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Bacterial cell size: multifactorial and multifaceted

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Abstract

How cells establish, maintain, and modulate size has always been an area of great interest and fascination. Until recently, technical limitations curtailed our ability to understand the molecular basis of bacterial cell size control. In the past decade, advances in microfluidics, imaging, and high throughput single cell analysis, however, have led to renewed interest in the topic and a flurry of work revealing size to be a highly complex trait involving the integration of three core aspects of bacterial physiology: metabolism, growth, and cell cycle progression.

Keywords

cell size; cell cycle; sizer; timer; homeostatic control

Introduction: The importance of being the right size

From a cell's perspective, size matters. A lot. Too small, and you will not have the resources necessary to reproduce or even to carry out basic housekeeping functions. Too large, and your surface area to volume ratio becomes too small for the efficient transport of nutrients in and waste out—starving your cytoplasm of essential molecules and poisoning it with toxic compounds. How bacterial cells modulate size to preserve viability and maximize reproductive efficiency in a constantly changing environment is the focus of this review.

Given the vast diversity of bacterial sizes and morphologies [reviewed in (60, and 110)] it is important to keep in mind that while the principles of cell size control are likely to remain the same, the mechanisms by which different organisms modulate and maintain size are equally likely to be divergent. In other words, while size itself must be regulated, how regulation is conducted at the molecular level is likely to be almost as diverse as bacteria themselves. Note that for the purpose of this review we are focusing on only a few bacterial models in which cell size regulation has been extensively studied. Even among the select rod shaped organisms addressed in this review—*Escherichia coli*, *Bacillus subtilis*, and *Caulobacter crescentus*—the mechanisms governing cell size can diverge substantially.

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Maintaining cell size: not as easy as it looks

Like many things, our understanding of bacterial cell size has been both clarified and distorted by how we measure it. Since the dawn of time—or at least the dawn of microbiology as field—the “party line” has been that bacteria double in size prior to division. This idea has its foundation in the two traditional approaches to studying bacterial growth and physiology: the qualitative assessment of the behavior of a limited number of dividing cells in the microscope and the quantitative assessment of the behavior of bacterial populations in culture. It is easy to see how either of these approaches might have led us astray. Observation of growing *E. coli* gives the distinct impression that cells double in mass before dividing producing two equivalent daughter cells, while measurements of optical density versus colony forming units in an exponentially growing culture suggests that the average (or “typical”) cell exhibits the same behavior.

The advent of state-of-the-art approaches for the analysis of statistically robust numbers of individual bacteria across multiple cell cycles however, has largely invalidated this long-standing paradigm. Analysis of time lapse images of tens of thousands of individual *E. coli*, *B. subtilis* and *C. crescentus* cells indicates that instead of doubling in mass each generation, individual cells cultured under steady state conditions add the same amount of volume independent of their size at birth (3, 16, 52, 92). Cells that are stochastically small at birth—due to variability in growth rate, division timing or both—add the same amount of volume () as those that are stochastically large. Put another way, what is true for the “typical” cell in a batch culture is not true for the individual bacterium. While the addition of a constant amount of volume prior to division does not immediately correct for differences in size, over multiple cell cycles the combination of constant coupled with repeated rounds of medial division results in a normalization of daughter cell size (Figure 1).

Despite having a clearer picture of WHAT cells do to maintain cell size under steady state conditions, HOW the accomplish this objective at the molecular level remains for the most part an open question. Retaining size under steady-state conditions requires that cells meet two criteria: divide at the right place to produce appropriately sized daughter cells and dividing at the right time to ensure the addition of sufficient volume. Below we review what is known about how cells meet each of these criteria.

Division site selection: precision engineering in the absence of a measuring tape

Bacteria set the location of their division site with remarkable precision, suggesting a highly regulated process. Division in both *E. coli* and *B. subtilis* occurs within ~2% of the cell’s geographical middle generating two identical daughter cells (48, 69, 111). Recent work generally supports a model in which establishment of the division site is an active process, while inhibitory factors prevent assembly of the division machinery at aberrant subcellular locations. Although functional equivalents have yet to be detected in *E. coli*, *B. subtilis* or *C. crescentus*, factors promoting assembly of the division machinery have been identified in several organisms including *Streptococcus pneumoniae* and *Myxococcus xanthus*. Positive regulators include PomZ (*M. xanthus*), which is recruited to the future division site where it subsequently promotes assembly of the bacterial tubulin homolog FtsZ and associated components of the cell division machinery proteins (96). Similarly MapZ (also known as

LocZ) is required for localization of FtsZ and other cell division proteins in *Streptococcus pneumoniae* (37). FtsZ is dispensable for hyphal growth in *Streptomyces coelicolor* (67), however it is absolutely required for sporulation, which involves the transformation of long syncytial filaments into individual exospores. In one of the few examples of a situation in which division sites are established apparently *de novo* in filamentous cells, SsgA localizes to internucleoid spaces, recruiting first SsgB and then FtsZ to this position to initiate assembly of the cytokinetic machinery (106). The signals guiding PomZ, MapZ and SsgA localization remain elusive.

