Supplementary Material for

Mechanical crack propagation drives millisecond daughter cell separation in *Staphylococcus aureus*

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Materials and Methods

Bacterial strains and growth conditions

*Staphylococcus aureus* strain Newman (kindly provided by P. Levin) was used for all experiments described in the main text. Strain RN6390Δ*spa* (kindly provided by A. Cheung) and ATCC 29213 were also used to validate results from Newman (Fig. S13). *S. aureus* strain RN4220 expressing GFP (encoded on plasmid pCM29) (33) was used for volume measurements using the cytoplasmic GFP signal. Strain UAMS-1 and UAMS-1 Δ*atl* (34) (kindly provided by J. Bose and K. W. Bayles) were used to characterize the role of Atl. Bacterial cultures were grown at 37 °C with aeration in either tryptic soy broth (TSB) or brain-heart infusion (BHI) medium. For all experiments, overnight cultures were diluted 1:1000 into fresh medium and grown until mid-exponential phase. Live cell imaging was performed on 1% agarose pads or in a microfluidic flow cell (CellASIC, plate B04A) with TSB or BHI.

Microscopy

2D time-lapse imaging was performed on a Nikon Eclipse Ti inverted fluorescence microscope with a 100x (NA 1.40) oil-immersion objective (Nikon Instruments) and MicroManager v1.4 (35). Cells were maintained at 37 °C (or 30 °C for single-cell tracking) during imaging with an active-control environmental chamber (HaisonTech). An iXon3 888 EMCCD camera (Andor Technology) was used for most experiments and a Zyla 5.5 sCMOS camera (Andor Technology) was used for millisecond imaging of *S. aureus* cell separation.

3D structured illumination microscopy (SIM) and deconvolution was performed on a DeltaVision OMX BLAZE system (Applied Precision-GE, Inc.) equipped with 3 EMCCDs (Evolve, Photometrics Inc.) using an Olympus UPlanApo 100x (NA 1.40) oil-immersion objective. Fixed samples were mounted on high-performance cover glass #1.5 (Zeiss) with SlowFade® Gold antifade reagent (Life Technologies). SIM excitation was done with 100-mW lasers (488nm, 568nm) and widefield epifluorescence excitation for deconvolution datasets was with InsightSSI™ illuminator (488) and standard emission filter sets (528/48 nm and 609/37 nm). 3D-SIM images were taken using a 125-nm z-step with 25 slices (total 375 images per channel) while deconvolution images were taken with 200 nm per slice (total 21 slices). SIM reconstruction and iterative deconvolution were computed using SoftWoRx (Applied Precision-
GE, Inc.). 1-µm Polybead® amino microspheres (Polysciences, Inc) labeled with both Alexa-Fluor-568 and Alexa-Fluor-488 carboxylic acid succinimidyl ester (Life Technologies) were used to correct the scale in z and the alignment between the two colors.

Oscillatory osmotic shock

A microfluidic flow cell (CellASIC, EMD Millipore) was used to exchange media with different osmolarities (18). Mid-exponential phase cells were diluted 200-fold into fresh medium and loaded into a CellASIC B04A plate and incubated at 37 °C in the microscope environmental chamber. The osmolarity of the medium in the chamber was switched between growth medium (medium A) and growth medium plus sorbitol (medium B) with the ONIX microfluidic perfusion platform (CellASIC, EMD Millipore) every minute. The modest change in osmolarity (with addition of 200 mM sorbitol) did not change the average rate of cell growth (Fig. S2E), and the period of oscillation (2 min) is less than 10% of the average doubling time (25 min, Fig. S2E). To monitor medium osmolarity during the osmotic shock, 0.5 µg/mL deactivated Alexa-Fluor-647 carboxylic acid succinimidyl ester dye (Life Technologies) was included with medium B as a tracer dye. Cell were imaged every 10 s with phase contrast to record division events and Cy5 excitation to monitor the dye intensity (medium osmolarity). Doubling time was measured by counting the number of frames from one popping to the next popping for each individual cell. Popping frequency was calculated by dividing the number of popping events by the total number of cells for each frame. Shift of the popping frequency was calculated as the average difference between the popping frequency during the low sorbitol phase and high sorbitol phase, and was normalized by the average popping frequency of the entire period.

Cell surface labeling

Exponentially growing cells were pelleted and resuspended in PBS with 2 µg/mL Alexa-Fluor-488 conjugated WGA (Life Technologies). After incubation at room temperature for 2 min, the cells were pelleted, washed with PBS, and resuspended in culture medium with or without 1 µg/mL FM 4-64FX (Life Technologies). At this point, the WGA-488-labeled cells were either mounted on an agarose pad supplemented with 1 µg/mL FM 4-64FX (Life Technologies) for time-lapse microscopy, or incubated at 37 °C with shaking for 0 to 10 min for 3D SIM. After the chase period, the cells were pelleted, washed with PBS twice, and
resuspended in PBS in 1/4 of the culture volume. Amine-reactive dye Alexa-Fluor-568 carboxylic acid succinimidy ester (Life Technologies) was added to a final concentration of 0.05 mg/mL. The cells were pelleted after 1 min, washed with PBS twice, and resuspended in PBS + 4% formaldehyde for fixation overnight at 4 °C.

