nature microbiology

Review article

https://doi.org/10.1038/s41564-024-01812-9

Cell envelope diversity and evolution across the bacterial tree of life

Received: 22 December 2023

Accepted: 16 August 2024

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The bacterial cell envelope is a complex multilayered structure conserved across all bacterial phyla. It is categorized into two main types based on the number of membranes surrounding the cell. Monoderm bacteria are enclosed by a single membrane, whereas diderm cells are distinguished by the presence of a second, outer membrane (OM). An ancient divide in the bacterial domain has resulted in two major clades: the Gracilicutes, consisting strictly of diderm phyla; and the Terrabacteria, encompassing monoderm and diderm species with diverse cell envelope architectures. Recent structural and phylogenetic advancements have improved our understanding of the diversity and evolution of the OM across the bacterial tree of life. Here we discuss cell envelope variability within major bacterial phyla and focus on conserved features found in diderm lineages. Characterizing the mechanisms of OM biogenesis and the evolutionary gains and losses of the OM provides insights into the primordial cell and the last universal common ancestor from which all living organisms subsequently evolved.

The bacterial cell envelope is a multilayered structure that encapsulates the cell and serves as a protective, adaptive and functional barrier to the environment[1]. Studying its composition and structure can explain how bacteria have adapted to different environments and stressors over time. Historically, bacteria were classified as Gram positive and Gram negative based on their ability to retain the Gram stain[2]. However, ultrastructural studies using cryo-electron tomography (cryo-ET) and cryo-electron microscopy of vitrified (frozen in a hydrated, glass-like state) thin sections of cells (a technique called CEMOVIS) have revealed that the number of membranes represents a conserved feature that can aid taxonomy and classification[3–5]. Two major cell envelope architectures have been characterized to date: monoderm and diderm (Fig. 1a). Monoderm bacteria are enveloped by a single cytoplasmic membrane and a thick layer of the structural polymer peptidoglycan, whereas diderm bacteria are surrounded by two membranes—an inner membrane (IM) and an outer membrane (OM)—with a thin layer of peptidoglycan between them[1]. Despite being a vital and ancient feature, many questions remain about the biogenesis and evolution of the bacterial cell envelope and, in particular, the OM.

With the advancement and accessibility of genome-resolved metagenomics, the number of microbial genomes has substantially increased in recent years, revealing dark matter of uncultivated organisms that encompass a large proportion of current databases[6–8]. Insights from these genomes have vastly expanded knowledge of bacterial physiology and helped in the identification of previously undescribed bacterial phyla, fuelling further questions about their diversification. Genomic data are particularly valuable for elucidating the biogenesis and evolution of the bacterial OM by following the distribution of conserved OM proteins (OMPs) across phyla. However, any inferences about the evolution of the OM on such large timescales require well-defined phylogenetic relationships and rooting of the bacterial tree of life to provide directionality to possible evolutionary trajectories. While phylogenomic studies have consistently supported the monophyly of individual bacterial phyla, intra-phyla relationships remain uncertain. This is partly due to the challenges associated with rooting of the bacterial tree of life, which include choosing an appropriate outgroup and avoiding artefacts such as long-branch attraction that can falsely relate two divergent phyla[9,10]. To overcome the need for an outgroup, Coleman et al.[9] conducted an outgroup-free rooting of the bacterial tree of life by using horizontal gene transfer (HGT) modelling along with probabilistic frameworks and species–gene reconciliation methods. Leveraging this approach, these findings support

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https://doi.org/10.1038/s41564-024-01812-9

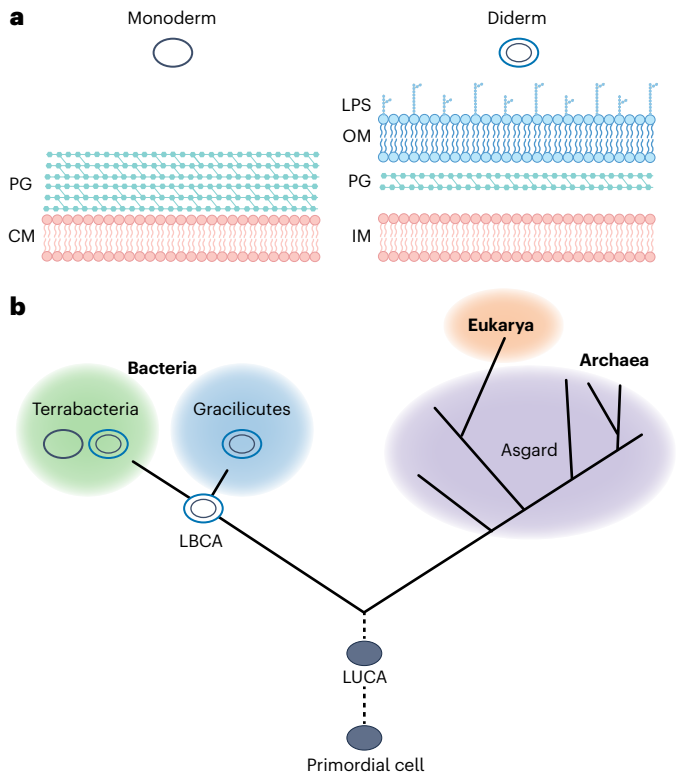

Fig. 1 | Monoderm and diderm cell envelope plans in bacteria. a, Typical monoderms have a cytoplasmic membrane (CM) and a thick layer of peptidoglycan (PG), whereas typical diderms are surrounded by an IM and an OM, separated by a thin layer of PG. **b**, Metagenomic and phylogenetic studies divide the domain of Bacteria into two major clades, Terrabacteria (monoderm and diderm) and Gracilicutes (strictly diderm). The domain Eukarya evolved within the Asgard superphylum of Archaea. Reticulation between domains is not shown.

a root between Gracilicutes and Terrabacteria and the hypothesis that the last bacterial common ancestor (LBCA) was a diderm[9,11–14]. In this Review, we examine the diversity of cell envelope architectures across major bacterial phyla, with focus on the distinct OM features found in diderm Terrabacteria. Furthermore, we discuss possible mechanisms of OM biogenesis and use the monoderm–diderm divergence to shed light on the nature of the primordial cell and last universal common ancestor (LUCA).

Gracilicutes and Terrabacteria represent an ancient divide

Phylogenetic analyses with the presence or absence of an outgroup have robustly indicated that the most ancient divide in the bacterial domain resulted in two major clades: Gracilicutes (including Proteobacteria and Bacteroidota) and Terrabacteria (including Firmicutes and Cyanobacteria) (Fig. 1b)[9–11,15,16]. These clades correlate with distinct cell envelope architectures: Gracilicutes form a monophyletic diderm group, whereas Terrabacteria contain both monoderm and diderm species with diverse cell envelope architectures[9,11,17]. Since the presence or absence of a second membrane represents a key evolutionary step, the ultrastructure and composition of the cell envelope can provide insight into intra-phyla relationships and the inheritance of ancient structural features across evolutionary timescales.

