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Sequential assembly of the septal cell envelope prior to V snapping in *Corynebacterium glutamicum*

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Abstract

Members of the Corynebacterineae, including *Corynebacterium* and *Mycobacterium*, have an atypical cell envelope characterized by an additional mycomembrane outside of the peptidoglycan layer. How this multilayered cell envelope is assembled remains unclear. Here, we tracked the assembly dynamics of different envelope layers in *Corynebacterium glutamicum* and *Mycobacterium smegmatis* by using metabolic labeling and found that the septal cell envelope is assembled sequentially in both species. Additionally, we demonstrate that in *C. glutamicum*, the peripheral peptidoglycan layer at the septal junction remains contiguous throughout septation, forming a diffusion barrier for the fluid mycomembrane. This diffusion barrier is resolved through perforations in the peripheral peptidoglycan, thus leading to the confluency of the mycomembrane before daughter cell separation (V snapping). Furthermore, the same junctional peptidoglycan also serves as a mechanical link holding the daughter cells together and undergoes mechanical fracture during V snapping. Finally, we show that normal V snapping in *C. glutamicum* depends on complete assembly of the septal cell envelope.

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Author contributions

X.Z., F.P.R.-R., C.R.B., and J.A.T. conceived the project and designed experiments. X.Z. performed the experiments and analyzed data. F.P.R.-R. synthesized and characterized the fluorescent probes. H.C.L. and T.G.B. constructed bacterial strains. J.C.B. contributed expertise to the FRAP experiments and analysis. X.Z. and J.A.T. wrote the manuscript. All authors discussed the results and interpretation of the data, and read and commented on the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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Most currently studied bacteria fall into one of the two groups according to their cell-envelope architecture and the results of Gram staining. One major difference between Gram-positive and Gram-negative bacteria, beyond the thickness of the peptidoglycan (PG) layer, is the presence of an additional membrane, the outer membrane (OM), in Gram-negative bacteria¹. A fascinating exception to this classification is bacteria in the Corynebacterineae suborder, which includes both the industrial amino acid producer *C. glutamicum* and the human pathogen *Mycobacterium tuberculosis*. Although this suborder resides phylogenetically within the high-G + C Gram-positive clade Actinobacteria, the cell envelope of Corynebacterineae species includes the mycomembrane (MM), an additional membrane outside of the PG^{2,3}, which is chemically and structurally distinct from the lipopolysaccharide-containing OM of Gram-negative bacteria. This group of bacteria is therefore also referred to as mycolata.

The major lipid components of the MM are mycolic acids (MA), 2-alkyl, 3-hydroxy long-chain fatty acids (20–40 carbon atoms for *Corynebacterium* and as many as 90 carbon atoms for *Mycobacterium*)⁴, which exist in two forms (Fig. 1a): either covalently linked to the PG layer (PGL) through arabinogalactan (AG) anchors or freely extractable glycolipids that are predominately trehalose based (such as trehalose monomycolate (TMM) and trehalose dimycolate)⁴. Differences in MA structure can lead to substantial differences in MM fluidity⁵. Similarly to the OM in Gram-negative bacteria, the MM also serves as an additional permeability barrier^{6,7} and is the target of several front-line antituberculosis drugs^{8,9}.

Given the atypical cell-envelope architecture, the question of how Corynebacterineae build their cell envelopes during growth and division has been a focus of research on these organisms¹⁰. To grow, rod-shaped *Corynebacterium* and *Mycobacterium* assemble a new cell envelope at the poles^{11,12}, which are organized by the polar scaffold protein DivIVA^{13–15}. After cytokinesis, despite having the MM, these bacteria build a flat septum similar to that in other Gram-positive bacteria. Remarkably, to separate the daughter cells, the septum is resolved through a fast and dramatic V snapping^{16–18}, which occurs within 10 ms and is a common trait widespread among the Actinobacteria¹⁹. However, the exact geometry of the envelope structure at the septum during cytokinesis and the relative time point at which the MM of the new poles is assembled, relative to septation and V snapping, remain unclear.

Results

The septal cell envelope is sequentially assembled during cytokinesis in *C. glutamicum* and *M. smegmatis*

To track cell-envelope assembly, we applied metabolic labeling²⁰, using a combination of fluorescent probes to simultaneously monitor the assembly dynamics of different envelope layers in *C. glutamicum* and *M. smegmatis*. These probes exploit the promiscuity of the enzymatic activity of the extracellular biosynthetic enzymes involved in this assembly process so that lipids derived from these probes can be incorporated into specific layers of the cell envelope and thus provide spatiotemporal profiles of corresponding envelope-assembly activities. We used three types of probes in this study (Fig. 1a,f): (i) fluorescent D-

amino acids (FDAA, such as TAMRA D-lysine (TDL) or NBD D-alanine (NADA)) that incorporate into the PGL¹²; (ii) fluorophore–trehalose conjugates (FTre, such as 6-fluorescein-trehalose (6-FITre) or 6-TAMRA-trehalose (6-TMR-Tre)) that label trehalose glycolipids in the MM^{5,21}; and (iii) fluorophore–TMM-mimic conjugates (*O*-TMM probes, such as *O*-TMM–Alexa Fluor 647 (*O*-TMM-647) or *O*-TMM-dibromofluorescein (*O*-TMM-DBF) that were modified from the alkyne-functionalized trehalose analog²² and incorporate into the MM both covalently (onto AG, as TMM mimics, and therefore report on the mycolation of AG) and noncovalently (into trehalose glycolipids, as trehalose derivatives similar to 6-FITre) (Supplementary Fig. 1). To monitor the incorporation of these probes throughout the cell cycle in individual bacterial cells, we used microfluidic chambers to culture cells in medium supplemented with fluorescent probes and briefly switched to medium without probes before each observation.

After the initial introduction of the probes to *C. glutamicum*, the PG probes showed a clear asymmetric polar localization consistent with the known growth pattern^{11,12}, whereas the MM probes were distributed evenly along the cell periphery, probably because of the high fluidity of the MM⁵ (Supplementary Fig. 2a,b,e). The underlying polar synthesis activity of the MM was revealed only when we removed free glycolipids in *C. glutamicum* cells labeled with a short pulse of *O*-TMM-647 (Supplementary Fig. 2f). In contrast, given the low fluidity of *M. smegmatis* MM⁵, both the PG and MM probes exhibited asymmetric polar localization patterns that resembled each other (Supplementary Fig. 2c–e), thus suggesting that different layers of the cell envelope are coassembled at the growing poles.