**Cell tracking in 2D with membrane labeling**

Custom MATLAB (MathWorks) image processing code was used to segment and track cells labeled with membrane dye FM 4-64FX to measure cell volume and surface area over time. Image regions with only one or two cells in the initial microcolony were selected for the analysis (the algorithm could not faithfully segment microcolonies with more than four cells). Cell group outlines were obtained by applying edge detection on the fluorescence images to select the outer boundary and were then segmented based on the concave regions of the outlines. Segmented outlines were each fitted with an ellipse to approximate the cell contour in 2D. To estimate the cell volume and surface area, the 2D ellipse was rotated along the long axis to generate a 3D prolate spheroid, with volume

\[
V = \frac{4}{3}\pi ac^2,
\]

where \(a\) and \(c\) are the radii along the long and short axes, respectively, and surface area

\[
S_{\text{prolate}} = 2\pi c^2 \left(1 + \frac{a}{ce} \sin^{-1} e\right), \quad \text{with} \quad e^2 = 1 - \frac{c^2}{a^2}.
\]

Cell identities were tracked primarily based on their centroids from right after the previous popping to the next popping event. The relative change in cell volume/surface area during popping was measured as follow:

\[
\Delta V_{\text{popping}} \% = \frac{V_{\text{daughter1}} + V_{\text{daughter2}} - V_{\text{mother}}}{V_{\text{mother}}} \cdot \frac{\Delta V}{V};
\]

\[
\Delta S_{\text{popping}} \% = \frac{S_{\text{daughter1}} + S_{\text{daughter2}} - S_{\text{mother}}}{S_{\text{mother}}} \cdot \frac{\Delta S}{S}, \quad S_{\text{mother}} = S_{\text{mother}} + 2\pi c_{\text{mother}}^2
\]

where \(\frac{\Delta V}{V}\) and \(\frac{\Delta S}{S}\) are the average growth rate derived from the pre-popping and post-popping states.
Cell tracking in 3D with cytoplasmic GFP

Growing RN4220 cells expressing GFP (encoded on plasmid pCM29) were imaged with 3D deconvolution microscopy every 30 seconds. The deconvolved z stacks were analyzed with custom MATLAB (MathWorks) image processing code. The algorithm goes through two rounds of thresholding to select voxels that belong to each individual cell from the z stacks: first, a global threshold (threshold 1) was applied to separate out the GFP intensity from the background; next, the points extracted with threshold 1 were clustered with k-means to segment each individual cell; then, a second, cell-specific threshold (threshold 2, to account for variations in GFP expression level within the cluster) was used to extract the voxels and calculate cell volume for each cell. To parameterize the two thresholds for the algorithm, spherical beads with uniform fluorescence and an average diameter of 1.08 μm (Fluoresbrite® YG Microspheres 1.00μm, Polysciences, Inc) were imaged and processed the same way as the cells with varying thresholds. Threshold 1 was set as 20% of the maximum intensity of the z stack to give robust performance of the algorithm. Threshold 2 for volume measurement was chosen as 40% of the maximum intensity of the individual cluster (cell or bead), which gives a close estimation of the bead volume based on the diameter measured from the middle slice. To compare the average GFP intensity of the mother cell and corresponding daughter cells for each popping event, a threshold was derived from the intensity distribution of the daughter cells and all the voxels above the threshold were selected to calculate the average intensity.

Measurement of cell surface area in 3D

Custom MATLAB (MathWorks) code was used to analyze the data. To measure the surface area, the positions (coordinates) of the cell surface were extracted from the 3D SIM data of the Alexa-Fluor-568 NHS channel with a threshold filter and the z coordinates were corrected based on calibration using spherical beads. The extracted points were then clustered with k-means to segment cells in 3D (Fig. S5). Each segmented group of points (corresponding to the surface coordinates of a single cell) was fitted to an ellipsoid iteratively to remove any septum points. For cells with a septum, the isolated septum points were fitted to a plane and the peripheral points were fitted to a spheroid that was symmetrical across the septum plane, which was centered to the y-z plane, defined by the equation
\[ \frac{x^2}{a^2} + \frac{y^2}{b^2} + \frac{z^2}{c^2} = 1, \text{ where } b=c \text{ if there is a septum.} \]

The total cell surface area was calculated based on the equation:

\[ S = 2\pi c^2 + \frac{2\pi ab}{\sin \phi} \left( E(\phi,\kappa) \sin^2 \phi + F(\phi,\kappa) \cos^2 \phi \right), \]

where \( \cos \phi = \frac{c}{a} \), \( \kappa^2 = \frac{a^2 \left( b^2 - c^2 \right)}{b^2 \left( a^2 - c^2 \right)} \), the identities of the radii were rearranged so that \( a \geq b \geq c \), and \( F(\phi,\kappa) \) and \( E(\phi,\kappa) \) are incomplete elliptic integrals of the first and second kind, respectively.

