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The RND-family transporter, HpnN, is required for hopanoid localization to the outer membrane of *Rhodopseudomonas palustris* TIE-1

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*Rhodopseudomonas palustris* TIE-1 is a Gram-negative bacterium that produces structurally diverse hopanoid lipids that are similar to eukaryotic steroids. Its genome encodes several homologues to proteins involved in eukaryotic steroid trafficking. In this study, we explored the possibility that two of these proteins are involved in intracellular hopanoid transport. *R. palustris* has a sophisticated membrane system comprising outer, cytoplasmic, and inner cytoplasmic membranes. It also divides asymmetrically, producing a mother and swimmer cell. We deleted genes encoding two putative hopanoid transporters that belong to the resistance-nodulation-division (RND) superfamily. Phenotypic analyses revealed that one of these putative transporters (HpnN) is essential for the movement of hopanoids from the cytoplasmic to the outer membrane, whereas the other (Rpal_4267) plays a minor role. CSR hopanoids, such as diploptene, are evenly distributed between mother and swimmer cells, whereas hpnN is required for the CSR hopanoid, bacteriohopanetetrol, to remain localized to the mother cell type. Mutant cells lacking HpnN grow like the WT at 30 °C but slower at 38 °C. Following cell division at 38 °C, the ΔhpnN cells remain connected by their cell wall, forming long filaments. This phenotype may be attributed to hopanoid mislocalization because a double mutant deficient in both hopanoid biosynthesis and transport does not form filaments. However, the lack of hopanoids severely compromises cell growth at higher temperatures more generally. Because hopanoid mutants only manifest a strong phenotype under certain conditions, *R. palustris* is an attractive model organism in which to study their transport and function.

H opanoids are bacterial lipids that exhibit structural and bio-synthetic similarity to eukaryotic steroids (Fig. 1A), with the exception that they can be synthesized under strictly anaerobic conditions whereas steroids cannot. Molecular fossils of steroids and hopanoids (steranes and hopanes, respectively) can be found in ancient sedimentary rocks that formed at least 2.7 billion years ago (1–3). Whereas steroids are well known to play important roles in eukaryotic membrane composition and curvature as well as intercellular signaling pathways (4–6), we know relatively little about the biological functions of hopanoids. Physiological studies have associated hopanoid production with changes in temperature, desiccation, pH, and cellular differentiation (7–11). The immense structural variation of hopanoids, including modification by methylation or the addition of diverse polar head groups (12, 13), suggests there may be specificity in their structures with regard to localization and/or function. Certain hopanoids, for instance, localize to the outer membrane of Gram-negative cells in the context of stress resistance (8), and other work has indicated that hopanoids are specifically found in the outer membrane of a variety of bacteria (8, 14, 15). However, the mechanism of intercellular hopanoid transport has not been explored.

Recently, an “unsolved mystery” featuring hopanoids was highlighted regarding the evolutionary history of proteins found in the Hedgehog signaling pathway (5). Haussmann et al. (5) argued that eukaryotic transporters (e.g. Patched, Dispatched, and the Niemann–Pick carrier protein 1 (NPC1)) involved in movement of cholesterol or cholesterol-modified morphogens likely arose from bacterial hopanoid transporters belonging to the resistance-nodulation-division (RND) superfamily. This hypothesis was based on sequence similarity between the eukaryotic proteins and a specific subfamily of bacterial RND transporters, termed HpnN, whose members are associated with hopanoid biosynthesis genes in bacterial genomes. The structural similarity between steroids and hopanoids—the putative substrates of these transporters—further supports this hypothesis. This model assumes that the HpnN subfamily plays a role in hopanoid transport, and predicts that the last common ancestor of the bacterial HpnN and eukaryotic transporters was also a hopanoid transporter.

To test this hypothesis, we chose to work with the Gram-negative bacterium *Rhodopseudomonas palustris* TIE-1, which has emerged in recent years as an attractive model organism in which to study hopanoid biology (10, 16–19). *R. palustris* can produce three different membrane types (outer, cytoplasmic, and inner cytoplasmic; these latter membranes are lamellar and are structurally reminiscent of the Golgi apparatus in eukaryotic cells) and also divides asymmetrically, producing distinct mother and swimmer cells. Its genome contains two putative RND transporters—Rpal_4254 (HpnN) and Rpal_4267—within the region of the *R. palustris* chromosome that encodes many of the hopanoid biosynthetic genes (Fig. 1B). By combining genetic and cell biological approaches, we identified one of these genes (*hpnN*) as being essential for hopanoid transport to the outer membrane. Here, we explore the evolutionary relationship of HpnN to steroid transporters, and demonstrate that it plays an important role in the growth of *R. palustris* at 38 °C.

