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Mechanisms of bacterial morphogenesis: Evolutionary cell biology approaches provide new insights

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Abstract

How Darwin's "endless forms most beautiful" have evolved remains one of the most exciting questions in biology. The significant variety of bacterial shapes is most likely due to the specific advantages they confer with respect to the diverse environments they occupy. While our understanding of the mechanisms generating relatively simple shapes has improved tremendously in the last few years, the molecular mechanisms underlying the generation of complex shapes and the evolution of shape diversity are largely unknown. The emerging field of bacterial evolutionary cell biology provides a novel strategy to answer this question in a comparative phylogenetic framework. This relatively novel approach provides hypotheses and insights into cell biological mechanisms, such as morphogenesis, and their evolution that would have been difficult to obtain by studying only model organisms. We discuss the necessary steps, challenges, and impact of integrating "evolutionary thinking" into bacterial cell biology in the genomic era.

Keywords

bacterial shape; evolutionary cell biology; morphological transitions; evolutionary developmental biology; co-option; tree-thinking; non-model organisms

Introduction

It is difficult not to marvel at the amazing diversity of shapes in the living world: we witness it every day when we encounter plants and animals of different shapes and sizes. How the diversity of organismal forms evolved remains one of the most fundamental and fascinating questions in biology. Since the dawn of microbiology, shape, in particular the classic rod, sphere, and spiral forms, has served as an important descriptor of bacterial species. A simple, but often overlooked, fact is that there is significant morphological diversity in the microbial world, hidden to the naked eye. Under the microscope, bacteria can be found in multiple shapes and sizes, from simple spheres, rods, and spirals to unconventional chains, coils, stars, and more complex shapes such as branching filaments or bacteria that radiate cell envelope extensions from the cell body [1] (Fig. 1).

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It is intuitive that the different shapes observed in the macroscopic world, for example fins, wings, or a long neck, confer specific advantages. Why a particular bacterium has a given shape is a difficult question to answer, one confounded by the fact that a single shape rarely dominates a given environment and simple shapes such as spheres, ovoids, and rods can be found in a variety of environments. Ultimately, shape will be influenced by a combination of factors, including, but not limited to, nutrient availability, attachment and dispersal strategies, motility requirements, and predation, and therefore more than one shape may provide advantages in a given environment [1]. While the advantages of most bacterial shapes are yet to be determined [1], the high fidelity of bacterial species morphology and the conservation of shapes spanning distant taxa (and hence long periods of time) suggest that their shapes confer specific advantages. For example, *Helicobacter pylori* is hypothesized to use its corkscrew shape to traverse the thick mucus layer that covers and protects the epithelial lining of the stomach mucosa, and shape mutants that have lost this characteristic helical twist exhibit attenuated stomach colonization [2–4]. Other examples come from aquatic bacteria living in oligotrophic environments. Oligotrophy is often connected with small coccoid bacteria, simply because this shape increases the surface/volume ratio [1]. Another morphological feature found in oligotrophic bacteria, albeit less frequently than the small coccoid shape, is known as the stalk, a thin cylindrical extension of the cell envelope that protrudes from the cell body and serves as a nutrient scavenging antenna thought to improve the efficiency of nutrient uptake [5] (Figs. 1 and 2). In addition, some bacterial species can vary their shape in order to optimize their ability to survive and reproduce in different environmental conditions or as a natural part of their life cycle, a process known as morphological plasticity [6]. When the filamentous soil bacterium *Streptomyces coelicolor* finds itself in a favorable environment, it forms a branched vegetative mycelium that allows it to spread and burrow deep into the surrounding substrate (Fig. 1F). However, when the environment becomes unfavorable, the branches extend upwards from the surface to form aerial hyphae, which differentiate further into a series of spores that are released into the environment to facilitate cell dispersal [7]. Morphological plasticity is also a hallmark of a number of pathogens. For example, uropathogenic *Escherichia coli* (UPEC) switches from non-motile rods to cocci, then to motile rods, and ultimately to a filamentous form whose size is thought to prevent phagocytosis during the course of infection [6]. *H. pylori* and *Campylobacter jejuni* have been shown to assume coccoid forms after starvation and, although these coccid-shaped cells are non-cultivable, research has shown that these cells are still able to infect hosts [8,9].

Phylogenetic analysis suggests that the last common ancestor of bacteria was probably rod shaped, giving rise at various times to cocci/ovococci or other shapes [10]. It has been suggested that there is a significant correlation between cell shape and the arrangement of the *dcw* cluster of genes involved in cell division and cell wall synthesis [11], but it remains to be seen if this correlation still holds, given the ever growing amount of available genomic data. Morphological variations are often found in closely related bacterial species: the diversity in the number and positioning of stalks or flagella in several phyla [12–16], the variation in the number and shape of endospores in the Firmicutes [17], the structural diversity of fruiting bodies of *Myxobacteria* [18], and the diverse helical shapes within the *Helicobacter* and *Campylobacter* genera [19,20] to name but a few. The mechanisms that

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control shape changes within a bacterial species are beginning to be understood, but the mechanisms by which new morphologies evolved from ancestral ones mostly remain to be described. However, while several studies have pinpointed the molecular mechanisms behind morphological transitions in multicellular eukaryotes, highlighting the importance of regulatory and functional sequence evolution in the plant and animal kingdoms [21–25], the mechanisms underlying the transitions leading to the morphological diversity of bacteria remain unknown. In this review, we describe the mechanisms of bacterial cell shape generation and evolution, and we discuss the importance of conducting future bacterial cell and developmental biology studies in a comparative phylogenetic framework.

The nature of bacterial shape

The bacterial cell wall plays a pivotal role in maintaining the shape of bacterial cells [26]. The most prevalent form of bacterial cell wall is the peptidoglycan (PG), a structure composed of glycan strands made of repeating disaccharide subunits composed of *N*-Acetylmuramic acid (MurNAc) and *N*-Acetylglucosamine (GlcNAc), which are further crosslinked by pentapeptide bridges attached to the MurNAc units (For details please see Fig. 3). The resulting mesh-like structure is rigid enough to maintain bacterial shapes, yet also is elastic and can be dynamically modified [26,27]. Indeed, disruption of PG structure or synthesis in *E. coli* and *Bacillus subtilis* can quickly lead to a round-shaped cell called a “spheroplast” [26,28,29].

The not-so-simple ways of generating simple sphere, rod, or spiral shapes

The morphogenesis of different bacterial shapes requires the spatiotemporal modulation of the PG synthesis machinery, the exact mechanisms of which remain largely unknown. Most studies have focused on a few model organisms, revealing some of the principles of how the basic sphere/ovoid, rod, and spiral shapes are generated.

Conceptually, there are two major PG synthesis modes whose combination likely leads to the majority of bacterial shapes: growth and cytokinesis, which may have a common ancestry [30]. 1) Growth. Growth can occur by PG synthesis evenly distributed throughout the cell (dispersed growth) or from one or many spatially restricted zones, leading to zonal growth. As explained below, zonal growth can be specified spatially by various molecular mechanisms to yield different shapes. 2) Cytokinesis (often called septation in bacteria). Cell division requires directing PG synthesis inwards, usually at the midcell, perpendicular to the long axis of the cell. Cell division is mostly governed by the tubulin homolog FtsZ [31], which assembles into filaments to form a ring-like structure (Z-ring) around the division plane (Fig. 4) [32–34]. FtsZ recruits, directly or indirectly, a large number of proteins involved in PG synthesis, as shown in various species [30]. It is still not clear if the Z-ring is the major driving force during cell division, or if the Z-ring simply serves as a scaffold for proteins that provide constrictive force [33–35]. FtsZ is conserved in all bacterial phyla except for the Tenericutes (Mollicutes), Planctomycetes and the Chlamydia group [36,37].

