and growth.

Experimental considerations

On a technical note, the relative democratization of microfluidic devices led to a substantial increase in the number of cells tracked over generations and provided us with the necessary statistics to evaluate first order correlations and estimate the degree of variability of the different variables (*e.g.*, generation time). The distinction between the different families of

models proposed may require the estimation of higher order statistics and greater numbers of cell cycles (Grilli et al., 2017).

In addition, the field may gain from establishing a common standard experimental set up, from the imaging procedures to the analysis pipeline, to gain in reproducibility. This standard set-up should take into consideration the limitations reported for the PDMS-based fluidic devices (friction forces can limit cell expansion) (Yang et al., 2018). It should also take into account the fact that the distribution of cell ages of cells at any given time during an experiment has consequences on the interpretation we can make of time averaged variables. The uniformity of cell age distribution allows for a better match with ergodicity assumptions (Rochman et al., 2018).

Toward molecular bases of the coupling between growth and the cell cycle

Microfluidic experiments provided us with the dynamics of single cell progressing through the cell cycle (Adiciptaningrum et al., 2015; Logsdon et al., 2017; Santi et al., 2013; Si et al., 2019; Wallden et al., 2016; Witz et al., 2019). The segmentation of inter-division period in B, C and D periods consistently led the authors to decouple the inception of the C period from cell division. The DNA cycle is coupled to size by a size-dependent mechanism triggering the initiation of DNA replication after the addition of a constant size (or mass) per origin of replication. The coupling between the DNA cycle with the division cycle and growth is more problematic as it requires specific correlations such as a negative correlation between single cell growth rate and their C+D period. Note that the concurrent processes model proposed by (Micali et al., 2018a) predicts this correlation instead of assuming it (Wallden et al., 2016). Since the C period was shown to be quite insensitive to size and growth rate (Adiciptaningrum et al., 2015), it suggests a role for late DNA cycle events (end of DNA replication or segregation) in triggering cell division. As we will see below, the existence of a crosstalk between cell division and DNA segregation has been established (Kennedy et al., 2008; Lesterlin et al., 2008), and a few molecular mechanisms may provide a molecular basis to this peculiar role of the D period. We may gain insights by separating the D period in sub-periods in the future, to capture the adaptation of the D period due to the effects linked to DNA segregation requirements from those related to cell constriction.

The requirement for two independent triggers to couple cell size with the cell cycle (2 adders or potentially one adder and a timer in some bacterial species (Santi et al., 2013)), reflects the relative independence of the processes driving the DNA and the division cycles (i.e., the fact that they are not strictly interdependent, see below) (Boye and Nordström, 2003; Nordstrom et al., 1991). To account for the added volume, the accumulation of a single protein (DnaA for DNA replication and FtsZ for cell division) (Ojkic et al., 2019; Si et al.,

2019), or of a cell pole precursor (Harris and Theriot, 2016), is proposed to trigger the commitment to the next cell cycle event in a growth dependent manner. This factor is synthesized at a rate proportional to the cellular growth rate and its activity depends on a given number of molecules rather than its concentration. These hypotheses are analogous to the auto-repressor model presented by Sompayrac and Maaløe (Sompayrac and Maaløe, 1973), one of the simplest circuit recapitulating the adder behavior. These models are strongly supported by the invariance of initiation size in *E. coli* and *B. subtilis* (Sauls et al., 2019; Si et al., 2017). However, as we have seen earlier, this invariance may not hold true, and as we shall see below, the regulatory network of the cell cycle is far more complex than monitoring the level of a single protein or metabolite. Understanding how a "simple" cellular behavior such as the adder phenomenon can emerge from a high molecular complexity constitutes a unique opportunity to close the gap between the observed dynamics of cellular proliferation and its mechanistic bases. A wealth of cell biology and molecular data describing the mechanistic bases of many aspects of cellular proliferation has been reported. In the following, we attempt to review these mechanisms through the lens of the required coordination between DNA and division cycles and cell growth.

The cell cycle pace makers and the checkpoint paradigm

The cell cycle is essentially defined by periods (i.e., temporal phases) corresponding to specific cellular processes, and by the mechanisms controlling the transitions between these periods. One cellular process such as DNA replication, DNA segregation or cytokinesis characterize each period, while molecular switches or cell cycle checkpoints control the transition between periods. However each process defining one period does not intrinsically trigger the following one, and the cell cycle is better viewed as a set of recurrent cellular processes that are synchronized via a heap of molecular mechanisms (Boye and Nordström, 2003; Nordstrom et al., 1991). Supporting this view, cell division can be genetically impaired without affecting DNA replication, segregation and cell growth, leading to the formation of filamentous cells (e.g., the *fts* mutants, for *f*ilamentous *t*hermo*s*ensitive, reviewed in (Donachie and Robinson, 1987)). Conversely, DNA replication and/or segregation can be stopped without temporarily inhibiting cell division. Even the inhibition of DNA segregation does not prevent further rounds of DNA replication in *E. coli* (Wang et al., 2008).

The concept of cell cycle checkpoint encapsulates the idea of control mechanisms enforcing dependency between cell cycle events (e.g., licensing mechanisms), as opposed to intrinsically coupled events . The former type of dependency may be bypassed with loss of function mutations and the possibility to genetically disrupt the normal succession of cell cycle events remains a gold-standard to define what is a cell cycle checkpoint or not

(Hartwell and Weinert, 1989). Checkpoints typically integrate information from surveillance systems monitoring the level of completion of the previous phase and the capacity of the cell to complete the next step (e.g., completion of the assembly of the division machinery, or the spindle in Eukaryotic cells, or the absence of DNA damage).

In the BCD bacterial cell cycle, the B period extends from cell birth to the initiation of DNA replication and the C and D period to the replicative phase and the time elapsed between the end of DNA replication and cell division, respectively. More periods were defined to include observables related to cell division and the dynamics of the nucleoid (*e.g.*, T- or U-period (Helmstetter, 1987)). Two classical checkpoints control the onset of the DNA cycle, and the activation of the division machinery (divisome). These two molecular switches integrate a multiplicity of information and are believed to set the pace of the cell cycle. These two switches crystalized thus far virtually all efforts to develop mechanistic models for cell size homeostasis.

Initiation of DNA replication

The control of the initiation of DNA replication has been extensively studied and is well described in recent and excellent reviews (Katayama et al., 2010, 2017; Kaur et al., 2014; O'Donnell et al., 2013). Briefly, the mechanistic switch in the regulation of the initiation of DNA replication at the unique replication origin, *oriC*, lies on two essential elements, the nucleotide-bound state of the DnaA protein and the topology of the *oriC* DNA region. DnaA binds to multiple sites (DnaA box motifs) within *oriC* in an ordered manner according to the affinity of each motif to DnaA in its ATP or ADP bound forms. The interplay between DnaA and *oriC* culminates in the formation of a structured orisome, promoting the opening of the replication bubble (Leonard et al., 2019).

It is often argued that a critical threshold of the initiator protein (DnaA) necessary for building the orisome must be attained to trigger the initiation of DNA replication. A large collection of studies highlight the preeminence of the control of the amount, not concentration (Boye et al., 1996), of ATP bound DnaA protein in the cell as the central regulatory mechanism (Hansen and Atlung, 2018; Hansen et al., 1991a). DnaA concentration has been reported to be nearly constant over a wide range of growth rate (Hansen et al., 1991b; Herrick et al., 1996). The amount of DnaA protein is therefore proportional to cell mass or size. A critical threshold of DnaA amounts would trigger initiation of DNA replication at a specific cell size, while an integral threshold of DnaA (amount of DnaA produced since the last replication initiation event) would result in an adder phenomenon between initiation events. This latter mechanism implies that all the DnaA protein is the limiting factor and that the DnaA molecules used for the previous initiation event cannot be involved in for the next. However,

increasing the levels of DnaA per cell by up to a 50% did not altered the timing of DNA replication, arguing that the DnaA protein is not limiting in the cell (Flåtten et al., 2015).

