# Dynamics of Escherichia coli's passive response to a sudden decrease in external osmolarity

# Dynamics of *Escherichia coli's* passive response to a sudden decrease in external osmolarity

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**For most cells, a sudden decrease in external osmolarity results in fast water influx that can burst the cell. To survive, cells rely on the passive response of mechanosensitive channels, which open under increased membrane tension and allow the release of cytoplasmic solutes and water. Although the gating and the molecular structure of mechanosensitive channels found in Escherichia coli have been extensively studied, the overall dynamics of the whole cellular response remain poorly understood. Here we characterize E. coli's passive response to a sudden hypo-osmotic shock (downshock) on a single-cell level. We show that initial fast volume expansion is followed by a slow volume recovery that can end below the initial value. Similar response patterns were observed at downshocks of a wide range of magnitudes. While wild type cells adapted to osmotic downshocks and resumed growing, cells of a double mutant ($\Delta mscL, \Delta mscS$) strain expanded, but failed to fully recover, often lysing or not resuming growth at high osmotic downshocks. We propose a theoretical model to explain our observations by simulating mechanosensitive channels opening, and subsequent solute efflux and water flux. The model illustrates how solute efflux, driven by mechanical pressure and solute chemical potential, competes with water influx to reduce cellular osmotic pressure and allow volume recovery. Our work highlights the vital role of mechanosensation in bacterial survival.**

osmotic downshock | mechanosensing in bacteria | single cell imaging

Abbreviations: PMF, *E. coli*, MSC

## Introduction

Biology offers an array of intriguing mechanical solutions, both active and passive, often exceeding what is currently possible with man-made methods. Understanding how biological systems achieve different functionalities under mechanical stimuli can inform new, thus far unexplored design principles. One such passive control system is the bacterial response to sudden decreases in external osmolarities.

A gram-negative cell's fluid cytoplasm is separated from the external environment by the inner membrane, the periplasmic space and the outer membrane. Ordinarily, the total solute concentration within the cytoplasm is higher than that of the environment, resulting in a positive osmotic pressure on the cell wall (termed turgor pressure) [1]. *Escherichia coli* is able to respond to both increases and decreases in external concentrations. An increase in external osmolarity (hyperosmotic shock or upshock) results in water efflux from the cell interior, causing cellular volume to shrink and osmotic pressure to drop to zero [2]. *E. coli* responds by actively accumulating specific solutes (osmolytes), such as potassium, proline and glycine-betaine [2]. Accumulation of osmolytes in the cell's cytoplasm causes re-entry of water, cell volume increase and recovery of osmotic pressure [3, 4]. A downward shift in external osmolarity (termed hypo-osmotic shock or downshock) causes fast water influx into the cell's cytoplasm. As a result, the osmotic pressure increases and the cell expands in a nonlinear fashion [5, 6]. Turgor pressure in *E. coli* has been estimated to lie between 0.3-3 atm [5, 7], rising up to 20 atm upon a large downshock [6]. An increase in the inner membrane tension, caused by the expansion, is thought to activate the nonspecific export of solutes through mechano-sensitive channels (MSCs), such as MscS and MscL (Fig. 1 A) [9]. As the solutes leave the cell, so does the cytoplasmic water, enabling the cell to recover original volume and pressure (Fig. 1 A).

Mechanosensitive channels are found in a wide range of cells [13–15], displaying great diversity. The precise gating mechanism of these pressure controlled channels has attracted a lot of attention from scientific community. Despite the efforts, it remains a challenge [16]. To our current knowledge *E. coli* possess seven different mechanosensitive channels [17]. Of those seven, four play the dominant role: the mechanosensitive channel of small conductance (MscS), the large mechanosesitive channel (MscL) [13, 18, 19], the mechanosensitive channel of miniconductance (MscM) [20] and the potassium-dependent mechanosensitive channel (MscK) [21]. Since their discovery in giant spheroplasts of *E. coli* [17, 22], crystal structures of some of the channels have been obtained [10–12], and channel function has been extensively studied *in vitro* [10, 17, 22–26]. The most widely used *in vitro* technique, electrophysiology, enabled measurements of channels' pressure sensitivity, open dwell-time, conductance, as well as ion selectivity [22, 27]. For example, *in vitro* measured opening time of MscS or MscL is on the order of 20-30 ms [28, 29] and the channels close immediately upon the decrease in tension [17].

### Significance

**Mechanosensation is central to life. Bacteria, like majority of walled cells, live and grow under significant osmotic pressure. By relying on mechanosensitive regulation, bacteria can adapt to dramatic changes in osmotic pressure. Studying such mechanical sensing and control is critical for understanding bacterial survival in a complex host and natural environment. Here, we investigate the fundamental design principles of Escherichia coli's passive mechanosensitive response to osmotic downshocks, by implementing single-cell high resolution imaging. We explain the observed cell volume changes by modeling flux of water and solutes across the cell membrane. A better characterization of bacterial mechnosensitive response can help us map their reaction to environmental threats, including antibiotic treatments.**

**Reserved for Publication Footnotes**

In contrast to *in vitro* studies, *in vivo* studies are rare and mostly focused on estimating bacterial population survival with or without MSCs present [17, 29, 30]. For example, we know that if either MscS or MscL alone is present in the cell membrane, populations of cells can easily survive the abrupt osmotic downshock [29]. When both channels are lacking the survival rate decreases [30, 31]. On a single cell level, a recent study looked at the nature of cells dying upon downshocks and found that it depends on the flow rate with which the shock is administered [30].

However, *in vitro* studies of mechanosensitive channel gating and population survival studies cannot be easily translated into insights on the passive control of the whole cell volume and pressure. Here, by looking at the response to hypoosmotic shocks on a single cell level, we show that the volume recovery after initial fast expansion proceeds on a much slower time scale, on the order of minutes. In addition, cellular volume can decrease below the initial value. We present a theoretical model that explains our experimental observations. A competition between water efflux and influx and solute efflux through mechanosensitve channels gives rise to the observed characteristic slower volume recovery. The chemical potential of water and solutes serve as effective 'control' variables in this passive dynamic system.

## Results

**Characterizing whole cell downshock response.** *E. coli's* response to downshocks has previously been characterized using light scattering in a stop flow device and within the first second post shock [29]. We applied a single cell microscopy assay previously developed [3], to extend the length of observation and investigate the response to downshocks in a range of shocks of different magnitudes.

