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PERSPECTIVE

Connecting the dots between bacterial biofilms and ice cream

Nicola R Stanley-Wall[1] and Cait E MacPhee[2]

[1] Division of Molecular Microbiology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK
[2] James Clerk Maxwell Building, School of Physics, University of Edinburgh, Edinburgh EH9 3JZ, UK

E-mail: n.r.stanleywall@dundee.ac.uk **and** cait.macphee@ed.ac.uk

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Abstract

Emerging research is revealing a diverse array of interfacially-active proteins that are involved in varied biological process from foaming horse sweat to bacterial raincoat formation. We describe an interdisciplinary approach to study the molecular and biophysical mechanisms controlling the activity of an unusual bacterial protein called BslA. This protein is needed for biofilm formation and forms a protective layer or raincoat over the bacterial community, but also has a multitude of potential applications in multiphase formulations. Here we document our journey from fundamental research to an examination of the applications for this surface-active protein in ice cream.

On 31st August 2015 we released a press statement with the headline: '*Slower melting ice cream in pipeline, thanks to new ingredient*'. The article ignited the interest of global news agencies resulting in prolific, high profile coverage across major national and international radio, electronic and print outlets. The fact that it was released on a Bank Holiday Monday in England no doubt helped, but does not explain the interest generated in Canada, Australia, India and Malaysia, among others. Here we present the scientific journey that instigated this international media storm.

The story is founded in a fundamental desire to understand the molecular and biophysical properties of biofilms; social communities of microbes that are encased in a self-produced sticky extracellular matrix (Hobley *et al* 2015). Biofilms, from a human's point of view, can have both positive and negative implications depending on the environment where they grow. For instance, multispecies biofilm consortia are required for sewage processing and bioremediation but conversely are the causative agents of the majority of biofouling incidences and chronic infections. From the microbe's perspective living in a biofilm is pretty much always a good thing. The structured community facilitates access to nutrients, protection from extracellular stress, and provides stability to the resident microbes in terms of their physical environment. For these reasons knowledge of how the biofilm matrix is assembled, and stabilised, is considered fundamental to the development of novel strategies to control biofilm formation and disruption (Hobley *et al* 2015).

Bacillus subtilis is a 'generally recognised as safe' gram-positive bacterium that is widely used by the agricultural and home care industries. The proteases and amylases found in 'Bio' laundry detergent are predominantly derived from *Bacillus* species. The consequences of biofilm formation are however a two-sided coin: in the agricultural sector it is a positive attribute that promotes plant growth and the induction of systemic resistance, while in the industrial sector biofilm formation is problematic as it results in biofouling of industrial pipelines. For these reasons, combined with the inherent genetic tractability, *B. subtilis* is a perfect organism to dissect the design principles that control biofilm formation. Our combined scientific background, bringing together soft matter physics and molecular microbiology, allows us to take an interdisciplinary approach to studying biofilm formation. We focus on the assembly of the biofilm matrix as it is this process that facilitates the establishment of the mature microbial community.

Researchers from Harvard University made the initial discovery that the *B. subtilis* biofilm is remarkably non-wetting (Epstein *et al* 2011) (figure 1(a)), to the extent that a water droplet placed on the biofilm has a higher contact angle than a water droplet placed on a Teflon film. This is now known to be due to the production of a protein called BslA (for **B**iofilm

