Donor-delivered cell wall hydrolases facilitate nanotube penetration into recipient bacteria

Amit K. Baidya¹, Ilan Rosenshine¹ & Sigal Ben-Yehuda¹✉

Bacteria can produce membranous nanotubes that mediate contact-dependent exchange of molecules among bacterial cells. However, it is unclear how nanotubes cross the cell wall to emerge from the donor or to penetrate into the recipient cell. Here, we report that Bacillus subtilis utilizes cell wall remodeling enzymes, the LytC amidase and its enhancer LytB, for efficient nanotube extrusion and penetration. Nanotube production is reduced in a lytBC mutant, and the few nanotubes formed appear deficient in penetrating into target cells. Donor-derived LytB molecules localize along nanotubes and on the surface of nanotube-connected neighbouring cells, primarily at sites of nanotube penetration. Furthermore, LytB from donor B. subtilis can activate LytC of recipient bacteria from diverse species, facilitating cell wall hydrolysis to establish nanotube connection. Our data provide a mechanistic view of how intercellular connecting devices can be formed among neighbouring bacteria.

¹Department of Microbiology and Molecular Genetics, Institute for Medical Research Israel-Canada, The Hebrew University-Hadassah Medical School, The Hebrew University of Jerusalem, POB 12272, 91120 Jerusalem, Israel. ✉email: sigalb@ekmd.huji.ac.il
Bacteria growing in close proximity exhibit networks of contact-dependent interactions, enabling the targeted delivery of DNA, proteins, and small molecules. By employing the Gram-positive soil bacterium Bacillus subtilis (Bs) as a model, we have previously described a type of contact-dependent molecular trade mediated by intercellular membranous bridges, termed nanotubes. Nanotubes were found to serve as conduits for intercellular exchange of metabolites, cytoplasmic proteins and even plasmids, hinting at their potential significance in shaping natural bacterial communities. Further, utilizing these intercellular bridges, Bs could deliver the WapA RNAse toxin to, and acquire nutrients from, the opponent species B. megaterium (Bm). Intriguingly, nanotube-like structures were shown to facilitate trafficking of nutrients among various species, including Gram-positive and Gram-negative bacteria, and even in a trans-kingdom manner between bacterial pathogens and mammalian host cells.

We previously provided evidence that a calcineurin-like phosphodiesterase, YmdB, is enriched in the nanotube biochemical fraction of Bs. In fact, cells lacking ymdB were perturbed in nanotube production, a deficiency that coincided with decreased molecular exchange, thereby providing the first genetic link between both processes. More recently, we have demonstrated that five conserved membrane proteins (FlhA, FlhB, FlfP, FlfQ, and FlfR), components of the flagellar export apparatus, designated CORE, serve as a platform for nanotube biogenesis. CORE-dependent nanotube production was shown to be conserved among distinct species, signifying nanotube as a ubiquitous bacterial organelle. In accord, COREs from diverse species could restore nanotube generation and functionality in bacteria lacking endogenous CORE.

Ultrastructural analysis indicated Bs nanotubes to be membranous in nature and to directly emanate from the cell membrane, cross the cell wall (CW) and protrude from the cell surface. The bacterial CW is primarily comprised of peptidoglycan (PG) layers wrapped around the cytoplasmic membrane. In most bacteria, the PG polymer is made of glycan strands cross-linked by peptide side chains which provide strength and rigidity to maintain the cell shape. The estimated thickness of the Gram-positive CW is ~30–100 nm, and the effective pore size is ~2 nm. This brings about the questions as to how the delicate membranous nanotubes emerge from the thick CW shield, and how do they penetrate the protective CW of nearby bacteria. Actually, opening of the PG layers wrapped around the cytoplasmic membrane, termed nanotubes, can serve as conduits for intercellular exchange of nutrients among various species, including Gram-positive and Gram-negative bacteria, and even in a trans-kingdom manner between bacterial pathogens and mammalian host cells.

Interestingly, the nanotube biochemical fraction of Bs was found to contain two major CW hydrolases, LytC (N-acetylmuramoyl-l-alanine amidase) and its activator LytB, encoded by the same operon, both shown to be part of the CW associated proteome. LytC harbors a catalytic domain, which is conserved among Gram-positive and Gram-negative bacteria, that cleaves the amide bond between the N-acetyl muramic acid and stem peptide joining the two glycan layers, an activity that is facilitated in the presence of LytB. Notably, LytC was found to impact Bs swarming motility, independently of flagella formation; however, the exact role of these CW hydrolases is still elusive.

Here we show that LytB and LytC assist nanotube extrusion from the surface of the producing bacterium. Furthermore, LytB molecules are found to localize over nanotube structures, suggesting that they can travel along nanotubes to reach the surface of adjacent bacterium of the same or different species. We further provide evidence that interspecies pairs of LytB, from a nanotube producer, and LytC, from a nanotube receiver, can cooperate to promote nanotube penetration to a recipient bacterium. Our results suggest that the repertoire of hydrolases possessed by the interacting bacteria determines the efficiency of interspecies nanotube formation.

