Disruption of Glycogen Metabolism Alters Cell Size in *Escherichia Coli*

Felix E. Walt

Stellenbosch University

Léo Bürgy

ETH Zurich

Lize Engelbrecht

Stellenbosch University

Rozanne C.M. Adams

Stellenbosch University

Jessica Stadler

Stellenbosch University

Lindi Strydom

Stellenbosch University

Gavin M. George

ETH Zurich

Samuel C. Zeeman

ETH Zurich

Jens Kossmann

Stellenbosch University

James R. Lloyd (✉ lloyd@sun.ac.za)

Stellenbosch University

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Abstract

Background: The availability of nutrients impacts cell size and growth rate in many organisms. Research in *E. coli* has traditionally focused on the influence of exogenous nutrient sources on cell size through their effect on growth and cell cycle progression. Utilising a set of mutants where three genes involved in glycogen degradation - glycogen phosphorylase (*glgP*), glycogen debranching enzyme (*glgX*) and maltodextrin phosphorylase (*malP*) - were disrupted, we examined if degradation of this energy storage compound affects cell size.

Results: It was found that mutations to *malP* increased cell lengths and resulted in substantial heterogeneity of cell size. This was most apparent during exponential growth and the phenotype was unaccompanied by alterations in Z-ring occurrence, cellular FtsZ levels and generation times. $\Delta malP$ mutant cells did, however, show abnormal nuclear staining patterns and accumulate increased DnaA amounts at late growth stages, indicating a potential effect on DNA replication.

Conclusion: This study reveals a link between glycogen metabolism and cell size in *E. coli*. A disrupted *malP* allele is associated with cell size heterogeneity and elongation at all stages of growth, without detectable changes to mass doubling time, FtsZ protein levels or division ring frequency.

Introduction

Robust adaptive survival strategies are employed by bacteria to promote fitness under varying environmental conditions. For example, when nutrients are abundant, bacterial cells demonstrate increases primarily in length, but also in width and growth rate [1]. This permits parental cells to promote vigour of future generations by giving rise to larger daughter cells [2]. Enhanced rates of macromolecular biosynthesis accompany nutrient-dependent size increases to ensure that larger cells are born with more DNA, RNA and protein [3–6]. This positive scaling relationship between cell size and external nutrient-imposed growth rate has historically been termed Schaechter's nutrient growth law [1]. *Escherichia coli* is known to passively correct deviant changes to cell size by adding a constant, growth rate-dependent volume of cellular material (unit cell) before the onset of division, regardless of size at birth [7, 8] which has led to the proposal of what is known as the adder model [9].

Several studies have reported that the volume of the unit cell proposed in the adder model is an invariable cellular mass per replication origin [5, 10, 11] that is proportional to the initiation mass [5, 12]. Regulation of replication initiation ensures that origin firing is triggered only when the cell has grown and accumulated protein factors, such as DnaA, to a threshold required to trigger this event. Whilst initiation mass, or unit cell volume, is important in cell size control it is the regulation of cell division, rather than DNA replication, that ensures cell size homeostasis [13]. Subsequently, division alone allows the cell to correct stochastic deviations in size and relies on the accumulation of factors – such as the division protein FtsZ – to a threshold concentration which allows formation of a Z-ring at midcell. Several other essential divisome proteins are involved in this process which eventually culminates in septation [14].

Cell size, growth and cell cycle progression are known to be influenced by extracellular nutrient abundance. However, the role of energy storage compounds in these processes is less clear. For example, it is unknown if the major carbon store in bacteria, glycogen, influences cell size. This polyglucan consists of short α -1,4 linked glucan chains joined by α -1,6 branchpoints. Three enzymes are known to be involved in its catabolism in the cytosol of *E. coli* and endogenous glycogen turnover has previously been linked to cell division [15]. It is initially degraded by glycogen phosphorylase (GlgP) [16] that removes external glucose moieties, synthesising glucose 1-phosphate (G1P). A glycogen debranching enzyme (GlgX) [17] cleaves the α -1,6 branchpoints and the resulting soluble malto-oligosaccharides (MOS) are further mobilised to G1P by GlgP and maltodextrin phosphorylase (MalP) [18]. G1P can then enter pathways involved in central carbon metabolism (CCM), such as glycolysis.

Recent work within our research group generated an *E. coli* $\Delta malP/\Delta glgP/\Delta glgX$ triple mutant strain capable of glycogen synthesis, but severely impaired in its ability to degrade this polymer [19]. In this study we assessed the effect of impairing glycogen breakdown on *E. coli* cell size. We demonstrate that a lesion in *malP* leads to substantial heterogeneity in cell length, revealing a potential link between glycogen turnover and DNA replication.

Results

Mutants inhibited in glycogen catabolism grow at the same rate, are equally viable, but show reduced culturability. A synthetic, nutrient-rich medium was employed for our analyses and we examined if growth was affected in isogenic mutant strains lacking *malP*, *glgP* and/or *glgX* [19, 20] (Table 1). We determined growth at 450nm as this wavelength permits more accurate optical density measurements in the synthetic medium we used [21, 22], than observations at 600nm would [23]. No significant differences in doubling time were observed between the strains (Additional File 1: Figure S1). We decided to examine the colony forming capacity at three different time points that coincide with early exponential ($OD_{450} \sim 0.05$), onset of stationary ($OD_{450} \sim 4.0$) and late stationary phase ($OD_{450} \sim 7.0$). Although cells from all strains grew at the same rate, all MalP-deficient strains showed significant reductions in bacterial titre during exponential growth when compared to strains with a wild type (WT) *malP* allele (Fig. 1a). By stationary phase, however, none of the mutants differed in culturability from the parental strain. Whilst some differences were detected between the mutant strains during late stationary phase, this was not linked to the presence of a specific mutant allele. The phenomenon of reduced culturability at the same optical density during exponential growth could indicate that bacteria were forming non-viable or anucleate cells, or were growing larger without dividing, so we examined these possibilities in our mutant strains.

Cell viability was assessed by confocal microscopy (Fig. 1b; Additional File 1: Figures S2-S4) and no differences were observed between any of the strains during exponential growth or at the onset of stationary phase. During late stationary phase, however, mutants with a disrupted *malP* allele produced significantly greater proportions of viable cells than both the parental strain and $\Delta glgP/\Delta glgX$.

