Determining the bacterial cell biology of Planctomycetes

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Bacteria of the phylum Planctomycetes have been previously reported to possess several features that are typical of eukaryotes, such as cytosolic compartmentalization and endocytosis-like macromolecule uptake. However, recent evidence points towards a Gram-negative cell plan for Planctomycetes, although in-depth experimental analysis has been hampered by insufficient genetic tools. Here we develop methods for expression of fluorescent proteins and for gene deletion in a model planctomycete, Planctopirus limnophila, to analyse its cell organization in detail. Super-resolution light microscopy of mutants, cryo-electron tomography, bioinformatic predictions and proteomic analyses support an altered Gram-negative cell plan for Planctomycetes, including a defined outer membrane, a periplasmic space that can be greatly enlarged and convoluted, and an energized cytoplasmic membrane. These conclusions are further supported by experiments performed with two other Planctomycetes, Gemmata obscuriglobus and Rhodopirellula baltica. We also provide experimental evidence that is inconsistent with endocytosis-like macromolecule uptake; instead, extracellular macromolecules can be taken up and accumulate in the periplasmic space through unclear mechanisms.
For decades, Planctomycetes appeared to blur the line between prokaryotes and eukaryotes. These unusual bacteria are ubiquitous and both environmentally and biotechnologically important. Planctomycetes were initially described as eukaryotes and later acknowledged as bacteria, and they were proposed to share some sort of evolutionary link with eukaryotes. This hypothesis was supported by conspicuous traits of the planctomycetal cell biology, traits which are usually associated with eukaryotes rather than with bacteria.

For example, Planctomycetes were thought to have a proteinaceous cell wall, while a peptidoglycan cell wall to maintain cell integrity is the bacterial hallmark trait. In addition, Planctomycetes are thought to comprise a compartmentalized cytosol that is separated via an intracytoplasmic membrane into a paryphoplasm and a pirellulosome. However, the cytosol of bacteria is, with few exceptions, an enclosed compartment that follows the outer shape of the cell; the cytosol of eukaryotes is divided into multiple compartments (Fig. 1). Furthermore, some species of Planctomycetes (the so-called anammox bacteria) contain an anammoxosome, a distinct compartment for energy production that has been called a bacterial mitochondrion. Other planctomycetal species such as Gemmatia obscuriglobus were reported to encode their highly condensed nucleoid in double membranes, which structurally resembled the eukaryotic nuclear membrane. Accordingly, a spatial separation of transcription and translation was suggested.

Uniquely, Planctomycetes and some other members of the Planctomycetes-Verrucomicrobia-Chlamydiae superphylum are the only organisms among bacteria and archaea whose genomes encode proteins with high structural similarity to eukaryotic membrane coat proteins (MCs). Paralleling their eukaryotic counterparts, planctomycetal MCs comprise β-propeller domains followed by stacked pairs of α-helices. A significant number of planctomycetal MC-like proteins are localized in close proximity to either the intracytoplasmatic membrane (Fig. 1) or to vesicles within the paryphoplasm. Eukaryotic MCs such as clathrins play a major role in the formation of coated vesicles during endocytosis and display similar localizations to those in Planctomycetes. Endocytosis is considered as a hallmark of eukaryotes because it is presumed to have paved the way for the acquisition of an endosymbiont. Interestingly, planctomycetal vesicles have been suggested to support endocytosis-like uptake of macromolecules into the paryphoplasm of the planctomycetal species Gemmatia obscuriglobus. This would be the only vesicle-based uptake system to be found outside of the eukaryotic domain. This served as a strong argument for an evolutionary link between Planctomycetes and eukaryotes.

Phylogenetically, the Planctomycetes are Gram-negative bacteria. Nevertheless, the previously proposed planctomycetal cell plan differs significantly from that of other Gram-negative bacteria and has been frequently revisited. For example, the Gemmatia obscuriglobus ‘nucleus’ has been questioned. Peptidoglycan—the hallmark of free-living bacteria—has been found in several Planctomycetes. Recent bioinformatic and chemical analyses support a more typical Gram-negative cell plan than previously thought. However, to the best of our knowledge, endocytosis-like uptake has not been experimentally tested again after the initial report. Furthermore, research on planctomycetal cell biology is hampered by a paucity of genetic tools.

Here, we revisit planctomycetal cell biology using both existing and new genetic tools, together with super-resolution light microscopy, bioinformatic predictions and proteomic analysis. Furthermore, we analyse frozen-hydrated planctomycetal cells with cryo-electron tomography and find further evidence for a Gram-negative cell plan that differs from that of other bacteria by the presence of an enlarged periplasmic space. Finally, we study the planctomycetal endocytosis-like process in detail.

