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Supplementary information

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Survival dynamics of starving bacteria are determined by ion homeostasis that maintains plasmolysis

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Supplementary Note: Mathematical Model

Introduction

In this section we describe the phenomenological simulation that we used in the main text in Fig. 2. The model is based on the idea that the bacterium must perform ion homeostasis in order to remain viable. The goal of the model is to understand a) why the loss of ion homeostasis is rapid and random c) why individual bacteria are in plasmolysis during starvation, despite energy for pumping being limited.

All cell have to deal with the problem that their inventory of impermeable molecules (proteins, RNA, DNA etc.) sets up an unstable osmotic condition that would lead to swelling of the cytoplasm in equilibrium[10]. This so-called Donnan effect, and how cells counteract it, has received much attention in the modelling world[11–14]. A full description of ion homeostasis is complex, parameter rich, and requires assumptions that, with the limit information available, would be impossible to get right. For example, we do not know which types of pumps are active during starvation, what the internal ion concentrations in starvation are, what the electric potential is, how permeable the cell is to the individual ions and how to correctly account for the periplasmic space.

For this reason, we focus on a minimal model that includes the Donnan effect and how cell counteract it by active pumping. We refer the reader to the literature for a complete discussion of multi-ion and electric effects.

Formulating the phenomenological osmoregulation model

Osmotic pressure

Osmotic pressure is given by the concentration difference of all solutes between the inside and the outside of a membrane

$$\Pi = RT\left(\sum c_{\mathrm{in},i} - \sum c_{\mathrm{out},i}\right), \tag{S1}$$

where R is the gas constant and T temperature. Many of the internal solutes are non-permeable, such as proteins, RNA, DNA and metabolites. These solutes, together with their positive counterions (because most macromolecules are net-negatively charged) form a set of solutes that are trapped in the cytoplasm of a viable bacterium, whose concentration we call c_{biomass}. The vast majority of solutes from the medium (more than 99%) on the other hand are ions such as K^+, Na^+, Cl^- or PO^{4-}, that can diffuse across the membrane. We do not know the precise nature of the permeability, and in particular we do not know the rate at which these metabolites permeate the membrane. But typically, any cell keeps the concentration of Na^+ low, while keeping K^+ high to balance charges from biomass. To avoid having to describe the dynamics of all ions, we instead subsume them into an effective 'ion concentration', that is outside c_{out} and inside c_{in}, such that we can write osmotic pressure as

$$\Pi = RT(c_{\mathrm{biomass}} + c_{\mathrm{in}} - c_{\mathrm{out}}). \tag{S2}$$

When the osmotic pressure in the cytoplasm becomes negative, e.g., if the bacterium is pumping ions out of the cytoplasm, the cytoplasm will contract such that the internal concentration with

the new, reduced volume matches the outside concentration, $c_{\mathrm{biomass}} + c_{\mathrm{in}} = (n_{\mathrm{biomass}} + n_{\mathrm{in}})/V_{\mathrm{cytoplasm}} = c_{\mathrm{out}}$. This state of contracted cytoplasm is called plasmolysis, and the steady state maintenance of concentration gradients between inside and outside is called ion homeostasis.

As a simplification, we will not describe osmotic pressures and cytoplasmic volume changes explicitly in our model but instead focus only on the dynamics of the internal solutes. This removes the need to keep track of cytoplasmic volume changes. We further use an approximation of the internal concentration as $c_{\mathrm{in}} = n_{\mathrm{in}}/V_{\mathrm{cytoplasm},0}$, where we normalize the internal ion abundance n_{in} by the volume of the cytoplasm in the non-contracted state, $V_{\mathrm{cytoplasm},0}$. We further disregard tension in the inner membrane.

Ion homeostasis

One fundamental problem of ion homeostasis is that ions can diffuse across the membrane and equilibrate the ion gradient. Thus, ion homeostasis requires transport of ions against the concentration gradient, which according to the second law of thermodynamics requires energy to be dissipated in the process. According to Fick's law, the diffusive influx j_{in} is set by the permeability P and ion gradient

$$j_{\mathrm{in}} = P(c_{\mathrm{out}} - c_{\mathrm{in}}). \quad \text{(S3)}$$

Here, we use the total influx of ions into over the bacterial surface, rather than using the definition of permeability being defined per area. Thus, permeability scales with cell surface area, $P \propto S$. As an important simplification we omit electrical terms in Eq. S3. Electric terms would be positive or negative, depending on the ion species they describe. This simplification allows us to describe the internal concentration of osmolytes with a single parameter, while at the same time not imposing assumptions on diffusion and active transport of the individual ions.