Furthermore, both $\Delta malP$ and $\Delta malP/\Delta glgP$ formed more viable cells at this stage than either $\Delta glgP$ or $\Delta glgX$, though these single mutants did not differ from either $\Delta malP/\Delta glgX$ or the triple mutant.

All mutant strains produce longer cells than the parental strain during exponential growth. Size parameters of each strain were determined at the same three growth stages using image analysis of bacteria captured by confocal microscopy (Additional File 1: Figures S2-S4). Width was found to be unaltered between strains (Additional File 2: Table S1). Individual lengths were sorted into defined intervals to create weighted histograms (Fig. 2a-c). Mutants with a lesion in *malP* demonstrated greater heterogeneity in length during early exponential growth with histograms revealing a marked shift in the prevalence of longer cells, with mean lengths increased from between 2.60–2.82 μm in strains with a WT *malP* allele to between 3.25–3.65 μm in $\Delta malP$ mutants (Fig. 2a). Variability in cell lengths was greater in $\Delta malP$ mutant strains, with the standard deviation being over 70% of the mean compared to less than 40% for the other strains during early exponential growth (Fig. 2a). Most cells from all strains ranged between 2 to 4 μm in length at this growth phase and the range of cell lengths was also broader here (Fig. 2a) than during stationary phase (Fig. 2b, c).

We classed cells longer than 4 μm as elongated. Using Bayesian inference, we examined the probability that each strain accumulates increased numbers of elongated cells by chance and constructed box plots to visualize differences between strains (Fig. 2d). During early exponential growth, all strains with a mutant *malP* allele were significantly enriched in elongated cells, compared to those strains with a WT *malP* allele (Fig. 2d). This was again observed at the onset of stationary phase, whilst at the final sampling point the $\Delta malP/\Delta glgX$ and $\Delta malP/\Delta glgP/\Delta glgX$ strains formed greater proportions of long cells than all the other strains. The $\Delta glgP$, $\Delta glgX$ and $\Delta glgP/\Delta glgX$ mutants accumulated more long cells than the WT during exponential growth (Fig. 2d). The $\Delta glgP/\Delta glgX$ double mutant differed significantly from the WT, $\Delta glgP$ and $\Delta glgX$ strains in its production of elongated cells at the onset of stationary phase, but this was not observed at the final time point.

It is well-known that disruptions to cell division may lead to cell elongation and filamentation in *E. coli* [24], so we examined whether the elongation observed in $\Delta malP$ mutant strains could be attributed to changes in FtsZ protein levels or Z-ring frequency.

Z-ring formation and FtsZ amounts are unimpaired in the mutant strains. Membrane-staining revealed invaginating septa at midcell in normal and elongated $\Delta malP$ mutant cells, along with invaginating septa forming at the $\frac{1}{4}$ or $\frac{3}{4}$ cell position in abnormally long bacteria (Additional File 1: Figure S2b). To assess whether Z-ring assembly was perturbed in the mutant strains, the percentage of cells with division rings during early exponential growth was determined by FtsZ immunolabelling and confocal microscopy. We found that Z-ring formation was unchanged in the different strains, with $\sim 70\%$ of cells from all strains showing visible Z-rings during this growth phase (Table 2). Whereas Z-rings were limited to the middle of the cell in bacteria with a WT *malP* allele (Fig. 3a), $\Delta malP$ mutants formed division rings at midcell in both normal and long cells and at the $\frac{1}{4}$ and/or $\frac{3}{4}$ cell position in elongated cells and filaments. Diffuse fluorescent signal was observed in areas between segregated nucleoids in elongated $\Delta malP$ mutants,

suggesting that FtsZ accumulated at putative division sites, but that Z-ring assembly was inhibited. Immunoblots showed that there were no differences in FtsZ levels between any of the strains at any of the time points examined (Fig. 3b; Additional File 1: Figure S5).

Since glycogen over-accumulation has been associated with cell size dysregulation upon entry into stationary phase in *E. coli csrA* mutants [25], we investigated whether our mutant strains accumulated excessive amounts of this biopolymer.

Glycogen and protein aggregates are observed in $\Delta glgX$ mutants. Glycogen amounts increased in all the strains upon entry into stationary phase, with cells in late stationary phase containing the most (Additional File 1: Figure S6). The triple mutant accumulated significantly more glycogen than the WT both at the onset ($p = 0.026$) and in late ($p = 0.038$) stationary phase, although these were the only significant differences detected at any time point.

We visualized glycogen aggregates in the strains as well as in a glycogen synthase ($\Delta glgA$) mutant [20]. As expected [26], no staining was observed in the $\Delta glgA$ strain (Fig. 4; Additional File 1: Figures S7, S8) demonstrating the specificity of the stain. We observed substantial glycogen bodies in all strains with a defective *glgX* allele during early exponential growth (Fig. 4). Whilst polar glycogen aggregates were observed in these strains, glycogen bodies were also noted in nucleoid-free regions and, infrequently, at midcell within elongated bacteria where both *malP* and *glgX* are mutated ($\Delta malP/\Delta glgX$ and $\Delta malP/\Delta glgP/\Delta glgX$). Polar polyglucan bodies appeared in all strains upon entry into stationary phase, with some diffuse signal detected along the cell peripheries (Additional File 1: Figure S7). Glycogen bodies were particularly pronounced in strains with a defective *glgX* allele and were again detected at inter-nucleoid zones within elongated $\Delta malP/\Delta glgX$ and $\Delta malP/\Delta glgP/\Delta glgX$ cells. This phenotype was less apparent in all strains cultured to late stationary phase (Additional File 1: Figure S8) as staining was restricted to the cell poles and periphery.

Protein aggregates, stained by the amine-reactive viability dye, were observed only weakly in *GlgX*-deficient strains during early exponential growth, and their spatial arrangement at midcell and polar regions coincides with that of glycogen bodies (Additional File 1: Figure S2). At later growth stages, protein granules of various sizes were observed mainly at the poles of all strains (Additional File 1: Figures S3-S4).