Beyond this simplistic view centered on a single protein, it appears that the regulation of the initiation of DNA replication is complex, and that multiple signals and modulators interfere with this minimal view (Riber et al., 2016; Ryan et al., 2004). In fact, beyond protein amount and concentration, the balance between ATP- and ADP-loaded DnaA seems to be the relevant molecular cue that integrates regulatory signals controlling the initiation of DNA replication. The nucleotide-bound state of DnaA (ATP versus ADP) is highly regulated along the cell cycle, through multiple protein-protein, protein-DNA (reviewed in (Katayama et al., 2017)), protein-phospholipids interactions (Sekimizu and Kornberg, 1988; Xia and Dowhan, 1995). Moreover, the topology of the *oriC* DNA region is influenced both locally and globally, for example through the control of the transcription of neighboring genes (*gidA* and *mioC*) (Theisen et al., 1993), and more globally by the structure of the chromosome in the cell (Magnan and Bates, 2015).

Our understanding of the large regulatory network controlling the initiation of DNA replication offers good insights on how a single round of DNA replication occurs for each *oriC* opening event or on the basis of the synchrony between initiation events in a single cell. However, it is still unclear how growth information is integrated in this checkpoint, leading to the coupling between cell mass/size and DNA replication presented in 1968 (Cooper and Helmstetter, 1968; Donachie, 1968). In *B. subtilis*, replacing *oriC* and *dnaA* by a plasmidic origin of replication, in the presence or not of a functional *dnaA* gene, unveiled multiple and independent connections between *oriC*, DnaA and respiration, central carbon metabolism, fatty acid synthesis, phospholipid synthesis, and protein synthesis (Murray and Koh, 2014). Furthermore, in *B. subtilis* and *E. coli*, suppressors of thermosensitive alleles of essential DNA cycle genes have been repeatedly mapped to genes related to the central carbon metabolism, pointing to a possible integration via yet-to-be described mechanisms (Jannière et al., 2007; Maciąg et al., 2011; Maciąg-Dorszyńska et al., 2012; Nouri et al., 2018; Tymecka-Mulik et al., 2017).

In a real tour de force, Camsund and colleagues combined single cell tracking and cell cycle dynamic analysis from video-microscopy in a microfluidic device with CRISPR-Cas9 RNAi (Camsund et al., 2020). This technology allows for the characterization of the alteration in cell cycle dynamics associated with the inhibition of expression of tens of genes in a single experiment. Following a fluorescent reporter for DNA replication, they clustered lineages according to their cell cycle and growth dynamics (e.g., small/large size at birth or at the time of initiation of DNA replication). Focusing on cells with an altered average initiation size, they

identified multiple genes known to be involved in the processes that are directly or indirectly regulating the balance between ATP- and ADP-loaded DnaA. These results highlight the central role of the balance between ADP- and ATP-loaded DnaA in integrating regulatory information, but also illustrate the multiplicity of mechanisms and sources of regulatory information that feed into the initiation of DNA replication. The effects of these multiple regulatory mechanisms are difficult to reconcile with models of replication initiation through the accumulation of DnaA protein up to a threshold. At the population level, the ATP- ADP-loaded DnaA balance may be a good descriptor for the average timing of initiation of DNA replication, but the multiplicity of regulatory signals suggests that the DnaA protein is not the limiting factor for DNA replication a finer description may be required at the single cell level.

The control of the initiation of DNA replication remains an active field of research, which has been influenced by the concept of invariance of the initiation mass. It will be crucial to reconsider the massive amount of molecular data in light of the results from Zheng and colleagues (Zheng et al., 2020).

Divisome assembly and activation of cytokinesis

The field of bacterial cell division has gained tremendous molecular insights on how cell division works and how it is controlled (for reviews, see for example (Du and Lutkenhaus, 2017; Mahone and Goley, 2020)). Our purpose here is to highlight a few elements that are relevant to our understanding of the coordination between cell size, cell growth and the cell cycle. The same models proposed for DnaA and the initiation of DNA replication were proposed to be applicable for the activation of cell constriction through the accumulation of FtsZ protein up to a critical or integral threshold. However, here again, the multiplicity of the regulation layers suggests that these models are too simplistic.

The divisome (*i.e.*, the multiprotein complex mediating cytokinesis) assembles progressively through a cascade of recruitments of proteins. The chain of recruitment ensures the maturation of the divisome so that cell constriction occurs in a timely manner, in concert at all 3 layers of the cell envelope (inner and outer membranes and the peptidoglycan layer) and in between the two copies of the genetic material. The highly conserved tubulin-like protein FtsZ assembles at mid-cell into short polymers that are anchored to the cytoplasmic membrane by ZipA (γ-proteobacteria specific protein) and FtsA proteins. The 'Z-ring' results from the treadmilling dynamics of FtsZ short poymers around the circonference of the cell (Bisson-Filho et al., 2017; Yang et al., 2017). FtsA is also able to polymerize. The polymeric state of FtsA may be a first control point in the assembly and dynamics of the divisome (Pichoff et al., 2012), although the signal remains unclear. FtsA promotes the recruitment of intermediate proteins that connect the division machinery to the cell envelope and the

chromosome, recruit functional modules and/or maintain the synthetic activity of the divisome in an “off” state until activation. Among them, the sub-complex FtsEX was shown to establish a physical link between the cytoplasmic FtsZ polymers at the membrane and the peptidoglycan. FtsEX mediates the recruitment of amidases (through the intermediate activator protein EnvC in *E. coli*) that denude the glycan strains of the peptidoglycan and promote the recruitment of late cell division protein (Pichoff et al., 2019; Sham et al., 2011; Yang et al., 2011). The next protein to be recruited, the essential division protein FtsK is also involved in chromosome segregation (Bigot et al., 2007). FtsK recruits the FtsQBL sub-complex by interacting at least, with FtsQ (Di Lallo et al., 2003; Dubarry et al., 2010). The role of FtsQBL is to hold in an “off”-state the synthetic activity of the FtsWI complex (transglycosylase and transpeptidase, respectively) (Boes et al., 2019). The impact on cell division of FtsK variants unable to fulfill chromosome related functions suggest a defect in the constriction process (Lesterlin et al., 2008; Stouf et al., 2013). These results naturally bring to mind the possibility of a checkpoint for the activation of the divisome dependent on the segregation status of the chromosome (Dubarry et al., 2010; Grainge, 2010).

Once the cell division machinery is activated through an as yet unknown mechanism, FtsZ seems to play the role of conductor by dynamically distributing the active sites of peptidoglycan synthesis around the division site (Bisson-Filho et al., 2017; Yang et al., 2017). The GTPase activity of the FtsZ protein promotes the treadmilling dynamics of short FtsZ filaments around the constriction site. FtsZ proteins may remain static in filaments, but the affinity of components of the peptidoglycan synthesis machinery for FtsZ filaments (FtsI?) may allow FtsZ dynamic structures to displace the sites of peptidoglycan synthesis away from the most constricted regions of the constriction ring. In *E. coli*, the rate of FtsZ treadmilling does not dictate cell constriction rate (Yang et al., 2017), while it does in *B. subtilis* (Bisson-Filho et al., 2017). This difference may be related to the constraints associated with the synthesis a septum in *B. subtilis* instead of two new poles ‘on the fly’ in *E. coli*. Regardless, the short and dynamic nature of FtsZ structures and their distributive function call for a revision of simple integral threshold models based on the accumulation of FtsZ up to a critical added amount. These models have the didactic advantage of relating the adder phenomenon at the cellular level to molecular elements, tut they require that the FtsZ molecules used in one septum should not be used in another one. Otherwise the added amount of FtsZ protein could not be linked to the initiation of cell division. A critical experiment would be to test whether FtsZ proteins can be used at multiple constriction sites, within the same cell or over generations.