How spheres are made: the perfect symmetry—Synthesizing a spherical cell body is intuitive, as it is physically the perfect shape for a membrane bound structure under osmotic

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pressure. Spherical cells are generated by uniformly growing inwards from the septum of the dividing cell. This mechanism ensures that both daughter cells are equally spherical (Fig. 4F) [38]. In addition, peripheral growth regions around the septum can elongate the sphere-shaped cell body, sculpting an oval-shaped cell (Fig. 4G) [38]. This so-called peripheral growth is coordinated by DivIVA and FtsZ in *Streptococcus pneumoniae* [38–40]. DivIVA binds preferentially to negatively curved regions of cells and drives different types of zonal growth (see below). Interestingly, most spherical or oval-shaped bacteria lack the actin homolog *mreB* gene (Fig. 5) that is required for lateral PG synthesis in many rod-shaped species (see below) [41]. Whether this is a secondary loss or the cause in the evolution from rod to sphere/ovococcus remains to be investigated.

How rods are made: many solutions to the same problem—Synthesizing a rod shape can be achieved through a number of mechanisms, including some variations on zonal growth (Fig. 4A–E). The actin-homolog MreB is required for rod-shaped cell elongation by dispersed PG synthesis along the cell body in a number of species, including the major experimental models *E. coli*, *B. subtilis*, and *C. crescentus* (Fig. 4A). MreB has been postulated to form membrane-associated filaments that rotate circumferentially inside the cell body [42–44]. Although it is still debated whether MreB can form extended filaments or simply local, discrete patches [45,46], lateral PG synthesis is clearly associated with MreB [47]. When MreB synthesis is disrupted, the localization of PG synthesis proteins is also disrupted, and cells display several growth defects leading to a rounded cell morphology [48]. Conversely, when PG synthesis is disrupted, either through the depletion of PG precursors or the addition of antibiotics, the rotation of MreB filaments is halted [42–44]. Simulations have predicted that the rotation of MreB may be critical to the morphogenesis of the rod shape by ensuring appropriately distributed PG incorporation throughout the cell body [49–51]. Feedback between cell geometry and MreB localization targets PG synthesis to regions of negative PG curvature to maintain the rod shape [51].

In addition to the dispersed mode of growth described above, some rod-shaped species, such as *E. coli* and *C. crescentus*, also elongate partly from the midcell using so-called pre-septal PG synthesis (Fig. 4A, purple bands). This FtsZ-dependent mode of PG synthesis occurs just prior to septation [52–54] and likely involves an interaction with MreB. These two proteins co-localize in *C. crescentus* [55,56] and *E. coli* [57], and they have recently been shown to interact directly in *E. coli* to transfer PG synthesis enzymes from the cell elongation machinery to the midcell for pre-septal and/or septal PG synthesis [57]. Therefore, the coordinated action of MreB and FtsZ apparently mediates a shift from dispersed to zonal PG synthesis in many species.

Alternatively, the rod shape can be achieved by cell elongation from one or both poles. For example, *Agrobacterium tumefaciens* grows unipolarly (Fig. 4B) and the actinobacteria *Corynebacterium glutamicum* and *Mycobacterium tuberculosis* grow bipolarly (Fig. 4C) [58–62]. Polar growth in *C. glutamicum* [58] and *M. tuberculosis* [62] requires DivIVA, the disruption of which leads to a rounded cell shape, similar to when dispersed elongation is disrupted in rod-shaped *E. coli* or *B. subtilis* cells. Interestingly, *A. tumefaciens*, *C. glutamicum*, and *M. tuberculosis* all lack MreB, suggesting that the polar elongation mechanism is functionally equivalent to MreB-directed dispersed elongation (Fig. 5). It

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remains to be determined if the evolution of polar growth machineries made MreB dispensable in these clades. Interestingly, in the case of *A. tumefaciens*, cells that have roughly doubled in length switch their zonal growth from a polar to a pre-septal mode analogous to the pre-septal mode described above for *E. coli* and *C. crescentus* (Fig. 4B, purple bands), which positions the elongation machinery at the new poles following division [59]. It is possible that polar growth arose in rod-shaped cells that lost the dispersed mode of growth but instead maintained pre-septal growth and repurposed it for growth at the poles. However, it remains to be determined which growth mode is ancestral. Since growth modes can now be readily detected with recently developed fluorescent probes for PG synthesis [63,64], the evolution of growth modes can be experimentally analyzed in a proper phylogenetic context to determine ancestral and derived states.

Intriguingly, the rod shape can also be generated by yet more mechanisms. *Lactococcus lactis*, an ovococcoid species, can form long rod-shaped filamentous cells in a synthetic medium (Fig. 4D). *L. lactis* also lacks MreB (Fig. 5), achieving its filamentous rod shape by forming a series of peripheral growth zones at Z-rings that are inhibited for cytokinesis (Fig. 4D, purple bands) [65]. Finally, perhaps the most eccentric way of synthesizing a rod is found in one ectosymbiotic gammaproteobacterium (not formally named) that attaches to the surface of the marine nematode *Laxus oneistus* [66]. This ectosymbiotic bacterium attaches to its host polarly and forms a monolayer biofilm that expands as the nematode grows in size. This bacterium grows in width and divides longitudinally, defying what is known in all previously studied rod-shaped bacterial species (Fig. 4E). This growth mechanism is well suited ecologically to maintain coverage of the nematode surface. Not surprisingly, the Z-ring is also positioned longitudinally to coordinate the cytokinesis of two daughter cells, bringing up the fascinating question of how FtsZ can localize in this fashion [66].

How spirals are made: the art of twisting—The spiral shape is a bit trickier to generate, and mechanistic studies are relatively scarce. The twisted spiral shape can be viewed as a summation of at least three distinct growth modes: 1) elongation, 2) curvature, and 3) twist [3,67]. There are at least two distinct mechanisms for generating a spiral, both of which utilize differential growth of the PG to induce either positive or negative curvature on one side of the cylindrical cell body. The first is an “active” mechanism in which genes are directly associated with generating the helical shape. *H. pylori*, a pathogenic species well known for its iconic helical shape, has been the major model organism for studying this mechanism. A number of genes have been shown to affect the helical nature of the cell body to varying degrees. It was suggested that the helical shape is achieved by local modification of PG crosslinks for every twist to create flexible regions that introduce the negative curvature, thereby forming the spiral shape (Fig. 4I) [3,49,68]. The alternative “passive” mechanism can be mediated by protein filaments “molding” the cell shape. In *C. crescentus*, the intermediate filament (IF)-like cytoskeleton protein CreS forms a protein bundle on only one side of the cell body [69]. By limiting the local lateral growth, possibly via MreB, this mechanism introduces positive curvature, resulting a crescent-shaped cell body [70]. However, the *C. crescentus* curved cell body is actually also twisted (Fig. 4J). This becomes obvious during prolonged growth in stationary phase, resulting in filamentous helices [71],

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indicating that a similar passive mechanism can also facilitate the synthesis of a spiral-shaped cell body.

Zonal growth is used to generate complex shapes

In the previous section, we briefly summarized decades of research on simple bacterial cell morphologies. Specifically, we have shown that simple rods can be achieved by at least five different mechanisms, some of which may be used to generate other shapes. However, the metaphorical "elephant in the room" question remains: what mechanisms are required to generate more complex or eccentric cell shapes? Unfortunately, we know little about how these shapes are generated at the molecular or even cellular level. Conceptually, these complex morphologies (Fig. 1) can be achieved by specifying zones of PG synthesis to target growth at specific subcellular locations [19,72].