**Results**

**LytB and LytC impact nanotube extrusion and penetration.** As LytB and LytC hydrolases were found to be associated with the nanotube biochemical fraction, we reasoned that they might facilitate the passage of nanotubes through the thick CW material, enabling their reach to the cell exterior. To investigate this possibility, we examined nanotube production in Bs mutants lacking lytB, lytC, or both (Supplementary Fig. 1A, B), grown on a solid surface, by employing Extreme-High Resolution Scanning Electron Microscopy (XHR-SEM). Although intercellular nanotubes were readily detectable in wild-type (wt) cells, their occurrence was significantly reduced in the inspected mutant strains, with the ΔlytBC mutant showing the most severe phenotype (Fig. 1a–c; Supplementary Fig. 1C). Consistently, these mutants were deficient in nanotube-associated intercellular molecular trafficking (Supplementary Fig. 2). Interestingly, the few nanotubes that eventually extruded from the ΔlytBC mutant cells, appeared as they failed to penetrate into the nearby recipient bacterium, exhibiting unusual "continual" nanotubes, extending over the surface of their neighboring cell (Fig. 1b, d; Supplementary Fig. 1C). Such continual nanotubes were rarely observed in wt cells (Fig. 1a, d; Supplementary Fig. 1C). Similar results were obtained when the amidase active site of LytC was mutated to abolish its catalytic activity in a strain lacking lytB, indicating that deficiency in CW degradation is associated with the observed phenotypes (Supplementary Fig. 3). These results hinted that LytB and LytC facilitate extrusion of nanotubes by the producer cell along with their penetration into the receiver bacterium.

**Revealing LytB and LytC localization pattern.** To explore the underlying process of Lyt-mediated nanotube formation, LytB and LytC were tagged with GFP and their localization pattern followed. Both protein fusions were undetectable by fluorescence microscopy (not shown), however, adding antibodies against GFP to live intact cells exposed LytB to be present on the cell surface, arrayed in a foci-like pattern (Fig. 2), while LytC-GFP was still observable on the bacterium surface by immuno-XHR-SEM, indicating that the protein could still reach the cell surface in the absence of nanotubes.

To explore the possibility that LytB surface localization is associated with nanotube generation, we aimed to co-visualize nanotubes and LytB molecules at high resolution. To this end, LytB-GFP expressing cells were subjected to immuno-XHR-SEM, utilizing primary anti-GFP antibodies followed by gold-labeled secondary antibodies. LytB molecules were observed along nanotube structures and over the bacterial surface, mainly close to nanotube sites (Fig. 3a; Supplementary Fig. 5A). These high densities of protein molecules likely correspond to the LytB foci observed on the bacterium surface by immunofluorescence microscopy (Fig. 2a). Similar results were obtained with LytB-
associated LytC, detected by western blot analysis, was significantly weaker than that of LytB (Supplementary Fig. 7D). Consistently, the signal from cell surface LytB molecules to be localized in clusters over the cell surface, whereas no gold signal was found on Bs surface grown alone (Fig. 3b; Supplementary Figs. 5B and 8B). Furthermore, these LytB molecules were arrayed over the Bs surface in vicinity to sites of interspecies connecting nanotubes, but scarce on Bs surface regions that were distant from these sites (Fig. 3b; Supplementary Figs. 5B and 8B). This phenomenon was emphasized when Bs

Bs-derived LytB can be delivered to other species via nanotubes. The localization of LytB molecules to intercellular nanotubes suggests that these molecules may travel via nanotubes to reach the target bacterium and assist in invading the neighboring recipient. To investigate this possibility, we examined whether LytB molecules can be deposited on the surface of a recipient bacterium belonging to a different species. Hence, we co-incubated Bs, producing LytB-GFP, with a wt Bm, lacking GFP, and carried out Immuno-XHR-SEM analysis to visualize single LytB molecules. Since Bm is significantly larger than Bs, we could readily distinguish between these two species in the co-culture (Supplementary Fig. 8A). Remarkably, LytB molecules were plentiful on the surface of Bm when co-incubated with Bs, whereas no gold signal was found on Bm surface grown alone (Fig. 3b; Supplementary Figs. 5B and 8B). Furthermore, these LytB molecules were arrayed over the Bm surface in vicinity to sites of interspecies connecting nanotubes, but scarce on Bm surface regions that were distant from these sites (Fig. 3b; Supplementary Fig. 5B). This phenomenon was emphasized when Bs

