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CHAPTER TWO

Stress-induced adaptive morphogenesis in bacteria

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Abstract

Bacteria thrive in virtually all environments. Like all other living organisms, bacteria may encounter various types of stresses, to which cells need to adapt. In this chapter, we describe how cells cope with stressful conditions and how this may lead to dramatic morphological changes. These changes may not only allow harmless cells to withstand environmental insults but can also benefit pathogenic bacteria by enabling them to escape from the immune system and the activity of antibiotics. A better understanding of stress-induced morphogenesis will help us to develop new approaches to combat such harmful pathogens.

1. Introduction

Evolutionary selection has created a large diversity of bacterial cell shapes, which benefit a particular bacterial life style, environment or reproduction cycle. In addition to some well-characterized bacterial shapes such as rods, cocci and spirals, several others are more exotic, including mycelial, coryneform, bulbiform, fusiform and even square and star shapes (Caccamo & Brun, 2018; Walsby, 1980; Young, 2006). Cells can furthermore be equipped with various appendages that contribute to cell shape, such as flagella, pili or stalks. The environmental conditions to which a bacterium is exposed can dramatically fluctuate throughout its life. Bacteria may encounter many different types of stresses, ranging from changes in pH, osmotic pressure, temperature, exposure to antibiotics or nutrient availability associated with for example the invasion of a mammalian host or upon the encounter of extreme habitats or seasonal weather changes (Ewert & Deming, 2014; Fang, Frawley, Tapscott, & Vázquez-Torres, 2016; Trastoy et al., 2018). There is growing evidence that changing bacterial shape and size, a process referred to as morphological plasticity or adaptive morphogenesis, is an active response to counter environmental stresses and promote survival (Justice, Hunstad, Cegelski, & Hultgren, 2008; Kysela, Randich, Caccamo, & Brun, 2016; Yang, Blair, & Salama, 2016).

Stress-induced adaptive morphogenesis is associated with major physiological and morphological changes in the cell. In this review, we will specifically focus on morphological changes brought about by changes in the cell envelope structure, including their consequences and benefits. We will also describe the alterations in nucleoid morphology that often co-occur with stress-induced cell shape changes. As the morphology of

harmful pathogenic bacteria can also dramatically change in the host, a better understanding of this morphological plasticity may be used for our benefit to efficiently combat pathogens.

2. Bacterial cell morphology in favorable environments

2.1 The bacterial cell envelope

Bacterial cell morphology was historically of importance to identify species of interest. Despite today's prevalence of genomics and proteomics approaches, the characterization of the cells' morphology remains an important characteristic of bacteria that can give important insight into their lifestyle and behavior. Bacterial morphology is largely dictated by the cell envelope. This structure is the first line of defense against environmental challenges, and the port of communication between the cell and the outside world (Silhavy, Kahne, & Walker, 2010). Based on the composition of the cell envelope, bacteria are categorized as Gram-negative or Gram-positive. The Gram-negative, or diderm cell envelope, is composed of a fluid cytoplasmic membrane, thin cell wall layer and a rigid outer membrane. The compartment between the two membrane layers is termed the periplasm and holds the cell wall layer and a variety of protein structures (Silhavy et al., 2010). Not only the cell wall, but also the outer membrane layer is important to sustain mechanical forces that act on the cell (Rojas et al., 2018). The rigid outer-membrane contains covalently bound lipopolysaccharides (LPSs) which provides the cell envelope with a negative charge and is endotoxic for host species (Raetz & Whitfield, 2002). The Gram-positive, or monoderm, bacteria have only one cytoplasmic membrane but a thick cell wall, which contains lipoteichoic acids and a high percentage (as much as 60%) of wall teichoic acids (Swoboda, Campbell, Meredith, & Walker, 2010). The teichoic acids are long anionic polymers and give the cell envelope an overall negative charge. Therefore, these are considered to the LPS-equivalent in Gram-positive bacteria.

The cell wall is, with few exceptions, an essential part of the cell envelope and dictates the shape of the bacterium (Höltje, 1998; Vollmer, Blanot, & de Pedro, 2008). An important constituent of the cell wall in both Gram-positive and negative cells is peptidoglycan (PG). The PG forms a meshwork-like structure that envelopes the cell, and which in essence

forms a single gigantic molecule called the murein sacculus (Höltje, 1998). Peptidoglycan strands are composed of monomers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). These strands are covalently cross-linked via peptide stems that emerge from the MurNAc subunits. The composition of the peptide stem and the degree of cross-linking varies tremendously among bacterial species and can also vary along with changes in environmental conditions (Cava & de Pedro, 2014; Vollmer, Blanot, et al., 2008).

The PG biosynthetic pathway can be spatially divided into three phases: a cytoplasmic phase, a membrane-associated phase and a periplasmic phase. In the cytoplasmic phase, precursor molecules UDP-GlcNAc and UDP-MurNAc-pentapeptide are formed. All steps are regulated by a cascade of mainly Mur enzymes: a family of transferases (MurA), dehydrogenases (MurB), amino acid ligases (MurC, MurD, MurE, MurG, and Ddl), racemases (Alr, DadX, and MurI) and glycosyltransferases (MraY, MurG) (Barreteau et al., 2008; Typas, Banzhaf, Gross, & Vollmer, 2012). In the next phase, the cytoplasmic product UDP-MurNAc-pentapeptide is tethered to the lipid membrane via the transport lipid bactoprenol (C55- isoprenoid undecaprenyl phosphate), thereby forming the precursor lipid I. Attachment of UDP-GlcNAc to lipid I via MurG leads to the formation of lipid II. Lipid II is then transported to the other side of the cytoplasmic membrane. The identity of the transporter or flippase in the PG biosynthesis pathway remains controversial, although studies highlight FtsW (Mohammadi et al., 2011), MurJ (Ruiz, 2008; Sham et al., 2014) and/or Ami (Meeske et al., 2015) as possible candidates.

In the final phase of PG synthesis, lipid II monomers are polymerized into PG strands by glycosyltransferases in the periplasmic space (Typas et al., 2012). These glycan strands are then incorporated into the pre-existing sacculus structure and cross-linked by transpeptidases. These glycosyltransferases and transpeptidases are referred to as penicillin binding proteins (PBPs) after their affinity for the antibiotic penicillin, which inhibits the activity of these PG synthases (Sauvage, Kerff, Terrak, Ayala, & Charlier, 2008; Vollmer & Bertsche, 2008). Recently, a new family of PG synthases was described: the SEDS (shape, elongation, division and sporulation) proteins (Cho et al., 2016; Leclercq et al., 2017; Meeske et al., 2016). In contrast to the PBPs, the SEDS proteins are relatively insensitive to antibiotics known to inhibit PBPs. Instead, the SEDS are affected by a different class of bioactive compounds, and thus may be an interesting target for novel antibiotics (Emami et al., 2017).

2.2 Elongation and cell division

The cell wall is a highly dynamic structure. In order for cells to grow, incorporation of new cell wall material into the pre-existing PG needs to be spatially and temporally controlled. The growth of the sacculus of a rod-shaped bacterium is orchestrated by two multiprotein complexes: one is responsible for elongation of the cell whereas the other synthesizes the PG septum for cell division (Fig. 1).

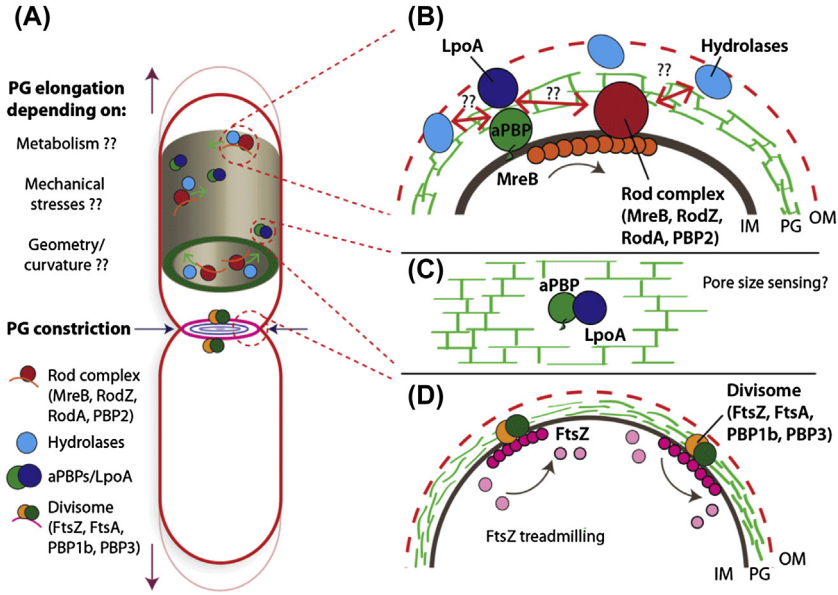

Fig. 1 ***Overview of cell wall synthesis in rod-shaped bacteria.*** (A) Schematic overview of a rod-shaped cell in which the rod complex, hydrolases, penicillin-binding proteins (aPBPs) and the divisome are indicated. Cell growth is affected by several factors, including the metabolic state and geometry of the cells, and various forms of stress. (B) Inset showing the cell wall synthesis machineries involved in cell elongation. These include the Rod complex, containing the transglycosylase RodA and the transpeptidase PBP2, which function in concert with the MreB filaments. In addition, the aPBPs are shown, which were recently shown to work in a partially independent manner from the rod complex (Cho et al., 2016). Hydrolases may contribute to the synthesis process by cleaving old layers of peptidoglycan. It remains unknown if the hydrolases interact with members of the Rod complex or the aPBPs. (C) The bifunctional aPBPs are activated by the outer membrane proteins LpoA and LpoB (Paradis-Bleau et al., 2010; Typas et al., 2010). The activated aPBPs may detect holes in the PG. (D) Cell division is mediated by the divisome (containing PBP1b and PBP3), which is guided by treadmilling FtsZ filaments. *This figure was reproduced with permission from van Teeffelen, S., & Renner, L. D. (2018). Recent advances in understanding how rod-like bacteria stably maintain their cell shapes.* F1000Research, *7, 241.*

Here we provide an overview of these complexes, for detailed information on all components involved and their interactions the reader is referred to other excellent reviews (den Blaauwen, de Pedro, Nguyen-Distèche, & Ayala, 2008; Egan, Cleverley, Peters, Lewis, & Vollmer, 2017; Typas et al., 2012).