For homeostasis to be maintained, this influx must be balanced with an outflux of equal magnitude, $j_{\mathrm{out}} = j_{\mathrm{in}}$.

Permeability of plasma membranes to inorganic ions increases if the membrane is stretched [15–17]. Because from TEM images we know that the cytoplasmic membrane in starvation is under tension – the shape follows a half-sphere in the area where the membrane is detached from the pole (Extended Data Fig. 2C) – and we know any increase in internal solute concentration will lead to an increase of cytoplasmic volume and thus stretching of the membrane, we implemented stretch-activated permeability. Because stretching will increase with cytoplasmic volume, and cytoplasmic volume depends on the abundance of internal ions, n_{in}, we expect permeability P to be a function of n_{in} or alternatively $c_{\mathrm{in}} = n_{\mathrm{in}}/V_{\mathrm{cytoplasm},0}$. Early in starvation, where there is an excess in membrane material (Extended Data Fig. 2C), we see permeability stain PI increasing linearly (Extended Data Fig. 3D), so we suspect the functional form of P to be constant for low c_{in} and increasing around some critical value when stretch-activation sets in. Eventually, once the membrane is stretched beyond such that holes form, it should be fully permeable. For this reason we implemented permeability as a Hill function with constant offset,

$$P(c_{\mathrm{in}}) = P_0 + P_1 \frac{c_{\mathrm{in}}^n}{K_1^n + c_{\mathrm{in}}^n}. \tag{S4}$$

The choice of using a Hill function allows us to adjust the steepness using the Hill parameter n in the simulation. We further make the simplifying assumption that permeability for a given internal ion concentration is constant over time.

Cannibalistic nutrient recycling

To balance the diffusive influx of ions from the medium, the cell must do active transport, which requires ATP by consuming nutrients. These nutrients can either come from a limited amount of storage or from recycling of biomass from perished bacteria. The latter, the 'cannibalistic' recycling, is a central aspect of starvation survival[5]. If we assume all bacteria to be equal, then we can describe the nutrient resource of a single cell ν as being supplied by the death of bacteria and consumed by viable bacteria. If α is the amount of nutrients, called the recycling yield [5], that can be recycled from a perished cell, then the total nutrient supply will be $-\alpha\dot{N}$ where $-\dot{N}$ is the number of bacteria that die per time. Because bigger cells contain more nutrients, shown in Ref. [5], the recycling yield scales with cell volume, $\alpha \propto V$. These nutrients will be divided equally among all viable cells N if the cell density is high enough to ensure that nutrient concentrations lie above the transport affinity. We experimentally found that this assumption only breaks down below a density of around 10^5CFU/ml, as evident by a faster death compared to higher concentrations. Because we perform our experiments in the range of 10^9 to 10^7 CFU/ml, and in this regime it was shown that no nutrients accumulate in the medium[5] we can safely assume complete nutrient recycling in our experiments.

The provided nutrients are consumed by maintenance at rate β. Taken together, we can describe the dynamics of the nutrients as

$$\frac{\mathrm{d}\nu}{\mathrm{d}t} = -\alpha \frac{1}{N}\frac{\mathrm{d}N}{\mathrm{d}t} - \beta\,. \tag{S5}$$

We assume that a fraction ϕ of the maintenance rate is used for pumping ions against the gradient. To account for pumping to cease if c_{in} gets too small, we implement that $\phi(c_{\mathrm{in}})$ decreases linearly to zero at low concentrations following a Michaelis-Menten type function. At high concentrations, on the other hand, we suspect that once the membrane gets stretched, pumping ceases as ATP and other energy-rich molecules gradients disappear. To have this ceasing of pumping coincide with stretch-activated permeability, because both effects depend on the same physical stretching of the membrane, we choose the same dependence on c_{in}

$$\phi(c_{\mathrm{in}}) = \phi_0 \frac{c_{\mathrm{in}}}{K_2 + c_{\mathrm{in}}}\left(1 - \frac{c_{\mathrm{in}}^n}{K_1^n + c_{\mathrm{in}}^n}\right). \tag{S6}$$