Strains lacking MalP produce multinucleate filamentous cells, but there is no alteration in numbers of anucleate cells. During early exponential growth, DNA staining demonstrated no obvious phenotypic differences between $\Delta glgP$, $\Delta glgX$ and $\Delta glgP/\Delta glgX$ mutants and the WT (Figs. 3a, 4; Additional File 1: Figure S2a). Genetic material within elongated cells from $\Delta malP$ mutants appeared as unsegregated masses or as regularly spaced nucleoids along the length of the cell (Figs. 3a, 4; Additional File 1: Figure S2b). During stationary phase, MalP-deficient mutants again demonstrated aberrant nucleoid partitioning, characterized by clumps of DNA (Additional File 1: Figures S3b, S4b, S7, S8), whilst this was not observed in the other strains (Additional File 1: Figures S3a, S4a, S7, S8).

We determined the percentage of anucleate cells formed by the strains over time and observed no significant differences in anucleate cell production between any of the strains during either exponential growth, or at the onset of stationary phase (Additional File 1: Figure S9). In late stationary phase, the $\Delta glgX$ mutant produced significantly fewer anucleate cells than the WT ($p = 0.035$) but no other differences were detected between groups.

DNA concentrations are unaltered in the mutant strains, while stationary phase DnaA levels are elevated in $\Delta malP$ mutants. DNA concentrations relative to the WT (Table 3) were assessed during early exponential growth. The DNA and protein contents of individual cells were measured by flow cytometry. Total protein of an *E. coli* cell may be used as a measure of its mass [27], however, the substantial variability in our data meant that we were unable to resolve any differences between strains.

Immunoblots revealed no marked differences in DnaA levels between any of the strains during exponential growth (Fig. 5; Additional File 1: Figure S10), though levels of this initiator protein were elevated in $\Delta malP$ mutants upon entry into stationary phase with these differences becoming greater by late stationary phase.

Discussion

One of the major carbon stores in bacteria is glycogen and its breakdown is linked to CCM. Here, we examined if glycogen degradation influences bacterial cell size using a set of mutants impaired in their ability to degrade this polyglucan. The three enzymes examined in this study catalyse different steps of glycogen degradation. GlgP and GlgX release respectively G1P or MOS from the glycogen polymer, while MalP acts downstream of GlgX, degrading the maltodextrins that it releases to G1P.

During early exponential growth, all strains accumulated cells which were longer than those found during stationary phase (Fig. 2a-c), and strains with a mutant *malP* allele were significantly enriched in elongated cells (Fig. 2d). No detectable differences in viability (Fig. 1b) or anucleate cell formation (Additional File 1: Figure S9) were found between strains at this growth phase, which indicates that the reason $\Delta malP$ mutants show reduced culturability during early exponential growth (Fig. 1a) is partially caused by the formation of elongated and filamentous cells.

Similar changes in length have previously been observed in an *E. coli* $\Delta malP$ mutant strain grown on maltodextrins, where length increases were ascribed to the accumulation of intracellular MOS [18]. However, our results (see Additional File 2: Table S3 for an overview of our data) indicate that this cannot be the entire explanation. In the current study, the pathway of glycogen synthesis from exogenous maltose/maltodextrin [26, 28], which involves substantial MOS turnover, will be repressed due to the presence of glucose in the medium [29]. During glycogen breakdown, GlgX supplies MalP with substrate by releasing MOS from branched glycogen [17]. If MOS accumulation were solely responsible for cell size increases, then disruption of both *glgX* and *malP* should reduce cell size compared with the *malP* single mutant and this was not the case at the first two sampling points (Fig. 2d; Additional File 1: Figures S2b, *glgX* and *malP* formed greater proportions of long cells than all

the other strains by late stationary phase (Fig. 2d). Because of this we decided to explore other possible explanations.

Delays in cell cycle progression are known to alter cell length. For example, arresting initiation of DNA replication [13, 30] or delaying the onset of septation [12] have been shown to result in significant increases in cell length, whilst width is either unchanged or reduced [31]. On the other hand, disruptions to fatty acid metabolism alter both cell length and width [32, 33]. In our experiments, cell width remained invariable (Additional File 2: Table S1) indicating that the observed alteration in cell length is more likely the result of some downstream effect on the cell cycle, rather than on fatty acid metabolism. Hence, we investigated aspects of the cell cycle.

Whilst mutant *ΔmalP* strains showed substantial heterogeneity (Additional File 1: Figure S2b) and enhanced lengths (Fig. 2a, d) during early exponential growth, mass doubling times were unchanged between strains (Additional File 1: Figure S1). Genetic alterations that lead to a contravention of Schaechter's nutrient growth law exist, including moderate delays in division that have no observable effect on mass doubling times, but enhance cellular lengths [12, 34]. Invaginating septa were observed forming at midcell or one cellular unit away from a cell pole in filamentous and elongated, exponentially growing MalP-deficient bacteria (Additional File 1: Figure S2b). Size homeostasis in filamentous cells is ensured by employing adder-like mechanisms alongside the Min protein system to sense absolute size and direct division rings to form one cell unit away from the pole, and two from another ring [35]. This allows progeny of even the longest cells to converge at a population average after multiple generations, as was observed in the MalP-deficient strains by late stationary phase (Fig. 2a-c). Given the lack of alteration in division plate numbers (Table 2), it is unsurprising that FtsZ amounts were similar in all strains at all growth stages (Fig. 3b). Taken together this demonstrates that the impairment of division is not caused by decreased capacity to form division rings but is instead the result of some mechanism(s) delaying septation.

Elongated, early exponential phase MalP-deficient cells contain multiple nucleoids which include poorly separated masses of genetic material (Figs. 3a, 4; Additional File 1: Figure S2b). Hindering the normal progression of DNA replication is expected to delay cell constriction and suggests that maternal cells experience difficulties coordinating DNA replication with nutrient availability, cell size and mass doubling. Stochastically large daughter cells are thus likely born to maternal *ΔmalP* mutants cells that are unable to regulate synchronous progression of cell cycle events [31, 33, 35] – a situation which has been reported in other mutants affected in CCM [31].

