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PERSPECTIVE

Origin of diderm (Gram-negative) bacteria: antibiotic selection pressure rather than endosymbiosis likely led to the evolution of bacterial cells with two membranes

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Abstract The prokaryotic organisms can be divided into two main groups depending upon whether their cell envelopes contain one membrane (monoderms) or two membranes (diderms). It is important to understand how these and other variations that are observed in the cell envelopes of prokaryotic organisms have originated. In 2009, James Lake proposed that cells with two membranes (primarily Gram-negative bacteria) originated from an ancient endosymbiotic event involving an *Actinobacteria* and a *Clostridia* (Lake 2009). However, this Perspective argues that this proposal is based on a number of incorrect assumptions and the data presented in support of this model are also of questionable nature. Thus, there is *no reliable* evidence to support the endosymbiotic origin of double membrane bacteria. In contrast, many observations suggest that antibiotic selection pressure was an important selective force in prokaryotic evolution and that it likely played a central role in the evolution of diderm (Gram-negative) bacteria. Some bacterial phyla, such as *Deinococcus-Thermus*, which lack lipopolysaccharide (LPS) and yet contain some characteristics of the diderm bacteria, are postulated as evolutionary intermediates (simple diderms) in the transition between the monoderm bacterial taxa and the bacterial groups that have the archetypal LPS-containing outer cell membrane found in Gram-negative bacteria. It is possible to distinguish the two stages in the evolution of diderm-LPS cells (viz. monoderm bacteria → simple diderms lacking LPS → LPS containing archetypal diderm bacteria) by means of conserved inserts in the Hsp70 and Hsp60 proteins. The insert in the Hsp60 protein also distinguishes the traditional Gram-negative diderm bacterial phyla from atypical taxa of diderm bacteria (viz. *Negativicutes*, *Fusobacteria*, *Synergistetes* and *Elusimicrobia*). The Gram-negative bacterial phyla with an LPS-diderm cell envelope, as defined by the presence of the Hsp60 insert, are indicated to form a monophyletic clade and no loss of the outer membrane from any species from this group seems to have occurred. This argues against the origin of monoderm prokaryotes from diderm bacteria by loss of outer membrane.

Keywords Bacterial cell envelopes · Origin of the outer cell membrane · Gram-negative bacteria · Lipopolysaccharides · Monoderm and diderm bacteria · Endosymbiotic hypothesis · Antibiotic selection pressure · Conserved inserts in Hsp60 and Hsp70 proteins · Simple monoderms

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Introduction—complexity of the bacterial cell envelopes

The prokaryotic organisms are traditionally divided into two main groups i.e. Gram-positive and Gram-negative, based on their Gram-stain retention characteristics (Gram 1884; Stanier et al. 1976). Although the Gram-staining has not proven to be a reliable criterion for the higher-level division or classification of prokaryotic organisms, a more important structural characteristic that generally distinguishes these two types of organisms is the nature of their cell envelopes (Stanier et al. 1976; Murray 1986). Most Gram-positive bacteria *are bounded by a single cell membrane* and they generally contain a relatively thick peptidoglycan layer that is responsible for retaining the Gram-stain. In contrast most "true" Gram-negative bacteria are surrounded by two different cell membranes and they contain only a thin peptidoglycan layer in the *periplasmic compartment* that is bounded by the inner and outer membranes (Stanier et al. 1976; Murray 1986; Truper and Schleifer 1992; Gupta 1998b; Sutcliffe 2010). Although these differences in the cell envelope characteristics of Gram-positive and Gram-negative bacteria have long been known, due to the variability of Gram-staining response and polyphyletic branching of these two groups of bacteria in the 16S rRNA gene and other phylogenetic trees (Olsen and Woese 1993; Ludwig and Klenk 2005), the possibility that the cells with two membranes (diderm bacteria) might be phylogenetically distinct from monoderm prokaryotes was not recognized until 1998. This recognition came from the identification of a 21–23 aa long conserved insert in the Hsp70 family of protein that was uniquely shared by different phyla of diderm bacteria but absent in all other prokaryotes including Archaea (Gupta and Singh 1994; Gupta 1998b, c). The absence of this indel in bacterial lineages such as *Mycoplasma* that stained Gram-negative (as they lack the peptidoglycan layer) but contained a single membrane, provided evidence that the presence or absence of the outer cell membrane, rather than the Gram-staining response constituted a useful phylogenetic characteristic (Gupta 1998b, 2000; Cavalier-Smith, 2002).

The division of prokaryotic organisms into two distinct groups viz. "monoderms" and "diderms" based upon the presence or absence of the outer membrane and the large insert in the Hsp70 protein (Gupta 1998a, b, c) led to the question regarding which of these two lineages was ancestral and which was derived. Insights into this question was provided by our observation that Hsp70 and another protein, MreB, which is also present in different prokaryotic lineages, have evolved from an ancient gene duplication in the common ancestor of prokaryotes (Gupta and Golding 1993; Gupta 1998b). Thus, the presence or absence of the insert in the MreB protein could be used to determine whether the indel in the Hsp70 protein is an insert or a deletion. Because the MreB protein from different lineages, similar to the Hsp70 from monoderm prokaryotes, did not contain this indel, the absence of the indel was inferred to be the ancestral character state of the Hsp70 protein (Gupta and Golding 1993; Gupta 1998b). Thus, the large indel in the Hsp70 protein was an insert that occurred in an ancestor of the diderm bacteria (Gupta 1998b, c). This observation and a number of other observations (reviewed in Gupta 1998b) indicated that the cells with one membrane are ancestral and that the cells with two membranes originated from them (Gupta and Golding 1993; Gupta 1998b; Koch 2003). A subsequent study by Lake also supported this inference (Lake et al. 2007). Some authors have suggested that cells with two membranes evolved prior to those with one membrane (Cavalier-Smith 2006; Griffiths 2007; Valas and Bourne 2009). However, Valas and Bourne place the root of the prokaryotic tree in the *Chloroflexi*, which are now indicated to have a monoderm rather than a diderm cell envelope (Valas and Bourne 2009; Sutcliffe 2010, 2011). It is also difficult to conceive of any simple model where a cell with both an inner and outer membrane can directly evolve without the initial development of a cell with only a single membrane.