Fig. 1 B shows a typical volume recovery trace of a single wild type cell subjected to a large osmotic downshock ($\Delta$c=1130 mOsmol), delivered with a local flow rate of 0.68 $\mu$l/min. At this rate, full transition to the lower osmolarity media is completed within 0.8 seconds (see *Methods*). Cytoplasmic volume was monitored via cytoplasmically expressed GFP, sampled at a frame every 0.2 seconds for initial 15 min, and at a frame every 5 seconds for the rest of the 75 minute recording. Characteristic phases were identified and indicated with different background colors. (I) Expansion Phase, observed immediately after downshock. (II) Decrease Phase of volume recovery, observed post expansion, lasting several minutes. As the volume decreases in this phase, a characteristic 'overshoot' below the initial volume is often observed. (III) Increase Phase of volume recovery, observed after minimum volume ($V_{min}$) has been reached, and lasts until initial volume is reestablished, i.e. approximately 30 min. (IV) Cell growth phase, observed post volume recovery. Fig. 1 C gives raw images corresponding to different phases shown in Fig. 1 B.

We analyzed volume changes in 609 wild type cells before, during and post downshock for the following shock magnitudes: 103, 190, 460, 790, 960, 1130 and 1337 mOsmol. Fig. 2, left shows average traces with standard deviations of 103 - 1130 mOsmol shocks. All cells quickly expand in Phase I and show characteristic slow volume recovery in Phase II. As the shock increases, the length of Phase II and the 'overshoot' increase. SI Appendix, Fig. S1, left shows average traces with standard deviations over longer time periods; Phase IV, i.e. growth, is visible for all shock magnitudes. SI Appendix Fig. S3, left shows average trace with standard deviations of our largest shock, 1337 mOsmol. We observe expansion in Phase I, however, only small recovery in Phase II is visible, with no characteristic 'overshoot' and no Phase IV. In fact, a large number of cells in 1337 mOsmol condition lyse during our recording (see also SI Appendix, Fig. S10).

To confirm that the characteristic slow volume recovery after expansion is due to mechanosensitive channels, we characterized the response of a mutant strain lacking the channels contributing the most to downshock response [31], MscS and MscL (double mutant). Fig. 2, right shows average traces with standard deviations of the double mutant cells exposed to downshocks of same magnitudes as in Fig. 2, left. In total 480 cells were analyzed. The double mutant strain quickly expands for all shock magnitudes, similarly to the wild type. The response of the double mutant strain in Phase II and Phase III is similar to that of the wild type for shocks $<$ 390 mOsmol. For shocks $\geq$ 390 mOsmol, the double mutant shows a smaller decrease in volume post expansion and no 'overshoot'. In addition, Phase IV was not observed within 35 min post downshock for any of our shocks (SI Appendix, Fig. S2). SI Appendix, Fig. S3, right shows average traces with standard deviations of the double mutant exposed to the largest shock. The response is similar to that of the wild type, where the number of lysing cells during our recording in 1337 mOsmol condition increased for the double mutant (see also SI Appendix, Fig S10 and analysis of observed lysis later in the text).

During sample preparation we attached individual cells to the cover slip surface. To investigate if the observed characteristic response to a sudden downshock is specific to surface attached cells only, we repeated the experiment on freely floating cells (*Methods* and SI Appendix, Fig. S4). We were particularly interested in Phase II and III, i.e. slow volume recovery and 'overshoot'. In the case of free cells, the shock was completed in microfuge tubes. Imaging began 3-4min post shock and continued at specific time points, giving a population averaged volume (SI Appendix, Fig. S4 and *Methods*). SI Appendix, Fig. S4 shows that characteristic phases II-IV are similar to those observed for the individual cell given in Fig. 1 B. Phase I, i.e. expansion, could not be captured with this method as it was completed before the imaging began.

In Fig. 1 and 2 the shock was induced by removing a given osmolarity of NaCl. To examine whether the characteristic response observed is specific to the solute that causes the downshock, we have induced the shock by removing sucrose in SI Appendix, Fig. S5. The magnitude of the downshock in SI Appendix, Fig. S5 was 590 mOsmol and the response observed is very similar to that of the 790 mOsmol NaCl induced shock shown in Fig. 2.

**Maximum volume expansion occurs fast, on the order of seconds.** To quantify the extent of post downshock volume expansion in the wild type strain, in Fig. 3 A (blue) we plot maximum volumes, $V_{n,max}$, against the magnitude of the downshock. $V_{n,max}$ increases with the shock magnitude up to 790 mOsmol, at which point it reaches $\approx$15% and expansion saturates. The time it takes to reach $V_{n,max}$ $\approx$30 sec (Fig. 3 B, blue) for all shock magnitudes.

We compare the $V_{n,max}$ of the wild type to that of the double mutant. $V_{n,max}$ and $T_{max}$ for the double mutant are given in Fig. 3 A and B in red. Similarly to the wild type, the double mutant expands more with increasing shock magnitude. However, for shocks $\geq$ 790 mOsmol, $V_{n,max}$ of the double mutant saturates at a slightly higher value, $\approx$20 %. The time it takes to reach $V_{n,max}$ for the double mutant strain lasts $\approx$30 sec, similar to the wild type, with the only difference that at higher shock magnitudes full expansion is slightly faster, lasting $\approx$20 sec.

**Upon expansion volume recovery is slow, on the order of minutes, and volume can decrease below the initial value.** To determine the length of the time volume decreases from $V_{n,max}$ to $V_{n,min}$ (minimal, post shock value) in Phase II, we identified the time point, $T_{min}$, at which $V_{n,min}$ is reached. Fig. 3 C shows a box plot of $V_{n,min}$ and Fig. 3 D of $T_{min}$ against the downshock magnitude.Wild type cells (in blue) show increasing overshoot with increasing shock magnitude, reaching $\approx 0.9$ for the highest shock. In contrast, the double mutant (in red) does not overshoot in any of the conditions. The time it takes to reach $V_{n,min}$ increases with the shock magnitude both for the wild type and the double mutant, reaching $T_{min} \approx 8$ min for the wild type, and $\approx 5$ min for the double mutant. $T_{min}$ is consistently lower at different shock magnitudes for the double mutant compared to the wild type.

