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One or two membranes?

Diderm Firmicutes challenge the Gram-positive/Gram-negative divide

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Abstract

How, when, and why the transition between cell envelopes with one membrane (Gram-positives or monoderms) and two (Gram-negative or diderms) occurred in Bacteria is a key unanswered question in evolutionary biology. Different hypotheses have been put forward, suggesting that either the monoderm or the diderm phenotype is ancestral. The existence of diderm members in the classically monoderm Firmicutes challenges the Gram-positive/Gram-negative divide and provides a great opportunity to tackle the issue. In this review, we present current knowledge on the diversity of bacterial cell envelopes, including these atypical Firmicutes. We discuss how phylogenomic analysis supports the hypothesis that the diderm cell envelope architecture is an ancestral character in the Firmicutes, and that the monoderm phenotype in this phylum arose multiple times independently by loss of the outer membrane. Given the overwhelming distribution of diderm phenotypes with respect to monoderm ones, this scenario likely extends to the ancestor of all bacteria. Finally, we discuss the recent development of genetic tools for *Veillonella parvula*, a diderm

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Firmicute member of the human microbiome, which indicates it as an emerging new experimental model to investigate fundamental aspects of the diderm/monoderm transition.

The Gram-positive/Gram-negative divide

The origin of the classification of Bacteria into Gram-positives and Gram-negatives dates back to 1884 when Hans Christian Gram developed a staining technique for visualizing pneumococci cells in the lungs of pneumonia victims (Gram, 1884). This method consisted of four steps: gentian violet staining (violet color in gram-positive bacteria), iodine treatment, decolorization with alcohol and counterstaining with safranin (pink color in gram-negative bacteria). He noticed an outstanding resistance of some bacterial cells to decolorization, but he could not explain this phenomenon. The link between Gram-negative stain and the presence of a diderm envelope architecture did not occur until 1964, when Bladen and Mergenhagen obtained the first image of the double membrane of the Gram-negative bacterium *Veillonella* (Bladen and Mergenhagen, 1964).

Currently, the Firmicute *Bacillus subtilis* and the Gammaproteobacterium *Escherichia coli* represent the archetypes of monoderm and diderm envelopes, respectively (Silhavy et al., 2010). The monoderm envelope presents a thick peptidoglycan layer and a single cytoplasmic membrane, topped by teichoic or lipoteichoic acids. The diderm cell envelope shows instead a thin peptidoglycan layer and a second outer membrane (OM), often containing lipopolysaccharide (LPS) in its outer leaflet. The biogenesis of the classical LPS-diderm envelope is a complex process that requires a large number of dedicated cellular systems (Figure 1). These include the pathway for synthesizing LPS (Lpx and Kds enzymes), its flipping through the inner membrane (IM) (MsbA) and transport to the OM (Lpt system), the assembly and insertion of proteins in the OM (Bam and Tam systems), and the machinery responsible for lipid asymmetry between the IM and the OM (Mla system). In the case of machineries that are present both in monoderm and diderms, specific proteins are needed to anchor these to the OM (e.g. secretin for type IV secretion systems (PilQ), or P and L rings for flagella (FlgAHI)). Moreover, the distribution of different secretion systems in Bacteria seems to be linked with the structure of the cell envelope. While monoderm bacteria lack genes encoding diderm-like secretion systems proteins, the diderms show a wide diversity of secretion systems (Abby et al., 2016).

Diderm-first or Monoderm-first?

The existence of monoderm and diderm cell envelopes has led to different hypotheses on how and why they came into being, which can be generally grouped into two main alternative scenarios, Monoderm-first and Diderm-first (Figure 2). The Monoderm-first hypothesis posits that the

monoderm cell envelope, perceived as a more rudimentary and ancestral structure, would have preceded the emergence of the outer envelope. For example, Gupta proposed that antibiotic selection pressure was one of the main drivers for the origin of the OM and consequently the emergence of all diderm bacteria from monoderm ancestors (Gupta, 2011) (Figure 2). Under this scenario, bacterial groups lacking LPS would be evolutionary intermediates (simple diderms) in the transition between the monoderm phenotype and the classical OM-LPS cell envelope (Gupta, 2011) (Figure 2). However, while the OM confers particular resistance to antibiotics, it is not known how much impact antibiotics would have had on ancient bacterial evolution. In an alternative scenario of the monoderm-first hypothesis, Lake suggested that diderm bacteria arose from the fusion of two monoderm bacteria, a member of Firmicutes and a member of Actinobacteria (Lake, 2009). This scenario has been criticized on multiple bases regarding the assumptions on which Lake's analysis was based and the accuracy of the analyzed data (Gupta, 2011). Indeed, Lake defined the prokaryotes as composed of five main groups with no valid phylogenetic basis. Moreover, no unique molecular characteristics have been identified that are commonly shared by all or most species from diderms on the one hand, and all or most members from Actinobacteria or Firmicutes on the other hand. More recently, Tocheva and colleagues have put forward an alternative scenario for the origin of the OM, which is based on the fact that a transient outer envelope is formed during endospore formation in the Firmicutes, but is then lost during spore germination. Under this scenario, the OM would have originated once in an ancestral sporulating monoderm ancestor of all Bacteria (Errington, 2013; Tocheva et al., 2011, 2016) (Figure 2). However, to date this type of sporulation is specific of the Firmicutes and likely originated in this phylum, after the last bacterial common ancestor.

In contrast to these hypotheses, Cavalier-Smith proposed that the first bacteria were diderm (Diderm-first hypothesis) (Cavalier-Smith 2002). He suggested that the loss of OM was an accident that occurred in a diderm bacterium by mutation in the peptidoglycan (PG) cell wall growth machinery, leading to increase of its thickness as to break the OM attachments (Cavalier-Smith, 2006) (Figure 2). He also suggested that from this single loss of the OM, all monoderm Bacteria, Eukaryotes and Archaea would have evolved from a diderm ancestral clade which was identified in the Chloroflexi and suggested to lie at the root of the Tree of Life (Cavalier-Smith, 2006). However, Chloroflexi have since been shown to be monoderms (Sutcliffe et al., 2011), and are not the earliest emerging lineage in recent rooted phylogenies of Bacteria (Raymann et al., 2015).