Although the monoderm or diderm cell structures as exemplified by the model organisms *Bacillus subtilis* and *Escherichia coli* are the most common types of cell envelopes present within the bacterial domain, several bacterial taxa are now known that contain atypical outer cell envelopes (or layers) that do not correspond to these model organisms (Sutcliffe 2010, 2011). Sutcliffe (2010) has recently reviewed the work on this subject and it illustrates

that the distinction between the monoderm and diderm cell structures is not clear-cut and that the observed differences are important in terms of understanding the origin of the outer cell membrane. For example, the bacteria belonging to the order *Corynebacterineae* (phylum Actinobacteria), although widely considered as monoderms, have an outer lipid layer composed of mycolic acid molecules which are arranged in a highly ordered form resembling an outer membrane (Brennan and Nikaido 1995; Sutcliffe 2010). Similarly, the bacteria belonging to the phylum *Thermotogae* contain an outer toga (envelope) consisting primarily of proteins rather than lipids (Reysenbach 2001). Although some characteristics of genes/proteins found in the *Thermotogae* genomes indicate that they are capable of synthesizing lipids and transporting them to the outer envelope (Sutcliffe 2010), their outer envelope is clearly distinct from all other monoderm and diderm bacteria. Several other phyla of bacteria that are considered as diderms (viz. *Chloroflexi, Deinococcus-Thermus*) lack LPS (Sutcliffe 2010), which is considered to be a defining characteristic of the archetypal outer cell membrane. Sutcliffe has also presented strong arguments that *Chloroflexi*, which are widely believed to have a diderm cell envelope, are monoderm (Sutcliffe 2011). They lack LPS (as well the genes for various key proteins involved in the synthesis of LPS) and proteins characteristic of outer membranes, such BamA family proteins and outer membrane components of secretion systems. Further, the cell envelopes of some of these species are indicated to be multi-layered with no evidence of lipids in the outer cell layer (Hanada and Pierson 2006). Hence, the outer layer in some *Chloroflexi* could be composed of polysaccharides or proteins (Sutcliffe 2011). These observations point to the complexity of the bacterial outer membrane structure and indicate that the distinction between monoderm and diderm cell envelopes is not quite simple or straightforward, at least by biochemical means. These observations are important in evaluating any model or hypothesis for the origin of the outer cell membrane. Moreover, it should be taken into account that the mycolic acid based outer membranes of the order *Corynebacterineae* (phylum Actinobacteria) provide evidence that lipid outer membranes have evolved more than once.

Have diderm bacteria originated via endosymbiosis?—critical evaluation of Lake's hypothesis and data

Lake has recently proposed that cells with two membranes are the result of an ancient endosymbiotic event involving two monoderm bacteria belonging to the phyla/taxa *Actinobacteria* and *Clostridia* (Lake 2009). He reached this inference based upon the presence or absence of a given dataset of proteins in different groups of bacteria. For his analyses, Lake made an important assumption that all prokaryotic organisms belong to one of the five *natural and phylogenetically well separated groups* viz. i.e. Diderm bacteria (D), *Actinobacteria* (A), Archaea (R), *Bacillus* and relatives (B) and *Clostridia* and relatives (C). Of these five groups, the distinctness of *Archaea*, and more recently *Actinobacteria*, is established based upon large numbers of molecular characteristics, including many signature proteins and conserved indels that are uniquely found in all species from these taxa (Olsen and Woese 1997; Gao and Gupta 2005; Walsh and Doolittle 2005; Gao et al. 2006; Gao and Gupta 2007; Gupta and Shami 2011). The clade D corresponding to diderm bacteria as defined by Lake contains different bacterial phyla including *Thermotogae, Fusobacteria, Deinococcus-Thermus* and *Chloroflexi*, whose outer cell envelopes are atypical and differ in important respects from archetypical bacterial cell with two membranes (Sutcliffe 2010). Significantly, there is no evidence from any source that different bacterial phyla that Lake places in Clade D form a monophyletic lineage. The presence of the large insert in the Hsp70 protein is indicated to be a marker that can distinguish monoderm and diderm prokaryotes (Gupta 1998b); however, this insert is not found in *Thermotogae* and *Fusobacteria* (Gupta 1998b; Singh and Gupta 2009) but is found in the monoderm *Chloroflexi* (see above). Phylogenetic studies on *Fusobacteria* indicate that they are more closely related to *Clostridia* than to any of the diderm bacteria (Griffiths and Gupta 2004; Mira et al. 2004; Karpathy et al. 2007). Therefore, the clade D as defined by Lake does not constitute a monophyletic group based upon either morphological or phylogenetic considerations, which is an essential requirement for analysis of this nature. Further, this clade also includes majority of the known bacterial phyla (including *Chloroflexi* which

are now indicated to be monoderms; Sutcliffe 2011) and the representation of this heterogeneous group by a single entity, as Lake has done, can lead to misleading results.

