The Response of *Escherichia coli* Growth Rate to Osmotic Shock

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Abstract

It has long been proposed that osmotic pressure plays an essential role during bacterial growth as a driving force for mechanical expansion of the cell wall. This is largely based on analogy to plant cells, where this mechanism has been established, and on classic experiments in which bacterial growth rate was observed to decrease as the osmolarity of the growth medium is increased. To test this hypothesis, and to distinguish the effect of osmotic pressure from other effects that osmolarity might have on cell growth, we monitored single-cell elongation of *Escherichia coli* while rapidly changing the osmolarity of their medium. We discovered that elastic strain within the cell wall decreased only moderately with increasing medium osmolarity. Furthermore, we found that *E. coli* cells maintained their original elongation rate for tens of minutes after hyperosmotic shock. Next, oscillatory hyperosmotic shock revealed that while temporary plasmolysis slowed cell elongation, the cells "stored" growth, such that once osmotic pressure was re-established the cells elongated to the length that they would have attained had they never been plasmolyzed. Additionally, in response to hypoosmotic shock, *E. coli* attained its steady-state elongation rate over the course of $\approx$5 min, and mutant strains lacking stretch-activated ion channels actually elongate slower after hyperosmotic shock than do wild-type *E. coli*. Finally, by monitoring MreB motion during osmotic shock, we found that cell wall biosynthesis was unaffected by osmotic pressure. These results lead us to the conclusion that peptidoglycan synthesis is the rate-limiting step in cell wall expansion, whereas osmotic pressure plays only a minor role.

Cell growth is the result of a complex system of biochemical processes and mechanical forces. For bacterial, plant, and fungal cells, growth requires both the synthesis of cytoplasmic components and the expansion of the cell wall, a polymeric network that encloses these cells and protects them from cytolysis. It is well established that plant cells use osmotic pressure in order to drive mechanical expansion of the cell wall during growth [1, 2]. On the other hand, we have a poor understanding of the physical mechanisms of cell wall expansion in bacteria. Furthermore, the bacterial cell wall is distinct from its eukaryotic counterparts, in both ultrastructure and chemical composition. In particular, the peptidoglycan cell wall of Gram-negative bacteria is extremely thin, comprising perhaps a molecular monolayer [3], raising the question of whether these organisms require osmotic pressure for cell wall expansion, or whether they employ a different strategy than organisms with thicker walls.

Osmotic pressure forces water into a cell according to the Morse Equation, $P = RT(C_{in} - C_{out})$, where C_{in} is the osmolarity of cytoplasm, C_{out} is the osmolarity of the extracellular medium, R is the gas constant, and T is the temperature. In the Gram-negative bacterium *Escherichia coli*, P is estimated to be 1-3 atm [4, 5]. A primary role of the cell wall is to bear this load by balancing it with mechanical stress within the wall. In 1924, Walter proposed a theory of bacterial growth based on the premise that mechanical stress, in turn, is responsible for continuously deforming the cell wall during growth [6]. In support of this theory, he and others found that the growth rate of a number of bacterial species, including *E. coli*, is inversely correlated with the osmolarity of the growth medium (Fig. 1A, [7, 8, 9]): this would be expected from the Morse Equation if growth rate were correlated with pressure. The decrease in growth rate did not depend on the chemical used to modulate the osmolarity, which demonstrated that this effect was not due to specific, toxic reactions. Since Walter, several theorists have offered plausible molecular mechanisms by which osmotic pressure could drive cell wall expansion. These theories range from ones in which the cell wall is irreversibly stretched by osmotic pressure when peptidoglycan cross-links are hydrolyzed [10, 11], to ones in which peptidoglycan synthesis is subject to osmotic pressure [12, 13]. Two of these theories explicitly predict the scaling between cell growth-rate and pressure [12, 13], providing possible explanations of the classic experiments [6, 7, 8, 9].