A wide diversity of cell envelopes in Bacteria

The main difficulty in testing these various scenarios arises from the fact that the evolutionary relationships among monoderm and diderm phyla are still ill resolved. Moreover, beyond these

various hypotheses, one important aspect is that the most studied model monoderm and diderm organisms are certainly not representative of the whole diversity of bacterial cell envelope architectures, for the majority of which we are completely lacking any characterization, as already remarked by Sutcliffe, who carried out in 2010 a survey of diderms and monoderms across 24 Bacterial phyla (Sutcliffe, 2010). A huge number of genomes covering a wide fraction of bacterial diversity has become since available, including whole new clades of uncultured bacteria. Notably, Antunes et al. remarked the presence of Lpx genes -and therefore an OM- in a large number of phyla (Antunes et al. 2016).

Here, we have updated and extended these previous observations in the light of these new genomic data and available experimental characterization from the literature (Figure 3). We reanalyzed the distribution of diderms and monoderms phenotypes in representatives of 36 major phyla by searching for BamA homologues as a proxy for the presence of an OM, as well as other markers involved in LPS synthesis and transport (LpxABCD, KdsABCD and LptACFG). It appears that the majority of bacterial phyla are actually diderms, in particular those forming the large clade referred to as Gracilicutes (Cavalier-Smith, 2006), with all including members having either well-characterized OM with LPS, or possessing the genetic potential for it. Some exceptions are nevertheless observed, such is the example of the PVC superphylum (Planctomycetes, Verrucomicrobia, Chlamydiae, Omnitrophica and Lentisphaerae). Their members present thinner PG walls compared to model diderms, and it was believed until recently that some of them completely lacked PG (van Niftrik and Devos, 2017). Moreover, some key LPS synthesis and transport genes are absent in many PVC taxa (Figure 3), and more experimental work is needed to support the presence or absence of LPS in these taxa. Another example is that of Fibrobacteres, where some key LPS synthesis and transport genes are also absent (Figure 3). In fact, it has been shown that the OM of *Fibrobacter succinogenes* lack typical LPS components lipid A and Kdo, and instead the exopolysaccharide is composed of two polysaccharide polymers and one glycolipid (Vinogradov et al., 2001). Finally, Nitrospinae and Tectomicrobia also seem to lack some genes involved in LPS biosynthesis (Figure 3), but very few genomes are available for these phyla and they are not complete. Another exception within the Gracilicutes is represented by the Spirochaetes. Only some members, such as *Leptospira* and *Brachyspira*, have LPS, in agreement with the presence of the corresponding genes (Bulach et al., 2000). *Leptospira* have a typical spirochaete cell structure, with a coiled cell body, or protoplasmic cylinder, comprising the cytoplasm, IM and the PG. This protoplasmic cylinder is surrounded by an OM. Two flagella are inserted at each end of the cell, and wrap around the protoplasmic cylinder in the periplasmic space. These flagella are essential components of the motility apparatus of spirochetes and are usually called “periplasmic flagella”. The

OM is loosely attached to the cell (Li et al., 2000). Other Spirochaetes, including *Treponema* and *Borrelia*, lack LPS in their OM. Instead, they contain an outer sheath, which encloses the protoplasmic cylinder. Flagella are also located in the area between the outer sheath and the protoplasmic cylinder (Cullen et al., 2004).

In contrast to Gracilicutes, the phyla belonging to the Terrabacteria clade appear much more variable in their cell envelopes (Figure 3). Members of the clade including Dictyoglomi, Cyanobacteria, Deinococcus-Thermus, and Thermotogae all present atypical diderm envelopes. In Cyanobacteria, the peptidoglycan layer is often considerably thicker than in the typical Gram-negative bacteria (Hoiczyk and Hansel, 2000). All contain BamA homologues, as well as all *lpx* and *lpt* genes, but the *kds* genes are more scattered (Figure 3). Dictyoglomi have a diderm phenotype (Hoppert et al., 2012), and possess BAM homologues as well as the genes involved in LipidA synthesis, but not *kds* genes (Figure 3). A distinguishing feature of the two current isolates of Dictyoglomi is the formation of rotund bodies as the culture enters the late logarithmic phase of growth. Cells form aggregates sharing a single OM, and these bundles continue cell division until a spherical body is formed containing up to 50 or more cells (Hoppert et al., 2012). Morphologically, they are similar to the rotund bodies described for cultures of the genus *Thermus*, representative of the phylum Deinococcus-Thermus (Brock and Edwards, 1970). Members of the Deinococcus-Thermus phylum also are another interesting example, as they present a diderm phenotype, but were thought not to have LPS (Thompson and Murray, 1982), consistent with the fact that the first sequenced representatives lacked the *lpx* genes (Sutcliffe, 2010). However, many members of this phylum have complete LipidA and Lpt pathways (Figure 3), and so the absence of LPS in some members is likely due to secondary loss. The tethering of the OM is also peculiar in this phylum: in *T. thermophilus* the SlpA protein acts as a scaffold, it was suggested to represent an ancestral type of structural protein that shows intermediate properties between S-layer proteins and OM porins, and insertion of SlpA depends on the OM beta-barrel assembly machinery BamA (Acosta et al., 2012). TamB was also suggested to be involved in the maintenance of cell envelope integrity through SlpA in *D. radiodurans* (Yu et al., 2017). In Thermotogae, a sheath-like structure surrounds the cell and is detached at the poles, and is commonly referred to as the “toga”. This atypical outer cell envelope differs from the classical outer cell membrane of Gram-negative bacteria as it lacks LPS (Raetz and Whitfield, 2002). OmpA1, an alpha-helical protein, serves to anchor the toga to the peptidoglycan (Petrus et al., 2012, Ranjit and Noll 2016). It plays a similar role as the *E. coli* OmpA porin, but they are not evolutionary or structurally related. OmpA1 remains associated to the OM through porin OmpB in the portion of the toga that it is detached from the inner envelope. OmpB is absent in *E. coli* (Petrus et al., 2012, Ranjit and Noll 2016). In agreement with the lack of LPS, no related genes are