The complex that is driving sacculus elongation or lateral growth is named the elongosome and is associated with the cytoskeletal protein MreB. The MreB protein forms short filaments, which are dynamically treadmilling circumferentially around rod-shaped cells (Dominguez-Escobar et al., 2011; Garner et al., 2011; van Teeffelen & Gitai, 2011). Treadmilling of MreB depends on the activity of the cell wall synthesis machinery and is hence abolished upon interruption of cell wall synthesis (Dominguez-Escobar et al., 2011; Garner et al., 2011; van Teeffelen et al., 2011). After a period of cell elongation, the process of cell division is initiated. Cell division starts with the positioning of the conserved FtsZ protein at midcell, guided by the activity of the Min system and nucleoid exclusion (Bernhardt & de Boer, 2005; Dajkovic, Lan, Sun, Wirtz, & Lutkenhaus, 2008; Wu & Errington, 2004). Polymerization of FtsZ at the future division site results in the formation of short filaments, which dynamically treadmill around the circumference of the cell and form a ring structure (the Z ring) (Bisson-Filho et al., 2017; van Teeffelen & Renner, 2018). Not the complete ring, but the short FtsZ filaments itself are sufficient to initiate constriction of the cell envelope and synthesis of septal PG (Yao et al., 2017). The FtsZ filaments are tethered to the membrane by FtsA and ZipA, the latter being an *Escherichia coli*-specific protein (Errington, Daniel, & Scheffers, 2003; Hale & de Boer, 1997; Jensen, Thompson, & Harry, 2005; Pichoff & Lutkenhaus, 2005). FtsZ functions as a scaffold for other members of the so-called divisome complex (Natale, Pazos, & Vicente, 2013). When the assembly of the divisome is complete, the division-specific PG synthases will make septal PG that will develop into the new poles of the daughter cells. The actual process of cell separation is mediated by various endopeptidases, amidases and hydrolases (Heidrich et al., 2001; Morlot, Uehara, Marquis, Bernhardt, & Rudner, 2010; Priyadarshini, Popham, & Young, 2006).

2.3 Bacterial shape determination

Bacterial shape is dictated by the structure of the sacculus. The growth of the sacculus is driven by elongation via the MreB protein, whereas cell division is orchestrated by FtsZ. The MreB filaments localize to regions with a

negative curvature, but they are excluded from the cell pole due to the presence of anionic phospholipids in this region (Hussain et al., 2018; Kawazura et al., 2017; Ursell et al., 2014). As cell wall insertion straightens the curved regions, it generates and maintains a rod-shaped cell in a self-correcting manner (Ursell et al., 2014). For example, MreB localizes at regions with negative curvature during the reversion of spherical wall-less cells (also referred to as L-forms, see Section 4.1 of this review) (Billings et al., 2014). When MreB is inhibited, the L-form cells revert to a walled cell but are unable to generate a rod-shaped cell (Billings et al., 2014).

However, MreB is not the only driver of PG localization. Several bacterial species such as *Streptomyces*, *Corynebacterium* and *Mycobacterium*, utilize an MreB-independent method for growth (Flärdh, 2010). These species have a polar growth mechanism, which relies on the localization of the protein DivIVA (Flärdh, 2003). This coiled-coil protein also has a preference for negatively curved membranes, but in contrast to MreB, it localizes to the cell poles and is required for polar cell wall synthesis (Edwards, Thomaides, & Errington, 2000; Flärdh, 2003; Lenarcic et al., 2009; Letek et al., 2008). In the filamentous bacterium *Streptomyces coelicolor*, DivIVA forms a multiprotein complex together with cytoskeletal proteins Scy and FilP, called the polarisome. This complex guides PG synthesis at existing hyphal tips and induces *de novo* tip generation during branching (Ausmees, 2013; Holmes et al., 2013).

Another shape-determining mechanism has been described for *Caulobacter crescentus*. This Gram-negative bacterium has a characteristic curved shape that is maintained by crescentin. This intermediate filament-like protein polymerizes and spans the length of the cell along inner curvature of the cytoplasmic membrane (Ausmees, Kuhn, & Jacobs-Wagner, 2003). Crescentin applies physical force onto the cell, which directs and distributes cell wall growth and hence a curved shape (Cabeen et al., 2009; Wolgemuth et al., 2005).

Besides redirection of PG synthesis machinery with cytoskeletal proteins, even small alterations in the sacculus can alter the cell shape, as is shown in *E. coli* where a lesser degree of crosslinking leads to curvature of the cell (Huang, Mukhopadhyay, Wen, Gitai, & Wingreen, 2008). Even PG polymerization itself can drive shape formation. It has been shown that the physical force accompanying PG polymerization can enable membrane invagination during endospore formation in *Bacillus subtilis* (Meyer, Gutierrez, Pogliano, & Dworkin, 2010). A combination of cytoskeletal elements, protein complexes, PG hydrolysis and synthesis can drive sacculus

(de)formation and thus determine bacterial shape. Morphogenesis or shape changes can therefore be induced by influencing the proteins and components mentioned above. As described throughout this review, it is likely that stress can affect these components and thus induce sacculus restructuring and shape alterations.

2.4 Genome organization

The genomes of bacteria are compactly folded and functionally organized inside a structure referred to as the 'nucleoid' (Dame & Tark-Dame, 2016; Travers & Muskhelishvili, 2005). This structure is not membrane-confined contrary to its eukaryotic equivalent, the nucleus. Analogous to eukaryotic chromatin, bacterial chromatin is also organized at different length scales by a variety of mechanisms, of biological or physico-chemical nature (Dame, 2005; Dame, Kalmykowa, & Grainger, 2011; Dame & Tark-Dame, 2016; Dillon & Dorman, 2010; Dorman, 2013; Luijsterburg, Noom, Wuite, & Dame, 2006; Travers & Muskhelishvili, 2005). Whereas the molecular details may differ, overall many features of genome organization are shared between different bacterial species. In *E. coli*, at the Mbp scale (0.5−1.5 Mbp), genomes are organized in 6 macrodomains: an Ori and a Ter domain encompassing the origin and terminus of replication, Left and Right domains on the left and right replichores respectively, and two non-structured domains flanking the Ter domain (Boccard, Esnault, & Valens, 2005). During recent years, domains of smaller size (Chromosomal Interaction Domains (CIDs); 30−500 kbp) have been identified in a variety of bacterial species: *E. coli* (Lioy et al., 2018), *B. subtilis* (Marbouty et al., 2015) (Wang et al., 2015), *C. crescentus* (Le, Imakaev, Mirny, & Laub, 2013), *Vibrio cholerae* (Val et al., 2016) and *Mycoplasma pneumoniae* (Trussart et al., 2017). Yet smaller domains, microdomains (∼10 kbp), have been shown to exist in *E. coli* (Postow, Hardy, Arsuaga, & Cozzarelli, 2004) and *Salmonella typhimurium* (Deng, Stein, & Higgins, 2004; Higgins, Yang, Fu, & Roth, 1996). The function of these different types of domains is to date unclear, but it has been shown that domains and their boundaries are affected by transcription and DNA supercoiling state (Le et al., 2013; Le & Laub, 2016), suggesting a functional organization of genes, as well as a possible role for transcription in shaping the genome.