3D SIM data from the WGA channel were treated similarly to extract surface coordinates and then clustered. For WGA data in each cluster (segmented cell), edge points were selected from each z slice and a boundary plane was fitted to all of the selected edge points (Fig. S5). The surface area covered by WGA was then calculated through surface integration on the surface segment generated by the boundary plane intersection.

The surface area of the double septa for pre-divisional cells was calculated as: \( S_{sep} = 2\pi c^2 \)

where \( c \) is the radius of the septum for cells with completed septa. A linear fit of the outer-wall fraction to aspect ratio was generated (Fig. S5C). For “ready-to-pop” cells with the average aspect ratio 1.31 (0.09 S.D.), the predicted outer-wall percentage is \(~72\%\). To account for the effect of cell wall thickness at the peripheral ring on the septum surface area calculation, the radius \( c \) was corrected by subtracting the outer wall ring thickness measured by cryo-EM (~30 nm (7)), which increases the average outer-wall percentage to 74%.

**Correlative light and scanning electron microscopy**

Correlative microscopy coverslips CMC34A with 0.1-mm grid size (Pyser-SGI, LTD) were used to localize cells. FM 4-64FX-labeled cells (with or without WGA-488 pulse) were pelleted and resuspended in cold PBS, and settled onto poly-L-lysine (Sigma-Aldrich) treated correlative coverslips for 2 min on ice. After three washes with cold PBS, the absorbed cells were fixed with 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) on the coverslip at 4 °C overnight. The fixed coverslips were washed with PBS and mounted onto a glass coverslip. Epifluorescence and phase contrast images were collected and the locations of
cells on the grids were recorded for later correlation. Imaged coverslips were post-fixed with 1% OsO₄ at 4 °C for 1 h, dehydrated in a series of increasing concentrations of ethanol (50%, 70%, 95%, and 100%), and inserted into an Autosamdri®-815 Series A Critical Point Dryer (Tousimis) to remove residual ethanol with carbon dioxide. The dehydrated samples were then sputter-coated with gold-palladium to ~60 Å-thickness and visualized with a Sigma series field emission scanning electron microscope (Zeiss). Surface structures (holes and cracks) were blindly classified (present or not) by three independent investigators without examining the corresponding fluorescence images.

**Deflation of cells by removing turgor pressure**

To provide parameter constraints for the mechanical model of the cell wall, we measured the decrease in cell volume upon removing the turgor pressure either by disruption of the cytoplasmic membrane with 5% sarkosyl (sodium lauroyl sarcosinate) (Fig. S1A) or by osmotic shock with 2 M sorbitol (Fig. S1B). Exponentially growing Newman cells were stained with FM 4-64FX and WGA-488 as described above and loaded into a CellASIC B04A plate in fresh TSB supplemented with 1 µg/mL WGA-488 and imaged in the same conditions as 2D time-lapse experiments. The medium in the chamber was switched from TSB to TSB plus deflating reagent (5% sarkosyl or 2 M sorbitol) and the cells were imaged during the process with manual refocusing before each acquisition. Cells were outlined to calculate cell volume as described above using the cell wall staining (WGA-488).

**Evaluation of prolate shape approximation**

To examine the performance of the prolate shape approximation used to calculate the volume and surface area in 2D cell tracking described above, we estimated the cell shape from 3D SIM data of fixed cells labeled with NHS-Alexa-568 (as in Fig. 1D and Fig. S5). Images were processed similarly as described above in Measurement of cell surface area in 3D. After segmentation by clustering, an isosurface mesh was generated with the convex hull algorithm in MATLAB (MathWorks). **Snake3D** (by Dirk-Jan Kroon, retrieved July, 2014) was applied to the initial isosurface to generate a smooth mesh. The volume and surface area of the mesh were calculated with **geom3d toolbox** (by David Legland, retrieved July, 2014). To approximate the mesh with a prolate spheroid, the mesh was first fit to an ellipsoid to identify the long axis
(thereafter defined as the $x$-axis). Subsequently, the mesh was fit to a spheroid symmetric about the $x$-axis. The percent error of the surface-area (SA) to volume (V) ratio due to the prolate spheroid approximation was calculated as:

$$\%bias = 100 \times \left( \frac{SA_{prolate}}{V_{prolate}} \right) - 1.$$

**Mechanical modeling of cell wall stress with finite element analysis**

We constructed mechanical models of the dividing cell, with the goal of determining the stress distribution in the cell wall, and the deformation accompanying growth and popping. Specifically, we built elastostatic models for three key states of the cell: (1) before septum formation, (2) just before popping, and (3) just after popping (Fig. S6). In all cases we assumed the cell wall and the septum are composed of the same isotropic, incompressible, linear elastic material. The models were inflated with an internal turgor pressure, and the deformation and stresses within the material were computed using an axisymmetric finite element method, with the aid of the COMSOL Multiphysics® software package, version 4.4 (COMSOL, Inc).