The divisome appears more than ever as a dynamic machinery that is assembled in a complex manner and that its activation can depend on external information (e.g., from the

cell envelope, the chromosome). To further illustrate the diversity of the sources of signals integrated by the divisome, let us just mention a few other examples. In a couple of seminal studies, Levin and co-workers identified a molecular link between enzymes related to UDP-glucose and cell division: in *B. subtilis* and *E. coli*, distinct enzymes (UgtP and OpgH, respectively) appear to inhibit FtsZ polymerization under nutrient-rich conditions, thus leading to an increase in cell size (Hill et al., 2013; Weart et al., 2007). In *E. coli*, the production of the cell division protein YmgF (Karimova et al., 2009) depends on the cell-cycle dependent expression of the two genes flanking *oriC*, *gidA* and *mioC* (Lies et al., 2015). The diguanylate cyclase YfiN acts as a cell division inhibitor in response to reductive and cell envelope stresses (Kim and Harshey, 2016). Lastly, mutations in amino-acid metabolism genes suggest that diverting the carbon flux from glycine, threonine and methionine biosynthetic pathways rescues some cell division defects associated with thermosensitive alleles of *ftsK*, *ftsQ* and to a lesser extent, *ftsI* (Vega and Margolin, 2018).

The complexity of the regulation of the divisome are unlikely to be determined by the accumulation of a single component as in the conceptually elegant integral threshold model developed around FtsZ (Ojkic et al., 2019; Si et al., 2019). An assumption of these models require that the amount of newly synthesized FtsZ constitutes the limiting factor for the activation of the division machinery, in spite of the large bundle of regulatory mechanisms that are necessary under a wide range of growth conditions. Making a parallel with the ATP-ADP-loaded DnaA balance, the focalization of FtsZ treadmilling at mid-cell remains the major hub integrating regulatory information for the division process. However FtsZ is clearly not the only relevant molecular player and probably not the rate limiting factor at each division cycle (Coltharp et al., 2016).

Driving the cell cycle via hierarchical transcription and protein degradation networks

A complex regulatory network driving the cell cycle in *C. crescentus* (reviewed in (Lasker et al., 2016)) sets the pace of both DNA replication and cell division. Owing to their short half-life, RNA turnover is most often tuned by *de novo* transcription, while protein turnover is largely controlled by their degradation. The cell cycle transcriptional network of the bacterium *C. crescentus* is a prominent example of cell cycle control through cyclic expression of cell cycle master regulators (Lasker et al., 2016). In parallel, the targeted degradation of the proteins is mediated by adaptor proteins so as to modulate protein amounts along the cell cycle (Joshi and Chien, 2016; Joshi et al., 2015; Lasker et al., 2016).

The control of the oscillations of cell cycle proteins is a knob that allows for the integration nutritional and environmental cues. In *C. crescentus*, DnaA activity drives the timing of the

initiation of DNA replication, while the oscillation in the amounts of the master regulator CtrA regulates the asymmetry of replication in the two asymmetric swarmer and stalked daughter cells (Jonas et al., 2011). A nutritional stress reduces the rate of translation of the DnaA, which quickly reduces the amounts of DnaA protein in the cell because of the constitutive Lon-dependent degradation of DnaA. It also stabilizes the master regulator CtrA which is a negative regulator of DNA replication. As a result, strong nutritional limitations quickly lead to cell cycle arrest in G1 phase (Leslie et al., 2015). In addition, stresses such as exposure to ethanol or high salt concentrations lead to the inactivation of the master regulator CtrA via its de-phosphorylation and subsequent degradation. In the absence of CtrA, DNA replication is positively regulated while cell division is blocked. Stressed cells therefore become filamentous, with multiple copies of their genome. This stress response allow for the maintenance of growth in mass during the stress period in presence of nutrients, which provides a clear growth advantage (Heinrich et al., 2016).

The existence of a gene expression network, with a defined temporal cycling, may be obscured in many bacterial species by the lack of synchronization method necessary to characterize systematically the temporal dynamics of gene expression along the cell cycle . However, such a regulatory network controlling both classical cell cycle checkpoints (initiation of DNA replication and cytokinesis) is difficult to envision in *E. coli* or *B. subtilis* because of their ability to manage overlapping cell cycles.

Organizing centers coordinate a multiplicity of mechanisms to synchronize cell cycle events

The mechanisms interlinking cell cycle events are variable from bacterium to bacterium. However, a common theme emerges where the DNA and division cycles are synchronized by a coherent cross-regulation network (see graphical abstract). At early stages of the DNA cycle, the assembly of the divisome is inhibited, while at late stages DNA segregation and cell division are involved in a crosstalk that precipitates the completion of both processes. It appears that the chromosome architecture at the cellular level plays a pivotal role in this interplay between DNA and division cycles (Haeusser and Levin, 2019).

Getting organized to coordinate cell cycle events

Bacteria are highly organized unicellular organisms (Hoppert and Mayer, 1999). This high degree of cellular organization is vital for the proper coordination of all cellular processes. Every aspect of DNA and division cycles and cell growth takes on an organizational dimension: from chromosomes, secondary replicons and their physical assembly as nucleoids, to protein patterning across the cytoplasm and the cell envelope. Cell cycle

progression is fundamentally based on dynamic spatial patterning of cell cycle regulators, and the cellular organization of the chromosome plays a central role in their localization as much as the regulators define the choreography of the chromosome along the cell cycle. In the model organism *C. crescentus*, the cell cycle has historically been studied through the dynamic patterning of cell cycle regulating proteins, while in *E. coli* and *B. subtilis* the cellular architecture of the chromosome has often taken the center stage. The latter chromosome centric view has the advantage of grouping bacteria in two major classes – chromosome dominantly organized around their (i) origin of replication region or (ii) terminally replicated region – and we will use this point of view to briefly describe how cellular organization promotes the coordination of multiple cellular processes.

Bacterial chromosomes are typically circular DNA molecules defined by three major features – (i) the origin of replication *oriC*, (ii) the recombination site *dif* on the opposite side of *oriC* (halfway through the circular DNA molecular starting from *oriC*), and (iii) *oriC*-*dif* oriented motifs such as KOPS (Bigot et al., 2005) and Chi sites (El Karoui et al., 1999) and base composition biases as the GC skew. Chromosomes are thus bipolarized from *oriC* to *dif*. These two sites are also part of large chromosomal domains (or macrodomains) displaying homogenous subcellular localization and dynamics (Niki et al., 2000; Valens et al., 2004). The *oriC* and *dif* sites together with their surrounding sequences thus occupy specific subcellular locations. These locations may vary between bacteria. Strikingly, each chromosome locus also occupy a typical location in the cell that follows their linear arrangement along the DNA molecule (Espeli et al., 2008; Viollier et al., 2004). Therefore, the bulk of chromosomal DNA, as the specific *oriC* and *dif*-carrying regions, carries both genetic and spatial information. It follows that proteins binding to specific chromosomal loci are spatially patterned in the cell.

The 3D organization of one part of the bacterial chromosome seems to be sufficient to direct the global conformation of the chromosome in the cell. Most bacteria rely on a ParAB*S* system to guide DNA segregation (Livny et al., 2007). Briefly, the ParB protein binds specifically the centromeric sequence(s) *parS* to nucleate the formation of a large complex containing other ParB protein copies bound dynamically and non-specifically to the DNA around *parS* over several kilobases (Breier and Grossman, 2007). The subcellular positioning of this partition complex and/or its bi-polar migration leading to DNA segregation depends on the cognate ParA ATPase protein (Lim et al., 2014; Sanchez et al., 2015). In organisms with a chromosome-borne ParABS system, *parS* sites are most often concentrated near the origin of replication, leading to long range organization of the *oriC* regions as macrodomains and to ordered chromosome positioning and segregation following the *oriC* to *dif* axis. In addition, an interplay between the ParB/*parS* and the SMC (Structural

Maintenance of Chromosomes) complexes resulting in ordered pairing of the two *oriC* to *dif* chromosome halves (or replichores (Blattner et al., 1997)) has been revealed in different organisms (*Caulobacter crescentus*, *Bacillus subtilis, Pseudomonas aeruginosa* (Le et al., 2013; Vallet-Gely and Boccard, 2013; Wang et al., 2017). It thus seems that the *oriC-parS* region contains the required information for global chromosome arrangement in these organisms.