2.5 Bacterial chromatin proteins

Already in the 1970s, numerous mostly small and basic proteins were found associated with bacterial genomes. For a long time, these proteins were

referred to as histone-like proteins due to their superficial similarity to eukaryotic histones and their proposed similar role (Drlica & Rouviere-Yaniv, 1987). To date, this family of proteins is referred to as bacterial chromatin proteins or nucleoid-associated proteins (NAPs) (Dame, 2005; Dame & Tark-Dame, 2016; Dillon & Dorman, 2010; Dorman, 2014). Even though this family of proteins includes over 10 different members, only a subset of these proteins has been extensively investigated in terms of their ability to organize genomes. We have to distinguish between proteins that predominantly bind unspecific to DNA (generally expressed at high levels, thousands of copies), and proteins with sequence specificity (generally expressed at much lower levels) (Ali Azam, Iwata, Nishimura, Ueda, & Ishihama, 1999; Ishihama et al., 2014). Depending on their DNA binding properties, these proteins 1) have generic roles in genome organization, modulating transcription of numerous genes by their global structural role, or 2) defined roles in transcription regulation by altering the local structure (generally at the promoters) of genes and operons, and less clear roles in genome organization. In *E. coli,* chromatin proteins H-NS and HU are expressed at high levels and lack sequence specificity, suggesting generic roles in genome organization. Other proteins, such as IHF and FIS, exhibit sequence specificity, are expressed at low to intermediate levels and are known to be involved in specific regulation of genes. Structurally, these proteins fall into two distinct classes: 1) DNA bridging proteins (H-NS) and 2) DNA bending proteins (HU, IHF and FIS) (Dame, 2005; Luijsterburg et al., 2006; Luijsterburg, White, van Driel, & Dame, 2008). The ability of H-NS to bridge multiple DNA segments suggests a role of this protein in genome organization involving the formation of loops (Dame, Wyman, & Goosen, 2000; Noom, Navarre, Oshima, Wuite, & Dame, 2007). DNA bending proteins reduce the effective volume occupied by a DNA molecule by introducing bends at many sites along the chain (Skoko, Wong, Johnson, & Marko, 2004; van Noort, Verbrugge, Goosen, Dekker, & Dame, 2004). Another important player in genome organization is condensin. Condensins can also bridge multiple DNA segments, but they function through an embrace-type mechanism (Gruber, 2017; Hirano, 2016). This results in DNA loops which are thought to actively grow in size in a process referred to as loop extrusion (Hassler, Shaltiel, & Haering, 2018; Nichols & Corces, 2018). This mechanism has been confirmed for yeast condensin (Ganji et al., 2018). Whether loop extrusion occurs *in vivo* in bacteria has yet to be established. At least for HU, it has already been demonstrated that the protein plays a role in global compaction of the genome *in vivo*, in line with its ability to bend DNA. This effect was shown directly

by fluorescence microscopy: by using fluorescent pairs of genomic markers, the physical distance was shown to be consistently larger in HU knockout strains in *C. crescentus* (Dame, Tark-Dame, & Schiessel, 2011; Hong & McAdams, 2011). These observations were recently further confirmed using Chromosome Conformation Capture (3C)-based technology in *C. crescentus* (Le et al., 2013) and in *E. coli* (Lioy et al., 2018).

Similarly, FIS was shown to play a role in global compaction of the *E. coli* genome. It remains to be established what structural role H-NS actually plays *in vivo*, as the effects of H-NS knockout are known to be at least partially compensated by upregulation of the paralogue StpA, which was not accounted for (Lioy et al., 2018). Different chromatin proteins operate at different length scale, based on the observations in different knockout strains. This may be related to a hierarchical organization of the folded genome over different length scales as discussed in Section 2.4. It is important to keep in mind that most of the bacterial chromatin proteins play direct or indirect roles in gene regulation, making it very hard to establish functional correlations and mechanisms of operation through protein knockout experiments. Note that of the proteins discussed above, only HU/IHF and SMC proteins are generally conserved. H-NS/StpA is poorly conserved at the sequence level, but appears to be conserved at the structural/functional level: the Rok-protein of *B. subtilis* (Smits & Grossman, 2010), the MvaT-proteins of *Pseudomonas* sp. (Tendeng, Soutourina, Danchin, & Bertin, 2003) and Lsr2-proteins of mycobacteria (Gordon, Imperial, Wang, Navarre, & Liu, 2008) all have DNA binding and regulatory properties very similar to H-NS.

2.6 Genome segregation during division

To ensure faithful propagation of genomic material to the daughter cells, the genome needs to be replicated and - at the time of cell division — be fully segregated. Replication initiates at the origin of replication (ori) and proceeds bi-directionally along the two arms of circular bacterial genomes until the terminus of replication (Ter). In rod-shaped cells, following replication, the two ori's split and move toward their new locations in each half of the growing cell (Badrinarayanan, Le, & Laub, 2015; Reyes-Lamothe, Nicolas, & Sherratt, 2012). It has been argued that no 'active' process is required for segregation, i.e. that it could be 'passively' driven by an increase in entropy in rod shaped cells (Jun & Wright, 2010). However, segregation can be actively driven by factors promoting tethering of the origin to the distant pole of a forming daughter cell. Such tethering has been demonstrated for

C. crescentus (Jensen & Shapiro, 1999), sporulating *B. subtilis* (see below) (Ben-Yehuda et al., 2005; Wu & Errington, 2003) and chromosome I of *Vibrio cholera* (Fogel & Waldor, 2005). No evidence for active chromosome segregation due to tethering has been detected in *E. coli* (Nielsen, Ottesen, Youngren, Austin, & Hansen, 2006; Wang, Liu, Possoz, & Sherratt, 2006) or in vegetative *B. subtilis* cells (Berkmen & Grossman, 2007; Teleman, Graumann, Lin, Grossman, & Losick, 1998).

3. Key morphological stress responses

3.1 Bacterial cell envelope stress response

The cell envelope of a bacterium is in direct contact with the cell's environment and the first line of defense against stressors. Sudden temperature shocks, changes in the pH or changes in the amount of osmolytes can stress the cell envelope. In both Gram-positive and Gram-negative bacteria, perturbations in the integrity of the cell envelope often initiate a so-called cell envelope stress response (CESR) (Jordan, Hutchings, & Mascher, 2008; Raivio, 2005). In Gram-negative bacteria, the CESR is also triggered when cell envelope proteins in the periplasm are damaged or malfunctioning (Raivio, 2005). Upon activation, the CESR-linked regulatory systems will try to counteract the induced damage. The regulatory systems underlying this stress response are conserved, and the best-known ones are two component systems (TCSs) and extracytoplasmic function (ECF) sigma factors. TCS are comprised of a membrane-associated sensor kinase that autophosphorylates before transferring the phosphoryl group to its cognate response regulator (Stock, Robinson, & Goudreau, 2000).

The best-known example of a two-component system involved in envelope damage is the Cpx stress response. It gets activated when the integrity of the cell envelope is affected by salt, metals, lipids, misfolded proteins and cell wall targeting antibiotics (Delhaye, Collet, & Laloux, 2016; Hunke, Keller, & Müller, 2012). This regulatory system controls over 100 different genes, many of which are involved in production of macromolecular surface structures, such as pili and fimbriae (Rowley, Spector, Kormanec, & Roberts, 2006). Constitutive activation of Cpx in *E. coli* leads to upregulation of PG-modifying enzymes YgaU and LdtD. This, in turn, leads to an increase in DAP-DAP cross links, which might add to stabilization of the cell wall (Bernal-Cabas, Ayala, & Raivio, 2015). Another example of how a TCS affects cell wall homeostasis, and thereby its morphology, has been

described for *Corynebacterium glutamicum*. Under influence of hyperosmotic conditions, the CESR is induced by MtrB kinase-dependent activation of the MtrA response regulator (Möker et al., 2004; Möker, Krämer, Unden, Krämer, & Morbach, 2007; Möker, Reihlen, Krämer, & Morbach, 2007). The MtrAB system directly or indirectly influences cell wall homeostasis, since its deletion strongly affected morphology. Additionally, these cells were more sensitive to lysozyme, vancomycin and penicillin (but not ethambutol) (Möker et al., 2004). Furthermore, microarray analyses performed with the *mtrAB* mutant strain determined that some known targets are involved in uptake of compatible solutes, while other targets relate to cell wall metabolism (Möker et al., 2004). Taken together, these findings demonstrate that the MtrAB system is a crucial regulatory system responding to cell wall damage.

In *B. subtilis*, several ECF sigma factors have been identified that are implied in responding to cell envelope stress, including SigW, SigM, SigX and SigV. Some of these sigma factors appear to have overlapping regulons (Helmann, 2016). These regulons were mostly defined by exposing cells to different antibiotics, many of which target steps in the biosynthesis of peptidoglycan. For instance, the regulon of SigX (σ^X) is relatively small and contains the *dltABCDE* and *pssA-ydfM-psd* operons, both of which are involved in cell wall modification (Cao & Helmann, 2004). Expression of these operons under influence of stress lead to the D-alanylation of teichoic acids and incorporation of phosphatidylethanolamine into the cell membrane, which together makes the cell envelope more positively charged. This change creates resistance against cationic antibacterial peptides (Cao & Helmann, 2004; Perego et al., 1995). Bacterial CESR systems can be activated by a large range of stresses targeting the cell envelope (Jordan et al., 2008). Even though several studies have shown a link between a CESR system and cell wall homeostasis, it remains to be elucidated if, and how CESR networks play a role in adaptive morphogenesis.

3.2 Bacterial nucleoid stress response

Bacterial genome organization is dynamic and susceptible to environmental cues, thus potentially playing a key role in translating environmental signals into a physiological response. Environmental stresses result in morphological changes of the nucleoid as seen by microscopy. In some cases, these changes can be correlated with alterations in the amounts or activity of bacterial chromatin proteins.