A classic example of how the positioning of zonal growth can generate complex morphologies comes from studies of branch formation in *Streptomyces*. As is the case for most, if not all, Actinomycetales, *Streptomyces* species grow polarly. In addition, *Streptomyces* species often initiate lateral growth to form long branched filaments, resulting in a complex network of branched mycelium (Fig. 1F). In *S. coelicolor*, the formation of new branches occurs behind the tip of growing hyphae [7,73]. The negative curvature binding protein DivIVA localizes to the poles of growing hyphae and forms a structure called the polarisome, which recruits the PG synthesis machinery. Phosphorylation of DivIVA by the kinase AfsK causes the disassembly of part of the apical polarisome. The resulting DivIVA foci left behind the growing tip initiate the formation of new polarisomes and therefore the formation of a new zone of growth (Fig. 4H) [7,74,75]. In Firmicutes, DivIVA is required to prevent Z-ring formation at the new cell poles after division in *B. subtilis*, and to coordinate midcell elongation in *S. pneumoniae* [76,77]. Interestingly, only DivIVA from Actinobacteria species (either *S. coelicolor* or *M. tuberculosis*), but not Firmicutes (*B. subtilis* or *S. pneumoniae*), can rescue the elongation defect of a *divIVA* mutant in the Actinobacterium *C. glutamicum* [58]. These results indicate that the function of DivIVA is different in Actinobacteria compared to the Firmicutes (Fig. 5), but the mode of growth has only been studied in a few species in these groups. It may be that the role of DivIVA has shifted from regulating septal PG synthesis in Firmicutes to coordinating polar growth and lateral growth for branching in Actinobacteria (Fig. 5). A robust phylogenetic study of the mode of growth and DivIVA function in this group will be required to resolve the ancestral state of DivIVA (see section 3).

The knowledge gained from studying branch formation in *S. coelicolor* provides a glimpse into the mechanisms that generate complex morphologies: nature extends and builds upon basic shapes. Compared to rod-shaped cells, *S. coelicolor* branching can be viewed as a result of controlled zonal growth at discrete localized positions along the cell body. This simple strategy is most efficient with a highly modular mechanism, in which a master regulator controls the activity and/or localization of the whole complex/pathway. Here, simply changing the location of DivIVA is both necessary and sufficient to recruit the PG synthesis machinery to new positions to from branches. However, DivIVA is not found in Gram-negative bacteria, which possess equal, if not more, morphological diversity.

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Therefore, a great variety of such modular mechanisms must exist, as demonstrated in the next example.

The mechanism and evolution of morphogenesis: the study of stalk synthesis and localization provides a model

The stalk is a thin, appendage-like extension of all three layers of the cell envelope (inner membrane, peptidoglycan, and outer membrane) found in phylogenetically diverse groups of bacteria [14] (Figs. 1 and 2). Not to be confused with the hypha structure in *Streptomyces*, the stalk is much narrower than the cell body and hence a morphologically distinct organelle, analogous to the cilium of the eukaryotic cells [78]. Stalk structure, synthesis, and function have been mostly studied in *C. crescentus*, a model organism for bacterial development and adhesion [79–81]. The stalk is synthesized from its cell-proximal region and is compartmentalized from the cell body by proteinaceous structures called crossbands [81,82]. The stalk increases cell buoyancy and can be used as a nutrient scavenging organelle [5,83,84]. In *C. crescentus*, the stalk grows precisely from the polar location bearing the adhesive holdfast, and therefore pushes the attached cell away from the surface. Because there is little to no flow at a surface, pushing the cell away provides access to flowing nutrients: increasing the distance from the surface from 1 to 10 μm would provide an ~10% increase in nutrient flux [5,83,85]. The stalk can even serve as a "birth canal" through which budding bacteria produce daughter cells in the families Hyphomonadaceae and Hyphomicrobiaceae [86]. Although studies have shown that certain genes involved in PG synthesis and its modulation play a role in the synthesis of the stalk, the exact molecular mechanism for stalk synthesis and positioning remains undetermined [87].

Recently, an evolutionary cell biology study has provided novel insights into the mechanisms of stalk synthesis and positioning. In the closely related *Asticcacaulis* genus, the number and location of the stalks drastically differs from that of *C. crescentus*, yet its structure appears identical in the two genera [13] (Figs. 1 and 2). In *C. crescentus*, the stalk is positioned at a single cell pole; in *Asticcacaulis excentricus*, the stalk is made at a subpolar position off-center of a cell pole; and finally, in *Asticcacaulis biprosthecum*, two stalks are positioned bilaterally on the cell body [12–14] (Figs. 1 and 2). Stalks are synthesized from their cell body-proximal region in all species, suggesting that a common molecular mechanism may exist to account for the positioning and growth of stalks [14]. To identify potential stalk morphogens, the localization of proteins known to localize polarly in *C. crescentus* was determined in *A. biprosthecum*, leading to the identification of two proteins that localize at the base of its bi-lateral stalks rather than the cell pole [14]. One of the proteins, SpmX, was shown to be required for stalk synthesis in the *Asticcacaulis* genus (Fig. 2D), whereas it is not required in *C. crescentus* [88]. Expression of SpmX in an exogenous species could drive stalk synthesis at alternative positions (Fig. 2E). These results show that SpmX is necessary and sufficient to drive stalk synthesis to specific positions, indicating that it functions in a modular manner, much like DivIVA in the localization of the required PG synthesis machinery for branch formation in *S. coelicolor*. Therefore, SpmX serves as a morphogen for stalk synthesis in *Asticcacaulis*, responsible for coordinating zonal growth at species-dependent locations to produce the stalk(s), ultimately contributing to the diversity of cell shape [14].

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How did stalk positioning evolve?

Stalks are faithfully reproduced at defined positions by a number of diverse species across multiple phyla, but the positions can vary between species. The evolutionary progression of stalk positioning, inferred from phylogeny, places the polar stalk of *C. crescentus* ancestral to sub-polar and bi-lateral stalks (Fig. 2B) [14]. This intuitive progression is in agreement with the evolutionary principle that more complex structures are typically built from simpler, yet similar ones [89]. SpmX regulates development in *C. crescentus*, but it is not required for stalk synthesis in this species, making it a surprising candidate for a morphogen for stalk positioning in *Asticcacaulis* [88]. These observations indicate that certain changes have occurred for SpmX to evolve new functions. The process of repurposing an existing biological unit (gene, pathway, organ, etc.) for a new function is referred to as co-option [90]. Based on similar evolutionary developmental biology (evo-devo) studies of eukaryotic multicellular organisms, such changes could be regulatory and/or functional. Coincidentally, the *Asticcacaulis* SpmX has expanded by as many as 400 amino acids to over 800, compared to the 435 amino acids of *Caulobacter* SpmX (Fig. 2C). Furthermore, this expansion is limited to a highly divergent intermediate region, as the N-terminal muramidase domain and C-terminal transmembrane domains remain conserved (Fig. 2C). To test the hypothesis that the expansion of this domain is the key to SpmX's role in stalk synthesis, a series of chimeric proteins, in which separate domains of SpmX from different species are fused together, were constructed to test their function in stalk synthesis and positioning in different species. The results indicated that through progressive changes in its divergent C-terminal domain, SpmX evolved the ability to synthesize, and then target, stalk synthesis at specific positions (Fig. 2E). Last but not least, it was shown that over-expression of SpmX in *A. excentricus* leads to the formation of multiple sub-polar stalks, hinting that an increase in the expression level of SpmX might be a prerequisite for bi-lateral stalk synthesis in *A. biprosthecum* [14].

In summary, functional evolution of a specific domain of SpmX is the key to the evolution of cell morphology in *Asticcacaulis* and *Caulobacter*. Although SpmX is not required for stalk synthesis in *C. crescentus*, it is certainly reasonable to assume that a protein analogous to SpmX exists in *C. crescentus* to coordinate stalk synthesis, and that the actual downstream stalk synthesis machineries are likely homologous in *Asticcacaulis* and *Caulobacter*, resulting in almost identical stalk ultrastructure. Furthermore, to generate complex cell morphologies, as is observed in some of the *Rhizobiales* (Fig. 1L) [59], which lack *spmX* orthologs, a morphogen analogous to SpmX may exist to coordinate the zonal growth that specifies distinct morphologies.