**Results**

**Phylogenetic Clues to the Function of Hopanoid-Associated RND Transporters.** The RND superfamily is widespread across bacteria, archaea, and eukaryotes. The hopanoid-associated RND transporters Rpal_4254 and Rpal_4267 share characteristic topological features with this superfamily, namely 12 predicted

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transmembrane helices with large extracytoplasmic loops between helices 1 and 2 and helices 7 and 8, as predicted by Krogh et al. (20). Unlike the well characterized tripartite efflux pumps of Gram-negative bacteria (e.g., AcrAB-ToIC), the hopanoid-associated proteins appear to lack ToIC docking domains and are not cotranscribed with a cognate adaptor protein. To gain insight into the function and evolutionary history of these proteins, we reconstructed their phylogeny relative to eight recognized families of RND transporters (Fig. 2). From the superfamily phylogeny, Rpal_4254 appears most closely related to family 8, whose sole characterized member is required for the accumulation of the pigment xanthomonadin in the outer membrane of Xanthomonas oryzae (21). Rpal_4267 is associated with family 7, whose members include archaeal and spirochete proteins of unknown function (22). Regardless of where the root is placed in our RND superfamily phylogeny (Fig. 2), the eukaryotic family 6 transporters and the HpnN-containing family 8 cannot be sister clades, indicating that the eukaryotic Patched family of proteins (family 6) is no more closely related to HpnN than to other families of bacterial RND transporters (5).

To assess how widespread hopanoid-associated RND transporters might be beyond just *R. palustris*, a second phylogeny was constructed; this time focused on proteins from families 7 and 8 (Fig. 3 and Fig. S1). Because most proteins in these two families come from organisms lacking squalene-hopene cyclase (Fig. 3, inner ring), the potential for hopanoid intracellular transport is relatively restricted. To further support a function in hopanoid transport, we asked whether the RND-encoding gene was near hopanoid biosynthesis genes on the chromosome (Fig. 3, outer ring). Several good candidates for hopanoid transport emerged: the HpnN-like clade, which includes Rpal_4254 and is found in diverse proteobacteria; a smaller Rpal_4267-like clade confined to closely related α-proteobacteria; and a lone archaeal isolate, Halorubrum lacusprofundi. Because the HpnN-like clade contains a diversity of known hopanoid producing bacteria spanning α-, β-, γ-, and δ-proteobacteria, we hypothesized that it might be involved in the intracellular localization of hopanoids in *R. palustris* (Fig. 3).

**Rpal_4254 (HpnN) Is Essential for Bacteriohopanopetalon Localization to Outer Membrane.** Hopanoids were found to comprise 12.5 ± 1 μg × mg total lipid extract (TLE)−1 of membrane fractions of *R. palustris* (Fig. 4). The most abundant hopanoid was bacteriohopanopetalon (BHT), which composed 8.1 ± 0.7 μg × mg TLE−1 of the whole cell (Fig. 4). The C30 hopanoids (e.g., diploptene; Fig. 1) were less abundant and collectively composed 3.8 ± 0.3 μg × mg TLE−1 and 8.7 ± 2.8 μg × mg TLE−1 of the whole cell and outer membrane fractions, respectively. Deletion of the genes encoding the two hopanoid associated RND transporters (Rpal_4254 and Rpal_4267) created mutant strains with different phenotypes. Whereas the Rpal_4254 mutant no longer contained any hopanoids in the outer membrane (Fig. 4), the Rpal_4267 mutant still contained a significant percentage (Fig. S2). Interestingly, the Rpal_4267 mutant produced more 2-methylhopanoids then observed in the WT or Rpal_4254 mutant, possibly indicating some role for Rpal_4267 in hopanoid homeostasis. Because the Rpal_4267 mutant was only mildly compromised in hopanoid transport, we focused our characterization efforts on the Rpal_4254 mutant, which we refer to henceforth as ΔhpnN. ΔhpnN contained elevated concentrations of hopanoids in the cytoplasmic and inner cytoplasmic membrane fractions. For example, BHT increased from 4.4 ± 1.9 μg × mg TLE−1 in WT to 11.89 ± 2.0 μg × mg TLE−1 in ΔhpnN (Summary Figure). To verify that the loss of hopanoids in the outer membrane was a result of the deletion of hpnN, we performed a complementation experiment. When the hpnN gene was expressed on a multicopy plasmid in the ΔhpnN mutant background, hopanoid transport to the outer membrane was restored. Interestingly, whereas the whole-cell hopanoid composition of ΔhpnN did not change significantly relative to WT, complementation of the ΔhpnN mutant stimulated C30 hopanoid biosynthesis and transport to the outer membrane (Fig. 4); this is potentiola a result of expression of hpnN from a multicopy plasmid.