**Fig. 1 LytB and LytC impact nanotube extrusion and penetration.** a, b Bs wt (GD215: Δhag) (a) and ΔlytBC (AB64: ΔlytBC, Δhag) (b) strains were grown to mid logarithmic phase, spotted onto EM grids, incubated on LB agar plates for 4 h at 37 °C, and visualized by XHR-SEM. Shown are typical fields of cells (left panels), enlargements of the boxed regions (middle panels), and schematics that depict the interpretive cell layout and highlight intercellular nanotube patterns (right panels). Arrows indicate intercellular nanotubes. Scale bars represent 500 nm.

c Quantiﬁcation of the average percentage of continual nanotubes displayed by Bs wt (GD215) and ΔlytBC (AB64) strains following XHR-SEM analysis as described in a, b. Shown are mean ± SEM and P values (unpaired Student’s t test) of at least three independent experiments (n_cells = 300).

d Quantiﬁcation of the average number of nanotubes displayed by Bs wt (GD215: Δhag), ΔlytB (AB232: ΔlytB, Δhag), ΔlytC (AB62: ΔlytC, Δhag), and ΔlytBC (AB64: ΔlytBC, Δhag) strains were subjected to XHR-SEM analysis as described in a, b. Shown are mean ± SEM and P values (unpaired Student’s t test) of at least three independent experiments. (n_nanotubes = 150). Source data are provided as a Source Data file.

HA producing strain stained with anti-HA antibodies (Supplementary Fig. 6A). As controls, gold signal was not obtained from cells lacking GFP or from cells expressing a cytoplasmic ribosomal protein fused to GFP (Supplementary Fig. 6B, C). Furthermore, cells harboring a YFP fusion to an unrelated surface exposed membrane protein yielded a scattered pattern, which did not coincide with nanotubes (Supplementary Fig. 6D). Inspecting LytB localization in a CORE deﬁcient mutant, revealed LytB molecules to be localized in clusters over the cell surface, consistent with the immunofluorescence results (Supplementary Figs. 4D and 7A). Visualizing LytC revealed fewer molecules in comparison to that of LytB, with the majority being scattered over the cell surface (Supplementary Fig. 7B). Very few LytC molecules were found to localize into nanotube structures (Supplementary Fig. 7B, C), suggesting that, unlike LytB, the LytC enzyme operates at a lower concentration and is not efﬁciently delivered via nanotubes. Consistently, the signal from cell surface associated LytC, detected by western blot analysis, was signiﬁcantly weaker than that of LytB (Supplementary Fig. 7D).
and Vc LytB molecules were detected on nanotubes formed between delivery possibly due to the low amount of this protein. Interspecies deposition of molecules was not apparent with LytC, penetration through the CW of Gram-positive bacteria. Such molecules are delivered along nanotubes to facilitate nanotube microscopy. Shown are phase contrast images (left panels) and respective displayed per cell. Shown are mean ± SEM and FITC-conjugated secondary antibodies and visualized by test) of at least three independent experiments (t data are provided as a Source Data wt (AB132: ΔymdB, ΔymdBOE ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB ΔymdB Δymb
Fig. 3 LytB molecules travel along intercellular nanotubes to reach the recipient cell surface. **a** Bs cells expressing LytB-GFP (AB144: lytB-gfp, ΔymdB, amyE::P<sub>hyper-spank</sub>-ymdB, Δhag) were spotted onto EM grids and subjected to immuno-gold XHR-SEM analysis, using primary antibodies against GFP and secondary gold-conjugated antibodies. Samples were not coated before observation. Shown is an overlay of XHR-SEM images acquired using TLD-SE (through lens detector-secondary electron) for nanotube visualization and vCD (low-kV high-contrast detector) for gold particle (white dots) detection (left panel), and the corresponding vCD image (middle panel). Schematic depicts the interpretive cell layout and highlights the region with gold signal captured by XHR-SEM (right panel). **b** Bs cells expressing LytB-GFP (AB170: lytB-gfp, ΔymdB, amyE::P<sub>hyper-spank</sub>-ymdB, Δhag, ΔwapA) were mixed with Bm (OS2, lacking GFP), spotted onto EM grids and subjected to immuno-gold XHR-SEM as described in **a**. Shown is an overlay of XHR-SEM images acquired using TLD-SE for nanotube visualization and vCD for gold particle (white dots) detection (left panel), and a corresponding vCD image of the boxed region (middle panel). Schematic depicts the interpretive cell layout and highlights the region with gold signal captured by XHR-SEM (right panel). **c** Bs cells expressing GFP tagged LytB (AB144: lytB-gfp, ΔymdB, amyE::P<sub>hyper-spank</sub>-ymdB, Δhag) were mixed with Sa (MRSA, lacking GFP), spotted onto EM grids and subjected to immuno-gold XHR-SEM as described in **a**. Shown is an overlay of XHR-SEM images acquired using TLD-SE for nanotube visualization and vCD for gold particle (white dots) detection (left panel), and a corresponding vCD image of the boxed region (middle panel). Schematic depicts the interpretive cell layout and highlights the region with gold signal captured by XHR-SEM (right panel). **d** Bs cells expressing GFP tagged LytB (AB144: lytB-gfp, ΔymdB, amyE::P<sub>hyper-spank</sub>-ymdB, Δhag) were mixed with Vc (N16961, lacking GFP), spotted onto EM grids and subjected to immuno-gold XHR-SEM analysis as described in **a**. Shown is an overlay of XHR-SEM images acquired using TLD-SE for nanotube visualization and vCD for gold particle (white dots) detection (left panel), and the corresponding vCD image (middle panel). Schematic depicts the interpretive cell layout and highlights the region with gold signal captured by XHR-SEM (right panel). Each experiment was repeated at least five times independently with similar results. Scale bars represent 500 nm. Arrows indicate intercellular nanotubes.
intact Sa cells (Fig. 5c, d). Taken together, these results show that LytB-Bs and LytC-Bs can act from the outside to degrade the CW of various species.