The other two proposed main taxa, B and C, are presently part of the phylum *Firmicutes* (Ludwig and Klenk 2005). This phylum is poorly characterized phylogenetically and no biochemical or molecular marker is known that is uniquely shared by all *Firmicutes* species. The division of this phylum into the two main prokaryotic taxa, B and C, which according to Lake are naturally and phylogenetically clearly separated, is not accurate and no evidence is presented to support that they form monophyletic lineages. Within the *Firmicutes*, the *Clostridia* species (taxa C) in particular are a very heterogeneous assemblage and it has proven difficult to circumscribe this clade by phylogenetic or any other means (Wiegel et al. 2006). Recently, several bacterial species that were previously part of the Class *Clostridia* have been placed in a separate phylum, the *Synergistetes* (Jumas-Bilak et al. 2009; Hugenholtz et al. 2009). The species from this phylum, similar to *Fusobacteria*, contain two membranes and also genes for the key LPS biosynthetic enzymes (Baena et al. 1998; Jumas-Bilak et al. 2009; Sutcliffe 2010). Moreover, the situation is further complicated by the recent delineation of the Class *Negativicutes* within the phylum *Firmicutes* (Marchandin et al. 2010), as many representatives of this apparently have outer membranes containing LPS (Sutcliffe 2010). Thus, if the Clade D is defined on the basis of presence of two cell membranes then these taxa should have been part of Clade D rather than Clade C. Therefore, the division of the prokaryotes into the 5 main groups as defined by Lake (2009), on which his entire analysis was based, was based on completely arbitrary considerations and it has no valid phylogenetic, taxonomic or morphological/biochemical basis.

Another serious problem with Lake's hypothesis (Lake 2009) relates to the quality and accuracy of the data on which his hypothesis was based. Lake examined the presence or absence of proteins from different families into the five proposed taxa and based on these results reached the conclusion that a tree like topology was not supported by the character states of many proteins and that their distribution can only be explained by a ring-like structure involving origin of taxon D by merger of taxa A and C. However, Lake provided no information how widely these proteins were distributed in different groups. To obtain information in this regard, I carried out Blast searches on 24 proteins corresponding to the first, third and fourth row in Table 1 of Lake's paper (Lake 2009). The results of these analyses, along with those reported by Lake for the same proteins, are presented in Table 1. The character states for these proteins as reported by Lake are also shown in the Table 1. Very surprisingly, the species distribution patterns (or character states) for most of these proteins were very different from those reported by Lake. For example, of the first three proteins in Table 1, which according to Lake supported the pattern [R (+), A (+), B (+), C (−) and D (−)], the first two were present in large numbers of *Clostridia* (C) as well as several diderm bacteria (D). The third protein (PTH2) was found to be largely specific for *Actinobacteria* (A) and only 4 hits for *Archaea* (R) and 1 hit for *Bacillus* (B), which are barely significant, were observed. Similar major discrepancies were noted for the 15 proteins that were reported to exhibit the pattern [R (−), A (+), B (+), C (+) and D (−)]. For two of these proteins MecA_N and RsbU_N, large numbers of hits from A, B, C and D were observed; For the proteins Cas_Csm6 and DUF624, all significant hits were from the *Bacillus* group (B); For two other proteins (Omega-Repress and SASP), only 1–3 hits from A were observed, but a similar number of hits were also seen for D (Table 1). For Lactococcin 972, no significant hit for C was observed. Of the 15 proteins in this category, only 5 proteins (DUF1048, DUF939, Etx_Mtx2, L.biotic_A and Phage_holin), at best, indicated the pattern noted by Lake (Lake, 2009). However, for three of these proteins, the total numbers of significant hits from all groups were in the range of 15–17 (including many hits for the same species) and for all 5 of these proteins very few hits were observed from the clade A and C species indicating that their species distribution was extremely limited and they do not provide reliable characteristics. Additionally, for 4 of the 15 proteins in this category, many significant hits were from bacteriophages, indicating that lateral gene transfer for these proteins should be common (Gogarten and Townsend 2005) and their species distribution patterns would not be reliable. Similar discrepancies between the observed and reported patterns were seen for 8 other proteins, which according to Lake supported the pattern [R (+), A

Table 1 Distribution patterns of various protein families in the indicated taxa

Protein name	Accession no.	Species distribution or character state pattern									
		Reported by Lake					Observed distribution pattern				
		R	A	B	C	D	R	A	B	C	D
DUF567	PF04525	+	+	+	–	–	+3	+>20	+>50	+>20	+3
FR47	PF08445	+	+	+	–	–	+15*	+>50	+>50	+>10	+>50
PTH2	PF01981	+	+	+	–	–	+4*	+>50	+1*	–	–
Cas_Csm6	YP_82039[a]	–	+	+	+	–	–	–	+5	–	–
DUF1048	PF06304	–	+	+	+	–	–	+7*	+>50	+9*	–
DUF348	PF03990[#]	–	+	+	+	–	–	+>50	+>50	+>50	4
DUF624	PF04854	–	+	+	+	–	–	–	+>50	–	–
DUF939	PF06081	–	+	+	+	–	–	+7	+>50	+11*	–
Etx_Mtx2	PF03318	–	+	+	+	–	–	+1	+11*	+7	–
G5	PF07501[#]	–	+	+	+	–	–	+>50	+>50	+>50	+4*
L.biotic_A	PF04604	–	+	+	+	–	–	+1	+12	+2	–
Lactococcin	PF09683	–	+	+	+	–	–	+5	+13*	–	–
MecA_N	PF05223	–	+	+	+	–	–	+>50	+>50	+>50	+>50
Omega Rep	PF07764	–	+	+	+	–	–	+1	+28*	+2	+3
Phage-holin	PF04688[#]	–	+	+	+	–	–	+3	+50*	+2	–
Phage_min2	PF06152[#]	–	+	+	+	–	–	+8*	+46*	+14*	+1*
RsbU_N	PF08673	–	+	+	+	–	+6	+>50	+>50	+16*	+>50
SASP	PF00269	–	+	+	+	–	–	+1	+>50	+>50	+2
DUF1002	PF06207	+	–	+	+	–	+2*	–	+>50	+>50	–
DUF1338	PF07142	+	–	+	+	–	–	–	+>50	+17*	–
DUF1646	PF07854	+	–	+	+	–	+12*	–	+2	+15*	–
DUF964	PF06133	+	–	+	+	–	–	–	+>50	+16*	–
DUF988	PF06177	+	–	+	+	–	+4	+7*	+>50	+>50	+5
Hth_MGA	PF08280	+	–	+	+	–	–	–	+>50	+1*	+1*
UPF0154	PF03672	+	–	+	+	–	–	–	+>50	+7*	–
YcH	PF07435	+	–	+	+	–	–	–	+>50	–	–