We further absorb the proportionality constant of the cost of pumping (called κ in the main text) into ϕ and write the active pumping flux of bacteria as

$$j_{\mathrm{pump}} = \phi(c_{\mathrm{in}})\beta. \quad \text{(S7)}$$

In principle, bacteria likely use nutrients for processes other than survival. For example, in Ref. [5] it was shown that *E. coli* performs proteins synthesis in starvation, but that this protein synthesis is not essential for survival, and preventing it does not decrease death rate. Therefore, it likely is not consuming significant amounts of resources, but in principle, any such processes will decrease the amount of nutrients available for maintenance of ion homeostasis. Since we do not know the functional form of these alternative processes, and we aim to keep the model lean, we omit them here.

Dynamics of cytoplasmic solutes

Using the combination of diffusive influx and active pumping, we can calculate the dynamics of the internal ion concentration c_{in}.

$$\frac{\mathrm{d}c_{\mathrm{in}}}{\mathrm{d}t} = P(c_{\mathrm{in}})(c_{\mathrm{out}} - c_{\mathrm{in}}) - j_{\mathrm{pump}} + D\xi(t) \quad \text{(S8)}$$

In addition, we assume that there is noise in the underlying biological processes that affect permeability or pumping. White noise $\xi(t)$ is the derivative of the Wiener process, $\xi(t) = \mathrm{d}W(t)/\mathrm{d}t$ and D is the diffusion constant. Here, we do not attempt to put noise on a physical basis, but use it as a parameter of the simulation.

Death

Finally, we need to define when a bacterium is called dead, in order to calculate death rate. Because death is due to lysis (Fig. 1), which is in turn due to an increase in internal ion concentration, we call a bacterium dead if there is a significant increase of the internal ion concentration, $c_{\mathrm{in}}/c_{\mathrm{out}} \gg 0$ that goes beyond just stretching of the cytoplasmic membrane $c_{\mathrm{in}} > K_1$.

Quasi-steady state of nutrient recycling

The result of the model is a two-dimensional system describing c_{in} and ν. It can be further simplified with a timescale separation.

In principle, nutrients will likely take time to be recycled and converted into ATP. Furthermore, uptake of nutrients will depend on the concentration of nutrients in the medium. But because we do not have microscopic knowledge about either of these, we make further coarse-graining assumptions to prevent unnecessary parameter bloat in our simulation. Metabolic reactions typically happen on the timescale of second to minutes, while the timescale of the population decrease is 1 day. Therefore, we assume that nutrient recycling is fast compared to the overall dynamics. Second, we coarse grain all nutrients (metabolites, proteins, DNA, RNA, lipids, etc) that are released by dying bacteria into a single nutrient ν and assume that the density is high enough such that these nutrients can be taken up efficiently. Evidence for this comes from the

observation that the decay of viability is exponential in the regime we are performing our experiments: In experiments where nutrients are washed away with microfluidics, death rate increases exponentially[18], presumably because of the inability of bacteria to perform maintenance. If in our case nutrient concentration where to become limiting, we would equally expect an increase in death rate. As this is not the case (see Fig. 1A), we believe that it is a reasonable assumption to omit the concentration dependence of nutrient uptake.

Given that nutrient release is slow, on the timescale of a day, while the death process is fast, loss of ion homeostasis, swelling and lysis happen on the timescale of an hour, we separate the timescales and assume that Eq. S5 is in quasi-steady state, during the dynamics, i.e., $\dot{\nu} = 0$. This allows us to omit parameters describing nutrient uptake rates and affinities and keeps the model lean. As a result of this timescale separation we can derive from Eq. S5 that $\beta = \alpha\gamma$, where $\gamma = -\dot{N}/N$ is death rate, which is the main result of Ref. [5]. Evidence that this separation of timescales is correct is the observation that nutrients contained in the entire population (viable cells, dead cells and supernatant) decrease linearly with viability as starvation progresses in Ref. [5]. Any violation of the steady state would lead to an accumulation of nutrients the supernatant and a non-linear relation between total nutrients and viability.