However, since bacterial cells possess a number of mechanisms to regulate their cytoplasmic osmolarity in response to changes in their osmotic environment [14], it is unclear whether raising medium osmolarity causes a decrease in osmotic pressure. The inverse correlation between growth rate and C_{out} could result from another, non-specific osmotic effect such as altered water potential within the cell (which could affect, for example, protein folding [15] or signaling [16]). We sought to distinguish between these possibilities by measuring the elastic strain within the cell wall as a function of medium osmolarity, and the time scale at which osmotic shock (acute changes in medium osmolarity) modulates growth rate and cell wall synthesis. If growth is pressure-mediated, then 1) elastic strain, which depends on osmotic pressure, should be highly correlated with growth rate; and 2) growth rate and/or wall synthesis should rapidly change upon osmotic shock. On the other hand, if medium osmolarity modulates growth rate solely through non-specific effects, these processes may adapt more slowly.

We found that elastic strain decreases only moderately with increasing medium osmolarity. By measuring the growth-rate response of *E. coli* across time scales that span four orders of magnitude, we concluded that altering osmotic pressure had little effect on growth rate, except in the case of plasmolysis (when the sign of P is negative, causing the cytoplasm to separate from the cell wall). *E. coli* growth-rate adapted slowly to changes in medium osmolarity, on the time scale of tens of minutes. Furthermore, if osmotic pressure was restored after slight plasmolysis, *E. coli* cells quickly elongated to the length that they would have attained had they never undergone plasmolysis. From this result, we inferred that peptidoglycan synthesis is insensitive to changes in osmotic pressure. In support of the latter conclusion, we found that the speed of MreB, a protein whose motion is dependent on peptidoglycan synthesis [19, 20, 21], is unaffected by osmotic shock. Therefore, our results rule out a mechanically based model of cell wall expansion in *E. coli*, and demonstrate that the rate of cell wall synthesis is the rate-limiting step in cell wall expansion.