Prior to popping, the cell wall is modeled as a prolate ellipsoidal shell of non-negligible thickness. In State 2, the septa form two parallel discs separated by a layer of linearly elastic “glue” material. The material properties of the glue were chosen to be significantly softer than the cell wall and septum. None of the results discussed in this manuscript were significantly altered by the specific elastic moduli selected for the glue layer, and this layer simply serves as a proxy for modeling the contact between the septal plates. Similar results were obtained by fixing the inner face of each septal plate to remain flat in the axial direction. Other geometric and physical parameters defining the models were selected to match the deformed geometry of septated cells as measured in cryo-EM studies (7) and cell dimension constraints from our light microscopy measurements; these parameters, and the rationale for their selection, are summarized in Table S1.

All length units in our models are non-dimensionalized such that the undeformed radius in State 1 (prior to septum formation) is $R_1 = 1$. All force and pressure units are non-dimensionalized such that the Young's modulus of the cell wall is $E = 1$. Other parameters are estimated from experimental measurements as detailed below. The qualitative stress distribution in the cell wall (Fig. 3) is determined by the geometry of the model and is not sensitive to exact
parameter values. Stresses in the peripheral ring are consistent with an approximate analytical model described in Supplementary Note 2. As cell growth is relatively slow compared to equilibration of osmotic pressure, we assumed the turgor pressure \( P \) is the same in both State 1 and State 2. For a given set of values for all other parameters, the pressure \( P \) and the undeformed radius in State 2 (just before popping) \( R_2 \) are solved for such that the overall cell volume in the deflated state is 77% that of the inflated state (as measured via deflating cells with 5% sarkosyl in Fig. S1) and the inflated volume in State 2 is 150% of the inflated volume in State 1 (septum formation starts at roughly 1/3 of the way through the cell cycle and cell volume doubles through the cell cycle as measured by time lapse microscopy, Fig 2B, C).

The aspect ratio \( A \) was chosen to match the inflated aspect ratio in both State 1 and State 2. We found that the same value of \( A \) was appropriate for both states, indicating that the observed increase in aspect ratio during cell growth can be attributed entirely to mechanical stretching rather than a change in the preferred shape of the cell wall material. Cell wall thickness \( H \) was selected such that in the deformed septated state (State 2), this thickness is 3.7% \( (7) \) of the deformed radius (average 512 nm measured by 3D SIM). For simplicity, we assume this thickness to remain the same before and after the septum forms. The septum thickness \( H_{sep} \) was chosen such that this thickness in the deformed state equaled that of the cell wall. Similarly, the separation between septa \( d_{sep} \) was chosen to be 68% of the cell wall thickness in the deformed state \( (7) \).

As the cell grows, its increasing girth \( (R_2> R_1) \) can be achieved through either material growth of the septum itself, thickening of the outer wall at the peripheral bridge, or some combination of the two (see Supplementary Note 1 for more discussion). We set the parameter \( R_{sep} \) (the undeformed radius of the completed septum) such that the thickness of the cell wall at the peripheral ring is 56% greater than elsewhere in the deformed state, consistent with prior cryo-EM observations \( (7) \).

Once the septum has started to form, the peripheral ring of the cell is not in direct contact with the cell membrane, and thus will not necessarily grow along with the rest of the cell (Supplementary Note 1). In our model we introduce a parameter \( R_p \) corresponding to the ground state outer radius of the peripheral ring. For simplicity, we set \( R_p = R_1 \), thereby assuming no growth of the peripheral ring after the septum begins to form. We note that the mismatch in ground state radius between the peripheral ring and the rest of the cell is crucial for the
development of high circumferential stress at the cell periphery (Fig. S8). The attachment of the peripheral ring to the cell is achieved in the finite element model by matching displacements and traction forces along the attached boundary.

Given the extremely short timescale for popping, we assumed that all geometric parameters remained constant before and after popping (State 2 and State 3). Furthermore, we assumed that this transition was sufficiently fast to preclude the equilibration of pressure by water flux. Thus, we set an altered turgor pressure in State 3 to satisfy the constraint that the internal volume contained within the wall remains constant before and after popping. This constraint is consistent with experimental measurements of the cell volume (Fig. 2D).

For visualization purposes, we created intermediate models of the cell and septum growth (Movie S6), interpolating between State 1 and State 2. In these models, the septum length, outer septum radius, and cell wall radius were assumed to increase linearly with time.
Supplementary Notes

1. Assumptions on mode of cell wall growth

To model the stress distribution in the cell wall prior to popping, we consider here the undeformed state of the cell wall (before applying any stress such as turgor pressure). There are three distinct cell wall segments once septation starts: the outer wall, the septum, and the peripheral ring (Fig. 1A). Since we observed that the S. aureus cell grows continuously throughout the cell cycle, we have to consider how each of the three cell wall segments might expand to accommodate the increase of cell radius.