The emerging field of bacterial evolutionary cell biology

We have briefly covered how basic sphere, rod, and spiral shapes can be generated in bacteria. Furthermore, we described the mechanisms underlying the synthesis of two distinct complex morphologies, cell branching and stalks. While it is clear that our knowledge of how bacterial shapes are generated is still very limited, we observe an emerging pattern in which complex shapes can evolve from basic shapes using a similar evolutionary mechanism – controlled zonal growth. Such mechanisms are only identifiable when we

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investigate multiple species that are morphologically distinct yet closely related. The importance of using evolutionary principles and phylogenetically informed comparative biology in the study of complex processes is nicely illustrated in the evo-devo (evolution of development) field [91]. Three important mechanisms have emerged from studies of morphological transitions in eukaryotes: 1) changes in cis-regulatory elements or protein sequences are frequently associated with morphological transitions; 2) modularity is usually present in the morphogenesis pathways (morphogen); 3) genes with existing functions can acquire new roles through evolution (co-option) [91–93].

Although evo-devo studies have historically focused on the shape of multicellular eukaryotes, their findings are potentially applicable to any evolutionary process. Conversely, any cell biological process can be studied using the approaches of evo-devo with minor modifications, as exemplified in the emerging field of evolutionary cell biology where these principles are beginning to be applied to the evolution of subcellular organization [94].

Why evolutionary cell biology in bacteria?

Two examples illustrate the potential benefits of studying evolutionary cell biology in bacteria: 1) The role of SpmX in stalk synthesis could not be inferred from its role in the much studied model *C. crescentus* and instead its discovery required its study in the closely related *Asticcacaulis* genus. In addition, the machinery for stalk synthesis remains to be identified in any genus. Now, SpmX can be used as a starting point to identify the downstream stalk synthesis machinery in *Asticcacaulis* and the acquired knowledge can be applied back to *C. crescentus*, where most of the stalk synthesis machinery is expected to be the same given the common ultrastructure of stalks in the two genera. 2) The rod-shaped *A. tumefaciens* had been assumed to elongate by incorporating new PG material in a dispersed manner along the side wall, similar to *E. coli* [95]. However, many phylogenetically closely related Rhizobiales species were known to grow polarly. In addition, the *A. tumefaciens* genome lacks *mreB*, which is essential for dispersed cell elongation in *E. coli* and *B. subtilis*. These evolutionary observations led to testing the mode of growth of *A. tumefaciens*, leading to the discovery that *A. tumefaciens*, and likely most species in the Rhizobiales order, grow polarly [59]. A more detailed study of growth modes in the Alphaproteobacteria will be required to answer this question.

Next generation sequencing paves the way for bacterial evolutionary cell biology studies

Recent advances in next generation sequencing (NGS) technologies have enabled the affordable sequencing of a large number of genomes [96]. At the time of this writing, approximately 22,000 bacterial genomes had been sequenced and deposited in public databases. However, most of the sequencing efforts have focused on pathogenic strains and a few selected model organisms, such as *E. coli* and *B. subtilis*, although efforts are underway to compile a phylogeny-driven genomic encyclopedia of bacteria [97]. The availability of genomic data provides an opportunity to study different organisms without experimental manipulation. For example, the presence or absence of metabolic pathways can be used to define the lifestyle of species of interest [98]. Alternatively, the evolutionary conservation of genes helps to predict whether a protein of interest may have broadly important or species-specific functions. For example, FtsZ and MreB are widely conserved

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in a majority of bacterial phyla, supporting their crucial roles as described above; whereas SpmX is only found in *Caulobacter*-related species, indicating that its role must be specific to this clade of bacteria, such as developmental regulation and/or stalk synthesis. Finally, genomic sequences enable the construction of rigorous phylogenies, which is essential in bacterial evolutionary studies, as we will discuss next.

The four key steps in bacterial evolutionary cell biology research

Mallarino et al. (2012) have summarized a research approach for evo-devo studies of animal morphological evolution, which includes three key steps: 1) quantification of morphological variation, 2) identification of candidate developmental mechanism, and 3) functional analysis of genes and pathways. Here we adapt these three steps for the bacterial evolutionary cell biology field, with one key ingredient added as the first step: rigorous phylogenetic analysis to identify closely related species with variations of interest (Fig. 6):

1. Rigorous phylogenetic analysis of bacterial species

   In eukaryotes, studies focusing on morphological evolution are always performed within phylogenetically closely related species. However, animals have numerous visible morphological features that can be used to reliably predict phylogeny most of the time (only birds look like birds). In bacteria, phylogeny cannot be reliably predicted based on morphological traits alone since there is insufficient character state variation in bacterial morphology, especially in the most common round, oval, and rod-shaped species. Furthermore, convergent evolution in bacterial shape complicates analysis (the coccoid form potentially arose multiple times). However, systematic analysis of cell size and shape in various bacterial species using recently developed automated quantitative image analysis tools could provide finer resolution to alleviate this problem [14,50,99,100]. Therefore, to reliably evaluate the phylogeny of bacteria, relevant molecular data are essential. Traditionally, the 16S rRNA sequence has been used to infer the phylogeny of bacteria, but in cases where the phylogeny is difficult to resolve based on a single gene, the best strategy is to sequence the genomes of interest, which is becoming increasingly affordable [101]. For example, to study stalk positioning, several closely related stalked and non-stalked species were sequenced [102] so that rigorous phylogenetic analysis could be performed to provide insights into the generation of morphological variation [14].

2. Quantification of phenotypic variation

   After rigorous phylogenetic analysis, it is essential to quantitatively characterize the morphological traits. In the case of stalk positioning, the difference may be obvious, but within the stalked species, there are also variations in the length or even the diameter of the stalks, which would require more thorough characterization. Alternatively, the spiral shape of diverse *Helicobacter* and *Campylobacter* species are inherently different and also require rigorous characterizations. Finally, from the perspective of cell biology, phenotypic variation can be observed in different forms: variation in function and localization of proteins of interest, changes in the arrangement of intracellular organelles, and

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even divergence of gene function after duplication, all of which require rigorous quantification.

3. Identification of candidate evolutionary mechanisms

   The identification of candidate evolutionary mechanisms usually requires a systematic and creative approach. One strategy is to take advantage of existing knowledge by branching out from established model organisms. For example, in the study of stalk synthesis in the *Caulobacter* clade, knowledge about the localization of developmental regulators was exploited to identify proteins that localize at the base of stalks in *Asticcacaulis*. Similarly, the mechanisms by which branches form in *Streptomyces* may have evolved from polar growth, as the branching morphogen, DivIVA, is also required for polar growth in the closely related rod-shaped *Corynebacterium* and *Mycobacterium* (Fig. 5). It would be interesting to sample more species in Actinobacteria to see if the function of DivIVA in branch formation is a derived state.

4. Functional studies of genes or pathways

   Once candidate genes or pathways are identified, the next critical step is to study the candidate genes in the model organism and closely related non-model organisms. For example, after SpmX was identified as a candidate morphogen for stalk synthesis, *spmX* mutants were constructed in both *Asticcacaulis* species and were found to be stalkless, confirming the prediction (Fig. 2D). Next, cross-complementation genetics provides a powerful test as to whether changes to the genes of interest generate phenotypic variation in closely related species. For example, different alleles of SpmX were expressed in different strains from the same xylose-inducible promoter, ensuring that the only variable was the SpmX protein. The results showed that exogenous SpmX could still drive stalk synthesis, albeit at a different position, indicating that SpmX evolution is the mechanism underlying the evolution of stalk positioning (Fig. 2E). This study also demonstrated yet another advantage of branching out from a model organism, since related genetic tools are much more likely to work in closely related non-model organisms.