present in currently available genomes of Thermotogae. Notably, we could not confirm the presence of the distant *kdsA* homologues previously reported in Sutcliffe (2010), and no other *kds* gene was found in all currently available genomes. Finally, in many members of the Dictyoglomi, Deinococcus/Thermus, Cyanobacteria, and Thermotoga clade, we observed the presence of genes for the LPS transport system Lpt despite the absence of *lpx* genes (Figure 3). This is intriguing and surely worth investigating further.

Apart from the well-known monoderm Actinobacteria and the Chloroflexi, only a few other phyla seem to lack an OM. Caldiserica and Coprothermobacterota are likely monoderm because their genomes lack all of the OM marker genes. Moreover, no OM was observed experimentally in *Caldisericum exile* (Mori et al., 2009). However, only one genome is currently available for Caldiserica and Coprothermobacterota, so their monoderm nature needs to be confirmed when more sequence and experimental data becomes available. It is worth noting that within the classical monoderm Actinobacteria, the cell envelope of Corynebacteriales (which include Mycobacteria) contains mycolic acid and forms a barrier on the outer surface of cells. However, this structure is not homologous to the classical diderm OM and likely arose independently (Vincent et al., 2018). Accordingly, no OM markers are found in this clade. Within the classical monoderm Firmicutes, Tenericutes are also an exception because they lack a PG wall. The absence of a cell wall imposes certain demands on their cytoplasmic membrane, particularly regarding osmotic resistance. For this reason, the incorporation of host-derived sterols into their membranes is needed in significant quantities. The composition of their membranes is therefore much more similar to animal cell membranes than to other bacteria membranes, as they also incorporate phospholipids from the host (Rottem et al., 1986).

To conclude, despite this diversity of cell envelope, two observations can be made: the first is that the vast majority of bacterial phyla appear diderm; the second is that all members of a bacterial phylum are homogeneous in having either a monoderm or a diderm phenotype. However, the Firmicutes represent an exception to this rule, as they contain both diderm and monoderm lineages. This provides a great opportunity to test the diderm-first or monoderm-first hypotheses.

Firmicutes with an outer membrane?

The Firmicutes are one of the largest bacterial phyla and the textbook example of monoderm cell envelope (Gibbons and Murray, 1978). They include well-studied models such as *Bacillus subtilis*, but also important pathogens (e.g. *Bacillus anthracis*, *Clostridium difficile*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*). Significant reorganization of the Firmicutes taxonomy was recently proposed, especially in the class Clostridia (Yutin and Galperin, 2013),

however, a wide fraction of diversity in this phylum remains largely uncharacterized. Surprisingly, two poorly studied clades, the Negativicutes and the Halanaerobiales, appear diderm under the microscope.

The Negativicutes have been identified in various anaerobic environments, such as soil and lake sediments, industrial waste, and animal and human digestive tracts (Vos et al., 2009). Most Negativicutes live in moderate temperature environments and some form endospores, which is a specific characteristic of the Firmicutes. When the class Negativicutes was proposed (Marchandin et al., 2010), Gram-negative members previously belonging to the class Clostridia were reassigned to the families Veillonellaceae and Acidaminococcaceae in the novel order Selenomonadales (Marchandin et al., 2010). Afterwards, they were divided into the four families Acidaminococcaceae, Veillonellaceae, Sporomusaceae and Selenomonadaceae (Campbell et al., 2015). The second clade of diderm Firmicutes is the Halanaerobiales, a poorly studied group of moderate halophilic and strictly anaerobic Firmicutes that were first isolated from saline environments such as lake and lagoon sediments (Zeikus et al., 1983; Oren et al., 1984; Rainey et al., 1995). Similarly to the Negativicutes, the Halanaerobiales display a diderm phenotype and some sporulate (Zeikus et al., 1983; Zhilina et al., 1992, 2012). Very little experimental data is available on the nature of the diderm cell envelope of Negativicutes and Halanaerobiales. The LPS of various Negativicutes has been characterized throughout the years, with the best characterized belonging to the beer spoilage bacteria *Pectinatus cerevisiophilus* (Helander et al., 1983). The LPS of *Pectinatus cerevisiophilus* was found to have unique features including extensive heterogeneity within strains, a non-repeating polysaccharide that replaces the O-antigen and an atypical lipid A (Helander et al., 2004). Another feature of the diderm Firmicutes envelope that has been studied is the mechanism of OM attachment, completely different from that of *E. coli* (Kojima et al., 2010). *E. coli* utilizes Braun's lipoprotein (Lpp) (Braun and Rehn, 1969) and other redundant systems such as Pal (Cascales et al., 2002) and OmpA (Park et al., 2011) for attachment of the OM, whereas *Selenomonas ruminantium* was shown to bind polyamine-modified peptidoglycan to the OM through a protein that contains an S-Layer homology domain (SLH) and a porin domain (OmpM) (Kojima and Kamio, 2012).