While a specific protein has yet to be identified as a beacon for assembly of the cell division machinery in *E. coli* or *C. crescentus*, in *B. subtilis*, there is substantial evidence suggesting that establishment of the medial division site is coupled to the initiation of DNA replication from a medially-positioned origin of replication (48, 72). The cell division machinery assembles off-center, adjacent to the unsegregated nucleoid in conditional mutants unable to initiate new rounds of DNA replication. Medial assembly is restored, however, in cells in which replication initiation is allowed to proceed but DNA synthesis is inhibited. The idea that division site selection—the earliest step in division—is linked to DNA replication is appealing for several reasons, not the least of which it ties division to the initiation of DNA replication, which a growing body of evidence suggests is a size dependent phenomenon, at least in *E. coli* (below).

Although dispensable for medial site selection, division inhibitors are critical to prevent septation at aberrant subcellular positions and to “corral” the division machinery at the nascent septal site to increase division efficiency. Defects in the widely conserved “*min*” proteins or the *B. subtilis* cell division inhibitor *ezrA*, not only lead to aberrant polar assembly of the cell division machinery but also increase cell length (25, 45, 61). Genetic analysis of these mutants suggests that the increase in length may be a consequence of diluting the concentration of key cell division proteins at the nascent septum and/or altering the dynamics of septal PG synthesis (20, 62, 63). While inhibitors such as the Min proteins—which prevent assembly of the division machinery at cell poles—or nucleoid occlusion factors (Noc in *B. subtilis* and SlmA in *E. coli*) which deter division over unsegregated chromosomes, have been suggested as potential mediators of division site selection in *E. coli* and *B. subtilis*, an increasing body of evidence suggests medial division site selection is independent of both sets of proteins (6, 84).

Sizers and timers, oh my

Assuming cells can accurately establish the location of their division site, the next hurdle coordinating division with cell growth and cell cycle progression to maintain size. In this regard there are two potential options: a “timer”—a means of assessing how long cells spend in a given cell cycle phase prior to initiation of the next—or a “sizer”—a means of assessing achievement of a specific size as a requirement for entry into a particular stage of the cell cycle (Figure 2A). In *E. coli*, there is significant data supporting the presence of a “sizer” functioning at the onset of replication initiation while a “timer” appears to be at work at the same step in the *B. subtilis* cell cycle.

Achievement of critical mass as a signal for entry into the cell cycle in *E. coli*

The idea of a sizer linked to the initiation of DNA replication has its roots in the work of William Donachie, who used DNA replication data from *E. coli* (22) and growth rate and Coulter counter cell size data from *Salmonella* (87) to conclude that an idealized average cell initiates DNA replication when it reaches a specific size (28). Specifically, Donachie observed that regardless of growth rate, his idealized cells initiate DNA replication only once they reach a particular mass. Significantly, the constant ratio of cell mass to replication origin at initiation held true not only for slow growing cells undergoing a single round of DNA replication, but also for faster growing cells cultured in nutrient rich medium. This was (and is!) an important distinction as fast growing bacteria including *E. coli* and *B. subtilis* compensate for replication times longer than the interdivision period by initiating new rounds of DNA replication prior to completion of the previous round (22). Although only one round of DNA replication is initiated per division cycle, new rounds are only completed in subsequent generations. At the fastest growth rates, cells can have more than a dozen replication forks proceeding simultaneously. To maintain a constant initiation mass during multifork replication, cells must increase size in response to increases in nutrient availability and growth rate. The mechanisms that allow them to do so are discussed in the next section.

Recent data from both batch culture and single cell experiments support Donachie's proposal that replication initiation is tied to achievement of a specific cell size, at least in *E. coli* (Figure 2A). In batch culture, *E. coli* mutants that are reduced 20–30% in size due to hyperactivation of the cell division machinery (see below and the next section for a discussion of these mutants) delay replication initiation until they reach the same size as their wild type peers (50). Such cells compensate for this delay by increasing the rate of replication fork progression to ensure they have a complete copy of the chromosome prior to division.

Replication initiation serving as a size dependent phenomenon, appears to be at play not only in the world of idealized “average” cells but also in the “real” world of individual cells. Single cell analysis suggests individual cells correct for stochastic reductions in size by delaying replication initiation until they reach the appropriate size (101). Moreover, measurements of tens of thousands of single *E. coli* cells cultured under a wide range of conditions, suggest the size of a “unit cell” containing just enough resources to grow and to initiate a single round of DNA replication is constant. Although the unit cell is insensitive to changes in nutrient availability, RNA or protein synthesis, cell division and a host of other environmental stress, genetic manipulation indicates that unit cell size is inversely related to the frequency of replication initiation (90). Replication initiation is sufficiently robust that it remains coupled to size even in when cell shape is perturbed by altering expression of MreB and FtsZ, cytoskeletal proteins that direct lateral and septal cell wall synthesis (101, 112).