It is important to point out that the four steps need not be ordered in a flow-chart fashion (Fig. 6). For example, following quantitation of morphological traits, sequenced strains might not encompass the needed variation at either the cellular or the molecular level, which would lead to further strain collection and sequencing efforts. Alternatively, after functional studies, the candidate genes or pathways may not prove sufficient to account for the variation observed in the chosen organisms, which would require either additional strain collection and/or a revised strategy to identify candidate genes or pathways. Therefore, it is more appropriate to perceive the four steps in a dynamic fashion, in which each step may require revisions based on the other step(s).

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Conclusion and outlook

Through billions of years of evolution, bacteria have achieved their dominant success in today's world [1]. As Theodosius Dobzhansky elegantly stated, "Nothing in biology makes sense except in the light of evolution" [103]. The benefits of understanding the mechanisms underlying the evolution of bacterial shape and other cellular processes include: 1) Confirmation of the knowledge acquired from model systems, often limited to studies of a single species, and expansion of what we cannot learn from model organisms; 2) Understanding how old genes can be co-opted for a new purpose by means of the evolution of regulatory and/or protein sequences; 3) Potential implications in medical science; as described earlier, many pathogenic species use shape to their advantage when invading hosts, some are even capable of transforming on the fly; 4) The ability to control the shape or metabolic processes of bacterial cells can be potentially useful in synthetic biology to maximize the efficiency of industrial applications such as fermentation, because the ability to take up nutrients at the micro-scale is limited by diffusion [5]; 5) Finally, studying the mechanisms underlying Darwin's "endless forms most beautiful" [104] provides an excellent opportunity for researchers to convey the beauty of evolution and science to the public and to inspire future generations of scientists.

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Abbreviations

PG	peptidoglycan
Evo-Devo	evolution of development

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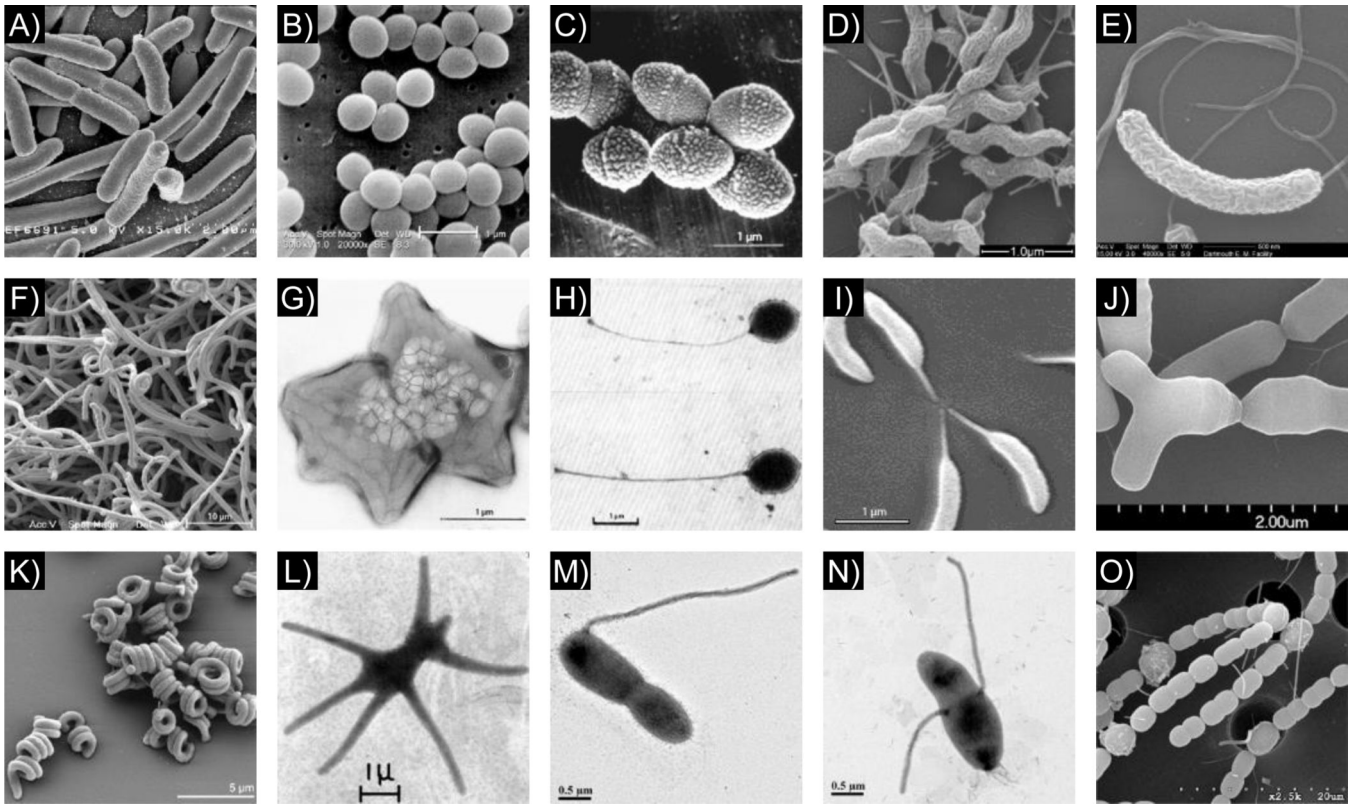

Figure 1.

The diversity of bacterial shapes. For each shape, a brief description and the name of one representative species is provided, followed by the image source in parenthesis. **A:** Rod, *Escherichia coli* (NIAID); **B:** Sphere, *Staphylococcus aureus* (Janice Haney Carr, CDC); **C:** Ovococcoid, *Streptococcus pneumoniae* [105]; **D:** Spiral, *Campylobacter jejuni* [106]; **E:** Crescent, *Vibrio cholerae* (Louisa Howard, Dartmouth College); **F:** Branched filaments, *Streptomyces coelicolor* (Paul Hoskisson, University of Strathclyde); **G:** Star, *Stella vacuolata* [107]; **H:** Stalked, *Planctomyces maris* [108]; **I:** Stalked and crescent, *Caulobacter crescentus* (Ellen Quardokus, Indiana University); **J:** Bifid/Y-shaped, *Bifidobacterium breve* (Daria Zhurina and Paul Walther, University of Ulm); **K:** Coil, *Spirosoma linguale* [109]; **L:** Multi-stalked, *Ancalomicrobium adetum* [110]; **M:** Stalked, *Asticcacaulis excentricus* (Chao Jiang, Stanford University); **N:** Stalked, *Asticcacaulis biprosthecum* (Chao Jiang, Stanford University); **O:** Chain and Heterocysts, *Anabaena variabilis* (Jinshun Zhong, University of Missouri-St. Louis) [111]. All images are reproduced with permission.