The first genomes of diderm Firmicutes to be sequenced were the Negativicute *Thermosinus carboxydivorans* (Skolova et al., 2004) and the Halanaerobiale *Halothermomix orenii* (Mavromatis et al., 2009), and both were shown to contain homologues of the genes involved in OM biosynthesis, consistently with their diderm appearance (Mavromatis et al., 2009). However, the origins and relationships between the OM of Halanaerobiales and Negativicutes have been unclear. While Tocheva et al., speculated that the OM is an ancestral character in the Firmicutes that was vertically inherited by the Negativicutes (Tocheva et al., 2011); Campbell et al., put forward the hypothesis of a

possible acquisition of the OM in Negativicutes by horizontal gene transfer (Campbell et al., 2014). Moreover, from the combined analysis of the genes coding for LPS, Mavromatis et al., proposed a horizontal gene transfer of the OM between Halanaerobiales and Negativicutes (Mavromatis et al., 2009). However, all these hypotheses relied on the analysis of a limited number of genomes and could not resolve the question about the origin of the OM in this phylum. In 2016, Antunes et al., (2016) reconstructed a robust reference phylogeny of the phylum based on concatenation of ribosomal proteins, which confirmed the polyphyly of Clostridia and robustly resolved the relative positions of Halanaerobiales and Negativicutes in the Firmicutes phylogeny (Figure 4A). In fact, the two diderm groups are not each other's closest relatives and instead branch with different monoderm lineages. In particular, Halanaerobiales appear to be the deepest branching lineage in the Firmicutes, while the Negativicutes are well embedded into Clostridia.

The presence of two distinct diderm lineages branching within the classically monoderm Firmicutes poses the important question of what might be their origin and provides an exceptional opportunity to clarify the monoderm/diderm transition.

Characteristics of the diderm Firmicutes OM

By comparing the genomes of more than 200 members of the Firmicutes including over 45 Negativicutes and Halanaerobiales, Antunes et al., (2016) sought to infer some of the characteristics of the cell envelope of diderm Firmicutes. Strikingly, it was observed that in diderm Firmicutes many homologues of the genes involved in the biogenesis of the OM are clustered in a large conserved genomic locus with similar organization (Antunes et al., 2016) (Figure 4B). These genes indicate the presence of some of the classical diderm OM systems described in Figure 1, such as a complete pathway for LPS synthesis and transport, or OM protein biogenesis. However, some specificities are apparent in diderm Firmicutes. In general, diderm Firmicutes seem to possess streamlined versions of the classical OM systems such as those present in *E. coli* (Figure 1). It is unclear if components are missing altogether, are present but not recognizable at the sequence level, or are replaced by non-homologous proteins. Concerning the OM components of the Lpt pathway, LptDE, only LptD was identified in Negativicutes and Halanaerobiales (Figure 4B). The OM gene cluster includes three genes coding for TamB, BamA and one or multiple copies of the periplasmic chaperone Skp. This three-component system for OM protein biogenesis is peculiar, was proposed to have preceded the classical BAM/TAM system, and might be involved in both autotransporter secretion and OMP assembly (Figure 1) (Heinz and Lithgow, 2014; Antunes et al., 2016). All diderm Firmicutes possess one to multiple copies of OmpM in the OM, suggesting a common OM tethering mechanism with some redundancy (Figure 4B).

The two diderm Firmicutes lineages also present their own specificities, in line with their evolutionary distance. For example, the OM gene cluster of Negativicutes contains homologues of genes coding for the Mla pathway for lipid asymmetry in classical diderm bacteria (Figure 1), but only some of the IM components (MlaEFD) (Figure 4B). It is unclear if the OM components (MlaAC) are present elsewhere in the genome and are not recognizable at the sequence level, or if Negativicutes use other non-homologous proteins. In contrast, no homologues were identified in Halanaerobiales, suggesting the presence of a different system in these organisms. Another difference concerns the Lol pathway for inserting lipoproteins in the OM (Figure 1), which appears totally absent in Negativicutes, whereas Halanaerobiales have a small three-gene cluster which may potentially code for a complete Lol system (*lolA*, *lolC/E*, *lolD*) (Antunes et al., 2016).

Phylogenomic analysis of diderm Firmicutes supports the diderm-first scenario

The similarities of the inferred characteristics of the OM of Halanaerobiales and Negativicutes indicates that they are homologous structures with an origin common with other diderm bacteria, and are not the product of convergent evolution. However, the phylogenetic distance among the two diderm lineages within the Firmicutes poses the question of how their OM might have appeared. One hypothesis is that they were obtained by horizontal gene transfer from a diderm bacterium, either twice independently or in one of the two diderm lineages and then from this to the other. This scenario may be suggested by the presence of the conserved large OM gene cluster in the genomes of both Halanaerobiales and Negativicutes. However, a number of considerations weaken this scenario. First of all, it is difficult to imagine the simultaneous and immediate expression/regulation of all the genes of the cluster to build a functional OM in a monoderm firmicute cellular setting. Second, some other OM systems lie elsewhere in the genome and should have been acquired at the same time. Third, the OM gene cluster seem to be specific of Halanaerobiales and Negativicutes and is not found in other sequenced diderm bacteria.

Nevertheless, Antunes et al., (2016) performed phylogenetic analysis to test this scenario. Whereas the poor conservation at the sequence level of most of the membrane proteins coded in the OM gene cluster prevented to produce robust phylogenies and understand their origin and evolutionary history, this is not the case of the cytosolic enzymes involved in LPS synthesis. Moreover, most of these enzymes are conserved in all diderm bacteria with LPS. A global phylogeny built on the concatenation of four of the most conserved proteins for LipidA synthesis (LpxABCD) indeed produced a phylogeny with overall consistency with the bacterial reference tree (Antunes et al., 2016). Importantly, Negativicutes and Halanaerobiales form a robust monophyletic clade with internal branching consistent with their species phylogeny, indicating that these *lpx* genes were not

acquired by horizontal gene transfer but were rather already present in their ancestor, which is by definition the ancestor of all Firmicutes. It is therefore likely that the whole OM gene cluster followed the same evolutionary history, and consequently that the ancestor of all Firmicutes already had an OM with LPS. As a consequence, the monoderm phenotype in the Firmicutes would have originated multiple times independently during the diversification of this phylum through loss of an ancestral OM (Antunes et al., 2016) (Figure 4A).

