Minireview

Stress responses go three dimensional – the spatial order of physiological differentiation in bacterial macrocolony biofilms

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Summary
In natural habitats, bacteria often occur in multicellular communities characterized by a robust extracellular matrix of proteins, amyloid fibres, exopolysaccharides and extracellular DNA. These biofilms show pronounced stress resistance including a resilience against antibiotics that causes serious medical and technical problems. This review summarizes recent studies that have revealed clear spatial physiological differentiation, complex supracellular architecture and striking morphology in macrocolony biofilms. By responding to gradients of nutrients, oxygen, waste products and signalling compounds that build up in growing biofilms, various stress responses determine whether bacteria grow and proliferate or whether they enter into stationary phase and use their remaining resources for maintenance and survival. As a consequence, biofilms differentiate into at least two distinct layers of vegetatively growing and stationary phase cells that exhibit very different cellular physiology. This includes a stratification of matrix production with a major impact on microscopic architecture, biophysical properties and directly visible morphology of macrocolony biofilms. Using *Escherichia coli* as a model system, this review also describes our detailed current knowledge about the underlying molecular control networks – prominently featuring sigma factors, transcriptional cascades and second messengers – that drive this spatial differentiation and points out directions for future research.

Introduction
In their natural habitats, bacteria preferably adopt a sedentary lifestyle in communities known as biofilms (Costerton *et al*., 1987). Interest in biofilms increased considerably when microbiologists began to appreciate bacteria as ‘social’ organisms and, in particular, when it was recognized that such social conduct was also the cause of severe medical and technological problems (Costerton *et al*., 1999; Beech and Sunner, 2004). Still unsolved problems associated with biofilms, which can be as important and dissimilar as their function as environmental reservoirs of pathogens (Hall-Stoodley and Stoodley, 2005), their resistance against antibiotics (Hoiby *et al*., 2010) or key roles in chronic infection (Bjarnsholt *et al*., 2013) and biocorrosion (Beech and Sunner, 2004), but also various beneficial aspects of biofilms have kept microbiologists busy trying to elucidate the fundamental principles driving biofilm formation.

The sessile and multicellular biofilm lifestyle is accompanied by the production of an extracellular matrix composed of exopolysaccharides, proteins, amyloid fibres and even extracellular nucleic acids, which promotes surface and intercellular adhesion and provides protection to the resident bacteria (Flemming and Wingender, 2010). Actual matrix composition is highly diverse among species and subject to intricate environmental regulation and sometimes mutational variation. Some species seem to strongly rely on combinations of exopolysaccharides. Thus, small colony variants of *Pseudomonas aeruginosa* produce high levels of the ‘aggregative’ exopolysaccharides Pel and Psl with distinct roles in early biofilm formation, while mucoid variants overproduce alginate (Yang *et al*., 2011; Mann and Wozniak, 2012). Other species combine amyloid protein fibres, which are prominently found in biofilms (Larsen *et al*., 2007), with exopolysaccharide(s) and/or specific additional proteins. Examples include amyloid curli fibres and cellulose in *Salmonella* spp., *Escherichia coli* and other enterobacteriaceae (Römling, 2005), or the TasA amyloid, the EPS exopolysaccharide and BslA hydrophobin in the...
Gram-positive *Bacillus subtilis* (Ostrowski *et al*., 2011; Vlamakis *et al*., 2013). On the other hand, *Vibrio cholera* relies on a specific combination of matrix proteins – RbmA, RbmC and Bap1, which have distinct functions during early biofilm formation – and the VPS exopolysaccharide (Yildiz and Schoolnik, 1999; Fong *et al*., 2006; Fong and Yildiz, 2007). Extracellular DNA in biofilm matrices has only recently gained attention. It derives from cell lysis mediated by prophages and various autolysins (Das *et al*., 2013), contributes to viscoelasticity of biofilms (Peterson *et al*., 2013) and facilitates twitching motility-related expansion at the leading edge of colonies and interstitial biofilms (Gloag *et al*., 2013).

The high diversity of natural biofilm settings has been experimentally mimicked using a series of biofilm models, which differ in the nature of the surface support of the biofilm as well as in the spatial distribution of nutrients, oxygen and water (Fig. 1). Thus, biofilm growth in microtiter plates or flow cells represents submerged biofilms growing on solid inert surfaces typically found in natural aquatic environments or on catheters (Christensen *et al*., 1999; O’Toole, 2011). Subaerial biofilms, which often contain cyanobacteria together with fungi, grow also on solid support (e.g. rocks in deserts or on buildings), which may provide micronutrients, while oxygen, carbon dioxide and ambiental water is provided by the surrounding air (Gorbushina and Broughton, 2009). Pellicle biofilms – as first stunningly described by Ferdinand Cohn (1876) in the 19th century – grow on liquid–air interfaces with nutrients coming from the liquid phase (Ostrowski *et al*., 2011; Vlamakis *et al*., 2013). Finally, macrocolony biofilms on nutrient-providing semi-solid agar plates reflect conditions of biofilms growing on decaying organic material such as soil or human food materials. Unlike classical colonies that arise from single cells, macrocolonies are inoculated by spotting a cell suspension (a few microlitres) on agar-solidified medium and are grown for extended times (Aguilar *et al*., 2007).

The mechanisms by which gradients of nutrients, oxygen, waste products or signalling compounds that build up within a biofilm as well as additional external stress conditions shape biofilm structure and drive physiological differentiation have only recently emerged as a major topic in biofilm research [summarized by Haussler and Fuqua (2013)]. Two major attributes make macrocolonies a highly valuable model to study physiological differentiation and architectural development in biofilms: (i) at the microscale, macrocolonies exhibit a remarkably high level of organization, where it is even possible to identify distinct cell lineages; and (ii) macrocolony morphogenesis involves the elaboration of macroscopic complex three-dimensional forms – sharing characteristics with tissues of higher organisms – that are strongly modulated by different environmental or stress conditions.