Plugging in Eq. (S7), we obtain

$$\frac{\mathrm{d}c_{\mathrm{in}}}{\mathrm{d}t} = P(c_{\mathrm{in}})(c_{\mathrm{out}} - c_{\mathrm{in}}) - \alpha\phi(c_{\mathrm{in}})\gamma + D\xi(t). \tag{S9}$$

To show that the assumption is indeed reasonable, and that we are not missing any dynamics, such as limit cycles, with our timescale separation, we ran the simulation also with explicit nutrient recycling (Extended Data Fig. 5). We found that the decrease of viability is indeed exponential and that nutrient resources ν are constant during the dynamics (Extended Data Fig. 5A-B). If we externally supply nutrients for a brief period, and let the system relax back (Extended Data Fig. 5C-D), we see that viability returns to the exponential decrease and nutrients return to their steady state. The characteristic lag in viability is akin to experimental results[5]. After the lag, the decrease of viability returns to an exponential decrease and constant ν, showing that the decay is a stable state.

We therefore conclude that, at least for the parameters chosen for our simulation, we can neglect the dynamics of the nutrients and focus on the internal ion concentration.

Simulation of the model

To run a stochastic simulation of the model, we implemented noise as a normal-distributed contribution to concentration of every cell i, where at each time-step $c_t^i = c_{t-1}^i + \Delta t \frac{\mathrm{d}c^i}{\mathrm{d}t} + \xi(D, \Delta t)\Delta$, where $\xi(D, \Delta t)$ is a random, normal distributed number with mean 0 and standard deviation $\sqrt{2D\Delta t}$. In addition, we simulated the nutrient resource for each individual cell by consuming all available nutrients at each time-step $\nu_{t-1} = \beta\Delta t$, and adding nutrients of the

number of bacteria that have died in the time-step, $-\Delta N$, proportionally to the remaining fraction of viable bacteria, $\nu_t = -\alpha\,\Delta N/N$.

The simulation was run using Eqs. S4 to S9 using the following parameters:

Parameter	Comment	Value	Unit
c_{out}	All concentrations normalized to c_{out}	1	1
P_0	Permeability times surface area, unstretched	5	d^{-1}
P_1	Permeability times surface area, stretched-activated contribution	15	d^{-1}
n	Hill coefficient	10	-
K_1	Half-maximum of stretch-activation	1/3	c_{out}
K_2	Half-maximum of pumping	1/6	c_{out}
$\alpha\phi_0$	Recycled nutrients that are used for pumping, divided by cost of pumping	16	c_{out}
D	Diffusion coefficient	0.1	$\frac{c_{\mathrm{out}}^2}{\mathrm{d}}$
c_{tresh}	Threshold at which a cell is called dead and its nutrients are distributed.	0.35	c_{out}

The results are shown in Fig. 2. With our parameter set, one can see that it is possible that individual bacteria have a low internal ion concentration during starvation, only to cross over rapidly and randomly towards equilibrium. Death rate is constant in the simulation, too, see Fig. 2. This closely matches our experimental observations that cells are in plasmolysis and seemingly randomly lose their polarization, swell and lyse (Fig. 1).

The fact that our simulation can capture the dynamics shows that our assumptions and simplifications were within reason. Considering electric terms, multiple ion species, delays in nutrient recycling and other factors are not essential to explain the dynamics, mainly because the Donnan-effect and stretch-induced permeabilization are independent of the species of the osmolytes. There is certainly room for follow-up theoretical works and experimental characterizations of these physical quantities, to allow a more thorough physical understanding of the effects.

Analytical approximation of death rate

One theoretical question remains. How does the system find its collective steady state and what determines the death rate?