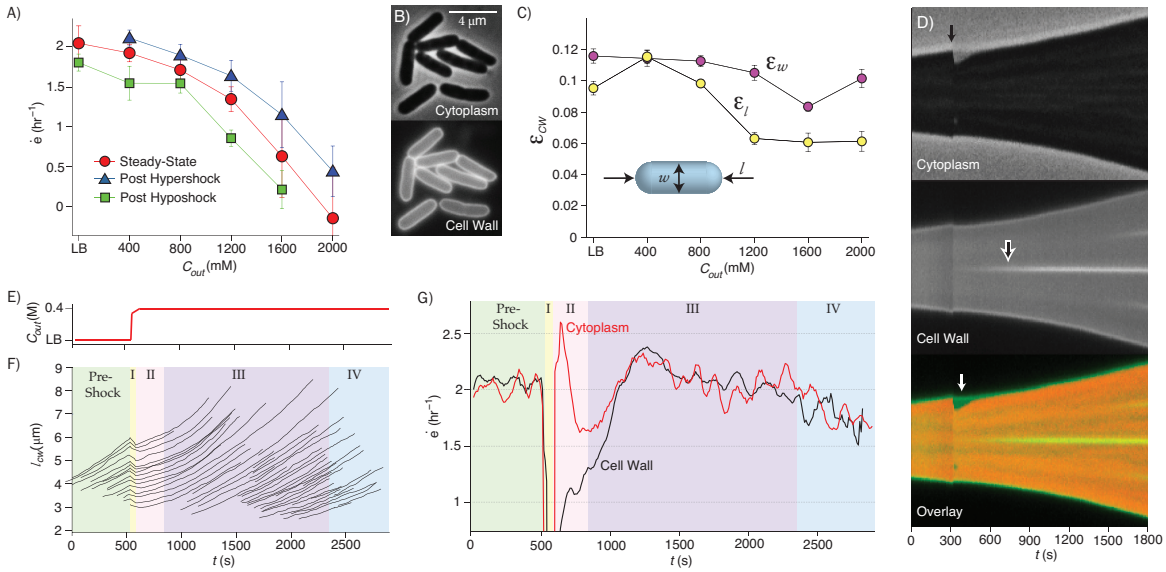

Figure 1: ***Escherichia coli* cells maintain their elongation rate after hyperosmotic shock**. (A) The population-averaged steady-state elongation rate of *E. coli* as a function of the concentration of sorbitol in the growth medium (red circles). Also shown are the population-averaged elongation rates after recovery from a 400 mM hyperosmotic shock (blue triangles) and immediately after a 400 mM hypoosmotic shock (green squares). Each data point is the average over 20-100 cells. Error bars indicate ± 1 s.e. (B) *E. coli* stained with fluorescent WGA, imaged in both phase and epifluorescence. (C) Longitudinal strain, ε_l, and radial strain, ε_w, as a function of medium osmolarity. Error bars indicate ± 1 s.e. (Inset) Diagram illustrating length and width of a rod-shaped cell. (D) Kymographs of a cell elongating during a 400 mM hyperosmotic shock. The black arrow indicates the time of the shock. The white arrow indicates the period after the shock during which the cytoplasm is shorter than the cell wall. The white/black arrow indicates the formation of a septum during cell division. (E) The concentration of sorbitol in the growth medium versus time during a hyperosmotic shock. The osmolality of the LB growth medium alone is 260 mMol/Kg. (F) The length of the cell wall of representative cells during the same shock. Each line represents one cell. The background colors indicate the four phases of response. (G) The population-averaged elongation rate versus time of both the cytoplasm and the cell wall during the shock. The traces are the average over 42 cells.

Results

Because *E. coli* cells elongate exponentially [22], their growth rate can be expressed as a relative rate of elongation, $\dot{e} = (dl/dt)/l$, where l is the length of the cell (Fig. 1B). This empirical quantity accounts for reversible elongation of the cell, in which the cytoplasm acquires water and the cell wall stretches elastically, as well as irreversible elongation, in which uptake of water is accompanied by synthesis and/or hydrolysis of peptidoglycan. While reversible elongation will result from changes in osmotic pressure, irreversible elongation occurs during steady-state growth. Our goal was to determine whether irreversible elongation, like reversible elongation, is pressure-dependent.

Cell-wall strain varies weakly with medium osmolarity

We used phase microscopy to measure the steady-state elongation rate of single *E. coli* cells as a function of the osmolarity of the extracellular medium and found a negative correlation between these quantities (Fig. 1A), in agreement with the classic bulk measurements [11, 12, 13]. Steady-state elongation was achieved by culturing the cells in chemostatic conditions using a microfluidic flow-cell.

In order to determine whether the dependence between elongation rate and medium osmolarity was mediated by osmotic pressure, or whether it was due to other, non-specific effects of osmolarity on elongation rate, we first inquired whether osmotic pressure was also negatively correlated with medium osmolarity. Although the small size of bacterial cells precludes direct measurement of osmotic pressure with a capillary probe, we could measure the degree to which pressure stretches the cell wall by plasmolyzing the cell and quantifying the resulting contraction of the wall. To do this we labeled the cell wall with a fluorescent dye and recorded time-lapse images of cells using phase and epifluorescence microscopy (Fig. 1B) during plasmolysis. We found that longitudinal strain within the cell wall, $\varepsilon_l = (l - l_{eq})/l_{eq}$, decreased moderately with medium osmolarity (Fig. 1C), where l_{eq} is the length of the cell wall after plasmolysis. On the other hand, the radial strain, $\varepsilon_w = (w - w_{eq})/w_{eq}$ decreases very little. Notably, there is significant elastic strain in both dimensions at a medium osmolarity that

quenches cell elongation. These results suggest that elastic strain within the cell wall does not determine elongation rate, and argue against a role for osmotic pressure in cell wall expansion. However, from these measurements, we could not rule out the possibility that non-linear dependences between pressure, elastic strain, and elongation-rate yield the observed relationship between medium osmolarity and elongation rate. Thus, as an independent means of probing this relationship, we next measured the time scale over which elongation rate relaxes to its steady-state value in response to osmotic shock.

E. coli maintains its elongation rate after hyperosmotic shock

Since hyperosmotic shock dehydrates *E. coli* cells within seconds (Fig. S1, [23]) we expected that if osmotic pressure drives cell-wall expansion, then the elongation rate would rapidly attain its steady-state value after hyperosmotic shock, or would fall below this value until solute transporters upregulate osmotic pressure to its steady-state value.