### Results

**Light microscopy of the planctomycetal membrane organization.** To analyse the planctomycetal cell plan with fluorescence microscopy, we constructed a constitutive gfp-expressing Planctomycetes limnophilia strain (formally known as Planctomyces limnophilus), which was then stained with DAPI (DNA) and FM4-64 (lipid membrane). The DNA of P. limnophilia was always condensed (Fig. 2a–j, DAPI: blue) while FM4-64 staining varied between individual cells. After analysing 1,838 bacteria, two different membrane staining patterns could be distinguished (representative overview shown in Fig. 2a–j; detailed analysis in Supplementary Fig. 1a,b): type 1 cells (27.6%) displayed a membrane staining pattern comparable to E. coli which served as ‘typical’ Gram-negative control (Fig. 2b,e,h and Supplementary Fig. 1c). Only the outer rim of these cells was stained red, indicating close proximity of the outer- and cytoplasmic membranes (Fig. 2e). In contrast, type 2 cells (72.4%) comprised additional red foci either within the cell or attached to the outer membrane (Fig. 2c,d,f,g). The GFP protein localized within the cytosol (Fig. 2c,d,f,g, GFP: green); cells were framed by a red stained membrane, while their interior was mostly green with some yellow areas (Fig. 2d,i). The yellow regions resulted from green cytosolic GFP and red membrane signals within the same area of the cell which becomes evident if intensity histograms of the three representative overlays are plotted. The additional maxima of such curves likely originate from invaginations of the innermost membrane into the cytosol (Fig. 2f,g, green and red arrowheads). If such membrane invaginations were smaller in diameter than the diffraction limit of light (about 200–250 nm), they would be visible as red dots in wide field (WF) microscopy. Furthermore, since FM4-64 preferentially stains the cytoplasmic membrane, this result implies that the innermost membrane is consistent with the cytoplasmic membrane. To prove this view, we treated both P. limnophilia and E. coli cells with high sucrose concentrations, so that the ensuing plasmolytic effect could be observed in phase-contrast. We could reproduce these findings in a gfp-expressing E. coli mutant (Supplementary Fig. 1c,d). After the treatment with 30% sucrose, additional staining revealed a significantly shrunken nucleoid (Supplementary Fig. 1d,i, t-test: \( P = 0.0001 \)) and cytoplasm (Supplementary Fig. 1d,j, t-test: \( P = 0.0001 \)) while the periplasmic space was enlarged (Supplementary Fig. 1d: phaco). When the same treatment was applied to P. limnophilia, the proposed invaginations of the innermost membrane into the cytosol increased significantly (Supplementary Fig. 1i, t-test: \( P = 0.0001 \)) and they became visible as distinct indentations (Supplementary Fig. 1f,h). Remarkably, the condensed nucleoid was even further compressed (Supplementary Fig. 1k, t-test: \( P = 0.0009 \)) and an empty area became visible within the green GFP signal that illustrates an extreme enlargement of the periplasmic space (Supplementary Fig. 1f,h). This observation became even more evident when intensity histograms of untreated and treated P. limnophilia cells were compared (Supplementary Fig. 1g,h). While untreated cells comprise a maximum of the GFP signal at mid cell (Supplementary Fig. 1g), treated cells show a local minimum at the same position (Supplementary Fig. 1h). To further verify our model (Fig. 2h–j; Model), we performed structured illumination microscopy (SR-SIM). This technique roughly doubles the resolution of WF microscopy (Supplementary Fig. 2a–c) and accordingly resolved the invaginations of the innermost membrane (Fig. 2k–m).
Two other model Planctomycetes, Gemmatum obscuriglobus and Rhodopirellula baltica, were analysed with similar staining experiments (Supplementary Fig. 2d–g). These species were chosen since they cover the planctomycetal phylogeny in a representative manner20. Given the larger size of G. obscuriglobus, the phase contrast images revealed more detail compared to P. limnophila and SR-SIM resolved the invaginations of the innermost membrane more clearly (Supplementary Fig. 2d,e). Similar results were obtained for R. baltica (Supplementary Fig. 2f,g). Taken together, our results, showing that FM4–64 and Nile Red stain preferentially the innermost membrane of Planctomycetes, supports the conclusion that this membrane represents the cytoplasmic membrane. This finding was further supported by SEM analysis (Supplementary Fig. 2h,i).

Cryo-electron tomography of P. limnophila. P. limnophila cells investigated by cryo-electron tomography displayed the two types of membrane invaginations, i.e., cells with a Gram-negative typical shape of the cytoplasmic compartment (Supplementary Fig. 3a–l) and cells with distinct invaginations, with a complex membrane organization (Fig. 3 and Supplementary Fig. 3g–l). Selected slices of the tomograms showed cytoplasmic invaginations, but we did not find any distinct 50–200 nm endocytic vesicles. Instead, these invaginations were still connected below and/or above in z-direction. Sometimes connections were formed by small cytoplasmic ‘bridges’ (about 50 nm in diameter), or by thin tube- or disk-like invaginations of the cytoplasmic membrane (Fig. 3, Supplementary Fig. 3 and Supplementary Movie 1). The latter could even convey the impression of a double membrane structure (Fig. 3). Yet, we did not observe distinctly isolated membranous compartments in our tomograms in addition to the common compartments cytoplasm and periplasm.

We would expect to find such endocytic vesicles if they are regular or frequent cellular structures, particularly as the cells were randomly oriented and we should not have systematically missed characteristic cellular zones. Moreover, we found only two types of density patterns in tomograms, either originating from the cytoplasm, including various structures of high contrast, or from the periplasm. Given that most of the reconstructed cells were thinned by focused ion beam (FIB) micromachining (remaining thickness 250–600 nm), we cannot absolutely exclude the possibility of other rare diversifications of the cellular membrane system.

The 3-D data illustrate how the complicated membrane shape is stabilised (Fig. 3m,n). The crateriform structures in the outer membrane, or at least some of them, are connected to the cell membrane and appear to fasten it in a position close to the cell wall. Where crateriform complexes are missing, the membrane can invaginate. Sometimes only a small membrane patch and a tubular volume of cytoplasm remained attached. The density of crateriform structures was 29.1 ± 12.8 per m² for the surface area where the cell membrane was not invaginated (data from 5 tomograms with 141 crateriform structures in total). The extrapolated number of protein complexes per cell was 104 ± 17.

Membrane potential staining and ATPase localization. If the innermost membrane is the genuine cytoplasmic membrane (as indicated by the FM4–64 staining experiments), it should be energized and contain the proton-translocating ATPases. To test this hypothesis, we stained all three model Planctomycetes with DiOC₆(3) to visualize the membrane potential29; FM4–64 lipid staining was performed in addition to DAPI (DNA) staining. At least 50 cells per species were observed, and
representative micrographs are shown in Supplementary Fig. 4a–f. The green DiOC6(3) stain is visible within the cells and co-localizes with red signals of the membrane stain. However, the red membrane stain always surrounds the outer rim of the cells where no green signal is observed (Supplementary Fig. 4). Colocalization was further verified employing intensity blots and calculating Pearson’s correlations and Mander’s overlaps (Supplementary Fig. 4g–i). These results denote that only the innermost membrane is energized.