One way of conceptualizing the dynamics of the system is mapping the problem onto a potential $U(c_{\mathrm{in}})$, where

$$\frac{\mathrm{d}c_{\mathrm{in}}}{\mathrm{d}t} = -U'(c_{\mathrm{in}}) + D\xi(t). \tag{S10}$$

In a physics setting, $-U'(c_{\mathrm{in}})$ would be the restoring force acting on c_{in}. This approach is akin to the classical Kramers' escape calculation, where a stochastic particle escapes from a potential

well. The reason why we map the stochastic problem onto the potential well is because we designed the simulation such that bacteria are (1) fluctuating around a low c_{in} during starvation and (2) to randomly and rapidly increase towards a high concentration, meaning lysis. This dynamic means that the potential has a local minimum at low c_{in}, where noise $\xi(t)$ can push the cell out and across the barrier towards high c_{in} and thus lysis. This 'hopping rate' in the classic calculation is Kramers' escape rate, which equals death rate in our case.

To understand the dynamics, we will discuss the shape of U. By looking at the zeros of $U'(c_{\mathrm{in}})$ we can understand the maxima and minima of the system. For small c_{in},

$$U'(c_{\mathrm{in}}) \approx -Pc_{\mathrm{out}} + \frac{\alpha\phi_0 c_{\mathrm{in}}\gamma}{K_2} \quad \text{for } c_{\mathrm{in}} \ll c_{\mathrm{out}}, \tag{S11}$$

i.e., negative for $c_{\mathrm{in}} = 0$, but increasing. $U'(c_{\mathrm{in}})$ can become positive if the linear term outweighs the constant term before pumping is shut-off by stretch-activated permeability around $c_{\mathrm{in}} < K_1$. This the case for $Pc_{\mathrm{out}}K_2/\alpha\phi_0\gamma > K_1$, which depends on death rate γ.

In the regime of $c_{\mathrm{in}} \approx c_{\mathrm{out}}$, $\phi(c_{\mathrm{in}}) = 0$ and $P = P_0 + P_1$. Thus

$$U'(c_{\mathrm{in}}) \approx -(P_0 + P_1)(c_{\mathrm{out}} - \mathrm{c_{in}}) = 0 \quad \text{for } c_{\mathrm{in}} = c_{\mathrm{out}} \tag{S12}$$

At this zero, the second derivative $U''(c_{\mathrm{in}}) = (P_0 + P_1) > 0$, meaning that it is always a minimum of $U(c_{\mathrm{in}})$.

Thus, the potential $U(c_{\mathrm{in}})$ will always have one zero at $c_{\mathrm{out}} = \mathrm{c_{in}}$, and can have in addition one minimum at a low concentration and a maximum at an intermediate concentration.

Kramers' escape from the potential well
If the potential has a minimum at low concentrations, then the noise-mediated escape from the minimum at concentration $\mathrm{c_{min}}$ across the barrier at c_{max} can be understood with Kramers' escape theory [19,20]. If the concentration is in a quasi-steady state at low c_{in}, and the barrier $E_{\mathrm{b}} = U(c_{max}) - U(\mathrm{c_{min}})$ is bigger than the diffusion constant $E_{\mathrm{b}} > D$, then the escape rate can be approximated as

$$k \approx \frac{1}{2\pi}[U''(\mathrm{c_{min}})|U''(c_{max})|]^{\frac{1}{2}} \exp\left(\frac{E_{\mathrm{b}}}{D}\right) \tag{S13}$$

In our model we equate the escape rate and the death rate, $k = \gamma$, meaning that any cell that crosses the barrier has irreversibly died. But because the potential itself depends on the death rate, this leads to the interesting system where the escape rate across the barrier determines the height of the barrier.

Emergence of a steady-state death rate and exponential decay
The feedback loop in which the escape rate across the barrier affects the barrier height brings about a self-adjusting system with a unique steady-state death rate. We restrict ourselves here to cases where, in addition to the requirements for Kramers escape theory, we can assume that the concentrations at which the pseudo-potential achieves its minimum and maximum do not

significantly change with death rate and can be approximated as constants. While this assumption breaks down with very large fluctuations in the death rate, it is reasonable given the small fluctuations that usually occur.