Increasing DnaA concentrations in *E. coli* has been shown to cause over-initiation and filamentation with replication fork stalling observed under run-out conditions and flow cytometry examination [36], however, we observed no differences in DnaA amounts between strains during early exponential growth (Fig. 5). This indicates that alterations in exponential phase DnaA levels do not provide an explanation for the aberrant chromosome staining patterns observed during this growth phase (Figs. 3a, 4; Additional File 1: Figure S2b). The cause and implication of elevated stationary phase DnaA levels on DNA replication in

ΔmalP mutants remains unclear. However, it points to a potential effect on DNA replication and it is evident that these strains still experience nucleoid partitioning defects at the later growth stages (Additional File 1: Figures S3b, S4b, S7, S8).

Length increases were not exclusive to cells from strains with a mutated *malP* allele as the *ΔglgP*, *ΔglgX* and *ΔglgP/ΔglgX* mutant strains formed greater proportions of longer cells more frequently than the WT during exponential growth (Fig. 2d) alongside no obvious alteration to DNA staining (Figs. 3a, 4; Additional File 1: Figures S2-S4, S7, S8). Discrepancies in the proportion of elongated cells produced by mutant strains with a lesion in *glgX* (especially in combination with a mutant *malP* allele) became more pronounced during stationary phase (Fig. 2b, c). This is typified by the observation that *ΔmalP/ΔglgX* and the *ΔmalP/ΔglgP/ΔglgX* strains accumulate enhanced proportions of long cells compared to the *ΔmalP* and *ΔmalP/ΔglgP* strains by late stationary phase (Fig. 2d).

Disruptions to *glgP* and/or *glgX* have been reported to increase glycogen concentration as turnover rates are diminished [16, 17, 19, 37]. Previously, we observed significant increases in stationary phase glycogen amounts in all GlgX-deficient strains cultured in LB broth with glucose [19]. However, the only difference we could detect using the current nutritional regime was between the WT and triple mutant during stationary phase (Additional File 1: Figure S6), when cells are expected to accumulate substantial amounts of this polymer. The reason for this discrepancy is most likely due to differences in media composition that are known to affect bacterial glycogen accumulation [38], but the media used in this study allows us to exclude the presence of increased glycogen amounts as being the cause of filament formation in the mutants.

Glycogen deposits were observed microscopically at polar sites in all strains at the onset of stationary phase. This was most clear in *ΔglgX* mutant strains and deposits also formed at inter-nucleoid zones when *malP* was concurrently disrupted (Additional File 1: Figure S7). Despite the low amounts of glycogen detected during exponential growth (Additional File 1: Figure S6) we were able to visualize glycogen bodies in all strains containing a lesion in *glgX* (Fig. 4), again appearing at inter-nucleoid zones and potential division sites (Fig. 3a) and not only at the cell periphery as reported previously for *E. coli* [39–41]. This indicates that small amounts of glycogen remain within these cells upon exiting lag phase [42, 43], possibly due to structural changes that are known to increase short chains within this polymer [17, 19, 37] and which would be expected to delay turnover [37].

Protein inclusion bodies were observed from viability micrographs in GlgX-deficient strains during exponential growth (Additional File 1: Figure S2), and their occurrence at polar or inter-nucleoid sites mimics the spatial distribution of glycogen bodies (Fig. 4). In *E. coli*, sites of protein aggregation are spatially dictated by nucleoid occlusion [44], where the bacterial nucleoid sets a zone of high macromolecular crowding around itself. Damaged proteins are passively sequestered to these sites [44, 45], effectively restricting protein inclusion bodies to DNA-free regions within the cytosol. Excess glycogen, like polypeptides, are likely targeted passively to sites of low macromolecular crowding, determined by nucleoid occlusion [44] following synthesis at the cell periphery. It seems plausible that

glycogen granules with altered structure may act as aggregation seeds, permitting co-aggregation with proteins at designated sites in the cell [46]. Within the context of endogenous metabolism, such sequestration mechanisms may protect macromolecules, like proteins, from turnover, as the cell will preferentially degrade glycogen for carbon and energy [47, 48].

Strains where *glgP* and *glgX* are concurrently disrupted ($\Delta glgP/\Delta glgX$ and $\Delta malP/\Delta glgP/\Delta glgX$) showed the most conspicuous glycogen (Fig. 4) and protein inclusion bodies (Additional File 1: Figure S2) during exponential growth. At the onset of stationary phase, the $\Delta glgP/\Delta glgX$ double mutant, which displayed prominent glycogen deposits (Additional File 1: Figure S7) was significantly enriched in long cells compared to the WT, $\Delta glgP$ and $\Delta glgX$ strains (Fig. 2d). Together with the observation of distinct glycogen bodies in the $\Delta malP/\Delta glgX$ and $\Delta malP/\Delta glgP/\Delta glgX$ strains at the onset of stationary phase (Additional File 1: Figure S7), and the finding that these two strains produced significantly more long cells than the $\Delta malP$ and $\Delta malP/\Delta glgP$ strains at the final sampling point (Fig. 2d), it is plausible that the accumulation of glycogen deposits may, independently of cell cycle progression, enhance cell lengths. However, a link between glycogen turnover and cell cycle progression cannot entirely be excluded. This might be in the form of physical inhibition of cell division through the presence of glycogen granules at putative division sites, or through an alteration in the polymer structure leading to decreased G1P release [37] resulting in less intracellular ATP. It has been shown previously that *E. coli* cells rely on glycogen breakdown for energy production during cell division as glucose uptake ceases at this stage [15].

Although our results do not demonstrate a direct mechanistic link between the genetic disruptions and alteration in cell size, they provide novel insight into how cell lengths are altered in mutants affecting glycogen degradation. A lesion in *malP* is associated with cell size heterogeneity and elongation at all stages of growth, without detectable differences in growth rate, FtsZ protein levels or division ring frequency. Whilst cell cycle progression is clearly affected in these mutant strains, it is unclear whether this results from disturbing carbon flux leading to altered energy levels, or the levels of specific metabolites that synchronize cell cycle events. Apparent disruptions to the regulation of DNA replication may be the result of unidentified protein-protein interactions between enzymes involved in glycogen catabolism or CCM and those involved in controlling aspects of the cell cycle. A high-throughput study of protein-protein interactions in *E. coli* revealed that MalP interacts with diverse proteins known to regulate the SOS response, DNA damage repair and metabolism [49] although the reason for this is unknown. Links between CCM and DNA replication have been demonstrated previously [50–53] though how MalP helps regulate this fundamental process remains unclear. Phosphorylases produce substantial amounts of G1P during glycogen degradation, which may be fed into CCM after being converted to G6P. Disruptions to CCM and UDPG levels are known to affect cell size [31, 54], so it is tempting to speculate that these may be affected pleiotropically in the mutants in this study and our future work will examine these hypotheses.