This review will first point out the many links between stress responses and biofilm physiology that have emerged over the past years. It will then proceed to describe how stress conditions such as nutrient and oxygen limitation that occur heterogeneously in virtually every biofilm give rise to specific patterns of physiological stratification within macrocolonies. Special emphasis will
be put on the role extracellular matrix components and the link to environmental stresses that so far have been studied in a handful of bacterial model organisms such as *E. coli*, *Salmonella spp.*, *Pseudomonas aeruginosa* and *B. subtilis*. Finally, taking *E. coli* as an example, this review describes how already existing detailed knowledge of global stress regulatory networks that control bacterial physiology in liquid cultures also provides a conceptual framework to understand the molecular basis of physiological stratification and biofilm microarchitecture.

**Links between stress responses and biofilm physiology**

Even early biofilm studies already indicated that community life can be stressful for bacteria. Due to cellular crowding in biofilms, bacteria obviously have to cope with nutritional competition, non-optimal oxygen supply and the accumulation of waste products (such as acids produced by fermentation in oxygen-depleted zones of a biofilm). Not surprisingly, biofilm cells in general were thus found to grow relatively slowly (Moller et al., 1996; Anderl et al., 2003). This raised the question why biofilms then seem to be the dominant form of bacterial life in natural environments. However, slow or even no growth is generally associated with pronounced stress resistance induced by general stress responses such as those triggered by σ^S (RpoS) in *E. coli* and other gamma-proteobacteria (Hengge, 2011) or by σ^H and σ^K in low GC Gram-positives such as *B. subtilis* (Price, 2011). Bacteria in a biofilm in general also exhibit increased antibiotic resistance, which e.g. in *P. aeruginosa* has been observed to depend on nutrient starvation signalling by the stringent response alarmone (p)ppGpp (Nguyen et al., 2014). Moreover, matrix-surrounded community life can provide protection against protozoan grazing and other forms of predation in natural environments (Matz et al., 2005).

With the advent of gene expression profiling approaches, a large number of known stress-induced and stationary-phase genes were found to be activated in biofilm cells of different bacterial species (Schembri et al., 2003; Beloin et al., 2004; Ren et al., 2004; Hentzer et al., 2005; Domka et al., 2007). These and several other evidences suggested an overall picture of biofilm cells resembling stationary phase cells (Steedley et al., 2002; Beloin and Ghigo, 2005). In fact, in *E. coli*, the expression of many regulatory and structural genes involved in the biosynthesis of curli fibres and cellulose, i.e. key biofilm matrix components, depends on the general stress response master regulator σ^S (Römling et al., 1998; 2000), whose own expression requires (p)ppGpp (Hengge, 2011). Also the expression of many enzymes involved in the synthesis and degradation of the biofilm-promoting second messenger c-di-GMP is under σ^S control (Weber et al., 2006; Sommerfeldt et al., 2009). Production of flagella, which contribute to the initial stages of biofilm formation (Pratt and Kolter, 1998; Wood et al., 2006), requires the second messenger cAMP (Soutourina et al., 1999), which accumulates under conditions of non-optimal carbon source supply (Botsford and Harman, 1992). Furthermore, highly complex stress/starvation-triggered developmental processes such as the formation of endospores in *B. subtilis* have been found to naturally occur within biofilms with regulatory circuits of sporulation and biofilm formation being tightly interconnected (Brandt et al., 2001; Vlamakis et al., 2013). In social bacteria such as *Myxococcus*, starvation-induced sporulation is obligatorily coupled to the formation of a highly structured biofilm that culminates in the formation of fruiting bodies (Kroos, 2007).

Nevertheless, the simplistic view of biofilms representing aggregates of highly stress resistant and slowly growing or even stationary phase cells has been debated on the basis of the recognized heterogeneity of microenvironments within biofilms (An and Parsek, 2007). Recent more comprehensive experimental designs have actually brought heterogeneity into context by identifying distinct cell subpopulations and starting to establish links to global stress regulatory networks (Vlamakis et al., 2008; Williamson et al., 2012; Serra et al., 2013b).

**Physiological differentiation within the three-dimensional space of macrocolony biofilms**

**Chemical gradients and patterns of physiological stratification**

Residing at different locations within structurally complex communities implies for bacteria to be non-uniformly exposed to growth resources and extracellular signalling molecules that diffuse along gradients. Such chemical gradients impact on gene expression, which ultimately determines the formation of physiologically distinct subpopulations (Stewart and Franklin, 2008).

Nutrients and oxygen gradients are particularly crucial in physiological differentiation. These gradients occur in any biofilm and result from the dynamic balance between diffusion and consumption. For *P. aeruginosa*, oxygen is critical to keep cell metabolism active in the absence of alternative electron acceptors. In regions close to the surface of *P. aeruginosa* macrocolonies, oxygen is consumed faster than it can diffuse into deeper layers, thereby becoming inaccessible for the rest of the community (Werner et al., 2004). Thus, acting as a growth-limiting resource, oxygen defines a two-layer pattern with a top layer of cells (about 60 μm thick) that exhibit active protein synthesis and a thick middle and bottom layer of cells (about two third of the macrocolony) that are...
metabolically inactive (Werner et al., 2004; Williamson et al., 2012). Due to this inactivity, cells in the lower layer allow nutrients to diffuse from the agar interface through the colony bottom layer to the upper layer where they are actually consumed.