In contrast to the apparently synchronous incorporation of different probes at the poles, we observed a sequential incorporation of the probes at the septum during cytokinesis for both *C. glutamicum* (Fig. 1b, Supplementary Fig. 3a and Supplementary Video 1) and *M. smegmatis* (Supplementary Fig. 4a), results indicating that different layers of the cell envelope are not coassembled at the septal plane. Specifically, the FDAA signal reporting PG biosynthesis always appeared first at the septum and tracked with the septation process, as indicated by the invagination of the cytoplasmic membrane visualized with FM4–64 (Supplementary Fig. 5); next were the *O*-TMM probes, which trailed behind FDAA during septation (Supplementary Fig. 1d), and then the FTre probes, which typically were the last to appear.

By analyzing the accumulation of septal signals for each probe (Fig. 1c; details in Methods and Supplementary Fig. 6), we quantified the delay in septal appearance between the FDAA and FTre probes (Fig. 1d, τ_1). The two FTre probes, 6-FITre and 6-TMR-Tre, showed slightly different incorporation dynamics in *C. glutamicum* (Supplementary Fig. 3d,e), probably because of the differences in their fluorophore structures; however, for both probes and both species, we observed a notable delay ($\tau_1 > 0$, Supplementary Figs. 3c and 4c) confirming the observation that the septal cell envelope is sequentially assembled from PG to MM (Supplementary Fig. 7).

Finally, we noticed that in *C. glutamicum*, FTre probes often exhibited a rapid increase in septal signal (RISS) before V snapping (Fig. 1b, Supplementary Figs. 3a and 8a and Supplementary Video 1) especially for 6-FITre, a result probably attributable to its higher

incorporation at the cell periphery relative to the septum (Supplementary Fig. 3e). In agreement with these results, the duration of septal 6-FITre accumulation was much shorter than that for TDL (Fig. 1e, in most cases within 10 min), thus suggesting that the RISS might correspond to a novel cell-cycle event distinct from the de novo cell-envelope biogenesis at the septum.

The mycomembrane of *C. glutamicum* becomes confluent before V snapping

Given the high fluidity of the *C. glutamicum* MM⁵, we hypothesized that the RISS might be a manifestation of an inflow of the labeled trehalose glycolipids in the peripheral MM into the septum. To test this possibility, we used pulse–chase experiments in which we prelabeled *C. glutamicum* cells with FTre and followed the labeled cells as they grew and divided in the absence of FTre probes. We initially focused on cells that did not have septal labeling of FTre, to determine whether and when labeled MM glycolipids from the cell periphery might relocate into the septum (Fig. 2a). Indeed, we observed RISS before V snapping in the chase experiment with all three trehalose-based probes (Fig. 2b and Supplementary Fig. 8c).

The observed inflow indicated that glycolipids in the peripheral MM can diffuse into the septum after RISS. That is, the MM becomes confluent between the septum and cell periphery at that point. To confirm the confluency of the MM, we performed fluorescence recovery after photobleaching (FRAP) experiments on prelabeled cells with septal 6-FITre signal (post RISS). We observed a rapid recovery after photobleaching of the labeled septum, with recovery dynamics similar to that after photobleaching of the centers of cells with no septal labeling (before RISS) (Fig. 2c–f). Both the half-time of recovery and the mobile-fraction measurements revealed that the labeled trehalose glycolipids are highly mobile and that the septum is contiguous with the cell periphery post RISS, in which the labeled trehalose glycolipids freely diffuse in and out of the septum.

Interestingly, we found that MM confluency in *C. glutamicum* occurred beyond single cells and was sometimes observed between physically touching neighbor cells. In FRAP experiments on 6-FITrelabeled cell pairs (recently V-snapped daughter cells), we observed a rapid recovery of the fluorescence signal in the photobleached cells, which eventually reached the same fluorescence intensity as that in unbleached cells (Fig. 3a). In addition, we observed transfer (Fig. 3b and Supplementary Video 2) and exchange (Fig. 3c) of fluorescently labeled glycolipids between *C. glutamicum* cells that came into physical contact due to growth and V snapping, results possibly revealing an unknown community aspect of the MM fluidity.

Peripheral PGL in *C. glutamicum* forms a diffusion barrier for lipids in the MM

Despite being highly fluid, *C. glutamicum* MM becomes confluent between the septum and cell periphery only shortly before V snapping rather than during septation, thus suggesting the presence of an effective diffusion barrier that prevents trehalose glycolipids from entering the septum before the completion of septation. This presence was confirmed by chase experiments with the exogenous fluorescent phospholipid analogs fluorescein 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (FDHPE) and Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rDHPE), which have been shown to

specifically stain the MM²³. In labeled *C. glutamicum* cells, these fluorescent phospholipid analogs had similar diffusion dynamics (Supplementary Fig. 9a–c) and displayed the same inflow (RISS) (Supplementary Fig. 9d,e) as that observed for trehalose glycolipids, thus supporting the existence of a diffusion barrier for lipids at the peripheral ring.

What forms the diffusion barrier, and how is it resolved before V snapping? To identify the diffusion barrier, we used transmission electron microscopy (TEM) to examine the ultrastructure of the cell envelope at the T junction between the septum and cell periphery. Conventional TEM preparation with nonspecific staining of *C. glutamicum* typically reveals a multilayered cell envelope²⁴ (Fig. 4a), which from inside out is composed of a thick, electron-dense layer (considered to correspond to the PGL and probably to the attached AG layer); a thin, electron-transparent layer (assumed to be the MM); and an electron-dense outer layer (a complex protein–carbohydrate matrix with some lipids).

To facilitate the assignment of the layers observed in conventional TEM, especially the MM, we adapted a recently developed click-EM²⁵ method to specifically enhance the EM contrast of the MM through metabolic labeling. We attached the singlet-oxygen-generating fluorophore DBF to trehalose to form an *O*-TMMDBF conjugate, which incorporates into the MM. Illumination of *O*-TMM-DBF-labeled *C. glutamicum* cells generated singlet oxygen, which locally polymerized diaminobenzidine (DAB) into an osmiophilic product and resulted in increased electron densities after staining with osmium tetroxide (Supplementary Fig. 10). With this method, the MM appeared as the darkest (most electron-dense) layer, whereas the less electron-dense layer underneath was probably the PGL (Fig. 4a). However, no contrast enhancement for the MM was observed at the septum, possibly because of the limited access of DAB to the septum.