Because the mother’s outer wall makes up about three quarters of the new daughter’s surface (Fig. 2F), we concluded that there must be growth (new wall material insertion) in the outer wall during the cell cycle. Also consistent with this hypothesis, we note that, if the undeformed radius of the outer wall did not grow, then the turgor pressure would have to increase substantially during the cell cycle to make up for the increase of inflated cell radius and volume. Under this scenario, there would be a large range of relative changes in cell volume upon deflation by detergent (up to 50%) and a strong dependency on pre-deflated cell volume (state in cell cycle), none of which we actually observed in our deflation experiment (Fig. S1D).

Next, we considered the possible modes of coupling between growth of the septal disk and the outer (mother cell) wall. Like many other Gram-positive bacteria, septation in S. aureus is FtsZ-dependent and we first considered a scenario where cell wall synthesis (new wall material insertion) only happens at the Z-ring associated leading tip (inner ring) of the septal disk. In this mode, the septum disk takes an undeformed radius of the pre-septated cell (Fig. 7A top) and would have to expand mechanically as the cell radius increases. A model based on this geometry predicts a heavily invaginated cell shape (Fig. S7A bottom) that is not consistent with the typical shape we observed of a septated S. aureus cell. We thus propose that the septum disk has to grow in undeformed radius as well along with the outer wall.

Finally, for the peripheral ring, we modeled two scenarios: one in which the peripheral ring grows (increases its undeformed radius) along with the rest of the cell (Fig. S7B) and one where it keeps the same undeformed radius throughout septum construction (Fig. S7C). The cell shape and geometry predicted by the two models are almost identical since the peripheral ring constitutes a relatively small portion of the overall cell wall, so our experimental measurements
cannot easily distinguish between these two possibilities. However, the stress on the peripheral ring is much higher in the second model where the ring is stretched out to match the radius of the rest of the cell. We believe that the second model more closely represents the true pattern of growth based on the fact that the peripheral ring is not adjacent to the cytoplasmic membrane (which carries the enzymes and precursors necessary for insertion of newly synthesized peptidoglycan) and thus cannot grow by direct insertion of new wall materials. It is possible that there is some degree of relaxation of the peripheral ring through cell wall hydrolase activities (which could lower the wall stiffness and rearrange the cell wall organization) and possibly plastic deformation (yielding to the high circumferential stress) to increase the undeformed radius of the ring and lower the circumferential stress.

2. Approximate analytical model

Using a purely linear model for deformation of the system, we can approximate the geometry and stresses of the pre-popped state as described here. For simplicity, in this section we approximate the cell as a spherical (rather than ellipsoidal) thin shell, connected to a thin plate at the equator (the septum), with a thin ring of material attached just below the plate (the peripheral ring) as illustrated in Fig. S8A.

A spherical thin shell of radius $a$ and thickness $t$ inflated with pressure $p$ will expand and develop material stresses of magnitude $\frac{pa^2}{2t}$ in the tangential directions. The radial expansion of the inflated sphere is given by $d_o = \frac{pa^2(1-v)}{2Et}$ where $E$ is the Young's modulus and $v$ is the Poisson ratio of the shell (36). A circular plate of the same radius with the same material properties ($E$, $v$) and with thickness $t_p$, subjected to an axisymmetric radial tensile force $T$ (per unit area), will expand radially by an approximate amount $d_p = \frac{Ta(1-v)}{E}$. We note that under the same external pressure ($T = p$) the radial expansion of the plate is a factor of $\frac{t}{a}$ smaller than the expansion of the sphere. Thus, the septal plate joined to the cell wall at the equator acts to constrain the radial expansion shell.

Since the thin septal plate exerts a narrowly localized force on the expanding shell, bending stresses as well as stresses due to stretching must be taken into account when analyzing the joint
system. For a hemispherical dome constrained to a radial expansion \( d \), and assuming no rotation of the shell at the equator, the shear stress (averaged over the shell thickness) at the equator is given by

\[
\sigma_{rc} = \frac{pa(1-\nu) - 2Eid}{2\lambda t}, \quad \text{where} \quad \lambda^2 = \sqrt{\frac{3(1-\nu^2)}{a}}.
\]

The quantity \( 2\pi at\sigma_{rc} \) is an approximation of the total radial force exerted by the shell on the plate, for a given radial displacement \( d \). Balancing this with the total radial force \( 2\pi at_p T \) exerted by the plate on the shell allows us to solve for the radial displacement at equilibrium:

\[
d = \frac{d_0}{1 + \frac{\lambda}{(1-\nu)} t_p}, \quad \text{where} \quad d_0 \text{ is the displacement for the empty sphere, as given above.}
\]

The hoop stress at the mid-surface of the shell then ranges from

\[
\sigma_o = \frac{Ed}{a} + \frac{vpa}{2} = \frac{pa}{2t} \left[ \frac{(1-\nu)t + \nu \lambda t_p}{(1-\nu)t + \lambda t_p} \right]
\]

at the equator to \( \sigma_z = \frac{pa}{2t} \) far from the septal plate. The axial stress is not influenced by the presence of the septal plate and is given by \( \frac{pa}{2t} \) throughout the shell. Thus, in this simple dome and plate system, the overall stress will be lower at the equator than elsewhere in the shell.