**LytB and LytC from diverse species can couple to facilitate nanotube penetration.** Bm harbors homologs of both LytB-Bs (LytB-Bm) and LytC-Bs (LytC-Bm), but Sa contains only a LytC-Bs homolog (LytC-Sa) (Supplementary Fig. 13C). We next sought to explore whether pairing of LytB and LytC from diverse species could synergize to facilitate CW cleavage. Therefore, we monitored the degradation activity of LytB-Bs on isolated Bs sacculi when combined with LytC-Bm or LytC-Sa. RBB and XHR-SEM analyses showed a significant synergy between LytB-Bs and LytC-Bm that could not be detected with LytC-Sa (Fig. 6a; Supplementary Fig. 13D). Consistent results were obtained using the PI penetration assay with Bs, Bm, or Sa cells (Fig. 6b; Supplementary Fig. 13E, F). The strong synergy between LytB-Bs and LytC-Bm raises the likelihood that these two enzymes can collaborate in nature to degrade the bacterium CW and facilitate nanotube penetration. To mimic such a scenario, we treated wt Bm or mutated Bm lacking lytC with purified LytB-Bs, and tested the ability of the cells to acquire PI. A sharp decrease in the PI uptake was seen for Bm ΔlytC in comparison to Bm wt (Fig. 6c). In accord, PI uptake was decreased in Bs cells deficient for lytC treated in a similar manner (Fig. 6c). These results support the view that LytB delivered via nanotubes can interact with the recipient’s native LytC to locally facilitate CW degradation and hence nanotube invasion.

We have previously shown that Bs kills Bm cells grown in proximity by delivering the tRNase toxin WapA via nanotubes. We thus examined the ability of Bs to inhibit Bm growth, as a readout for establishment of interspecies nanotubes. As expected, Bs lacking both lytB and lytC was impaired in killing Bm, most likely due to the reduced number of nanotubes (Figs. 1 and 7a; Supplementary Fig. 14A). Nevertheless, Bs lacking solely lytC was capable of efficiently killing its Bm opponent, an activity that was dramatically decreased when the recipient Bm was lacking its...
Fig. 4 CWB domains are not required for LytB localization to nanotubes. a A schematic model illustrating the N-terminal SP site, three CWB domains, and amidease enhancer region of LytB, based on NCBI BLAST CD-search, KEGG GenomeNet database MOTIF, and EMBL-EBI InterPro. b Western blot analysis of intracellular and cell surface levels of LytB fused to GFP from Bs wt (AB278: lytB-gfp, gapA-HA<sub>c</sub>), ΔCWB (AB284: lytB-ΔCWB-gfp, gapA-HA<sub>c</sub>), and ΔSP (AB285: lytB-ΔSP-gfp, gapA-HA<sub>c</sub>) strains. Initially, surface proteins were isolated using 1.5 M LiCl from equal number of cells from each strain, followed by intracellular protein isolation by treating the cells with lysozyme and lysis buffer. Shown is a western blot analysis utilizing anti-GFP antibodies to indicate the levels of intracellular (I) and surface (S) LytB (left panel). Intracellular GapA-HAx2 levels, detected on a separate western blot with anti-HA antibodies, serve as a loading control (right panel). The experiments were repeated at least three times independently with similar results. c, d Bs cells expressing GFP tagged LytB-ΔSP (AB263: lytB-ΔSP-gfp, amyD<sub>c</sub>, amyE<sub>c</sub>hyper-spark-ymdB, Δhag) (c) or GFP tagged LytB-ΔCWB (AB250: lytB-ΔCWB-gfp, amyE<sub>c</sub>hyper-spark-ymdB, Δhag) (d) were spotted onto EM grids and subjected to immuno-gold XHR-SEM analysis, using primary antibodies against GFP and secondary gold-conjugated antibodies. Samples were not coated before observation. Shown is an overlay of XHR-SEM images acquired using TLD-SE for nanotube visualization and vCD for gold particle (white dots) detection (left panels), and the corresponding vCD image of the boxed region (middle panels). Schematics depict the interpretive cell layout and highlight regions with gold signal captured by XHR-SEM (right panels). Scale bars represent 500 nm. Arrows indicate the intercellular nanotubes. e Bs cells expressing GFP tagged LytB-ΔCWB (AB250) were mixed with Sa (MRSA, lacking GFP), spotted onto EM grids and subjected to immuno-gold XHR-SEM as described in c, d. Shown are (left to right): an overlay of XHR-SEM images acquired using TLD-SE for nanotube visualization and vCD for gold particle (white dots) detection, an enlargement of the boxed region displayed in the left panel, a corresponding vCD image of the left panel, and a schematic depicting the interpretive cell layout, highlighting the region with gold signal captured by XHR-SEM. Scale bars represent 500 nm. Arrows indicate the intercellular nanotubes. f Quantitation of cell surface display of the number of gold-labeled LytB molecules per cell in wt (AB144), ΔCWB (AB250), and ΔSP (AB263) strains following immuno XHR-SEM as described in c, d. Shown are mean ± SEM and P values (unpaired Student’s t test) of at least three independent experiments (n<sub>cells</sub> = 50). g Quantitation of the number of gold labeled LytB molecules displayed per Sa cell surface when mixed with wt (AB144) or ΔCWB (AB250) Bs strains following immuno XHR-SEM analysis as described in e. Shown are mean ± SEM and P values (unpaired Student’s t test) of at least three independent experiments (n<sub>cells</sub> = 30). Source data are provided as a Source Data file.