Homologous OMPs among diderm Gracilicutes and Terrabacteria indicate that the OM is an ancient feature preceding the divergence of the two clades. This OM (hereafter referred to as the typical OM) necessitates machineries for synthesis and maintenance, including β-barrel assembly machinery (BAM), the lipopolysaccharide transport system (LPT), the localization of lipoprotein export (Lol) pathway and membrane-tethering proteins (Fig. 2). The OM components of these pathways are characterized by a distinct β-barrel fold[18]. For example, BamA of the BAM complex is a 16-stranded β-barrel porin with varying numbers of polypeptide transport-associated (POTRA) domains, and LptD from the LPT system is a 26-stranded β-barrel porin[19,20]. The exclusivity of β-barrel proteins to the OM is attributed to differences in protein folding thermodynamics between the IM and OM[21]. As integral membrane proteins, the highly hydrophobic surfaces of the β-barrels pose a challenge for folding and insertion into the OM[21–23]. Thus, the use of the BAM complex (comprising BamA and accessory lipoproteins BamBCDE) has evolved to lower the kinetic energy of protein insertion without the use of exogenous energy[24–26]. Although the presence of the different BAM components can vary among species, BamA is conserved across all typical diderms and is essential for OMP insertion[3,27,28]. The LPT system is responsible for the export of lipopolysaccharides (LPSs) to the outer leaflet of the OM (Fig. 3)[20]. Although not all diderm species contain LPS in their OM, LptD is conserved among most typical diderms[3,20]. Homologues of LptD have been shown to be essential for lipoprotein translocation across the OM and transport of glycerolipids in plants, indicating the possibility of functional diversification of LptD in bacteria[29–31]. Lastly, the Lol pathway lacks an integral OM component and is found exclusively in Gracilicutes, with the exception of Deinococcus–Thermus, where the pathway was acquired through HGT (Figs. 2 and 3)[17].

A notable distinguishing feature found between typical diderm Terrabacteria and Gracilicutes is the tethering system used for anchoring the OM to the peptidoglycan (Fig. 2)[17]. In Gracilicutes, Lpp (also known as Braun's lipoprotein), Pal and the β-barrel OmpA are used to bind the OM to the peptidoglycan, whereas in Terrabacteria tethering is accomplished by the SlpA/OmpM-like proteins (Fig. 2)[17,32]. SlpA/OmpM assembles into homotrimers and consists of a β-barrel porin, a coiled-coil domain that extends into the periplasm and a conserved amino-terminal S-layer homology domain that binds organic acids or secondary cell wall polymers within the peptidoglycan[17,33].

Across Gracilicutes and Terrabacteria, many diderm and monoderm species have additional surface layers, such as proteinaceous S-layers and carbohydrate capsules[34,35]. With a wide array of functions, S-layers are two-dimensional crystalline arrays formed by repeating units of (glyco)proteins that cover the entirety of the cell surface[34,36]. Although present across diverse bacteria and archaea and sharing little to no sequence homology, characterized S-layer proteins exhibit a limited set of protein folds, including β-sandwich, β-helix and immunoglobulin (Ig)-like domains[35,37,38]. Ig-like domain-containing proteins are also found on the surface of eukaryotic cells, highlighting the evolutionary importance of the Ig-like fold[38].

The presence or absence of key OM machineries such as BAM, LPT, Lol and membrane-tethering systems illustrates that Gracilicutes have retained most of these markers, whereas Terrabacteria display pronounced differences (Fig. 3). For example, diderm Terrabacteria lack the Lol pathway (except HGT in Deinococcus–Thermus) and encode for the clade-specific SlpA/OmpM tethering proteins[17,39]. Despite the apparent distinctions between diderm Gracilicutes and Terrabacteria, the presence of BamA, LptD and β-barrel membrane proteins is regarded as a conserved feature of a typical OM. Consistent with sequence-based homology searches, Gracilicutes have retained the diderm cell plan, whereas Terrabacteria display remarkable cell envelope diversity (Fig. 3).

Cell envelope architecture of major Terrabacteria phyla

Based on the cell envelope architecture of the major bacterial phyla described here, few are monophyletic with respect to the monoderm cell envelope plan. In addition to Chloroflexi and candidate phyla radiation (CPR) described below, two other major phyla, Caldiserica and Coprothermobacterota, lack homologues of the key OM components and are thus proposed to be monoderm (Fig. 3). However, as shown

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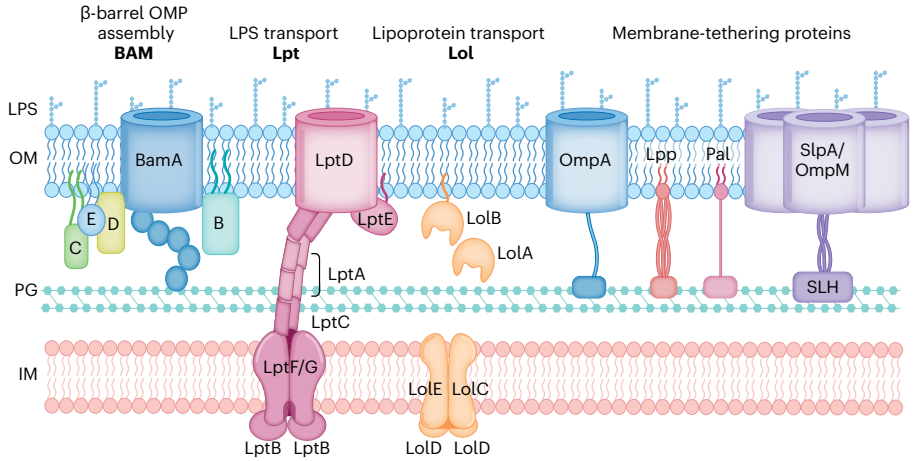

Fig. 2 | Key components of a typical OM. The BAM complex inserts OMPs into the OM with BamA conserved in all typical diderms. The LPT complex transports LPS molecules across the periplasm and into the outer leaflet of the OM, with LptD highly conserved among typical diderms. Lipoproteins are trafficked to the OM by the Lol pathway, where the IM transporter LolCDE and conserved chaperone LolA transport lipoproteins across the periplasm for insertion into the OM by LolB. Lpp, Pal and the β-barrel OmpA are the main OM–PG tethering systems found in Gracilicutes. The SlpA/OmpM family of proteins specific to Terrabacteria tether the OM to the PG via an amino-terminal S-layer homology (SLH) domain.

for Actinobacteria, the absence of typical OMPs does not exclude the possibility of a unique OM in these phyla. Therefore, ultrastructural and biochemical characterization is needed to define the cell envelope architectures of these monoderm phyla. Below we discuss recent studies characterizing the major Terrabacteria phyla and the evolutionary implications stemming from the distribution of monoderms and diderms with typical and unique OMs.