Interestingly, less oxygen-dependent bacterial species can completely reverse this physiological stratification. As described in further detail below, E. coli macrocolonies feature actively growing cells in the lower layer close to the nutrient-providing agar support, while the upper layer is inhabited by non-growing starving cells (Serra et al., 2013b). For E. coli cells, oxygen limitation (or the absence of alternative respiratory electron acceptors) is not crucial for growth since they can obtain energy through fermentation. Therefore, nutrients are in this case the growth resource that becomes limiting in the upper zone and determines the physiological switch that results in stratification.

An interesting example of macrocolonies combining these stratification patterns was reported for Staphylococcus aureus and Staphylococcus epidermidis. Based on the detection of DNA and protein biosynthesis, staphylococcal macrocolonies can show a three-layer pattern with cells growing aerobically and fermentatively in zones adjacent to the air and agar interfaces, respectively, and non-growing cells found in a relatively thick middle zone (Rani et al., 2007). The interpretation of this pattern is that the cells at the macrocolony bottom consume fermentable substrates such as glucose, fructose or serine. No further growth is possible except along the top zone close to the air interface, where oxygen and non-fermentable substrates diffusing up from the bottom zone are available. The absence of both oxygen and fermentable carbon sources makes the large inner zone a growth-inactive area (Rani et al., 2007).

Importantly, the formation of such a growth-inactive zone is a key determinant of antibiotic resistance of the biofilm. Beta-lactamase-negative Klebsiella pneumoniae macrocolony biofilms exhibit an essentially similar three-layered pattern of physiological differentiation as Staphylococcus biofilms (Anderl et al., 2003). When macrocolonies were subject to ampicillin treatment for 12 h, cells were killed in the bottom and top layers, i.e. where cells were growing fermentatively or aerobically, respectively, but not in the central part of the macrocolony inhabited by cells that grow slowly or not at all (Anderl et al., 2003). It should be noted that while in macrocolonies nutrients and oxygen diffuse in opposite directions, in submerged biofilms grown on inert surfaces, both resources come from the surrounding liquid medium (Fig. 1). This logically explains the finding of growing cells in the outer layers and starving cells inside the ‘mushroom’-like structures of submerged biofilms (An and Parsek, 2007; Stewart and Franklin, 2008).

The question of the existence of distinct physiological states within macrocolony biofilms was also addressed in B. subtilis, where fluorescence reporters under the control of gene promoters specific for motile, matrix-producing and sporulating cells were used to visually localize these three cell types in cross-sections of a macrocolony (Vlamakis et al., 2008). The results demonstrated that cellular differentiation within a biofilm follows a precise spatiotemporal order. In terms of spatial organization, flagellated cells were predominantly found at the bottom and outer edge of the macrocolony. Matrix-producing cells were present in patches throughout the macrocolony, while sporulating cells were primarily found at the top of the macrocolony (Vlamakis et al., 2008). The latter was in agreement with earlier studies showing sporulation to take place preferentially in aerial projections, i.e. at the tip of colony wrinkles (Branda et al., 2001; Veening et al., 2006). In terms of temporal order, flagellated bacteria primarily differentiated into matrix-producing cells, with those located in the most nutrient-deprived zones ultimately initiating sporulation (Vlamakis et al., 2008). Thus, cell fate within B. subtilis macrocolonies also seems dictated by a ‘nutritional state’-dependent logic with the final transition to sporulating cells occurring not only far away from the nutrient source, but also after an energy-expensive process such as matrix production probably intensified nutrient depletion. Furthermore, the dynamics of these local differentiation events seems strongly modulated by self-produced small molecules. The ComX pheromone, which stimulates B. subtilis natural competence, also triggers differentiation to surfactin producers (Lopez et al., 2009a). Surfactin, in turn, acts paracrinally on a different subpopulation stimulating both matrix biosynthesis and cannibal toxin production (Lopez et al., 2009a,b). Thus, acting like ‘cannibals’, matrix-producing cells thrive at the expense of other cell types, as they grow on the nutrients released by cannibalized cells which delays their differentiation into sporulating cells. Promoting the increase and maintenance of the matrix-producing population seems therefore a strategy to prioritize nutrient utilization for structural consolidation and protection of the community.

Nutrient-dependent physiological differentiation in E. coli macrocolonies

The extensively studied physiology of the E. coli growth cycle in liquid cultures was recently shown to provide also a unique basis for understanding physiological differentiation within a biofilm (Serra et al., 2013b). With abundant carbon/energy sources, as for instance present in a complex medium such as the commonly used Luria–Bertani broth (LB), E. coli initially grows exponentially with short division cycles. When composition and quality of these resources become suboptimal due to ongoing
consumption, bacteria start getting ‘hungry’ and enter a phase of post-exponential growth with decreasing growth rates (Amsler et al., 1993; Ferenci, 2001; Liu et al., 2005). To optimize nutrient scavenging, post-exponential-phase E. coli cells, which are still rod-shaped and divide, not only express additional high affinity transport and metabolic systems, but also produce flagella and become highly motile (Adler and Templeton, 1967; Amsler et al., 1993; Liu et al., 2005; Zhao et al., 2007). However, when this ‘foraging’ strategy is no longer successful and available nutrients decrease further and insufficient to support growth, E. coli cells undergo a global physiological switch to concentrate on maintenance and survival (Lange and Hengge-Aronis, 1991a). The molecular basis of this switch is a sigma factor exchange at RNA polymerase, with σ^52 now taking the lead (see below for details). Cells stop the costly production of flagella and undergo a couple of reductive cell divisions, which generates smaller and ovoid cells (Lange and Hengge-Aronis, 1991b). Finally, they enter into stationary phase with cells arresting growth and becoming multiple stress resistant. If this happens below 30°C, much of the remaining resources are spent on producing extracellular matrix components like amyloid curli fibres and cellulose [summarized in Hengge (2011)].