endogenous lytC gene (Fig. 7b; Supplementary Fig. 14A). Furthermore, a clear reduction in interspecies nanotubes was monitored when Bs ΔlytC was mixed with Bm ΔlytC (Supplementary Fig. 14B). However, absence of LytB from Bs, Bm, or both had no effect on elimination of Bm (Fig. 7c; Supplementary Fig. 14A). Together, these results reinforce the view that compatibility between LytB and LytC can determine the multiplicity of interspecies interaction.

Discussion

Bacteria produce various apparatuses to conduct intercellular molecular exchange, including transfer of DNA, toxin delivery, as well as nutrient acquisition<sup>6,9,32,33</sup>. However, it remains mysterious how an organelle that physically bridges two adjacent cells is being established to execute these functions. In line with this question, the tactics by which the thick bacterial CW is invaded to pave the way for an intercellular device is yet unknown. Here we report that CW hydrolases can be utilized by the bacterium not only for the emergence of an intercellular nanotube device from the producer cell surface, but also to penetrate the neighboring recipient to establish an intercellular route. We show that the Gram-positive bacterium Bs uses the CW remodeling enzymes, LytC-amidase, and its enhancer LytB, to cross the donor and the recipient CW barriers. Our data suggest that LytB, and to a lesser extent LytC, migrate along nanotubes and reach the surface of neighboring bacteria. Remarkably, such delivery of LytB-Bs molecules could occur in an interspecies manner, as LytB-Bs molecules were deposited over the surface of foreign species in proximity to nanotube penetration sites, suggesting that they act to locally remodel the recipient CW to enable nanotube access. Such delivery of CW hydrolases along with the synergy observed between interspecies pairs of LytB and LytC in situ that nanotube penetration through the CW might be dependent on interspecies compatibility between these hydrolases, among other enzymes with a similar function. This could be a crucial parameter in determining the extent of nanotube-mediated cytoplasmic sharing in heterogenous bacterial communities.

The LytC amidase hydrolyses the highly conserved amide bond present between the N-acetyl muramic acid residue and the l-alanine of the PG backbone, indicating that LytB and LytC pairs potentially have the capacity to modify the CW of multiple species, yet the efficiency of their delivery could vary. The observation that LytB lacking its CW binding domains can still localize to nanotubes, but was rarely observed over the cell surface, raises the possibility that LytB molecules are transported through the membrane nanotubes in a SP domain-dependent manner, and employ the CW binding domains to fasten to the recipient surface. This possibility is strengthened by the findings that LytB-Bs molecules failed to be deposited onto the outer membrane surface of the Gram-negative Vc. Henceforth, our data indicate that CW lytic enzymes could be a preferential mechanism for interactions among Gram-positive species, as such they are likely to be one of the earliest nanotube-delivered cargo. It is tempting to assume that following CW perturbation of a nanotube recipient Gram-positive bacterium, a membrane fusion event is likely to take place. This could be also applicable to interactions involving Gram-negative bacteria or even between bacteria and their mammalian host cells.