Possibly one of the most common stresses encountered by bacteria is the exhaustion of nutrients. Such a situation is encountered during laboratory growth condition when the culture transitions from exponential growth to stationary phase, where growth ceases. In *E. coli,* this transition coincides with large changes in expression levels of a subset of NAPs. Specifically, during fast growth, the expression level of the DNA bending protein FIS is high (Ali Azam et al., 1999), which warrants high transcription and translation potential by its activation of stable RNA operons (Nilsson et al., 1992; Ross, Thompson, Newlands, & Gourse, 1990). Upon entry in stationary phase, Dps is highly expressed. This protein is thought to protect against harsh environmental conditions by physically sequestering the DNA inside a dense liquid crystalline structure (Frenkiel-Krispin et al., 2004; Wolf et al., 1999). This is thought to render the DNA less prone to damage induction, as well as by its ability to quench oxygen radicals (Karas, Westerlaken, & Meyer, 2015; Zhao et al., 2002). Similarly, the nucleoid in *Bacillus* and *Clostridium* spores is dramatically reshaped into a ring-like structure through the association of α/β-type SASP proteins, which are synthesized in developing spores late in sporulation. These proteins form about 5%—15% of the total protein content of spores (Setlow, 1988), and are degraded by a specific protease early in spore outgrowth. This yields amino acids as protein building blocks for novel proteins when macromolecular synthesis resumes (Sussman & Setlow, 1991; Traag, Pugliese, Setlow, Setlow, & Losick, 2013). In addition, spores contain significant amounts (50.000 copies/genome) of the HU protein, which is present in equal amounts in vegetative cells and spores (Ross & Setlow, 2000).

4. Morphological plasticity upon exposure to stress

Historically, bacterial species were identified and characterized by their shape. Nowadays we have come to a better understanding that bacteria can alter their morphology depending on their cell cycle stage, their surrounding environment and the presence of stressors. We now know that stressors can indeed initiate morphogenesis of cells. The change into specific bacterial shapes can be beneficial to overcome the stressor and survive in new environments. The bacterial cell envelope as well as the nucleoid respond to a variety of external stresses by activating protein cascades or metabolic pathways, which can in turn change the physiological state of a bacterium. These changes can directly or indirectly lead to an altered

morphology. In the following sections we will discuss examples of well-studied morphological responses that result from exposure to environmental insults (Table 1, Fig. 2).

4.1 Cell wall-deficient cells

As discussed in the previous sections, the cell wall is a highly conserved structure that protects cells from environment stresses, while also resisting the high outward-facing turgor pressure in the cells (Typas et al., 2012; Vollmer, Blanot, et al., 2008). As nearly all bacteria have a cell wall, countless antibiotics target this protective layer. Interestingly, several species are able to evade such stresses by adopting a cell wall-deficient life style as so-called L-forms. The first L-forms were described in 1935 and named in honor of the Lister Institute where they were discovered (Klieneberger, 1935). Following the first studies on L-forms, many terms have been used interchangeably to describe morphologically similar cells such as L-phase bacteria, L-variants, and cell wall defective forms.

L-forms can be broadly described as a *mutant* form of a walled bacterium that is able to grow and divide indefinitely without a cell wall. These cells adopt a spherical shape, which is their most energetically favorable conformation. L-form bacteria require an isotonic environment since they lack the turgor-withstanding cell wall. A few clinical reports have described L-form cells in patient samples (Onwuamaegbu, Belcher, & Soare, 2005), and some studies have reported bacterial L-forms in plant tissue (Ferguson, Booth, & Allan, 2000). L-forms are generated in laboratory settings by cultivation of walled cells in osmoprotective media together with agents that degrade or interfere with cell wall synthesis, such as lysozyme and penicillin G (Allan, Hoischen, & Gumpert, 2009; Errington, 2013; Errington, Mickiewicz, Kawai, & Wu, 2016). Prolonged exposure in such media can cause bacterial cells to accumulate mutations which promote a cell wall-deficient (CWD) life style (Allan et al., 2009; Errington et al., 2016; Leaver, Dominguez-Cuevas, Coxhead, Daniel, & Errington, 2009; Mercier, Kawai, & Errington, 2014; Ramijan et al., 2018). These mutations fall in two separate classes: some mutations lead to the upregulation of membrane synthesis, while other mutations alleviate oxidative stress levels in the cells. Whether the wall-less bacteria identified in patient and plant samples had accumulated these mutations or contained a natural adaptation response system has not been explored.

Unlike walled bacteria, L-forms are able to propagate independently of the FtsZ-based division machinery (Leaver et al., 2009). Instead, these cells

Table 1 Overview of stress-induced bacterial cell morphologies. See text for further explanation, details and references.

Cell type	Signature morphology	Examples	Examples of inducing condition(s)
L-forms	Cell wall-deficient cells	*Escherichia coli* *Bacillus subtilis* *Staphylococcus aureus* *Listeria monocytogenes* *Pseudomonas aeruginosa* *Mycobacterium bovis* *Filamentous actinomycetes*	Cell wall targeting antibiotics, lysozyme, osmotic stress, cryogenic stress, nutrient starvation, hyperosmotic stress
Filamentous cells	Elongation of cells	*Escherichia coli* *Mycobacterium tuberculosis* *Salmonella typhimurium* *Caulobacter crescentus* *Campylobacter jejuni* *Legionella pneumophila* *Burkholderia pseudomallei*	Host-induced DNA-damage, starvation, stationary phase, antibiotics
Spores	Dormant state, multiple structural layers, dehydration and DNA condensation	*Streptomyces,* *Bacillus,* *Clostridium*	Starvation
VBNC	Change from rod-shaped to coccoid morphology	*Escherichia coli* *Vibrio cholerae,* *Helicobacter pylori* *Campylobacter jejuni* *Enterococcus faecalis* *Vibrio vulnificus* *Salmonella typhimurium*	Starvation, osmotic shock, low or high temperatures, acidity, aerobiosis
Swarmer cells	Increased number of flagella, elongation	*Escherichia coli* *Salmonella typhimurium* *Proteus mirabilis* *Vibrio parahaemolyticus*	Encounter of solid surface or viscous environment
Stalked cells	Transition from motile into sessile cells with one or multiple stalks and holdfast	*Caulobacter crescentus* *Hyphomonas* species *Brevundimonas* species *Planctomycetes* *Verrucomicrobia*	Integral part of the life cycle, (phosphate) starvation

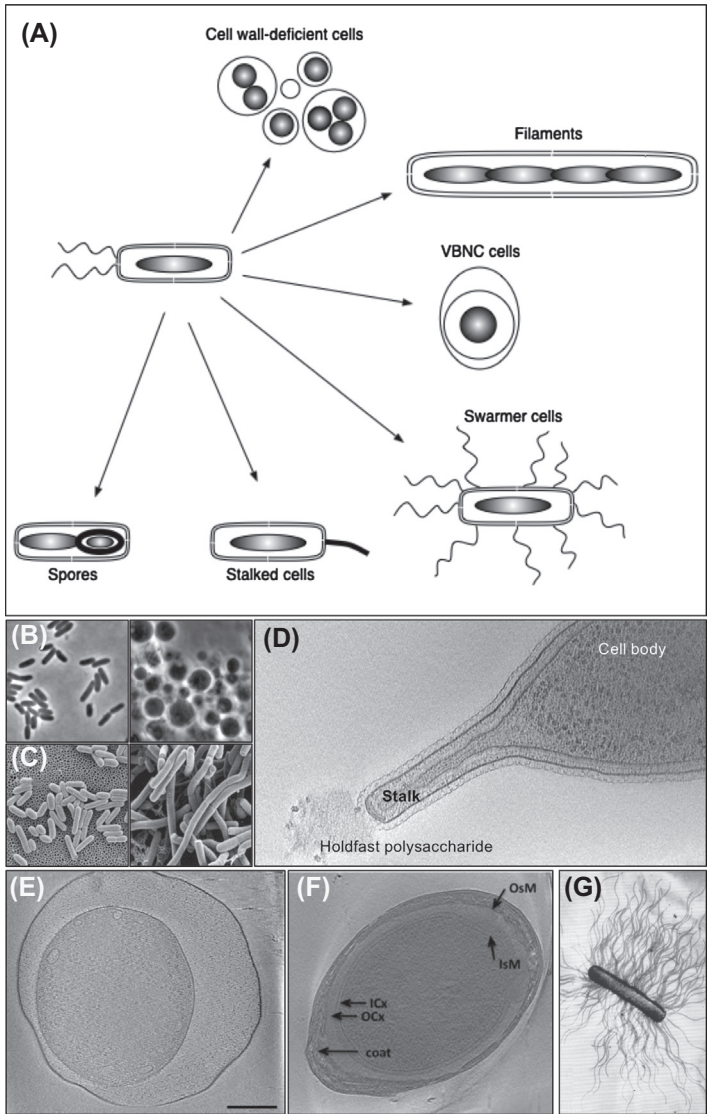

Fig. 2 ***Examples of stress-induced morphological adaptations in bacteria.*** (A) Schematic overview of the morphological adaptations of a bacterial cell to various stresses, including the formation of cell wall-deficient cells, filaments, viable but non-culturable (VBNC) cells, swarmer cells, stalks and spores. (B) Morphology of *E. coli* MG1655 in the absence (left) or presence (right) of D-cycloserine. Addition of this antibiotic causes *E. coli* to become cell wall-deficient. (C) Morphology of *Listeria monocytogenes* grown at pH 7.0 (left) or pH 5.0 (right). The low pH induces filamentation. (D) Stalk formation in *Caulobacter crescentus*. Please note the holdfast polysaccharide at the end of the stalk. (E) Viable but non-culturable cell of *Vibrio cholerae* from salt water. (F) Spore of