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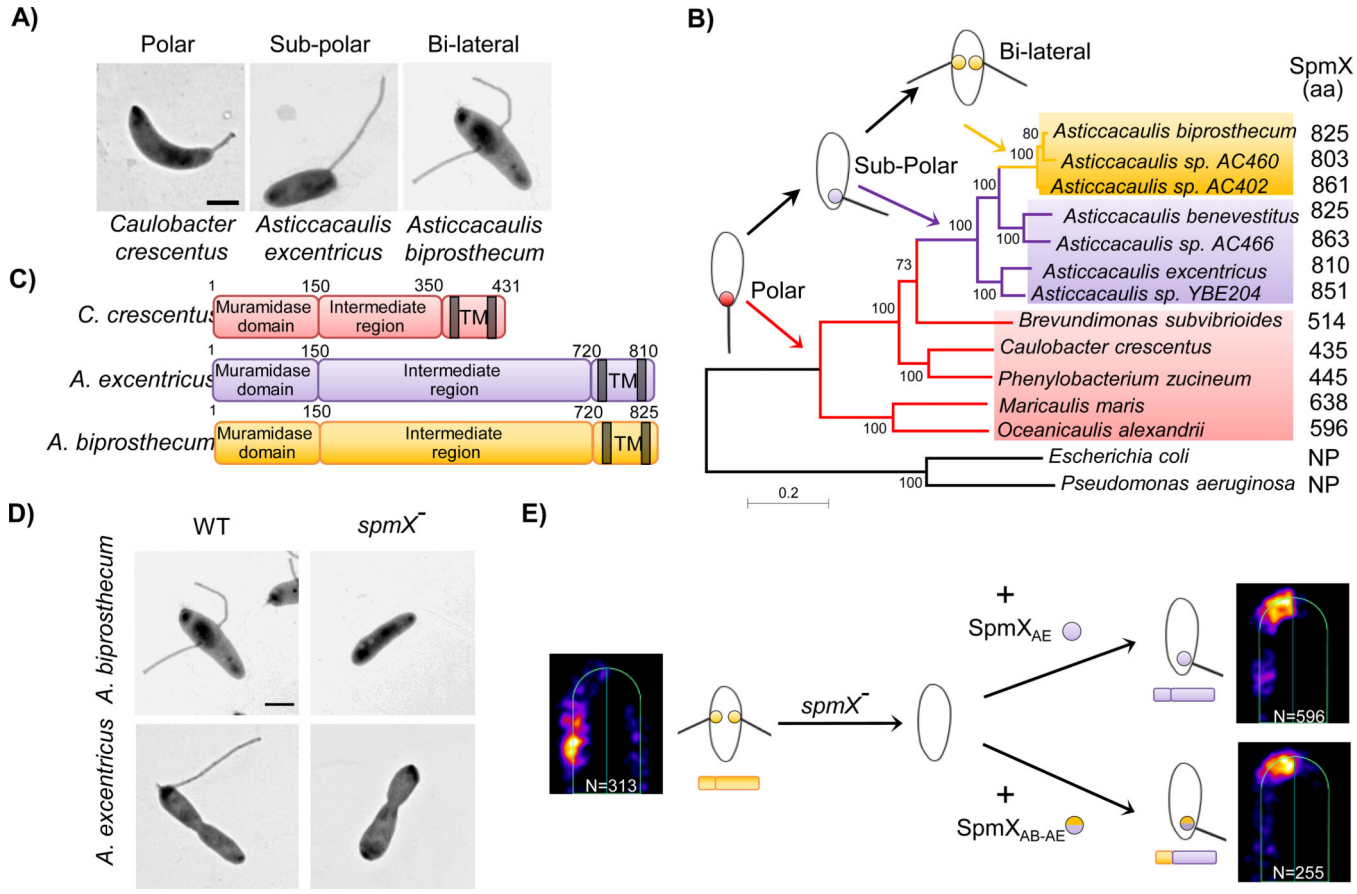

Figure 2.

SpmX is the evolving morphogen of stalk synthesis. **A:** Transmission Electron micrographs of three species with distinct stalk positioning. **B:** Phylogenetic tree and inferred evolutionary trajectory of stalk positioning. Colors of shading, branches, and SpmX (filled circles) denote the polar (red), sub-polar (purple), and bi-lateral (yellow) stalk positioning, respectively. Arrows point to the origin of respective morphologies. The size of SpmX is indicated in amino acids (aa). NP, orthologs not present. Scale bar, number of substitutions per site. **C:** Domain organization of SpmX. Transmembrane domains (TM) are shown as grey bars. All versions of SpmX share a conserved N-terminal putative muramidase domain and two C-terminal transmembrane domains. However, the intermediate region is highly variable in both length and sequence. **D:** SpmX is required for stalk synthesis in *Asticcacaulis*. Transmission electron microscopy images of *Asticcacaulis* species and their respective *spmX*$^{-}$ stalkless mutants. **E:** Heat maps of SpmX localization in the *A. biprosthecum spmX*$^{-}$ mutant expressing $SpmX_{AB}$-EGFP (left), $SpmX_{AE}$-EGFP (Right, top) or the chimeric $SpmX_{AB\text{-}AE}$-EGFP, with the N-terminal *A. biprosthecum* muramidase domain fused to the C-terminal *A. excentricus* intermediate and TM domains (Right, bottom). Notice that both $SpmX_{AE}$-EGFP and the chimeric $SpmX_{AB\text{-}AE}$-EGFP are able to drive morphological transitions from bi-lateral to sub-polar predominantly. “N” indicates the number of foci analyzed. Figure adapted from Jiang et al (2014) with permission.

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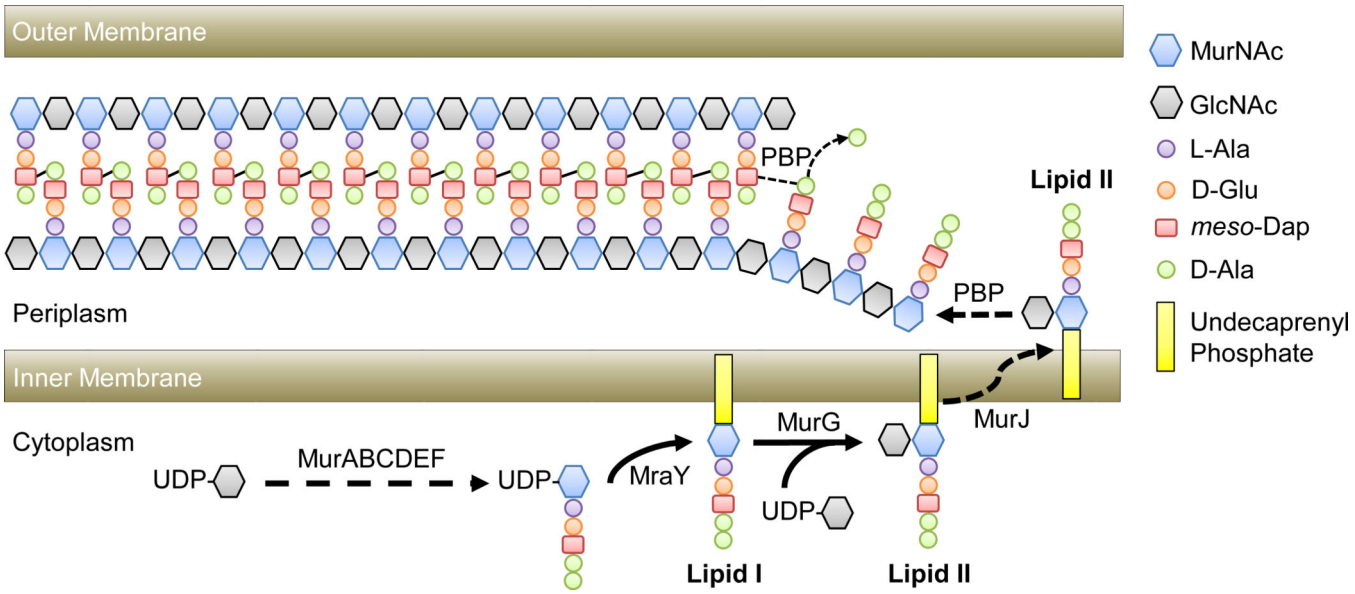

Figure 3.