Enterobacteriaceae are a noticeable example where SMC and the chromosomal ParAB*S* systems seem to have been lost through evolution. Although it is still unclear how the organization and bipolar migration the *oriC* regions is achieved, the whole organization of the chromosome seems to have switch from an *ori*-centric to a *dif*-centric mode into which the terminal part of the chromosome (*ter* domain) containing *dif* seems to hold the information necessary for chromosome structuration. These bacteria contain a specific cluster of genes including genes encoding the condensin-like complex MukBEF and the MatP protein (Brezellec, 2006). MatP specifically binds *matS* sites scattered along a large *ter* region (800 kb in *E. coli* K12) (Mercier et al., 2008). It interacts with divisome-borne proteins (see below), keeping *ter* regions at midcell during the D period and with the MukBEF complex, acting in the global cellular positioning of the chromosome (Nolivos et al., 2016). Importantly, recent work identified functional homologs of the *E. coli* components outside *Enterobacteriaceae*, promoting the necessary integration of the late events of chromosome segregation with cytokinesis (Ozaki et al., 2020; Woldemeskel et al., 2017) (see below).

Mechanistic coupling between DNA replication and segregation

A common feature in bacteria is that DNA replication and segregation are two largely overlapping events. These two processes forming the DNA cycle are not intrinsically coupled, but are made interdependent by two types of mechanisms. The initiation of DNA replication integrates the capacity of the cell to perform DNA segregation as a signal (*i.e.*, sensing the presence of a complete partition complex). In addition, the completion of DNA replication can be facilitated by a proper segregation of newly synthesized sister chromatids.

The DNA replication initiation factor DnaA and segregation system ParA/ParB-*parS* (similar to Soj/Spo0J-*parS* in *Bacillus subtilis,* RctA/RctB-*parS* in *Vibrio cholerae*) interlink DNA replication and segregation. Deletion of *spo0J*/*parB* in *B. subtilis* or of *parA1*, *parB1* or *parS1* in *V. cholerae* led to abnormal localizations of *oriC* and a dysregulation of DNA replication (Lee et al., 2003; Yamaichi et al., 2011). Scholefield et al. showed in *B. subtilis* that the partition mechanism seems to regulate DNA replication through the dimerization of Soj (ParA) and its fixation to Spo0J (ParB) : the monomeric form of Soj is able to depolymerize oligomers of DnaA both *in vitro* and *in vivo*, thus imposing a delay in DNA replication

initiation. The physical interaction of Spo0J with Soj limits the regulatory activity of Soj on DnaA (Scholefield et al., 2011, 2012). In *V. cholerae*, the two chromosomes regulate differently their replication. The replication initiation factor RctB of chromosome II is able to bind specifically to *parS*, leading to the titration of RctB and to a delay in the initiation of DNA replication (Gerding et al., 2015). A similar behavior has been described in *C. crescentus*, where DnaA also exhibits the capacity to bind *parS*. However, in *C. crescentus* recent evidence suggested that DnaA might promote DNA segregation (Mera et al., 2014), while in *B. subtilis* and *V. cholerae*, the partition system modulates the activity of the initiator protein. It is tempting to speculate on the role of this regulatory inversion (DnaA acts on ParA). The initiator protein can induce the accumulation of ParA at the new pole as replication is initiated and may therefore drive the assembly of a new polar hub as ParA will help forming a second PopZ matrix at the new pole (Laloux and Jacobs-Wagner, 2013).

DNA replication provokes topological constraints on the DNA with two consequences: accumulation of positive supercoiling ahead of the replication forks provokes their arrest and the transmission of the constraints behind the forks leads to interwoven sister chromatids. The release of these topological constraints by the type II topoisomerases is thus essential for the completion of DNA replication as well as for subsequent segregation of sister chromatids. This release is tightly controlled, suggesting its timing is important. This is the case in the two following examples. In *C. crescentus*, the high-fitness cost (nearly essential) gene *gapR* encodes a nucleoid-associated DNA binding protein with peculiar DNA binding dynamics (i.e., a very low dissociation constant). GapR accumulates in front of the replication forks (Arias-Cartin et al., 2017) and drives the activity of gyrase, the type II topoisomerase with prominent swivel activity in front of the forks (Guo et al., 2018). This release of topological constraints ahead of the forks controlled by GapR is essential for the completion of DNA replication under fast growth conditions. In *E. coli*, the resolution of precatenanes (interwoven nascent chromatids) by TopoIV appears controlled by an orchestrated delay in methylation of newly synthetized DNA (Joshi et al., 2013; Lesterlin et al., 2012; Wang et al., 2008). This is thought to promote a 5 to 8 min (about 400 kb) period of post-replicative cohesion behind progressing forks and shortening this time provokes global segregation defects. Note that in *Enterobacteriaceae* this does not apply to the *ter* region, into which catenane resolution is primarily controlled by MatP and cohesion times are longer (Nolivos et al., 2016).

DNA segregation regulates negatively cell division at early stages and positively at final stages.

Cell division is synchronized with DNA segregation in multiple ways. Yet, the combination of these mechanisms generates a coherent coordination of DNA and division cycles. DNA replication and early DNA segregation stages inhibit cell division, while late segregation steps are positively coordinated with cell constriction.

The dynamics of the cell cycle at the single cell level, in agreement with earlier results, revealed that the D period plays a peculiar role in cell size homeostasis (see above). The mechanisms described below could play a pivotal role in coupling the cell cycle with growth by modulating the duration of the D period. Most mechanisms are involved with focalizing FtsZ treadmilling at mid-cell. One mechanism involving the large essential division protein FtsK stands out and seems to mediate a crosstalk between cell division and DNA segregation.

Cellular Patterning of cell division inhibitors prevents premature cell division

Multiple mechanisms grouped under the term Nucleoid Occlusion (NO) are known to prevent the assembly and activation of the division machinery over unsegregated chromosomes (Figure 2A, B). The DNA binding protein SlmA (*E. coli*) and Noc (*B. subtilis*) exemplify the first NO mechanism discovered, based on the cellular patterning of FtsZ polymerization inhibitors. The global organization and dynamics of the chromosomes in these organisms establish an inhibitor free zone at mid cell as soon as the bulk of DNA is segregated toward each daughter cell and cleared away from mid-cell (Bernhardt and de Boer, 2005; Cho et al., 2011; Tonthat et al., 2011; Wu and Errington, 2004; Wu et al., 2009). Beyond SlmA in *E. coli* and Noc in *B. subtilis*, the Nucleoid Occlusion phenomenon is not fully understood and may be achieved by diverse mechanisms. The controlled activity of the RocS protein in time and space in *Streptococcus pneumoniae* cells seems to accomplish a very similar function, termed Nucleoid Protection (Mercy et al., 2019). The position of the division site is selected very early on (in the preceding division cycle) in *S. pneumoniae* cells. Taking advantage of the spatial information carried by the global structure of the chromosome, RocS interacts with the centromeric region of the chromosome and the protein ParB and localizes at the future division sites at the ¼ and ¾ positions of *S. pneumoniae* cells (Mercy et al., 2019). Thus, RocS localizes to the division site to prevent septum closure over the chromosome instead of being distributed over the chromosome (Figure 2C). The regulatory activity of RocS on the division machinery remains elusive.