Consider now the case where we take a thin ring that has a ground state (unstressed) radius \( a_b \), where \( a_b < a \). The thickness of this ring is assumed to be much smaller than its radius. The ring is stretched out radially by a distance \( d + (a - a_b) \), so that its final radius is the same as the dome plate system. This is an approximate model for the case where the peripheral ring of the cell wall does not grow as quickly as the rest of the cell. The radial strain in such a ring is dictated by its hoop stress \( \sigma_{ob} \) and its axial stress, which we assume to be equal to the axial stress in the dome at the equator. That is, we have

\[
\frac{d + (a - a_b)}{a_b} = \frac{\sigma_{ob}}{E} - \frac{vpa}{2Et}
\]

which can be solved for the hoop stress in this peripheral ring to give:

\[
\sigma_{ob} = \frac{E(d + (a - a_b))}{a_b} + \frac{vpa}{2t}
\]
We note that this approximation is accurate only in the linear (small displacement) limit – that is, at low pressure and small difference in ground state radii $a - a_\nu$. Nonetheless, it is useful for elucidating the dependence of the stress at the peripheral ring on the different material parameters. These approximate calculations demonstrate that the septal plate limits the radial growth of the cell (leading to an increased aspect ratio) and that high stress in the peripheral ring is dependent on the existence of a length mismatch between the ring and remainder of the cell wall (Fig. S8B).

3. Periodic necking of the peripheral ring under circumferential stress

In the idealized case of a purely plastic thin rod necking under tension, the dominant wavelength $\lambda_0$ of the periodic thinning is $\lambda_0 / R_0 = \pi$, where $R_0$ is the radius of the rod (28).

By considering the peripheral ring as a curved rod, the thickness of the cell wall at the peripheral ring corresponds to the diameter of the rod. A crude measurement of the spacing between the perforations in the peripheral ring indicates an average peak wavelength of about 50 nm (Fig. S11B, ~65 nm if corrected for the dehydration during SEM sample preparation), while the thickness of the cell wall at the peripheral ring is about 30 nm, corresponding to $R_0 \sim 15$ nm (7). This gives a ratio of 3.3 (~4 after correction), close to $\pi$, suggesting that a mechanical origin for the spacing between perforations is quite plausible.
Table S1. Summary of parameters used in the mechanical model of the cell wall.

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<th>Description</th>
<th>Value (dimensionless)</th>
<th>Rationale</th>
<th>Reference or figure</th>
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<td>(7)</td>
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<tr>
<td>$d_{\text{sep}}$</td>
<td>septum separation</td>
<td>0.033</td>
<td>$d_{\text{sep}}^<em>/H^</em> = 0.68$</td>
<td>(7)</td>
</tr>
<tr>
<td>$R_{\text{sep}}$</td>
<td>septum radius</td>
<td>1.071</td>
<td>$H_{\text{mid}}^<em>/H^</em> = 1.56$</td>
<td>(7)</td>
</tr>
<tr>
<td>$R_p$</td>
<td>peripheral ring radius</td>
<td>1#</td>
<td>$R_p=R_1$</td>
<td></td>
</tr>
<tr>
<td>$P_3$</td>
<td>inflation pressure in state 3</td>
<td>0.0084</td>
<td>$V_3^{\text{in}} = V_2^{\text{in}}$</td>
<td>Fig. 2D</td>
</tr>
</tbody>
</table>

Subscripts refer to the three states illustrated in Fig. S6.

* Quantities measured in the deformed (inflated) state. Where no subscript is specified, the deformed values are measured from state 2.