In addition, we localized the ATPase within cells using WF fluorescence and direct stochastical optical reconstruction microscopy (dSTORM, Fig. 4a–d). ATPases were identified using an anti-Na\(^{+}\)-F\(_{1}\)F\(_{0}\)-ATPase rabbit antibody. The secondary anti-rabbit IgG (labelled with Alexa Fluor 647) led to rather diffuse red foci in WF micrographs (Fig. 4a). Given the size of both antibodies and the WF diffraction limit, fluorescence signals within 400 nm of a cell’s outer rim cannot distinguish between ATPase localization at the innermost and outermost membrane. However, 60.79% of the detected signals came from the middle of the cell and are thus most likely related with ATPases that sit in the innermost (cytoplasmic) membrane which invaginated into the cytoplasm. 39.21% of the fluorescence signals resulted from the outer rim of cells and thus originates from either an ATPase localization at the outer membrane, or from a localization at the innermost cytoplasmic membrane (Fig. 4a,b, Supplementary Fig. 5a,b). dSTORM experiments revealed that the fluorescence signal was localized within the cell, and not exclusively around the outer rim as one would expect if the outermost membrane were energized (Fig. 4c,d). We further verified the localization of ATPases employing
Figure 3 | Cryo-electron tomography of Planctopirus limnophila cells. (a–f) Representative slices of a whole cell tomogram with inter-slice distances of 71, 71, 64, 71 and 64 nm from top left to bottom right (Supplementary Movie 1). The membrane is heavily invaginated and creates a huge periplasmic space. Crateriform structures (‘pits’) in the cell wall are always in close contact to the cell membrane (arrowheads) and are missing at sites of invagination (asterisk). (g–l) Slices of a tomogram of a cell thinned to 320 nm by focused ion beam (FIB) micromachining; inter-slice distances are 41 nm throughout. Example of a cell with complex membrane invaginations creating a ‘channel system’ of the periplasm inside the inner cell volume. Scale bars, 250 nm. (m,n) Surface-rendered reconstruction of the cell in (g–l) visualized in two different angles (m,n). Colour code: cell membrane (light blue), PG (orange), outer membrane (green), crateriform structures (purple), granules (yellow), ribosomes (dark blue).
immunogold labelling of high-pressure frozen, freeze substituted slices of *G. obscuriglobus* cells, and found that significantly more gold particles were located at the innermost membrane (40.5%) than close to the outermost membrane (26.5%, *t*-test: *P* = 0.0001) or to arbitrarily distributed gold particles within (21.4%, *t*-test: *P* = 0.0001) or outside of cells (11.6%, *t*-test: *P* = 0.0001) (Fig. 4e, Supplementary Fig. 5c,d and Supplementary Table 1).

In conclusion, although we cannot exclude the presence of ATPases at the outer membrane, our results show that most ATPases are situated at the energized innermost (cytoplasmic) membrane.

The membrane proteome of *P. limnophila*. If the innermost membrane is the cytoplasmic membrane, then the outermost membrane should represent a Gram-negative outer membrane (OM, Fig. 1). To test this hypothesis, we analysed the existence of characteristic OM proteins by two different approaches. First we employed bioinformatics to identify homologues of well described OM proteins with a focus on secretion systems in *P. limnophila*, *G. obscuriglobus*, and *R. baltica*. We found many proteins encoded in all three planctomycetal genomes that belong to various secretion pathways (see Supplementary Fig. 6 for summary). While all three genomes encoded nearly all proteins of the Sec secretion pathway, only the genome of *R. baltica* contained a high number of Type VI secretion system-related genes.

In a second approach, we analysed the *P. limnophila* cell envelope proteome. Using the PSORT algorithm, 31 putative outer membrane proteins were predicted to be encoded in the genome, which is similar to the average number predicted for other Gram-negatives (34,8 according to PSORTdb39). LC-MS analysis of the *P. limnophila* proteome membrane fraction confirmed 77% of the predicted proteins (24 in total, Supplementary Table 2). 16 of these proteins are related to secretion and transport systems and fit our bioinformatic localization prediction (Supplementary Fig. 6b). In addition, for 11 of these proteins a localization in the outer membrane had been previously predicted by others21. In addition to the 24 outer membrane proteins, we detected 24 extracellular proteins, 34 periplasmic proteins, 366 cytoplasmic membrane proteins and 399 proteins of unknown localization within the membrane fraction of the *P. limnophila* proteome. Among proteins with unknown localization, a putative TolC homologue and a previous predicted OMP21 where identified by manual inspection (ADG67044.1 and ADG67856.1 in Supplementary Table 2). That is, we identified typical Gram-negative cell envelope proteins (Supplementary Table 2).

Macromolecule uptake in Planctomycetes. Endocytosis-like uptake is key for the hypothesis that Planctomycetes and eukaryotes share some sort of evolutionary link6, but it conflicts...
with the existence of a typical bacterial outer membrane. We therefore revisited previously performed GFP uptake experiments with *G. obscuriglobus*<sup>16</sup>. In 10.15% of the 1,281 analysed cells, we observed GFP uptake and the formation of GFP-containing foci in WF fluorescence microscopic experiments. Treatment of the cells with CCCP prevented uptake (0% uptake among 1,294 analysed cells), indicating an active process (Supplementary Fig. 7a–d) as previously described<sup>16</sup>. However, when *G. obscuriglobus* cells were analysed with super-resolution structured illumination microscopy (SR-SIM), the GFP signals were located within the invaginations of the cytoplasmic membrane (Supplementary Fig. 7h). Three-dimensional reconstructions illustrated the internalization of GFP into *G. obscuriglobus* cells, and the overlay with stained membranes (Nile Red) supported the localization of GFP molecules in the enlarged periplasmic space (Supplementary Fig. 7i and Supplementary Movie 2). Applying corresponding experimental settings, we did not observe GFP internalization by *P. limnophila* cells (Supplementary Fig. 7e–g).

Since GFP is an artificial substrate for Planctomycetes, we then looked for a more physiologically relevant experimental setting. Planctomycetes can utilize various carbon compounds<sup>2</sup>, and we recently found that they can live on high molecular weight dextran as sole carbon source<sup>3</sup>. We fed *G. obscuriglobus* cells with 30 kDa-sized fluorescently labelled dextran and observed the same distinct foci as in the GFP feeding experiments within 23% of the 1,065 analysed cells (versus none of the 816 analysed CCCP-treated cells, Fig. 5a–d). But again, super resolution microscopy (dSTORM) revealed an accumulation of dextran (10 kDa Alexa 647) in the periplasmic space only (Fig. 5e). Cluster analysis of dSTORM images revealed that dextran did not accumulate in 50–200 nm sized vesicles.
but rather is distributed in the periplasmic space (Supplementary Fig. 8). This finding was further corroborated by SR-SIM microscopy which allowed parallel staining of membranes and DNA (Fig. 5f). Three-dimensional reconstructions gave analogous results compared to GFP uptake experiments (Fig. 5g and Supplementary Movie 3). Most importantly, with dextran as substrate, similar results were obtained also with P. limnophila, where 22.17% of the 15,557 analysed cells took up the substrate (Supplementary Fig. 9 and Supplementary Movie 4).

Since genetic tools exist for *P. limnophila* but not for *G. obscuriglobus*, we explored the potential role of membrane coat-like proteins (MCs), expected to function in planctomycetal endocytosis, in *P. limnophila*. First, we used the MC-like protein gp4978 suggested to play a key role in GFP uptake in *G. obscuriglobus* to detect its homologue in *P. limnophila*. In addition to classical sequence-based comparison, we employed structure prediction as previously used to identify MCs in Planctomycetes (Supplementary Fig. 10). While structure-based analysis revealed ten putative MC-like proteins, only one of them, Plim_1972, comprised high sequence similarity to the *G. obscuriglobus* MC-like protein (40% identity versus 29%-22% for other putative MCs) above thresholds previously used to determine homology among planctomycetal proteins.