The MinC/D/E proteins form a tri-partite system controlling the positioning of the division site through the pole-to-pole oscillation of MinC, which inhibits off-center cell division and FtsZ

polymerization (Adler et al., 1967; Lutkenhaus, 2007) (Figure 2A, B). Interestingly, the Min systems has also been shown in *B. subtilis* (Kloosterman et al., 2016), and proposed in *E. coli* (Di Ventura et al., 2013), to participate to the efficient segregation of the chromosome. In this hypothesis, the pole-to-pole oscillation of the MinD protein, which also binds DNA, may help the segregation of chromosomes by dynamically tethering DNA to the membrane in a biased manner toward the poles. Different proteins of the same family fulfill related functions in different organisms. In *C. crescentus*, the MinC functional homolog protein MipZ interacts with the partitioning protein ParB (Mohl et al., 2001). The tethering of the ParB-*parS* partition complex at the poles drives the formation of the bipolar gradient of MipZ once segregation is completed, allowing cytokinesis at mid-cell (Thanbichler and Shapiro, 2006). In *Myxococcus xanthus*, the Pom system (PomX/Y/Z) performs a similar function by localizing PomZ, a positive regulator of FtsZ localization , at mid cell through oscillation over the nucleoid (Schumacher and Søgaard-Andersen, 2017; Schumacher et al., 2017; Treuner-Lange et al., 2013). In *S. pneumoniae*, division plane and site selection is mediated by the protein MapZ and the segregation of the origins of replication *via* its ParAB*S* system (Fleurie et al., 2014; Raaphorst et al., 2017).

ParB proteins provide yet another link between DNA segregation and the control of cell division. As mentioned in the previous paragraphs, in *S. pneumoniae*, ParB is involved in the division site selection and in the recruitment of the nucleoid protection factor RocS. Moreover, the segregation-related ParB homolog Spo0J from *B. subtilis* has also been shown to act synergistically with the Min system and Nucleoid Occlusion (Haeusser and Levin, 2019; Hajduk et al., 2019; Kloosterman et al., 2016)) to couple DNA segregation with division site selection and divisome dynamics (Haeusser and Levin, 2019; Hajduk et al., 2019; Raaphorst et al., 2017).

In *E. coli*, DNA segregation also depends on cell size. The duration of the D period positively correlates with cell size when cell length increases upon *ftsZ* depletion, or cell width increases upon *mreB* depletion (Zheng et al., 2016). In addition, the segregation of bulk DNA in the cell, a step referred to as nucleoid splitting and corresponding to the apparition of bilobed nucleoids (Bates and Kleckner, 2005), is dependent on cell size: the larger the cell, the earlier the nucleoid splits (Campos et al., 2018). Furthermore, the distribution among bacteria of the mechanisms linking the DNA and division cycle is remarkably dependent on cell morphology. For example, cocci generally do not possess homologs of the Min and Noc/SlmA proteins (Pinho et al., 2013). Without poles, the Min system is unlikely to provide the correct information about the position of the division site.

The early inhibition of cell division by DNA segregation can take many forms and is also cell

shape dependent. Importantly, the relation between cell size and the DNA cycle is not limited to the D period. In some *E. coli* mutants with reduced size (by ~30%) growing at the same rate as the wild type strain, it is not the D period but the C period that is shortened, although the mutated genes (*ftsA* and *pgm*) are unlikely to have a direct effect on the speed up of about 25% of DNA replication (Hill et al., 2012).

Late segregation events promote divisome assembly and activate cell division

Multiple molecular mechanisms are at play to coordinate the late stages of DNA segregation with cell division (Figure 2). The late stages of segregation occur during the D period. They include the resolution of 2 major types of physical links between sister chromosomes. (i) The bidirectional replication of circular DNA molecules results in covalently interlocked chromosomes. This molecular architecture of replicated chromosomes, called catenanes, is resolved by TopoIV. (ii) Chromosome dimers (the joining of the two daughter chromosomes into a single circular DNA molecule), frequently formed by recombinational repair, are resolved by XerCD-mediated site-specific recombination at the *dif* site. Catenanes and dimer resolution appear to be linked in time and space and both controlled by an interplay between MatP and the FtsK protein (El Sayyed et al., 2016; Stouf et al., 2013). Two direct connections between late segregation and cytokinesis have been reported (Mannik and Bailey, 2015), both of them involving global chromosome structure and dynamics.

MatP interacts with ZapB, an abundant small protein that interacts indirectly with FtsZ *via* ZapA (Espeli et al., 2012). ZapA and ZapB are thought to form a large highly dynamic structure localizing both at the *ter* region of the chromosome and at the divisome (Buss et al., 2017). They help focalize FtsZ at mid-cell and induce the co-localization of the *ter* region with the divisome (Bailey et al., 2014). Since MatP is also required for normal cell division, it has been proposed that it helps localizing ZapB and ZapA at mid-cell depending on the positioning of the *ter* region, mediating a positive control on divisome assembly referred to as the *ter*-linkage (Bailey et al., 2014). Note that the *ter* region is devoid of SlmA binding sites (Cho and Bernhardt, 2013), preventing contradictory signals between positive and negative signals mediated by DNA-bound MatP-ZapAB and SlmA, respectivelly. Note that without MatP, cells constrict faster (Coltharp et al., 2016). Taken together, these results suggest that the *ter*-linkage can both promote the assembly of the divisome and slow down its activity once activated, if MatP remains at mid-cell with unsegregated *ter* regions at the time of cell constriction. As mentioned earlier, functional homologs of the components of the *ter*-linkage have been found outside of the *Vibionaceae* and *Enterobacteriaceae* (Ozaki et al., 2020; Woldemeskel et al., 2017) – ZapA was initially identified in *B. subtilis* (Gueiros-Filho and Losick, 2002)). The functional homolog of MatP in *C. crescentus*, ZapT, was found to preferentially bind DNA around the *dif* site and to help localize the *ter* domain of the

chromosome with the division machinery (Ozaki et al., 2020). The same study reports the presence of ZapT homologs in a diverse set of proteobacteria, highlighting the importance of connecting late segregation steps with division, even when the chromosome adopts an *ori*-centric organization.

The FtsK protein is an obvious link between late segregation and cell division since it is physically involved in both processes (Figure 2A, B). This highly conserved protein is large, multifunctional, multi-domain and broadly organized in three spatial domains (Bigot et al., 2004, 2007; Crozat et al., 2014). The N-terminal side of the protein anchors FtsK to the inner membrane specifically at the divisome. In *E. coli*, this domain is essential to cell division (Begg et al., 1995; Dubarry et al., 2010). The highly conserved C-terminal region is organized in three sub-domains: α and β form a ATP-fueled DNA translocation motor (Massey et al., 2006; Sivanathan et al., 2006), while γ controls translocation (Bigot et al., 2005; Ptacin et al., 2006; Sivanathan et al., 2006; Yates et al., 2006). The central portion separating the N- and C-terminal domains is a highly variable linker containing interaction interfaces with proteins of the divisome (Di Lallo et al., 2003; Dubarry et al., 2010). The DNA-translocation activity of FtsK is oriented by recognition of the KOPS DNA motifs, which orientation most preferentially follow the *oriC* to *dif* axis of the chromosome, by the γ subdomain (Bigot et al., 2005; Levy et al., 2005). Although KOPS motifs are present and their orientation biased on the whole chromosome, *E. coli* FtsK most preferentially acts in a restricted region around *dif*, roughly corresponding to the *matS*-containing region (Deghorain et al., 2011). This region displays an ordered segregation pattern, *dif* being segregated last, which depends on MatP and the KOPS-reading activity of FtsK (Stouf et al., 2013). It has been proposed that MatP, by keeping the *ter* region at midcell, creates a substrate for FtsK that in turn removes MatP while translocating (Graham et al., 2010), allowing segregation to complete (Stouf et al., 2013). Translocation stops at *dif* upon interaction of the γ domain with XerCD, which also induces recombination to resolve dimers (Graham et al., 2010). Both FtsK and XerCD positively control the activity of TopoIV (El Sayyed et al., 2016). All events of late chromosome segregation thus appear coupled in time and space: ordered segregation by FtsK and final chromosome untangling, including resolution of dimers and removal of catenanes.

In *E. coli*, late segregation events occur concomitantly with cell constriction or slightly before, *i.e.*, at late steps of divisome assembly (Galli et al., 2017; Kennedy et al., 2008; Steiner and Kuempel, 1998; Stouf et al., 2013). The current model posits that this is due to activation of FtsK-mediated DNA translocation at the time of division. It has been proposed that this activation relies on the hexamerisation of the N-terminal domain in the septum (Bisicchia et al., 2013). This concomitance of events appears under selection pressure during evolution.