To test this hypothesis, we monitored the elongation of the cytoplasm and the cell wall during 400 mM hyperosmotic shock (Fig. 1D-G). The elongation-rate response of the cells was characterized by four phases (Fig. 1F,G). I) During the shock, both the cytoplasm and the cell wall rapidly shrank due to dehydration of the cell and consequent elastic contraction of the cell wall (Fig. 1D,E). The cytoplasm shrank more than the cell wall (Fig. 1D). In agreement with recent observations [24], separation between the cytoplasm and the cell wall was most commonly observed at pole(s) of the cell. II) During the following several minutes, the cytoplasm rapidly elongated while the cell wall elongated at a slower rate (Fig. 1G). III) During the tens of minutes after recovery from plasmolysis, the cells elongate at a rate approximately equal to the pre-shock elongation rate, and therefore *faster* than their steady-state value at the new osmolarity (Fig 1A,G). IV) Finally, the cells decelerate to their steady-state value. This general behavior is independent of the pre-shock osmolarity (Fig. 1A,S2), except that for high pre-shock osmolarities, the majority of the cells did not plasmolyze (Fig. S2).

The fact that cells resume their pre-shock elongation rate immediately after recovery from plasmolysis suggests that cell wall expansion is not regulated by mechanical stress. Whereas osmotic pressure changes within seconds upon osmotic shock, elongation rate is apparently de-

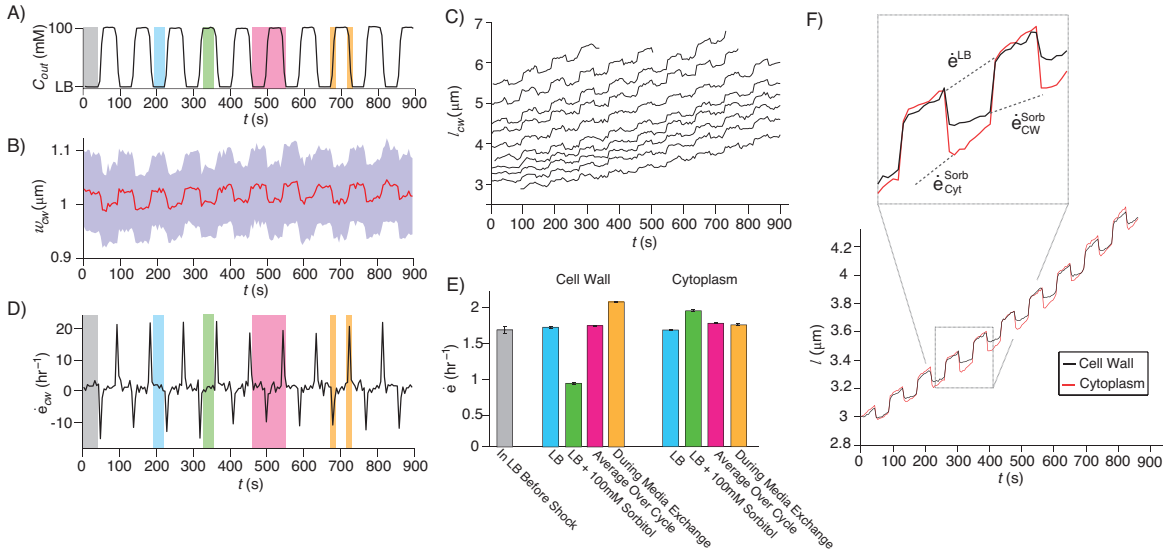

Figure 2: **Hyperosmotic shock causes stored growth.** (A) The concentration of sorbitol in the growth medium during a 100 mM oscillatory hyperosmotic shock with a 90 s period. The various phases of the oscillatory cycle are highlighted in different colors. (B) The population-averaged width of the cell wall during the shock (n=37). The confidence intervals indicate ± 1 s.e. (C) The length of the cell walls of representative cells during the shock. (D) The population-averaged elongation rate of the cell wall during the shock. (E) The population-averaged elongation rate of both the cytoplasm and the cell wall during each phase of the oscillatory cycle. Error bars indicate ± 1 s.e. (F) The effective population-averaged length of the cytoplasm and cell wall obtained by integrating their average elongation rate (see *Methods*). (Inset) Zoom-in of two cycles, labeled with the growth rates of the cytoplasm and cell wall in LB+sorbitol ($\dot{e}_{\mathrm{CW}}^{\mathrm{Sorb}}$ and $\dot{e}_{\mathrm{Cyt}}^{\mathrm{Sorb}}$, respectively) and in LB ($\dot{e}^{\mathrm{LB}}$).

termined by variables that vary slowly, on the order of tens of minutes after a shock. Strikingly, we observe a significant rate of cell wall elongation even during plasmolysis (Fig. 1G, Phase II), which demonstrates that mechanical stress is not even required for cell wall elongation. This elongation may reflect cell wall synthesis in the absence of turgor pressure.