Plim_1972 was subsequently deleted from the *P. limnophila* genome, resulting in the *P. limnophila* AMC mutant. The deletion was verified through PCR experiments targeting gDNA of both mutant and wild type (WT) cultures (Supplementary Fig. 11a,b). Furthermore, we used synthesized oligopeptides to produce an antibody against Plim_1972. Western blot analysis of the WT in comparison to the mutant revealed that the protein was absent in the latter (Supplementary Fig. 11c). The *P. limnophila* ΔMC mutant was then used in dextran feeding experiments. In three independent experiments with three technical replicates each, no significant differences were detected in the uptake of labelled dextran between the mutant and the WT after analysing 15,556 and 19,502 cells respectively (t-test: *P* = 0.21, Supplementary Fig. 11d–f). Thus, our results demonstrate that Plim_1972 is not required for dextran internalization. However, we cannot exclude the possibility that other MC-like proteins may be involved in this process.

**Planctomycetal appendages.** To study how dextran passed through the outer membrane of Planctomycetes, we analysed the surface of *P. limnophila* and *G. obscuriglobus* by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). We found that the outer membrane of *P. limnophila* displays two different types of crateriform structures. Large crateriform structures were distributed all over the cell surface (Fig. 6a and Supplementary Fig. 3), with some restrictions (Fig. 3 and Supplementary Movie 1). In some cases, pil-like fibres with a diameter of 12 nm were attached to the large crateriform structures (Fig. 6b). In contrast, small crateriform structures were exclusively found at the pole and were associated with the holdfast-structure and the stalk, which measure 6 nm in diameter (Fig. 6c). Light microscopic analysis allowed visualization of both types of fibres, the stalk and the pil-like in *P. limnophila* and *G. obscuriglobus* (Fig. 6d,g). Feeding experiments with gold-labelled dextran molecules revealed gold particles primarily at pil-like fibres associated with the large crateriform structures of *P. limnophila* and *G. obscuriglobus* (Fig. 6e,f,h,i). Some pil-like fibre bundles were longer than the entire cell body (Fig. 6j). Some gold particles appeared to be internalized in such experiments (Fig. 6j). Thus, these results indicate that dextran binds to fibres that originate from large crateriform structures (Fig. 6k).

**Discussion**

Planctomycetes have challenged our concepts of microbial structure and function because of unusual characteristics such as apparent cytosol compartmentalization and ability to perform endocytosis-like macromolecule uptake (Fig. 1; for review see ref. 1). While recent work challenged this view producing definitive evidence has been hampered by the paucity of genetic tools. In this study, we developed methods for *gfp* expression and gene deletion in a model planctomycete to study its cell plan and macromolecule uptake.

Construction of a *gfp*-expressing *P. limnophila* strain in combination with membrane staining and (super-resolution) microscopy revealed that the innermost membrane possesses features of a cytoplasmic membrane. In contrast to other bacteria, the planctomycetal cytoplasmic membrane can comprise multiple invaginations, leading to enlargements of the periplasmic space (Fig. 2). Plasmolysis experiments demonstrated that such enlargements can be further expanded (Supplementary Fig. 1). Freeze-fracturing (Supplementary Fig. 2h,i) and cryo-electron tomography (CET) of frozen-hydrated *P. limnophila* cells (Fig. 3 and Supplementary Movie 3) showed a typically Gram-negative cell envelope consisting of an outer membrane, a peptidoglycan layer and a cytoplasmic membrane. Furthermore, tomographic reconstructions revealed that the membrane invaginations are interconnected (Fig. 3, Supplementary Fig. 3 and Supplementary Movie 1). DioC₃(3) staining supports that the innermost cytoplasmic membrane is energized (Supplementary Fig. 4). Similar results have been previously obtained for *G. obscuriglobus*, but the ability of DioC₃(3) to visualize the membrane potential was not acknowledged. Our results are consistent with the ATPase localization at the innermost membrane of *G. obscuriglobus* (Fig. 4). Previous studies on anammox Planctomycetes proposed a different ATPase localization, either on all three membranes, or only on the anammoxosome membrane. However, this might have been caused by ‘moonlighting’ of F1-ATPase domains, or may be explained by effects of specific anammox structures leading to mislabelling. Our microscopic observations are consistent with bioinformatic predictions of the outer membrane secretion systems and with the analyses of the *P. limnophila* membrane proteome (Supplementary Fig. 6). Both methods revealed proteins typical for the outer membrane of Gram-negative bacteria such as porins, as well as typical periplasmic proteins and proteins that are associated with the cytoplasmic membrane (Supplementary Table 2). Our findings are also in line with the recent report of lipid A in Planctomycetes, a key component of lipopolysaccharides in outer membranes.