The proteins in this Table correspond to those described by Lake (2009) in his Supplementary Tables S2H (first 3 protein), S2J (next 15 proteins) and S2C (last 8 proteins). Blastp searches on these proteins were conducted using the default parameters and information for the first 500 hits (or maximum number of hits observed, if this number was <500) was obtained and a lineage report of these hits, which indicate both the Blast scores as well as their taxonomic affiliation was generated (a feature of the Blast program). This table lists all of the hits with Blast score of 40 or more. A blast score of 40 generally corresponds to Expect (or E) value of 0.1 or higher and in most cases indicates very weak or no significant sequence similarity between the query protein and the observed hit

* Indicate that several of the observed hits have blast score in the range of 40–45, which may or may not be significant. However, the exclusion of these hits or using a higher blast score (viz. 45 or 50) as a criterion for significant hits does not qualitatively change the nature of the observed results. The complete results of Blast analyses for these proteins (performed in October 2009) and the taxonomic lineages of different hits are provided in the Supplemental file

[a] The indicated accession number (PF09659) was not correct. Hence, blast searches were carried out with the top entry (accession number is indicated) using the protein name

[#] Many significant hits for bacteriophages were observed for these proteins

(−), B (+), C (+) and D (−)]. Although, comprehensive analyses have not been conducted on all of the proteins that were analyzed by Lake, the results for the 26 proteins presented in Table 1, which correspond to three of the important character states, raise serious concerns regarding the quality and

accuracy of the data that was used to infer that the cells with two membranes (clade D) evolved by a merger of taxa A and C.

In addition to these important concerns regarding the critical assumptions on which Lake's analysis was based and the accuracy of his data, the endosymbiotic origin of diderm bacteria by merger of an *Actinobacteria* and *Clostridia* is also not supported by several other important observations. First, in all established cases of endosymbiosis (viz. origin of mitochondria from *Alphaproteobacteria*, or origin of plastids from *Cyanobacteria*) (Margulis 1993), numerous genes that are distinctive characteristics of the original endosymbiont(s) are commonly retained by *all* of the derived organisms (Gray 1999). Thus, all plants and photosynthetic eukaryotes contain numerous genes and other characteristics that they uniquely share with cyanobacteria (Gupta et al. 2003; Mulkidjanian et al. 2006; Gupta and Mathews 2010). Similarly, all eukaryotic organisms, without any exceptions, contain notable fractions of their genes that are derived from either alpha proteobacteria or archaeal ancestors (Gupta 1998b; Rivera and Lake 2004). However, for the two prokaryotic taxa, *Actinobacteria* and *Clostridia*, whose merger is postulated to have given rise to the diderm bacteria, no unique molecular or other characteristics have been identified that are commonly shared by all or most species from either taxa A and D or by taxa C and D (Gao et al. 2006; Gupta and Gao 2009), which are expected to be very common patterns if the mergers of the taxa A and C gave rise to the taxon D.

Bacterial lineages that might be intermediates in the monoderm-diderm transition

Although the distinction between the monoderm and diderm prokaryotes is very meaningful, it does not represent a major evolutionary transition, such as that seen between prokaryotes and eukaryotes (Szathmary and Smith 1995; Margulis 1996; Mayr 1998; Gupta 1998b). Unlike the latter transition, where no clear intermediates are found, a number of bacterial groups could represent possible intermediates in the transition from monoderm to diderm bacteria. As noted earlier, some bacterial phyla such as *Deinococcus-Thermus* and *Thermotogae*, although they contain some features of the diderm bacteria, they lack LPS which is considered to be a defining characteristic of the archetypical diderm or Gram-negative bacteria (Sutcliffe 2010). In the case of *Deinococcus* species although they contain an outer membrane, they also possess a thick peptidoglycan layer ($\sim$50 nm) and stain Gram-positive similar to various monoderm bacteria (Murray 1992; Gupta 1998b). This observation indicates that in the transition from monoderm to diderm bacteria the outer membrane likely evolved first and this was followed by a reduction in the thickness of the peptidoglycan layer (Gupta 1998b, 2000). The biochemical, structural and phylogenetic characteristics of *Deinococcus-Thermus* taxa indicate that the cell envelope in them may represent an intermediate stage in the development of archetypical diderm cell envelope that is characteristic of the traditional Gram-negative phyla. I will refer to this bacterial group lacking LPS and containing some features of the diderm bacteria as "Simple Diderms" in contrast to the LPS-containing archetypical diderm bacteria. The cell envelopes of Thermotogae species may represent an alternate attempt to develop an outer cell membrane. In addition to the above taxa that contain some features of the diderm-prokaryotes, recent work has revealed that a number of bacterial phyla that are either part of the *Firmicutes* phylum or branch in its proximity (viz. *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia*) also contain an outer membrane and the genomes of these species contain genes encoding for LPS biosynthesis (Mira et al. 2004; Karpathy et al. 2007; Herlemann et al. 2009; Sutcliffe 2010). Because these bacterial phyla are distantly related to the other phyla of traditional Gram-negative bacteria, the relationships of the outer cell envelopes in these two groups is presently unclear (see below).