Utilization of CW hydrolases for the emergence of extracellular appendages from the producer cell has been documented in various bacterial species, for example the EtwA from <i>E. coli</i> in type 3 secretion system<sup>34</sup>, FlgI from <i>Salmonella</i> in flagellum<sup>35</sup>, VirB1 from <i>Agrobacterium tumefaciens</i> in type 4 secretion system<sup>36</sup>, EssH from <i>Sa</i> in type 7 secretion system<sup>37</sup>, TraG from <i>Enterococcus faecalis</i>, and CwI from <i>Bs</i> in type 4 conjugation plasm<sup>38,39</sup>. Likewise, prophage-encoded endolysins are capable of poking holes within the PG layer, leading to membrane vesicle release by the host<sup>40</sup>. Intriguingly, Anaabaena sp. utilizes a CW hydrolase to assemble an array of nanopores on the bacterial division septum to connect daughter bacteria residing within the same chain<sup>41</sup>. Notably, in all of these mentioned cases, CW hydrolases were found to assist the formation of extracellular structures by the producing bacterium. Similarly, we observed that the lack of LytB and LytC reduces the emergence of nanotubes from the generating bacterium. However, the requirement for these hydrolases to penetrate the CW of the nearby bacterium highlights another dimension for their functionality. It is tempting to speculate that additional intercellular organelles, such as conjugation systems, cross the recipient CW with the aid of dedicated CW hydrolases.

In light of our findings, we propose that Lyt proteins act as keys that enable penetration of nanotubes to recipient bacteria, with the recipient’s own array of CW lytic protein reservoir
contributing to the efficiency of this interaction. Given that nanotubes may be used for both cooperation and competition, our data suggest that bacteria might exploit Lyt compatibility to selectively design their dwelling niche, by physically connecting and exchanging cytoplasmic molecules preferentially with favored species. In line with this view, bacteria might develop resistance mechanisms for such connections to defend from potential delivered toxins and to protect their nutritional resources from being grasped by competing bacteria.

Fig. 5 LytB-Bs and LytC-Bs can exogenously cleave CW of foreign species. a CWs were isolated from Bs (PY79), Bm (OS2), or Sa (MRSA), labeled with RBB, mixed with purified LytB-Bs, LytC-Bs or both. The cleaved products were separated by centrifugation and absorbance of supernatant was measured at 595 nm. Shown are mean ± SEM of at least three independent experiments. b Bs sacculi derived from PY79 cells were incubated for 30 min with purified LytB-Bs, LytC-Bs or both, spotted onto poly-L-lysine coated coverslips and visualized by XHR-SEM. Scale bars represent 500 nm. c Bs (PY79), Bm (OS2) or Sa (MRSA) cells were incubated for 10 min with purified LytB-Bs, LytC-Bs or both, stained with PI and visualized by fluorescence microscopy. Shown are overlay images of phase contrast (gray) and fluorescence from PI staining (red). Scale bar represents 5 μm. d Quantitation of PI uptake by Bs (PY79), Bm (OS2), or Sa (MRSA) cells treated as described in c. Shown is the percentage of PI-labeled cells and mean ± SEM of at least three independent experiments (n_cells = 400). NT indicates no treatment (samples contained only buffer). The experiments were repeated at least three times independently with similar results. Source data are provided as a Source Data file.
Methods
Bacterial strains and general methods. Bs are derivatives of the wild-type strain PY79. Bm is a wild-type soil isolate (OS2) and its derivatives, Sa is MRSA strain and Vc is O1 El Tor N16961 strain. Bacterial strains and plasmids are listed in Supplementary Data 1, and primers are listed in Supplementary Data 2. All general methods were carried out as described previously42. Transformation into Bm OS2 cells was conducted as described43. In brief, Bm OS2 cells were grown up to 1.0 OD600, washed with electroporation buffer (25% PEG 6000 and 0.1 M sorbitol) and resuspended in 1 ml of electroporation buffer. Electroporation was carried out with 0.1 ml of cells supplemented with 500 ng of linear DNA at 1500 V (Bio-Rad). Cells were then diluted into 1 ml of LB and plated on selective antibiotic plate.

Site-directed mutagenesis. Site-directed mutagenesis was carried out to introduce point mutations into the LytC amidase active sites. Four amino acid residues, critical for the amidase activity of LytC (histidine-328 and 395, glutamic acid-342 and 461),