Firmicutes

For decades, the well-established phylum Firmicutes was considered classically monoderm, with members, such as the model organism *Bacillus subtilis*, having a cell envelope composed of a single cytoplasmic membrane and a thick layer of peptidoglycan (Fig. 4). Discovery of the diderm class Negativicutes revealed that the phylum is not monophyletic with respect to the monoderm cell plan[40,41]. Structural and biochemical characterization of the Negativicute *Acetonema longum* confirmed the presence of a typical OM with conserved BamA, LPS and β-barrel proteins, and phylogenetics and genomic profiling validated that the OM is an ancient feature (Fig. 3)[40]. A unique capability of the Firmicute phylum is the ability to produce endospores—a dormant cell form characterized by the presence of a multilayered protective envelope[42]. Following asymmetric cell division, a prespore compartment is engulfed by the mother cell and undergoes several modifications, such as formation of the cortex (a thick protective layer of modified peptidoglycan with a low number of crosslinks compared with vegetative peptidoglycan) and a proteinaceous coat[43]. Importantly, both monoderm (for example, *B. subtilis*) and diderm (for example, *A. longum*) mother cells produce a diderm spore that is surrounded by two IM-like membranes[44]. Upon germination, monoderms shed their outer spore membranes, whereas diderm sporulators remodel it from an IM-like membrane into a typical OM[40,44]. Thus, comparing endospore formation between monoderm and diderm Firmicutes revealed that both can synthesize thin and thick peptidoglycan and form diderm spores[44]. Highlighting the similarities between the monoderm and diderm Firmicutes led to the hypothesis that the ancestor of the phylum was a diderm sporulator and the monoderm species evolved through multiple OM losses[40,44]. The identification of additional diderm lineages (Halanaerobiales and Limnochordia) with conserved OM components corroborates the hypothesis that the last common ancestor of all Firmicutes was a diderm, and loss of the OmpM membrane-tethering protein was proposed as a mechanism for the diderm-to-monoderm transition[17,45,46].

Actinobacteria

Similar to Firmicutes, Actinobacteria were historically regarded as classically monoderm; however, it is now well established that this phylum contains monoderm and diderm species[47]. Exemplified by the filamentous *Streptomyces*, the monoderm species are surrounded by a cytoplasmic membrane and a thick layer of peptidoglycan (Fig. 4). The diderm members of Actinobacteria possess a unique second OM—the mycomembrane (MOM) (Fig. 4). The presence of a MOM in the order Corynebacteriales (including the pathogenic genus *Mycobacterium*) was initially supported by biochemical characterization and freeze fracture imaging[48,49] and was subsequently confirmed by cryo-ET and CEMOVIS[5,50]. The MOM comprises an inner leaflet of long-chain mycolic acids (C_{60}–C_{90}) and an outer leaflet consisting of non-covalently bound lipids, proteins and glycopeptidolipids and exhibits a high degree of variability in the type, modifications and branch length of its mycolic acids and surface-exposed lipids[47,51–53]. In addition to the MOM, the cell envelope of diderm Actinobacteria consists of an IM and a thin peptidoglycan layer covalently bound to a distinct arabinogalactan polymer[47]. Lastly, the cell envelope is surrounded by a weakly attached capsule, which consists mainly of exopolysaccharides, proteins and a small number of lipids[54].

Notably, the MOM has no discernable shared ancestry with the typical OM found in diderm Gracilicutes and Terrabacteria. It therefore serves as an example of de novo OM biogenesis within the otherwise monoderm Actinobacteria phylum[47]. Based on the presence or absence of key cell envelope features, stepwise gene acquisition was proposed as the mechanism for MOM biogenesis[47]. The novelty and complexity of the MOM raises questions about the types of proteins it supports. Similar to typical OMPs, the mycobacterial OMPs (mOMP) probably include transenvelope complexes, proteins essential for uptake and secretion and proteins involved in maintaining the structural integrity of the MOM. Currently, MspA, OmpATb and CpnT are the best-studied mOMPs, with MspA representing the only structurally characterized mOMP[55–58]. This protein is involved in hydrophilic molecule transport across the MOM in *Mycobacterium smegmatis* and oligomerization of eight subunits results in the formation of a β-barrel porin[55,59,60]. Since MspA lacks sequence homology to any known OMP, it serves as an example of convergent evolution of the β-barrel fold (Box 1). Porin activity for OmpATb from *Mycobacterium tuberculosis* and its homologues in other Corynebacteriales have also been documented; however, their tertiary structures remain to be elucidated[57,61,62]. Recent studies of the mammalian cell entry

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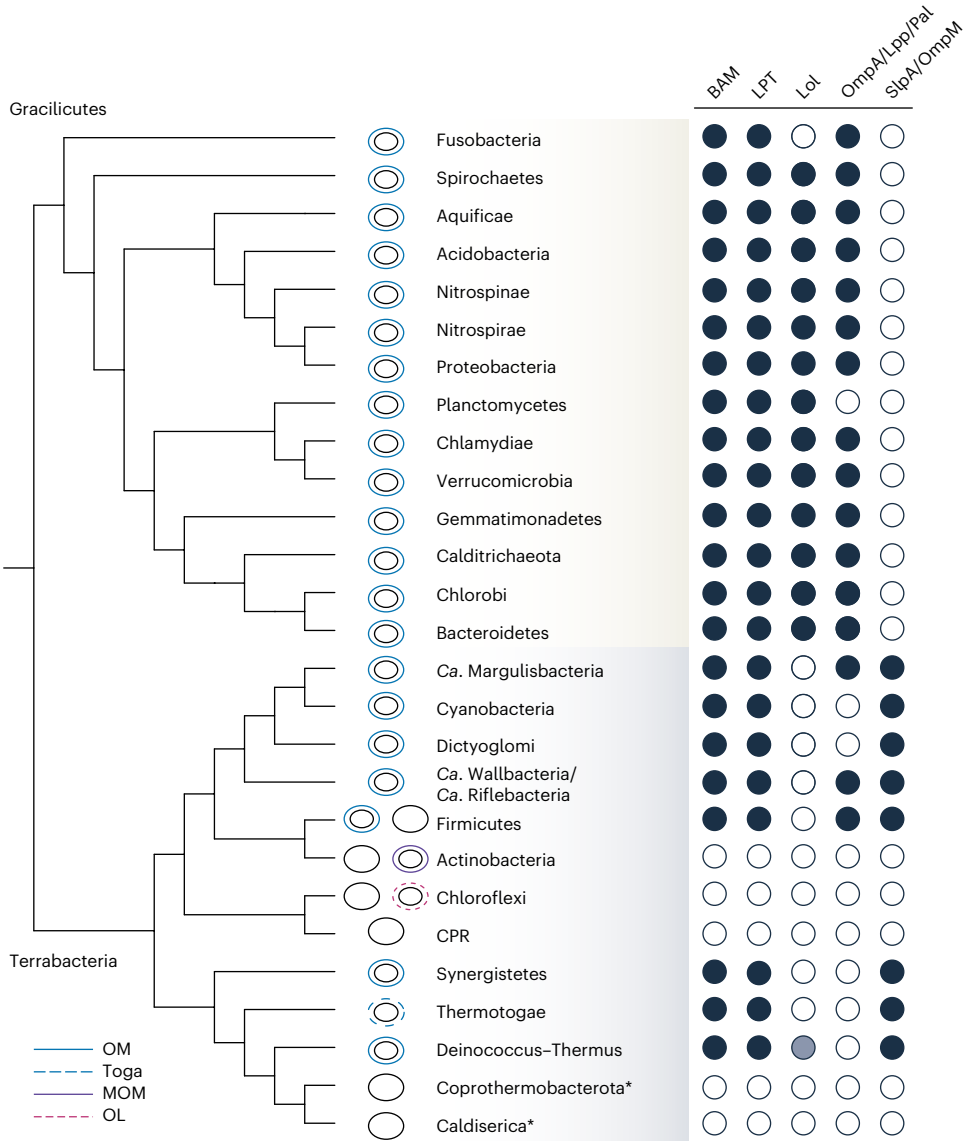