The mechanism through which the OM would have been repeatedly lost in the Firmicutes is unclear. However, two elements may link it to the specific sporulation process of the Firmicutes, where an OM is formed transiently from the inner membrane during sporulation, and is subsequently lost during maturation. Eliza Tocheva and Grant Jensen have nicely shown that in the sporulating Negativicute *Acetonema longum* the transient OM is indeed formed from the inner membrane but is not lost during maturation (Tocheva et al., 2011). This evidence led them to propose that the OM would have originated during sporulation through this process (Errington, 2013; Tocheva et al., 2011, 2016). We propose an alternative hypothesis: that sporulation allowed instead the loss of the OM. This could have occurred by an accident that produced instability of the OM attachment, but did not lead to immediate lethality if compensated rapidly by strongly selected compensatory mutations such as those producing a thicker cell wall (Figure 4C). The rest of the genes related to the biogenesis of the OM would have been subsequently lost or repurposed for other cell-envelope functions. In brief, the loss of the OM would not have been selected, but just tolerated in very specific conditions. The involvement of sporulation in the loss of the OM would explain why this occurred specifically in the Firmicutes and not in other diderm phyla, which do not have this process.

The hypothesis of a diderm ancestor of the Firmicutes is also supported by the fact that the large majority of bacterial phyla are actually diderm (Figure 3). Therefore, it is likely that the ancestor of all bacteria was itself already a diderm, and that the OM was lost multiple times independently in the ancestors of the few current monoderm phyla, supporting the Diderm-first scenario. We do not emit any hypothesis on how the OM came into being in the first place at this stage. Whether sporulation was involved will need to be investigated by further analysis of the origin of this process in Bacteria.

***Veillonella parvula* as a new experimental model of the bacterial cell envelope**

The inferences on the characteristics of the OM in diderm Firmicutes, as well as the consequent hypotheses on the diderm/monoderm transition now need to be confirmed by experimental data, and ideally in a genetically tractable model.

Veillonella parvula (Negativicutes) was described for the first time at the end of 19th century (Veillon & Zuber 1898). It is a nonmotile nonsporulating small (0.4 μm) coccus. This species is one of the most abundant constituents of the oral bacterial community, being an early secondary colonizer, and associating strongly with *Streptococcus* species, which provide it with lactate which is its primary carbon source, as *Veillonella* is unable to feed on carbohydrates (Aas et al., 2005, Rogosa 1963, Palmer et al., 2006; Chalmers et al., 2008; Dewhirst et al., 2010). Relatively aerotolerant anaerobe, *V. parvula* is able to protect obligate anaerobes from oxidative stress in oral microbiome (Rogosa 1963; Zhou et al., 2017). Its occurrence is not limited to the buccal cavity, as *V. parvula* colonizes also the gastrointestinal tract, especially the small intestine (van den Bogert et al., 2013). It has been recently suggested that the early colonization of the infants' gut by *V. parvula* is important for the correct training of the immune system (Arrieta et al., 2015). On the other hand, *V. parvula* has been associated with oral cavity diseases, such as caries or periodontitis (Mashima et al., 2015; Damé-Teixeira et al., 2018), and some rare non-oral infections with formation of abscesses (for a review, see Hirai et al., 2016). The first genome of *V. parvula* DSM 2008 strain was sequenced in 2010, its size is 2,13 Mb, it encodes 1859 predicted proteins, its GC content is of 38.6% (Gronow et al., 2010). It possesses the OM gene cluster typical of diderm Firmicutes (Figure 4B).

V. parvula was recently used to obtain the first outer membrane proteome of a diderm firmicute (Poppleton et al., 2017). By combining bioinformatics prediction with experimental data, this study could confirm the existence of a number of OM systems previously only inferred on the basis of homology and synteny (Antunes et al., 2016) (Figure 5A). The elements of the ancestral beta barrel assembly system BamA/TamB were detected, as well as the associated chaperone proteins Skp. Consistently with the presence of the Bam/Tam systems, many porins were also detected. Among these are the OmpM proteins mentioned above and responsible for the tethering of the outer membrane to peptidoglycan in diderm Firmicutes. While four OmpM homologues are encoded in the *V. parvula* DSM 2008 genome, one of them is the most abundant protein in the OM and another one is the third most abundant protein, which stresses the importance of this OM attachment system in *V. parvula* cell physiology. TonB dependent receptors and TolC efflux pumps were also detected in abundance. Ten different trimeric autotransporter adhesins were detected at the cell surface, along with other types of adhesins, which reflects the lifestyle of this organism in the dental plaque community. Some proteins containing an SLH (S-layer homology) domain proteins were also present. The presence of a complete LPS transport Lpt system was equally confirmed. Finally, a large number of uncharacterized, hypothetical proteins were detected, some of which are encoded by genes located in the OM diderm gene cluster and could be potentially involved in novel systems for the biogenesis and maintenance of the OM of diderm Firmicutes. It is worth noting that the proteins

identified in the OM, most of which are conserved in all diderm Firmicutes, are scattered all over the *V. parvula* genome and not only the OM gene cluster (Figure 5B). This weakens the hypothesis that they were acquired through a single horizontal gene transfer. Having said that, it is still possible -and even likely- that the fact of possessing an OM could have favored subsequent acquisition of some genes from other diderm bacteria.