From his initial observations Donachie inferred the existence of a sizer; a positive regulator of replication initiation that accumulates in a manner proportional to cell size, reaching critical levels only when cells attain a specific mass (28). To be effective, this sizer would then be inactivated after replication initiation, such that it would have to accumulate de novo during the next replication cycle. Donachie also acknowledged the alternative possibility that

growth dependent dilution of an inhibitor to sub-critical concentrations might result in a similar outcome.

A good—although not perfect—candidate for a potential sizer controlling replication initiation is the highly conserved AAA+ ATPase, DnaA (55, 56, 66). DnaA-ATP binds cooperatively to the origin of replication (*oriC*) and drives open complex formation, facilitating loading of the replication machinery (31, 32, 34, 73). While *dnaA* is expressed throughout the *E. coli* cell cycle, several mechanisms including titration by DnaA binding sites outside *oriC* and activation of DnaA's intrinsic ATP hydrolysis activity are thought to ensure that the ratio of free DnaA^{ATP} to *oriC* increases in a growth rate dependent manner until it is high enough to support initiation (27, 76).

There are arguments both for and against DnaA serving as a sizer linking replication initiation in *E. coli* to achievement of a specific cell mass. In support of sizer role, depletion of DnaA delays the initiation of DNA replication and leads to dose dependent increases in *E. coli* and *B. subtilis* cell length (66, 75). Similarly, a ~30% increase in [DnaA] is sufficient to correct the replication delay in short *E. coli* mutants (50). At the same time, however, overexpression experiments suggest that DnaA is not limiting for either DNA replication or cell size. A 50% increase in DnaA concentration does not detectably alter the timing of DNA replication initiation and cell size is only reduced ~20% in response to a 5-fold increases in DnaA concentration (36, 66). One possibility is that accumulation of DnaA to critical levels is just one of several mechanisms responsible for coordinating the initiation of DNA replication with cell size. Importantly, depletion of essentially any factor required for either DNA replication or division results in increases in cell size, raising the possibility that a wide range of cell cycle proteins could serve a similar function [e.g. (15, 48, 51)].

Although DnaA may very well serve as a sizer in *E. coli*, this role does not appear to be universally conserved. Like *E. coli*, wild type *B. subtilis* cells cultured under steady state conditions initiate replication at a stereotypical size, however, data from short cells supports the potential for a time-dependent rather than a size-dependent mechanism governing replication initiation in this organism (Figure 2A). In contrast to *E. coli*, small *B. subtilis* cells initiate DNA replication at the same time as their longer wild type counterparts, despite quantitative data indicating that the absolute amount of DnaA is ~30% lower in these cells in accordance with their reduced size (total DnaA concentration is unchanged) (50). This difference is not necessarily surprising, given both the extensive evolutionary distance between *E. coli* and *B. subtilis* and accumulating data suggesting the regulatory networks governing the initiation of DNA replication are not conserved between these two model organisms. For example, *B. subtilis* does not have a homolog of SeqA, which sequesters hemi-methylated origin-proximal DNA immediately following replication initiation (74). Nor does it appear to have an equivalent of HdaA, which stimulates DnaA-ATP hydrolysis in *E. coli* to convert it to its inactive ADP bound form subsequent to initiation. The putative *B. subtilis* HdaA homolog YabA, does not impact either DnaA mediated ATP hydrolysis or DnaA concentration (19, 44). Divergent means of controlling replication initiation highlights not only the value of studying fundamental control mechanisms in different model systems, but also serves as a cautionary tale about the hazards of extrapolating universal principals from a single model organisms.

Cell size as a balance between elongation and division

In addition to replication initiation, an argument can be made for the presence of at least one if not several size control mechanisms operating at division; the literal last chance for cells to correct any defects prior to daughter cell production. The most likely candidate for a cell division dependent size appears to be FtsZ, a highly conserved tubulin-like GTPase that forms a discontinuous ring at the nascent division site [reviewed here (46)]. FtsZ is the first of the conserved cell division proteins to localize to the division site where recent work suggests it forms a treadmilling platform for assembly and function of the cell division (35, 108).

Like DnaA, FtsZ concentration is more or less constant over the course of the cell cycle in *E. coli* and *B. subtilis* but division requires accumulation of FtsZ to a critical concentration. The best illustration of this phenomenon are experiments from the Vicente lab in which a modest, ~20% reduction in FtsZ levels is sufficient to increase *E. coli* cell length by ~2-fold without detectably impacting growth rate (77). As a structural protein that requires accumulation to critical levels to support assembly of the division machinery, it is straightforward to imagine a scenario in which growth dependent accumulation of FtsZ to critical levels serves as an additional means of tying cell cycle progression to achievement of a specific size. At the same time, however, like DnaA, FtsZ overexpression has an extremely modest impact on cell size; 2-fold overexpression reduces square area by only ~10%, suggesting either the presence of an upstream regulator that prevents assembly of the division apparatus too early in the cell cycle or the requirement for a promoter of division with a similar function (40, 104).