E. coli stores growth during oscillatory hyperosmotic shock

Based on single-shock experiments alone, we could not rule out the possibility that osmotic pressure re-equilibrates or even overshoots its initial value within minutes after hyperosmotic shock, potentially accounting for the resumption of the initial elongation rate. *E. coli* responds to hyperosmotic shock by importing potassium, as well as compatible solutes such as proline and glycine betaine, if they are present [25]. Potassium takes several minutes to accumulate in the cytoplasm [26], while the time for activation of the broad-specificity compatible solute

transporter ProP is 1-2 min [27]. In addition, *E. coli* recovers from plasmolysis within several minutes after large hyperosmotic shocks (Fig. 1G, [23]), suggesting that this is the time scale of osmoregulation. Therefore, we wished to determine whether a pressure-dependence of elongation rate would be evident at shorter time scales, < 1 min after hyperosmotic shock, before osmoregulatory mechanisms are able to achieve osmotic homeostasis.

It is difficult to measure elongation rates at sub-minute time scales due to the small size of bacteria: in lysogeny broth (LB), *E. coli* cells elongate at ≈ 2 hr^{-1} (Fig. 1A), which is equivalent to ≈ 2 nm/s. In order to precisely measure elongation rates on short time scales, in spite of the optical diffraction limit, we combined three strategies: 1) we determined cell boundaries to sub-pixel resolution and then were able to discern changes in cell length below the diffraction limit (see *Methods*); 2) we averaged the elongation rate over a population of cells; and 3) we averaged the elongation-rate of these populations over many successive identical osmotic shocks. To achieve the latter, we subjected the cells to oscillatory hyperosmotic shock, during which, after an initial period in LB, the medium was repeatedly exchanged for LB concentrated with sorbitol (Fig. 2A, Movie S1). We examined the effects of relatively small-amplitude osmotic shocks, $<$300 mM, in order to avoid significant plasmolysis, and because within this range the variation in steady-state elongation rate is negligible (Fig. 1A). Thus, any variations in elongation rate across this range of osmolarity will be due solely to changes in osmotic pressure.

We found that the elongation rate of *E. coli* cells during oscillatory hyperosmotic shock depends on the phase of the oscillatory cycle. During the phase of the cycle when the medium osmolarity is being raised or lowered, the cells acutely shrink and swell due to osmosis (Fig. 2B,C). The cell-wall width decreases upon each hyperosmotic shock, and this decrease is reversed only when the cells are re-immersed in LB, suggesting that osmotic pressure is indeed being reduced for the duration of each hyperosmotic shock (Fig. 2B). The cell-wall length also undergoes acute deformation during the phases of media exchange, which leads to sharp peaks and valleys in the the time-dependent cell-wall elongation rate (Fig 2D).

During the phase of the cycle when the cells are in LB, their cell walls elongate at the same rate as they did before the oscillatory shock, when they were also growing in LB (Fig. 2E).

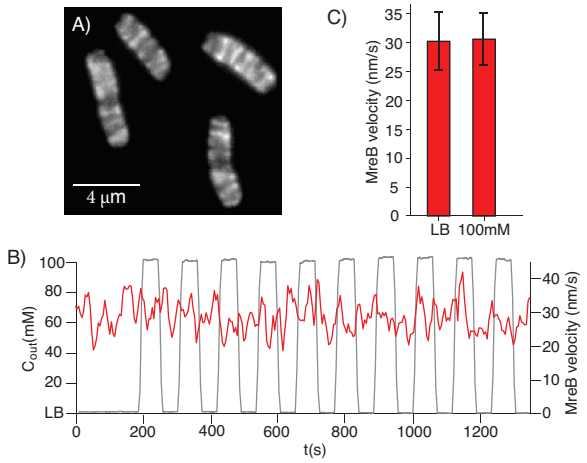

Figure 3: **Hyperosmotic shock does not affect MreB motion.** (A) Average intensity projections over a 100 s interval of *E. coli* expressing an MreB-msfGFP fusion protein, illustrating circumferential tracks. (B) The concentration of sorbitol in the growth medium (gray) and the average velocity of MreB puncta (red) during oscillatory hyperosmotic shock. (C) The average velocity of MreB puncta versus phase of the oscillatory cycle. Error bars indicate ± 1 s.e.