**Hopanoid Expression Is Correlated to Cell Cycle of *R. palustris*.** The cell cycle of *R. palustris* is obligately bimodal with each cell division resulting in the production of a mother cell and a flagellated swarmer cell. Because these cell types have different biological functions and different membrane structures (e.g., mother cells produce buds from one end of the cell, which become swarmer cells), we decided to explore the expression of hopanoids in the context of the cell cycle. Synchronous cultures of *R. palustris* were created using the sucrose density gradient pro-
tocot described by Westmacott and Primrose (23). We followed the growth of swarmer cells into mother cells by light microscopy and observed the loss of motility (i.e., conversion to mother cell), the budding growth of the swarmer cell from the mother cell, followed by cell division (Fig. 5A). Cultures doubled in direct cell count between hours 5 and 6, suggesting growth had been successfully synchronized (Fig. 5B). The relative DNA content of cells was followed by using the PicoGreen dsDNA stain, and a doubling of DNA staining at hour 5 confirmed that more than 95% of the cells in the synchronized culture contained a second copy of the chromosome (Fig. 5B). At the start of our synchronized growth experiment, swarmer cells were devoid of BHT to the limit of detection of approximately 1 μg BHT × mg TLE⁻¹ (Fig. 5C). In contrast, C₅₀ hopanoids were found in equal amounts in mother and swarmer cells. This may imply that different hopanoids have different cellular functions. The hopanoid content of the synchronized cultures increased to 20 μg BHT × mg⁻¹ TLE within 2 h of incubation in complete medium (Fig. 5C). BHT synthesized during the growth of swarmer cells into mother cells was exclusively produced in the desmethyl form. Following cell division, swarmer cells were separated from mother cells by density gradient centrifugation. The new population of swarmer cells contained trace amounts of BHT (possibly resulting from imperfect separation from mother cells) whereas the mother cells contained 20 μg BHT × mg⁻¹ TLE. These data demonstrate that the synthesis of BHT is correlated to the cell cycle in R. palustris. Interestingly, both BHT and 2-methylBHT were detected in swarmer cells of ΔhpnN (Fig. S3). Because total hopanoid abundance was unchanged in ΔhpnN, the occurrence of BHT in swarmer cells is unlikely to result from up-regulation of hopanoid biosynthesis. Instead, diffusion of BHT and 2-methylBHT from the cytoplasmic membrane of mother cells to that of swarmer cells seems probable.

**Permissive and Selective Growth of Mutant Strains in Response to Temperature.** Separate from the question of whether hopanoid production is cell cycle-associated, we were interested in exploring whether specific environmental conditions might elicit their production. Because hopanoid production by other bacteria is stimulated at elevated growth temperatures (7, 11), we set out to determine whether this is also the case for R. palustris. When the growth temperature was increased from 30 °C to 38 °C, whole-cell BHT content increased by approximately 25%. Accordingly, we predicted that the growth of ΔhpnN would be conditionally sensitive to temperature. To test this hypothesis, two control strains were constructed: one that cannot produce hopanoids (Δshc) and one that neither produces hopanoids nor contains the hpnN gene (ΔshcΔhpnN). Increasing the temperature from 30 to 38 °C slightly increased the growth rate of the WT, but ΔhpnN, Δshc, and ΔshcΔhpnN all grew more slowly and to lower OD₆₀₀ at 38 °C than at 30 °C (Fig. 6A and B). The expression of hpnN from pDMD5 restored normal growth to the