Materials And Methods

All strains (Table 1) affected in glycogen metabolism are derivatives of the BW25113 [55] and form part of the isogenic Keio collection [20]. They were obtained either from the *E. coli* Genetic Stock Centre (CGSC, Yale University) or were manufactured as described previously [19].

Cells were cultured at 37°C in AB medium [56] supplemented with 10 µg/mL thiamine, 25 µg/mL uridine [57], 0.4% (w/v) glucose and 0.5% (w/v) casamino acids (ABTGCU medium). To achieve balanced growth, cells were grown overnight from a single colony and then diluted 1:1000 into fresh medium. Bacteria were cultured until an OD₄₅₀ ~ 0.2 - 0.4 was reached, at which point cells were back-diluted to an OD₄₅₀ ~ 0.005 into fresh medium. Growth was followed spectrophotometrically at 450 nm at 30-minute intervals for the first three hours of growth and doubling times calculated from OD values using an online tool (http://www.doubling-time.com/compute_more.php).

Colony forming units were assessed by serially diluting an aliquot of cells in GTE (50 mM Glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) and plating 10 µL on LB agar plates. Plates were incubated for 12 hours at 37°C, at which point colonies were counted manually.

Glycogen and protein quantification

Duplicate samples of either 45 mL (exponential phase cells) or 2 mL (stationary phase cells) were harvested by centrifugation (20 000 x g, 10 minutes, 4°C) and cell pellets frozen at -80°C until glycogen levels were determined using a previously published method [19].

For determination of total protein, cell pellets were re-suspended in an equal volume of protein extraction buffer (PEB, 17 mM Tris-HCl pH 8.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM β-mercaptoethanol) and lysed through sonication (10 bursts lasting 15 seconds each, at 5 W output, with 1-minute cooling between bursts). Cell suspensions were clarified by centrifugation (20 000 x g, 10 minutes, 4°C) and total soluble protein quantified [58] using a kit (Bio-Rad Protein Assay Kit) and BSA as a standard.

Immunoblotting

Cells were harvested, washed once in TE buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA) and re-suspended in PEB, before being lysed by sonication and using soluble protein concentrations determined as described above. Samples containing 10 µg total protein were separated by 10% (w/v) SDS-PAGE and transferred onto a PVDF membrane by semi-dry blotting using standard procedure [59]. Membranes were probed either with α-FtsZ (1:2000 dilution) serum (Agrisera) or α-DnaA (1:1500 dilution) serum followed by goat anti-rabbit secondary antibody, conjugated to alkaline phosphatase. Immunoblots were developed by incubating in BCIP/NBT solution (Sigma) for 10 minutes before imaging. Images were subsequently converted to black and white using Adobe Photoshop CS6 (Adobe Inc).

A mutant *dnaA46* strain (CM742 [60]; Table 1), was used as a negative control in the blots assessing DnaA protein amounts. This strain was cultured at 30°C in LB broth until early exponential phase (OD₆₀₀~

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0.1 – 0.2) and then shifted to 42°C (non-permissive temperature). Growth continued for 1.5 hours at which point cells were harvested and soluble proteins extracted and quantified as described above.

Flow cytometry

Cells in early exponential phase ($OD_{450\sim}$ 0.1 – 0.2) were directly harvested by centrifugation (3 300 x g, 5 minutes, 4°C) and re-suspended in ice-cold TE buffer before being fixed by diluting tenfold in 77% (v/v) ice-cold ethanol [61].

Samples were kept at 4°C for at least 12 hours before being pelleted by centrifugation and air dried to remove any residual ethanol [62]. Samples were washed once in 0.1 M phosphate buffer (pH 9.0) and re-suspended in the same buffer containing fluorescein isothiocyanate (FITC) at a final concentration of 5 µg/mL to stain total cellular protein [63]. After overnight staining at 4°C, cells were washed twice in TBS (20 mM Tris-HCl pH 7.5, 130 mM NaCl) and genetic content stained with 1.5 µg/mL of Hoechst 33258 (Sigma) in the same buffer [64]. Hoechst staining proceeded for at least 30 minutes on ice, prior to flow analysis.

Flow cytometry was performed using a BD FACS Aria™ IIu, equipped with a violet (407 nm) and blue (488 nm) laser, and data recorded using BD FACSDIVA™ 8.0.1 software. Hoechst 33258 fluorescence was captured using a 450/40 bandpass filter, whilst FITC fluorescence was collected through a 530/30 bandpass filter. Average cell mass was taken as median FITC fluorescence, whilst average DNA content per cell was determined as median Hoechst fluorescence. The relative DNA per mass (DNA concentration) (ratio) was calculated by dividing average DNA content by average cell mass [65]. Data obtained from flow experiments were processed by FlowJo v10.6.1 (©Tree Star, Inc) software.

Membrane and vital staining techniques

An aliquot of cells corresponding to $\sim 10^5$ CFU/mL were collected at the indicated growth stages by centrifugation (3 300 x g, 5 min, 22°C) and re-suspended in 1 mL 1X PBS (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4 , 1.76 mM KH_2PO_4). Cell envelopes were stained by adding 5 µL of 1 mg/mL FM™ 4-64FX (Thermo Fisher Scientific) and non-viable cells stained with 1 µL of the LIVE/DEAD™ cell dye (Thermo Fisher Scientific). Samples were incubated at room temperature for 5 minutes and then fixed using an established method with slight modifications [66], through the addition of 200 µL ice-cold fixative (16% (v/v) formaldehyde, 0.06% (v/v) glutaraldehyde in 1X PBS). Following incubation at room temperature for 15 minutes with slight agitation (100 RPM), cells were kept on ice for another 30 minutes before being collected by centrifugation (20 000 x g, 1 minute, 4°C) and washed three times with 1X PBS containing 1 mM EDTA (PBS-E). Bacteria were re-suspended in GTE and stored at 4°C. Mounting and imaging occurred within 48 hours of sample preparation.