We define the function

$$f(c_{\mathrm{in}}) \equiv P(c_{\mathrm{in}})(c_{\mathrm{out}} - c_{\mathrm{in}}) - j_{\mathrm{pump}} = -U'(c_{\mathrm{in}}). \tag{S14}$$

This means that Eq. S13 is equivalent to

$$k \approx \frac{1}{2\pi}[-f'(\mathrm{c}_{\mathrm{min}})f'(c_{\mathrm{max}})]^{\frac{1}{2}} \exp\left(\frac{\int_{c_{\mathrm{min}}}^{c_{\mathrm{max}}} f(c)\mathrm{d}c}{D}\right) \tag{S15}$$

Noting that form of j_{pump} depends linearly on the death rate, we can define functions f_{influx} and f_{pump} independent of the death rate such that

$$f(c_{\mathrm{in}}) = f_{\mathrm{influx}}(c_{\mathrm{in}}) - f_{\mathrm{pump}}(c_{\mathrm{in}})\gamma. \tag{S16}$$

Then, if we define the constants

$$\begin{aligned}
&A_0 \equiv -f'_{\mathrm{influx}}(c_{\mathrm{min}}),\\
&A_1 \equiv f'_{\mathrm{pump}}(c_{\mathrm{min}}),\\
&B_0 \equiv f'_{\mathrm{influx}}(c_{\mathrm{max}}),\\
&B_1 \equiv -f'_{\mathrm{pump}}(c_{\mathrm{max}}),\\
&C_0 \equiv \tfrac{1}{\mathrm{D}}\textstyle\int_{c_{\mathrm{min}}}^{c_{\mathrm{max}}} f_{\mathrm{influx}}(c)\mathrm{d}c, \text{ and}\\
&C_1 \equiv \tfrac{1}{D}\textstyle\int_{c_{\mathrm{min}}}^{c_{\mathrm{max}}} f_{\mathrm{pump}}(c)\mathrm{d}c,
\end{aligned} \tag{S17}$$

we can rewrite the Kramers escape rate as

$$\mathrm{k} \approx \tfrac{1}{2\pi}[(A_0 + A_1\gamma)(B_0 + B_1\gamma)]^{\frac{1}{2}} \exp(\mathrm{C}_0 - \mathrm{C}_1\gamma). \tag{S18}$$

The physical constraints of the system can then impose qualitative restrictions on the behavior of the escape rate as a function of the death rate. Because we are working in a regime where c_{min}, the equilibrium corresponding to healthy cells, is low enough that there is very little stretch-induced permeability while pumping is still increasing, we can conclude that A_0 is positive since the permeability will remain relatively constant while the concentration gradient will decrease if the internal ion concentration increases, and A_1 is positive since the pumping rate will increase with greater ion concentration. Similarly, since the maximum occurs where stretch-induced permeability is very high and pumping becomes more difficult, B_0 and B_1 are both positive. Finally, since influx and pumping are always positive whenever c_{in} is positive, C_0 and C_1 must both be positive. Under these conditions, the graph of k as a function of γ is always defined, infinitely differentiable, and positive for nonnegative γ, and it approaches zero as γ approaches infinity.

Steady-state death rates are found by finding values such that

$$k(\gamma) = \gamma. \tag{S19}$$

Using the expression for k and rearranging, we find that this requirement is equivalent to

$$2\pi\gamma[(A_0 + A_1\gamma)(B_0 + B_1\gamma)]^{-1/2} = \exp(C_0 - C_1\gamma). \tag{S20}$$

Because

$$\frac{\mathrm{d}}{\mathrm{d}\gamma}\left[2\pi\gamma[(A_0 + A_1\gamma)(B_0 + B_1\gamma)]^{-1/2}\right] = \frac{\pi(2A_0B_0 + A_0B_1\gamma + A_1B_0\gamma)}{[(A_0 + A_1\gamma)(B_0 + B_1\gamma)]^{3/2}}, \tag{S21}$$

which is always positive for nonnegative γ, which requires that an increasing function of γ must intersect with a decreasing function to achieve equilibrium. Furthermore, since the LHS is less than the RHS for very small γ and greater than the RHS when γ is large, there exists a unique death rate γ^* at which these two curves intersect.

Under perturbations from the steady-state death rate γ^*, the system will adjust itself back toward equilibrium. The above analysis implies that $k > \gamma$ if $\gamma < \gamma^*$ and $k < \gamma$ if $\gamma > \gamma^*$, which means that the rate of escape from the pseudo-potential will correct itself toward γ^* if the death rate at any moment is above or below the steady-state death rate. Thus, following random fluctuations or changes to the death rate by outside factors, the system will relax back into the steady-state equilibrium.

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