V. parvula has a high potential to become a new experimental model of the bacterial cell envelope. It is an aerotolerant anaerobe, which eases manipulation, and its generation time is 1.5 hours. Moreover, *Veillonella* is currently the only diderm Firmicute for which genetic tools are available (Liu et al., 2011). These include an *Escherichia/Veillonella* shuttle vector (Liu et al., 2012), a suicide vector for gene disruption (Zhou et al. 2015a), and a scarless deletion system based on two consecutive crossing-overs using a suicide vector with a counterselectable marker (Zhou et al. 2015b). Finally, naturally competent strains of *V. parvula* were recently isolated. Very high efficiency of transformation with linear DNA combined with equally high efficiency of crossing-over facilitated the construct design for gene deletion (Knapp et al., 2017). The availability of these tools now opens the way to characterization of many of these potential novel systems involved in the biogenesis and functioning of the OM in diderm Firmicutes.

Conclusions and Perspectives

The steady increase in availability of genome data from a wide diversity of microorganisms, combined with new bioinformatic tools to analyze them, provide an exciting opportunity to address fundamental questions in prokaryotic evolution. In this respect, while there has been a lot of recent attention on the evolution of Archaea and their involvement in the origin of Eukaryotes (Adam et al., 2017), many open questions still remain to be investigated in Bacteria. The transition between monoderms and diderms is clearly one of them. Moreover, despite the importance of the cell envelope, it is surprising how little we still know about its real diversity in the vast number of bacterial lineages. The existence of diderm Firmicutes adds an important piece to the puzzle. It indicates that a phylum considered as the classical example of monoderm bacteria does actually contain a mixture of monoderm and diderm cell envelopes, blurring the long-time sharp divide between Gram-positives and Gram-negatives. It also strongly suggests that, at least in this phylum, the OM is an ancestral character and the monoderm cell envelope is a derived cellular structure.

The study of diderm Firmicutes may also help to better understand the biogenesis and functioning of the cell envelope of important monoderm Firmicutes pathogens. It will also be necessary to explore further the diversity of Halanaerobiales, both from a genomic and experimental point of view, as they are a deep lineage in the Firmicutes and may provide key information on the ancestral

diderm cell envelope of the Firmicutes. It cannot be excluded that additional diderm lineages will be found within the large number of yet uncharacterized uncultured Firmicutes lineages. Further comparative genomic analysis of diderm Firmicutes, combined with the characterization of mutant strains in *V. parvula*, will surely provide additional elements on the nature of their atypical cell envelope and its evolutionary relationship to that of other bacterial diderms as well as their monoderm relatives, and may eventually allow to test experimentally the diderm/monoderm transition.

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Figures

Figure 1. Schematic diagram of a diderm cell envelope and a few classical OM systems. The systems mentioned in the text are shown schematically. Adapted from Antunes et al., (2016).

Figure 2. Evolutionary hypotheses for the origin and evolution of OM proposed by Gupta (2011), Lake (2009), Tocheva et al., (2016), and Cavalier-Smith (2002). Peptidoglycan layer (PG) is in pink; Outer membrane (OM) in purple and inner membrane (IM) in black. For detailed discussion, see text.

Figure 3. Experimental or inferred presence of an OM in Bacteria. The experimental identification of an OM, containing LPS or not, was retrieved from the literature. Additionally, the genomes of 203 bacteria representative of 36 major phyla were examined for the presence of homologues of OM markers: BamA, LpxA-D, KdsA-D, and LptACFG. Inference of the LipidA, KDO and LPT pathways is based on the presence of all marker genes. Full white circles indicate complete absence of the pathway, full black circles indicate presence of a complete pathway, half black circles indicate presence of either a complete or a partial pathway, full grey circles indicate presence of a partial pathway or complete absence, and half grey circles indicate presence of a complete or partial pathway, or complete absence. The complete absence of a pathway in a given phylum was verified by checking all available genomes in NCBI. *The OM of Corynebacteriales has a secondary independent origin (see text for discussion). The grey circle in the monoderm Chloroflexi indicates presence of

distant homologues in one or two genomes only, whose homology and function needs to be verified experimentally.

Figure 4. (A) Schematic representation of the Firmicutes phylum-level phylogeny from Antunes et al., 2016, onto which the presence of OM is mapped. An OM was present in the ancestor of all Firmicutes and was retained in diderm lineages (Negativicutes and Halanaerobiales), while it was lost at least five times independently (crosses). Electron microscopy images of *Veillonella parvula* (Negativicutes) and *Halanaerobium saccharolyticum* (Halanaerobiales) are from Poppleton et al., 2017 and Antunes et al., 2016, respectively. **(B) A large OM gene cluster in diderm Firmicutes lineages.** Genes for LPS synthesis and transfer, OMP biogenesis and OM tethering are in purple; Flagellar genes are in yellow; and genes for lipid asymmetry are in green. **(C) Hypothetical scenario of OM loss in Firmicutes.** The ancestor of Firmicutes was a sporulating diderm. The OM was lost multiple times during sporulation giving rise to the monoderm phenotype in Firmicutes.

Figure 5. Outer membrane proteome of *V. parvula* DSM2008. (A) Proteins detected in the OM are in bold while surface exposed ones are indicated by bold and italics. Reproduced from Poppleton *et al.* (2017). **(B)** Genomic localization of the 78 proteins from the OM proteome on *V. parvula* DSM2008 chromosome.

