Micro**Commentary**

The protective layer of biofilm: a repellent function for a new class of amphiphilic proteins

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Summary

Bacteria can survive harsh conditions when growing in complex communities of cells known as biofilms. The matrix of the biofilm presents a scaffold where cells are attached to each other and to the surface. The biofilm matrix is also a protective barrier that confers tolerance against various antimicrobial agents. In this issue of *Molecular Microbiology*, Kobayashi and Iwano (2012) show that the liquid permeability of *Bacillus subtilis* biofilms is determined by a small secreted protein, i.e. BslA (formerly called YuaB). BslA is important for the proper development of biofilms, but unlike exopolysaccharide and TasA, is not directly involved in cell cluster formation, and is synthesized following the production of exopolysaccharide and amyloid fibres. The amphiphilic BslA protein forms a polymer *in vitro* and localizes *in vivo* to the surface of the biofilm. The microstructures of the biofilm wrinkles are reduced in the *bslA* mutant strain and the liquid repellency of the biofilm surface is diminished. Exogenously added BslA$_{42-181}$ protein complements the *bslA* mutation and restores not only water repellency, but also the formation of aerial structures. This study demonstrates that amphiphilic proteins have an important role in liquid repellency of biofilms and it suggests that these polymers contribute to antimicrobial resistance.

Bacteria that attach to surfaces often organize into highly differentiated populations of cells called biofilms (O'Toole *et al.*, 2000). Biofilms are regarded as the most common lifestyle of bacteria in nature. Cells encased in an extracellular matrix differentiate into specialized cell types discriminated by distinct transcriptional regulation (Abee *et al.*, 2011). One part of the consortium produces the structural components of biofilm, the extracellular polysaccharides and proteins, while other cells differentiate into resister cell types, spores or persistent cells with no or low metabolic activity. Also, part of the population undergoes cell lysis, resulting in extracellular DNA. Cells in the biofilm that produce extracellular matrix benefit from a higher resistance against environmental insults, like desiccation or the presence of antimicrobials. Even when part of the population produces extracellular matrix, the biofilm as a whole is being protected against these insults. The increased resistance against antimicrobials is suggested to come from the lower antimicrobial penetration, changed expression of resistance genes in biofilms, and the development of so-called persister cells (Davies, 2003). These cells have reduced metabolic activity in the middle of biofilms. Persister cells not only increase the resistance against antimicrobials, they are also a potential source of dispersion. However, a recent article suggests that matrix production might actually reduce the dispersal efficiency of these cells (Nadell and Bassler, 2011). The antimicrobial resistance mechanism provided by biofilms forms a long lasting and relevant research question (Costerton *et al.*, 1999). A recent publication of *Bacillus subtilis* biofilm liquid repellency by Epstein *et al.* (2011) gives new insights on this topic. Targeted modification of water repellency of biofilms might provide a novel way of increasing antimicrobial sensitivity.

In the natural environment, the Gram-positive model organism *B. subtilis* is soil dwelling and thereby exposed to water that possibly contains heavy metals, antimicrobials and other toxins. Under these conditions, it is plausible that biofilms are selected for their enhanced capacity to repel water, which protects them against environmental pollutants or other aggressive compounds. This putatively explains why *B. subtilis* often functions as a biocontrol
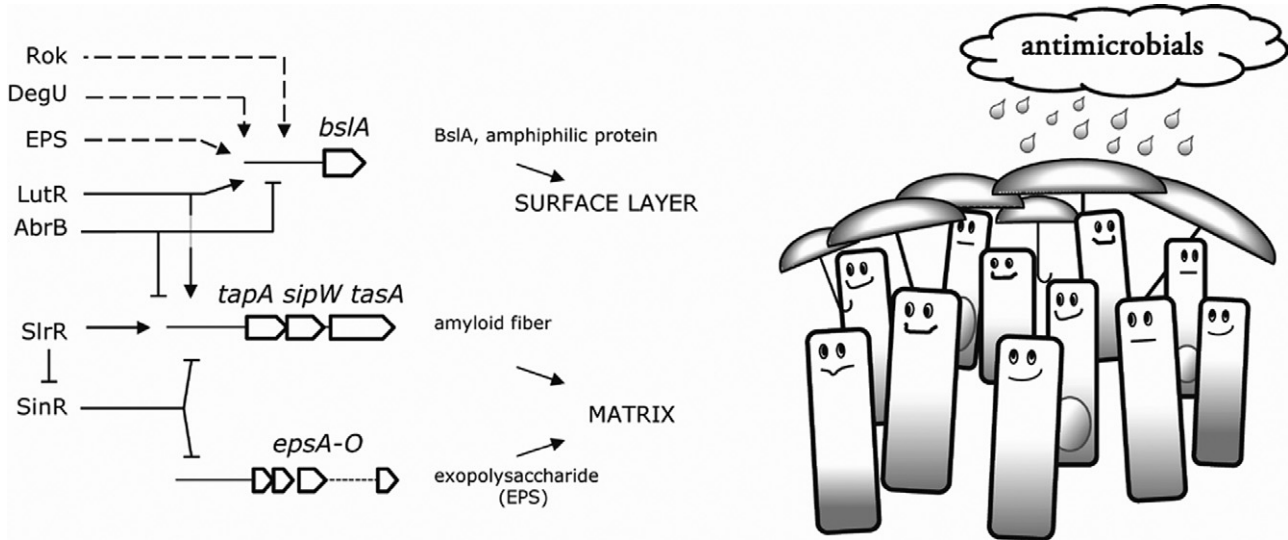
Fig. 1. Intertwined regulation of the *bslA* gene, *eps* and *tapA* operon. Arrows and T-bars depict activation and negative regulation respectively. Direct and indirect interactions are indicated with solid and dashed lines respectively. Right side of the figure symbolizes the 'umbrella effect' of BslA (e.g. liquid repellency).