Examination of the T junction with both TEM methods revealed a Π -shaped structure of the PGL with the two separated septal PGLs attached to the peripheral PGL (Fig. 4a), a result consistent with TEM observations made previously in *Mycobacterium*²⁶. In light of this structure, we propose that the peripheral PGL at the T junction may serve as the diffusion barrier for the MM glycolipids. Only in a subset of cells with fully formed septa did we occasionally find examples in which the junctional PGL was less thick than the adjacent peripheral PGL (Fig. 4b), thus suggesting that the junctional PGL remains contiguous throughout septation. Interestingly, we found that the thinning typically appeared to advance outward from the septum to the cell periphery (Fig. 4b). This thinning of the junctional PGL resembled the perforations along the peripheral ring that we previously observed in *C. glutamicum* through scanning electron microscopy (SEM)¹⁹ (Fig. 4c), which are probably responsible for resolving the junctional PGL and thus the diffusion barrier for the MM glycolipids.

To test for the connection between the perforations at the peripheral ring and the dissolution of the diffusion barrier for glycolipids, we performed correlative light and scanning electron microscopy (CLSEM) on 6-FITre-labeled *C. glutamicum* (Fig. 4d). We chose 6-FITre because its presence at the septum best correlated with RISS, and all cells with septal 6-FITre labeling examined in the FRAP experiments displayed confluent MM, thus indicating that septal 6-FITre labeling is a good marker for the dissolution of the diffusion barrier. Of

the 154 cells imaged, 46 had detectable septal labeling of 6-FITC, and 32 of the 46 cells (~70%) had clear visible surface perforations, whereas all the cells with visible surface perforations had septal 6-FITC labeling ($n = 32$ cells). It is possible that the cells with septal 6-FITC labeling but no visible perforations might have had perforations that were too small to be detected, obscured by other surface features, or on the underside of the cells.

Together, our results indicate that the PGL at the septal junction forms a diffusion barrier for the trehalose glycolipids separating the MM at the cell periphery from the septum, and this diffusion barrier is resolved by perforations in the junctional PGL (typically after completion of septation) giving rise to a confluent MM, which completes the daughter cell envelopes.

Peripheral PGL undergoes mechanical fracture during V snapping in *C. glutamicum*

We previously established that daughter cell separation (V snapping) in *C. glutamicum* as well as several other *Actinobacteria* species occurs within 10 ms, exhibits a hinged geometry between the daughter cells¹⁹, and resembles the fast mechanical popping in *Staphylococcus aureus*²⁷. Here, with improved temporal resolution, we observed that V snapping in *C. glutamicum* was completed within 2 ms (Fig. 5a), a result supporting the notion that V snapping probably occurs through a mechanical-fracture mechanism.

However, given the complex cell-envelope composition of *Corynebacterium*, it was unclear which layer of the envelope undergoes the mechanical fracture during V snapping and links the daughter cells afterward. The MAs or the S layer have been suggested to act as the linking material joining the two daughter cells after snapping and giving rise to the V-shaped arrangement^{28,29}. The *C. glutamicum* strain that we used (ATCC 13032) is incidentally deficient in S-layer production, owing to the absence of the S-layer promoter PS2 (ref. ³⁰), yet it still undergoes V snapping, thus ruling out the possibility that the S layer might be required for V snapping. To evaluate the contribution of MAs to V snapping, we took advantage of a natural isolate, *Corynebacterium amycolatum*, that is devoid of MAs³¹. We found that *C. amycolatum* V snapped just like *C. glutamicum*, a process that occurred within 10 ms and resulted in surface perforations resembling those in *C. glutamicum* and a hinged geometry of daughter cells afterward (Fig. 5b). Therefore, we concluded that V snapping in *Corynebacterium* is independent of MAs and the S layer.

Additionally, by performing an oscillatory osmotic shock experiment, we found that V snapping in *C. glutamicum* is driven by turgor pressure (as in *S. aureus*) (Fig. 5c). By changing the osmolarity of the medium with 200 mM sorbitol (which corresponded to an ~20% change in turgor pressure in *C. glutamicum*; Supplementary Fig. 11) at 4-min intervals, we were able to modulate the turgor pressure, and we recorded V-snapping events as a function of medium osmolarity (Fig. 5c). The occurrence of V snapping was strongly dependent on the phase of the osmotic shock; high medium osmolarity (low turgor) suppressed V snapping, whereas low medium osmolarity (high turgor) induced V snapping.

We thus propose that the peripheral PGL provides the mechanical strength to hold the daughter cells together against the turgor pressure before V snapping and undergoes the mechanical fracture during V snapping. Consistently with this possibility, the Π -shaped structure of the PGL at the T junction revealed by TEM (Fig. 4a,b) resembles the septal cell

wall geometry in *S. aureus*^{32,33}, and in both species, the junctional PGL remains contiguous throughout septation. To demonstrate the specific effect of turgor pressure on V snapping, we exposed the septa of TDL-labeled *C. glutamicum* cells to turgor pressure by killing one of the daughter cells with laser ablation³⁴, and we observed that the septa changed from flat to curved outward after ablation (Fig. 5d), similarly to the septal shape changes during V snapping. We propose that this turgor-driven shape conversion of the septum drives the mechanical fracture of the junctional PGL along the perforations (probably initiated from one of the perforations shown in Fig. 4c), thereby allowing the daughter cells to separate and rotate around the hinge.

Finally, to visualize the fracture of the peripheral PGL after V snapping, we followed *C. glutamicum* cells prelabeled with NADA to track the fate of PGL. Following cells that started without septa, we found that after V snapping, the PGL was fractured at one side, whereas the other side remained connected and served as the hinge point around which the daughter cells rotated (Fig. 5e). This hinge structure was also observed with contrast-enhanced TEM (Fig. 5f).

Normal V snapping in *C. glutamicum* depends on complete assembly of the septal cell envelope

Our findings suggest that the peripheral PGL is responsible for both the diffusion barrier for MM glycolipids and the mechanical support to hold the two daughter cells together before V snapping, and the perforations formed along the peripheral ring relieve both constraints. Given the difference in the critical size of the perforations for allowing diffusion of glycolipids versus initiating mechanical fracture, the perforations might serve as a built-in mechanism to ensure completion of the daughter cell envelope at the new poles before exposure to the environment during V snapping. We therefore decided to assess the dependency of the two processes (septal cell envelope assembly and V snapping) on each other.

First, we sought to test whether inhibiting V snapping might affect septal cell envelope assembly. To this end, we constructed a deletion mutant of a gene encoding a NlpC/P60-like PG hydrolase in *C. glutamicum* strain MB001 (*cgp_1735*), which has an evident defect in daughter cell separation, thus leading to multiple septa within a cell in *C. glutamicum* R (*cgr_1596*)³³; we then followed the septal-cell-envelope assembly of that mutant with a combination of fluorescent probes (TDL, *O*-TMM-647, and 6-FITre) (Fig. 6a and Supplementary Video 3). We found that this mutant did not appear to have a notable delay in the timing of the rapid septal appearance of 6-FITre (RISS/MM confluency) relative to septation ($\tau_1 = 36 \pm 18$ min, mean $\pm$ s.d., $n = 33$ cells) but displayed a severe delay in V snapping relative to that of septal 6-FITre ($\tau_2 = 87 \pm 53$, mean $\pm$ s.d., $n = 26$ cells). Given the nature of the mutant (deletion of a PG hydrolase), we propose that this mutant is probably deficient in the enlargement or growth of the perforations rather than in their initial formation.