# Parameters that appear in the approximate model and are critical to the overall physics of the system.
Figure S1. *S. aureus* cell walls are under significant tensile stresses. (A, B) Representative images of “ready-to-pop” *S. aureus* Newman cells: before and after disruption of cytoplasmic membrane by 5% sarkosyl (A); before and after osmotic shock with 2 M sorbitol (B). Scale bars: 1 μm. (C) Relative changes in cell volume upon addition of 5% sarkosyl or 2 M sorbitol to the medium in the CellASIC flow chambers. (D) Relative volume changes upon sarkosyl treatment plotted against the initial cell volume.
Figure S2. Oscillatory osmotic shock by media exchange does not affect cell overall growth rate but induces an amplitude-dependent popping frequency shift. (A-D) Average popping frequency, defined as number of popping events per cell per frame (10 s), as a function of the sorbitol concentration for osmotic shock amplitude of 0 mM (A, n=61 cycles and 469 popping events total), 100 mM (B, n=59 cycles and 501 popping events), 200 mM (C, n=69 cycles and 400 popping events, same data as in Fig. 1C) and 500 mM (D, n=59 cycles and 492 popping events).
events). Error bars denote standard errors. (E) Doubling time distribution in the oscillatory osmotic shock experiments with 0 mM (A) or 200 mM sorbitol (C). Doubling time was measured as the time span from immediately after the each popping event to the next popping event for each individual cell. 200 mM sorbitol shock does not affect the average doubling time but does increase the dispersion, consistent with the finding that osmotic shock affects the likelihood and thus relative timing of popping. (F) Effect of sorbitol concentration during the oscillatory shock on popping frequency distribution. Shift of the popping frequency was calculated as the difference between the average popping frequency during the low sorbitol phase and high sorbitol phase, and normalized by the average popping frequency of the whole period. Error bars denote standard errors.
Figure S3. Cell volume does not significantly change during popping. (A) Scheme for measuring cell volume of *S. aureus* RN4220 expressing cytoplasmic GFP with 3D deconvolution microscopy. Cells were imaged every 30 seconds and two representative time points that span a popping event are shown here. Voxels were extracted from the 3D stack (Movie S5, middle slices from the *z* stack shown at the left) and clustered to calculate cell volume for each individual cell. Scale bars: 1 µm. (B) Traces of cell volume as a function of time for the cells numbered in (A). Dashed black line denotes the total cell volume of all the cells present at a given time. (C, D) Distribution of relative changes in cell volume (C) and averaged GFP intensity (D) during popping after correction. Red dashed lines denote the average (2% ± 5% SD for volume, -5% ± 2% SD for GFP intensity, *n*=28).
Figure S4. The prolate approximation slightly underestimates surface area to volume ratio for small cells. (A) 3D SIM images (top) and the corresponding cell shape mesh extracted with Snake3D (bottom) of representative late stage (ready-to-pop, left) and early stage (recently popped, right) cells labeled with NHS-Alexa568. (B) Overlay of the shape mesh (gray) and the prolate spheroid fit (blue) of the blue cell above shown in xy and xz (the long axis of the spheroid defined as x-axis). (C) Surface area to volume ratio of the spheroid versus surface area to volume ratio of the extracted shape (dashed line denotes the identity line). Data are colored based on the cell volume (increase from blue to red). (D) % bias in surface area to volume ratio by assuming a prolate shape as a function of volume for cells at various stages of the cell cycle: no detectable septum (blue); with a septum (red); with a completed septum (solid red). The approximation performs worse for early stage (small) cells and gives a relatively lower surface area to volume ratio, which may result in a slight underestimation of the surface area of popped cells and thus an overestimation of the decrease of surface area upon popping, but in all cases the error due to the simplified shape assumption is less than 3%.
Figure S5. The outer wall of the mother cell makes up about three quarters of the new daughter’s surface. (A) Illustration of the data analysis procedures for measuring surface area with 3D SIM data on WGA pulse-chase labeled cells. Scale bars: 1 µm. (B) The percentage of old surface (black in the inset) in the new daughter cell’s surface shows little dependence on cell volume (blue open circles). (C) The percentage of outer surface (black in the inset) in total surface area with completed septum (red) is proportional to cell aspect ratio in the spheroid cell model. Red line is a linear fit to the data (blue open circles) and the dashed lines represent the predicted outer-wall% (0.72) for average aspect ratio of “ready-to-pop” cells (1.31).
Figure S6. Model geometries for three different states: before septum formation (state 1), immediately before popping (state 2), and immediately after popping (state 3). Top images show the undeformed geometry with the relevant geometric and physical parameters labeled. Purple regions are made of isotropic, incompressible, linear elastic cell wall material. Gray region is made of a compressible linear elastic “glue” material. Parameter values are summarized in Table S1. Middle images show the deformed (inflated) geometry of each state, with the undeformed boundaries shown in black for comparison. Bottom images show von Mises stress (color scale is shown at right; warmer colors indicate higher stress magnitudes).
Figure S7. Observed cell geometry constrains models of cell wall growth. Top images show the undeformed geometry of “ready-to-pop” cells based on different modes of cell wall growth and bottom images show the corresponding cell shape and von Mises stress after inflation with turgor pressure. (A) The septum and the peripheral ring do not expand their undeformed (ground state) radius during septation (i.e. new wall synthesis only happens at the tip of the septum). (B) Both the septum and the peripheral ring expand their undeformed radii along with the growth of the outer wall. (C) Only the peripheral ring does not expand its undeformed radius during septation.
Figure S8. Approximate analytical model. (A) Illustration of the three elements considered in the approximate analytic model: a spherical thin shell (the outer wall), connected to a thin plate at the equator (the septum), with a thin ring of material attached just below the plate (the peripheral ring). (B) Circumferential stress (blue) and axial stress (green) at the peripheral ring as a function of the peripheral ring radius mismatch ($a - a_b$ in the approximate model and $R_z - R_p$ in the cell model). Plain lines are approximate analytical solutions and lines with dots are result from the COMSOL model parameterized as in Table S1. The pressure was set as 0.02 $E$. 