A simplified model of peptidoglycan synthesis in *E. coli*. PG synthesis is a complex process coordinated by a number of proteins that are conserved in almost all bacterial species. Due to the scope of this review, we will only briefly cover the biochemical fundamentals of PG synthesis using *E. coli* as an example (for detailed reviews see [26,27]). PG synthesis begins with the synthesis of PG precursors in the cytoplasm. The nucleotide sugar uridine diphosphate *N*-Acetylglucosamine (UDP-GlcNAc) is converted to uridine diphosphate *N*-Acetylmuramic acid (UDP-MurNAc) by MurAB. MurCEDF then catalyze the addition of an amino acid side chain to UDP-MurNAc through the sequential addition of L-alanine (L-Ala), D-glutamic acid (D-Glu), *meso*-diaminopimelic acid (*meso*-Dap; a derivative of lysine), and two D-alanines (D-Ala). This UDP-MurNAc-pentapeptide is then anchored to the inner membrane via the transport lipid undecaprenyl phosphate by MraY to form Lipid I. A UDP-GlcNAc moiety is attached to Lipid I via glycosidic bond by MurG to form the disaccharide-pentapeptide precursor known as Lipid II, the basic building block of the PG. The disaccharide-pentapeptide is flipped across the inner membrane to the periplasmic space by a flippase (MurJ) where it is incorporated into the nascent PG chain by penicillin-binding protein (PBP) transglycosylase activity. Once incorporated, PBP transpeptidases crosslink *meso*-Dap of one pentapeptide to D-Ala of an opposing pentapeptide, concomitant with the cleavage of the terminal D-Ala, thus incorporating a new chain into the PG sacculus. Due to the crucial roles of these enzymatic activities in PG synthesis, PG transpeptidases, also known as Penicillin-Binding Proteins (PBPs), remain the best targets for antibiotics. In addition to the synthesis machinery, numerous enzymes also exist to remodel the existing PG structure (for a review, please see [27]). It is important to note that the PG is not essential for organisms to form distinct shapes, as seen in some intracellular parasitic bacterial species that lack PG, such as *Mycoplasma* and *Spiroplasma* in Tenericutes (Mollicutes), and free-living bacteria like the Planctomycetes [114,115]. Interestingly, recent work has shown that in the Chlamydia group, where the presence of a PG was uncertain, at least two species have a detectable PG structure [64,116]. These findings suggest that rigorous re-examination of other presumably PG-deficient bacterial species, such as the

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Planctomycetes, is crucial to our understanding of the evolution of cell wall synthesis in bacteria.

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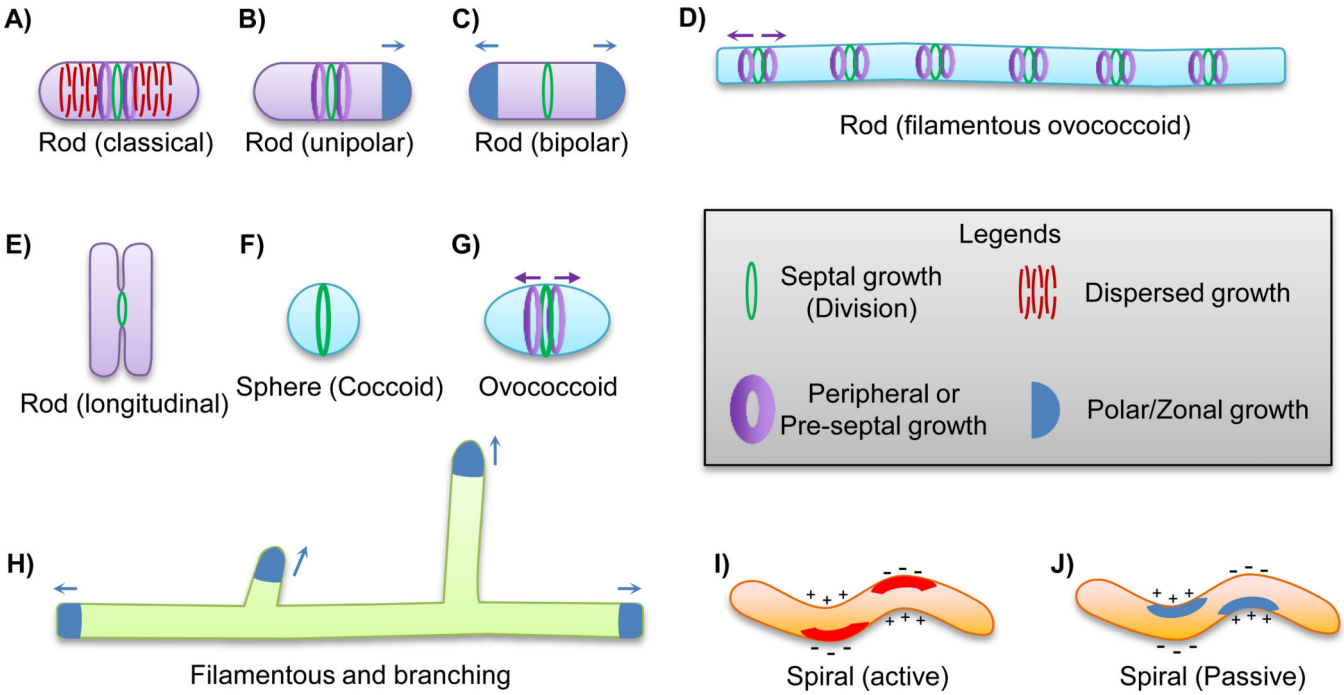

Figure 4.

Mechanisms underlying the synthesis of various bacterial shapes. Arrows indicate the direction of the various zonal growth mechanisms. **A–E**: The various ways of making a rod-shaped cell body. Septation is required to resolve two or more daughter cells in each case (Green ring). **A:** The dispersed elongation model in which new material is uniformly incorporated into the side wall (Red dashed rings). In *E. coli* and *C. crescentus*, a specialized type of growth called pre-septal growth also contributes to elongation (Purple bands). **B:** Unipolar growth elongates the cell body in a "budding" fashion (Blue cap). In *A. tumefaciens*, pre-septal elongation occurs and defines the future sites of active polar growth (Purple bands). **C:** Some Actinobacteria species elongate the cell body in a bi-polar fashion (Blue caps), driven by DivIVA as detailed in the text. **D:** Filamentous ovococcoid cells may also achieve a rod-shaped cell body by a combination of inhibition of cell division (Green rings) and persistent peripheral growth around the septal region (Purple bands), as seen in *Lactococcus lactis*. **E:** Strikingly, one ectosymbiotic Gammaproteobacterium that attaches to the surface of the marine nematode *Laxus oneistus* grows in width and divides longitudinally. The Z-ring is also positioned longitudinally to divide the cell (Green ring). **F–G:** The sphere (coccoid) and oval shape (ovococcoid) utilize septal growth (Green ring) to synthesize the hemispheres of two respective daughter cells. In addition, regions of peripheral growth (Purple bands) can elongate the sphere-shaped cell body to sculpt an oval-shaped cell. **H:** The long branched filaments of *Streptomyces coelicolor* are achieved by tip growth at discrete positions directed by the protein DivIVA, (Blue caps), as detailed in the main text. **I–J:** The spiral shape can be achieved in at least two different ways. An "active" mechanism in which proteins localize to one side of the cell cylinder and induce negative curvature formation by relaxing cross-links of glycan strands (**I**, red stripes). Alternatively, cytoskeleton proteins can induce the formation of positive curvature by physically molding one side of the cell body (**J**, blue stripes). The positive and negative curvature of the cell

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body are indicated by + and − signs. The Z-ring positioning is imprecise in the only studied spiral-shaped organism *Helicobacter pylori*, hence it is not depicted in the schematics.