To test this hypothesis, we directly inspected the perforations in the mutant with CLSEM (Fig. 6b) and indeed found that visible perforations along the peripheral ring were much rarer in the mutant, even for cells with septal 6-FITre labeling: 23% of the mutant cells with

septal 6-FITre labeling ($n = 86$ cells) had visible perforations, as compared with 61% for the wild-type strain MB001 ($n = 44$ cells). In addition, we observed that perforations were more likely to be found in mutant cells with multiple septa: 41% of cells with multiple septa had visible perforations ($n = 43$ cells), whereas only 5% of cells with a single septum that had 6-FITre labeling ($n = 39$ cells) had visible perforations. These results are consistent with our hypothesis that the mutant is primarily deficient in perforation enlargement, with the observed delay in V snapping relative to the rest of the cell cycle. However, albeit delayed, the mutant still V snapped quickly, generating hinged daughter cells as the wild type did.

One conceivable consequence of delaying daughter cell separation for a polar-growing bacterium such as *C. glutamicum* is that the center compartments created by secondary septation ('trapped daughter cells') do not have a free pole and therefore cannot elongate via the usual polar growth processes. Indeed, we noticed that the mutant tended to have greater cell width than that of the wild type (Supplementary Fig. 12a,b) and occasionally had curved secondary septa associated with the trapped daughter cells (Supplementary Fig. 12c); both observations are probably due to increased turgor pressure as a result of mass accumulation in the absence of cell elongation. Consistently with this possibility, the curved septa became flat when the built-up turgor pressure was relieved (Supplementary Fig. 12c,d). Interestingly, the delay in V snapping for secondary septa ($\tau_2 = 64 \pm 33$ min, mean $\pm$ s.d., $n = 16$ septa) was much shorter than that for primary septa ($\tau_2 = 124 \pm 59$ min, mean $\pm$ s.d., $n = 10$ septa), a result probably related to the higher turgor pressure in the trapped daughter cells. Together, our observations with this mutant confirmed that V snapping is downstream of completion of the septal cell envelope assembly/MM confluency, and that the timing of these two processes can be decoupled by inhibiting the enlargement of the perforations.

Next, we inhibited cell envelope assembly with the anti-TB drug ethambutol (EMB) to determine whether V snapping might be affected. EMB targets arabinose transferase³⁵, which elongates arabinogalactan chains³⁶, and thus drug treatment decreases the number of sites that anchor the inner leaflet of the MM³⁷. We treated *C. glutamicum* cells with 50 μ g/mL EMB (approximately ten times the minimum inhibitory concentration)²³ in the presence of fluorescent probes and monitored changes in cell growth and cell-envelope assembly. After EMB treatment, we first noticed an increase in FITre signal at the poles (Fig. 6c, Supplementary Fig. 13a and Supplementary Video 4), probably a manifestation of the MM glycolipids accumulating and releasing from the compromised cell envelope, owing to the lack of anchoring points at the growing cell poles. Consistently with this possibility, we observed membrane fragments stemming from the poles of EMB-treated cells with negative-staining TEM (Supplementary Fig. 13g).

Subsequently, we found that cell elongation was severely slowed (nearly stalled after 1-h treatment, Supplementary Fig. 13b) while septation continued, thus giving rise to a more rounded cell morphology, in agreement with findings from previous reports for EMB treatment^{23,38}. We observed an increase in cell width (Supplementary Fig. 13c,d) that was similar to the results for the mutant described above and probably resulted from the cell-elongation inhibition, which exacerbated the morphological changes. Interestingly, we found that after 1 h of drug treatment, only the FDAAs were incorporated at the septum during septation, O-TMM-647 showed a weak signal, and FITre probes were nearly absent from the

septum (Fig. 6c and Supplementary Fig. 13a), results supporting our conclusion that the septal cell envelope is assembled sequentially.

Most notably, we found that after the disrupted septation, V snapping was appreciably slowed from milliseconds to tens of minutes, and intermediates were readily observed where the two newly exposed poles were devoid of FTre labeling (Fig. 6c and Supplementary Video 4). In some extreme cases, the daughter cells adopted a nonhinged geometry or even delayed their separation, thus giving rise to multiple septa (Supplementary Fig. 13e). To rule out the possibility that the observed lack of septal FTre signal and delayed/affected V snapping might have been a result of defects in perforation formation, we performed CLSEM and found that most cells after 3-h EMB treatment displayed visible perforations (Fig. 6d), probably because of the enrichment of a 'ready-to-separate' cell population as a result of delayed separation. In addition, we observed incomplete separation, in which the daughter cells appeared to adhere to each other at the septum/new poles in addition to the hinge (Fig. 6d), probably as a consequence of the disrupted septal cell envelope. Therefore, we conclude that the fast daughter cell separation (V snapping) in *C. glutamicum* depends on the complete and correct assembly of the septal cell envelope.

Discussion

We found that the multilayered cell envelope of *C. glutamicum* and *M. smegmatis* is assembled sequentially at the septum from PG to MM during cytokinesis, unlike the apparently synchronous assembly observed during polar growth, thus suggesting that *C. glutamicum* and *M. smegmatis* use two different mechanisms (and machineries) for envelope assembly during polar growth versus cytokinesis. This conclusion was further supported by our observations with EMB treatment, after which polar PG synthesis and cell elongation were stalled while septal PG synthesis and septation were not affected, in agreement with the observations in a recent report³⁹. The sequential assembly of septal PG and MM in the mycolata contrasts substantially with the synchronous assembly of septal PG and OM in Gram-negative bacteria. Recently, several studies have found that LCP family proteins, which link the wall teichoic acids to PG in model Gram-positive bacteria^{40,41}, are responsible for the ligation of AG to PG in mycolata^{42–44}, thereby suggesting a potentially shared evolutionary origin of the assembly process for those two PG-anchored polymers. Thus, the assembly of the PG–AG–MM cell envelope in the mycolata is more similar to that in other Gram-positive bacteria without an additional membrane than to that of Gram-negative bacterial cell envelopes with an OM, and the geometry of the Π -shaped septum is a direct consequence of the sequential septal envelope assembly, which prevents premature exposure of the new cell poles while they are still under construction.