Two conserved inserts that are present in the Hsp70 and Hsp60 proteins provide important insights into the development of outer cell envelopes in bacterial groups. The large insert in the Hsp70 protein that was referred to earlier is a shared characteristic of all bacterial phyla that are traditionally considered to be Gram-negative including the *Chloroflexi* (likely monoderm, see above) and *Deinococcus-Thermus* (Gupta 1998b; Singh and Gupta 2009). However, except for isolated exceptions, this insert is absent from virtually all *Actinobacteria, Firmicutes* (including *Negativicutes*), *Thermotogae*, *Fusobacteria* and *Synergistetes* (as well as *Elusimicrobium*) (Gupta

1998b; Griffiths and Gupta 2004; Singh and Gupta 2009) (unpublished results). The rare genetic change responsible for this conserved insert was introduced at a very early stage in the evolution of diderm bacteria. This insert provides evidence that the bacterial groups such as *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia* that contain an outer cell envelope with LPS are distantly related to the traditional phyla of Gram-negative (diderm) bacteria. Besides the Hsp70 insert, another conserved insert that we have identified in the Hsp60 protein is uniquely present in different phyla of traditional Gram-negative bacteria whose outer cell envelopes contain LPS but it is not found in *Deinococcus-Thermus, Chloroflexi* as well as the above noted phyla of bacteria (Fig. 1). This insert, in addition to further confirming that *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia* are distantly related to the traditional Gram-negative bacteria, also provides evidence that the *Chloroflexi* and *Deinococcus-Thermus* branched prior to all of the phyla of traditional Gram-negative bacteria. Thus, based upon the species distribution patterns of the Hsp70 and Hsp60 inserts, it is possible to infer that the phyla consisting of *Chloroflexi* and *Deinococcus-Thermus* species branched immediately prior to the clade consisting of different phyla of traditional Gram-negative bacteria (Fig. 2). It should be noted that the conserved insert in the Hsp60 protein is a unique and distinctive property of different species from various phyla of traditional LPS-containing Gram-negative bacteria and this insert provides a reliable molecular marker to identify and circumscribe this clade in molecular terms (Fig. 2). Although the cellular function of this conserved insert is not known, our recent work shows that it is essential for the group of species where it is found as deletion of this insert or any significant changes in it leads to the failure of cell growth (Singh and Gupta 2009).

The bacterial groups consisting of *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia* that are also indicated to have an outer membrane with LPS are distinguished from the traditional phyla of Gram-negative bacteria by the absence of the insert in the Hsp60 protein (Fig. 1). It has been reported that *Synergistetes* species, although they contain an outer membrane, lack the genes for the TolAQR-Pal complex that is required for assembly and maintenance of outer membranes (Hugenholtz et al. 2009). Hence, the nature and the role of the outer membrane in these species could be different from the traditional phyla of Gram-negative bacteria defined by the presence of the Hsp60 insert. Hence, I will refer to these taxa of bacteria as “Atypical diderms” to distinguish them from “Traditional or archetypal” Gram-negative diderm bacteria.

Antibiotic selection pressure as a driving force for the evolution of diderm bacteria

The question can be asked what selective forces were responsible for the evolution of diderm bacteria from monoderm bacteria. Lake speculates that the acquisition of photosynthetic ability from *Clostridia* may have been important in this regard (Lake 2009). However, photosynthetic ability within the *Clostridia* (phylum *Firmicutes*) is only found within a single family *Heliobacteriaceae* that contains a total of 7 species (Madigan 1992). Of these, the genome of *Heliobacterium modesticaldum* has been sequenced and the different genes/proteins from it show no specific affiliation to the diderm bacteria (Sattley et al. 2008). Additionally, photosynthetic ability within diderm bacteria is found in only 4 of the more than 20 phyla (Blankenship and Hartman 1998; Gupta 2003), which argues against it being the main selective force for the development of outer membrane. In contrast to Lake’s proposal, I have suggested that the outer membrane of diderm bacteria has evolved as a defense mechanism in response to the evolution of antibiotic selection pressures (Gupta 1998b, 2000). The main arguments in support of this view are as follow: (i) The monoderm bacteria, which include *Streptomyces*, are the main producers of most of the known antibiotics (Davies 1994; Wright 2007); (ii) The production of antibiotics by some organisms gives them tremendous selective advantage over non-producing or antibiotic-sensitive bacteria (Cavalier-Smith 1992; Davies 1994); (iii) Resistance to antibiotics can develop by a variety of mechanisms including: changes in their target genes; inactivation of antibiotics by different enzymes; reducing antibiotic entry into cells by different mechanisms; and expulsion of the antibiotics by drug efflux pumps. (Nikaido 1989; Davies 1994; Spratt 1994; Wright 2007); (iv) Gram-positive bacteria in general display higher sensitivity to antibiotic than Gram-negative bacteria (Nikaido 1989; Spratt 1994).