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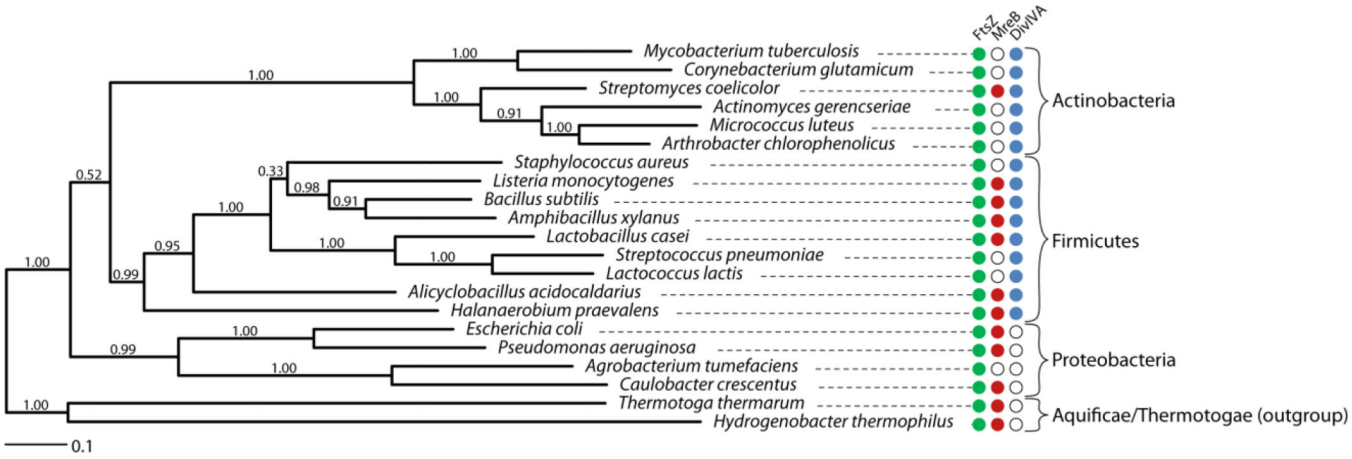

Figure 5.

The distribution of FtsZ, MreB, and DivIVA in selected Gram-positive bacteria. We randomly chose 138 genera across different orders and then chose 1–3 species in each genus, preferably with finished genomes. The Aquificae/Thermotogae outgroup is a deep-branched bacterial group usually found in extreme environments. Overall, the phylogenetic tree is representative of the result of 186 sampled genomes: 1. FtsZ is found in all species tested. 2. MreB is missing in most Actinobacteria and coccoid species. However, we note exceptions to this “rule” as several coccoid species in the Actinobacteria and Firmicutes have identifiable MreB. 3. DivIVA is almost strictly restricted to Gram-positive bacteria (Actinobacteria and Firmicutes). The phylogenetic tree of representative species (selected based on the scope of this review) belonging to Actinobacteria, Firmicutes, Proteobacteria and the outgroup deep branching extremophiles was calculated based on the alignment of the GyrA protein using the maximum likelihood method based on the LG model in MEGA 6 [117], supported by 100 bootstrap replicates. A discrete Gamma distribution with invariant positions was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The low support value for grouping Actinobacteria and Firmicutes together agrees with a previous report [118]. Filled or open circles indicate the genomic presence or absence of FtsZ (Green), MreB (Red), and DivIVA (Blue) detected by the Bi-directional Best Hit method (BBH) [119], respectively. Note that DivIVA is only present in Gram-positive bacterial species (Actinobacteria and Firmicutes), although its function diverges significantly in different species. Specifically, DivIVA is required for polar elongation in Actinobacteria but not in Firmicutes. MreB is absent in characterized coccoid/ovococcoid species [38]. In addition, MreB is also absent in polarly growing species (including *A. tumefaciens* in Proteobacteria), except for *S. coelicolor* in which MreB is required for sporulation, but not elongation [73].

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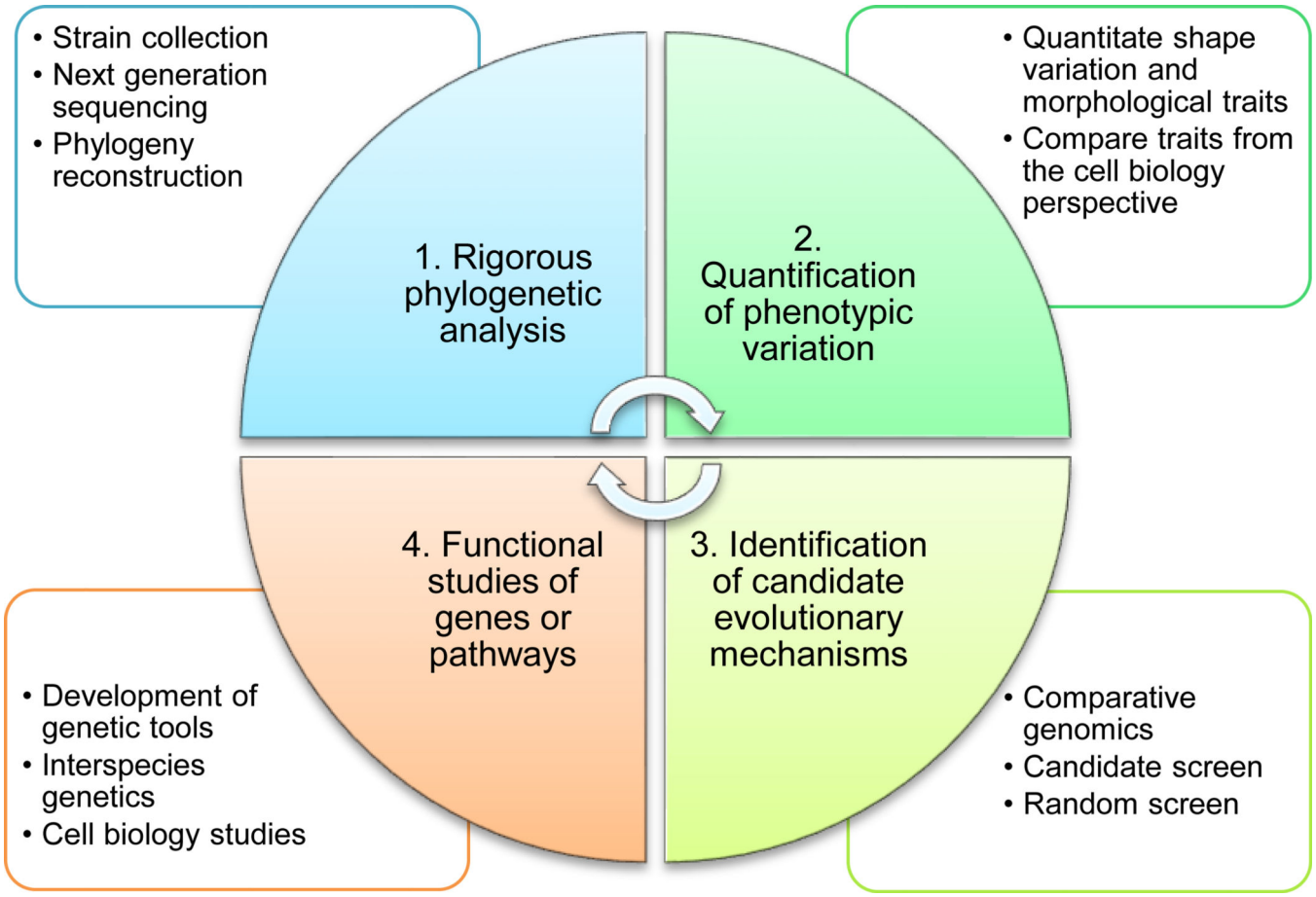

Figure 6.

Steps of evolutionary cell biology research in bacteria. Each of the four steps is indispensable to identify the evolutionary mechanisms underlying variation in different species from either a cell or developmental biology perspective. Construction of a rigorous phylogeny is the foundation of evolutionary cell biology studies, in that an unreliable phylogeny will lead to misinterpretations of the evolutionary history of species/traits and mistakes in experimental design and implementation. Development of genetic tools in non-model organisms serves as the other technical barrier for this type of studies; however, certain biochemical approaches, such as the use of antibiotics, universal or specific molecular probes, or specific antibodies, can be exploited to study organisms where genetic experiments are not feasible.