However, sequential assembly poses an additional challenge for coordination of the envelope assembly as well as coordination with other cellular processes, such as the daughter cell separation that occurs downstream of septation. In the case of *C. glutamicum* with a fluid MM, we discovered that the septum geometry of PGL might play a role in this coordination. We found that the peripheral PGL at the septal junction serves both as a diffusion barrier that physically separates the MM at the cell periphery from the septum and as a mechanical link holding the daughter cells together. Resolution of this junctional PGL through perforations

leads to confluency of the MM of daughter cells and V snapping. Our characterization of a PG hydrolase mutant *cgp_1735*, primarily deficient in perforation enlargement, revealed the different perforation size requirements for diffusion of MM glycolipids and for initiating the mechanical fracture that results in V snapping. Therefore, the peripheral PGL and the difference in critical perforation size provide a potential mechanism for the coordination of cell-envelope assembly and daughter cell separation. Although how the perforations are initially formed and regulated spatiotemporally remains unclear, according to our TEM study, they appear to start from inside the septum compartment, the only region of the peripheral PGL that is neither adjacent to the plasma membrane nor decorated by MM (Supplementary Fig. 14).

Furthermore, treatment with EMB revealed an additional layer of dependency of daughter cell separation on septal cell envelope assembly in *C. glutamicum*. We found that V snapping was notably slowed and delayed after EMB treatment, unlike the results for the *cgp_1735* mutant, in which V snapping was delayed but still occurred with similar speed and geometry to that in wild-type cells. The observation that daughter cells typically remained attached at the septum after the disrupted daughter cell separation caused by EMB suggests that high adhesion between the two septal plates probably contributed to the resistance for separation. The decreased incorporation of *O*-TMM and FTre probes at the septum/new poles, which displayed a smooth surface distinct from the rough surface at the cell periphery observed by SEM (Fig. 6d), indicated a lack of mycolylation consistent with the known mode of action for EMB. The EMB-treated cells might possibly have continued to secrete mycolates to the septum but, owing to the lack of anchoring points, became disorganized and either directly resulted in high adhesion or indirectly resulted in disrupted enzyme activities in the septal compartment.

Intriguingly, the MM fluidity varies dramatically between *C. glutamicum* (fluid) and *M. smegmatis* (waxy), a finding likely to have substantial implications for cell-envelope assembly and cell physiology. High fluidity would favor homeostasis of the MM despite the polar growth mode (Supplementary Fig. 14) and thus could provide a buffer to tolerate defects during assembly, whereas low fluidity would require tighter control to maintain cell-envelope integrity but could promote heterogeneity within a cell population. Indeed, we observed a relatively shorter delay between septal PG- and MM-assembly activities in *M. smegmatis* than *C. glutamicum* (Supplementary Figs. 4d and 7). Finally, our finding that the hydrophilic PGL forms a diffusion barrier for the MM glycolipids (a result consistent with the ordered multilayer envelope structure observed by EM) also poses a challenge for the transport of those mycolates made in the cytoplasm to the cell surface^{45,46}. Future studies identifying and characterizing the molecular players involved not only in the biosynthesis but also in the transport pathway would help shed light on the assembly of the atypical cell envelope in Corynebacterineae.

Methods

Bacterial strains and growth conditions

C. glutamicum strain ATCC 13032, *C. amycolatum* ATCC 49368, and *M. smegmatis* strain mc²155 (ATCC 700084) were used for this study. Corynebacteria were grown with BHI at

30 °C, and *M. smegmatis* was grown with 7H9 supplemented with 0.5% glycerol, 10% ADC, and 0.05% Tween-80 at 37 °C. For all experiments, overnight cultures were diluted 1:100 into fresh medium and grown until midexponential phase. Live-cell imaging was performed on 1% agarose pads prepared with fresh medium or in CellASIC B04A plates (EMD Millipore). 1 µg/mL FM4-64 (Life Technologies) was added in culture or agarose pads when needed to stain the membrane for time-lapse microscopy.

Construction of MB001 *cgp_1735*

For markerless deletion of *cgp_1735* in MB001 (a prophage-free variant of *C. glutamicum* ATCC 13032)⁴⁷, we amplified two DNA fragments corresponding to 750 bp upstream and downstream of *cgp_1735* from MB001 genomic DNA with primers 206_1735F and 1735upR and 1735doF and 206_1735R (Supplementary Table 1), respectively. The fragments were ligated into the vector pCRD206 (ref. ⁴⁸), whose backbone was amplified by isothermal assembly with primers CRD206F and CRD206R, thus yielding pHCL67. pHCL67 was introduced into MB001 by electroporation. Transformants (MB001/pHCL67) were selected by plating the cells on BHI plates supplemented with 15 µg/mL kanamycin (BHI + 15Kan) at 26 °C. To induce integration of pHCL67 into the genome, we plated MB001/pHCL67 cells on BHI + 15Kan and incubated them at 37 °C. Single integrants were plated on BHIS plates supplemented with 10% (wt/vol) sucrose and incubated at 37 °C to select for cells that had undergone double crossover. MB001 *cgp_1735* clones were identified through diagnostic PCR with primers 1735upupF and 1735endR.

Synthesis of fluorescent probes

6-FITre⁵, 6-TMR-Tre⁴⁹, *O*-alkTMM²², NADA¹², TDL¹², BTAA⁵⁰, and DBF-N₃ (ref. ²⁵) were synthesized as previously reported. *O*-TMM probes were obtained from click chemistry of *O*-alkTMM and azide fluorophores. *O*-alkTMM was dissolved at 25 mM in doubly distilled water and filtered through a 0.2-µm membrane. Stock solutions of BTAA and CuSO₄ at 50 mM, in DMSO and water, respectively, were prepared and stored at -20 °C. A 100 mM sodium ascorbate solution was freshly prepared before the click-chemistry reaction. Copper-click cocktail was prepared by mixture of 0.5 µL of 50 mM BTAA, 3 µL of 50 mM CuSO₄, 2.5 µL of 10 mM azide fluorophore (Alexa Fluor 647-N₃ or dibromofluorescein-N₃), 12.5 µL of 100 mM sodium ascorbate, and 1 µL of 25 mM *O*-alkTMM. Reactions were allowed to proceed at room temperature with rapid mixing for 30 min. Copper-clicked products at a final concentration of 1.28 mM were used immediately for cell-labeling experiments or stored at -20 °C. 6-TreAz-AF647 was prepared similarly by mixture of 0.5 µL of 50 mM BTAA, 3 µL of 50 mM CuSO₄, 0.5 µL of 25 mM 6-TreAz, 12.5 µL of 100 mM sodium ascorbate, and 2.5 µL of 10 mM Alexa Fluor 647 alkyne.