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**Fig. 3.** Maximum likelihood phylogeny of two families of RND transporters associated with hopanoid transport. Concentric rings depict co-occurrence of squalene hopene cyclase in the genome (inner ring) and proximity to genes known to be involved in hopanoid biosynthesis (outer ring). Nodes with bootstrap support greater than 95% are marked with a gray circle. For clarity, sequence names have been omitted and closely related clades have been collapsed; the full version is shown in Fig. S1.
ΔhpnN mutant compared with that of an empty vector control in the WT background; the empty vector control did not complement ΔhpnN. ΔhpnN was not as significantly affected as Δshc, suggesting that hopenoids can partially protect cells from elevated temperature when localized to the cytoplasmic and inner cytoplasmic membrane(s). Although we cannot rule out the possibility that the growth defect in the Δshc mutant is caused by the buildup of squalene, when succinate was supplied to stationary-phase cultures at 38 °C, it stimulated the growth of all strains. This, together with the fact that both the Δshc and ΔhpnN mutants appear to shed membrane material more than the WT, suggests that carbon limitation may underpin the growth defect as opposed to squalene being toxic per se. A growth handicap at 38 °C was most pronounced for ΔshcΔhpnN, suggesting that HpnN has additional roles besides transporting hopenoids.

Temperature-Dependent Cellular Filamentation Results from Hopenoid Mislocalization. We predicted that the slow growth and low yield of the hopenoid transport and/or biosynthesis mutant strains at elevated temperature might correlate with specific morphological deformities. To test this, we visualized the WT and mutants by light and transmission EM. R. palustris TIE-1 formed single cells at both 30 °C and 38 °C (Fig. 6 C and D), whereas mutant strains displayed diverse morphologies. ΔhpnN formed single cells when grown at 30 °C (Fig. 6 E), yet filamented when grown at 38 °C (Fig. 6 F); complementation of ΔhpnN with hpnN expressed on pDMD5 allowed the strain to divide normally at 38 °C, whereas an empty vector control did not. In contrast to ΔhpnN, Δshc and ΔshcΔhpnN formed single cells at both 30 °C and 38 °C but sometimes had small blebs localized to one pole at 38 °C (Fig. S4), suggesting that the observed temperature-dependent growth and morphological phenotypes may be caused by separate phenomena. Because the lack of filamentation in the Δshc and ΔshcΔhpnN mutants could have resulted from poor growth, we added 20 mM succinate to these cultures to stimulate their growth to an OD600 of 0.4, comparable to that of WT. Under these conditions, neither mutant filamented. This indicates that hopenoid mislocalization, rather than the absence of hopenoids in the outer membrane, is responsible for the filamentation phenotype observed at 38 °C. However, we cannot yet rule out the possibility that the growth rate, even in the presence of added succinate, was below that needed to trigger filamentation.

Relative DNA concentration was measured by flow cytometry to verify the chromosome was replicating in the filamented cells. When synchronous cultures of ΔhpnN were grown at 30 °C, the DNA content of the cell population doubled just before cell division (Fig. S5 A). In contrast, when ΔhpnN was grown at 38 °C, the DNA content increased well above that seen for swarmer or predivisional cells at 30 °C (Fig. S5 B), indicating that the 38 °C cells were replicating their chromosome. Interestingly, when
PicoGreen dsDNA dye was used to visualize DNA within the cells by fluorescence microscopy. Fluorescence was typically confined to one end of the filament (Fig. S4 C–E). These results suggest that although chromosome replication had occurred, chromosome partitioning was impaired in the filament. Because cellular morphology often tracks with that of the cell wall, we tested whether there were differences in the peptidoglycan sacculi of the WT and $\Delta$hpnN (Fig. 6 G–I). The sacculi of WT cells were consistent with their whole cells’ dimensions, whereas the sacculi of filamented $\Delta$hpnN cells were regularly constricted (Fig. 6J). These data indicate that cell division at the level of peptidoglycan synthesis or hydrolysis was detrimentally affected in $\Delta$hpnN at 38 °C.