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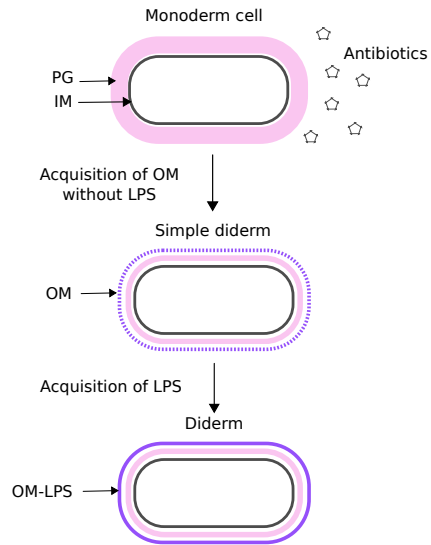
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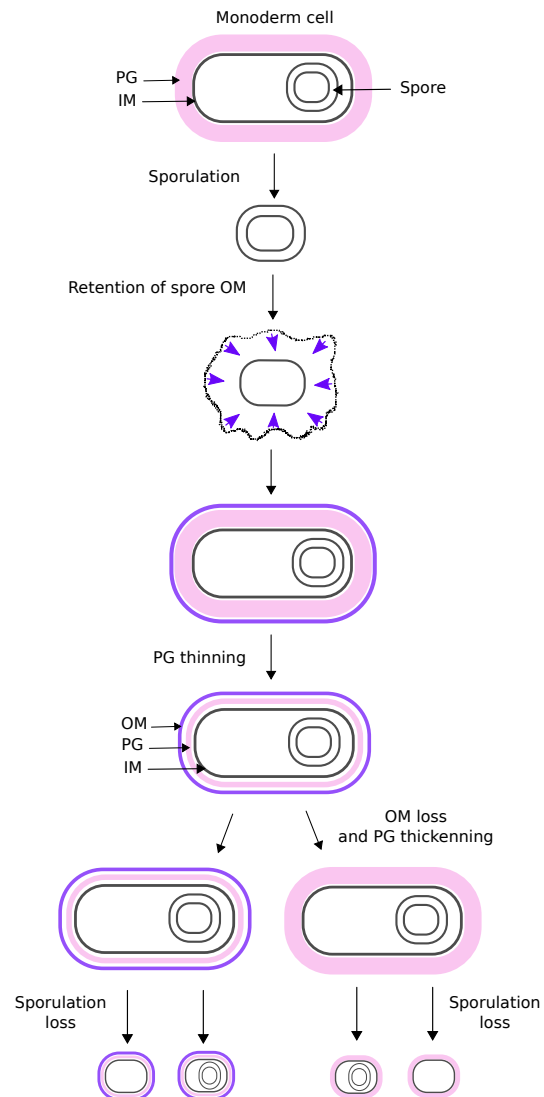
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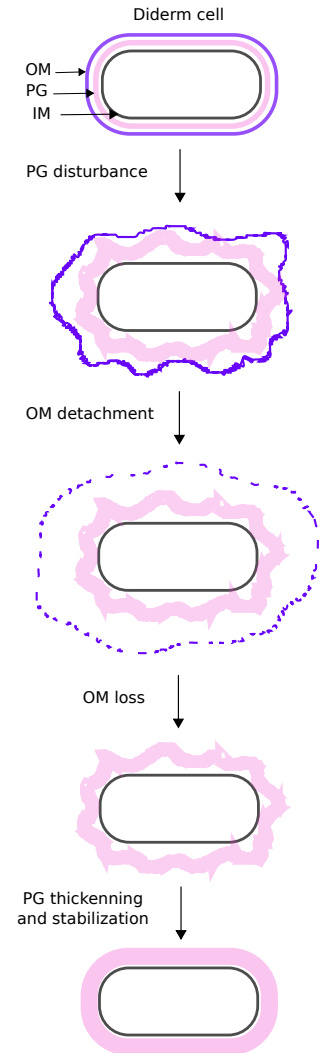
Gupta, 2011



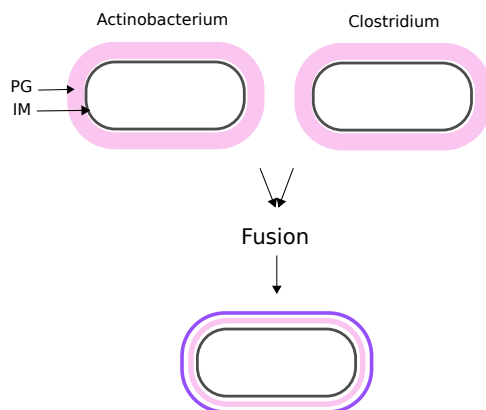
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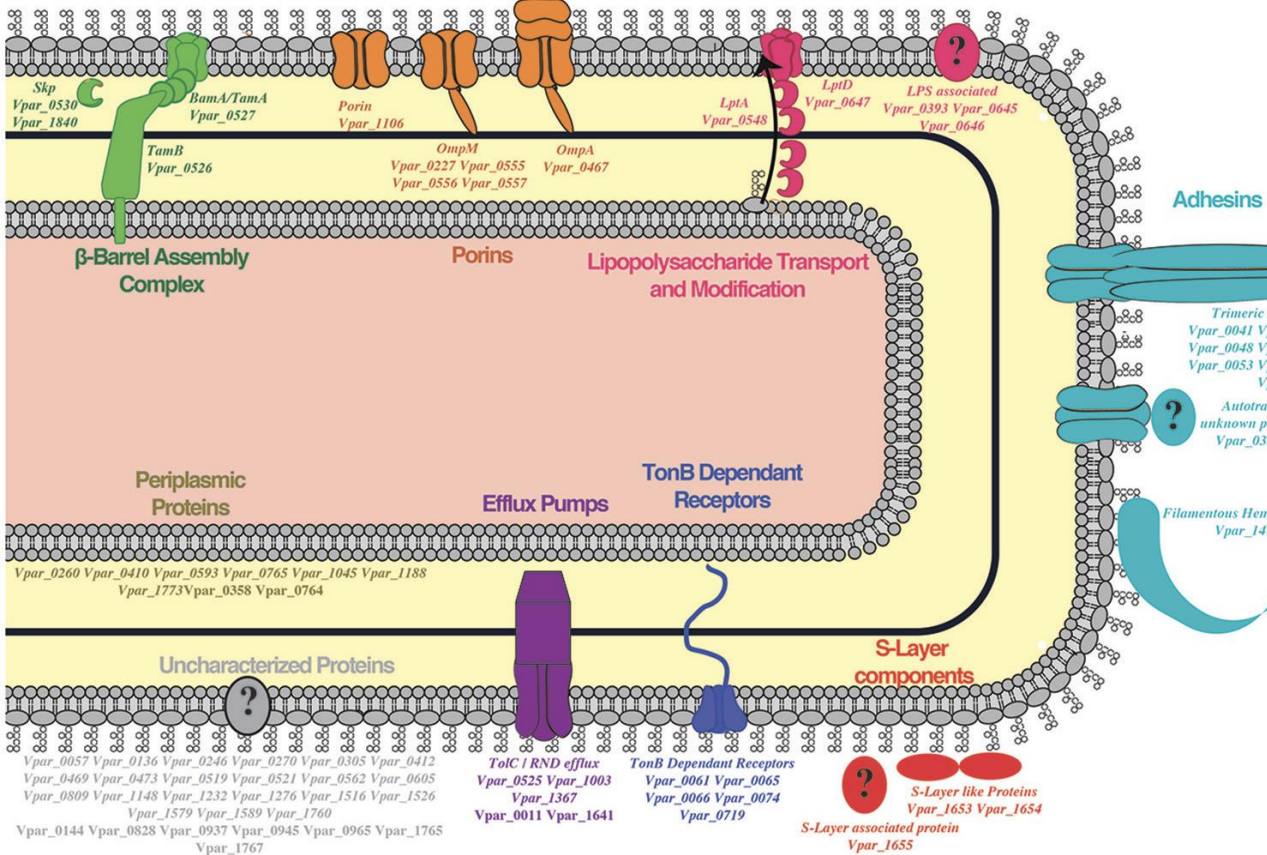
Cavalier-smith, 2002