Group	Species	Accession	144		178
Gamma-Proteo (>500/0)	Escherichia coli	P06139	IAQVGTISA	N	SDETVGKLIAEAMDKVGKEGVITVE
	Pseudomonas aeruginosa	P30718	---------	-	---SI-QI-----E----------
	Legionella pneumophila	YP_094724	---------	-	---AI-AI-----E----------
	Xylella fastidiosa	AE003849	----A----	-	---SI-NI-----K--------I-
Beta-Proteo (>180/0)	Neisseria meningitidis	P42385	-----S---	-	---Q--AI-----E----------
	Ralstonia solanacearum	17545361	-----A---	-	---SI-AR----------------
Alpha-Proteo (>300/0)	Rickettsia prowazekii	3861167	--------S	-	G-KEI-EK--K--EE---------
	Caulobacter crescentus	P48211	---------	-	G-KE--EM--K------N------
	Brucella abortus	P25967	V--------	-	AEAEI--M-----Q--N-------
Delta-Epsilon Proteo (>150/0)	Geobacter metallireducens	ZP_00082424	---------	-	N-K-I-DI--Q--E----------
	Desulfovibrio vulgaris	ZP_00129431	---------	-	--S-I-NI-----S----------
	Campylobacter jejuni	e1287421	----A----	-	---KI-N---D--E----D-----
	Helicobacter pylori	P42383	-T--A----	-	--HNI-----D--E----D-----
Aquificae (9/0)	Aquifex aeolicus	2984379	-E--A----	-	N-PEI--I--D--EE---D-----
	Hydrogenivirga sp.128-5	ZP_02176593	-E--AA---	-	N-PEI--I--D--EA---D-----
Chlamydiae-Verrucomicrobiae-Planctomycetes (>30/0)	Chlamydia muridarum	AAF39243	----A----	-	N-AEI-N------E----N-S---
	Protochlamydia amoebophila	YP_0081791	----A----	-	N-AEI-EM--Q-IE---RD-T---
	Lentisphaera araneosa	ZP_01875439	VK-IA----	-	G--EI-SI--D-------D-T-A--
	Verruco. spinosum	ZP_02926970	----A-V--	-	W-TEI-NI--D-------D-T---
	Planctomyces maris	ZP_01855423	-----A---	-	N-SVI-N---D-VE---RD-----
	Blastopirellula marina	ZP_01094293	V-H--A---	-	N-HAI-E-L---LYR---D-----
Acidobacteria, Chrysiogenetes, Nitrospira (8/0)	Acidobacterium capsulatum	225872384	---------	-	--A-I-TI-----K----D-----
	Terriglobus saanensis	320107401	---------	-	--AQI-TI-----K----D-----
	Desulfurispirillum indicum	317050928	---------	-	----I-EIL----E----D----I-
	Thermodesul. yellowstonii	206889461	---------	-	N-VSI-E-----------D-----
	Candidatus Nitrospira	302036732	-S-------	-	N-K-I-D------E----D-----
Gemmatimonadates, Dictyglomi and Deferribacteres (5/0)	Gemmatimonas aurantiaca	226226942	---------	-	N-PEI-N------E----D-----
	Dictyoglomus thermophilum	206900291	--H-AA---	-	N-TEI-N-----------D-----
	Denitrovibrio acetiphilus	291288717	-S---A---	-	N-QEI-DI--D-------D----I-
Bacteroides-Chlorobi-Fibrobacter (>130/0)	Chlorobium tepidum	AAM71772	---------	-	N-PEI-E-----------D-----
	Fibrobacter succinogenes	AY017380	----A----	-	N-PEI-E-L-N--E---ND----I-
	Cytophaga aquatilis	AF335327	-K-IAS---	-	N--VI-E---T-FA----------
	Bacteroides forsythus	CAB43992	-EH-AK---	-	G--GI--------Q--K--------
Spirochaetes (19/0)	Borrelia burgdorferi	P27575	----AS---	-	N-SYI-EK----------D-----
	Treponema pallidum	AAC65026	V-H-ASV--	-	N-KEI-RIL-S-IE---ND---D-D
	Leptospira interrogans	L14682	--N-AS---	-	N-N-I-N---D-------D-----
Cyanobacteria (>90/0)	Synechocystis sp. PCC6803	2506274	-----A---	G	N--E--QM--N------Q----SL-
	Nostoc punctiforme	17132795	-----A---	G	N--E--QM--Q-----------SL-
	Thermosyn. elongatus	11131866	----AA---	G	N--E--RM--D-----------SL-
Chloroflexi (0/10)	Chloroflexus aurantiacus	AAD33468	--H-A-N--		A-SEI-E----V-A----D-----
	Roseiflexus castenholzii	YP_001431326	--H-AA---		A-SEI-D----V-E----D-----
	Herpetosiphon aurantiacus	YP_001546443	-SS-A----		A-SSI-D----V------D-----
Deinococcus-Thermus (1/9)	Deinococcus radiodurans	G75499	-KK-AG---		N-----QE--S-----------I-
	Deinococcus grandis	AAS10491	-KK-AG---		N--Q--QE--S-----------I-
	Thermus aquaticus	P45746	-EE-A----		N-PE------D--E------I---
Thermotogae (0/9)	Thermotoga maritima	H72367	--H-AA---		NSAEI-E----------ED-----
	Fervidobacterium nodosum	YP_001411043	--H-AS---		NS-EI--------E---ED-----
	Petrotoga mobilis	YP_001568199	--H-AS---		NN-EI-NI---------ED-----
Actinobacteria (2/>150)	Mycobacterium leprae	P09239	--ATAA---		G-QSI-D----------N------
	Corynebacterium glutamicum	19553910	--N-A-V-S		R--V--EIV-A--E----D--V---
	Bifidobacterium longum	24850323	--A-A----		A-PE--EK----L----QD--V---
	Streptomyces coelicolor	8248795	--STAS---		A-TQI-E-----------------
	Arthrobacter ureafaciens	AAY233611	--ATAS---		G-QEI-A-----L-----------
Fusobacteria (0/23)	Fusobacterium nucleatum	ZP_02273152	-S--AS---		G--EI-----Q--A---ET-----
	Ilyobacter polytropus	310779165	----AS---		---EI-A------Q---ET-----
Synergistetes, Elusimicrobia (0/9)	Aminomonas paucivorans	312880436	----AS---		N-KR--E---------TED-----
	Therma. acidaminovorans	269792840	----AS---		N-KRI-E---------TED-----
	Aminobacterium colombiense	294101583	----AA---		N-SGI-Q------E---QD-----
	Elusimicrobium minutum	187251332	--IA----		N-RM--E------E---H------
Negativicutes (0/15)	Selenomonas ruminantium	51103893	----A---S		AN-ET-E------E----D-----
	Veillonella parvula	282850322	----ASV--		A--EI-G------E---ND-----
Firmicutes (6>250)	Clostridium perfringens	P26821	--R-AA---		A--KI-----D--E---N------
	Staphylococcus aureus	15927604	-----A---		A--EI-RY-S---E---ND----I-
	Bacillus subtilis	16077670	----AA---		A--E--S------ER--ND----I-
	Streptococcus pneumoniae	2545870	----AAV-S		RS-K--EY-S---E----D----I-
	Mycoplasma genitalium	12045254	-E--AA--S		GSKEI-----Q--AL---N----TD