Factors that impact the activation of downstream components of the cell division machinery can also impact cell size homeostasis. Assembly of the cell division machinery is a multistep process involving at least two sets of proteins: FtsZ and other “early” proteins which are the first to arrive at the future division site and the “late” proteins which include enzymes required to catalyze synthesis of septal peptidoglycan (22, 37, 25, 68, 79, 102). There is a delay lasting up to 20% of the cell cycle between FtsZ ring formation and assembly of late protein assembly, however there is no apparent delay between late protein assembly and division (1). Gain-of-function mutations in *ftsA*, a highly conserved early protein, *ftsL*, a functionally conserved late protein, and *ftsN*, encoding the last essential cell division protein to arrive at nascent septum in *E. coli*, strongly implicate late protein assembly dynamics in the homeostatic control of cell size (41, 64, 98). Most importantly, hypermorphic mutations in FtsA (FtsA*), FtsL (FtsL*), and overexpression of FtsN all reduce cell size by ~10–25% (41, 64, 98).

Genetic analysis suggests that recruitment of the late proteins is triggered by interactions between FtsA and FtsN which in turn enhance assembly of FtsL and its partner proteins FtsQ and FtsB (FtsL, DivIB, and DivIC in *B. subtilis*) (13, 14, 30). Serving what appears to be a primarily scaffolding function for late division protein recruitment, FtsQLB localize as a group and are required for recruitment of all downstream cell division proteins including FtsN, as well as the putative transglycosylase FtsW, the transpeptidase FtsI, and FtsN, which are all recruited to the division site subsequent to FtsQLB (12, 17, 24, 83). FtsN is unique in that it is both required for late protein recruitment, via its interactions with FtsA, as well as

for its own recruitment, by stimulating recruitment of its late protein interaction partners FtsQLB, FtsW, and FtsI (30, 64). Although it has no apparent enzymatic activity, as the last essential cell division protein to arrive at the nascent septum in *E. coli*, FtsN is often thought of as the “trigger” for cytokinesis. Genetic data suggest that in *E. coli* the ABC-transporter-like complex, FtsEX, plays an important role in the timing of late protein recruitment, mediating the interaction between FtsA and FtsN, driving FtsA into the ‘on’ conformation and stimulating interaction with other components of the division machinery including FtsQLB (30). What triggers FtsEX activity and whether activation it is tied to achievement of a specific size, remains an open question. (FtsN and FtsEX are less widely conserved than other cell division proteins, suggesting organisms like *B. subtilis* possess alternative means of coordinating division with cell growth).

Since division and late protein recruitment are temporally coupled, the reduction in size in the FtsA* and FtsL* hypermorphic mutant strains supports one of two potentially overlapping models. In the first, the late proteins are recruited earlier in the cell cycle, advancing the timing of division relative to elongation and thus reducing cell size. Alternatively, the timing of late protein recruitment may be preserved, but the rate of cytokinesis—which takes ~10–15% of the cell cycle in *E. coli*—is increased. While experimental evidence supporting the former is limited (38, 1, 23), there is significant data supporting the potential for an enhanced rate of cytokinesis contributing to the reduced size of *ftsA** and *ftsL** mutants. Most importantly, in *E. coli* conditional mutations in the FtsI transpeptidase, a late protein dependent on both FtsA and FtsL for localization, significantly reduce the rate of septal wall synthesis and increase *E. coli* cell length, suggesting that septal wall synthesis is rate limiting for division (21, 108). Gain-of-function mutations in periplasmic domains of the FtsI and its putative cognate transglycosylase, FtsW, have also been shown to reduce the size of *C. crescentus* cells (70).

Competition between the divisome and the elongasome for a limited pool of peptidoglycan precursors, may additionally exacerbate the size defect of *E. coli* FtsA* and FtsL* cell division mutants (21, 97, 105, 107). Increasing the rate of cross wall PG synthesis, necessarily sequesters PG precursors from the elongation machinery, further inhibiting elongation. Along these lines, Harris and Theriot have recently proposed a model in which division is triggered by accumulation of a critical amount of excess cell envelope precursors, including presumably the building blocks for PG, to threshold levels (47). Their model is supported by data indicating that partial inhibition of UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase, the enzyme coupling phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine and the first committed step in PG synthesis, leads to elongation (47). However, given our current understanding of late cell division protein recruitment and activation (above), the mechanism by which excess PG and other cell envelope precursors might trigger division is unclear. Importantly, there is evidence for a “hand-off” between the elongation and division machinery mediated by interactions between MreB and FtsZ (33), suggesting an additional integration point for cell size homeostasis.