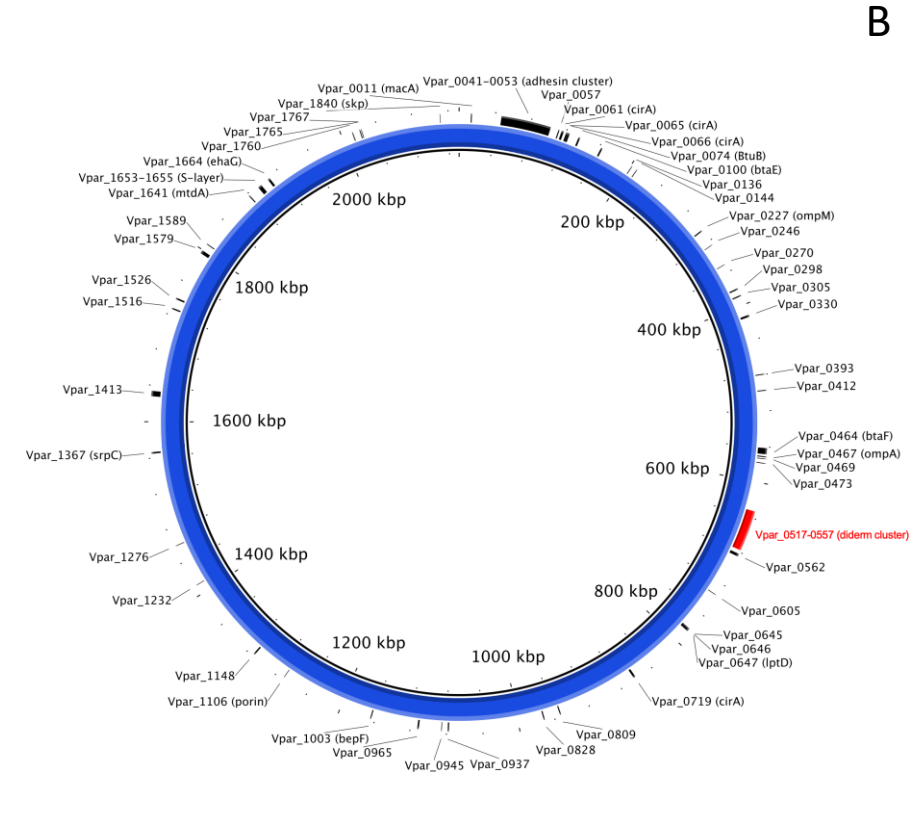
Lake, 2009



Phyla		Experimental identification of OM	Gene presence			
			BAM	LPX	KDS	LPT
Terrabacteria	Actinobacteria	Absent, except in Corynebacteriales*				
	Armatimonadetes	Present				
	Caldiserica	Not clear				
	Chloroflexi	Absent				
	Coprothermobacterota	Not reported				
	Cyanobacteria	Present				
	Deinococcus-Thermus	Present				
	Dictyoglomi	Present				
	Firmicutes	Presence in Negativicutes and Halanaerobiales				
	Synergistetes	Present				
	Thermotogae	Present				
Gracilicutes	Acidobacteria	Present				
	Aquificae	Present				
	Bacteroidetes	Present				
	Calditrichaeota	Present				
	Chlamydiae	Present				
	Chlorobi	Present				
	Chrysiogenetes	Not reported				
	Cloacimonetes	Not reported				
	Deferribacteres	Present				
	Elusimicrobia	Present				
	Fibrobacteres	Present				
	Fusobacteria	Present				
	Gemmatimonadetes	Present				
	Ignavibacteriae	Present				
	Lentisphaerae	Not reported				
	Nitrospinae	Present				
	Nitrospirae	Present				
	Omnitrophica	Not reported				
	Planctomycetes	Present				
	Alphaproteobacteria	Present				
	Betaproteobacteria	Present				
	Deltaproteobacteria	Present				
	Epsilonproteobacteria	Present				
	Gammaproteobacteria	Present				
	Zetaproteobacteria	Present				
	Spirochaetes	Present				
	Tectomicrobia	Not reported				
	Thermodesulfobacteria	Present				
	Verrucomicrobia	Present				
	Zixibacteria	Not reported				



A



B