**Growth at high osmolarities, together with downshock, do not change membrane permeability to water.** To gain more information on the physical mechanism behind the slow volume decrease in Phase II, we considered the possibility that, despite fast gating of mechanosensitive channels (within tens of ms [28, 29]), water exits the cell slowly. This could be the case if the cell envelope changes during growth at high osmolarities in such a way that water can no longer pass as fast. The change in envelope permeability properties with respect to water could be caused by changes in lipid composition of the bilayers, number of porins present in the outer membrane, aquaporin numbers in the inner membrane (AqpZ), as well as changes in the number of any other channels that might be facilitating water transport across the cell envelope. Alternatively, a change in water flux for any (or a combination of) the above named reasons could occur after initial expansion immediately post downshock. Fig. 4 A shows average volume of 13 cells grown in media of 1370 mOsmol subjected to a sudden upshock of 1272 mOsmol. Fig. 4 B shows the average volume of 30 cells grown at 1370 mOsmol, subjected to a 1130 mOsmol downshock followed by an immediate 2160 mOsmol upshock. In both cases, upon the upshock, the cytoplasmic volume shrinks within seconds. Fast reduction of volume shows that water can exit the cell fast in a post downshock expanded cell. SI Appendix, Fig. S6 shows cells grown in media of a different osmolarity, 1200 mOsmol, subjected to the same sequence of upshocks as in Fig. 4 A. Cell volume shrinks fast for these cells as well. Thus, the membrane permeability with respect to water does not change with the osmolarity of the growth media or the downshock magnitude.

**Model of *E. coli* response to downshock explains experimentally observed volume changes.** To understand the cellular response to a sudden downshock we observed experimentally, we developed the following model. An *E. coli* cell is separated from its environment by the semi-permeable membrane. Normally, the solute concentration in a cell is higher than that of the environment, giving rise to osmotic pressure:

$$\Pi = -\phi(c_i - c_e)RT \qquad [\mathbf{1}]$$

Here $c_i$, $c_e$, $R$, $T$ denote solute concentration inside the cell, solute concentration in the environment, ideal gas constant, and thermodynamic temperature. The constant $\phi$ is the molar osmotic coefficient [32] (we use osmotically active solute concentrations and set $\phi$ to 1).

Osmotic pressure in Equation [**1**] strains the cell wall, giving rise to mechanical pressure. To quantify it, we consider the elasticity of the cell wall, defined as:

$$E = \frac{True \quad stress}{True \quad strain} = \frac{d\sigma/l}{dr/r} \qquad [\mathbf{2}]$$

where $\sigma$ is the cell wall tension. We note that in our model we take into account only the elasticity of the cell wall, under the assumption that it is significantly larger compared to bilayer(s) elasticity.

For a bacterial cell represented as a cylinder with a thin wall of thickness $l$, cell wall tension $\sigma$ is given as:

$$\sigma = P \cdot r \qquad [\mathbf{3}]$$

where $r$ denotes the cell radius and $P$ is the pressure applied to the cylindrical cell wall. At a given point in time and for a pressurized cell, the volume is set by the balance between osmotic pressure ($\Pi$) and mechanical pressure (P) derived from the cell wall strain.

Water moves across the semi-permeable cell membrane in accordance with the chemical potential gradient, therefore water flux is given as:

$$j \propto -\Pi - P \qquad [\mathbf{4}]$$

We assume that the periplasmic space is in equilibrium with the external environment throughout our considerations (the outer membrane contains large number of porins [33]).

Given that osmotic pressure is proportional to the solute concentration difference, we can write:

$$\frac{dV}{dt} = V_m j A_c = V_m K \cdot (-\Pi - P) \qquad [\mathbf{5}]$$

Here $t$ denotes time, $V_m$ the molar volume of water and $A_c$ the superficial area of the cell. $K$ $(mol/(Pa \cdot s))$ is an effective 'conductivity' coefficient that characterizes the relationship between pressure difference and flow speed in moles. It depends on the nature of the flow process, which we expect to have both diffusive and quasi-laminar components [34]. Here we assume that water flows mainly through porins (AqpZ) and that the total number of AqpZ does not change. Under these assumptions changes in the surface area of the cell will not increase the water conductance and we keep $A_c$ constant.

To derive the expression for mechanical pressure, $P$, we take into account recent experiments depicting cell wall stress stiffening [5]. Deng et al. found that the elasticity of the cell wall in *E. coli* behaves as $E = E_0(P/P_0)^\gamma$, where $\gamma$=1.22 [5]. Here we assume $\gamma$=1, in rough agreement with experimental data and to simplify our model. We note that the assumption does not change the model behavior:

$$E = E_0 P/P_0 \qquad [\mathbf{6}]$$

where $E_0$ and $P_0$ denote elasticity of the cell wall and pressure when cell is at its original volume before the downshock ($V_0$). From equations [**2**] to [**6**] follows:

$$\frac{E_0 l}{P_0} \cdot \frac{dr}{r^2} = \frac{dP}{P} \qquad [\mathbf{7}]$$

Combining [**1**] to [**7**] we get (see also SI Appendix):

$$P = e^{\sqrt[3]{\frac{10}{3}\pi}\frac{E_0 l(V^{\frac{1}{3}} - V_0^{\frac{1}{3}})}{\Delta c_0 RT V^{\frac{1}{3}} V_0^{\frac{1}{3}}}} \cdot \Delta c_0 RT \frac{V_0^{\frac{1}{3}}}{V^{\frac{1}{3}}} \qquad [\mathbf{8}]$$

$V$ denotes the cell volume and $\Delta c_0$ is the initial, osmotically active solute concentration difference across the cell membrane, i.e. before the downshock.

The expression for osmotic pressure ([**1**]) and mechanical pressure ([**8**]), as well as Equation [**5**] enable us to fully characterize cell volume changes caused by the water flux in and of the cell.

Upon a sudden downshock and before the activation of MSCs, the environmental solute concentration is greatly smaller than the cellular solute concentration. Here we assume the membrane thickness and the cell surface area do not change considerably, thus the cell will expand at a fast rate and cell volume conforms to:

$$\frac{dV}{dt} = V_m K RT \left[ (\frac{n_i}{V} - c_e) - e^{\sqrt[3]{\frac{10}{3}\pi}\frac{E_0 l(V^{\frac{1}{3}} - V_0^{\frac{1}{3}})}{\Delta c_0 RT V^{\frac{1}{3}} V_0^{\frac{1}{3}}}} \cdot \Delta c_0 \frac{V_0^{\frac{1}{3}}}{V^{\frac{1}{3}}} \right] \quad [9]$$

Upon reaching a critical value, $V_{th}$, mechanosensitive channels open and cell volume can be described as:

$$\frac{dV}{dt} = (A+1) \cdot \frac{dV'}{dt} \quad [10]$$

Where $\frac{dV'}{dt}$ is the $\frac{dV}{dt}$ given in [**9**] and we use $A$ to characterize the relative conductivity of the membrane with opened channels. For example, $A = 2$ means 2 times higher conductivity compared to the cell membrane with closed mechanosensitive channels.