Periodic Acid-Schiff (PAS) staining

An aliquot of cells corresponding to $\sim 10^5$ CFU/mL were collected by centrifugation (3 300 x g, 5 min, 22°C) and re-suspended in 1 mL PBS. Cells were fixed essentially as described above, except that glutaraldehyde was omitted from the fixative [67]. Following the third wash step with PBS-E, cells were instead collected and resuspended in 100 μ L ice-cold TE buffer and membranes permeabilized by mixing cells with 1 mL ice-cold 77% (v/v) ethanol. Suspensions were kept on ice and periodic acid (Riedel-de Haën) in ddH₂O was added to the samples to a final concentration of 10 mg/mL.

Cells were kept on ice for at least 30 minutes, then harvested by centrifugation and re-suspended in 1 mL PBS-E following two wash steps with the same buffer. Suspensions were kept on ice prior to the dropwise addition of 25 μ L Schiff's reagent (Sigma) heated to 90°C. Following three inversions, samples were incubated at room temperature with slight agitation (150 RPM) for 30 minutes. Excess dye was removed by washing the cells three times with PBS-E and bacterial pellets were finally re-suspended in GTE and stored at 4°C. Imaging occurred within 48 hours of staining.

Immunofluorescent labelling of FtsZ

Z-rings were labelled using an established protocol, with minor modifications [68]. Briefly, 15 mL exponentially growing cells (OD₄₅₀ ~ 0.1 – 0.2) were fixed directly [66] by placing the cells in tubes containing 400 μ L 1 M NaPO₄ pH 7.4, and 3 mL ice-cold fixative (16% (v/v) formaldehyde, 0.06% (v/v) glutaraldehyde in PBS). Fixation was carried out at room temperature for 15 minutes with agitation (100 RPM), after which samples were placed on ice for another 30 minutes. Fixed cells were harvested by centrifugation (4 500 x g, 5 mins, 4°C), washed three times with PBS-E and stored in the same buffer at 4°C. Immunolabelling procedures were carried out within 48 hours of fixation. All subsequent steps were performed in 150 μ L volumes and centrifugation steps were at 4 500 x g for 5 mins at 4°C.

Permeabilization was achieved by re-suspending fixed cells in 150 μ L PBS containing 0.1% (v/v) Triton X-100 (PBS-T). Bacterial cells were incubated at room temperature for 45 minutes, washed three times with PBS-E and re-suspended in PBS containing 100 μ g/mL lysozyme and 5 mM EDTA. Samples were incubated for 45 minutes at room temperature before cells were collected and washed three times in PBS-E.

Cells were incubated in blocking buffer (2% BSA (w/v) in PBS) at 37°C for 30 minutes. They were collected by centrifugation and FtsZ was labelled using α -FtsZ serum (Agrisera) diluted in blocking buffer (1:500). Incubation in primary antibody occurred at 37°C for 1.5 hours with slight agitation (200 RPM). Cells were collected and washed three times in PBS-T, and then incubated in goat anti-rabbit IgG conjugated to Alexafluor 488 fluorophore (Invitrogen) diluted 1:200 in blocking buffer. Samples were incubated at 37°C for 30 minutes with slight agitation (200 RPM), after which cells were washed three times in PBS-T, washed once in PBS and finally re-suspended in 100 μ L GTE. Bacteria were kept at 4°C and were imaged within 24 hours of labelling FtsZ.

Sample mounting

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Approximately 20 μL of cells were air-dried either on agarose pads (1% (w/v) in PBS) or on slides coated with 0.1% (w/v) poly-L-lysine solution (Sigma) and bacterial nucleoids stained by topping cells with a drop of Fluoroshield™ containing 1.5 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI, Sigma). Cover slips were placed over the samples and kept at room temperature for ~ 30 minutes before cells were imaged.

Confocal image acquisition

Cells were visualized using a Zeiss LSM780 ElyraPS1 confocal microscope equipped with a 100X alpha Plan-Apochromat (N.A. = 1.46) M27 objective (Carl Zeiss). For nucleoid, membrane and viability staining the 405 nm, 488 nm and 561 nm laser lines were used to excite DAPI, FM 4-64FX and the fixable LIVE/DEAD dye, respectively. Glycogen bodies were examined by exciting PAS-stained polysaccharides with a 488 nm laser, which was also used for the excitation of Alexa 488 fluorophores used for Z-ring visualizations.

Emission was detected with a GaAsP detector in the spectral ranges of 415 nm to 498 nm ($\lambda_{\text{em}} = 450$ nm) for DAPI, 572 nm to 733 nm ($\lambda_{\text{em}} = 650$ nm) for FM 4-64FX, 498 nm to 555 nm ($\lambda_{\text{em}} = 530$ nm) for the LIVE/DEAD dye, 570 nm to 695 nm ($\lambda_{\text{em}} = 630$ nm) for PAS-stained polyglucans and 499 nm to 570 nm ($\lambda_{\text{em}} = 535$ nm) for Alexa 488-conjugated secondary antibodies. ZEN 2 (Black edition, Zeiss) was used for image capturing, analysis and manipulation.

Determination of viable and achromosomal cells

The frequencies of viable and achromosomal cells were manually determined using multichannel confocal images which were contrast-corrected and superimposed in ZEN 2. Cells completely devoid of DAPI-associated fluorescence were scored as achromosomal and bacteria were classified as non-viable only if signal from the vital stain was detected throughout the entire cell. Cells showing only polar fluorescent foci were considered viable. The frequencies of cells with Z-rings were manually determined in ZEN 2 from multichannel images and cells were scored positive if they displayed a solid band of fluorescence across the width of the cell, two adjacent fluorescent foci, or a single fluorescent focus, signifying a constricting septum.

Image Processing for Cell Size Analysis

The individual dimensions of membrane-stained bacteria were determined from confocal pictures. We devised a custom Python script (coliseg) that integrates several open-source Python packages (OpenCV [69] bindings, NumPy [70], Pandas [71] and Matplotlib [72] with seaborn [73]) to permit excellent delineation of cell borders and determination of size, being particularly effective in the analysis of adherent and filamentous cells.