use a so-called extrusion-resolution system for proliferation, during which numerous progeny cells are more or less simultaneously released from the mother cell. Additionally, L-form cells may propagate by means of blebbing, tubulation and vesiculation, which give rise to a morphologically heterogeneous population of cells (Briers et al., 2012; Cambré et al., 2014; Dell'Era et al., 2009; Koonin & Mulkidjanian, 2013; Leaver et al., 2009; Mercier, Dominguez-Cuevas, & Errington, 2012; Mercier et al., 2014; Ramijan et al., 2018; Studer et al., 2016). The mode by which L-forms proliferate is conserved in Gram-positive and Gram-negative bacteria and appears to be based solely on biophysical principles. The excessive membrane production in L-form cells drives an imbalance between the growth of the cell surface area and internal volume, leading to vesicle extrusion (Mercier et al., 2014).

Apart from exposure to cell wall-targeting agents, other stress-inducing conditions can lead to a transient or permanent cell wall-deficient state. The filamentous bacterium *Kitasatospora viridifaciens* and related actinomycetes, are able to extrude CWD cells when exposed to hyperosmotic stress (Ramijan et al., 2018). These so-called S-cells (for Stress-induced cells) are not able to proliferate in the CWD state and will revert to their walled state when stress is removed. However, prolonged exposure to hyperosmotic stress can lead to the acquisition of mutations and the consequent formation of variants that are indistinguishable from L-forms. Likewise, the rod-shaped *Mycobacterium bovis* undergoes a dramatic morphological transition when exposed to harsh conditions such as cryogenic stress and

Bacillus subtilis, which reveals the outer and inner spore membranes (OsMs and IsM, respectively), the inner and outer cortex (ICx and OCx, respectively), and the coat. (G) Swarmer cell of *Proteus mirabilis*. *All images were reproduced with permission from Mercier, R., Kawai, Y., & Errington, J. (2014). General principles for the formation and proliferation of a wall-free (L-form) state in bacteria.* Elife, *3. Jones, T. H., Vail, K. M., & McMullen, L. M. (2013). Filament formation by foodborne bacteria under sublethal stress.* International Journal of Food Microbiology, 165*(2), 97–110. Bharat, T. A. M., Kureisaite-Ciziene, D., Hardy, G. G., Yu, E. W., Devant, J. M., Hagen, W. J. H., et al. (2017). Structure of the hexagonal surface layer on Caulobacter crescentus cells.* Nature Microbiology, 2*, 17059. Brenzinger, S., van der Aart, L. T., van Wezel, G. P., Lacroix, J. M., Glatter, T., & Briegel, A. (2018).* Structural and proteomic changes in viable but non-culturable Vibrio cholerae. *bioRxiv, p. 433326. Tocheva, E. I., López-Garrido, J., Hughes, H. V., Fredlund, J., Kuru, E., Vannieuwenhze, M. S., et al. (2013). Peptidoglycan transformations during* Bacillus subtilis *sporulation.* Molecular Microbiology, 88*(4), 673–686. Hoeniger, J. F. (1965). Development of flagella by* Proteus mirabilis. Journal of General Microbiology, 40*(1), 29–42 for panels B, C, D, E, F and G, respectively.*

nutrient starvation, resulting in giant filamentous structures which appear to produce and release spherical L-form cells via budding and fission (Slavchev, Michailova, & Markova, 2013). These morphological variances offer an alternative route for the cell to survive and reproduce while exposed to stress.

4.2 Filamentation

Bacteria elongate and divide, but stressful circumstances can interrupt the cell separation process and give rise to long filamentous cells. Filamentation, the process by which generally bacillary, rod- or coccoid shaped cells transition into long cells containing one or multiple chromosomes, is often associated with entering a host environment. These filamentous cells can reach a length of more than 30 μm for *Legionella pneumophila* (Prashar et al., 2012) or even 70 μm as described for uropathogenic *E. coli* (UPEC) (Justice et al., 2004).

Filamentation of cells can be induced via the so-called SOS response that senses DNA damage. The SOS response system was discovered in the model bacterium *E. coli* and involves the proteins RecA and LexA (Gudas & Pardee, 1975; Janion, 2001; Little & Mount, 1982). RecA is a DNA recombinase that effectively binds to single-stranded DNA gaps and other perturbations in the DNA that result from DNA damage caused by for instance pH changes or starvation (Cox, 2003). DNA-bound RecA proteins are then able to assemble into filaments and thereby acquire protease activity (Cox, 2003; Menetski & Kowalczykowski, 1989). Such activated RecA catalyses the cleavage of the transcriptional repressor protein LexA (Horii et al., 1981; Little, 1984). LexA represses the SOS-genes, which encode proteins involved in DNA damage control and repair. The LexA-binding motifs have been studied in bacteria across different phyla, but appear not universally conserved and highly complex, as a result the downstream effectors could differ between bacterial species (Erill, Campoy, & Barbé, 2007). In *E. coli* for example, the LexA-repressed SOS-genes encode DNA-polymerases for the DNA repair process, but also the cell-division inhibitor SulA. The presence of SulA inhibits polymerization of FtsZ and thereby blocks cell division, avoiding transmission of the damaged DNA to the daughter cells (Janion, 2001; Mukherjee, Cao, & Lutkenhaus, 1998; Trusca, Scott, Thompson, & Bramhill, 1998). This SulA-dependent inhibition of cell division explains the long filamentous cells (Justice et al., 2008). Filamentation as a result of the stress-activated SOS-response is also involved in virulence. UPEC employ the SOS response to differentiate into filamentous

cells as part of their life cycle inside the mammalian host (Justice, Hunstad, Seed, & Hultgren, 2006). This SulA-mediated filamentation is required for virulence and supports survival in both macrophages and polymorphonuclear leukocytes (PMNs), which preferentially target non-filamentous UPEC cells (Horvath et al., 2011; Justice et al., 2004). The filamentous cells cannot be engulfed by the phagocytotic cells. Only when septation and division of the filamentous cells resumes are the daughter cells phagocytosed (Justice et al., 2004). The pathogenic *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) also initiates filamentation via the SOS response system. This intracellular pathogen infects macrophages, where it adopts a filamentous morphology caused by the phagocyte's oxidative radicals that induce DNA damage and trigger the SOS response (Eriksson, Lucchini, Thompson, Rhen, & Hinton, 2003; Rosenberger & Finlay, 2002). Filamentation has also been observed in *Mycobacterium tuberculosis*, the causative agent of tuberculosis, as a result from exposure to intracellular stresses inside macrophages (Chauhan et al., 2006).

Filamentation is not only restricted to intracellular pathogens: environmental stresses can also lead to the formation of filaments. The morphologically diverse *C. crescentus* is typically found as a motile swarmer or as a sessile, stalked cell. Additionally, they can also adopt an elongated, helical morphology (Wortinger, Quardokus, & Brun, 1998). In the late stationary phase, when nutrients become scarce, the cells elongate and become more resistant to pH fluctuations, hydrogen peroxide and heat compared to the shorter cells (Wortinger et al., 1998). When filamentous *C. crescentus* cells are resuspended in fresh media, they revert to their normal shape. Similarly, the helical shaped pathogen *Campylobacter jejuni* adopts a filamentous morphology when cells enter the stationary phase (Ghaffar, Connerton, & Connerton, 2015; Thomas, Hill, & Mabey, 1999). For *C. jejuni* and related food-borne pathogens, however, it has been hypothesized that filamentation could be a strategy by which cells are prepared for rapid division and multiplication, thereby maximizing their ability to spread and cause disease (Jones, Vail, & McMullen, 2013).

In addition to oxidative radicals and starvation-related stresses, filamentation can also be induced by antibiotics. Non-filamentous *Burkholderia pseudomallei* bacteria that were exposed to the clinical antibiotics ceftazidime, ofloxacin, and trimethoprim at a concentration below the minimal inhibitory concentration (MIC), turned into viable filaments. Upon removal of antibiotic pressure, the cells reverted back to a normal cell shape (Chen, Sun, Chua, & Gan, 2005).

Filamentation can have another important consequence. In *E. coli*, administration of sub-MIC concentrations of the genotoxic antibiotic ciprofloxacin also induces the SOS response and subsequent filamentation (Bos et al., 2015). These filaments were shown to contain multiple chromosomes and could create bacillary progeny via budding. Strikingly, these progeny cells showed an enhanced resistance to ciprofloxacin as a result of chromosomal recombination that had occurred within the filaments. These results stress the implications of sub-MIC administration and the role of filamentous cells in the development of antibiotic resistance (Bos et al., 2015).