At the point of channel opening the water chemical potential difference is still large, with the inside lower than the outside. Thus, more water rushes into the cell through newly opened holes (Fig. 5 B, left). We note that water can flow both in and out of the cell through the channels, whose individual size reaches $\approx 3$ nm upon opening [35, 36]. During this period the osmotic pressure inside the cell further increases, as the inward flow of water exceeds the outward flow of cytoplasmic solutes (Fig. 5 B, left). At the critical point at which Equation [**10**] equals zero, inward pressure starts pushing both the water and the solutes out of the cell.

Simultaneously, the opening of mechanosensitve channels rendered the cell membrane permeable to solutes, which causes solute flux down the solute chemical potential:

$$j_s = D_s \frac{\partial c_s}{\partial x} \quad [11]$$

where $D_s$ is the diffusion coefficient of solutes and $c_s$ solute concentration. Taking into account the number of mechanosensitve channels ($N_{MSC}$) and the cross-sectional area of a representative mechanosensitive channel ($a_{MSC}$) we get the inner solutes outward flow (see also Fig. 5 B and SI Appendix):

$$\frac{dn_i}{dt} = -AV_M K \cdot \frac{n_i}{V} \cdot e^{\sqrt[3]{\frac{10}{3}\pi}\frac{E_0 l(V^{\frac{1}{3}} - V_0^{\frac{1}{3}})}{\Delta c_0 RT V^{\frac{1}{3}} V_0^{\frac{1}{3}}}} \cdot \Delta c_0 RT \frac{V_0^{\frac{1}{3}}}{V^{\frac{1}{3}}} - AD_s N_{MSC} a_{MSC} \cdot \frac{\frac{n_i}{V} - c_0}{l_M} \quad [12]$$

where $n_i$ denotes the total amount of internal solutes in moles and $l_M$ is the thickness of the cell membrane. We substitute the unknown parameters of the solute diffusion ($D_s$) and channel number ($N_{MSC}$) with a combined chemical flow parameter alpha normalized to the initial volume $V_0$:

$$\alpha = \frac{D_s N_{MSC} a_{MSC}}{l_M \cdot V_0} \quad [13]$$

We thus have:

$$\frac{dn_i}{dt} = -AV_M K \cdot \frac{n_i}{V} \cdot e^{\sqrt[3]{\frac{10}{3}\pi}\frac{E_0 l(V^{\frac{1}{3}} - V_0^{\frac{1}{3}})}{\Delta c_0 RT V^{\frac{1}{3}} V_0^{\frac{1}{3}}}} \cdot \Delta c_0 RT \frac{V_0^{\frac{1}{3}}}{V^{\frac{1}{3}}} - A\alpha V_0 \cdot (\frac{n_i}{V} - c_0) \quad [14]$$

As the solutes exit, the outward flow becomes larger than the inward flow of water and the cell begins to shrink. At the same time the overall concentration difference decreases further (Fig. 5 B).

$V_{th}$, the volume at which MSCs open and close is expected to be the same [17]. Thus, our model predicts that in order to reach the cell volume that is smaller than initial, as experimentally observed in Fig. 2 and 3, $V_{th}$ needs to be small.

The qualitative predictions of our model are given in Fig. 5 B and results of the fit to the equations [**10**] and [**14**] in Fig. 5 D, top and SI Appendix, Table S2. We chose a representative trace of 960 mOsmol downshock and used four parameters during the fitting routine (*Methods*): $A$, $V_{th}$, $\alpha$ and $K$. Some of the parameters in our equations are physical quantities that have been experimentally estimated and we used these estimates to fix them (SI Appendix, Table S2). For example, we experimentally measured $V_0 = 1.3 \pm 0.1\ \mu m^3$. The initial difference between internal and external concentration we used, $\Delta c_0 = 0.04$ Osmol/l, is based on the experimental estimates of turgor pressure and the thickness of the cell wall, $l$. Measured values for turgor pressure are 300 kPa [7] and 29 kPa [5], so we use the in-between value, 100 kPa, to fix $\Delta c_0$. Thickness of the cell wall was measured to be $\approx$ 5nm [37]. For the normalized volume at which the mechanosensitive channels open, best fit yields $V_{th} = 1.04 \pm 0.01$. Fig. 5 C gives predictions of our model in a scenario where one of the model parameters changes, while others are kept fixed. For example, the double mutant strain is expected to have smaller $A$ and $\alpha$, as these parameters describe the water and solute conductivity of the membrane with mechanosensitve channels opened. Our model predicts that with smaller $A$ and $\alpha$, the cell volume expansion increases and 'overshoot' decreases (Fig. 5 C), as is seen in the double mutant's response to the downshocks (Fig. 2, right). To test our model predictions further, we performed the fit to a representative trace of the double mutant strain, considering the same shock magnitude as for the wild type (960 mOsmol). Fig, 5 D, bottom and SI Appendix, Table S3 show the results of the fit. In line with our expectations, $A$ and $\alpha$ obtained from the best fit are smaller in comparison to the wild type. The best fit yields $V_{th}$ for the double mutant is $1.083 \pm 0.001$. Increase in $V_{th}$ in Fig. 5 C results in higher cell volume expansion, but smaller overshoot, consistent with double mutant response in Fig. 2, right. SI Appendix, Fig. S7 shows the fit to all of the average traces obtained for the wild type and the double mutant at different shock magnitudes (Fig. 2). Good agreement between the experimental results and the fits are visible across all the conditions. Fit parameters are plotted against the shock magnitude at the bottom of SI Appendix, Fig. S7. $A$ and $\alpha$ are higher for the wild type than the double mutant across all shock magnitudes. The inverse is true for the $V_{th}$, which is higher for the double mutant when compared to the wild type. $V_{th}$ obtained for the wild type at higher shock magnitudes saturates, in agreement with expectations.

Fig. 6 B, left shows a prediction from the fit against the experimental data of the representative, wild type cell volume trace shown in Fig. 5 D at later time points, i.e. minutes after

the downshock. At later time points experimental data show disagreement with the fit predictions.