Image pre-processing

To convert 1144×1144×3 RGB images to binary ones containing bacterial outlines, they were first

Loading [MathJax]/jax/output/CommonHTML/jax.js e contrasts were stretched using histogram equalisation and

were then convolved with a 3×3 circular Gaussian kernel to decrease the rate of false positive contour detection. We then binarized the images with an intensity threshold $T = 8$; the contours were set with pixel intensity = 255, the background = 0. The images were convolved with an adaptive threshold in which the threshold value was derived from a weighted sum of the block size (block size = 15) region. Contours were subsequently dilated and then eroded to remove spurious blobs using the same kernel as for the initial Gaussian blurring. Images were then thinned such that contours are reduced to a 1-pixel wide thickness followed by a dilation. The resulting images contain the segmented bacteria.

Contour extraction

To extract the bacterial outline from the binary images into a table of lists representing raw contours, we used the function `findContours` using a two-level hierarchy retrieval mode without chain approximation. We opted for this retrieval mode as it better separated clumped/dividing bacteria; the outer contour is usually the clump itself whereas the inner contours are the actual bacteria.

Since the inner contours are extracted without distinction between agglutinated/isolated bacteria, we can reasonably assume that it does not create a bias. The potential holes were then discriminated on the base of their abnormal shape and surface area. We kept only contours with a ratio convex hull length / contour length greater than .9 and a contour area greater than 110 pixels. We finally modelled the bacteria shape by fitting the contours into rectangles. The length, the width of the fitted rectangle as well as the area of the raw contour were saved into a set of tuples (length, width, area).

Length analysis

To evaluate the spread of cell lengths in each strain during a specific growth phase, we used weighted histograms with a bin size of 1 μm , ranging from 0 to 8 μm . We overlaid the mutants on the wild type to identify which length bins were enriched or depleted.

Analysis of the proportions of elongated cells

We considered the probability of observing elongated cells (length > 4 μm) and investigated how this probability varies between strains. Since the average cell length is not sensitive to the presence of only a few elongated cells, we, instead, sought to evaluate the probability of observing one elongated cell. Thus, at each time point, we fit the proportion of elongated cell with a logistical regression.

$$y_i \sim \text{Binomial}(N_i, p_i)$$

$$\text{logit}(p_i) = \alpha_i$$

$$\alpha_i \sim \text{Normal}(\mu, \sigma)$$

$$\mu \sim \text{Normal}(-2, 1)$$

$$\sigma \sim \text{half-Normal}(0, 1)$$

Where

$$y_i$$

is the number of observed elongated cells,

$$p_i$$

is the successful probability of observing an elongated cell,

$$N_i$$

is the total number of observed cells, and

$$i$$

denotes the strain. For computational reasons, we used the

$$\textit{logit}$$

link function. Therefore, the intercepts

$$\alpha_i$$

can be modelled using a normal prior, whose mean is centred at -2 ($\text{expit}(2) = .1$) - a sensible value for a small probability of elongated cell.

We used Stan [74] and its Python interface PyStan [75] to draw samples from the posterior joint distribution. We ran four independent Markov chains during 4 000 iterations. To compare the proportions of elongated cells, we computed the $\log_2$ of ratios of each pair of strains. The expected proportions are computed back using the inverse logit function, also called “expit” function.

In Stan, the mathematical model turns into

```
data {  
  int<lower=0> N; // Number of observations  
  int<lower=0> G; // Index encoding the strain  
  int n[N]; // Number of observations in strain  
  int y[N]; // Number of elongated cells in strain  
  int<lower=1, upper=G> genotype[N]; // Encoding for the strain  
}
```

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```

parameters {

real mu;

real<lower=0> sigma;

vector[G] alpha;

}

model {

mu ~ normal(-2, 1);

sigma ~ std_normal();

alpha ~ normal(mu, sigma);

y ~ binomial_logit(n, alpha[genotype]); // Likelihood in unconstrained space

}

generated quantities {

real<lower=0,upper=1> p[G];

for (i in 1:G)

p[i] = inv_logit(alpha[i]);