### Discussion

In this study, we have shown that HpnN is required for hopanoid transport. Together with our phylogenetic analysis, our phenotypic data supports the hypothesis that eukaryotic cholesterol and lipidated-morphogen transporters are evolutionarily related to lipid-translocating bacterial RND transporters. However, we believe it is premature to conclude that the eukaryotic transporters arose specifically from a bacterial hopanoid transporter. Based on our phylogenies, this scenario would require that the families containing HpnN and the eukaryotic steroid transporters descended from a common hopanoid-transporting ancestor. Two issues complicate this scenario. First, the potential for hopanoid transport within the HpnN-containing family of the RND transporters—as judged by the ability of the bacteria with HpnN-like proteins to make hopanoids—is relatively limited, and indeed another member of the family has been shown to transport pigments instead (21). Thus, it is possible that the ancestor of this family transported a substrate other than hopanoids. Second, regardless of where the root is placed in our RND superfamily phylogeny (Fig. 2), the eukaryotic family 6 transporters and the HpnN-containing family 8 cannot be sister clades (24). This evolutionary complexity notwithstanding, both our functional and phylogenetic data support the use of bacterial model systems to gain insight into sterol trafficking in eukaryotes.

The asymmetric distribution of certain hopanoids between mother and daughter cells is intriguing, and given recent indications of subcellular lipid localization in bacteria (25, 26), it is telling that homologs of hopanoids may segregate at an even smaller scale. By analogy to studies demonstrating cholesterol’s role in generating protein–lipid microdomains with specific cellular functions (27), it is conceivable that asymmetric hopanoid distribution might promote cell division at elevated growth temperatures by participating in the formation of protein–lipid microdomains, resulting in the recruitment of cell division machinery to the proper subcellular region. Hopanoids might not be needed to organize such domains at lower temperatures because of a more rigid bacterial membrane in which protein organization might be more stable. Alternatively, hopanoids themselves might interact directly with cell division machinery, much as sterols have been shown to serve as allosteric effectors of other proteins (28). Related to this, it is noteworthy that the genome of R. palustris TIE-1 contains two putative sterol-binding proteins that also contain metallo-β-lactamase domains. Such domains are found in proteins that catalyze peptidoglycan hydrolysis (29). Interestingly, Escherichia coli outer membrane lipoproteins are known to activate cell wall polymerases (30, 31) and possibly also are found in proteins that catalyze peptidoglycan hydrolysis (29). Related to this, it is noteworthy that the genome of R. palustris contains two putative sterol-binding proteins (28). Based on our phylogenies, this scenario would require that the families containing HpnN and the eukaryotic steroid transporters descended from a common hopanoid-transporting ancestor. Two issues complicate this scenario. First, the potential for hopanoid transport within the HpnN-containing family of the RND transporters—as judged by the ability of the bacteria with HpnN-like proteins to make hopanoids—is relatively limited, and indeed another member of the family has been shown to transport pigments instead (21). Thus, it is possible that the ancestor of this family transported a substrate other than hopanoids. Second, regardless of where the root is placed in our RND superfamily phylogeny (Fig. 2), the eukaryotic family 6 transporters and the HpnN-containing family 8 cannot be sister clades (24). This evolutionary complexity notwithstanding, both our functional and phylogenetic data support the use of bacterial model systems to gain insight into sterol trafficking in eukaryotes.

### Methods

#### Phylogeny of HpnN.

The phylogeny of the entire RND superfamily was inferred based on the transmembrane domains, which are more conserved than the extracytoplasmic loops. We identified 8,999 proteins containing two instances of the structural domain SCO 82886 (multidrug efflux transporter AcrB transmembrane domain) in the MicrobesOnline public database (36). These domains were aligned by using HHM MER3 (http://hmmer.org) to the profile HHM of SCO 82886, and the tree was reconstructed using FastTree 2.1.3 (37). The resulting unrooted tree shown in Fig. 2 largely recovers the ancient molecular fossils. For example, structural modiﬁcations of sterols can have a dramatic impact on their biological function in higher organisms as well as inﬂuencing membrane curvature (32). In this context, it is noteworthy that hopanoid expression peaks in mother cells as they bear swarmer cells, a time when dramatic changes in membrane curvature occur. Of relevance is the fact that eukaryotic RND transporters similar to HpnN have been shown to be important in facilitating tubulation in eukaryotic cells, presumably through lipid trafficking (33–35). For example, in the nematode Caenorhabditis elegans, the proper assembly of a tubular channel through which sensory neurons access the outside environment is controlled by the HpnN-like proteins Daf-6 and CHE-14 (35). Although the precise mechanism whereby these proteins affect tubulation is not understood, it is thought that they contribute to tuning the balance between exocytosis and endocytosis to permit vesicle coalescence and the generation of a tube through the cell (35). It is unclear if HpnN bears mechanistic similarity to the Daf6 protein, but given that the budding growth (i.e., tube) bridging R. palustris mother cells and swarmer cells is at a similar scale to the tubular channel in C. elegans (35), it is worth investigating whether hopanoids localize to this part of the preswarming cell body in an HpnN-dependent manner.