To examine the possibility that the observed disagreement is due to the contribution of active transport processes, we exposed *E. coli* grown in media of 1130 mOsmol to a sudden downshock by transferring them into sodium phosphate buffer supplemented with 5 mM potassium chloride (Fig. 6 A). As *E. coli* can not grow in 5mM KCl only, the increase of volume observed in Phase III in Fig. 6 A is likely due to active potassium import, presumably by some of the components of the osmoregulatory network responsible for the recovery from a hyperosmotic shock, such as Trk, Kup and Kdp pumps [2]. We also hypothesize that if the disagreement between our model predictions and the experimental observation is due to active transport processes, cells subjected to a downshock in buffer media will not show an increase in cell volume upon reaching $V_{min}$. Traces in SI Appendix, Fig. S8 show the downshock response of cells in rich MM9 media (taken from Fig. 2) against the cells subjected to the same shock magnitude in the buffer only. Four different shock magnitudes were selected and in each Phase III of the recovery response is absent in the buffer, in agreement with our hypothesis. We further examined if the addition of active transport component into our model (SI Appendix) recovers Phase III observed in the experimental trace in Fig. 5D. Fig. 6B, right shows the result of the fit to the extended model. The fit is in very good agreement with the representative experimental trace.

**Growth rate upon downshock does not depend on the shock magnitude.** SI Appendix, Fig. S9 shows growth rates of individual cells after experiencing and recovering from the downshock of a given magnitude (Phase IV, SI Appendix, Fig. S2), as well as population growth curves and growth rates of cells grown at high osmolarities (up to OD 0.25-0.4) and subjected to a sudden downshock (*Methods*). In both cases growth rate does not depend on the magnitude of the downshock. Doubling time of individual cells observed in the microscope tunnel slide and the population growth rate measured in the plate reader are similar, $0.6\ h^{-1}$. SI Appendix, Fig. S9, C shows lag time of population growth curves upon a downshock of a given magnitude. Both the wild type and the double mutant exhibit longer lag times with higher shock magnitudes, where the double mutant lag time sharply increases for the two largest shocks.

We examined the survival rate of the wild type and the double mutant strain in order to compare them with previous reports [30, 31]. We consider a cell to be lysed if during the experimental time its total fluorescent intensity drops to background levels (*Methods*). SI Appendix, Fig. S10 A shows probability density of a cell lysing during 75 min experimental recording time, for each shock magnitude. In SI Appendix, Fig. S10 B we classify the wild type, and in SI Appendix, Fig. S10 C double mutant cells, into bursters, faders, rupturers and blebers following previously described definitions [30]. The percentage and type of cell lysis at our local flow rate of 0.68 $\mu$l/min is consistent with previously published studies [30]. The number of lysed cells is larger for the double mutant and for the double mutant lysis starts at lower downshock magnitudes in agreement with previous reports [30,31].

## Discussion

By monitoring changes in volume of individual cells we were able to explain *E. coli's* response to sudden decreases in external osmolarity. We found that upon a sudden downshock cell volume expands within≈ 30 sec irrespective of the shock magnitude. The volume expansion increased with shock magnitude and saturated at ≈ 15%. Previous estimates of the material properties of the cell wall used atomic force microscopy (AFM) to show peptidoglycan expansion of ≈12% per 1 atm [39]. This estimate is in agreement with our observation. Our results indicate that *E. coli's* membrane can expand beyond what is expected for a lipid vesicle. The result is in line with a recent study that showed *E. coli's* spheroplasts can increase their volume by more then three times, presumably by maintaining membrane reservoirs [40].

Despite the fact that mechanosensitive channels open on millisecond time scales, as observed in *in vitro* experiments [28, 29], the total cell volume recovery is significantly slower, taking minutes to complete. We reasoned that this could be caused by either hindered water transport across *E. coli's* membrane, as a result of growth at high osmolarity or as a consequence of the downshock, or by slow solute efflux. We found evidence for the latter. Our mathematical model considers post-downshock water and solute transport according to chemical potential difference, and takes into account cell wall stress stiffening properties. The model is in agreement with experimental data and suggests that post downshock, water rushes into the cell cytoplasm down the chemical potential gradient. Cell volume expands and increases tension in the cell wall, which results in opening of MSCs. At the point of channel opening, even more water flows into the cell further increasing wall tension. This effectively pushes the solutes, as well as the water out of the cell. Furthermore, opening of the channels renders the membrane permeable to the solutes, which now move down the solute chemical potential as well. At a critical point water no longer enters the cell, but starts to exit.

Our experimental traces show clear 'overshoot' during volume recovery that increases with shock magnitude. The observation suggests relatively low threshold for channel opening and closing. The fit of the representative trace in Fig. 5 D to the equations in our mathematical model predicts that MSC channels open/close at 4% volume expansion in the wild type strain. This is a relatively small value given the extent of maximum volume expansion we observe (15%). In the model we have assumed, guided by the *in vitro* studies, that MSCs open and close at the same lipid bilayer tension [17]. The $V_{th}$ value predicted by the model is thus a result of balancing the observed $V_{n,max}$ with $V_{min}$. However, it is possible that in a live cell the $V_{th}$ at which the channels open and close is not the same. In particular, since the response is the combined effect of seven different MSCs, where the number of individual channels of a given type can vary, and cooperative channel gating effects are possible [41–43].

When comparing the volume expansion of the mutant strain lacking MscL and MscS to that of the wild type, we found no difference for shocks up to 790 mOsmol, at which point the double mutant strain expanded slightly more (≈ 20%) and slightly faster. Larger and faster expansion is consistent with our model. In the wild type strain, the peak volume expansion is reached several seconds later compared to the double mutant, as upon opening of MSCs water influx competes with solute efflux and water efflux. In the double mutant there is far less of such competition. Consequently, the water influx upon the downshock is larger and the cell volume expands more.

Overall, we found the most obvious differences between the double mutant and the wild type response at large shocks. The result is in agreement with previous population studies [31]. However, small differences between the response of the wild type and the double mutant strain are visible even for smaller shocks. For example, some 'overshoot' is observed even for smaller shocks in the wild type strain, but none is visible in

the double mutant response. For shocks $\geq$ 790 mOsmol double mutant showed no growth within the 35 min observation period. At this point, we do not understand the nature of the damage caused by the absence of MscS and MscL, nor if it is reversible. However, our results show that lack of MscS and MscL does not result solely in instant rupture or bursting. In fact, most of the non growing double mutant cells that were subjected to 960 mOsmol and 1130 mOsmol downshock do not show any obvious damage as observed by fluorescent microscopy. It is possible that these cells continue growth, but at a later time.