Together, our results support a Gram-negative cell plan for planctomycetes, with a notable difference. In contrast to most Gram-negative bacteria, the planctomycetal periplasm appears to be variable among individual cells, sometimes leading to great enlargements of the periplasmic space caused by invaginations of the cytoplasmic membrane. Interestingly, such membrane invaginations have been previously reported in pathogenic Chlamydia, which belong to the same Planctomycetes-Verrucomicrobia-Chlamydiae superphylum. A planctomycetal Gram-negative cell plan challenges the existence of an endocytosis-like process. While Gram-negative bacteria frequently produce outer membrane vesicles, invagination of their outer membrane and formation of periplasmic vesicles is hindered by its asymmetric lipid architecture and the peptidoglycan cell wall. Furthermore, the periplasmic space is devoid of high-energy molecules such as ATP or GTP that are required for clathrin-mediated endocytosis in eukaryotes. Thus, the formation of endocytic vesicles by MC-proteins at the outer membrane of
Figure 6 | Planctopirus limnophila and Gemmata obscuriglobus possess fibres that bind to dextran. (a-c) TEM analysis of P. limnophila cells revealed multiple distinct appendages. The flagellum (a, white arrow) with a diameter of 22 nm is typical for bacteria. In contrast, large- (a,b, white arrowheads) and small crateriform structures (a,c, asterisk) are unique to the Planctomycetes and form pili with diameters of 12 nm (b) 6 nm (c) respectively. The polar thinner fibres (a,c, asterisks) form a stalk that attaches the cell to a surface or is involved in cell aggregation. Scale bars, 0.2 μm. (d-i) Light microscopic phase contrast (d,g, Phaco) and TEM (e,f,h,i) micrographs of P. limnophila (d-f) and G. obscuriglobus cells (g-i) fed with gold-labelled dextran (four individual experiments). In the light microscope (d,g) either the stalk of P. limnophila (asterisk), that is associated with the small crateriform structures (c), or the fibres of G. obscuriglobus associated with the large crateriform structures (b, white arrowheads) are visible. TEM analysis revealed dextran binding to fibres of both species (e,f,h,i). (j) Backscattered electron detection in the SEM micrograph shows electron dense gold particles as bright dots and illustrates that fibre bundles can even be longer than the entire cell body. Some gold particles appear to be internalized by the cell (j, black arrowheads). The scheme (k) shows the flagella/attachment pole of a P. limnophila cell, which is opposite to the reproduction pole where cell division through budding takes place. While small crateriform structures are localized only close to the flagellum and produce the stalk (asterisk), fibres associated with the large crateriform structures are distributed throughout the cell (white arrowheads). Scale bars, 0.5 μm.
Planctomycetes appears puzzling. We revisited *G. obscuriglobus* GFP uptake experiments\(^{16}\) and obtained similar results using WF microscopy (Supplementary Fig. 7a–d). However, when GFP uptake was analysed with SR-SIM, we found GFP in close proximity to invaginations of the cytoplasmic membrane (Supplementary Fig. 7h.i and Supplementary Movie 2). Similar results were obtained for the active uptake of fluorescein labelled dextran\(^{22}\) in *G. obscuriglobus*, and *P. limnophila* (Fig. 5; Supplementary Fig. 8). Super resolution microscopy suggested that dextran is homogeneously stored in the enlarged periplasm and not in large endocytic vesicles as previously proposed\(^{16}\) (Fig. 5, Supplementary Figs 8 and 11, Supplementary Movies 3 and 4). Comprehensive CET analysis did not reveal any of the previously proposed 50–200 nm endocytic vesicles in *P. limnophila*, but demonstrated continuity of the innermost cytoplasmic membrane (Fig. 3, Supplementary Fig. 3 and Supplementary Movie 1); the innermost membrane was sometimes heavily invaginated, which might be misinterpreted as vesicles. These observations are in line with the finding that the uptake of macromolecules was not affected in the *P. limnophila* ΔMC mutant compared to *P. limnophila* wild type cells. This mutant lacks a membrane coat-like protein that is similar to the one previously proposed to localize at endocytic vesicles in *G. obscuriglobus\(^{16}\). However, since *P. limnophila* possesses more than one MC-like gene, we cannot exclude that other MC-like proteins may be involved in macromolecule uptake. Based on our results and our interpretation of previous work, we conclude that Planctomycetes do not seem to use a vesicle-mediated mechanism for macromolecule uptake into the periplasm.

Nevertheless, both dextran and GFP must somehow cross the planctomycetal outer membrane to accumulate in the enlarged periplasm. Nonspecific pores, formed by typical outer membrane porins (similar to the ones identified in our proteomic approach, Supplementary Table 2), can facilitate uptake of ions and small macromolecules for macromolecule uptake into the periplasm.

In summary, our findings further support the idea that the planctomycetal cell architecture is Gram-negative. Yet, Planctomycetes remain peculiar, with complex cytoplasmic membrane invaginations, enigmatic crateriform structures and an unusual macromolecule uptake system that has still to be characterized.

**Note added in proof.** A recent publication reports the presence of nuclear pore-like structures in the planctomycete *Gemmatima obscuriglobus\(^{45}\).**

### Methods

**Cultivation conditions.** *Planctomycetes limnophila* DSM 3776 (the bacterium formerly known as *Planctomyces limnophila*) and *Gemmatima obscuriglobus* DSM 5831 were cultivated at 28 °C in limnic medium 3 (M3) composed of 1 g l\(^{-1}\) peptone, 1 g l\(^{-1}\) yeast extract, 1 g l\(^{-1}\) glucose, 5 ml vitamin solution (double concentrated) and 20 ml l\(^{-1}\) mineral salt solution buffered with 10 mM HEPES at pH 7.5. *Rhodopirellula baltica* SH1 DSM 10527 was cultivated at 28 °C in marine medium 2 (M2) composed of 1 g l\(^{-1}\) peptone, 1 g l\(^{-1}\) glucose, 10 ml vitamin solution (double concentrated), 250 ml double-concentrated artificial sea water (46.94 g l\(^{-1}\) NaCl, 7.84 g l\(^{-1}\) Na\(_2\)SO\(_4\), 21.28 g l\(^{-1}\) MgCl\(_2\) \(\times 6\) H\(_2\)O, 2.86 g l\(^{-1}\) CaCl\(_2\) \(\times 2\) H\(_2\)O, 0.384 g l\(^{-1}\) NaHCO\(_3\), 1.384 g l\(^{-1}\) KCl, 0.192 g l\(^{-1}\) KBr, 0.052 g l\(^{-1}\) H\(_2\)BO\(_3\), 0.08 g l\(^{-1}\) SrCl\(_2\) \(\times 6\) H\(_2\)O, 0.006 g l\(^{-1}\) NaF) and 20 ml l\(^{-1}\) mineral salt solution buffered with 5 mM Tris/Cl at pH 7.5. *Escherichia coli* K12 Top10 was cultured at 37 °C in lysogeny broth composed of 5 g l\(^{-1}\) yeast extract, 10 g l\(^{-1}\) trypoton and 10 g l\(^{-1}\) NaCl at 7.0 pH.

**Construction of a GFP Planctopirus limnophila strain.** The constitutive GFP expression system consists of a TNS transposon, harbouring the GPMut2 (ref. 46) gene along with a kanamycin resistance cassette under the transcriptional control of the GAPDH promoter from *P. limnophila*. Briefly, the construction was based on the pmOD3 \(\times\) R6K ori/MCS/> vector (Epitector). This pmOD3 vector and the pKen GFP mut2 plasmid (Addgene) were both cut with XmaI and PstI (Fermentas). Vector backbone and the GFP insert were ligated (NEB quick ligation) to construct pCJ0001. This plasmid and the PCR product of plasmid pM304-2 (primer GemCAGAAGAGAGACCAGACGACGAAAGTGTAATTGATCTAACT-3') were cloned with CiaI and EcoRI. Subsequently, the pCJ0001 backbone was ligated to the kanamycin resistance cassette obtained from pCR2.1 to construct pCJ0002. Then, the GAPDH promoter was amplified (Phusion DNA polymerase, NEB) from *P. limnophila* gDNA (Supplementary Table 2), and SCJ0036-2.2) showed similar cytosolic GFP localization and identical growth characteristics compared to the *P. limnophila* wild type. Strain SCJ0036-2.2 was used for this study.