Indeed, in bacteria with multiple chromosomes, replication of the individual chromosomes is tuned so that each termination of replication events is coupled with cell division in the same manner (Du et al., 2016; Frage et al., 2016).

Several observations suggest that a reciprocal control, i.e. exerted by FtsK on cell division depending on the progression of segregation, also exists. Indeed, FtsK catalytically mutants unable to translocate (FtsK ATPase mutants) display strong cell shape defects and a lysis phenotype suggesting a defect in the control of cell envelope synthesis (Lesterlin et al., 2008; Stouf et al., 2013). Strains with large chromosome inversion altering the *oriC* to *dif* symmetry show a cell division delay phenotype that turns lethal, involving massive cell lysis, when FtsK translocation is impaired (Lesterlin et al., 2008). In addition, an FtsK variant unable to recognizes KOPS provoke both late *ter* segregation and a delay in cell division (our unpublished results). Taken together, these data support the idea that FtsK activities on the chromosome modulate the divisome synthetic activity. It thus appears that the dynamics of the *ter* region, primarily controlled by MatP, and FtsK are at the core of a positive feedback loop leading to the concomitant closing of the septum with the translocation of the terminal region of the chromosome out of the division site. Interestingly, FtsK is genetically linked to PBP5 (*dacA*) (Begg et al., 1995) and interacts physically with PBP3 (Di Lallo et al., 2003), two enzymes involved in peptidoglycan synthesis at the division septum.

All the mechanisms presented above could potentially play a role in the control of the duration of the D period to achieve the adequate coupling between cell size and the DNA and division cycles. These mechanisms are expected to influence the age-dependence of the division process (Osella et al., 2014), although the tight correlation between nucleoid and cell size (Campos et al., 2018; Gray et al., 2019; Wu et al., 2019) may introduce another size-dependent sensing element to couple the DNA and division cycles to cell size. In any case, the dynamics of the 3D organization of bacterial genomes is proposed to be of paramount importance for the coordination of the cell cycle with cell size and growth (*i.e.*, for cell proliferation).

External signals: modulation and control of cell proliferation

Beyond the metabolic information integrated at both the initiation of DNA replication and divisome maturation, growth and metabolism deeply influence cell cycle progression through multiple molecular mechanisms operating at different time and space scales.

Constant survey by secondary messengers

Small molecules derived from nucleotides are widely distributed secondary messengers involved in cell morphogenesis and cell differentiation (Jenal et al., 2017). Despite high

regulatory potential, those small molecules received little interest so far as potential instantaneous coupling signals between cellular processes driving cell growth and the DNA and division cycles. Some of them have been shown to alter cell cycle progression and their role in the control of cell proliferation may have been underestimated.

Cyclic-di-GMP has been recognized as a major effector involved in cell differentiation in multiple bacterial species (*C. crescentus*, *Pseudomonas aeruginosa*, *Myxococcus xanthus*, *Bdellovibrio bacteriovorus* – reviewed in (Jenal et al., 2017)). It is also involved in the control of cell cycle progression, at the very least in the α–proteobacterium *C. crescentus*, via interactions with ATPase domains of cell cycle regulators (Jenal et al., 2017; Lori et al., 2015)

The Stringent Response mediated by the synthesis of ppGpp (guanosine tetraphosphate) by the RelA and SpoT proteins, 'adapts' the protein and lipid biosynthetic flux to the corresponding biosynthetic capacities. Low synthetic fluxes compared to synthetic capacities signal an impoverishment of growth conditions and the Stringent Response, mediated by an increase in ppGpp level in the cell, induces a global change in gene expression, eventually resulting in cell growth and cell cycle arrest (Ferullo and Lovett, 2008). The ppGpp molecular has been found to inhibit DNA replication by directly interacting with the DNA primase DnaG in *E. coli* and *B. subtilis* (Maciąg et al., 2010; Maciąg-Dorszyńska et al., 2013; Wang et al., 2007). Basal ppGpp levels have also been found to be critical for relationship between growth rate and the DNA cycle. In the absence of ppGpp, the initiation mass and the positive correlation of the rate of initiation of DNA replication with growth rate are changed, and DNA segregation seems impaired (Fernández-Coll et al., 2020). This impact of the cellular concentration of ppGpp may be largely explained by the ppGpp-dependent regulation of the expression of the DNA gyrase. In the absence of ppGpp, at least one of the gyrase genes (*gyrA*) is over-expressed (Fernández-Coll et al., 2020). The overproduction of the DNA gyrase is expected to induce a high degree of negative supercoiling which may inhibit the initiation of DNA replication (see above) as well as DNA segregation.

The secondary messenger Ap4A (di-adenosyl tetraphosphate) has been shown to modulate the timing of cell division in *E. coli* (Nishimura et al., 1997). Oxydative stress has been shown to induce the synthesis of Ap4A (among other di-nucleotidyl polyphosphate molecules) in *Salmonella typhimurium* (Bochner et al., 1984). It is tempting to speculate on the existence of a redox sensing mechanism modulating cell cycle progression in γ-proteobacteria.

In the α-proteobacterium *C. crescentus*, the oxidoreductive state of the cell constitutes a regulatory signal monitored by the proteins KidO and GdhZ and that modulates the cell cycle

progression both at an early stage, by controlling the decatenation of DNA by Topo IV (KidO) (Narayanan et al., 2015), and at later stages, by modulating the FtsZ polymerization at mid-cell (KidO and GdhZ) (Beaufay et al., 2015; Radhakrishnan et al., 2010).

Small molecules allow for controls with short time-scales. Their role in the control of cell growth and the cell cycle is well documented. It is tempting to envision a more general role, beyond the specific examples reported here. Secondary messengers may provide a constant coupling between cellular processes by tuning up or down all physiological processes in response to general signals (e.g., metabolic capacity, oxidative state)

Do finite resource effects enslave cell cycle progression to cell growth?

At the cellular scale, an intrinsic feedback between all cellular processes is imposed by a finite resource effect. This finite resource effect may be yet another possible mechanism at the origin of the proportional coupling between the rates of passage through the D period and the growth rate.

The notion of finite resources is perhaps best understood through the ribosome autocatalytic synthesis paradigm. The number of ribosomes per cell can be optimized by balancing translation capacity with the associated flux of amino-acids (Kafri et al., 2016; Scott and Hwa, 2011; Scott et al., 2014). Too few ribosomes would reduce the cellular growth rate while too many ribosomes would consume too much amino acids for autocatalytic ribosome synthesis and reduce growth rate as well. Resource allocation models recapitulate the correlation observed between the ribosomal content of a cell and growth rate (Scott and Hwa, 2011; Scott et al., 2010). Proteomics data support this proteome allocation model (Hui et al., 2015). Assuming proportionality to enzyme production rates, metabolic fluxes can be used to optimize energy allocation to proteome sectors in a genome-scale metabolic model, thereby interlinking metabolism with the SMK growth law through protein costs (Mori et al., 2019). Reframing the resource allocation as a self-replicating machine explicitly introduces a cell duplication program in the model (Jun et al., 2018; Pugatch, 2015) and will offer a versatile framework to explore, support or disprove cell cycle and growth control models in a more holistic manner, at the cellular scale (see (Groot et al., 2020; Pandey et al., 2020)).

Finite resources effectively impose correlations between all cellular processes, including the ones underlying cell growth and the DNA and division cycles. As a result, all processes display homeostatic behaviors, even if they are not the controlling ones. As an illustration, protein amounts per cell follow the cell size homeostatic behavior, without being the controlling element (Susman et al., 2018). As a consequence, we cannot take the homeostatic behavior of a phenotype (e.g., cell size, DnaA/FtsZ amounts) under steady state conditions as a proof that it is homeostatically controlled (Amir, 2017).