agent in the rhizosphere against many plant pathogens. For example, it has been shown that *B. subtilis* biofilm formation protects the roots of Arabidopsis against water-born plant pathogens (Bais *et al.*, 2004). The degree of water and low surface tension liquid repellency and gas penetration has been examined on architecturally complex colonies and pellicles of *B. subtilis* (Epstein *et al.*, 2011). Biofilm colonies of *B. subtilis* are extremely non-wetting, even surpassing the repellency of Teflon towards water. The non-wetting conditions are not only caused by the chemical properties of the surface molecules, but also by the complex microstructure of the biofilm (Roach *et al.*, 2008). Several studies present examples of liquid repellency in nature (e.g. super hydrophobic plant leaves and insects) (Aussillous and Quere, 2001; Feng *et al.*, 2002; Gao and Jiang, 2004), and these suggest that microstructures are important for the repellency. By mimicking the biofilm topography of *B. subtilis* biofilms using functionalized polymer replicas, it has been shown that the non-wetting behaviour towards water depends on both topographic and surface chemistry features (Epstein *et al.*, 2011). The paper of Kobayashi and Iwano in this issue of *Molecular Microbiology* shows that BslA (formerly YuaB) contributes both chemically and structurally to the water and low surface tension liquid repellency of *B. subtilis* biofilms (Kobayashi and Iwano, 2012).

The amphiphilic BslA protein is localized at the surface of the complex colonies. The *B. subtilis* strain lacking BslA has an altered biofilm microstructure and the development of biofilm micro- and macrostructures is reduced in the *bslA* mutant. BslA seems to fulfil its role after the production of exopolysaccharides and amyloid fibres,

thus the function and possibly also the expression of *bslA* might be temporally separated from that of the exopolysaccharide and amyloid fibre. Interestingly, the expression of *bslA* is altered by the presence of exopolysaccharide (Verhamme *et al.*, 2009; Kovacs and Kuipers, 2011). Even though the matrix components and BslA act sequentially during biofilm development, their transcriptional regulation shares many features (Fig. 1). Both the *eps* and *tapA* (including *tasA*) operons are regulated directly by SinR and AbrB. On top of this, the *tapA* operon is directly regulated by SlrR, while *eps* and *tapA* is indirectly affected by SlrR via SinR. The expressions of *abrB* and *sinI*, the antagonist of SinR, are both influenced by the level of phosphorylated Spo0A protein (Chai *et al.*, 2010). As such, Spo0A plays an important role in *B. subtilis* for the developmental timing and spatial expression of cell types during biofilm formation (Lopez *et al.*, 2009a). This function of Spo0A adds to its role in the sporulation pathway, for which Spo0A was originally well known. The intertwined regulatory pathways ensure that the genes are expressed under the appropriate conditions and in the correct subset of the population. Interestingly, the expression of *bslA* is also modulated by several regulators: AbrB acts directly on the regulatory region of *bslA*, while Rok and DegU indirectly affect *bslA* expression (Kobayashi, 2007; Verhamme *et al.*, 2009; Kovacs and Kuipers, 2011; Ostrowski *et al.*, 2011). Additionally, the transcriptional regulator of the biofilm-related *lutABC* genes, LutR (Chai *et al.*, 2009) directly regulates both the *tapA* operon and the *bslA* gene (Ö. Irigul *et al.*, unpubl. results). Therefore, in addition to the exopolysaccharide-affected expression of *bslA* (Verhamme *et al.*, 2009;

Kovacs and Kuipers, 2011), the matrix genes and *bslA* share this complex transcriptional regulation.