Based on our fit parameters in the case of the wild type trace given in Fig. 5D, MSCs will open at 4% volume increase, which corresponds to an increase in cell wall and membrane tension of $\approx$ 7 mN/m, which is in rough agreement with the *in vitro* measurements of channel opening membrane tensions, usually 5-15 mN/m [44,45]. At our measured $V_{max}$, and based on our fit parameters, the cell wall and inner, outer membrane tension increases by $\approx$ 30 mN/m.

Our study reveals the main characteristics of *E. coli's* passive response to downshock, namely fast volume expansion followed by a slow volume recovery that can 'overshoot' below the pre-shock volume. Additionally, we observed a degree of cell to cell variability, not only for cells exposed to a given downshock, but also when comparing the trends between different shock magnitudes. The observed heterogeneity may be expected, as recent studies show significant variation in MscL numbers within the population, as well as between different growth conditions (including growth on glucose in different external osmolarities) [41]. Apart from MscL, the case could be similar for the the six other MSCs in *E. coli*. Additionally, for the large number of MscL channels measured (up to $\approx$ 1000) [41], cooperative gating is possible [42], and could further increase cell to cell variability in the overall downshock responses.

**Fig. 1.** Characteristic cell volume response to a sudden downshock. (A) Upon a sudden decrease in external concentration cell volume expands, which leads to opening of mechanosensitive channels. Consequently, solutes exit the cell, allowing recovery of cell volume through loss of cytoplasmic water. (B) A characteristic single cell volume response for a 1130 mOsmol downshock. The trace was normalized by the initial volume, i.e. the volume before the downshock. Different phases of the recovery response are indicated with different colors. In gray is the expansion phase (Phase I), followed by two volume recovery phases. Phase II in orange is characterized by volume decrease and Phase III in green, by volume increase upon reaching the minimum volume. Phase IV in purple indicates recommenced growth. Initial 15 min are sampled at 5 Hz and an additional 1 h at a frame every 5 seconds. (C) Still images from different phases in (B). $T_0$ is the very beginning of the recording, prior to the downshock. $T_1$=45 sec, $T_2$=4 min, $T_3$=35 min and $T_4$=70 min. Red lines are drawn to indicate the size of the cell prior to the downshock. In comparison, the cell size at $T_1$ is slightly larger (Phase I), at $T_2$ smaller (Phase II), at $T_3$ it reaches the initial size (Phase III) and at $T_4$ it is significantly larger (Phase IV).

**Fig. 2.** Cell volume response of the wild type cells at different downshock magnitudes shows slow volume recovery and an 'overshoot'. We use descriptive statistics to present our data sets and plot average volume traces with standard deviations constructed from 609 (wild type) and 480 (double mutant) traces as a function of time. A zoom in to first five minutes of downshock response sampled every 0.375 sec is given. 64 (wt) and 66 (dm) cell were used for 103 mOsmol, 94 (wt) and 52 (dm) cell were used for 190 mOsmol, 66 (wt) and 54 (dm) for 390 mOsmol, 56 (wt) and 80 (dm) for 460 mOsmol, 90 (wt) and 68 (dm) for 790 mOsmol, 116 (wt) and 50 (dm) for 960 mOsmol and 106 (wt) and 77 (dm) cells for the 1130 mOsmol downshock. Volume expands in all conditions and increases with the shock magnitude. Slow recovery follows volume expansion. For the wild type cells volume drops below the initial value, increasingly so with the larger shocks.

**Fig. 3.** Analysis of maximum and minimum volume and time. (A) Box plot of maximum volume, $V_{n,max}$, and (B) the time at which maximum volume is reached, $T_{max}$, as a function of shock magnitude. The wild type is shown in blue and the double mutant in red. The upper/lower whiskers indicate 1.5 times the standard deviation value. The upper/lower edge of the boxes indicate the $3^{rd}/1^{st}$ quartile. The black line indicates the median and the yellow line the average value. $V_{n,max}$ increases with the shock magnitudes and saturates at and above 790 mOsmol. $T_{max}$ is independent from the shock magnitude for the wild type (blue) and slightly smaller for the double mutant (red) for the two largest shock magnitudes. (C) $V_{n,min}$ and (D) $T_{min}$ plotted against the shock magnitude for wild type (blue) and double mutant (red). $V_{n,min}$ in (C) is slightly below 1 for the wild type and decreases with the shock magnitude. $V_{n,min}$ for the double mutant stays above 1 in all conditions. $T_{min}$ increases with the shock magnitude for both wild type and double mutant cells.

**Fig. 4.** Cells, either grown at high osmolarities or subjected to a downshock, were exposed to a subsequent upshock. Normalized, average volume of (A) 13 and (B) 30 cells plotted against time in seconds. Standard deviation is given as a shaded area in light purple. (A) Cells grown in media of high osmolarity, 1370 mOsmol, were subjected to a further increase of external osmolarity (upshock of 1272 mOsmol). Arrow indicates the time at which shock was administered. Cell volume decreased within seconds post hyperosmotic shock. (B) Cells grown in the same media were subjected to a 1130 mOsmol downshock indicated with the first arrow. Upon the downshock volume expanded. $\approx$ 1min after the downshock cells were exposed to a strong upshock of 2160 mOsmol, indicated by the $2^{nd}$ arrow. Upon the upshock cell volume decreased within seconds.