◀ **Fig. 1** Partial sequence alignment of the Hsp60 protein showing a 1 aa insert (*boxed*) in a conserved region that is mainly specific for different bacterial phyla corresponding to traditional Gram-negative bacteria that have an outer cell membrane containing lipopolysaccharide. The presence or absence of this insert in all available sequences from different bacterial groups is indicated along with their names. For example, for Gamma-proteobacteria >500 hits corresponding to Hsp60 were observed and all of them contained this insert (i.e. >500 with insert, 0 without insert). Similarly, for the Actinobacteria phylum, >150 hits were observed and of these only 2 contained the insert (2/>150). Only representative sequences from different bacterial phyla are shown here. The absence of this insert in the *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia* distinguishes these atypical diderm taxa from all of the phyla of traditional Gram-negative bacteria that contain this insert. The dashes in the alignment indicate that the same amino acid as that found on the top line (i.e. *E. coli* protein) is present in that position. The accession numbers of sequences are given in the second column. The numbers on the top indicate the position of this sequence in *E. coli* protein

Based upon these observations, it is easy to conjecture that early in the evolutionary history of microbes when one group of Gram-positive bacteria (viz. *Streptomyces*) developed mechanisms to produce antibiotics, survival of most of the other bacteria that were sensitive to these antibiotics was at stake. To survive in this strongly selective environment, sensitive bacteria evolved a number of strategies to protect themselves from the cytotoxic effects of these antibiotics (see Fig. 2) (Spratt 1994; Gupta 2000). One of these strategies that was likely employed by Archaea was to mutationally change the target sites of different antibiotics, as various processes that are normally inhibited by antibiotics such as protein synthesis, RNA synthesis and cell wall biosynthesis are resistant to their effects in Archaea (Gupta 1998b, 1998c, 2000). The emergence of Archaea from Gram-positive bacteria in response to antibiotic selection pressure is also supported by a recent detailed study by Valas and Bourne (2011). Another important strategy to escape from the effects of antibiotics was to develop an outer protective layer (membrane) that would retard the entry of antibiotics into the cells (Nikaido 1989; Gupta 2000). In Gram-negative bacteria, many enzymes involved in the inactivation of antibiotics are localized in the periplasmic compartment (or intermembrane space), which further aids in antibiotic resistance (Nikaido 1989, 2003; Davies 1994; Spratt 1994). This strategy was independently employed by a number of bacterial groups leading to development of outer envelopes of differing biochemical properties (Fig. 2). Thus, the layered outer cell envelopes of the *Chloroflexi* and the diverse diderm cell envelopes of the *Corynebacterineae, Thermotogae*, *Deinococcus-Thermus, Negativicutes, Fusobacteria, Synergistetes* and *Elusimicrobia* could represent various attempts of developing an outer protective barrier (Fig. 2). Moreover, the outer cell envelopes in some of these lineages (viz. *Deinococcus-Thermus* and *Chloroflexi*; *Negativicutes, Fusobacteria, Synergistetes* and *Elusimicrobia*) could also be related or derived from each other. The absence of the Hsp70 insert in *Thermotogae*, *Corynebacterineae, Negativicutes, Synergistetes, Fusobacteria* and *Elusimicrobia* indicates that the outer cell membranes or layers in these taxa represent earlier stages (or alternative attempts) to develop a protective barrier in comparison to the outer envelopes of *Chloroflexi* and *Deinococcus-Thermus*, which have the Hsp70 insert. Of these different evolutionary experiments to develop an outer cell envelope, the structural characteristics of the outer cell envelope in *Deinococcus-Thermus* were apparently most successful and this lineage led to the eventual development of the archetypal diderm membranes that are found in different phyla of traditional Gram-negative bacteria (Fig. 2).