}

```

Statistical analyses

A minimum of three independent replicates were performed for all experiments, unless stated otherwise. Statistical tests were performed using Prism 6 (GraphPad Software) or Statistica 13 (Dell Software). Normality of data was assessed by Shapiro-Wilk tests and homoscedasticity demonstrated using Brown-Forsythe variance tests. Significant differences ($\alpha = 0.05$) between group means were determined by performing a one-way ANOVA with Tukey's post hoc test. A $\log_{10}$ -transformation was applied to culturability data prior to performing an analysis of variance to correct for heteroscedasticity and skewness of data.

Declarations

Acknowledgements

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions

JRL conceived the project and FEvdW conceptualized parts thereof. LB created coliseg and performed cell sizing analyses. LS generated mutant strains and FEvdW performed all experimental procedures. LE and RCMA assisted with experiments relating to confocal microscopy. JdS and FEvdW manually interpreted confocal micrographs. JRL and FEvdW wrote the manuscript. JRL and JK provided funding. GMG and SCZ provided critical feedback of the original paper. All authors were involved in reviewing and editing the final manuscript.

Ethics approval and consent

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Tables

Table 1

Genotypes of bacterial strains used in the study. Strains were either acquired from the E. coli Genetic Stock Center or manufactured in our laboratory.

Strain	Description	Reference
Wild type (BW25113)	$\Delta(araD-araB)567 \Delta lacZ4787(::rrnB-3) \lambda^- rph-1 \Delta(rhaD-rhaB)568$ <i>hsdR514</i>	[55]
$\Delta malP(kan)$	BW25113 $\Delta malP::kan$	[20]
$\Delta glgP(kan)$	BW25113 $\Delta glgP::kan$	[20]
$\Delta glgX(kan)$	BW25113 $\Delta glgX::kan$	[20]
$\Delta malP/$ $\Delta glgP(kan)$	BW25113 $\Delta malP::\Delta glgP::kan$	[19]
$\Delta malP/$ $\Delta glgX(kan)$	BW25113 $\Delta malP::\Delta glgX::kan$	[19]
$\Delta glgP/$ $\Delta glgX(kan)$	BW25113 $\Delta glgP::\Delta glgX::kan$	[19]
$\Delta malP/$ $\Delta glgP/$ $\Delta glgX(kan)$	BW25113 $\Delta malP::\Delta glgP::\Delta glgX::kan$	[19]
$\Delta glgA(kan)$	BW25113 $\Delta glgA::kan$	[20]
CM742	F ⁻ , <i>ara-19</i> , <i>fhuA1</i> , <i>lacY1</i> or <i>lacZ4</i> , <i>tsx-33</i> , <i>glnX44(AS)</i> , <i>galK2(Oc)</i> , λ^- , <i>Rac-0</i> , <i>hisG4(Oc)</i> , <i>rbcC1</i> , <i>rpsL8</i> , <i>mtl-1</i> , <i>dnaA46(ts)</i> , <i>metE46</i> , <i>thiE1</i>	[60]

Table 2

Analysis of Z-rings in cells. Confocal images of immunolabeled early exponential phase cells were inspected for the presence of Z-rings. Two independent replicates were performed, assessing ≥ 450 bacteria per experiment.

Percentage of cells with a Z-ring (%)		
Strain	Replicate 1	Replicate 2
Wild type	70.7	68.4
$\Delta glgP$	69.8	65.9
$\Delta glgX$	67.5	66.3
$\Delta glgP/\Delta glgX$	67.8	64.2
$\Delta malP$	72.7	67.1
$\Delta malP/\Delta glgP$	73.2	67.4
$\Delta malP/\Delta glgX$	67.7	67.5
$\Delta malP/\Delta glgP/\Delta glgX$	67.8	67.4

Table 3

DNA amounts and cell mass. Mean DNA content, cell mass and DNA/mass of untreated mutant *E. coli* strains relative to the wild type during early exponential growth. Data represent means $\pm$ standard deviation calculated from four independent replicates surveying $\sim 20\,000$ cells per run.

Strain	Relative DNA content	Relative mass	DNA/mass
Wild type	1.00 $\pm$ 0.35	1.00 $\pm$ 0.36	1.00 $\pm$ 0.51
$\Delta glgP$	1.04 $\pm$ 0.38	1.01 $\pm$ 0.37	1.04 $\pm$ 0.52
$\Delta glgX$	1.02 $\pm$ 0.41	1.05 $\pm$ 0.39	0.98 $\pm$ 0.56
$\Delta glgP/\Delta glgX$	1.00 $\pm$ 0.43	1.18 $\pm$ 0.41	0.85 $\pm$ 0.53
$\Delta malP$	1.48 $\pm$ 0.40	1.37 $\pm$ 0.40	1.08 $\pm$ 0.45
$\Delta malP/\Delta glgP$	1.08 $\pm$ 0.40	1.09 $\pm$ 0.40	0.99 $\pm$ 0.53
$\Delta malP/\Delta glgX$	1.06 $\pm$ 0.41	1.16 $\pm$ 0.40	0.91 $\pm$ 0.55
$\Delta malP/\Delta glgP/\Delta glgX$	1.06 $\pm$ 0.52	1.21 $\pm$ 0.42	0.86 $\pm$ 0.61

Figures

Figure 1

Bacterial culturability and viability. (a) Cell counts were ascertained by diluting cells harvested at the indicated times and plating suspensions on LB plates, which were incubated overnight. Colonies were manually scored, and plotted values are the means from three independent replicates $\pm$ SEM. (b) The percentage of viable cells were determined from confocal images, with plotted values being the means $\pm$

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experiment assessed ≥ 550 cells. Different letters in (a) and

(b) denote significant differences ($p \leq 0.05$) between group means as determined by a one-way ANOVA with Tukey's post hoc test.

Figure 2

Cell lengths of strains. (a-c) Distributions of cell length in each mutant strain (green) against the wild type strain (violet) during (a) early exponential, (b) onset stationary and (c) late stationary phase. The mean cell lengths (μm) $\pm$ standard deviation are displayed in the inset. (d) Box plots of the ratios of proportions of elongated cells ($>4 \mu\text{m}$). An equal proportion of elongated cells in both strains results in a $\log_2$ ratio of "0" (dashed line). The black middle bar of the box indicates the median and the whiskers span 95% of the data. The higher the box plot, the more elongated cells are in strain 1, with respect to strain 2. Significant increases in elongated cells (pstrain1 > pstrain2) are coloured grey (at one time point), blue (at two time points) or gold (at all time points). Data were obtained from three independent replicates where ≥ 1500

bacteria were measured per experiment. Inference was carried out using a binomial logit regression in Stan, where successes represent elongated cells. For numerical comparisons of the 95% credible intervals between individual proportions, see Additional File 2: Table S2.

Figure 3

Z-ring formation and FtsZ amounts. (a) Bacteria harvested at OD450 of ~ 0.15 were fixed and phase contrast and confocal microscopy images depict the

positions of Z-rings in cells during early exponential growth. These structures are shown in yellow, whilst DAPI-stained nucleoids are displayed in light blue. White arrows indicate the positions of Z-rings. (b) Immunoblot analysis of relative FtsZ levels. Ten micrograms of total crude protein were separated by 10% (w/v) SDS-PAGE and visualized following labelling with an anti-FtsZ antibody. Cropped blots are shown here whilst original blots are displayed in Additional File 1: Figure S5.

Figure 4

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Staining of glycogen and DNA in exponentially growing bacteria. Cells were cultured to early exponential phase and then harvested, fixed and stained for polyglucan and DNA before being imaged by confocal and phase contrast microscopy. DAPI-stained DNA is shown in light blue and PAS-stained glycogen in orange. White arrows indicate the positions of glycogen bodies.

Figure 5

Bacterial DnaA levels. Immunoblot examination of relative DnaA levels. Each lane was loaded with 10 μ g total protein and separated by 10% (w/v) SDS-PAGE, before being blotted onto nitrocellulose membranes and visualized following probing with an α -DnaA antibody. Cropped blots are shown here whilst original blots are displayed in Additional File 1: Figure S10.

Supplementary Files

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- [AdditionalFile1.pdf](#)
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