Fig. 3 | Conserved OM markers across major bacterial phyla. Black marks the presence of homologues of the BAM (BamA), LPT (LptD) and Lol pathways, OmpA/Lpp/Pal and SlpA/OmpM[3,17,20,39,45,46,90,139–141]. Gracilicutes are strictly diderm with a typical OM (blue). Terrabacteria show diverse cell envelope architectures, including monoderm, diderm with a typical OM (including toga) and diderm with de novo MOM (purple). Monoderm and diderm architectures are based on structural characterization and sequence-based homology[3,5,17,40,45,46,81,99,113,125,141]. Asterisks signify that the monoderm architecture was inferred from the absence of conserved OM markers. Lineages are denoted using National Center for Biotechnology Information nomenclature (for alternative nomenclature, see refs. 109,142). *Ca.*, *Candidatus* refers to candidate phyla. OL, outer layer. HGT is shown in grey. Tree adapted from ref. 17, Springer Nature Ltd.

complex from mycobacteria reveal that it forms a needle-like structure that spans the periplasm to facilitate lipid transport, although the MOM components of the complex remain to be elucidated[63].

Deinococcus–Thermus

Members of the phylum Deinococcus–Thermus (also known as Deinococcota) are diderm with an OM that lacks LPS[64,65]. The cell envelope of *Deinococcus radiodurans* has been well characterized and reveals a thick peptidoglycan layer (~40 nm, comparable to canonical Gram-positive bacteria) and a proteinaceous S-layer on the surface of cells (Fig. 4)[64–68]. Homology searches have detected the presence of a BamA homologue with five POTRA domains and no other components of the BAM complex[3,69]. BamA was shown to be essential for the proper assembly of the OM and S-layer, but was deemed non-essential for viability, probably due to the presence of a thick cell wall that can ensure resistance to osmotic pressure[69,70]. Despite lacking LPS, the presence of LptD and some LPT components in the Deinococcus–Thermus species suggests that LPS synthesis was probably present in the ancestor of the phylum and was replaced by alternative lipids over time (Figs. 3 and 4)[20,46]. The lipid composition of the IM is remarkably similar to that of the OM, with both sharing the same type of polar lipids but in varying proportions[65,71,72]. Notably, the polar lipids of *D. radiodurans* are largely unique and do not include any of the common phospholipids (phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol and phosphatidyl choline) found in most bacteria[72]. In addition, some members of the phylum contain more complex lipids and glycolipids that may involve ester linkages[72–74].

Recently, the in situ architecture of the native cell envelope of *D. radiodurans* was visualized with cryo-ET and showed the presence of an IM, thick peptidoglycan, an OM and an S-layer[64]. The S-layer is formed by the hexagonally packed intermediate layer (HPI) protein composed of six consecutive Ig-like domains[68,75,76]. High-resolution structural studies further revealed that the HPI protein forms a hexameric paracrystalline lattice with a high degree of interconnectivity

facilitated by the Ig-like domains[68]. The S-layer is thought to anchor to the OM by post-translational lipidation of the amino terminus of the mature HPI protein, whereas the OM harbours the highly abundant tethering protein SlpA—a 30-stranded β-barrel homologous in structure and function to OmpM[39]. This cell envelope model is supported by numerous biochemical and structural studies, including the following observations: (1) the subtomogram average of the repeating unit on the S-layer on the surface of cells is consistent with the cryo-EM structure of the HPI protein[64,68]; (2) deletion of SlpA does not abolish S-layer formation but rather results in cell envelope instability and OM blebbing, indicating that the protein is integral to the OM[39,66]; and (3) densities (~50 nm long) are visible in cryotomograms connecting the OM and peptidoglycan, consistent with the length and function of the coiled-coil domains of SlpA[39,68].

Thermotogae

Thermotogae possess the most atypical cell envelope among diderm Terrabacteria. Early transmission electron microscopy images of the hyperthermophilic bacterium *Thermotoga maritima* revealed the presence of an outermost layer with pronounced bulging around the poles of cells, referred to as the toga[77]. This layer appeared as an extended proteinaceous sheath of hexagonally arranged β-barrel trimers, and two proteins—Ompβ (predicted β-barrel) and Ompα (predicted coiled-coil)—were proposed as the main components of the toga[78–80]. Despite lacking LPS, genomic and proteomic analyses revealed the presence of BamA and LPT homologues in all members of the phylum (Fig. 3)[20,46,81]. Altogether, these observations created uncertainty as to whether the toga represented a unique OM with a functional BamA or a proteinaceous sheath formed by Ompβ and Ompα[3,78,80]. Direct visualization of the cell envelope of *T. maritima* with cryo-ET provided much-needed insight into the structure of the toga and revealed that it is formed by a two-dimensional hexagonal array of β-barrel trimers with interspersed membrane patches (Fig. 4)[81]. Although Thermotogae do not possess SlpA/OmpM, proteomic analyses identified several highly abundant β-barrel proteins and three S-layer homology domain-containing coiled-coil Ompα homologues as the major components of the toga[81]. Based on domain homology and structure prediction, these observations suggested that the phylum uses a bipartite tethering system by combining different β-barrel trimers with three conserved Ompα homologues[17,39,81]. Notably, Ompβ was not enriched in the toga of *T. maritima*, and homology searches revealed poor conservation among other Thermotogae species, indicating that Ompβ is not the main component of the toga[81]. The surprisingly high number and diversity of putative β-barrel toga proteins across the phylum could represent an evolutionary adaptation[81]. The unique structure of the toga represents the first characterized instance of a combined proteinaceous surface layer with membrane patches, demonstrating the possibility of such a structure as an evolutionary intermediate between the monoderm and diderm cell plans[81].