Using flagella, curli fibres, cell size and shape and the abundance of cell division as ‘anatomical’ hallmarks in high-resolution microscopic studies, these physiological changes known to occur in a temporal sequence in liquid cultures were found to translate into a precise spatial order within E. coli K-12 macrocolonies (Serra et al., 2013b) (Fig. 2A). This order is determined by the relative distance of bacteria from the nutrient source. Thus, bacteria at the bottom layers as well as at the thin outer edges of mature macrocolonies, i.e. close to the nutrient-providing agar interface, exhibit morphological features of post-exponential growth physiology: cells are elongated and rod-shaped; they divide and produce flagella, which form a tight mesh at the bottom of the macrocolony (Fig. 2D). By contrast, bacteria in the upper layer of the macrocolony, i.e. far away from nutrients, show morphological features characteristic of stationary phase: small ovoid cells that only very rarely show division are encased in a network of curli fibres that form ‘custom-moulded baskets’ around the producing cells (Fig. 2B). Similarly, curli-producing and H2O2-resistant bacteria (also the latter a phenotype dependent on σ^52) were found exclusively in the upper layer of macrocolonies of a uropathogenic E. coli strain (DePas et al., 2013). In conclusion, the basic architecture of E. coli macrocolonies has a two-layer structure with a zone of vegetative growth at the bottom and the outer edges and a zone of σ^52-determined stationary phase physiology in the top layer.

Between these two layers of E. coli K-12 macrocolonies, a transition zone of physiological heterogeneity can be discerned, in which small cells that already have switched to the stationary-phase mode and thus have turned on curli fibre production are found right next to elongated curli-free and flagellated cells (Fig. 2C). Remarkably, the curli-covered bacteria in this middle zone are found arranged in small chains (often consisting of four cells) and patches, suggesting that once cells turn on curli production, then this state is maintained in their progeny (Serra et al., 2013b). Since these adjacent curliated and curli-free cells are exposed to the same environmental conditions, this heterogeneity points to a stochastic element and possibly bistability in the outcome of sigma factor competition and/or the control of curli fibre production (see below).

Unlike many commensal or pathogenic E. coli ‘wild-type’ strains (Bokranz et al., 2005), E. coli K-12 laboratory strains do not produce cellulose as a matrix component (Da Re and Ghigo, 2006). This defect could be repaired by changing an E. coli K-12-specific chromosomal single nucleotide polymorphism that generates a stop codon in the bcsQ gene, which is polar on the expression of the downstream genes encoding the subunits of cellulose synthase (Serra et al., 2013a). The production of curli fibres and cellulose in this ‘dedomesticated’ strain resulted in pronounced phenotypic changes (Fig. 2E). At the high-resolution microscopic level, curli fibres and cellulose form a composite material that not only covers but also firmly connects cells at the surface and in the upper part of the top layer of the macrocolony. In addition, cellulose forms long filaments and extended sheets at the interface between the top and bottom layers (Fig. 2F). Macroscopically, colony morphology is drastically different from a curli-only producing strain: whereas the latter generates a colony pattern of concentric rings (Fig. 2A) that form by breakage of the brittle non-elastic curli fibre network of the top cell layer, the ‘dedomesticated’ strain produces very large and flat macrocolonies with complex patterns of long ridges and wrinkles (Fig. 2E). It seems that the cell-connective properties of the cellulose-curli composite interfere with vertical expansion, thus forcing the growing colony into lateral expansion. These macrocolonies in fact show tissue-like, i.e. cohesive and elastic properties, which results in their buckling up into macroscopic structures when cellular crowding generates lateral tension. However, even though matrix structure and physical properties as well as macrocolony morphology differ rather strikingly from the ‘classical’ curli-only producing E. coli K-12, the basic stratification into two layers with growing flagellated cells at the bottom and the outer edges and matrix-producing stationary phase cells in the top zone is essentially the same (Serra et al., 2013a).
Stationary phase:
- small ovoid cells
- no cell division
- major σ factor in RNAP: σ^5
- no ribosome synthesis
- multiple stress resistance/survival
- synthesis of amyloid curli fibres (at T<30°C)

Post-exponential phase:
- rod-shaped cells
- cell division
- major σ factors in RNAP: σ^70, σ^32
- reduced ribosome synthesis
- expression of C-scavenging systems
- synthesis of flagella

Physiological differentiation (heterogeneity)
This two-layer architecture was also found in mutant macrocolonies that produce cellulose only or that even did not produce curli and cellulose at all (Serra et al., 2013a). Thus, production of the biofilm matrix itself does not cause physiological and spatial differentiation of _E. coli_ macrocolonies, but rather is a consequence of the differentiation into layers of vegetatively growing and starving stationary phase cells. However, once stationary phase cells start to produce matrix components, then this process can indeed modulate differentiation further by accelerating the use of remaining carbon/energy sources and by affecting macrocolony morphology, which involves relocation of entire regions by tissue-like folding and buckling and thereby impacts on nutrient and oxygen gradients.