All examples of filamentation of bacterial cells mentioned above give an indication of the various stressors or changes in the physiological state of the bacterium that can lead to filamentation. For many bacterial species, the SOS response system is an important mechanism by which filamentation is established, for others the underlying mechanism remains to be elucidated. Nevertheless, filamentation appears a widespread phenomenon among bacteria by which their change in morphology enhances the survivability of the bacteria.

4.3 Viable but non-culturable cells

In response to a large variety of environmental stresses, some bacteria transit in to a so-called viable but non-culturable (VBNC) state (Pinto, Santos, & Chambel, 2015). In this state, the cells remain minimally metabolically active but do not proliferate when transferred to nutrient rich environments. The VNBC state of *E. coli* and *V. cholerae* have been described as early as in 1982 (Xu et al., 1982). Ever since, there have been many species described that are able to adopt the VNBC state (Oliver, 2005; Pinto et al., 2015). For some species, this transition is accompanied by a transition from a rod-shaped morphology into a coccoid morphology (Brenzinger et al., 2018, p. 433326; Chaiyanan et al., 2007; Pianetti et al., 2009). Transition into coccoid or spherical cells has been discussed to be potentially beneficial for nutrient uptake, as the cells generate a large surface area compared to a small cellular volume (Krebs & Taylor, 2011).

Bacteria in environmental reservoirs can transition into the VBNC state, but also pathogenic bacteria can change into VBNCs within their host (Yang et al., 2016). For instance, the gastric disease and ulcer-causing bacterium *Helicobacter pylori* shifts from its characteristic spiral shape into VBNC coccoid cells, under influence of stress conditions such as starvation, aerobiosis, and acid stress (Azevedo et al., 2007; Mizoguchi et al., 1998). Some studies report structural modifications of the peptidoglycan

that accompany this morphological transformation. Analysis of PG architecture revealed a substantial accumulation of the dipeptide monomers (N-acetylglucosaminyl-N-acetylmuramyl—L-Ala—D-Glu), and a decrease of tripeptide monomers (N-acetylglucosaminyl-N-acetylmuramyl—L-Ala—D-Glu-mesoDAP) in coccoid cells compared to the spiral form (Costa et al., 1999). The PG hydrolase AmiA is responsible for the dipeptide monomer accumulation. This PG modification provides an escape from the immune sensor (nucleotide oligomerization domain) Nod1, which senses only murein tripeptides or longer, and is unable to bind dipeptides (Chaput et al., 2006).

Modifications in the PG architecture were also described for *Enterococcus faecalis* cells in the VBNC state had an increased number of cross-links in its PG structure and double the amount of lipoteichoic acids compared to actively growing cells (Signoretto, Lleò, Tafi, & Canepari, 2000). The increased cross-linking is thought to result from the activity of PBP1 and the enterococcal specific PBP5, which remain active in the VBNC state. These modifications in VBNC cells generate a more rigid cell wall structure that is less prone to lysis when compared to that of actively growing cells (Signoretto et al., 2000). Cells in the VBNC state do not only modify their cell wall structure but can also have an altered membrane composition. *Vibrio vulnificus* cells enter the VBNC state when exposed to a temperature downshift. To maintain membrane fluidity the cells were found to produce more unsaturated and short chain fatty acids (Day & Oliver, 2004). When *de novo* fatty acid synthesis is inhibited, the *V. vulnificus* cells are not able to go into transition and do not survive (Day & Oliver, 2004). Alterations of the cell envelope thus contribute or are even required for the transition of bacteria into a VBNC state.

As previously mentioned, the food-borne pathogen *C. jejuni* has been shown to transform into filamentous cells upon entering the stationary phase. When the culture enters the decline phase, however, the filamentous cells will bleb off coccoid shaped cells with a decreased culturability (Thomas et al., 1999). Not only the age of the culture, but also exposure of the culture to heat (48 and 55 °C) induced a coccoid morphology and reduced culturability upon longer exposure times (Klančnik, Vučković, Jamnik, Abram, & Možina, 2014). *C. jejuni* also shifts into the coccoid form upon exposure to starvation, salinity and acidity (Jackson et al., 2009). This also results in a decreased infectious potential of *C. jejuni* (Klančnik et al., 2014). It remains to be elucidated how this morphological shape is regulated and whether it is advantageous for immune response escape.

So far, no universal molecular mechanism underlying entry of cells into the VBNC state is known. Few reports provide some information on this process in different species. One study showed that a *Salmonella* strain with a nucleotide polymorphism in the *rpoS* gene, which encodes the major stress regulator RpoS, was delayed in osmotic-shock induced VBNC morphogenesis, in comparison to strains without this mutation (Kusumoto, Asakura, & Kawamoto, 2012). In addition, disruption of *rpoS* results in the rapid induction of the VBNC state (Boaretti, Lleò, Bonato, Signoretto, & Canepari, 2003; Kusumoto et al., 2012). These results indicate that RpoS plays a major role in regulating cell fate in *Salmonella* bacteria. In for example *C. jejuni*, it was shown that polyphosphate kinase 1 (PPK1) plays an important role, as deletion of *ppk1* showed reduced numbers of VBNC formation under stress (Gangaiah, Kassem, Liu, & Rajashekara, 2009). In *E. coli* on the other hand, the osmosensor EnvZ is important for the transition into the VBNC state. *E. coli* bacteria lacking *envZ* could not enter the VBNC state when exposed to osmotic stress, pH and starvation (Darcan, Özkanca, Idil, & Flint, 2009).

Some bacteria can leave the VBNC state when conditions improve, and environmental stresses are removed. *E. coli* VBNCs can for example revert to their culturable state upon temperature change and amino acid supplementation (Pinto, Almeida, Almeida Santos, & Chambel, 2011). It has recently been shown that *E. coli* K12 VBNCs can be resuscitated by the take-up and usage of pyruvate as a carbon source (Vilhena et al., 2018). However, conditions that allow resuscitation have not been found yet for most species that can enter the VBNC state. The underlying molecular mechanisms of entry and exit from the VBNC state thus remains to be fully characterized and understood. Nevertheless, it is evident that the VBNC state is used by various species as an adaptation to survive stress.

4.4 Swarmer cells

Motile bacteria utilize their flagella to swim across an environment, searching for nutrients or favorable environments. When cells are challenged by dry surroundings or increased viscosity, they can morphologically differentiate into swarmer cells which are elongated and have typically an increased number of flagella (Kearns, 2010; Verstraeten et al., 2008). Compared to other types of surface-associated motility forms, swarming motility is the most rapid movement known to bacteria. It allows the cells to spread to the edge of a colony in order to find nutrients. Swimming is another flagella-driven behavior, the difference between swimming and swarming

is the execution of the behavior by an individual cell or as a group (Kearns, 2010). As swarming is a group-based behavior, it is not surprising that swarmer cell differentiation can not only be induced by challenging surfaces but can also be regulated via quorum sensing signals (Daniels, Vanderleyden, & Michiels, 2004).

Swarmer cell differentiation is associated with an increase in the number of flagella. Bacteria that swarm over robust surfaces typically generate more flagella than bacteria that differentiate into swarmer cells when swarming over viscous surfaces or soft agar (Partridge & Harshey, 2013). The viscous or temperate swarmers include peritrichously flagellated species as *E. coli* and *Salmonella*, which double their flagella number when differentiating into swarmer cells (Harshey & Matsuyama, 1994). The robust swarmers include polarly flagellated bacteria such as *Vibrio parahaemolyticus*, which revert to peritrichous flagellated cell type during swarm cell morphogenesis (M. R. Belas & Colwell, 1982). In peritrichous bacteria such as *E. coli* and *S. typhimurium* it remains unclear how this hyperflagellation is regulated as changes are subtle. For bacteria such as *V. parahaemolyticus,* the increase in flagella number is differently regulated as it concerns the activation of lateral flagella system in addition to their polar flagellum (Partridge & Harshey, 2013). In *V. parahaemolyticus* bacteria, the transcription of the *laf* gene cluster is activated upon encountering solid environments, resulting in the formation of peritrichous lateral flagella and swarming motility (R. Belas, Simon, & Silverman, 1986; McCarter & Silverman, 1990; Shinoda & Okamoto, 1977). The transcription of the *laf* genes appears to be linked to the rotation rate of the polar flagellum. The change from liquid to a more viscous environment can lead to a slower flagellar rotation rate, subsequent induction of the *laf* gene cluster and synthesis of lateral flagella (Belas et al., 1986; Kawagishi, Imagawa, Imae, McCarter, & Homma, 1996). *Proteus mirabilis* is another example of bacteria able to switch to swarming cells by utilizing the flagella to sense a solid surface and subsequently induce hyperflagellation (Belas & Suvanasuthi, 2005; Rather, 2005). In contrast, the flagella of *S. typhimurium* serve as sensors for the wetness of its surroundings and switches to swarm cell morphology accordingly (Wang, Suzuki, Mariconda, Porwollik, & Harshey, 2005). Upon transfer of these swarmer bacteria to liquid environments, they will readapt to their regular morphology.