Continuous monitoring of cell physiology and the finite resource effects open the intriguing possibility for an as yet poorly explored type of cell proliferation control. Time and size scales at the cellular level would be set by the continuously adjusted balance of the cell cycle progression and growth rates. This type of control is coherent with the remarkable number of genetic evidences linking the cell cycle and cellular metabolism (Jannière et al., 2007; Maciąg et al., 2011; Maciąg-Dorszyńska et al., 2012; Nouri et al., 2018; Tymecka-Mulik et al., 2017; Vega and Margolin, 2018). The complexity of such a connection between metabolism and cell proliferation may seem a little dizzying. However, similarly to the growth laws describing the dependence of cell size with growth (see Equations (1) and (2)), the connection between cell growth and metabolism can be described with a very low number of variables. Up to a growth rate of about 0.7 doubling per hour, growth rate is linearly related with carbon intake (Groot et al., 2019). These results suggest that a limited number of constrains shape the resource allocation strategy, at least at relatively slow growth rates. It is therefore tempting to ask how resources are allocated to the cell cycle and cell growth and whether this allocation can explain the nearly reciprocal relationship observed between growth and cell cycle progression rates, both at the population and single cell level (Wallden et al., 2016; Zheng et al., 2020).

Conclusions

Maybe not so surprisingly, many molecular mechanisms feed into cell cycle progression and its coordination with cytokinesis and cell growth. These mechanisms can be globally classified and ordered in a logical set of rules underpinning cell proliferation. The cell cycle checkpoints are the pace makers. In *C. crescentus*, a transcriptional regulatory network drives the activity of DnaA and FtsZ. In *E. coli*, it is believed that cell growth dictates the rate of accumulation of these two proteins up to a threshold that triggers the transition toward the next step. Following the rhythm pulsed by the checkpoints, cells commit to DNA replication, segregation and cytokinesis and make use of organizing centers to coordinate them. Polar hubs (*C. crescentus* and *V. cholerae*) or chromosomal domains (*ori* in *B. subtilis* and S. pneumoniae, *ter* in *E. coli*) concentrate key regulators interactions to coordinate the different cell cycle phases. DNA replication and segregation are coupled through DnaA-ParA interactions and the resolution of topological structures. Then, cell division and DNA segregation are coupled through multiple mechanisms (depending on the organism) that, in essence, prevent FtsZ polymerisation at mid-cell at early stages of DNA segregation and activate cell constriction at final stages (Figure 2). Finally, a number of mechanisms signal metabolic information, or other external signal to the different machineries driving the cell cycle and cytokinesis. Among them, cell growth may play a special role. The necessary

resource allocation to the different cellular processes prescribes a growth rate. This growth rate enslaves the rates of metabolite, RNA and protein productions, and may thereby set the same tempo to all cellular processes (provided that the resource allocation strategy is optimal).

At the cellular scale, the tracking of single cells progressing through their cycle strongly suggests that more than once cell cycle event must be coupled to cell size. Typically, at least one cell division event and one DNA cycle event are linked to cell size via an adder between consecutive events to couple the cell cycle with cell growth. The coupling between the division and DNA cycles remains unclear (Figure 1). The molecular mechanisms outlined in this review would rather support a model where the D period may be variable because of a coupling between DNA segregation and cell constriction.

The complexity and the multiplicity of mechanisms involved in the coordination of cell proliferation events preclude any chance of a comprehensive understanding without the help of models. Intriguingly, models derived from cell size homeostasis studies elegantly couple the DNA and division cycles to growth with simple rules. It is an exciting prospect to explore how the complexity of the regulatory network vanishes out at the cellular scale. In this pursuit, the profound knowledge of the molecular mechanisms underpinning cell proliferation is necessary to construct meaningful coarse-grain models of the cell cycle and cell proliferation. Knowing how *E. coli* cells grow, replicate and segregate their DNA or assemble their division machinery allowed for the interrogation of the SMK growth law by introducing independent perturbations in each variable of the model through manipulations of the different molecular mechanisms at play (Si et al., 2017; Zheng et al., 2016). In this respect, it appears that introducing more molecular complexity in cellular scale models of cell size homeostasis will be necessary. Neither DnaA nor FtsZ protein amounts per cell can account for the molecular complexity of the regulation of the initiation of DNA replication and the inception of cell constriction. At this time, more caution should be taken when naming the possible controlling factors responsible for sensing growth and added size and include other factors such as ZapA or FtsK and the related mechanisms (Kleckner et al., 2018; Zheng et al., 2020).

Our conception of the coupling between cell growth and the cell cycle has profoundly changed over the last decade, from a pure size threshold to a couple of independent triggers that depend on added size, rather than cell size/mass/volume itself. A number of different models have been proposed where either the DNA cycle or the division cycle is limiting, or both. The differences between these models reside in their ability to describe all the variability and coupling parameters between cell cycle events that we can measure. We also

anticipate that these differences will also emerge from their ability to describe the behavior of cells progressing through other cell cycle periods such as the inception of cell constriction, nucleoid splitting, or the initiation of synthesis of pre-septal peptidoglycan. Accordingly, it appears to us that a better temporal definition of the bacterial cell cycle, beyond the initiation and termination of DNA replication, will be required. Exploring the role of late steps of the DNA cycle in coupling DNA and division cycles seems to be the next frontier and may lead to unexpected parallels with the coupling of cytokinesis with chromosome segregation during the anaphase and telophase stages of mitosis. Note that the variability of the average initiation mass across growth conditions and the precise relationship between the C and D period durations and growth rate (Zheng et al., 2020) will also constrain models on the explicit coupling between DNA and division cycles with cell growth.

We also anticipate that the exploration of the law describing the dependency of the C+D period duration on growth rate will constitute one of the next goals in the field. The nearly reciprocal relationship between these two variables has strong implications on the coupling between cell growth and the cell cycle. A remarkable feature of this relationship is that it seems to hold at the population level (Michelsen et al., 2003; Zheng et al., 2020) and at the single cell level (Wallden et al., 2016). This tight relationship between the growth rate and the rate of progression through DNA replication and segregation reminds us that, beyond the coupling of one or more cell cycle stages with cell size, the relative control of the rates (growth and cell cycle progression) is likely another crucial element underpinning cell proliferation efficiency. Cell size, growth and the cell cycle may be coupled not only via isolated cell cycle events, but also through a controlled balance of the rates of growth and progression through cell cycle periods.

It appears that a ‘core’ cell cycle and cell division machinery drives the basic cell duplication process. For instance, DnaA, FtsZ as well as ParA and ParB are widely conserved proteins in the bacterial kingdom. This core machinery is then modulated by a substantial number (on the order of 10 in *E. coli*) of mechanisms that may vary among bacterial organisms (Pinho et al., 2013). These differences reflect, at least in part, functional and evolutionary constraints (e.g., cell shape) and that these different organisms have adopted different growth strategies. For instance, *C. crescentus* rely on a proliferation program that would be difficult to adapt to overlapping cycles as seen in *E. coli* or *B. subtilis*. Making sense of the diversity of proliferation strategies among bacterial strains and species will undoubtedly offer new perspectives on the evolutionary relevant mechanisms driving and controlling cell proliferation.

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Conflict of interest

The authors declare no conflict of interest.