**Stress response networks controlling cellular differentiation in _E. coli_ macrocolony biofilms**

Overall, these recent studies also suggest that bacterial biofilm formation should not be considered as a programme of specific physiological differentiation. Rather, cells generating a biofilm respond to their self-conditioned local microenvironment by adapting their gene expression to suit their specific needs similarly as planktonic cells do in liquid cultures. The major difference is that bacteria residing in biofilm do so in a stable spatially structured environment. At the molecular level, the regulatory mechanisms are provided by already well-studied stress responses (Storz and Hengge, 2011), which essentially guide bacteria in making the decision between two fundamentally different life strategies – either to opt for growth and proliferation or for maintenance and survival. Using _E. coli_ as a model system, we show here how the regulatory networks that control the transition from post-exponential to stationary phase provide a conceptual framework to understand spatial physiological differentiation in structured macrocolonies. The principle components of these networks are different sigma subunits of RNAP, several master regulators controlling transcriptional cascades and the nucleotide second messengers cAMP, (p)ppGpp and c-di-GMP.

Sigma subunits competing for RNAP core provide the basis for physiological differentiation into post-exponentially growing and stationary phase cells

With available resources always being limited, bacteria cannot maximize proliferation and general stress resistance at the same time (Ferenci, 2001; 2005; Nystrom, 2004; Hengge, 2011). At the molecular level, this is reflected by a limited cellular amount of RNAP core enzyme for which the various sigma factors, which activate different spectra of target genes, have to compete (Ishihama, 2000; Grigorova et al., 2006). While in growing cells the master sigma subunit is the vegetative σ^70 (RpoD) which essentially drives expression of ‘housekeeping’ genes involved in growth and metabolism, such a global role is taken over by σ^6 (RpoS) in stationary phase cells, which redirects transcription to hundreds of genes involved in multiple stress resistance, maintenance metabolism and other stationary phase functions (Weber et al., 2005). These global activities of σ^54 and σ^5 are complemented and – sometimes transiently replaced if need be – by several alternative sigma factors that control relatively smaller sets of stress genes. In _E. coli_, these are σ^24 (RpoE), σ^2 (RpoS) and σ^0 – drives the expression of genes involved in assembling and operating flagella during the post-exponential growth phase, as well as σ^2 (RpoH), σ^F (σ^24 or RpoE) and σ^4 (RpoN), which control sets of genes involved in coping with heat shock, extracytoplasmic stress and nitrogen limitation respectively (Storz and Hengge, 2011).
The cellular levels of cAMP and (p)ppGpp are enhanced in cells entering the post-exponential state, with cAMP accumulating in response to quantitatively and qualitatively low carbon/energy sources and increasing (p)ppGpp being associated with decreasing growth rates. While (p)ppGpp redirects vegetative RNAP away from ribosomal gene expression towards the expression of other genes, many of which have biosynthetic functions (a process called the ‘stringent response’), cAMP bound to cAMP, oxidized components of the respiratory chain and reduced ATP levels) and low concentrations of phosphorus or nitrogen sources. Despite its complexity – involving multiple signal integration, complex homeostatic feedback as well as positive feedbacks – the switch from a state of low production rates of unstable σ^S in growing cells to high synthesis and stabilization of σ^S during entry into stationary phase is now well understood. In addition, a series of different acute stress signals – such as sudden exposure to high osmolarity, acid, radiation, etc. – can rapidly throw this switch even in fast growing cells in case of emergency, which makes σ^S also a general stress sigma factor [for reviews focusing on different aspects of this control network, see Hengge (2009a; 2011), and Klauke and Hengge (2011)].

Notably, the threshold for switching to σ^S-driven physiology can be shifted by mutation [reviewed in Hengge (2011)]. Thus, when E. coli is kept under constant long-term carbon limitation in a chemostat (in the absence of additional stress) or during long-term stationary phase in LB medium, where balanced cryptic growth is established (i.e. still viable cells grow slowly on the debris of lysed cells), mutants with attenuated σ^S levels or activity emerge. These mutants have a competitive advantage by remaining in the growth state under the very limiting conditions, where the parental strains already switch to stationary phase physiology (Finkel, 2006; Ferenci, 2008).

The second messengers cAMP and (p)ppGpp and the transcriptional master regulators CRP and FlhDC determine the physiology of post-exponentially growing cells

Competition for RNAP core of the vegetative σ^70 and the stationary phase σ^S, with each one – when in the dominant role – operating at the expense of the other, constitutes the fundamental framework, in which mutually exclusive transcriptional cascades can drive biofilm-relevant properties such as flagella production in growing cells and curli and cellulose production in stationary phase cells (Pesavento et al., 2008). These regulatory cascades involve several key transcription factors as master regulators, which in turn respond to the crucial second messengers cAMP, (p)ppGpp and c-di-GMP (Fig. 3).

The cellular levels of cAMP and (p)ppGpp are enhanced in cells entering the post-exponential state, with cAMP accumulating in response to quantitatively and qualitatively low carbon/energy sources and increasing (p)ppGpp being associated with decreasing growth rates. While (p)ppGpp redirects vegetative RNAP away from ribosomal gene expression towards the expression of other genes, many of which have biosynthetic functions (a process called the ‘stringent response’), cAMP bound to
the master regulator CRP activates the expression of numerous carbon scavenging systems (Grainger et al., 2005; Shimada et al., 2011). In addition, the production of flagella is under control of cAMP-CRP (Soutourina et al., 1999). This global regulator activates flhDC, which in the hierarchical flagellar transcriptional cascade represents the single class 1 operon and encodes the master regulator (Liu and Matsumura, 1994; Wang et al., 2006). FlhDC and the $\sigma^70$-containing RNAP then activate the class 2 flagellar operons that encode proteins for the components and assembly of the hook-basal body, the flagellar sigma factor $\sigma^{fla}$, its antisigma factor FlgM and many other proteins. Once the hook-basal body is assembled, FlgM is secreted which relieves inhibition of $\sigma^{fla}$ to permit transcription of class 3 genes, including those encoding flagellin ($fliC$), i.e. the major subunit of the flagellar filament, and the flagellar motor proteins (Chilcott and Hughes, 2000; Chevance and Hughes, 2008).