Swarmer cell differentiation encompasses not only an increase in flagella number, but also generates elongated cells. Although this change in morphology is often described, exact mechanisms are not reported in detail. An early transposon study in *P. mirabilis* identified mutations responsible for

elongation in swarmer cells, and divided them in four categories: affecting either flagellar biosynthesis, LPS and cell wall biosynthesis, proteolysis of peptides or cell division (Belas, Goldman, & Ashliman, 1995). As the inhibition of cell division can cause filamentous cells (Section 4.2), it seems likely that suppression of cell division can lead to the elongation of swarmer cells. A more recent study on *P. mirabilis*, shows that differentiation into elongated swarmer cells can be controlled via the Rcs phosphorelay system, a cell envelope stress response (CESR) (Little, Tipping, & Gibbs, 2018). Disruption of the cell envelope integrity, by deleting the *rffG* gene which produces LPS-associated structures, inhibits swarmer cell differentiation. This phenotype is rescued by RcsB, a response regulator in the Rcs phosphorelay system which can activate the genes encoding for the MinCDE proteins involved in cell division (Howery, Clemmer, Şimşek, Kim, & Rather, 2015; Little et al., 2018). The Min proteins prevent the formation of the Z ring in rod-shaped cells and could thereby be involved in the production of elongated cells when activated by RcsB (Howery et al., 2015). It is therefore proposed that the CESR can guide the bacterial cells into swarmer cell morphogenesis, to help adapt and protect the cells against stressful conditions.

4.5 Stalked cells

Some bacteria belonging to the alpha-proteobacteria are able to synthesize one, or multiple tubular extensions of their cell envelope, called prosthecae or stalks (Jiang, Brown, Ducret, & Brun, 2014; Lawler & Brun, 2007; Stovepoindexter & Cohen-Bazire, 1964). The process of stalk formation has been best studied in *C. crescentus*, which synthesizes a single stalk on one of the cell poles (Curtis & Brun, 2010). Stalk formation in *C. crescentus* is an integral part of the dimorphic life cycle of this bacterium. The stalked cells elongate and replicate their DNA. In this late predivisional state, a flagellum is being assembled at the pole opposite of the stalk pole. Cell division and cell separation then yield two distinct cell types: one newborn, flagellated swarmer cell, and the progenitor stalked cell. The swarmer cells do not replicate DNA. To progress through the cell cycle, the cells eject their flagellum and synthesizing a stalk at the same cell pole (Curtis & Brun, 2010). Subsequently, the newly formed stalked cell will duplicate DNA and bud off a new swarmer cell.

Stalk formation is a morphological adaptation that may provide benefits for life in nutrient-poor environments. Indeed, stalks are more elongated under phosphate-starvation conditions (Lawler & Brun, 2007; Schmidt & Stanier, 1966), which led to the widely believed hypothesis that these

structures could act as nutrient scavenging antenna by increasing the cellular surface-to-volume ratio (Gonin, Quardokus, O'Donnol, Maddock, & Brun, 2000; Schmidt & Stanier, 1966). More specifically, phosphate would be sequestered from the environment and shuttled via the PstS protein through the lumen of the stalk to the cell body (Wagner, Setayeshgar, Sharon, Reilly, & Brun, 2006). However, this model is challenged by the discovery of protein complexes that form cross-bands in stalks-one per cell cycle, which limit diffusion of large molecules (Schlimpert et al., 2012). While this finding does not entirely disprove the assumption that stalks are involved in phosphate uptake, it makes it less likely that phosphate is transported in association with PstS through the stalk, as diffusion of proteins is restricted. However, small molecules may be able to traverse the proteinaceous cross-bands.

Stalks are also involved in surface colonization. More specifically, a strong adhesive substance, called holdfast, is secreted at the end of the stalk, allowing cells to firmly attach to a surface (Hernando-Pérez et al., 2018; Tsang, Li, Brun, Freund, & Tang, 2006). The holdfast is a complex polysaccharide containing N-acetylglucosamine (Merker & Smit, 1988). The holdfast and stalks enable the cells to position the majority of their cell body above an existing biofilm. Longer stalks could therefore contribute to exposing cells to richer nutrient environments, which, as a consequence, would be able to acquire nutrients more efficiently (Wagner & Brun, 2007). One further morphological adaptation that contributes to fitness to the sessile state is the crescent shape of *Caulobacter* cells (Persat, Stone, & Gitai, 2014). While straight *Caulobacter* cells had no apparent fitness loss in laboratory conditions (Ausmees et al., 2003), the cells' curvature appeared beneficial when cells were exposed to flow (Persat et al., 2014). The fluid flow orients the stalked cell such that it arcs over the surface to which the cell is attached. The curved shape of the cell thereby decreases the distance from the non-attached cell pole to this surface. As the cells progresses through the cell cycle, the newborn daughter cell has a more likely chance of sticking itself to this surface via pili that are present at the daughter cell pole. This demonstrates how multiple morphological adaptations contribute to fitness in the environment.

While *Caulobacter* are at present the best-studied stalk forming bacteria, other examples exist. For example, in the case of *Brevundimonas subvibrioides*, the single stalk appears less compartmentalized than in *Caulobacter* and can also be disassembled in case they are no longer needed (Curtis, 2017) For example, *Hyphomonas neptunium* also forms a single stalk. New

flagellated progeny is formed at the tip of the stalk, and the DNA has to be transported through the thin stalk into the new daughter cell (Cserti et al., 2017). Several other species have also reported to contain other stalk variants, such as for example slime or metal encrusted stalks (Kysela et al., 2016).

4.6 Spore formation

Nutrient scarcity is a common stressor in bacterial life. While flagellated bacteria may try to escape from such adverse conditions by swimming to more favorable environments, other bacteria will enter a developmental program by which they generate spores. Due to the presence of several additional cell surface layers, spores are well equipped to withstand the extreme conditions and can remain in their dormant state for hundreds of years (Gould, 2006; Henriques & Moran, 2007). Based on how spores are formed, two distinct spore types can be discriminated: endospores and exospores. Endospores are produced within the mother cell and occurs for instance in Firmicutes, while exospores are produced by numerous actinomycetes, and myxobacteria (Claessen, Rozen, Kuipers, Søgaard-Andersen, & van Wezel, 2014).

One of the earliest morphological changes that reveals the onset of endospore formation in *B. subtilis* is the formation of the so-called axial filament, whereby the segregating sister chromosomes adopt an elongated structure that stretches from pole-to-pole (Bylund, Haines, Piggot, & Higgins, 1993; Glaser et al., 1997). The axial filament is formed by capturing of the GC-rich centromere regions of the (partially) duplicated chromosomes by the RacA protein, which in turn is anchored to the DivIVA protein localized at the cell poles of the mother cell (Ben-Yehuda, Rudner, & Losick, 2003; Wu & Errington, 2003). This extended chromosome conformation was shown to be critical for trapping a complete chromosome into the future spore. Initially, the region around the origin of replication is captured in the developing forespore by RacA (Ben-Yehuda et al., 2003; Wu & Errington, 2003). The remaining part still resides in the mother cell and is actively translocated into the forespore by the activity of the DNA pump SpoIIIE, which resides at the leading edge of the closing septum (Bath, Wu, Errington, & Wang, 2000). In this manner, chromosome segregation is accurately coupled to completion of the asymmetric cell division event. Recent evidence indicates that SpoIIIE forms a coaxially paired channel, one for each chromosome arm, which ensure that the two arms of the forespore chromosome are transported simultaneously (Yen Shin et al.,

2015). As a result of this DNA translocation process, the forespore also inflates to its normal size (Lopez-Garrido et al., 2018).

Following chromosome segregation, the mother cell engulfs the forespore in a manner resembling phagocytosis, which is driven by cell wall remodeling (Ojkic, López-Garrido, Pogliano, & Endres, 2016; Tocheva et al., 2013). During this process, the forespore gets surrounded by a second membrane. In between these membranes, a thick layer of peptidoglycan is assembled, while on the outside of the developing spore a thick, multilayered coat is assembled (Henriques & Moran, 2000; Popham & Bernhards, 2015). This coat consists of dozens of different proteins that are deposited by the mother cell. In the final stages of the sporulation process, spores further maturate before lytic enzymes disrupt the mother cell (Foster, 1992; Vollmer, Joris, Charlier, & Foster, 2008).