**Fig. 5.** Model of cellular response to a sudden downshock. (A) A sudden decrease in external osmolarity leads to cell volume expansion and opening of mechanosensitive channels (panel 1 and 2). Upon channel opening, the water flux into the cell increases, as the water now flows inwards through the channels as well (panel 3, blue arrow). Consequently, solutes exit the cell down the solute chemical potential and due to increased pressure inside the cell (panel 3, blue and red arrows). Solute efflux through the channels tips the competition between water influx and efflux towards efflux, which allows the recovery of cell volume to proceed (panel 4, blue arrow). (B) Cell volume (black), water influx (blue), water efflux (orange) and solute efflux (red) are given against time for the wild type (left) and the double mutant (right). All are solutions to the mathematical model equations using four free parameters obtained from the best fit to the representative cell volume trace in 960 mOsmol downshock condition. Equation $\mathbf{[10]}$ was used to plot the cell volume. First part of Equation $\mathbf{[10]}$ was used for the water influx and second part of Equation $\mathbf{[10]}$ for the water efflux. Equation $\mathbf{[14]}$ was used for solute efflux. Vertical gray lines indicate following events in sequential order: osmotic shock, opening of the mechanosensitive channels, the point in time when $V_{max}$ is reached and closing of the channels. (C) Cell volume as predicted by the mathematical model given as a function of time. Parameters used in the best fit to the average cell volume in 960 mOsmol downshock condition were varied by $\pm$100% for all parameters. Only one parameter is varied at a time, the others are kept fixed. Green color indicates the lowest value used and red the highest (color scale is given on the right). Increasing $V_{th}$ increases $V_{max}$, but lowers $V_{min}$. Increasing $\alpha$ and $A$ decreases $V_{max}$ but increases $V_{min}$, while increasing $K$ and $\Delta C_0$ increases $V_{max}$ and decreases $V_{min}$, with a stronger effect on the $V_{min}$ reduction. $E_0 l$ increase has little effect on $V_{max}$ but it decreases $V_{min}$. (D) Representative trace of the wild type (top) and the double mutant (bottom) for the 960 mOsmol condition is given in black. Blue line shows the result of best fit to the average trace. Shaded orange regions show fit confidence intervals, from darker to lighter orange these are: 50%, 90%, 95%, and 99% . There is a good agreement between the model and the experimental data.

**Fig. 6.** Active response and post-shock growth rates. (A) Black line shows average volume against time of 36 wild type strains exposed to a 1310 mOsmol downshock. Cells were grown in MM9 supplemented with NaCl and transferred into sodium phosphate buffer supplemented with 5 mM KCl. Shaded orange region indicates standard deviation. Cell volume expanded and recovered, dropping below the initial volume. Within the last 30 min volume increase is visible. Inset shows an example of an individual trace, where volume increase occurs after $V_{min}$ is reached at $\approx$ 60min post downshock. (B) Left: Wild type representative trace (black) and the result of the global fit (blue) taken from Fig. 5D and shown on a longer time scale. Right: The fit (blue) is performed with the addition of the active pumping component (see SI Appendix for details on the extended model) and plotted against the same wild type representative trace shown on the left and in Fig. 5D (black).

## Materials and Methods

**Construction of E. coli strains.** Strains BW25113 with pWR20 and $\Delta$MscL$\Delta$MscS (double mutant) with pWR21 plasmid were used in the study for single cell fluorescent microscopy experiments. BW25113 is the Keio collection parent strain [46]. The plasmids pWR20 and pWR21 carry genes for constitutive expression of enhanced green fluorescent protein (eGFP) and kanamycin (kan) or ampicilin (amp) resistance respectively [3]. $\Delta$MscL$\Delta$MscS strain was constructed from BW25113 single knockout strains with the **mscL** or **mscS** genes replaced with kan cassette (available from Keio collection) [46]. After PCR verification of both single mutants, kan resistant **mscL** mutant was transformed with pCP20 plasmid to eliminate the antibiotic resistance [47]. The deletion of the kan cassette and the plasmid curing was verified by kanamycin, chloramphenicol and ampicillin sensitivity tests. Subsequently, P1 vir lysate was prepared from $\Delta$MscS strain carrying kan cassette, here used as the donor, for the transfection of the recipient strain, $\Delta$MscL. P1 tranductants were plated onto selective media (LB containing 50 $\mu$gmL$^{-1}$ of kanamycin). $\Delta$MscL$\Delta$MscS strain was verified by PCR and transformed with pWR21 plasmid.

**E. coli Growth and culturing.** **E. coli** strains were grown from frozen stocks (made from single colonies) in MM9 medium with 0.3% glucose, MEM essential amino acids (Sigma Aldrich) and supplemented with 0, 50, 125, 225, 300, 450, 550, 650 or 750 mM NaCl at $37\,^{\circ}$C to an OD of 0.2-0.4, aerobically with shaking (see also SI for media osmolarities). MM9 (**M**odified M9) is of the same composition as M9 [48] except sodium phosphate buffer only was used, and the media were supplemented with 1 mM KCl. MM9 was chosen over M9 to allow adjustment of potassium concentration from zero to the desired value. Media was supplemented with 50 $\mu$g/mL kanamycin. Upon reaching OD of 0.2-0.4 cells were kept at room temperature and used for sample preparation for up to 4 hours (up to maximum OD of 0.65). For SI Appendix, Fig. S5 cells were grown as above except in MM9 supplemented with 450mM sucrose. Growth curves in SI Appendix, Fig. S9 were obtained as follows: 300 $\mu$l of MM9 medium supplemented with 50, 125, 225, 300, 450, 550, 650 or 750 mM NaCl was inoculated with 2 $\mu$L of wild type and double mutant cells from frozen stock. Optical density was measured in a plate reader (BMG, Germany) every 7.5 min at $37\,^{\circ}$C, until stationary phase. To obtain growth curves after the downshock cells were initially inoculated as above. At an early exponential phase (OD 0.2-0.35) 2 $\mu$l of cells from each growth media were transferred into a well with MM9 medium with no additional salt. Optical density was measured every 7.5 min until stationary phase.

**Microscopy.** Cells were observed in epifluorescence using a Nikon Eclipse Ti microscope with perfect focus [4] at $21\,^{\circ}$C . At the beginning of each experiment, a field of view with 10 or more 'flat' cells was chosen as described in [3] (see also SI Appendix). Images of cells expressing eGFP were acquired at an exposure time of 0.1 seconds using a $512 \times 512$ pixel back-thinned electron-multiplying charge-coupled device camera (Andor Technologies). Epi-illumination light was shuttered in between image recordings to reduce photobleaching (SI Appendix, Fig. S11). Images were captured at every 0.2 s or 0.375 s during the first 15 min and every 5 s for the rest of the recordings. Total recording time was up to 75 min.

**Sample preparation and osmotic shock.** Microscope tunnel slide was prepared as in [49]; cells were attached to the cover slip surface as in [50]; and to administer a sudden osmotic shock (either downshock or upshock) the tunnel is flushed with 25 $\mu$l of the low or high osmolarity medium, with an average rate of 2.5$\mu$/l as in [3, 49]. To characterize the characteristic noise in the downshock experiment we performed a control flush. 3D histogram in Fig. S11, obtained from cells grown in MM9 and flushed with MM9, shows that the noise magnitude is small for the scale of volume changes we are observing. Osmotic shocks in microfuge tubes were performed in two different ways as follows. (1) Cells from grown cell culture were imaged in a tunnel slide before the downshock, no attachment was used. 1.3 ml of cell culture was then spun down and growth media was removed. Tunnel slide was prepared as above and placed in the microscope. Cells were downshocked by adding 0.1 ml of MM9. 10 $\mu$l of downshocked cells was flushed into the tunnel slide. Thirty different fields of view were chosen and cells were imaged at a frame every 1 min for 1.5 hours. (2) Several different tunnel slides were prepared before start of the experiment. Cells from the growth culture were imaged prior to the downshock with no attachment. 1 ml of cell culture was spun down and growth media was removed. Cells were shocked into 1 ml of MM9 and kept in the microfuge tube. At 2.5, 10, 20 and 30 min post downshock samples were added to the prepared tunnel slides for imaging.