Is there evidence for a sizer operating at division?

Despite substantial evidence supporting division as a key integration point in the nutrient dependent regulation of cell size, it is debatable whether *E. coli* or *B. subtilis* couple division to achievement of a specific size. In contrast to replication initiation, there is little in the way of direct evidence suggesting the presence of a size dependent “checkpoint” coupling division to cell size in *E. coli* or *B. subtilis*. In fact, analysis of single cell data from *E. coli* suggest that division is particularly sensitive to stochastic forces and there is at best a limited correlation between division and cell length (3, 16, 93). Whether division is a size dependent phenomenon, or even if some steps in division are more sensitive than others to cell growth, thus remains a significant open question.

The nutrient dependent control of cell size

Arguably the most striking finding of the classic 1958 study by Schaechter, Maaløe and Kjeldgaard is the observation that *S. typhimurium* size varies linearly as a function of growth rate (87) (Figure 3A). *S. typhimurium* cultured in nutrient rich medium are up to 3 times the size of those cultured in nutrient poor medium. Significantly, while size varies with growth conditions, the ratio of nucleic acid and protein content to cell size is constant (87). Dubbed “the growth law” the relationship between nutrient availability, cell size and composition applies equally well to both *E. coli* and *B. subtilis* [although intriguingly not *C. crescentus* whose size is largely independent of growth rate and medium (16)] (78, 86, 87). Single cell analysis of *E. coli* and *B. subtilis* indicates that nutrient dependent increases in size are achieved by increasing the amount of volume cells add in each generation (), with being greatest during growth in nutrient rich conditions. Nutrient-dependent increases in size are achieved in *B. subtilis* through increases in cell length, while *E. coli* and *Salmonella* increase both length and width upon transfer to nutrient rich medium.

Although Schaechter and colleagues determined that size varies linearly with growth rate regardless of the medium used to achieve that growth rate, additional experiments indicate that it is nutrient availability that ultimately dictates cell size. Most importantly, curtailing growth by reducing temperature has no impact on cell size as long as other conditions, particularly growth medium, remains constant (87). Moreover, growth rate and size can easily be uncoupled from one another as in the case for diminutive *E. coli ftsA** mutants which exhibit mass doubling times indistinguishable from their wild type relatives and filamentous *B. subtilis ftsZ* mutants that are severely curtailed for growth (5, 50).

Nutrients impact size in multiple ways

Nutrient dependent increases in cell size are almost certainly mediated through changes in both cell cycle progression and biosynthetic capacity (Figure 3B). It is easy to conceptualize how two such disparate processes could impact size if one imagines the cell as a length of pipe being made in a factory. In this analogy the rate at which the pipe is extruded from the fabrication machinery is equivalent to cellular growth rate, itself a product of biosynthetic capacity. In this analogy, the blade responsible for cutting lengths of pipe is the functional analog of the cell division machinery and the length of time between each cut is equivalent to length of the entire cell cycle. If the extrusion rate increases but the cutting rate remains

constant, the length of the pipes will increase. Conversely, increasing the rate of cutting but maintaining the extrusion rate reduces pipe length. Altering the rate of both extrusion and cutting permit the operator to fine-tune the length of pipe to precise specifications. In the same manner, changes in flux through central carbon metabolism should differentially impact cell cycle progression and cell growth, rendering daughter cell size exquisitely sensitive to changes in nutrient availability. While nutrient dependent changes in biosynthetic capacity directly impact growth rate, nutrient dependent changes in cell cycle progression do not.

Nutrient availability and cell cycle progression: nucleotide sugars and moonlighting enzymes