Fig. 6 Interspecies LytB and LytC can pair to enhance CW degradation. a CW was isolated from Bs (PY79), labeled with RBB and mixed with purified proteins in the various indicated combinations. The cleaved products were separated by centrifugation and absorbance of supernatant was measured at 595 nm. Shown are mean ± SEM of at least three independent experiments. b Bs wt (PY79) cells were incubated for 10 min with purified LytB-Bs with or without LytC from Bm or LytC from Sa, stained with PI, and visualized by fluorescence microscopy. Shown are overlay images of phase contrast (gray) and fluorescence from PI staining (red). Scale bar represents 5 μm. Graph presents quantitation of PI uptake by the indicated cells following the above treatments. Shown is the percentage of PI-labeled cells and mean ± SEM of at least three independent experiments (n_cells = 400). c Bm wt (OS2) or ΔlytC (ABm2) and Bs wt (PY79) or Bs ΔlytC (AB3) strains were incubated for 10 min with LytB-Bs, stained with PI and visualized by fluorescence microscopy. Shown are overlay images of phase contrast (gray) and fluorescence from PI staining (red). Scale bar represents 5 μm. Graph presents quantitation of PI uptake by the indicated cells following the above treatments. Shown is the percentage of PI-labeled cells and mean ± SEM of at least three independent experiments (n_cells = 400). Source data are provided as a Source Data file.
were selected following NCBI Conserved Domain annotation, and as described previously for the Bs sporulation amidase CwC, which is homologous to LytC44. The histidine (H) residues were replaced by leucine (L), and glutamic acid (E) residues were replaced by glutamine (Q), as these replacements have most severe effect on CwC amidase activity44. For mutagenesis, DNA fragments were amplified using primers LytC-SDM (P1-P10) that are listed in Supplementary Data 2. The fragments were then joined using Gibson assembly mix and transformed into the indicated Bs strains. The point mutations were confirmed by DNA sequencing.

**Molecular exchange assay.** For detecting molecular exchange, antibiotic transfer assay was carried out as described previously3,12 and in the legend to Supplementary Fig. 2. Donor and recipient strains used for the molecular exchange assay are listed in Supplementary Data 1. Respective donor and recipient strains were grown to mid logarithmic phase, after which cells were mixed in 1:1 ratio (OD600 = 0.8 or 0.08) and incubated in LB supplemented with 1 mM IPTG for 4 h at 37 °C with gentle shaking. Equal numbers of cells were spotted onto either double selective LB agar plates containing Chloramphenicol and Kanamycin for detection of protein exchange or triple selective LB agar plates containing Chloramphenicol, Kanamycin, and Lincomycin for detection of plasmid exchange and incubated overnight at 37 °C. Cells were spotted onto LB agar plates as a control. Fluorescent colonies were detected using Bio-Rad ChemiDoc MP Imaging System.

**Bacterial competition assay.** Overnight cultures of Bs and Bm were diluted to OD600 0.1, mixed and mounted onto a metal ring (A-7816, Invitrogen) filled with

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**Fig. 7 Inhibition of Bm by Bs is mediated by interspecies pairing of LytB and LytC.** a Bs wt (OS2) was mixed with GFP expressing Bs wt (AR16: P<sub>rrnE-gfp</sub>) or Bs ΔlytBC (AB264: ΔlytBC, P<sub>rrnE-gfp</sub>) and subjected to time lapse microscopy. Shown are overlay fluorescence from GFP (green) and phase contrast (red) images, captured at the indicated time points. Bs cells are shown in black while Bs cells are shown in green. Graph presents colony forming unit (CFU/mL) of Bs cells grown alone or in a mixture with the indicated Bs strains. Each time point represents an average value and SD of three independent experiments. b Bs wt (OS2) or Bs ΔlytC (ABm2) were mixed with GFP expressing Bs wt (AR16: P<sub>rrnE-gfp</sub>) or Bs ΔlytC (AB227: ΔlytC, P<sub>rrnE-gfp</sub>) and subjected to time lapse microscopy as presented in a. Graph presents colony forming unit (CFU/mL) of Bs cells grown alone or in a mixture with the indicated Bs strains. Each time point represents an average value and SD of three independent experiments. c Bs wt (OS2) or Bs ΔlytB (ABm1) were mixed with GFP expressing Bs ΔlytB (AB265: ΔlytB, P<sub>rrnE-gfp</sub>) and subjected to time lapse microscopy as presented in a. Graph presents colony forming unit (CFU/mL) of Bs cells grown alone or in a mixture with the indicated Bs strains. Each time point represents an average value and SD of three independent experiments. Time lapse microscopy experiments were repeated at least three times independently with similar results. Scale bars represent 5 μm. Source data are provided as a Source Data file.
Protein extraction and western blot analysis. Cells were centrifuged and incubated into the fresh LB at OD$_{600}$ 0.1 and incubated for 3 h at 37 °C with gentle shaking. The cell pellets were harvested and boiled in 4% SDS solution for 30 min. The cell lysates were then centrifuged at 10,000 x g for 10 min, and the supernatant containing the protein was collected. The proteins were precipitated with 25% TCA and 4% SDS solution for 30 min. The precipitated proteins were then washed twice with 1X PBS and subsequently treated with 0.1% SDS for 10 min. The cell pellets were then resuspended in buffer containing 1.5 M LiCl, 25 mM Tris-HCl (pH 8.0) and 0.5% Triton X-100, and 50 mM Na$_2$HPO$_4$. Next, the lysates were treated using Fastprep (FastPrep MP 6.5, 30 s, ×3) and the lysates were centrifuged at 10,000 x g for 10 min. The supernatant was then added and cells were incubated in ice for 30 min and treated using Fastprep (FastPrep MP 6.5, 30 s, ×3) and the lysates were collected after centrifugation at 10,000 x g for 20 min. The lysates were then mixed with Ni-NTA slurry (Quanta) and incubated at 4 °C for 1 h with gentle mixing. The mixtures were then poured into Poly-Prep chromatography column (Bio-Rad) and washed three times with wash buffer (50 mM Na$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted in elution buffer (50 mM Na$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted fractions were pooled and used for investigation.