As the *Deinococcus-Thermus* species lack the genes for LPS biosynthesis, it can be hypothesised that the subsequent evolution of these genes in either some species from this group or a closely related bacterium led to the development of an archetypical LPS-containing outer cell envelope characteristic of various traditional Gram-negative phyla. This development and other changes that accompanied the evolution of this new diderm-LPS cell were apparently evolutionarily highly successful as it led to the emergence of much of the microbial diversity (i.e. majority of the bacterial phyla) that is seen today (Ludwig and Klenk 2005; Sutcliffe 2010). It is important to note that the evolution of this archetypical LPS-containing diderm cell envelope, whose presence shows excellent correlation with the presence of the insert in the Hsp60 protein (see Fig. 1 and Table 1 in Sutcliffe 2010), was an important and apparently irreversible evolutionary development, as none of the species from this clade defined by the Hsp60 insert have lost the outer membrane. The fact that the outer cell membrane has not been lost from

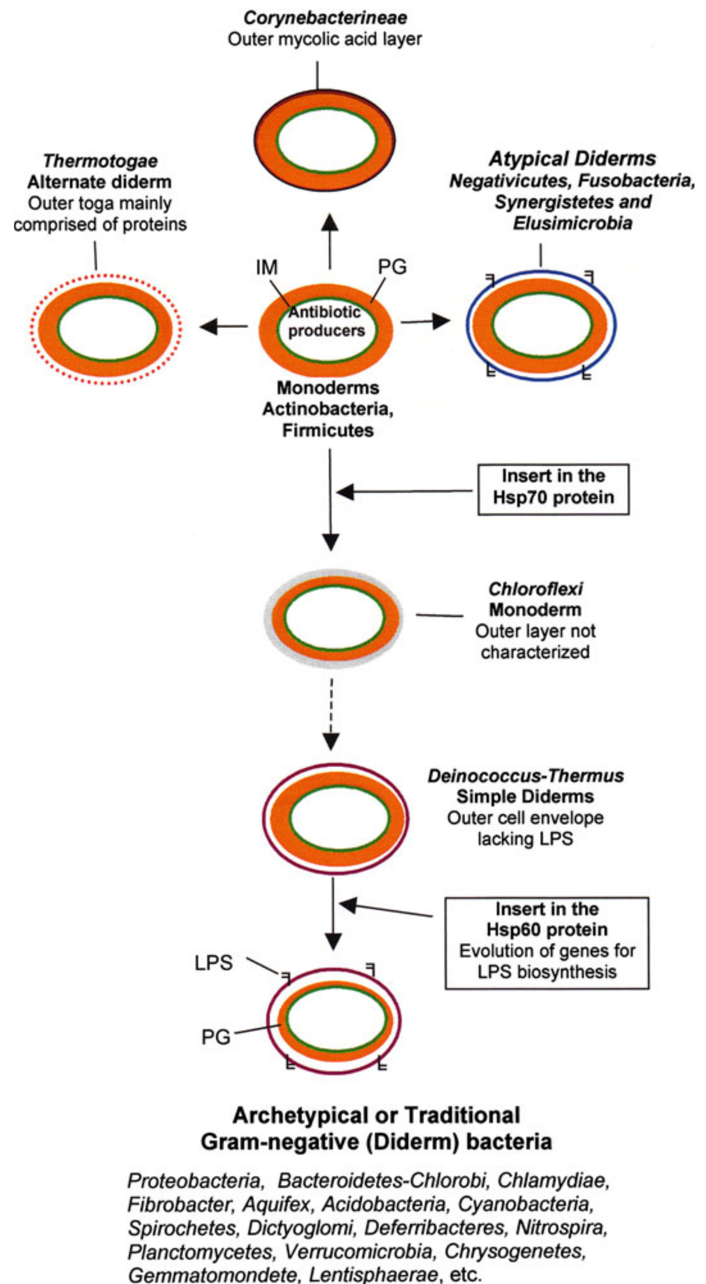

Fig. 2 A cartoon showing the development of outer cell envelopes in various bacterial lineages in response to antibiotic selection pressure (Gupta 2000). The outer cell envelope in *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia* (atypical diderm taxa) is distinguished from traditional diderm Gram-negative bacteria by the absence of the Hsp60 insert. The cell membrane from atypical and traditional Gram-negative bacteria are postulated to show significant differences in their biochemical and functional characteristics. The outer cell envelopes of the archetypical Gram-negative phyla are indicated to have evolved from the *Chloroflexi* and *Deinococcus-Thermus* groups of species. Information regarding species distribution of Hsp70 inserts for most bacterial phyla is provided in earlier work (Griffiths and Gupta 2004; Lake et al. 2007; Singh and Gupta 2009). Abbreviations: *PG* peptidoglycan, *IM* inner membrane, *LPS* lipopolysaccharides

any of the >1000 of species that are part of the archetypical diderm clade encompassing the majority of bacterial phyla (Fig. 1), also argues strongly against the origin of monoderm prokaryotes from diderm bacteria by the loss of outer membrane and the hypothesis that the cells with two membranes evolved prior to those with one membrane (Cavalier-Smith 2006; Griffiths 2007; Valas and Bourne 2009).

The possible relationship of the taxa consisting of atypical diderms (viz. *Negativicutes, Fusobacteria*, *Synergistetes* and *Elusimicrobia*) to the traditional LPS-diderm Gram-negative bacteria is presently unclear. It is quite likely that all of these atypical diderm taxa that show close affiliation to the *Firmicutes* are related to each other and therefore the diderm-LPS characteristics exhibited by them has a common origin. Although the presence of a

diderm-LPS phenotype in these two groups (i.e. atypical diderm and traditional diderms) can be explained by lateral transfer of various genes that are involved in the formation of outer cell membrane as well LPS biosynthesis between these groups, it is also possible that the outer membranes in these two groups have evolved independently and that the cell membrane organization and function in these two groups of prokaryotes may differ from each other in important aspects. Hence, further comparative studies on the biochemical and functional characteristics of the outer membrane characteristics from these two groups of bacteria should be of much interest.

In conclusion, the data presented here represent a significant criticism of the recently proposed 'prokaryotic endosymbiosis' hypothesis (Lake 2009). During the preparation of this Perspective, other criticisms of this hypothesis based on other grounds also appeared (Swithers et al. 2011). Alternative mechanisms for the evolution of outer membranes therefore need to be proposed and, as hypothesized here, it is plausible that antibiotic selection pressure was one of the main drivers in this important step in bacterial evolution.

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