Unlike endospores, exospores are separated from the mother cell by formation of a septum. Some of the best-studied species that form exospores are filamentous actinomycetes. These multicellular bacteria thrive in soil environments, and form branching networks of filaments (Claessen et al., 2014; van der Meij, Worsley, Hutchings, & van Wezel, 2017). Upon nutrient starvation, specialized filaments are formed that grow upward toward the air and which are destined to form spores. Unlike vegetative hyphae, these aerial hyphae contain at least two additional surface layers that render these hyphae hydrophobic and which probably also serve to protect spores (Claessen et al., 2003; Claessen et al., 2002; Yang et al., 2017). These growing aerial hyphae contain DNA, which in the process of sporulation condenses and segregates. Meanwhile, a highly orchestrated cell division process is initiated whereby tens of FtsZ-rings are formed simultaneously (Willemse, Borst, de Waal, Bisseling, & van Wezel, 2011). This requires the SsgB protein, which localizes to sites where cell division septa will be formed in a SepG-dependent manner (Zhang, Willemse, Claessen, & van Wezel, 2016). Strikingly, SsgB then recruits the cell division protein FtsZ, leading to the formation of multiple FtsZ rings (Willemse et al., 2011). Following assembly of the other required divisome members, a thick cell wall is synthesized. In addition to the cell surface layers present on the outside of the spores, the thick cell wall contributes to spore integrity (Mazza et al., 2006). In contrast to endospores however, exospores are generally less resistant to harsh environmental insults. This may be compensated for, in part, by the large number of exospores that are formed when compared to the single spore that is formed in the process of endospore formation.

5. Implications for pathogenicity

Bacterial plasticity gives rise to a large diversity of bacterial cell types, which are induced by a wide variety of stressors ranging from nutrient depletion to osmotic pressure (Table 1). Especially pathogenic bacteria will encounter various such stressors during infection of a mammalian host organism: upon invasion, the bacteria are exposed to huge variations in acidity, temperature, oxygen and nutrient availability, and presumably the host-immune system (Fang et al., 2016). Additionally, cells may become exposed to antibiotic compounds. All these circumstances require that the bacteria quickly adapt in order to survive, and some bacteria may even exploit this morphological plasticity to evade from the host immune system. Growing evidence suggests that the cells employ a range of morphological changes that allow them to thrive.

5.1 Evading and resisting the host-immune system

Shape plasticity allows bacteria to change their morphology, enabling them to evade immune cells such as phagocytic macrophages and neutrophils. As described above, filamentous cells provide a simple example: the evasion of phagocytes. These immune cells inactivate pathogens by trapping the bacteria at one of their poles. Since the filamentation of the pathogen makes it more difficult for the macrophages to find the cell poles, the pathogens have an increased chance of remaining undetected (Justice et al., 2004; Möller, Luehmann, Hall, & Vogel, 2012). Additionally, the length accompanying the filamentous bacterial shape ensures a long residence time in the phagocyte prior to phagosomal sealing (Prashar et al., 2013). Furthermore, the filamentation also gives an advantage even if the cells are engulfed by macrophages, as for example in the case of the intracellular pathogen *L. pneumophila*. Once inside the macrophage, these filaments reside in a vacuolar compartment where they differentiate into multiple rod-shaped cells that produce infective progeny cells (Prashar et al., 2012).

Another example of how morphological plasticity can help to overcome the host-immune system is demonstrated by the pathogen *Streptococcus pneumoniae*. *S. pneumoniae* are coccoid, chain-forming bacteria, which are cleared from the mammalian host via detection by the complement system. In this process, complement proteins identify and bind to the cells, and facilitate their internalization by phagocytic cells such as neutrophils. As a defense mechanism, S. *pneumoniae* produces a capsular polysaccharide layer to hide

from the complement system and phagocytoses by neutrophils (Hyams, Camberlein, Cohen, Bax, & Brown, 2010). Interestingly, the length of *S. pneumoniae* bacterial chains is variable and has a great impact on the potential virulence: smaller coccoid chains are more resistant to phagocytosis compared to longer chains, both *in vitro* and *in vivo* (Dalia & Weiser, 2011). This explains why *in vivo,* only the characteristic diplococci (chains of two cocci) are found, while longer chains are typical in *in vitro* systems. Shorter chains are produced by the activity of LytA, a N-acetylmuramyl-L-alanine amidase, which is involved in the separation of daughter cells (Sanchez-Puelles et al., 1986). These results imply that LytA plays an important role in length-mediated complement evasion.

However, in some instances the chaining of *S. pneumoniae* cells can also be beneficial. Similar to the filamentous forms of UPEC described above, such elongated structures are less likely to be engulfed by phagocytic cells compared to shorter ones. Longer chains are also more successful in the adherence and colonization of human epithelial cells *in vitro* (Rodriguez, Dalia, & Weiser, 2012). Taken together, both short and long coccoid chains may be advantageous for survival. Therefore, the morphological heterogeneity of *S. pneumoniae* is beneficial for infection: longer chains for the adherence and colonization phase, and small chains for the invasion phase. These examples illustrate how morphological plasticity facilitates establishing infections of pathogens.

5.2 Antibiotic treatment and morphological plasticity

The innate immunity plays an indispensable role in combating invading pathogens. It recognizes pathogens by their so-called pathogen-associated-molecular-patterns (PAMPs) on their cell surface via pathogen recognition receptors. This suggests that, theoretically, a lack of these patterns in wall-deficient cells may prevent efficient recognition by the immune system. Are pathogens able to transiently morph into a wall deficient state and thus evade the immune system and cause diseases? Some experimental studies support this tantalizing idea: to date, a range of diseases which have been associated with wall-less pathogens include urinary, cardiovascular, respiratory and cerebrospinal infections to rheumatic fever, osteomyelitis and arthritis (Errington et al., 2016). The potential role of transiently wall-less bacteria in the development of various diseases poses an important research question. However, even though several studies have correlated diseases to the presence of L-forms in patients and animals, the lack of lack statistical power and proper control experiments causes doubt of their

importance. These early studies were mostly based on microscopic observations where distinguishing between L-forms and host structures is ambiguous. Additionally, it the historical background of the patients is unclear with respect to for instance exposure to antibiotics, making such studies inconclusive. However, we know that many, if not all, bacteria can be converted to L-forms by exposing these cells to high levels of antibiotics that target cell wall synthesis (Mercier et al., 2014; Ramijan et al., 2018). Such cells ultimately will acquire mutations that allow them to proliferate in the wall-deficient state at least under laboratory conditions. Furthermore, some bacteria are able to transiently shed their wall. Both laboratory strains and clinical isolates of the pathogen *Pseudomonas aeruginosa* were found to transition from rod-shaped to spherical cells upon exposure to beta-lactam antibiotics (Monahan et al., 2014). In this state, cells generally become insensitive to cell wall-targeting antibiotics. These wall-deficient cells also become insensitive to the activity of lysozyme, which bacteria encounter in phagocytotic immune cells. In fact, recent evidence indicates that bacteria may evade from macrophages by using the endogenous lysozyme of the immune cells to switch to the wall-deficient state (Kawai, Mickiewicz, & Errington, 2018). Even more interesting, it was shown that this interaction with the macrophages and the subsequent L-form reversion protected the cells from beta-lactam induced lysis (Kawai et al., 2018). While it is unlikely that CWD cells themselves are as virulent as their walled relatives, it allows bacteria to reside in the host until the conditions have improved and a cell wall structure can be reassembled. These findings emphasize the potential involvement of CWD cells in infectious diseases and clearly require more scientific research.

Antibiotics may also induce other morphological changes in bacteria that contribute to their survival. Some antibiotics that target cell division cause filamentation, which, as we have discussed, may dramatically increase invasiveness in the host environment. One other consequence of filamentation is the increased likelihood of developing antimicrobial resistance (Bos et al., 2015). Filamentation may lead to the formation of multinucleated cells, which provide cells with the opportunity to mutate and possibly recombine chromosomes, thereby selecting for beneficial alleles and decreased sensitivity to antibiotics. This represents a novel mechanism how pathogens survive antibiotic treatment and adds another challenge in fighting disease. Other known responses to antibiotic treatment may include the formation of VBNC cells. Such cells are not resistant, but in this state, the cells can tolerate antibiotic treatments. Such cells are in several ways similar to

so-called persister cells (Lewis, 2010). The VBNC and persistency state both represent dormancy strategies during which cells are largely metabolically inactive and difficult to culture. Some recent papers even describe VNBC cells and persisters as a similar bacterial stress state, where VBNC cells representing an even more dormant state (Kim, Chowdhury, Yamasaki, & Wood, 2018).

6. Future perspectives

Cells have a remarkable flexibility to change morphology under conditions of stress. We have only recently started to dissect the potential consequences of these morphological changes and how they may contribute to the cells' survival. Except for a handful of examples, little is currently known about how bacteria adapt their morphology inside host organisms. However, this research question is highly relevant: gaining more insight may enable us to develop new strategies to combat diseases. For instance, the formation of CWD cells by pathogenic bacteria could perhaps be used as an opportunity to prevent their reversion from the wall-deficient to the walled state, and thereby preventing their ability to regain virulence. This could, for example, be accomplished by targeting the activity of specialized enzymes involved in PG synthesis and/or remodeling. Likewise, we may utilize molecules that interfere with nucleoid reorganization and dynamics when cells change between environments. By targeting major cellular stress pathways, we furthermore may be able to develop entirely new strategies to combat serious infections. However, we first need to identify which of these stress responses are used by bacteria in the host, which in itself represents a major challenge. But we have now reached the point where it becomes feasible to visualize bacteria in association with host tissue in unprecedented detail and in a native state by imaging techniques such as advanced microscopy methods (Briegel & Uphoff, 2018; Mahamid et al., 2015). This represents the important first step in bringing the study of morphological plasticity from a purely experimental to a more clinical setting.

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