CW purification and RBB labeling. CW purification was performed using NIS Elements AR version 4.5 (Nikon) or MetaMorph 7.4 (Molecular Devices). System control and image processing were performed with NIS Elements AR 4.3 (Nikon, Japan).

**XHR-SEM analysis.** grids were incubated for 2 h at 25 °C with rabbit anti-GFP or anti-HA antibodies in normal saline (0.15 M NaCl, 0.01% SDS and 0.01% NaN$_3$) (Jackson, USA) diluted 1:200 in PBSx1 containing 1% BSA. Grids were then washed three times with PBSx1 and incubated for 1 h at 25 °C with gold-conjugated goat-anti-rabbit antibodies (Jackson, USA) diluted 1:100 in PBSx1. Grids were washed three times with PBSx1 and fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 h at 25 °C. Grids were then washed gently with water, and cells were dehydrated by exposure to a graded series of ethanol washes [25, 50, 75, and 100%] for 10 min each, followed by overnight incubation in vacuum. Samples were imaged without coating by Magellan XHR-SEM (FEI). Immuno XHR-SEM analysis. grids were incubated for 2 h at 25 °C with rabbit anti-GFP or anti-HA antibodies in normal saline (0.15 M NaCl, 0.01% SDS and 0.01% NaN$_3$) (Jackson, USA) diluted 1:200 in PBSx1 containing 1% BSA. Grids were then washed three times with PBSx1 and incubated for 1 h at 25 °C with gold-conjugated goat-anti-rabbit antibodies (Jackson, USA) diluted 1:100 in PBSx1. Grids were washed three times with PBSx1 and fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 h at 25 °C. Grids were then washed gently with water, and cells were dehydrated by exposure to a graded series of ethanol washes [25, 50, 75, and 100%] for 10 min each, followed by overnight incubation in vacuum. Samples were imaged without coating by Magellan XHR-SEM (FEI) using TLD-SE and VCD modes. At first, the image of cells and nanotube was acquired in secondary electron mode using TLD-SE and then the same field was imaged using vCD (low-kV, High contrast) detector.

**Quantitation of LytB molecules on Sa cell surface.** Gold particles denoting the LytB molecules on Sa cell surface were quantified by dividing the cell into two halves– proximal and distal. The proximal half contained the nanotube attachment site and the half opposing the nanotube attachment site was designated as distal. Gold particles were counted and the ratio between proximal and distal side was designated as the half opposing the nanotube attachment site was designated as distal. Gold particles were counted and the ratio between proximal and distal side was plotted. The background signal was estimated in individual images by counting the particles in the cell-free regions from multiple areas that were equal in size to Sa cells.

**Protein extraction and western blot analysis.** Cells at mid-logarithmic phase were centrifuged, inoculated into fresh LB at OD$_{600}$ 0.1 and incubated for 4 h in 37 °C with gentle shaking. The cell pellets were harvested and washed with 10 mM Tris-HCl, pH 8.0. Cell pellet was then resuspended in buffer containing 1.5 M LiCl, 25 mM Tris-HCl at pH 8.0, and kept in ice for 10 min. The suspension was then centrifuged and supernatant containing the protein was collected. The proteins were precipitated using 10% TCA (w/v). The remaining cell pellet was then used to isolate the intracellular proteins following treatment with lysozyme (20 μg ml$^{-1}$) and subsequent with lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris pH 8.0). Next, proteins were separated by SDS-PAGE on 4, 6, 8, 10, 12, 14%, and 18% gels, and stained with 0.25% Coomassie Brilliant Blue R-250. Band intensities were quantified using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). The source data for Figs. 1c, 2b, 4b, 5a, 6a, 7b, 8a are provided as a Source data file. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Statistical analysis.** Statistical analysis was performed using the MS office excel and GraphPad Prism softwares. P-values obtained are indicated in the respective figures.

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

**Data availability.** The source data for Figs. 1c, d, 2b, 4b, 5a, 6a, 7c–e, 8c and Supplementary Figs. 1A, 2A–D, 7C, D, 9B, 10A, 11B, 13, and 14A, B are provided as a Source data file. All data supporting the findings of this study are available from the corresponding author upon request.

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Author contributions

A.K.B. performed the experiments. A.K.B., S.B.-Y, and I.R. conceived the experiments and managed the project.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to S.B.-Y.

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