**Staining of planctomycetal cells.** Membranes of *P. limnophila*, *R. baltica*, *G. obscuriglobus* and *E. coli* cells were stained with (N-3-Threonylarnmonium-propyl)-4-((6-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide (EM–64/EM–464-EM64)\(^{25}\) (ThermoFisher) or Nile Red (Sigma-Aldrich) at a final concentration of 3 μg ml\(^{-1}\). Samples were incubated for 10 min at room temperature (RT). In addition, DAPI was added at a final concentration of 1 μg ml\(^{-1}\) for 10 min at RT. Cells were washed twice with 1 ml tap water and centrifuged at 2,500 \(\times\) g for 1.5 min. For DiOC\(_6\)(3) staining of *P. limnophila*, *R. baltica* and *G. obscuriglobus*, cells were incubated for 45 min at 28 °C at a final concentration of 5 μg ml\(^{-1}\) DiOC\(_6\)(3) (ThermoFisher). To induce plasmolysis of *P. limnophila* and *E. coli*, cells were treated as previously described\(^{26}\). In brief: sucrose concentrations of 30% were applied for 3 min at RT respectively. Subsequently, cells were fixed with 1% glutaraldehyde for 1 h at RT. Afterwards, cells were washed with Fm4–64/Fm4-64FX (ThermoFisher) or Nile Red (Sigma-Aldrich) at a final concentration of 3 μg ml\(^{-1}\). For sample immobilization, MatTek Glassbottom Microwell Dishes (35 mm dish, 14 mm microwell with No. 1.5 cover-glass P35G–1.5–14–C) were used. To minimize drift and cell movement, 1% agarose pads were placed on top of 3 μl samples. To prevent evaporation, but to achieve optimal phase contrast imaging, the plastic lid was removed and the agarose pads were covered with an additional high-precision coverslip (LH24.1 Carl Roth GmbH). The coverslip was sealed against the plastic dish with grease (Vaseline, Lenhart Kosmetik). WF fluorescence- and Phaco samples were visualized on a Nikon Eclipse Ti microscope equipped with DAPI (570–440/40), GFP (485/20/525/50) and Fm4–64 (525/30–705/72) filters. Fluorescence z-stacks and bright-field images were visualized on a Zeiss LSM 780 with ELYRA PS.1 (Carl Zeiss AG) with 561, 488 and 405 nm lasers and BP 570–650 and BP 450–480 + 480–525 nm beam splitters. Z-Stack images were taken using a Nikon Eclipse Ti microscope equipped with DAPI (570–440/40), GFP (485/20/525/50) and Fm4–64 (525/30–705/72) filters. Fluorescence z-stacks and bright-field images were taken using a Nikon N Plan Apochromat \(\times\) 100 1.45 oil objective and the ORCA Flash 4.0 HAMMATSU or Nikon DS–R2 cameras, respectively. Images were pseudo-coloured using the NIH–Elements imaging software V4.2 and V4.3 (Nikon) software ZEN2011 (Carl Zeiss AG). Images were post processed using the Adobe Photoshop CS2 software (Adobe Systems) and tiled using the Adobe Photoshop CS2 software (Adobe Systems).

**Super resolution structured illumination microscopy (SR-SIM).** Samples were visualized on a Zeiss LSM 780 with ELYRA PS.1 (Carl Zeiss AG) with 561, 488 and 405 nm lasers and BP 570–450 + 525/50, BP 495–575 + 525/50 and BP 420–480 + 480–525 nm beam splitters. Z-Stack images were taken using a Plan Apochromat \(\times\) 100 1.45 oil DIC M27 objective and processed using the software ZEN2011 (Carl Zeiss AG). Images were post processed using the Amira 6.0 3D image analysis software (FEI). To compare WF and SR-SIM...
resolution in biological samples, intensity maxima of Fm4–64 or Nile-Red stained membranes or 15 nm colloidal gold solution (Aurion), and placed on holey carbon-coated 200 mesh copper grids (R2/1, Quantifoil, Jena, Germany) immediately before thin-film vitrification by plunge-freezing in liquid propane (63%)/ethane (37%) (ref. 47). Typically, grids with frozen-hydrated samples were mounted in Autogrids63 and 200 nm thin lamellae were then milled with a defined distance of 27,500 nm from the replication of a protective platinum layer in a dual-beam (FIB/SEM) instrument (Quanta 3D FEG, FEI, Hillsboro, OR, USA) equipped with a Quorum cryo-stage maintained at –185°C (PP2000T, Quorum, Essex, UK). Milling was carried out at nominal incident beam angles of 16° to 20° (9° to 13°) effectively using gallium beam currents of 300, 100 and 30 pA in sequential milling steps. Afterward tomographic tilt series were recorded under low dose conditions (total dose typically 150 e⁻/Å²) on a Tecnai G2 Polara (FEI, Eindhoven, the Netherlands) equipped with a post-column energy filter and a 2 k CCD camera (MultiScan) or a k2 sum direct electron detector (Gatan, Pleasanton, CA, USA). For tilt series recorded with the direct detection device, dose fractionation mode was employed and subframes of each projection were sampled, which were then aligned to compensate for beam-induced object drift, using an in-house implementation of the algorithm from the study by Li et al.20 Typically, tilt series were recorded at a nominal defocus of –5 or –6 μm, and a primary magnification of ×27,500 (corresponding to pixel sizes on the object level of 0.427 nm (K2) and 0.805 nm (2 k CCD)), and covered an angular range of ±60° in increments of 1.5° or 2°, respectively. IMOD v4.7.8 (ref. 51) was used for 3D reconstruction, and MatLab (MathWorks) incorporating the TOM toolbox52 for all image processing. Segmentation of three binned volumes was done in Amira v6.0.1 (FEI, Eindhoven, the Netherlands) with specific automatic membrane segmentation53.

Freeze-etching. Freeze-etching was performed on cells taken from a P. limnophila culture at two different time points. The cells were concentrated (centrifugation 800 g for 4 min) and inserted either in a gold dome-shaped carrier (1.7 μl per carrier) or in a jet-freeze carrier sandwich with a grid in between (3 μl for the total sandwich). The carriers were plunge frozen in liquid nitrogen. The frozen samples were introduced into a Balzers BAF400 freeze-etch machine precooled to −150°C with pressure below 10⁻⁷ bar. The samples were kept at −97°C for 7 min before being fractured, after they were allowed to freeze-etch (sublimation of water) for 4 min. The samples were shadowed with 1 nm Pt-C (angle 45°) and 10 nm C (angle 90°). Biological membranes were removed from the replicas by overnight incubation in 70% sulfuric acid. The replicas were washed twice with ddH₂O and picked up with 700-mesh copper hexagonal grids. The grids were investigated at 60 kV in a JEOL JEM-1010 TEM instrument.