The same lipid species are found in the IM and toga of *T. maritima*, with certain lipids differentially enriched[81]. In addition, membrane-spanning phosphoglycerol–ester and –ether lipids, which have been hypothesized to stabilize membranes at high temperatures, were found equally in the IM and toga[81,82]. A putative ABC permease with fused domains that shares homology with LptD, LptA and LptF/G (TM1735) was identified and proposed as the main lipid transport system from the IM to the toga[81]. Commonly found in Archaea, membrane-spanning lipids are rare among bacteria and are thought to be a thermal adaptation due to their ability to form rigid monolayers with low permeability[83]. Similarly, ether-derived lipids have only been detected in a few other bacterial phyla (including Firmicutes, Aquificae, Acidobacteria and Planctomycetes), although several others encode the potential for converting ester-linked lipids to ether-linked lipids[84–86]. The existence of different biosynthetic pathways in bacteria and archaea suggests that the synthesis of ether-derived lipids is a result of convergent evolution[85–87]. Recent phylogenetic evidence indicates that LBCA-encoded ester-type membrane lipids, and ether- and ester-derived lipids among extant bacteria and archaea, respectively, may be a result of HGT between domains[9,85,86]. Outstanding questions remain surrounding the membrane lipid composition of the LUCA, whether it was a mixed state of ether- and ester-linked lipids, which biosynthetic pathways were involved and the vertical or horizontal nature of their inheritance[86,87].

Cyanobacteria

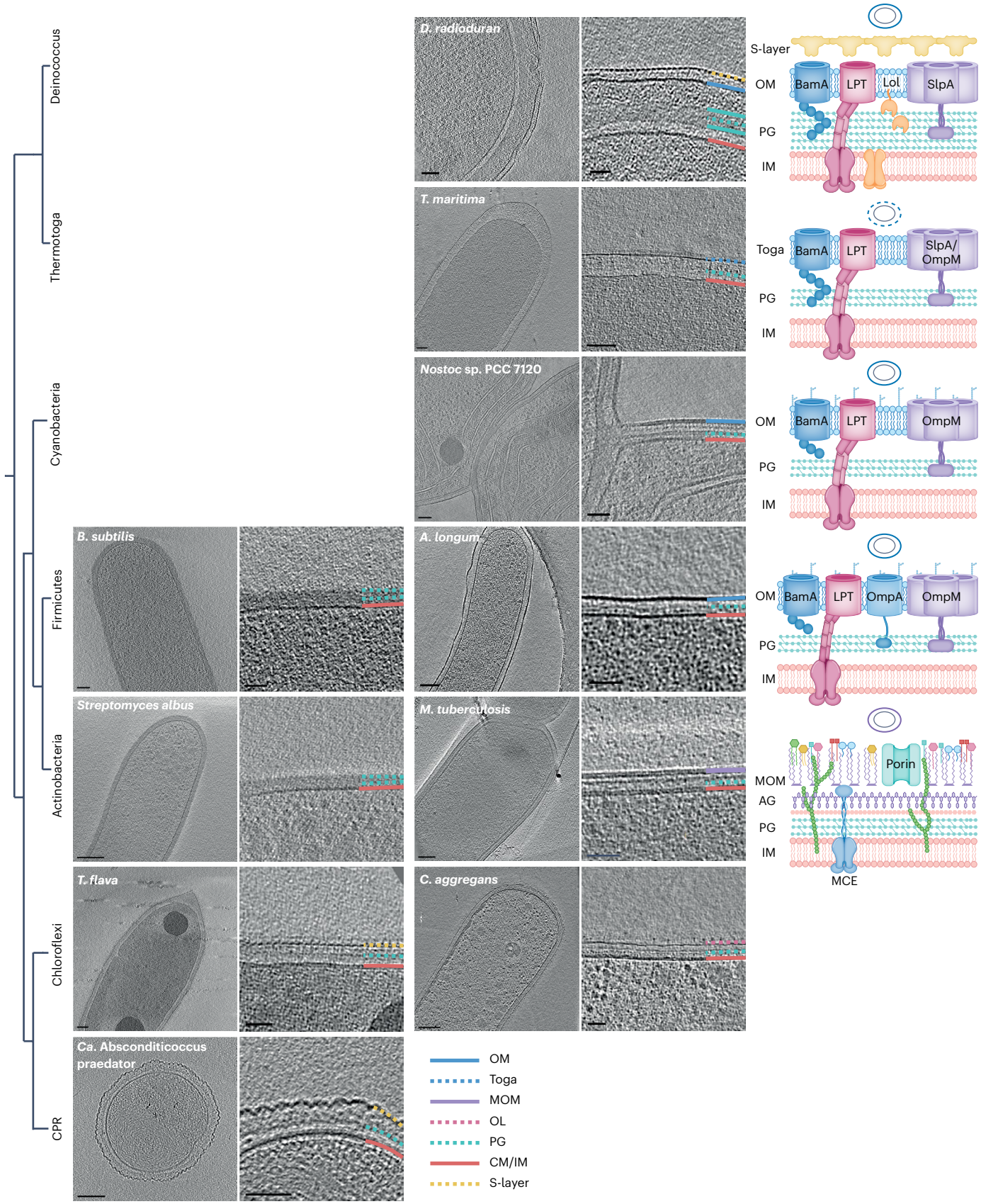
Cyanobacteria exhibit enormous diversity in morphology, physiology and metabolic capabilities. Members are unicellular or filamentous diderms, with an LPS-containing OM, although their envelopes feature unique variations of components, including carotenoids and β-hydroxypalmitic acid in the lipid A moiety of LPS[88]. Cyanobacteria encode BamA and distinct LptD homologues, although conservation of additional LPT components varies across the phylum[20,89,90]. Notably, the peptidoglycan in Cyanobacteria differs substantially from both typical monoderms and diderms[91]. For example, the peptidoglycan thickness is ~10 nm in unicellular strains such as *Synechococcus*, 15–35 nm in filamentous species such as *Phormidium uncinatum* and can be >700 nm in large members such as *Oscillatoria princeps*[91,92]. Although Cyanobacteria have an SlpA/OmpM membrane-tethering system and share secondary cell wall polymer characteristics with diderm Terrabacteria, the high degree of peptidoglycan crosslinking is similar to that of typical monoderms[39,88,93].