Already in parallel to these processes and under the positive influence of (p)ppGpp, rpoS transcription increases and in response to dwindling carbon/energy sources – $\sigma^5$ protein, which in growing cells is degraded by the ClpXP protease with the help of the targeting factor RssB, becomes stabilized. Consequently, $\sigma^5$ accumulates, but it still remains inefficient in outcompeting $\sigma^{fla}$ for core RNAP binding (Hengge, 2011). Moreover, the FlhDC-controlled cascade activates its prioritization over $\sigma^5$-controlled gene expression via FliZ, an abundant nucleoid-associated protein expressed from the same operon as $\sigma^{fla}$ that shows a DNA-binding specificity with a strong sequence overlap with the $\sigma^5$ consensus promoter, thus acting as a global repressor at many $\sigma^5$-dependent promoters (Pesavento et al., 2008; Pesavento and Hengge, 2012).

Overall, $\sigma^70$, cAMP-CRP, FlhDC, $\sigma^{fla}$ and FliZ thus establish a transcriptional pattern that includes flagella production and allows cells to optimize growth under the no longer optimal conditions in the post-exponential phase. In an *E. coli* macrocolony biofilm, this physiologically state is found in the bottom layer, which is a highly crowded environment but nevertheless close enough to the nutrient-providing agar support to still support growth and proliferation.

**Activation of $\sigma^5$ and the $\sigma^5$/MtrA/CsgD transcriptional cascade in the switch to stationary phase physiology**

As nutrient conditions worsen – i.e. later in post-exponential phase in liquid culture or further away from the agar support in a macrocolony – the *flhDC* operon is no longer transcribed and, due to proteolytic degradation of FlihDC and $\sigma^{fla}$, expression of the entire flagellar cascade is shut down (Barebruch and Hengge, 2007; Pesavento et al., 2008). In the macrocolony, this is apparent as a ‘dilution’ of flagella per cell with increasing distance from the agar support (Serra et al., 2013b). This is an effect of increasing levels of (p)ppGpp (Traxler et al., 2011), which interferes with flagellar expression (Lemke et al., 2009), further drives $\sigma^5$ expression (Lange et al., 1995) and shifts sigma factor competition for RNAP core in favour of alternative sigma factors such as $\sigma^5$ (Jishage et al., 2002). Binding to RNAP core and thus activation of $\sigma^5$ is further promoted by the $\sigma^5$-binding Crl protein and the anti-$\sigma^70$ factor Rsd, a process that includes a positive feedback due to expression of Rsd being further enhanced by $\sigma^5$ itself (Jishage and Ishihama, 1998; 1999; Bougdour et al., 2004; Typas et al., 2007; Banta et al., 2013). In addition, the negative homeostatic feedback in the control of $\sigma^5$ levels provided by the $\sigma^5$-dependent expression of its own proteolytic targeting factor RssB (a response regulator that is rate limiting for $\sigma^5$ degradation in growing cells) no longer operates for two reasons: (i) $\sigma^5$-containing RNAP levels get higher than required to maximally activate the rssAB promoter (Pruteanu and Hengge-Aronis, 2002), and (ii) energy limitation (in the presence of oxygen) leaves the quinones of the respiratory chain in an oxidized state, which inactivates the histidine sensor kinase ArcB that is required to phosphorylate and thus activate RssB (to drive $\sigma^5$ proteolysis) and ArcA (a globally acting transcription factor, which also represses rpoS transcription) (Malpica et al., 2004; Mika and Hengge, 2005). All these highly integrated regulatory processes allow $\sigma^5$ to strongly accumulate and basically ‘take over’ genome-wide gene expression which results in multiple stress resistance, switching to a maintenance metabolism and cells becoming smaller and ovoid due to ‘reductive’ cell division [summarized in Hengge (2011)].

Another major consequence of this switch is that $\sigma^5$ now deploys essentially the entire regulatory machinery for the synthesis of biofilm matrix components such as curli fibres and cellulose (Fig. 3). This includes the expression of a $\sigma^5$-controlled feedforward cascade consisting of the transcriptional regulators MtrA and CsgD (Brown et al., 2001; Weber et al., 2006) as well as several diguanylate cyclases (DGC) and phosphodiesterases (PDE) that produce and degrade the second messenger c-di-GMP, respectively, which is essential for the expression of CsgD (Weber et al., 2006; Pesavento et al., 2008; Sommerfeldt et al., 2009; Lindenberg et al., 2013) (see also below). CsgD expression and therefore curli and cellulose production occurs below 30°C in most *E. coli* strains (Bokranz et al., 2005). In some strains, however, this still uncharacterized temperature regulation does not operate and for some pathogenic *E. coli* curli fibres contribute to adhesion and even to invasion into host cells (Gophna et al., 2001).