Conversely, during the phase when they are in concentrated media, their walls elongate at a significantly slower rate (Fig. 2E). Even though the cells spend equal time in each media, the cell-wall elongation rate averaged over the period of the entire oscillatory shock is not equal to the average of the elongation rates in the two media, but rather, is equal to the steady-state elongation rate in LB alone. This result is explained by the fact that the cell-wall elongation rate averaged over the phases of media exchange is higher than the steady-state elongation rate in LB (Fig. 2E).

In contrast to the cell wall, the cytoplasm elongates at a nearly constant rate that is independent of phase, and that is equal to the steady-state elongation rate in LB (Fig. 2E,S3). These data suggest that the cell wall and the cytoplasm can elongate independently during oscillatory shock. To examine this in more detail, we calculated effective population-averaged length of the cell wall and of the cytoplasm by integrating their average elongation-rate over time (Fig. 2F; see *Methods*). This calculation reveals that the cytoplasm shrinks 10-20 nm more than the cell wall upon each hyperosmotic shock, and proceeds to elongate faster than the wall until the length of the two are equal. Thus, as in the single-shock experiments, the elongation of the cell wall is only retarded when the cell is plasmolyzed. Since the separation of the cell wall from the cytoplasm is well below the diffraction limit we could not resolve this plasmolysis in single cells, which demonstrates the value of averaging the elongation rate over a population of cells.

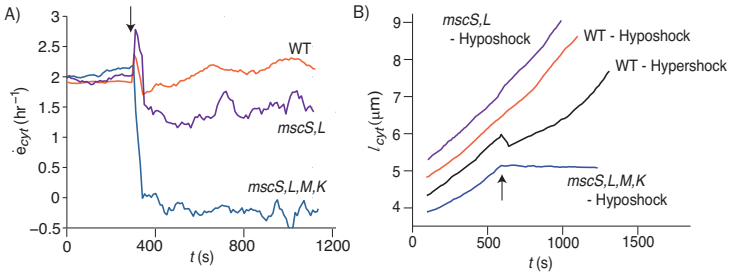

Figure 4: **Elongation rate equilibrates slowly after hyposmotic shock.** (A) The population-averaged elongation rate of three strains of *E. coli* during a hypoosmotic shock (from LB+400 mM to LB): MG1655 (wild-type, orange), MJF465 (Δ*mscS* Δ*mscL*, purple), and MJF612 (Δ*mscS* Δ*mscL* Δ*mscM* Δ*mscK*, blue). Arrow indicates time of shock. Each trace is the average elongation rate of 10-20 cells. (B) Comparison of the length of representative cells during a 400 mM hypoosmotic shock (black) and 400mM hyperosmotic shock. Arrow indicates time of shock.

All of the above behavior was observed over the entire range of oscillation periods that we were able to apply (>60 s, Fig. S4) and across a range of shock magnitudes (<100 mM). As a whole, these data suggest that the cell-wall elongation rate is independent of osmotic pressure unless the cell is plasmolyzed, i.e. $P \leq 0$. In addition, we propose that even during slight plasmolysis, in which the inner membrane recedes from the cell wall at the pole(s) of the cell, the cells continue to incorporate peptidoglycan into the lateral cell wall in an unextended state that makes only a partial contribution to elongation of the cell wall. A corollary to this proposal is that cell-wall biosynthesis is independent of osmotic pressure. These facts would explain why there is a non-zero cell-wall elongation rate during plasmolysis, and also why when pressure is re-established, the cell wall extends to the length that it would have attained had pressure never been depleted in the first place. This behavior is reminiscent of so-called "stored growth," long observed in certain plant tissues [28] and more recently in unicellular algae [29].

For shock magnitudes between 100-300 mM, cells undergo severe, visible plasmolysis during each cycle. Although they are still able to store growth, their average elongation rate across the oscillatory shock cycle is lower than the steady-state rate in LB (Fig. S4). To explain this decrease, we hypothesize that severe plasmolysis causes the inner membrane to recede from the polar and lateral cell wall, yielding reduced incorporation of peptidoglycan over the cycle.