Nostoc sp. PCC 7120 (also known as *Anabaena* sp.) is a model organism for the study of filamentous Cyanobacteria and cell–cell communication[94]. Along a filament, this organism differentiates into two cell types: single heterocysts separated by 10–20 photosynthetic vegetative cells[95]. The heterocysts carry out nitrogen fixation (a process highly sensitive to the presence of oxygen), whereas the vegetative cells depend on oxygen for photosynthesis[95]. These two cell types are mutually interdependent on nutrients such as fixed carbon and nitrogen[96,97]. The ability of heterocyst-forming Cyanobacteria to form long filaments, oftentimes with specialized cells with differentiated functions, has been shown to be dependent on LPS and peptidoglycan synthesis genes[98]. Recent cryo-ET studies revealed several notable features of the cell envelope of these bacteria[99]. For example, the peptidoglycan thickness of the cell wall appeared to be ~20 nm, whereas the peptidoglycan in the cross walls between neighbouring cells was much thicker (~40 nm) (Fig. 4)[99]. During cell division, only the peptidoglycan and IM form the septum, whereas the OM surrounds the entire

Fig. 4 | Cell envelope diversity in Terrabacteria. Cell envelope ultrastructure of representative species revealed by cryo-ET. Firmicutes and Actinobacteria contain monoderm and diderm species; Cyanobacteria, Thermotoga and Deinococcus are diderm with typical OM markers; Chloroflexi and CPR are monoderm. The images show a cell overview with the species name (left) and an enlarged cell envelope (right). Schematic envelope models (far right) show the presence of key cell envelope components. Scale bars, 100 nm (left) and 50 nm (right; magnified). The phylogenetic tree (far left) represents relationships among phyla[17]. AG, arabinogalactan; MCE, mammalian cell entry complex.

https://doi.org/10.1038/s41564-024-01812-9

filament[99,100]. Therefore, the septal junctions, that facilitate molecular exchange between cells, must have evolved to span two IMs and a thick layer of peptidoglycan. Notably, disruption of the OM integrity in unicellular *Synechocystis* sp. PCC 6803 was non-lethal and resulted in metabolically active cells that released photosynthetic by-products directly into the supernatant[101]. These observations provide a speculative link between the theory of OM loss via OM destabilization and cell survival of monoderms due to the presence of thick peptidoglycan.



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BOX 1

The β-barrel fold

Bacterial diderms with typical OMs, mitochondria and chloroplasts contain BamA/Omp85 family proteins with a conserved β-barrel fold indicative of shared ancestry. Exemplified by the mycobacterial β-barrel porin MspA, structural studies show that the β-barrel fold has evolved independently more than once, signifying the evolutionary advantage of this transmembrane fold as a cellular gatekeeper[55,60]. Although used as a proxy, it is important to note that the presence of a β-barrel protein alone is not sufficient to indicate the presence of a second membrane. For example, the monoderm Firmicute *Staphylococcus aureus* secretes a soluble toxin (α-haemolysin) that oligomerizes into host membranes as a heptameric pore-forming β-barrel and it shares no homology with typical OMPs[145]. Regardless of their primary structure and function, all β-barrel OMPs are thought to have evolved from a repeating β-hairpin and depend on BamA for membrane insertion[146]. BAM-independent assembly has been characterized for secretins (large multimeric β-barrel proteins), which can insert into the OM through the Lol pathway or by chaperone-assisted assembly[147–149]. Mechanisms of mOMP insertion into the MOM of mycobacteria remain uncharacterized.

Chloroflexi

Chloroflexi, also known as green non-sulfur bacteria, were often considered one of the most ancient phyla[102,103]. Comprising up to 30% of the bacteria in marine and freshwater environments, Chloroflexi members are ubiquitous across a wide range of environments and, accordingly, exhibit diverse metabolic abilities, including the capacity to contribute to carbon cycling and organic matter degradation in sediments[104–108]. This phylum is considered monoderm and is currently divided into several classes with cell morphologies, including filaments, filaments with branched mycelia, irregular cocci and rods[109,110].

Ultrastructural studies of the organohalide-respiring *Dehalococcoides mccartyi* revealed that the species is unique among monoderm Chloroflexi since its cell envelope lacks peptidoglycan and only comprises a cytoplasmic membrane and an S-layer[111]. The cytoplasmic membrane interacts directly with an S-layer that is enriched in filamentous appendages, reminiscent of an archaeal cell envelope[111]. Despite being a member of the peptidoglycan-lacking *Dehalococcoidia*, the newly characterized *Tepidiforma flava* was found to have a thin layer of peptidoglycan in addition to an S-layer (Fig. 4)[108,112]. Ancestral state reconstructions indicate that the peptidoglycan is ancestral to the *Dehalococcoidia* class and was lost among marine members such as *D. mccartyi*, demonstrating the complex evolutionary history within the phylum[108].

Isolates from several classes stain Gram negative and reveal multilayered cell envelope architectures, which has led to the notion that some Chloroflexi might be diderm[113–116]. Transmission electron microscopy studies of thin sections of the filamentous *Thermoflexus hugenholtzii* revealed three distinct surface layers, and cryotomograms of *Chloroflexus aggregans* revealed highly variable multilayered cell envelopes among closely related species (Fig. 4)[113,117]. In addition to a thin peptidoglycan layer, species displayed a surface monolayer termed the outer layer and an extra amorphous layer of unknown composition[113]. Since the phylum lacks homologues of BamA and LptD, as well as β-barrel proteins, members are considered monoderm[113,118]. The possibility of a unique OM and polysaccharide- or protein-based outer layer has been discussed previously; however, biochemical characterization is required to define the nature of the outer layer[3,113,118,119].

CPR

A sister clade to the Chloroflexi is the CPR, composed of mainly uncultivated bacterial phyla that are estimated to represent up to one-quarter of all bacterial diversity[7,8,10,120]. Classified as monoderm symbionts or parasites, CPR members have undergone large-scale and ancient genome reduction events[121]. Members have a core set of 106 genes that are not conserved among all other bacterial phyla, which raises questions about the evolutionary trajectory of the CPR phyla in relation to other bacteria[121]. Imaging studies of the few cultivated species have shown that they lack an OM and appear monoderm with a peptidoglycan layer and an S-layer, consistent with the presence of peptidoglycan synthesis genes and the absence of OM markers in their genomes (Fig. 4)[45,122–125]. CPR bacteria were recently found to have a high proportion of unique transmembrane and signal peptide-containing proteins compared with other bacteria and archaea[126]. Interestingly, the species characterized to date show similar biology to obligate parasites, which could explain their reduced genomes and the absence of metabolic and biosynthetic pathways[127]. CPR, along with other uncultivated clades, house a vast array of previously unknown proteins and synapomorphic gene families[126]. Given the number of uncultivated taxa, further efforts may uncover potentially diverse envelope architectures, S-layer anchoring machineries and metabolic adaptations.