In both *E. coli* and *B. subtilis*, changes in carbon availability are communicated to the division machinery via interactions between a nucleotide sugar, UDP-glucose and moonlighting sugar transferases that together mediate assembly of the tubulin-like cell division protein FtsZ (49, 103). UDP-glucose is synthesized in two steps from glucose-1-phosphate by a highly conserved phosphoglucomutase (*pgm* in *E. coli* and *pgcA* in *B. subtilis*) and a similarly well-conserved pyrophosphorylase (*galU* in *E. coli* and *gtaB* in *B. subtilis*). Its close proximity to glycolysis makes UDP-glucose an excellent intracellular signal for carbon availability that is sensitive to both glycolytic and gluconeogenic carbon sources. In *E. coli*, information about UDP-glucose concentration is conveyed to the division machinery by the glucosyltransferase OpgH, while the same function is served in *B. subtilis* by an unrelated glucosyltransferase, UgtP (49, 103). OpgH's normal function is to transfer the glucose moiety from UDP-glucose to the periplasm where it is converted into a lipid linked osmoprotectant, the osmoregulated periplasmic glucans (OPGs), in a series of subsequent enzymatic reactions (10, 11). UgtP is required for adding two glucose molecules to diacylglycerol, to generate a modified linker for lipoteichoic acid (LTA), an anionic polymer that is a major component of the Gram-positive cell wall (53). Although LTA is essential, the glucose modification of its diacylglycerol anchor is not (58, 103). Defects in OpgH, UgtP, or their cognate phosphoglucomutases and pyrophosphorylases, all reduce *E. coli* and *B. subtilis* cell size by between 15% and 30% (49, 103). For reasons that are unclear, defects in the phosphoglucomutases have a significantly more severe impact on cell size than defects in either of the glucosyltransferases with pyrophosphorylase mutants exhibiting an intermediate phenotype. Intriguingly both OPG and LTA synthesis are the primary generators of diacylglycerol, although the significance of this relationship is not known. Importantly, defects in OpgH and UgtP or their cognate phosphoglucomutase and/or pyrophosphorylase impact size without substantially interfering with growth rate, consistent with serving as part of a signal transduction pathway coupling carbon availability to cell size (49, 103).

Genetic and biochemical data suggest that UDP-glucose binding results in a conformational change that stimulates interaction between OpgH/UgtP and FtsZ. The cytoplasmic N-terminal domain of OpgH interacts directly with FtsZ to reduce the pool of FtsZ subunits available for assembly, and delay maturation of the cytokinetic ring (49). The mechanism by which UgtP inhibits FtsZ assembly is less clear, although affinity studies indicate that UDP-glucose causes concentration dependent changes in UgtP's affinity for itself and for FtsZ

(18). In carbon rich conditions, where UDP-glucose levels are presumably high, UgtP favors interaction with FtsZ, inhibiting assembly of the division machinery and thereby increasing cell size. In carbon poor conditions, apo-UgtP favors interaction with itself, forming large oligomers that sequester it from the cell division machinery.

Other nutrient-dependent signals and effectors

In *C. crescentus*, GdhZ, encoding glutamate dehydrogenase, and KidO, an NADH binding protein, interact directly with FtsZ to coordinate cell division with glutamate availability (9, 82). Like OpgH and UgtP, GdhZ is moonlighting protein that is required for the synthesis of α -ketoglutarate from glutamate and NAD^+ . Defects in *gdhZ* disrupt division, resulting in a wide and heterogeneous range of cell sizes (9). *In vitro* experiments indicate that Gdh interacts directly with FtsZ in a glutamate-dependent manner to inhibit division. KidO is proposed to facilitate Gdh mediated inhibition of FtsZ assembly by interfering with stabilizing interactions between single stranded FtsZ polymers (9). KidO activity is stimulated by the presence of NADH, a byproduct of α -ketoglutarate synthesis by Gdh. Specifically why cells coordinate cell cycle progression with glutamate availability is not immediately obvious, however, glutamate is utilized in multiple biosynthetic pathways, including synthesis of peptidoglycan precursors. Linking FtsZ assembly to glutamate availability via Gdh and KidO thus provides an additional means of coordinating nutrient availability with both cell wall synthesis and cell cycle progression.

Genetic data supports the presence of signal transduction pathways linking nitrogen metabolism and pyruvate production to division, although the molecular mechanisms have yet to be determined. Glutamine metabolism has been linked indirectly to regulation of FtsZ assembly in *E. coli*: defects in the nitrogen stress response pathway suppress a heat sensitive allele of *ftsZ* during growth in nitrogen limiting conditions (80). In *B. subtilis*, defects in pyruvate kinase (*pykA*) suppress the heat-sensitivity of a condition allele of *ftsZ* and lead to the formation of extra FtsZ rings at aberrant polar locations, supporting a role for this metabolic step in mediating FtsZ assembly (71). Although genetic analysis and feeding studies suggest that accumulation of pyruvate, rather than *pykA*, is responsible for promoting FtsZ assembly, the molecular mechanism is unknown (71). The impact of both glutamine and pyruvate on cell size homeostasis under steady state conditions has yet to be determined.

Biosynthetic capacity and cell size

While the lion's share of efforts to illuminate the mechanisms controlling cell size have focused on cycle progression, there is considerable data to suggest a major role for biosynthetic capacity as a primary determinant of size. , the volume of material that *E. coli* cells add each generation, is higher in nutrient rich conditions and lower in carbon poor conditions, consistent with changes in biosynthetic capacity shifting the balance between growth rate and rate cell cycle progression (16, 92).