**Speed of local flow for downshock delivery.** A green fluorescent dye (10$\mu$M Sodium Green (Molecular Probes) prepared in 10mM Tris buffer) was used to determine the local speed of downshock delivery. Microscope tunnel slide was prepared as described above. A sudden shock was administered by flushing 25$\mu$l of Sodium Green dye through the channel at an average flow rate of 2.5 $\mu$l/s while recording at a frame every 0.2 second. The dye was both flushed in and subsequently flushed out several times. To obtain the local flow rate close to the cover slip surface we started with transforming fluorescent intensity in arbitrary units to $\mu l$ by taking into account the size of the field of view, the number of pixels in the image frame and the size of the channel. Next, the difference in intensity between two adjacent frames was calculated to get the flux across a unit surface. We obtained the local flow rate of 0.68 $\mu$l/min as an average of the linear fits of the intensity profiles given in SI Appendix, Fig. S12.

**Image analysis.** Data analysis was performed on cells uniformly attached to the cover slip surface ('flat' cells) by a process of background subtraction and thresholding as described in [3] and SI Appendix. Cells stuck to the surface were assumed to be a spherocylinder (2 $\mu$m long and 1 $\mu$m in diameter) and cell area obtained from image analysis was converted to volume according to the formula $V(t) = S(t)^{\frac{3}{2}} \cdot \frac{\frac{10}{3} \cdot \pi}{(4+\pi)^{\frac{3}{2}}}$, see also SI Appendix, Fig. S11. When analyzing cells that were downshocked in the microfuge tube and imaged with no surface attachment, cells that were close to the cover slip surface and did not move significantly were chosen for time lapse analysis.

**Singe cell data analysis.** Traces recorded at a frame every 0.2 s were re-sampled to 0.375 s and further analyzed with the traces recorded at 0.375 s. Volume traces obtained upon image analysis were normalized. We used average volume of first 5 data points, corresponding to the first 1.8 s of recording. Next, normalized traces were passed through a median filter with the width of 5 points per window frame. Normalized and filtered traces were aligned by $T_{max}$ in Phase I. Total of 609 cells were analyzed for the wild type and 480 for the double mutant. To obtain the growth rate from single cell measurements in SI Appendix, Fig. S9 B, we analyzed the Phase IV part of the average traces given in SI Appendix, Fig. S1. The part of the trace starting at the beginning of Phase IV, i.e. when cell volume reaches 1, was fitted to an exponential. To obtain the cumulative bar diagrams in SI Appendix, Fig. S10 we used definitions of cell lysis types described before [30]. We assumed a cell to be lysed if the cell's fluorescent intensity dropped to background level during our observation. The cells that lysed during our recording were included in the $V_{max}$ analysis only, but excluded from the rest of our analysis. $V_{n,max}$ is the average value around the absolute maximum value identified in each trace (we averaged 5 points either side of the maximum value). $T_{max}$ is the difference between the start of the shock and the point in time $V_{n,max}$ is reached. To identify $V_{n,min}$ and $T_{min}$ in the wild type traces we apply a running window (10 points wide) starting from $T_{max}$ and identify

the minimum position within the trace. The double mutant traces often leveled off rather then continuously decreased. Thus, we modified our algorithm and identified if the average value of the current running window decreases below the average value plus three standard deviations of the neighboring window. Once the condition is no longer satisfied $V_{n,min}$ (and $T_{min}$) in the trace has been reached.

**Growth curve analysis.** OD measurements were converted to cell density as follows. 30 wells of 200 $\mu$l bacterial culture were grown to OD 0.15 in MM9 medium. The wells were pooled and 125$\mu$g/ml chloramphenicol was added to inhibit further cell division or growth. Cells were then concentrated to approximately 20X initial concentration, diluted down to 0.0625, 0.125, 0.25, 0.5, 1, 2, 3, 5, 7, 10 and 11 times initial concentration. OD was measured in the plate reader using the same conditions as the growth curves. Cells at 1x concentration were counted using bright-field illumination and in a tunnel slide of known height (100 $\mu$m) to give the true cell density. Obtained calibration curve is given in SI Appendix, Fig. S13. A second degree polynomial, was used to convert the growth curve ODs to cell number ($N = 2.2 \cdot 10^8 OD^2 + 1.45 \cdot 10^9 OD + 2 \cdot 10^6$) [51]. Calibrated growth curves were fitted using a Gaussian fitting algorithm [52] to give both the maximum growth rate and lag time as defined by the tangent to the inflection point in each condition shown in SI Appendix, Fig S9.

**Model fitting procedures.** To simulate the model, Equations **[10]** and **[14]** were used to calculate the volume and moles of internal solute during the downshock. To provide the initial volume for the model we used the measured $V_0$, and the initial value for $n_i$ was based on previously published results [7]. The opening of mechanosensitive channels was simulated by a conditional statement such that when $\frac{V}{V_0} > V_{th}$ A is positive, and zero otherwise. An adaptive Markov chain Monte Carlo sampling procedure [53] was used to fit the model to the averaged wild type and double mutant traces for the 960 mOsmol downshock. During fitting, simulation of the model was performed by the ode45 function in Matlab [54]. Fig. 5 D shows the posterior confidence intervals of 50,000 simulations that comprised the Markov chain for the final parameters listed in SI Appendix, Table S2 and S3. Upon obtaining t he best fit, we used the fit parameters to generate Fig. 5 B. Water influx was obtained from the first part of the equation **[9]** before channels open and **[10]** after. Similarly, water efflux was obtained from the second part of **[9]** and **[10]**. Solute efflux was obtained from **[14]**. To obtain Fig. 5 C we have varied the parameters obtained from the best fit to the wild type data by $\pm$ 100% for all parameters, with a step size of 10%. Total cellular volume was plotted by solving equation **[10]**.

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