![Figure S8](image.png)
Figure S9. **Turgor pressure contributes to the stress in peripheral ring.** From top to bottom: cell geometry, von Mises stress distribution in the cell wall, circumferential and axial stress in the peripheral ring without (deflated) and with (inflated) turgor pressure.
Figure S10. Enrichment of WGA in septal region correlates with perforations observed in SEM. (A-D) Correlative microscopy of Newman cells labeled with WGA-488 and FM 4-64FX prior to fixation. (A-B) Cells with enrichment of WGA in septal region (white arrowheads) as well as perforations on cell surface observed with SEM. (C-D) Cells with a complete septum but without enrichment of WGA in the septal region and no perforations were observed on the surface with SEM. Scale bars: 1 μm.
Figure S11. Perforations (holes and cracks) along the peripheral ring show a limited size distribution and characteristic spacing. (A) Histogram of the hole size distribution measured by SEM ($n=206$). Hole size is represented by the length of perforation along the septal periphery. (B) Histogram of hole spacing measured by SEM ($n=122$), spacing is measured as center to center distance between neighboring holes (or cracks). The data are collected from 52 cells with an average cell diameter of $776 \pm 50$ nm (average cell circumference of $2.4 \, \mu$m) measured by SEM.
Figure S12. Atl is not necessary for the ultra-fast popping and peripheral ring perforation formation in *S. aureus*. (A, D) Snapshots of typical 2-ms “popping” of *S. aureus* strain UAMS-1 (A) and UAMS-1 Δ*atl* (D). (B, E) Histograms of daughter cell separation duration of UAMS-1 (B) and UAMS-1 Δ*atl* (E) captured by phase contrast microscopy at 1000 frames/s (*n*=20). (C, F) SEM images of UAMS-1 (C) and UAMS-1 Δ*atl* (F). Inset shows the magnified image marked by the red box to highlight the surface perforations at the peripheral ring. Scale bars: 1 μm.
Figure S13. The observations on Newman are consistent with other strains of *S. aureus*. (A) Snapshots of *S. aureus* strain RN6390Δspa “popping” captured by phase contrast microscopy with 1.3 millisecond per frame. (B) Histogram of daughter cell separation duration measured at 1.3 millisecond per frame (*n*=10). (C) Distribution of cumulative counts of popping events in *S. aureus* strain ATCC 29213, red solid line denotes the concentration of sorbitol in the medium and the dashed line denotes average popping counts assuming a uniform distribution (*n*=117). (D, E) Correlative SEM on RN6390Δspa (D) and RN4220 (E) show the split open geometry of daughter cells as well as holes and cracks on the surface. Scale bars: 1 μm.
**Figure S14. Model of *S. aureus* cell cycle.** As the cell proceeds with the construction of the septum (red), both cell volume and cell surface area increase continuously and the septum is split during construction. After completion of the septum, holes and cracks form and grow gradually at the peripheral ring region as a result of necking instability due to a combination of hydrolase activities and high circumferential stress induced by differential growth of the peripheral ring. These perforations serves as stress hot spots to initiate a propagating crack that resolves the peripheral ring in a few milliseconds, leaving the two daughter cells attached by a hinge point. During this process, the previously flat septa become curved after detaching from each other but are still less curved than the older wall (often the two septa do not fully detach and thus only partially round up, see Fig. 1D-E). Gradually (in minutes to tens of minutes) the daughter cells become more round and more separated (Movie S4 and Movie S7). Dotted lines illustrate possible future septum planes. The time axis (right) is drawn approximately to scale.
Movie S1. Ultrafast popping of *S. aureus* strain Newman. Representative time-lapse movies of *S. aureus* strain Newman “popping” captured by phase-contrast microscopy at 1 ms/frame.

Movie S2. Deflation of *S. aureus* Newman cells. Time-lapse movies of “ready-to-pop” *S. aureus* Newman cells treated with 5% sarkosyl as in Fig. S1A.

Movie S3. 3D Visualization of the old-wall labeling pattern after popping. Volume view of the 3D SIM images of fixed Newman cells pulse labeled with WGA-488 and followed by 0 or 10 min chase growth in the absence of the dye (Fig. 1D).

Movie S4. Illustration of the 2D cell outline tracking. Time-lapse movie of *S. aureus* cells stained with FM 4-64 (left) and outlined by fitting with ellipses (right) as in Fig. 2A. Scale bar: 1 μm.

Movie S5. Tracking cell separation with cytoplasmic GFP in 3D. Time-lapse movie of *S. aureus* cells expressing cytoplasmic GFP acquired with 3D deconvolution microscopy (only one z slice was shown) and the corresponding z stacks before and after a popping event. Scale bar: 1 μm.

Movie S6. Illustration of cell growth during septum construction. Time-lapse movies of intermediate models of the cell and septum growth, interpolating between state 1 and state 2 (Fig. S6). The von Mises stress at the peripheral ring connecting the two daughter cells (arrow) becomes higher than elsewhere in the outer wall as the cell grows. In addition, the aspect ratio increases as the cell and septum grow.

Movie S7. Daughter cells do not fully round up during popping. Time-lapse movie of daughter cell separation captured at 3 min/frame (same cell as in Fig. 1E left). Cells were labeled with WGA-488 before mounted and the cell membrane was stained with FM 4-64.
References and Notes