Unlike eukaryotic steroids, hopanoids and their transporters are not essential in R. palustris TIE-1 (10), providing us with the opportunity to study the functional consequence of hopanoid transport and localization in a living cell. In the years to come, it will be interesting to identify the similarities and differences between hopanoids and steroids with respect to their biophysical properties and cellular functions. Given that hopanoids are remarkably geo-stable, a better appreciation of their biological functions will improve our evolutionary interpretations of their ancient molecular fossils.

#### Culture Conditions.

Bacterial strains used in this study are listed in Table S1. Medium and culturing protocols for the growth of R. palustris and E. coli were conducted under phototrophic growth conditions as described previously (17). Synchronized cultures of R. palustris were created using a sucrose density gradient to isolate swarmer cells, as described by West-macott and Primrose (23).

#### DNA Methods, Plasmid Construction, and Transformation.

All plasmids and primers used in this study are described in Table S2. The construction of clean deletion mutants of the Rpal4254 and Rpal4267 genes was carried out by using the gentamicin selection, sucrose counter-selection method described previously (43). The generation of complementing plasmids was carried out as previously described (43).
Membrane Preparations of *R. palustris*. The outer membrane of *R. palustris* was removed by gentle sonication in a 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS, pH 7.2) buffer and 5 mM EDTA. Cells were broken down by centrifugation at 5,000 × g for 20 min at 4 °C, and the supernatant containing outer membrane was collected. Aliquots of the outer membrane containing supernatant were layered on top of a 40% sucrose solution and subjected to ultracentrifugation at 50,000 × g for 10 min, resuspended in 10% SDS, and boiled for 30 min. The SDS solution was allowed to cool and was then subjected to ultracentrifugation at 50,000 × g for 2 h. Following ultracentrifugation, saccule formed a pellet at the bottom of the tube, and were resuspended in 10% SDS and boiled for another 30 min. Saccule were again harvested by ultracentrifugation and resuspended in water. Staining and visualization was accomplished as described for SI Methods.

Lipid Extractions and Hapanooid Quantification. Hapanooids were quantified by the high-temperature GC-MS protocols developed by Welander et al. (10, 17), except silica gel columns were used to distinguish variation in the abundances of hydrocarbon and alcohol structures. 2-Methyl-hopanooids could not be detected in the hexane fraction from the silica gel columns (Fig. S6). These results suggest 2-methylhopanooids are formed from 2-methyl dipolyolper during some of the steps of the analytical protocol. In the present study, we do not distinguish between diplopterol and diploptenes; rather, we extended the incubation of lipid extracts with pyridine and acetic anhydride to 30 min at 70 °C, only diplopterol and 2-methyldiploptene could be detected, and they were quantified as described previously (8). The extended hapanooids BHT, 2-methylBHT, and bacteriopahopanaemaminotriol were detected as described previously (10, 17).

**EM.** For transmission EM of whole cells, *R. palustris* cultures were diluted to a concentration of 1 × 10^6 cells mL⁻¹ by using sterile water. A drop of the diluted culture was placed on Parafilm and grids were floated on the surface for approximately 10 min. Grids were then stained by transferring them onto droplets of 2% (v/v) uranyl acetate (UA) (36) for 30 s. Grids were washed with deionized water and blotted dry. For the visualization of peptidoglycan saccule, cultures were harvested by centrifugation 5,000 × g for 10 min, resuspended in 10% SDS, and boiled for 30 min. The SDS solution was allowed to cool and was then subjected to ultracentrifugation at 50,000 × g for 2 h. Following ultracentrifugation, saccule formed a pellet at the bottom of the tube, and were resuspended in 10% SDS and boiled for another 30 min. Saccule were again harvested by ultracentrifugation and resuspended in water. Staining and visualization was accomplished as described for the whole cells.

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