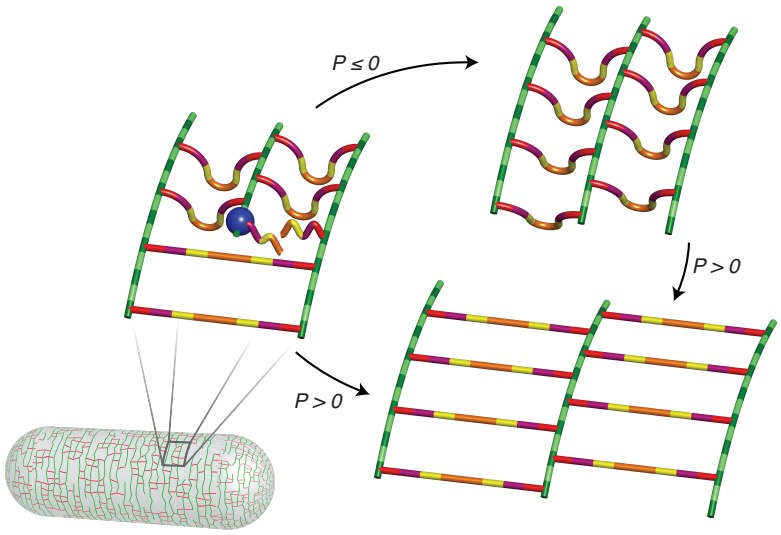

Figure 5: **Model of stored growth in *E. coli*.** Green bars represent glycan strands while red bars represent peptide cross-links. The blue sphere represents a peptidoglycan synthetic complex, which includes MreB. New glycan polymers are inserted such that cross-linking peptides buckle out of the plane of the cell wall. These cross-links are subsequently extended if osmotic pressure is positive. During periods of plasmolysis, elongation is "stored" in these polypeptide loops, which become fully extended when positive osmotic pressure is restored.

Hyperosmotic shock does not affect MreB motion

We sought to test our prediction that osmotic pressure does not affect the rate of cell-wall synthesis. Although it is not possible to measure this quantity directly, we can infer it by tracking MreB (Fig. 3A), a protein whose circumferential motion is dependent on cell-wall synthesis in *E. coli* [19]. When cells expressing an MreB-msfGFP fusion protein were subjected to oscillatory hyperosmotic shock, the average speed of MreB puncta was independent of the phase of the oscillatory cycle (Fig 3B,C), suggesting that cell-wall synthesis is independent of osmotic pressure and mechanical stress in the cell wall.

Hypoosmotic shock does not cause swelling of *E. coli*

To confirm that medium osmolarity modulates elongation rate on the time scale of minutes, we next measured elongation-rate response of *E. coli* cells to hypoosmotic shock. As with hyperosmotic shock, *E. coli* cells do not immediately obtain their steady-state elongation rate after hypoosmotic shock, but gradually accelerate to this value over $\approx$ 5 min (Fig. 1A,4A), indicating that this acceleration is not pressure-mediated. However, stretch-activated ion channels,

which down-regulate osmotic pressure by passively releasing cytoplasmic solutes, are activated by mechanical stress in the membrane within fractions of a second [30]. Thus, hypoosmotic shock may not actually alter osmotic pressure on a time-scale over which we can measure cell elongation. This could explain why cells show virtually no swelling during hypoosmotic shock, in sharp contrast to the drastic shrinking that cells undergo during hyperosmotic shocks of the same magnitude (Fig. 4B).

If elongation rate is indeed independent of osmotic pressure, diminishing the bacterium's ability to down-regulate pressure should not decrease the time it takes *E. coli* cells to accelerate to their steady-state elongation rate after hypoosmotic shock. To test this prediction, we utilized strains of *E. coli* that lacked two or more of the genes encoding the seven known stretch-activated ion channels. After hypoosmotic shock, bacterial cells that lacked the two major channels MscS and MscL actually elongated at a slower rate than did wild-type cells, and accelerated more slowly to their steady-state elongation rate (Fig. 4A), suggesting that medium osmolarity modulates elongation rate in a pressure-independent manner. Like their wild-type counterparts, the mutant cells showed negligible swelling during hypoosmotic shock (Fig. 4B); the lone signature of swelling is a small peak in the elongation rate (Fig. 4A). Whereas 22/26 of $\Delta mscS\Delta mscL$ cells continued to elongate after 400mM hypoosmotic shock, 0/13 of cells lacking four of the ion channels (MscS, MscL, MscM, MscK) continued to elongate after such a shock (Fig. 4C,D), and many of these cells underwent visible lysis. Therefore, it appears that osmotic pressure cannot extend the length of *E. coli* cells above the length prescribed by cell wall synthesis without causing lysis.