Characterization of *O*-TMM probes

To characterize the routes of incorporation for *O*-TMM probes, we fractionated labeled *C. glutamicum* cells to quantify the incorporation of probes into free glycolipids (noncovalent) versus the mycolylarabinogalactan peptidoglycan (mAGPG) polymer (covalent). Bacterial cultures (5 mL) incubated with fluorescent probes were washed twice with growth medium and subjected to organic extraction by addition of 1 mL 2:1 CHCl₃/CH₃OH and vigorous stirring in conical glass tubes overnight. The resulting heterogeneous mixture was

centrifuged for 10 min at 3,700 *g*, the organic-layer supernatant was collected, and the cell pellet containing the insoluble mAGPG material was reextracted twice as described above. Combined organic layers were concentrated and dried by rotary evaporation. Both fractions were then resuspended in 1 mL 2:1 CHCl₃/CH₃OH and analyzed by fluorimetry in quartz cuvettes (1 cm × 0.4 cm). Fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 l-format scanning spectrofluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, an A-1010B lamp housing with an integrated igniter, a switchable 814 photon-counting/analog photomultiplier detection unit, and an MD5020 motor driver.

Cell-envelope labeling

All labeling experiments were performed by addition of the probes to the growth medium. Both the FDAA probes (NADA and TDL) and FTre probes (6-FITre and 6-TMR-Tre) were used at 100 μM, whereas the *O*-TMM probes (*O*-TMM-647 and *O*-TMM-DBF) were used at 25 μM. For in situ labeling, cells in log phase were loaded into a CellASIC B04A plate, and fresh medium with different combinations of fluorescent probes was pressurized into the chamber. 30 s before each image acquisition, fresh medium without fluorescent probes was switched in to wash away the unlabeled free dyes. For chase experiments, overnight cultures were diluted 1:100 into fresh medium with the fluorescent probes and were incubated at the appropriate temperature with shaking in the dark for approximately four doubling times when the culture reached log phase. Labeled cells were then pelleted and washed with fresh medium before being mounted onto a 1% agarose pad made with fresh medium. fDHPE (Life Technologies/ Thermo Fisher) and rDHPE (Life Technologies/Thermo Fisher) were dissolved in ethanol at 1 mg/mL and used at 1 μg/mL (for time lapse) or 10 μg/mL (for FRAP) by incubation of cells with dye in PBS at room temperature for 5 min. Stained cells were washed with PBS before imaging.

Ethambutol treatment

Ethambutol was dissolved in sterile deionized water at 50 mg/mL and used at 50 μg/mL by addition of the stock solution directly to medium or bacterial culture. Negative-staining TEM was conducted on EMB-treated cells. 5 μL cell suspension in PBS was applied onto carbon-coated copper grids (300 mesh) for 1 min and rinsed in deionized H₂O. Each grid was then negatively stained with 1% uranyl acetate (dH₂O) for 1 min and dried. The samples were examined with the same electron microscope used above.

Time-lapse microscopy

2D time-lapse imaging was performed on a Nikon Eclipse Ti inverted fluorescence microscope with a 100× (NA 1.40) oil-immersion objective (Nikon Instruments) and controlled with μ-Manager v1.4. Cells grown on agarose pads or in CellASIC microfluidic chambers were maintained at targeted temperature during imaging with an active-control environmental chamber (HaisonTech). An iXon3 888 EMCCD camera (Andor) was used for fluorescence time-lapse microscopy experiments and a Zyla 5.5 sCMOS camera (Andor) was used for millisecond phase imaging of cell separation. For fluorescence, a 49002-ET-EGFP filter set (Chroma, ET470/40, 495LP, ET525/50) was used to image NADA, 6-FITre, fDHPE, and *O*-TMM-DBF; an 49008-ET-mCherry filter set (Chroma, ET560/40, 585LP,

ET630/75) was used to image FM, TDL, 6-TMR-Tre, and rDHPE; and a 49006-ET-Cy5 filter set (Chroma, ET620/60, 660LP, ET700/75) was used to image *O*-TMM-647. Images were taken with a time interval of 3 or 5 min.

Image analysis

Oufiti⁵¹ was used to segment the cells and extract cell outlines as well as fluorescence-intensity profiles along the cell backbones (Supplementary Fig. 6a,b). Custom MATLAB scripts were used to analyze the intensity profiles. To extract septal signals for each probe, we aligned cells on the basis of cell center, and a reference septum location was first determined by identification of the center peak signal with the second derivative of the FDAA signal in the last frame before V snapping; then for each frame, if there was a peak detected above a preset threshold and in proximity to the reference location, the septum area was redefined accordingly, the corresponding nonseptal area was used to estimate the baseline with the script Baseline Fit, and the signals were then normalized to the mean value of the baseline after baseline correction (Supplementary Fig. 6b); finally, the septal signals were calculated by integration of the normalized and corrected signal within the septal area. To estimate the delays and duration of signal accumulation, septal signals were fit to sigmoid with the script *sigm_fit*:

$$I = I_{\text{bottom}} + \frac{I_{\text{top}} - I_{\text{bottom}}}{1 + 10^{-\left(t - t_{1/2}\right) \times \text{HillSlope}}}$$

where I_{top} and I_{bottom} are the top and bottom plateaus of the signal, and $t_{1/2}$ is the midpoint of the accumulation. The midpoints were used to calculate delays τ_1 and τ_2 , and the *HillSlope* was used to estimate the duration of accumulation (from 5% to 95% signal) D :

$$D = \frac{2 \times \log(19)}{\text{HillSlope}}$$

Fluorescence recovery after photobleaching

FRAP experiments were performed on a Nikon Eclipse Ti inverted fluorescence microscope with a 100× (NA 1.49) oil-immersion Apo TIRF objective (Nikon Instruments), an Andor iXon3 885 EMCCD camera, and an Mosaic II patterned illumination system (Andor) under the control of NIS-Elements. Photobleaching was performed with a 450-mW, 405-nm laser for 50 ms to 2 s with a circular spot with a diameter of ~1–2 μm and was followed by a series of acquisitions with a 492-nm (for 6-FITre and fDHPE) or 561-nm (for 6-TMR-Tre and rDHPE) laser at shallow-angle TIRF and 50-ms exposure. Two segments with different acquisition intervals (1 s and 5 s) were used to acquire the FRAP data. Labeled cells were mounted on 1% agarose pad with PBS.