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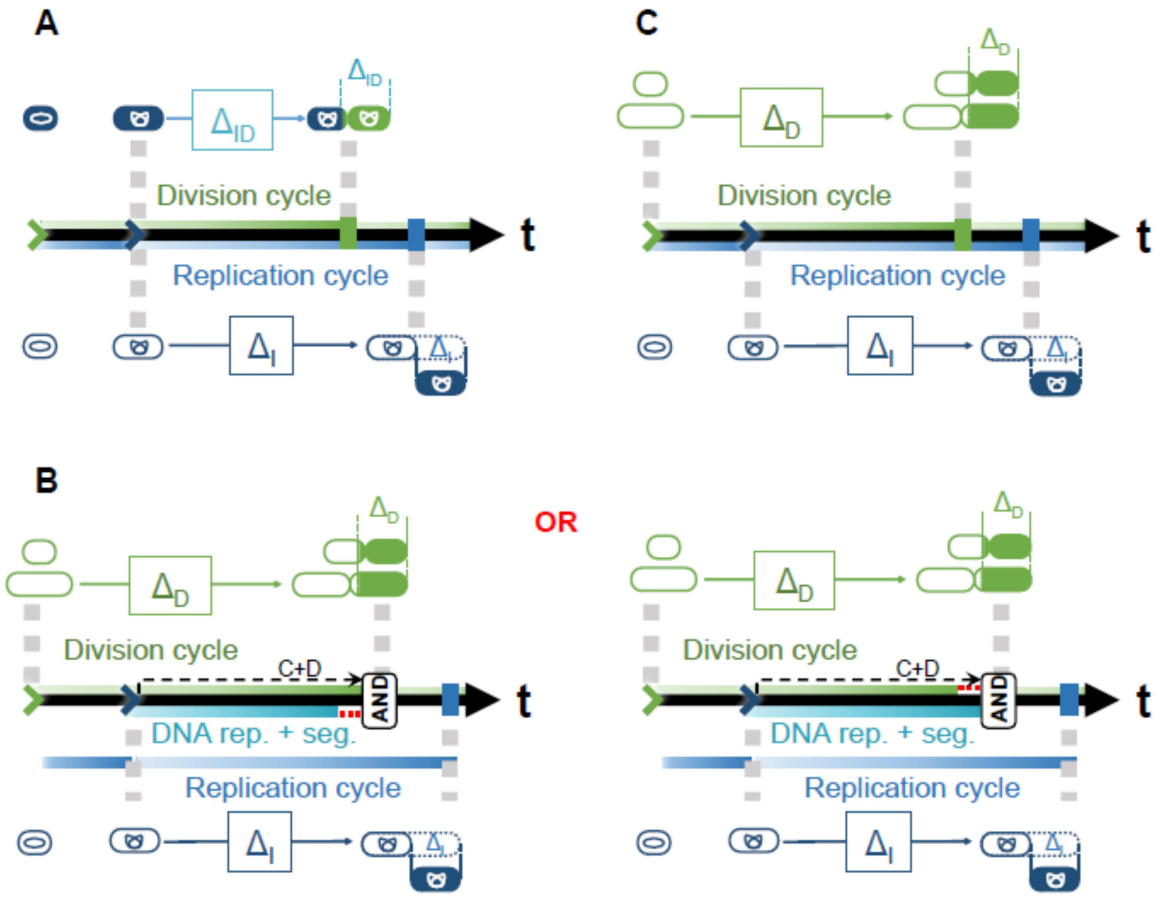

Figure 1. Models of parallel and concurrent processes driving cell size control. To ensure cell size homeostasis, cells must coordinate cell growth with the DNA and division cycles. At least two connections are therefore necessary to coordinate these three processes together. Double adder models seem to be able to recapitulate cell-scale observations by connecting the division (in green) and replication cycles (in blue) to themselves and to each other's. Note that the period covering DNA replication and chromosome segregation can be different from the C+D period has indicated in panel B, where they are represented with the turquoise color and a black dotted arrow. **A.** A replication centric model with one adder is running between two consecutive initiation of DNA replication (Δ_I) and the other interlinking the event of initiation of DNA replication with the following cell division event (Δ_{ID}) (Logsdon et al., 2017; Witz et al., 2019). **B.** The "AND gate" model where adders are running between two consecutive events of i) initiation of DNA replication (Δ_I) and ii) cell division (Δ_D), but in which the competition between the division process and the C+D period sets the timing of division. On the left panel, the division cycle is rate limiting while on the right panel right the

segregation and replication processes are the slowest. Note that by definition the D period does not necessarily ends with the end of DNA segregation, hence the difference between the C+D period and the time required for DNA replication and segregation processes on the right panel (Micali et al., 2018a, 2018b). **C.** Two-adder model where independent adders are running between two consecutive events of *i*) initiation of DNA replication (Δ_I) and *ii*) cell division (Δ_D) (Si et al., 2019).

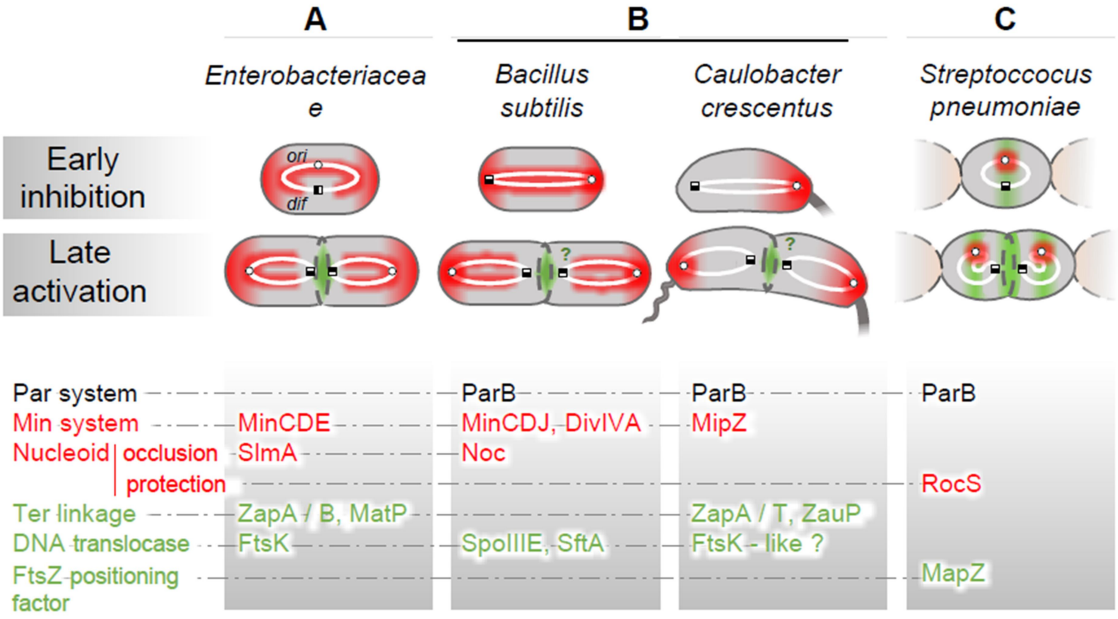

Figure 2. Phases of early inhibition and late activation drive the coordination between cell division and DNA segregation. The choreography of the chromosome along the cell cycle drives the temporal switch between inhibition and by spatially re-organizing key regulatory factors. On panels A., B. and C. are represented the positive (green) or negative (red) effects of key factors on the coordination between DNA segregation and cell division. Chromosomes are represented in white with their origin of replication, *ori* (white circle) and *dif* site (black and white square). The names of the major positive (green) and negative (red) regulators described in the text for each model organism is listed below each schematic. The black color for ParB indicates a dual role. **A.** In *Enterobacteriaceae,* the early inhibition of divisome assembly is mediated by the oscillatory Min system and the nucleoid occlusion factor SlmA. At later stages, SlmA has cleared away from mid-cell and multiple activators drive the localization of the *ter* macrodomain, centered around *dif*, at the division site and coordinate late segregation events with cytokinesis. **B.** In *B. subtilis* and *C. crescentus*, ParB, Noc and Min (ParB and MipZ in *C. crescentus*) organized at the pole(s) and around *ori* cooperate to inhibit Z ring formation at mid-cell. At later stages, the relocation of the two *ori* copies to both poles lifts the early inhibition. The crosstalk between late segregation steps and the division machinery via FtsK homologs (SpoIIIE and SftA in *B. subtilis*) remains to be established. **C.** In *S. pneumoniae*, the Min system is absent and poles do not serve as organizing centers. Instead, the segregation of the two copies of *ori* lifts the inhibition of the division machinery activity at mid cell and drives the assembly new division machineries at ¼ - ¾ positions in coordination with MapZ. The role the DNA translocase FtsK has not been

investigated in this organism. These new division sites are maintained inactive by the presence of RocS (functionally homologous to nucleoid occlusion). In *S. pneumoniae*, ParB plays a dual role by helping localize FtsZ and the nucleoid protection factor RocS. The position of the *dif* site schematized in *S. pneumoniae* cells does not reflect experimental observations and has been postulated for illustration purposes.