Immunofluorescence microscopy. For immunofluorescence microscopy 2 ml of an exponential-phase G. obscuriglobus culture was centrifuged at 2,500g for 2 min. The samples were fixed and stored in 3% paraformaldehyde (PFA). After incubation at 4°C overnight (ON) cells were washed three times with phosphate buffered saline (PBS). For permeabilisation, cells were resuspended in 150 μl ice cold resuspension buffer (50 mM Tris/HCl, 10 mM Na₂EDTA X 2H₂O pH 8.0, 0.1 mg ml⁻¹) and subsequently 150 μl disruption buffer (200 mM NaOH, 1% SDS) was added. Samples were five times gently inverted and incubated for 15 min at RT. Afterwards, cells were washed three times in PBS and for further treatment dissolved in 1 ml × 1 blockinning reagent (Roche, Germany) and incubated for 2 h at room temperature (RT) with agitation (300 rpm). The primary antibody (anti-Na⁺-F₁-F₀-ATPaseβ28) diluted 1:100 in × 1 blockinning reagent. Samples were incubated overnight at 4°C, washed three times with PBS and treated with the secondary antibody Alexa Fluor 488 goat anti-rabbit (1:1,000 diluted), 1:00 diluted and incubated for 3 h at RT in the dark. Finally, cells were washed three times with PBS and diluted in a corresponding volume of mounting medium (20 mM Tris pH 8.0, 0.5% N-propyl gallate, 90% glycerol) for WF fluorescence microscopy.

Membrane sectioning. Secretion system proteins in Planctomycetes. Essential secretion system proteins (secretion system 1 to VI, as defined by the KEGG database) were analysed with BLASTp and PSI-BLAST in the NCBI database against Planctomycetes limnophila, Gemmatum obscuriglobus and Rholodipithaea baltica genomes. Proteins with a lower identity than 1e⁻⁶ and with conserved domain architecture were assumed as correct. Only proteins with positive reciprocal blast verification are listed. If no homologue was found boxes are marked in white (Supplementary Fig. 6). In cases the protein sequence was notified by the KEGG database for one of the three analysed Planctomycetes this sequence was used as reference sequence for further analyses.

Proteome analysis. 500 ml of a P. limnophila culture, cultivated for 3 days at 28°C and 90 r.p.m. was harvested by centrifugation (5,000 × g for 15 min). The pellet was resuspended in 30 ml ice cold 20 mM MOPS buffer pH 8, 200 mM NaCl containing one dissolved EDTA-free protease inhibitor tablet (Roche). The cells were disrupted by cell homogenization (3 × 30 s 6.0 m s⁻¹; MP Biomedicals). Cellular debris was removed by centrifugation (5,000 × g for 20 min). Membranes were isolated from the soluble fraction by centrifugation at 4°C (100,000 × g). To isolate crude membrane fractions containing both cytoplasmic and outer membranes, cell extracts were subjected to isopycnic sucrose gradient centrifugation (1, 1.5, 2, and 2.5 M, 200,000 × g, 24 h, 12°C). Afterwards, membranes were treated with carbonate buffer to remove membrane associated and cytosolic proteins34–36 followed by a precipitation step to enrich the membrane proteins.58,59 Precipitated proteins were washed in 400 μl of ieccol methanol and pelleted by centrifugation (13,000 × g, 15 min, 4°C). The pellet was initially dried in a Speedvac (Eppendorf concentrator plus) for 8 min, air-dried in an extractor hood and finally dissolved in 8 M urea, 2 M thiourea. Aliquots of 12–30 μg protein were separated via one dimensional SDS-polyacrylamide gel electrophoresis50. In gel digestion of proteins was carried out as described60 by dividing each lane into eight subsamples with similar protein amounts which were densitometrically determined using AIDA software (Raytest Isotopenmeßgeräte GmbH). Extraction and desalting of the resulting peptides were done according to ref. 61.

For liquid chromatography—tandem mass spectrometry (LC–MS/MS) analysis a nanoACQUITY Ultra Performance Liquid Chromatography System (Waters Corporation, Milford, MA, USA) was coupled to an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific Inc.). Peptides from each gel piece were separated on a 8 cm, 10 μm AccQ urea C₁₈ column ( Waters, Milford, MA, USA) using a 176 min gradient starting with 3.7% buffer B (80% acetonitrile and 0.1% formic acid) and 96.3% buffer A (0.1% formic acid in 80% water), at a flow rate of 1 ml min⁻¹, from (30–51 min) 3.7–22.1% buffer B, (51–60 min) 22.1–27.0% B; (61–127 min) 27.0–48.3% B; 127–150 min 48.3–62.5% B; 150–163 min 62.5–99% B; 163–166 min 99% B; 166–171 min 99–3.7% B, 171–176 min 3.7% B. Primary MS scans were performed in the Fourier transformation mode scanning a m/z of 400–2,000 with a resolution (full width at half maximum m/z) of 40,000, and a mass lock of 445,1203. Primary ions were fragmented in a data-dependent collision induced dissociation mode for the 20 most abundant precursor ions with an exclusion time of 12 s and analysed by the LTQ ion trap. The following ionization parameters were applied: normalized collision energy: 35, activation Q: 0.25, activation time: 10 ms, isolation width: 2 m/z, charge state: +1 to +4. The signal to noise threshold was set to 2.
structure homology analysis. Subcellular protein localization was predicted using PSORTb 3.0 (http://www.psort.org/psortb)\textsuperscript{83} with the Gram-negative option. Detected proteins were further compared to our bioinformatic analyses, as well as to previous analyses\textsuperscript{28} (Supplementary Table 2).

**Construction of the G. obscuriglobus ΔMC mutant.** We searched for homologues of the G. obscuriglobus MC protein gp978 (WP_001384481) in the P. limnophila genome using BLAST. Plm_1972 (WP_013110233) was found to be most similar (E value 0, similarity 40%). In addition, the structure-based analysis for MC-like proteins was repeated for P. limnophila, as previously described\textsuperscript{12}, leading to the identification of other potential MC proteins in addition to Plm_1972 (Supplementary Fig. 9). Due to the highest sequence similarity, Plm_1972 (the only gp978 homologue with an NCBI BLAST alignment score above 200) was selected for gene deletion.