BslA has a SecA-dependent secretion signal and is present in the secretome of *B. subtilis* cells grown in suspension (Voigt *et al.*, 2009). Removal of the signal sequence abolishes the function of BslA, while exchanging the signal sequence with that of the cell-wall-associated WapA protein restores the action of BslA (Ostrowski *et al.*, 2011). While Kobayashi and Iwano (2012) show that BslA is mainly present in the matrix fraction of pellicles, Ostrowski *et al.* (2011) suggest that BslA is localized to the cell wall fraction. This controversy could arise from the differences in fractionation of cell and matrix, i.e. mild sonication (Branda *et al.*, 2006) versus boiling in SDS sample buffer (Kobayashi and Iwano, 2012). However, both papers present independent evidence for the observed localization. Immunogold labelling electron microscopy shows that BslA is associated to the cell wall of *B. subtilis* (Ostrowski *et al.*, 2011), while confocal laser scanning microscopy shows that exogenous labelled BslA covers the outlayer of complex colonies (Kobayashi and Iwano, 2012). It is possible that a part of the newly synthesized BslA localizes to the cell wall before being pushed to the surface of the biofilm.

There are several other examples where the surface property of microbes is modified at single cell level by amyloid proteins. Chaplins are produced by another soil-dwelling bacterium, *Streptomyces coelicolor* (Claessen *et al.*, 2003; Elliot *et al.*, 2003). The below-ground hyphae of *S. coelicolor* are hydrophilic, while the aerial hyphae and spores are covered by a thin layer of chaplins and become hyrdrophobic. The chaplin proteins form a fibre *in vitro* and decrease the surface tension at the air–water interface. Interestingly, similar to the complementation of water repellency by exogenous added BslA to the mutant *B. subtilis* strain, cell extracts containing chaplins also restore the formation of aerial hyphae in mutant *S. coelicolor* strains (Claessen *et al.*, 2003). Moreover, various fungi produce proteins that assemble to produce amyloid proteins *in vitro* and modify surface properties of aerial hyphae through their amphiphilic feature. The unrelated hydrophobins and repellents are produced by mycelial fungi and by the plant pathogen *Ustilago maydis* respectively (Wosten, 2001; Teertstra *et al.*, 2009). BslA accumulates on the surface of the biofilms (Kobayashi and Iwano, 2012), whereas the chaplins, hydrophobins and repellents all function at the cellular level and help the hyphae to grow into the air (Claessen *et al.*, 2006). This suggests that BslA might function to protect the biofilm as a whole. Although at the molecular level this difference might be minor, for evolution it makes a distinct difference whether the benefits of BslA are on the colony level or on the cellular level (West *et al.*, 2006). When benefits of BslA production are shared among all cells within the colony, its production can be exploited by cells that gain the benefits of BslA without paying the costs of BslA production. Therefore, additional mechanisms are required to explain its evolutionary maintenance, besides the direct benefits it delivers to the colony (e.g. group-level selection; Nowak, 2006).

Extracellular proteins produced by the cells in biofilms are shared among the entire population, while only a subset of the population is actually producing the exopolysaccharide and TasA components (Chai *et al.*, 2008; Kearns, 2008). Interestingly, strains that synthesize only the exopolysaccharide or only the TasA protein can complement each other when being mixed together, thereby producing normally developing pellicles (Branda *et al.*, 2006). By the same token, strains that only produce matrix or BslA also complement each other when grown together resulting complex colonies that resembles that of the wild type (Ostrowski *et al.*, 2011). Purified TasA amyloid fibre restores the formation of pellicles in the *tasA* mutant *B. subtilis* strain (Romero *et al.*, 2010). Similarly, adding of purified BslA to the mutant strain complements the ability to form wrinkle formation in pellicles and complex colonies. Exogenously added BslA also restores the surface roughness and the repellency of *B. subtilis* biofilms (Kobayashi and Iwano, 2012). This suggests that BslA when produced by part of the population can benefit the whole biofilm similar to the matrix components. In the soil or gut environment, *B. subtilis* grows in the vicinity of other microbes. Close relatives from the *Bacillus* genus were shown to induce the expression of genes for matrix production, suggesting that mixed biofilms might occur in nature (Shank *et al.*, 2011). Under these mixed conditions, the close relatives of *B. subtilis* might benefit from the production of amphiphilic proteins by *B. subtilis*, resulting in a single community that resides under the umbrella of liquid repellency (Fig. 1).

Finally, besides functioning as a protective layer against environmental stressors, BslA might also function to stabilize the internal conditions of the biofilm. Stable internal conditions facilitate a tight regulation of the cell differentiation process. One could argue that BslA resembles the epithelia of primitive multicellular organisms, which are required to sustain the internal developmental process and to protect the organisms from external stressors (Gerhart and Kirschner, 1997). Thus, BslA adds another important property to the list of multicellular characteristics that are associated with *B. subtilis* biofilm formation (Aguilar *et al.*, 2007; Lopez *et al.*, 2009b).

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