Mechanisms for OM biogenesis

Whether the LUCA had an OM and, if so, how it originated has been the subject of extensive discussion. Based on the distribution of conserved proteins, Woese and Fox[128] proposed that the LUCA was a monoderm, whereas Cavalier-Smith[129] and Blobel[130] reasoned for it being a diderm. Due to the seemingly simpler architecture, it is often assumed that the primordial cell had one membrane. This, coupled with the hypothesis that the LBCA was a diderm, denotes a membrane biogenesis event during the transition of a primordial cell to the LBCA. Several mechanisms for OM biogenesis have been put forward over the years (Fig. 5): Blobel[130] proposed that protein–protein interactions in lipid vesicles could have mediated the formation of a diderm ancestor; Cavalier-Smith[129] proposed that two diderm half-cells may have fused to make a protocell with a double-membraned envelope; Lake[131] proposed that diderm bacteria may have originated from an endosymbiotic relationship between a monoderm Clostridium and a monoderm Actinobacterium; Tocheva et al.[14] proposed that an ancient sporulation-like event in a monoderm primordial cell could have given rise to a diderm cell; and most recently, based on studies of Thermotogae, Sexton et al.[81] proposed a model for OM biogenesis by continuous buildup of lipids into an existing proteinaceous surface layer. Some of these hypotheses have been criticized (for example, Lake's analysis used a small group size with low diversity and Tocheva's model is not compatible with the lack of sporulation outside of Firmicutes)[119,132,133], whereas others would be challenging to test due to unattainable evolutionary scales and intermediates. Regardless, the basic principles of these models can be supported by studies of liposome dynamics in vitro or depicted in living biological systems. For example, IM invaginations characterized in members of the Planctomycetota–Verrucomicrobiota–Chlamydiota superphylum are consistent with the mechanism of OM biogenesis proposed by Blobel[134]. Studies of liposome dynamics using biophysical and structural methods have characterized the fusion of unilamellar vesicles to produce bilamellar ones analogous to the Cavalier-Smith model, whereas hemifusion between smaller and larger vesicles can result in multilamellar vesicles consistent with the model by Lake[135]. The formation of diderm endospores in bacteria and autophagosomes in eukaryotic cells serve as real-life examples analogous to the mechanism proposed by Tocheva et al.[14,136]. Lastly, the model proposed by Sexton et al. is based on direct observations of the toga architecture where a proteinaceous sheath can support lipid patches[137,138]. Altogether, these studies highlight the multiple

https://doi.org/10.1038/s41564-024-01812-9

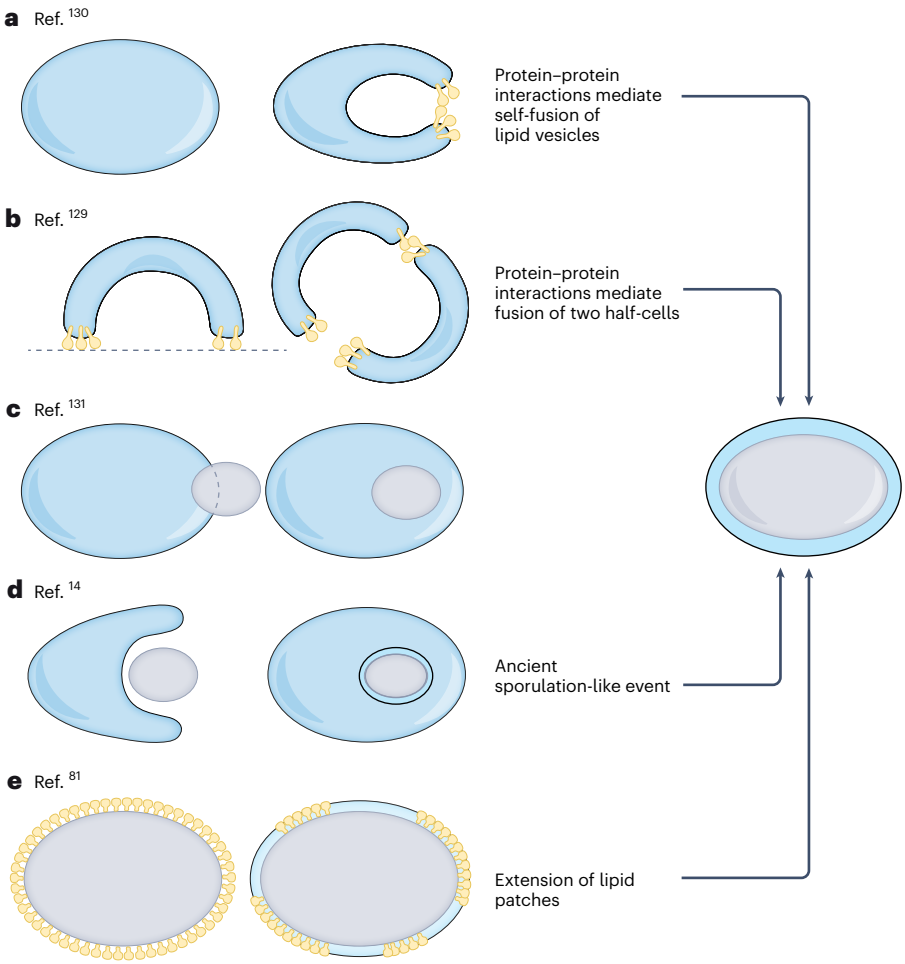

Fig. 5 | Proposed mechanisms for OM biogenesis. a, Protein–protein interactions on the surface of folded lipid vesicles mediate self-fusion[130]. **b**, Two diderm half-cells fuse to make a protocell with a diderm cell envelope[129]. **c**, The endosymbiotic relationship between monoderm Clostridia and Actinobacteria results in a diderm cell[131]. **d**, A sporulation-like event in a monoderm bacterium produces a diderm LBCA[14]. **e**, Sequential accumulation of lipids into a protein surface layer can extend into a second membrane[81]. Lipid bilayers are denoted with black lines and proteins are depicted in yellow.

topological states of lipid bilayers, indicating a range of possibilities for the evolution of a diderm cell plan.

Current evolutionary model supports a diderm LBCA

The current evolutionary model supports the hypothesis that the LBCA was a diderm and further highlights gains and losses of the OM throughout the domain (Fig. 6a)[11–14,123]. Assuming that the primordial cell was monoderm, the first de novo OM biogenesis event must have resulted in the LBCA, whereas a second OM gain is evident by the evolution of the MOM in the phylum Actinobacteria. Multiple OM losses have been confirmed in the phylum Firmicutes and were probably also responsible for the establishment of the monoderm phyla Chloroflexi, CPR, Coprothermobacterota and Caldiserica. Intrigued by the few monoderm phyla, we explored the possibility of a monoderm LBCA by placing Coprothermobacterota/Caldiserica or Chloroflexi/CPR as basal lineages while preserving the overall topology of the tree (Figs. 6b and 6c, respectively). Under both scenarios, the diderm phyla share a common diderm ancestor and, assuming that they never went through a diderm intermediate, the basal monoderm phyla would have retained an ancient monoderm cell plan. These observations, though highly speculative, provide a strong need for the characterization of monoderm phyla. Regardless of whether the LBCA was monoderm or diderm, multiple OM gains and losses are evident throughout the bacterial domain.