For Red/ET Recombination (GeneBridge) based deletion of the P. limnophila homologue, a fosmid library was prepared using EpicFos Fosmid Library Production Kit (Epicentre). Approximately 1,377 clones were screened for the presence of Plm_1972 (PLM_510200). Four positive clones were found, and clone P21H10 was further processed with the BAC Subcloning kit (GeneBridge). As linear template, the kanamycin resistance cassette from pCJ003 was amplified (primer: CJ590: 5'-GCG GCC GGT TCT AAT TGA AGT GCA TTA TTC TGA TGG AGA AGT TTG CAG GGA CAA GAT GTA CCA TTC TGA -3'; CJ591: 5'-TCT ACA GCA AGT TTG ATA AGC AGG TTA TTA TGA GAA TCT GCG GTT AGC GCA CAA GAT GTA GAA T-3'). After electroporation, five clones were selected for carrying the selective marker and the Plm_1972 gene. The insertion and insertion sides were tested using primers CJ590, CJ591 and CJ600 (TTT GAA TGG CAG ATC GGC), CJ601 (GGT TTC TTC CAT GAA GTC TTG ACA GCA AG-3'), the linear template, and the Plm_1972 gene. Cells were incubated on M3 plates (30 \( \mu \)g ml\(^{-1}\) kanamycin & 30 \( \mu \)g ml\(^{-1}\) chloramphenicol) for 7 days at 28 \( ^\circ \)C. Eight selected clones were incubated with M3 (30 \( \mu \)g ml\(^{-1}\) kanamycin and 30 \( \mu \)g ml\(^{-1}\) chloramphenicol) for 8 days at 28 \( ^\circ \)C at 90 r.p.m. Clone 6 (AP1972) was used for further investigations and subsequently named P. limnophila DMC.

In order to validate the genetic background of P. limnophila DSM 3776 in comparison to P. limnophila ΔMC, two different PCRs (Taq polymerase Qiagen), targeting either the flank regions of gene Plm_1972 (primers: CJ787_F 5'-TTC ACA TGT GTT TTC TCG ATA ATG AGC ATT TTT TGA TGA GAA -3'; CJ787_R 5'-GCC GCC GTT TCT AAT TGA ACT ATG CCA TTC TGA -3') and CJ592 (5'-GCG GTT TTA TTG ACA GCA AGT TTG CAG GGA CAA GAT GTA CCA TTC TGA -3'; CJ593: 5'-TCT ACA GCA AGT TTG ATA AGC AGG TTA TTA TGA GAA TCT GCG GTT AGC GCA CAA GAT GTA GAA T-3') respectively. Three positive clones were identified (pCJ1000-pCJ1002). pCJ1000 plasmid DNA was linearized using HinfII (Fermentas). Linear DNA (2–4 \( \mu \)g) was used for electroporation, as described before\textsuperscript{28}. Cells were incubated on M3 plates (30 \( \mu \)g ml\(^{-1}\) kanamycin & 30 \( \mu \)g ml\(^{-1}\) chloramphenicol) for 7 days at 28 \( ^\circ \)C at 90 r.p.m. Clone 6 (AP1972) was used for further investigations and subsequently named P. limnophila DMC.

To determine a positive uptake, treated cells were compared to the negative control. Macromolecule uptake experiment. For GFP uptake, 1 ml of a stationary phase liquid culture of G. obscuriglobus and P. limnophila were harvested and incubated as previously described\textsuperscript{28}. Cells were washed after incubation with 1 ml tap water and further analysed using WF fluorescence light microscopy and SR-SIM. As negative control, cells were poisoned at 50 \( \mu \)M Tris pH 7.5 for up to 4 h at 28 \( ^\circ \)C. Subsequently cells were washed twice in sterile tap water and then fixed in 1% formaldehyde for 1 h at 4 \( ^\circ \)C.

**Direct stochastic optical reconstruction microscopy (dSTORM).** dSTORM reconstruction was acquired with a Nikon Eclipse Ti (Nikon GmbH) inverse microscope in ‘Sedat’ configuration, with 647 nm Fibre Laser and 405 nm Argon laser and a mercury vapour and bright field LED. Differential interference contrast (DIC) was used for WF overlays. Images where taken with the Nikon \( \times 100 \) 1.49 APO TIRF objective and an ANDOR iXon3 camera. dSTORM-MEA-buffers were used as described previously\textsuperscript{7}. A time series of 5,000–20,000 frames per image was recorded at calibrated magnifications with dSTORM-SOF-Software (Nikon). Minimum peak height was determined according to the signal intensity. Shift correction was applied for all analysed images.

**TEM and SEM analysis of planctomycetes appendices.** For negative staining thin carbon support films were prepared by sublimation of carbon onto a freshly cleaned mica surface. Samples were negatively stained with 0.1%–2% (w/v) aqueous uranyl acetate and imaged at 50 kV in an JEM 1010 transmission electron microscope (Carl Zeiss AG) at an acceleration voltage of 80 kV. Images were taken at calibrated magnifications using a line replica. Images were recorded digitally with a Slow Scan CCD-Camera (ProScan, 1,024 x 1,024) with ITEM-Software (Olympus Soft Imaging Solutions).

**Data availability.** The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium database via the PRIDE (ref. 66) partner repository with dataset identifier PXD005738. The authors declare that all other data supporting the findings of this study are available within the paper and its Supplementary Information Files, or from the corresponding author on request.

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Author contributions

C.J. designed the study with help from C.B. and R.K. C.B., P.R. and D.B. cultivated all bacteria. M.J. constructed the GFP mutant. D.P.D. identified the MC protein homologues in *P. limnophilis* and helped selecting the gene for deletion. C.J. constructed the *P. limnophilis AMC* mutant, and C.B. and P.R. performed the verification. O.I. performed the bioinformatics analysis. C.B. did the staining- and dextran/GFP feeding experiments, the WF/dSTORM imaging and designed all figures together with C.J. and with help from all other authors. C.B. and D.B. performed the sucrose treatment and the immunofluorescence microscopy. C.B., G.R. and R.A. performed the SR-SIM and 3D reconstruction. Ma.S., Mi.S and H.E. performed the cryo-electron tomography including micromachining, segmentation and 3D reconstruction. M.R. accomplished the immunogold labelling and TEM/SEM imaging with help from C.B. and C.J. M.C.F.v.T. and L.v.N. performed the freeze-etching experiments and subsequent TEM analysis. C.B., M.K. and S.E. carried out the proteome analysis. C.J. and C.B. wrote the manuscript with help from all authors.

Additional information

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