The *csgD* gene (also called *agiD* in *Salmonella*) is in fact a key node or regulatory hub in this complex biofilm control network (Fig. 3). Its transcriptional regulation is remarkably

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complex and receives additional modulatory inputs from an entire series of transcription factors (including OmpR, CpxR and FruR) in response to diverse conditions such as changing osmolality, envelope stress or nutrient limitation (Prigent-Combaret et al., 2001; Brombacher et al., 2003; 2006; Gerstel and Römling, 2003; Jubelin et al., 2005; Ogasawara et al., 2010b; Lindenberg et al., 2013). In addition, csgD mRNA levels and translation are negatively modulated by several stress-regulated small regulatory RNAs (sRNA), including RprA, McaS and OmrAB (recently summarized by Mika and Hengge (2013)). In general, these sRNAs have multiple targets. Thus, McaS inversely regulates FlhDC and CsgD expression and thereby contributes to the mutual exclusion of flagella and curli/cellulose production (Jørgensen et al., 2012; Thomason et al., 2012). In a feedforward loop, RprA directly targets not only csgD mRNA, but also ydaM mRNA, which encodes a DGC that is essential for transcriptional initiation at the csgD promoter (Mika et al., 2012). Another intriguing and not fully characterized effect of this mRNA/sRNA interaction network is that high levels of csgD mRNA can also sequester these sRNAs and thereby affect the expression of their other targets (Mika et al., 2012; Mika and Hengge, 2013).

CsgD protein then drives the formation of curli fibres by directly activating transcription of the csgBAC operon (Römling et al., 2000; Weber et al., 2006), which encodes the major curli subunit CsgA, the nucleator protein CsgB and the small periplasmic protein CsgC that is proposed to play a role in subunit secretion (Evans and Chapman, 2013). As described below, CsgD control of cellulose production is indirect involving c-di-GMP signalling. Attempts to define a csgD regulon have yielded mixed results. Chromatin immunoprecipitation (ChIP) analysis of a CsgD-overproducing strain has detected around a dozen genes (besides csgBAC and yaiC) that may be positively or negatively regulated by CsgD protein (Ogasawara et al., 2011). However, using a csgD mutant in a microarray-based transcriptome analysis, a different and rather large csgD regulon (again including csgBAC and yaiC) has been observed, but currently, it is unclear which of these target genes are controlled by CsgD protein at the level of transcription initiation and which are indirectly regulated by csgD mRNA sequestering sRNAs which target the mRNAs of these genes (Mika et al., 2012).

In conclusion, σ54-dependent and, in particular, CsgD-dependent gene expression literally shapes the stationary phase upper layer of macrocolony biofilms. While this is most apparent for the matrix network surrounding the cells as well as for the size, shape and stress resistance of cells residing in this region of a macrocolony (Fig. 2), it may well extend to not yet characterized cellular and community properties regulated by σ54, CsgD and the sRNA network described above.

Role of the second messenger c-di-GMP in the inverse coordination of flagellar activity and biofilm matrix synthesis

c-di-GMP is a ubiquitous second messenger that in general promotes biofilm formation by driving the expression of adhesins and matrix components while usually downregulating expression and/or activity of flagella (for reviews focusing on different aspects, see Jenal and Malone (2006), Hengge (2009b; 2010), Krasteva et al. (2012) and Römling et al. (2013)). c-di-GMP is synthesized by DGCs characterized by the GGDEF domain, with this amino acid motif representing the active site (A-site) of these enzymes. The activities of most DGCs are also allosterically feedback-inhibited by their own product, with c-di-GMP binding to a secondary site (I-site). Dedicated PDEs containing EAL or HD-GYP domains degrade c-di-GMP (Schirmer and Jenal, 2009). These antagonistic enzymes regulate the intracellular levels of c-di-GMP, which is bound by effector components, which belong to diverse families of proteins or riboswitches in the 5′-untranslated regions of mRNAs. Acting for instance as transcription factors, as components of exopolysaccharide synthesis and excretion machineries or – in the case of riboswitches – in translational control, these effectors directly control the activity of highly diverse target components (Boyd and O’Toole, 2012; Krasteva et al., 2012).

The complexity of c-di-GMP signalling networks also relies on the often large numbers of different DGCs and PDEs that exist in most bacterial species. The E. coli K-12 genome encodes 12 proteins with a GGDEF domain, 10 proteins with an EAL domain and 7 proteins that contain both domains. In terms of enzymatic activity, these proteins represent a set of 12 DGCs, 13 PDEs and 4 ‘degenerate’ proteins, with the latter not displaying enzymatic activity and acting by direct protein–protein interactions (Hengge, 2010). Expression of these proteins is highly differentially controlled, but many – DGCs as well as PDEs – are induced during transition of E. coli from post-exponential to stationary growth phase (Sommerfeldt et al., 2009). In addition, some of these DGCs and PDEs seem to have highly specific functions that cannot be complemented by other DGCs and PDEs. This specificity has led to assumptions of ‘local’ action of some of these proteins in larger protein–protein complexes, with such interactions actually having been observed for many of these proteins [summarized in Hengge (2009b)].

A specific subset of these DGCs and PDEs becomes crucially involved in the operational switch between the flagellar and curli/cellulose control cascades (Fig. 3) (Weber et al., 2006; Pesavento et al., 2008). The first key player in this switch is YhiH, the most strongly expressed PDE in E. coli (Sommerfeldt et al., 2009), whose expres-
Downstream of CsgD expression, c-di-GMP is again necessary to allosterically activate cellulose synthase by directly binding to the regulatory PilZ-like domain of its larger subunit BcsA (Ross et al., 1987; Römling et al., 2005). For this process, the key player is the DGC YaiC (AdrA in Salmonella), which is expressed under the control of CsgD and $\sigma^S$-containing RNAP (Römling et al., 2000; Zogaj et al., 2001; Weber et al., 2006; Sommerfeldt et al., 2009). yaiC/adrA mutants do express CsgD and curli normally, but are specifically defective in producing cellulose (Römling et al., 2000; Simm et al., 2004). The reason for this high specificity of YaiC for activating cellulose synthase is unclear. Perhaps only YaiC — due to a high $K_i$ for product inhibition at its l-site — is able to drive c-di-GMP levels high enough to efficiently activate cellulose synthase, whose PilZ domain has a relatively high $K_d$ for c-di-GMP binding (Pultz et al., 2012).