Key conclusions and future directions

Understanding the diversity of cell envelopes, especially among Terrabacteria, can provide insight into OM biogenesis and major evolutionary events that have shaped extant bacterial phyla. Combining advanced structural biology approaches with phylogenomics and outgroup-free rooting of the bacterial tree of life has proven to be a powerful approach that has advanced our understanding, specifically: (1) the OM and β-barrel fold are ancient bacterial features; (2) the phyla Actinobacteria and Firmicutes are paraphyletic with respect to their cell envelope architectures and contain monoderm and diderm species; (3) the discovery of diderm lineages with a typical OM among Firmicutes indicates that the last common ancestor of all Firmicutes was diderm and the monoderm members formed as a result of multiple OM losses; (4) the discovery that the MOM has no discernable shared ancestry with the typical OM indicates that the MOM arose de novo, further supporting the hypothesis that the bacterial OM evolved more than once; (5) the MOM probably evolved through sequential gene acquisition, indicating that the OM was lost at the phylum level; and (6) the widespread distribution of SlpA/OmpM tethering proteins among diderm Terrabacteria represents an ancient anchoring system. Additionally, studies of diderm Firmicutes, Deinococcus–Thermus and Cyanobacteria demonstrate that disruption of SlpA/OmpM results in OM instability, providing a possible mechanism for OM loss.

https://doi.org/10.1038/s41564-024-01812-9

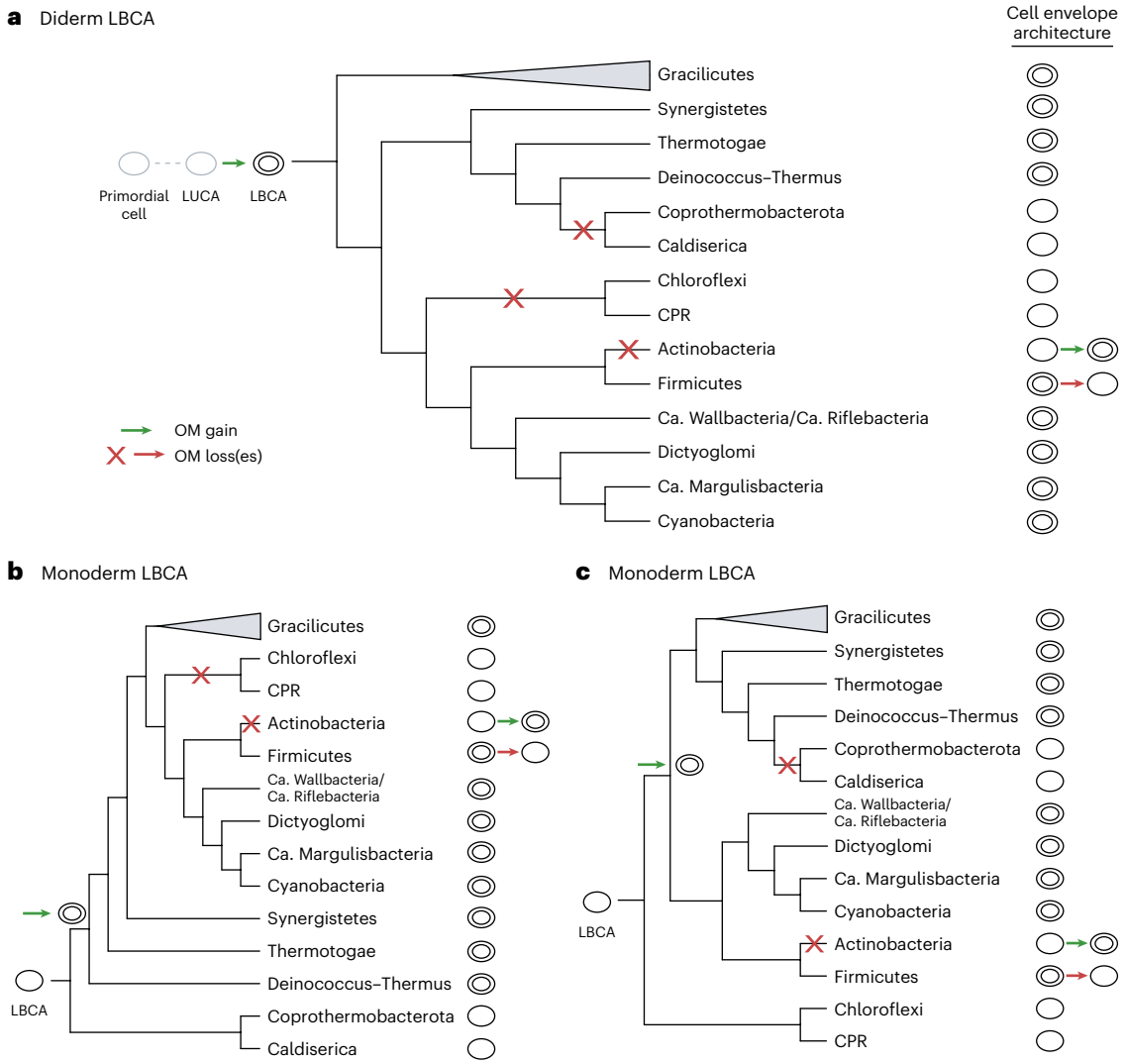

Fig. 6 | Evolutionary scenarios of OM losses and de novo biogenesis. **a**, Current model of the bacterial tree rooted between Gracilicutes and Terrabacteria, which supports a diderm LBCA. **b,c**, Rooting the tree at the monoderm phyla Coprothermobacterota and Caldiserica (**b**) or Chloroflexi and CPR (**c**) implicates their retention of the monoderm LBCA cell plan, whereas the divergence of the remaining phyla would have occurred after the evolution of the OM. Mapping the cell envelope architectures of modern phyla (shown on the right) highlights multiple OM gains and losses under all scenarios. Tree adapted from ref. 17.

Reconstruction of evolutionary events is highly dependent on the advancement of methodologies to assess diversity and infer phylogenetic relationships. It is tempting to reason that microbial dark matter in extreme environments that mimic early Earth conditions may hold the key to elucidating the evolutionary states of extant bacterial ancestors. Further efforts in cultivation techniques, imaging modalities, computational methods and targeted biochemical approaches will continue to uncover bacterial diversity.

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Acknowledgements

This project was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant to E.I.T. (RGPIN 04345). A.H. was supported by a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship (552674).

Author contributions

A.H. and E.I.T. conceived of the work and wrote the paper.

Competing interests

The authors declare no competing interests.

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Peer review information *Nature Microbiology* thanks the anonymous reviewers for their contribution to the peer review of this work.