Moreover, size is negatively correlated with accumulation of the bacterial alarmone guanosine tetraphosphate (ppGpp). ppGpp accumulates in response to nutrient starvation and is a global inhibitor of almost every major biosynthetic pathway including RNA and

protein synthesis, lipid synthesis, and DNA replication (65). Culturing *E. coli* in the presence of serine hydroxamate, a structural analog of L-serine that mimics amino acid starvation or overexpression of the ppGpp synthase, RelA, substantially reduces both growth rate and size (length and width) of cells cultured in nutrient rich medium (88, 94, 95, 109). The same is true in *B. subtilis*, where ppGpp accumulation leads to significant reductions in cell size and in *C. crescentus*, despite its generally limited response to changes in nutrient availability (81, 91).

Given ppGpp's role as a global regulator, it is impossible to know from these experiments if biosynthesis as a whole sets cell size or if particular anabolic pathways are more important than others. Experiments in which a single protein is massively overexpressed could be interpreted to indicate that protein synthesis is a key determinant of cell size (7, 47). However, shunting such a significant portion of cellular resources to synthesis of "useless" protein undoubtedly has pleiotropic effects that adversely impact all aspects of growth and physiology. Conversely, treatment of wild type *E. coli* and *C. crescentus* with the fatty acid synthesis inhibitor cerulenin results in significant reductions in cell size, however it also leads to significant increases in ppGpp concentration (8, 43, 89, 91). While data from *C. crescentus* suggests that ppGpp reduces size independent of fatty acid synthesis, it is unclear if the same is true in *E. coli*. Regardless, determining the molecular mechanisms underlying the relationship between biosynthetic capacity and cell size remains an important topic for future investigations.

Why increase size in response to increases in nutrient availability

While the rationale for maintaining size is obvious, less clear is why cells increase size in response to increases in nutrient availability rather than making more cells. While this questions, like most "why" questions, is likely not easily answered, analysis of *pgm* and *pgcA* mutants suggests that carbon dependent increases in size are important for ensuring there is sufficient space for the increased amount of DNA generated by multifork replication (49, 103). Although it does not appear to lead to deleterious effects in wild type cells cultured in the laboratory, *pgm* and *pgcA* mutants cultured under nutrient rich conditions exhibit a high frequency of FtsZ rings positioned over unsegregated nucleoids, a phenomenon that is unusual in wild type cells. The idea that nutrient-dependent increases in size may be an adaptation to multifork replication is further data indicating that *C. crescentus*, which does not undergo multifork replication, does not exhibit nutrient dependent increases in size (16). [For those interested in the topic, this hypothesis is addressed in depth here (4)]. An alternative hypothesis, is that increases in size provide a crude means of storing carbon and other nutrients in case nutritional fortunes suddenly change. Validation or refutation of either or both of these hypotheses will likely require population based approaches that permit assessment of competitive advantage of wild type and "short" mutant strains. Intriguingly, increases in size are a common feature of *E. coli* cells subjected thousands of repeated rounds of culture and back dilution in the long-term evolution (LTE) study of Lenski and colleagues suggesting there is a fitness advantage to increased size, at least under the conditions of the LTE (59).

Maintaining the proper dimensions

To maintain their distinctive rod shape, organisms like *E. coli* and *B. subtilis* must not only balance elongation and division—as touched on earlier in this review—but also deter dispersive modes of growth that might disrupt their cylindrical aspect. How bacteria accomplish this objective by modulating different modes of cell wall synthesis is the subject of numerous other reviews [e.g. (2)]. We would also refer the reader to a particularly delightful review by Kevin Young published in this journal that touches upon the spectacular diversity of shapes among the bacteria (110). At the same time, it would be remiss not to mention several aspects of cell shape maintenance that directly impact size in the rod shaped organisms that are the focus of this review.

The rod shape of both *E. coli* and *B. subtilis* is dependent in large part on the actin-like protein MreB which together with related Mre proteins forms dynamic platforms for assembly of the transpeptidases and transglycosylases required for lateral cell wall synthesis (15). Defects in the *mre* genes or the addition of the MreB inhibitor A22 quickly result in loss of rod shape and adoption of a rounded morphology (15, 42, 99). MreB moves via a treadmilling mechanism, sampling the surface of the cell in a generally circumferential manner (26, 39). Analysis of MreB in live *E. coli* supports a hypothesis in the orientation of MreB movement is a primary determinant of cell width (97). Whether or not differences in the MreB motion explain the wide variation of *E. coli* width observed across growth conditions has yet to be determined. *E. coli* cells are viable in the absence of MreB activity, relying instead on a more dispersive FtsZ-dependent mode of cell wall synthesis involving both a high molecular weight penicillin binding protein (PBP), PBP1b, and two low molecular weight PBPs, the dd-carboxypeptidases PBP5 and PBP7. PBP5 and PBP7 activity is likely to reduce the pool of available pentapeptides, suggesting a potential preference for tri-peptides in the dispersive mode of FtsZ mediated-cell wall synthesis (99, 100).