In summary, during transition from the post-exponential to the stationary growth phases cells essentially undergo a fine-tuned switch from a low to a high cellular c-di-GMP state. This switch, however, does not depend exclusively on isolated c-di-GMP signalling proteins, but rather on the more global regulatory networks directed by a few master regulators that precisely determine when and where the c-di-GMP signalling components are expressed and operate. Based on the stratification into post-exponentially growing and stationary phase bacteria within macrocolonies (Serra et al., 2013a,b), the known regulatory patterns of DGCs and PDEs (Sommerfeldt et al., 2009), and increasing cellular c-di-GMP level along the growth cycle in liquid cultures (Spangler et al., 2010), one can logically predict a corresponding heterogeneity of cellular c-di-GMP levels within these biofilms. An exciting challenge for the future will be to determine the precise levels of c-di-GMP and associated signalling components in single cells within structurally complex macrocolonies.

Short-range heterogeneity within macrocolonies and bistability of gene expression

The response to gradients of nutrients and oxygen, which extend over long distances within a macrocolony biofilm, establishes long-range heterogeneity of gene expression and global physiology as described above. In addition, short-range heterogeneity has been observed, with cells residing right next to each other displaying different morphology and physiology. In the physiological ‘transition’ zones between the bottom and upper strata as well as at the surface behind a certain distance from the outer growing edge of *E. coli* macrocolonies, shorter and highly curliated cells are found directly adjacent to ‘naked’, elongated and fully flagellated cells. In addition, expression of the CsgD regulator shows the same pattern of heterogeneity in these zones (Serra et al., 2013b). These
directly adjacent conditions must be exposed to identical environmental conditions, which points to an endogenous source of this micro-heterogeneity. Most likely, this is due to regulatory patterns that can generate bistability of gene expression. These patterns include the activity of antagonistic enzymes generating ultrasensitivity, long transcriptional cascades, mutual inhibition of regulatory components and positive feedbacks (Hooshangi et al. 2005; Dubnau and Losick, 2006; Lipshtat et al., 2006). All of these patterns are actually prominently present in the network that controls CsgD (Fig. 3). In particular, mutual inhibition is found at three levels, i.e. in (i) sigma factor competition, (ii) YciR degrading its own inhibitor and (iii) mutual inhibition of csgD mRNA and sRNAs. It will be a challenge for future work to sort out the contributions of these network motifs to bistable expression of CsgD and the matrix components curli and cellulose.

The combination of long-range heterogeneity of gene expression due to gradients of resources and short-range heterogeneity due to bistability of expression of a key regulator may be a general principle in biofilm architecture. Also in macrocolonies of B. subtilis, a stratification of expression of flagellar genes in the bottom layer and of matrix and sporulation genes in the upper layer was shown (Branda et al., 2001; Vlamakis et al., 2008) (and see above). In addition, biofilm matrix-producing cells are found side-by-side with non-producer cells, with this micro-heterogeneity based on bistability in the control of the regulator Spo0A activating expression of SinI, which in the producer cells antagonizes the repressor SinR that is expressed in all cells in the relevant regions of the macrocolonies (Chai et al., 2008).

Conclusions and perspectives

Based on a number of recent studies summarized here, it can be concluded that the heterogeneous cellular physiology and supracellular architecture of macrocolony biofilms is essentially established by stress responses that react to gradients of nutrients and oxygen that build up during growth of these biofilms. These stress responses require cells to make the decision between growth and proliferation or maintenance and survival, which – when happening along nutrient and oxygen gradients – results in a physiological stratification of the biofilm. An important consequence is a heterogeneous production of matrix components, which include various exoproteins, amyloid fibres and exopolysaccharides that provide protection, cohesion and even tissue-like elasticity to the biofilm. Overall, this also means that biofilm formation is not the result of a specific genetic programme of developmental differentiation, but rather provides a spatially differentiated multicellular environment, in which specific developmental processes such as sporulation can then be triggered.

As only shortly mentioned in this review, macrocolony biofilms also exhibit striking macroscopic morphological patterns of ridges, rings and wrinkles, a phenotype that has been termed ‘wrinkled’, ‘rugose’ or ‘r’dar’ (‘rough, dry and red’, with ‘redness’ depending on the use of the dye Congo Red), although these simple designations do not adequately reflect the complexity and diversity of these structures, which are drastically modulated by oxygen content, the presence of reactive oxygen species or salt or the humidity at the agar surface (Römling, 2005; Aguilar et al., 2007; Beyhan et al., 2007; DePas et al., 2013; Dietrich et al., 2013; Kolodkin-Gal et al., 2013; Serra et al., 2013a). A common signature of these structures is their strict dependence on extracellular matrix components (Friedman and Kolter, 2004; Römling, 2005; Romero et al., 2010; Colvin et al., 2012; Serra et al., 2013a,b), which confer the connectivity and elasticity that allow growing bacterial biofilms to essentially behave as tissues that buckle up under the spatial constraints and therefore tension generated by cellular crowding (Serra et al., 2013a; Trejo et al., 2013). It will be a major challenge for future research to characterize the underlying morphogenetic molecular mechanisms, i.e. how distinct matrix components, the network of multiple c-di-GMP-controlling DGCs and PDEs, the asymmetric expression of these components in different biofilm strata established by long-range nutrient and oxygen gradients as well as the role of additional stress signal input contribute to the microarchitecture and macroscopic morphology of biofilms.

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