A custom MATLAB script was used to analyze the FRAP data to extract the fluorescence-recovery kinetics. Briefly, the first image before photobleaching was used to generate a binary mask for the entire cell, and a second mask was generated during experiments to

mark the area of photobleaching. Total fluorescence intensities in both the whole cell area (I_{total}) and the bleached area (overlap between the cell mask and photobleaching-area mask, I_{FRAP}) were extracted and normalized with the following equations to correct for photobleaching of the dyes as a result of acquisition:

$$I(t) = \frac{I_{\text{FRAP}}(t)}{I_{\text{FRAP}}(0)} \times \frac{I_{\text{total}}(0)}{I_{\text{total}}(t)}$$

$$I_{\text{norm}} = \frac{I(t) - I(t_0)}{I(0) - I(t_0)}$$

where $t = 0$ is before bleaching, and $t = t_0$ is immediately after photobleaching.

The normalized fluorescence intensities of the bleached area I_{norm} (example traces in Fig. 2d) were then fitted to the equation below to extract the half-time and fraction of recovery:

$$Y = Y_{\text{max}} \left(1 - e^{\left(\frac{-\ln 2}{t_{1/2}} \right) (t - t_0)} \right)$$

where Y_{max} is the plateau of the recovery curve (fraction recovered), and $t_{1/2}$ is the half-time for the recovery.

Transmission electron microscopy with targeted contrast enhancement of MM

To label the MM, we grew *C. glutamicum* cells in BHI with 50 μM O-TMM-DBF for 4–6 h. Labeled cells were rinsed with 0.1 M sodium cacodylate buffer, pH 7.3, and fixed onto poly l-lysine (Sigma-Aldrich)-treated μ -Dish (35 mm, high Glass Bottom Grid-50, Ibidi) with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C overnight. The following steps were adapted from the click-EM protocol²⁵. Fixed and immobilized cells were first treated for 30 min on ice with blocking solution (0.1 M sodium cacodylate buffer containing 50 mM glycine, 10 mM KCN, 5 mM aminotriazole, and 1 μL of 30% H_2O_2 per 30 mL blocking solution) and were then imaged and photo-oxidized in the presence of freshly made DAB (Sigma Aldrich) solutions (dissolved in 0.1 N HCl to 5.4 mg/mL, which was then diluted tenfold into sodium cacodylate buffer and passed through a 0.2- μm filter) with the same setup as described above for time-lapse microscopy. Epifluorescence and phase-contrast images were taken first with minimum exposure, and regions of interest were illuminated with a GFP filter at full excitation capacity for 10 min.

Photo-oxidized samples were postfixed with 1% osmium tetroxide (Electron Microscopy Sciences) in water for 30 min on ice, washed with ice-cold water (4×2 min), and then dehydrated with an ice-cold graded ethanol series (30%, 50%, 70%, 90%, 100%, and 100%) for 4 min each and finally kept in room-temperature 100% ethanol for 4 min. Samples were then rinsed with acetonitrile and infiltrated with Epon (1:1 with acetonitrile for 2 h, 2:1

overnight and then 100% for 2 h with gentle rocking and then 100% for 2 h without rocking), and polymerized at 60 °C for 24 h. Subsequently, the coverslip and the plastic dish were removed by subjecting samples to approximately three alternating hot/cold cycles with a boiling-water bath and liquid-nitrogen bath. Then photo-oxidized areas of interest were identified and sawed out with a jeweler's saw. Finally, ultrathin (80-nm) sections were cut with a Leica Ultracut S ultramicrotome and viewed on a JEOL JEM-1400 transmission electron microscope at 120 kV.

For conventional TEM sample processing, *C. glutamicum* cells were fixed in 2% glutaraldehyde and 4% paraformaldehyde at 4 °C overnight. The fixed samples were pelleted and washed in sodium cacodylate buffer three times and resuspended in 20 µL of gelatin solution at 37 °C. The suspensions were allowed to solidify on ice and then cut into ~1-mm³ blocks and postfixed in 1% osmium tetroxide at 4 °C for 1 h. Samples were then washed with cold deionized water three times and stained with 1% uranyl acetate at 4 °C overnight. Samples were then dehydrated with a gradient series of ethanol and embedded in Epon in a process similar to that described above. Ultrathin (80-nm) sections were made with a microtome, stained with uranyl acetate and lead citrate, and viewed on the same electron microscope.

Correlative light and scanning electron microscopy

µ-Dish 35 mm, high Glass Bottom Grid-50 (Ibidi) imaging dishes were used to localize cells for CLSEM. Labeled cells (6-FITC + FM4-64 or TDL/O-TMM-647) were pelleted and resuspended in cold PBS and allowed to settle onto poly-L-lysine (SigmaAldrich)-treated correlative imaging dishes 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 cells on the imaging dish were washed with 0.1 M sodium cacodylate buffer and were imaged with light microscopy; first, epifluorescence and phase-contrast images were collected, and the locations of cells on the grids were recorded for later correlation. Imaged cells were postfixed with 1% osmium tetroxide at 4 °C for 1 h (after that step, the coverslips with the immobilized cells were cut out of the dish with a diamond knife), 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).

Oscillatory osmotic shock

A microfluidic flow cell (CellASIC, EMD Millipore) was used to exchange media with different osmolarities²⁷. Midexponential-phase cells were diluted 200-fold into fresh medium, loaded into a CellASIC B04A plate, and incubated at 30 °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 0–200 mM sorbitol (medium B) with the ONIX microfluidic perfusion platform (CellASIC) every minute. 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. Cells were imaged every 20 s with phase contrast to record division (V snapping) events and with Cy5 excitation to monitor the dye intensity (corresponding to medium osmolality).

Laser ablation

Laser-ablation experiments were performed with a MicroPoint laser system (Photonic Instruments/Andor) on an AxioObserver Z1 inverted fluorescence microscope (Carl Zeiss) with a 100× (Plan-Apo NA 1.46 Ph3) oil-immersion objective and back-illuminated EMCCD camera (C9100–14, Hamamatsu). A diffraction-limited laser spot was generated with a pulsed pumping laser through 5 mM coumarin dissolved in methanol. Both the MicroPoint system and fluorescence microscope were controlled with μ -Manager v2.0. Target cells were selected and ablated manually with ‘Point-and-shoot’ mode in the μ -Manager projector plug-in after initial calibration. Images before and after ablation were taken 5 s apart.