The SigmaS (RpoS) dependent transcription factor BolA is another well known mediator of cell size homeostasis and morphology in *E. coli*. Required for the transition from a rod to a shorter more rounded shape upon entry into stationary phase, BolA contributes to cell shape by mediating transcription of *pbp5* and *pbp6*, along with *mreB* (57). *bolA* over-expression results in a spherical morphology *bolA* is also induced by starvation, osmotic shock, and oxidative stress, though the impact of on size in this context has not been well-studied (85). BolA also plays a starring role in the transition between a planktonic lifestyle and a sessile biofilm via repression of genes required for motility and activation of biofilm specific genes (29). While biofilm formation involves significant changes in morphology, the contribution of BolA to cell shape in this context has yet to be investigated.

In an example of the close ties between cell size and other aspects of homeostatic control, recent work implicates the lipid carrier molecule, undecaprenyl phosphate (Und-P encoded by *uppS*), in *E. coli* morphology and size. Und-P is required for transport of cell envelope components including peptidoglycan precursors and enterobacterial common antigen (ECA), a non-essential outer membrane glycolipid, across the plasma membrane. Defects in *wecE*, encoding as sugar amino transferase required for ECA synthesis, but not other enzymes in the ECA lead to substantial increases in both length and width along with somewhat

irregular morphologies (54). Clever genetic analysis determined that the loss of *wecE* leads to accumulation of a so called dead end intermediate, ECA-lipid II depleting the cell of peptidoglycan-lipid II (PG-lipid II) and triggering the cell envelope stress response (54). In support of this idea, *wecE* morphological defects are suppressed not only by defects in upstream components of the ECA pathway, but also increasing by increasing expression of *uppS*, increasing Und-P recycling, and enhancing the interaction between PG and Und-P.

Conclusion: Cell size is a robust, multifaceted phenomenon

Like other biological phenomena, cell size is the product of multiple, complex processes that are each subject to their own homeostatic control mechanisms. As discussed above, tweaking any of these processes—be it central carbon metabolism, cell cycle progression, or cell envelope biogenesis—can dramatically impact cell size. At the same time, the mechanisms underlying cell size homeostasis are incredibly robust with the unit cell remaining insensitive to a host of environmental and physiological stresses (90). While we still have much to learn about the molecular nature of these mechanisms, we are confident that cell size will continue to fascinate investigators from a wide range of biological disciplines for many decades to come.

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Figure 1. The addition of a constant volume of material in each generation ensures bacterial cell size homeostasis under steady state conditions
nstead of doubling in size each generation, bacteria add a constant amount of volume regardless of their size at birth. Cells that are “born” too small for stochastic reasons (red) add the same volume of material as “normal” cells (purple) and cells that are born too large (green). Over several generations, the so-called adder mechanism dampens size variants within a population. refers to the amount of material added in each generation under a specific condition. The volume of increases with nutrient availability. Model based on: (3, 16, 52, 92).

Figure 2. Cell size is mediated in part through changes in cell cycle progression

(A) The initiation of replication is dependent on achievement of a critical size *E. coli* and a critical age in *B. subtilis*. (Left) Wild type *E. coli* and *B. subtilis* cells initiate new rounds of DNA replication upon achievement of a critical mass. (Middle) A “sizer” mechanism governs replication initiation in *E. coli*. Short *E. coli* mutants delay initiation (halo) until they reach the same size as their wild type counterparts. The delay is compensated for by an up to 30% increase in the rate of replication fork elongation, permitting cells to divide “on time” (50, 101). (Right) A “timer” mediates replication initiation in *B. subtilis*. Short *B. subtilis* mutants initiate DNA replication at the same time post-birth as their wild type counterparts (50). (B) A time line of the bacterial cell cycle. Information about size or time can be integrated at any one of three major events: the initiation of DNA replication, assembly of the tubulin-like cell division protein FtsZ and other “early” cell division proteins at the nascent division site, and recruitment of the “late” cell division proteins and the initiation of cytokinesis.

Figure 3. Cell size is a product of biosynthetic capacity and cell cycle progression

(A) Cell size and composition at steady-state increase linearly with increases in nutrient availability and biosynthetic capacity. In phase contrast images on the bottom of the graph, *E. coli* cultured in nutrient rich LB + 0.2% glucose (left) are approximately 3-fold larger than the same strain cultured in nutrient poor AB minimal salts + 0.2% succinate. Importantly, the ratio of cell mass to DNA, RNA, and protein remains constant under all conditions [Modified from Fig. 1 in (87)]. (B) Cell size is a function of cell cycle progression and growth. Nutrient flux through central metabolism releases energy and generates building blocks for biosynthesis. On the left, metabolic products serve as signals activating modulators of cell cycle progression to tune cell size in response to changes in nutrient availability. On the right, biosynthetic capacity dictates growth rate, impacting cell size at division. Changes in mass impact the timing of certain cell cycle events via a “sizer” mechanism (Green arrow). Cell cycle dependent signals impacting growth are inferred in the absence of experimental data (Red arrow).