Discussion and Conclusions

In order to measure the fundamental dependence of *E. coli* growth on osmotic pressure we monitored single-cell elongation rate and MreB dynamics during osmotic shock. Our several findings support the conclusion that osmotic pressure is not an essential driver of cell wall expansion in this organism. First, although cell growth can be quenched by increasing medium osmolarity, elastic strain within the cell wall decreases only moderately with osmolarity. Sec-

ond, the elongation rate of *E. coli* responds on slow time scales (>5 min) to both hyper- and hypo-osmotic shock (Fig. 1,4). Third, osmotic shock has no effect on MreB motion (Fig. 3). Fourth, although is it possible to slow cell wall expansion by plasmolyzing the cell, the bacteria can "store" growth such that when osmotic pressure is re-established the cell elongates to the length that it would have attained had plasmolysis not occurred (Fig. 2). Finally, hypoosmotic shock does not extend the cell wall beyond the length determined by steady-state elongation (Fig. 4).

As a whole, these data are consistent with a model in which the rate of cell wall synthesis is independent of osmotic pressure, and is the primary determinant of cell elongation rate. To account for stored growth, we propose that nascent peptidoglycan is inserted into the cell wall in an unextended state, and that positive osmotic pressure is required for extension (Fig. 5). Thus, during plasmolysis this material makes a smaller contribution to elongation than when the cell is turgid. However, once pressure is re-established, this material then contributes its full potential to cell elongation. Hence, according to our model, osmotic pressure does play a trivial role whereby a non-zero value is required to stretch out newly synthesized, un-extended peptidoglycan, but does not govern the rate of wall expansion.

Our data contradict two theoretical studies, which predict that bacterial elongation rate, peptidoglycan synthesis, and/or MreB speed scale directly with osmotic pressure [12, 13]. We also provide fundamental constraints with which to test other other mechanical models of bacterial growth [10, 11]. Because elongation rate does not respond rapidly to changes in the osmolarity of the growth medium, it is unlikely that the dependence of cell elongation-rate on medium osmolarity [6, 7, 8, 9] is mediated directly by osmotic pressure, although we can not rule out a scenario in which osmotic pressure is involved in a signaling cascade that modulates elongation rate.

The response of *E. coli* to osmotic shock contrasts with the wealth of evidence showing that plant cell elongation is directly tunable by osmotic pressure [1, 2]. However, *E. coli* share with plants the capability to store growth upon depletion of turgor pressure [28, 29]. In the case of *E. coli*, which has a very thin ($\leq$3 nm) cell wall [3], it is easy to imagine a mechanism for stored growth that depends on insertion of unextended wall material (Fig. 5), whereas in plants,

which have a much thicker cell wall (> 100 nm,[32]), the mechanisms of storing growth are more speculative [29].

A surprising finding of our study was that, upon hypoosmotic shock, *E. coli* cells do not display noticeable swelling or elastic expansion of the cell wall, even though there is significant contraction upon hyperosmotic shock (Fig. 4B). In fact, not even bacteria that lyse in response to the shock show swelling during lysis (Fig. 4B). This suggests that the cell wall may be bearing additional stress without stretching, perhaps due to non-linear mechanical properties. Indeed, the *E. coli* cell wall has been shown to strain-stiffen in response to AFM indentation [33]. Moreover, it is important to note that although the mechanical integrity of the *E. coli* cell is commonly attributed to the cell wall, it is possible that the outer membrane, which is rich in cross-linked lipopolysaccarides [34], is also load-bearing.

E. coli growth is evidently robust to changes in osmotic pressure. This could be an important adaptation for an enteric bacterium, which may regularly face drastic changes in its osmotic environment during entry and exit from the intestine. Similar studies on other bacterial species will reveal if the behavior we observe for *E. coli* is universal. In particular, it will be interesting to examine Gram-positive bacteria to see whether their thicker cell wall ($\approx$40 nm, [35]) is sufficient to endow them with a more plant-like elongation-rate response to changes in osmotic pressure.

Acknowledgments

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