Statistical analysis

Statistical analyses were performed with MATLAB (MathWorks, version 2014b). All *P* values in the study were determined with the nonparametric Wilcoxon rank-sum test. For most experiments, *n* refers to the number of cells analyzed unless noted otherwise.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability

All MATLAB scripts used to analyze microscopy images are available upon request.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files) or are available from the corresponding author on reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1 |. The septal cell envelope is sequentially assembled during cytokinesis in *C. glutamicum*.
a, Illustration of the mycolata cell envelope (not to scale) and targeted envelope layers for incorporation of the fluorescent probes used in this study. PM, plasma membrane. **b**, Representative time-lapse images of incorporation of fluorescent probes (TDL, O-TMM-647 and 6-FITre) and cell-envelope-assembly dynamics during cytokinesis. Yellow arrowheads indicate the rapid increase in septal signal (RISS). Microscopy results are representative of two independent experiments. **c**, Quantification of septal signal accumulation for different probes along the cell cycle of the cell shown in **b**. Solid lines for TDL and 6-FITre are fits to sigmoid function, and that for O-TMM-647 is based on smoothed raw data. τ_1 , relative delay in septal appearance between TDL and 6-FITre; τ_2 , delay between septal accumulation of 6-

FlTre and V snapping; D , estimated duration of septal signal accumulation (5–95%). AU, arbitrary units. **d**, Distribution of τ_1 (29 ± 12 min, mean $\pm$ s.d., $n = 33$ cells) and τ_2 (34 ± 24 min, mean $\pm$ s.d., $n = 40$ cells). **e**, Estimated duration of accumulation for the septal signal of TDL (48 ± 19 min, mean $\pm$ s.d., $n = 32$ cells) and 6-FlTre (10 ± 4 min, mean $\pm$ s.d., $n = 29$ cells), $P = 2.4 \times 10^{-11}$, as determined by a two-sided Wilcoxon rank-sum test. For the box plot: center line, median; box limits, upper and lower quartiles; whiskers, 1.5 $\times$ interquartile range extended to adjacent values; red plus signs, outliers. **f**, Structures of the fluorescent probes used in this study.

Fig. 2 |. The mycomembrane of *C. glutamicum* becomes confluent before V snapping.

a, Predicted outcomes of the chase experiment with labeled MM: no inflow (top) and inflow of labeled MM glycolipids into the septum (bottom). **b**, Montage of chase experiment on cells prelabeled with 6-FITre. The cell membrane was marked with FM4-64 (FM), which was present during the chase of 6-FITre. Yellow arrowheads indicate RISS. **c**, Representative FRAP profiles of 6-FITre-labeled *C. glutamicum* cells photobleached at the cell pole (top), cell center (middle), and septum (bottom). Yellow dashed circles indicate the areas of photobleaching. **d**, Fluorescence recovery traces for the cells shown in **c**. **e,f**, Quantification of the half-time for recovery (**e**) and the mobile fraction (**f**) for each of the subcellular

regions ($n = 22$, 11, and 18 cells for cell pole, center, and septum, respectively). P values were determined by a two-sided Wilcoxon rank-sum test. Microscopy results are representative of at least two independent experiments.

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Fig. 3 |. The mycomembrane is confluent between physically touching neighbor cells in *C. glutamicum*.

a, FRAP profile of 6-FITre-labeled *C. glutamicum* cell pairs. Images are contrast adjusted to show the distribution of the fluorescence signal. Yellow dashed circle indicates the area of photobleaching. **b**, Transfer of 6-TMR-Tre-labeled glycolipids between cells that came into contact during growth (V snapping of an adjacent cell). 6-TMR-Tre-labeled *C. glutamicum* cells and unlabeled cells were mixed 1:1 and loaded into a CellASIC B04A chamber to grow. **c**, Exchange of 6-FITre- and 6-TMR-Tre-labeled glycolipids between cells that came into contact during growth (V snapping). 6-FITre-labeled cells and 6-TMR-Tre-labeled cells were mixed 1:1 and loaded into a CellASIC B04A chamber to grow. Microscopy results are representative of two independent experiments.

Fig. 4 |. Peripheral PGL in *C. glutamicum* forms a diffusion barrier for lipids.

a, TEM images of *C. glutamicum* cells prepared with conventional staining (top) versus targeted metabolic labeling to enhance the electron density in MM (bottom). Zoom-in views of the septum-periphery T junction and the corresponding cartoon illustrations are shown on the right. OL, outer layer; ETL, electron-transparent layer. **b**, TEM image of the T-junction region with MM enhanced (left) and illustration of the corresponding cell-envelope geometry (right). Red arrows indicate thinning of the peripheral PGL. **c**, SEM image of a *C. glutamicum* cell with perforations (one example highlighted with a yellow arrow) formed along the peripheral ring. **d**, Correlative light microscopy (top) and SEM images (bottom) of *C. glutamicum* cells labeled with FM4-64FX and 6-FITC, captured at different stages of the cell cycle. Scale bars, 1 μ m. Both contrast-enhanced TEM and CLSEM results are representative of two independent experiments with at least 10 sections on multiple grids for TEM.

Fig. 5 |. Peripheral PGL in *C. glutamicum* undergoes mechanical fracture during V snapping.
a, Daughter cell separation (V snapping) of *C. glutamicum* captured with phase-contrast microscopy at 1 ms per frame. **b**, Daughter cell separation (V snapping) of *C. amycolatum* captured with phase-contrast microscopy at 10 ms per frame (top) and SEM image of *C. amycolatum* (bottom) showing the perforations at the periphery of presnapping cells. **c**, Cumulative counts of V-snapping events observed at different phases of the 4-min oscillatory-osmotic-shock period for *C. glutamicum*. Red line, concentration of sorbitol in the medium; dashed black line, average V-snapping counts, assuming a uniform distribution ($n = 60$ cycles with 87 V-snapping events). **d**, Illustration (left) and example images (right) before and after laser ablation of one of the daughter cells. **e**, Montage of chase experiment on *C. glutamicum* cells prelabeled with NADA. The cell membrane was marked with FM4-64. The yellow arrow points to the fractured PGL. Microscopy results are representative of at least two independent experiments. **f**, Contrast-enhanced TEM of a cell pair after V

snapping, with the hinge region highlighted in the inset. This image is representative of two independent experiments.

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Fig. 6 | V snapping in *C. glutamicum* depends on complete assembly of the septal cell envelope.
a, Cell cycle of *cgp_1735* monitored with fluorescent probes. Red dots, primary septa; yellow dots, secondary septa; yellow arrowheads, RISS of 6-FITre. **b,** Correlative light microscopy (top) and SEM (bottom) images of *cgp_1735* labeled with fluorescent probes. Yellow arrow highlights a perforation. **c,** Cellular changes during 50 µg/mL EMB treatment, as monitored with fluorescent probes. Green arrows, accumulation of 6-FITre signal at the pole; red arrows, intermediate states of the slow daughter cell separation. **d,** Correlative light microscopy (top) and SEM (bottom) images of representative *C. glutamicum* cells treated

with EMB for 2 h (left) and 5 h (right). Yellow arrow highlights one example perforation. Scale bars, 1 μm . Microscopy results are representative of two independent experiments, and CLSEM results are representative of one dataset in which three separate coverslip regions were examined, and at least 50 images were taken for each sample.

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