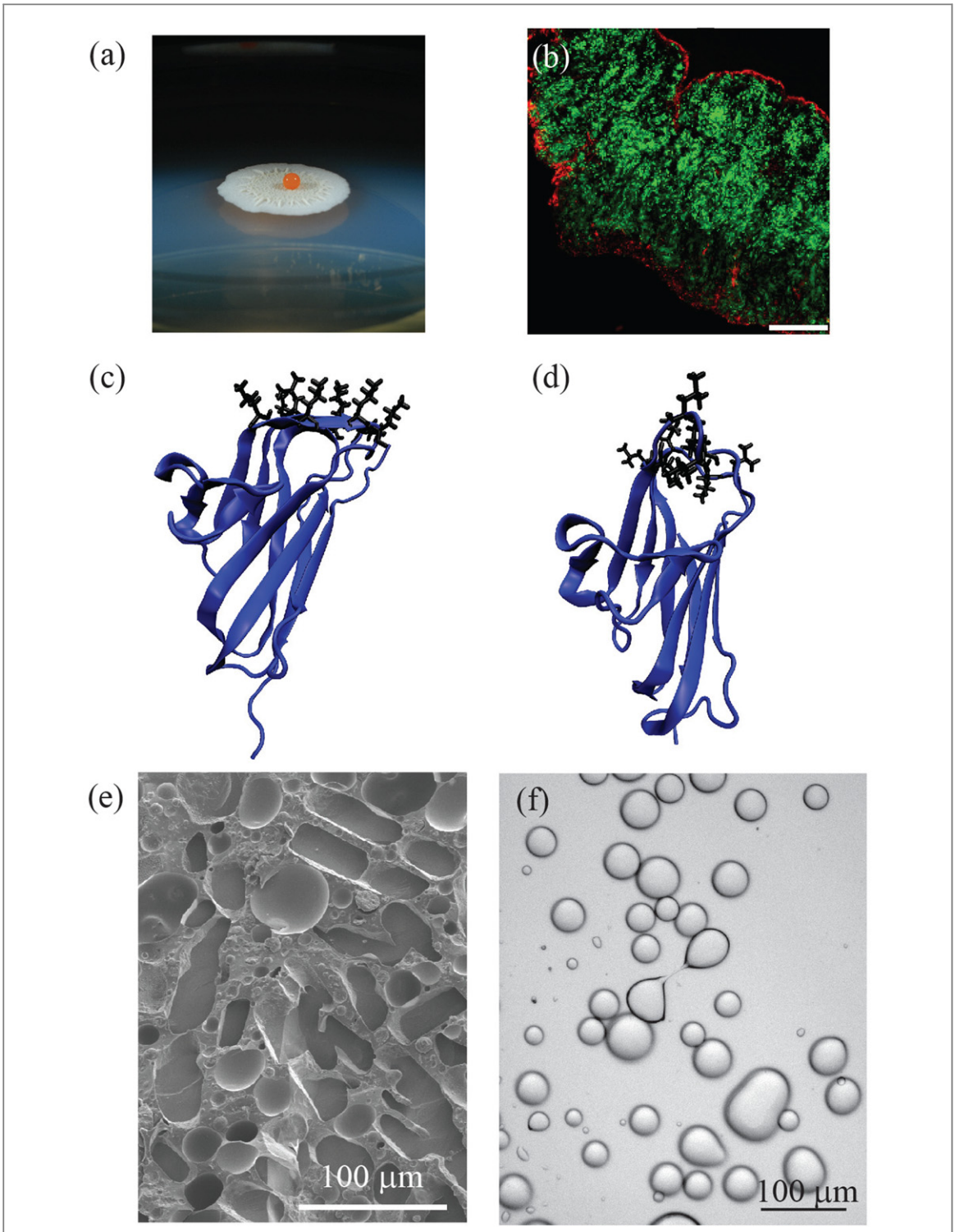

Figure. 1. BslA: a bacterial hydrophobin. (a) A *Bacillus subtilis* was biofilm grown for 48 h and a 5 μl droplet of orange-dyed water placed on top. The hydrophobicity of the bacterial community is apparent from the high contact angles of the droplet; (b) *in situ* analysis of BslA localisation in the mature wild type biofilm. Confocal scanning laser microscopy image of a cross-section where fluorescence from the bacterial cells is shown in green and the staining from BslA is shown in red. The scale bar is 50 μm. The image and legend is reproduced with permission from (Hobley *et al* 2013); (c) crystal structure of BslA at an interface with the leucine and isoleucine residues of the cap exposed; (d) crystal structure of BslA in solution with the leucine and isoleucine residues in the cap region protected from the aqueous environment. Both (c) and (d) were adapted with permission from (Bromley *et al* 2015); (e) a scanning electron microscope image of an ice cream formulation containing BslA as an emulsifying ingredient; (f) recombinant BslA stabilises oil-in-water emulsions, which are often anisotropic.

surface **l**ayer protein **A**). We knew BslA function had to be important since it fell under the control of one of the major regulators of biofilm formation (DegU) and deletion of *bslA* disrupted the biofilm (Kobayashi 2007, Verhamme *et al* 2007). However, at the primary amino acid sequence level BslA initially provided no clue as to its role in biofilm formation, let alone biofilm hydrophobicity. A breakthrough for

us came when a talented PhD student analysed the localisation of BslA within the mature biofilm, using confocal imaging microscopy coupled with immunofluorescence staining. He demonstrated that BslA was localised to the periphery of the biofilm (figure 1(b)): in short BslA appeared to form a 'protective' coat. Soon after, researchers from Japan directly linked BslA with biofilm hydrophobicity and showed that purified recombinant BslA was capable of forming flocs (Kobayashi and Iwano 2012).

Following this we used quantitative biophysical techniques to study BslA surface activity and (after a long journey!) solved the structure of BslA by crystallography (Hobley *et al* 2013) (figure 1(c)). Using these methods we were able to reveal how BslA was capable of imparting the extreme hydrophobicity to the biofilm. The core of the BslA protein is structurally similar to the widespread immunoglobulin superfamily and is therefore (apparently!?) nothing special; but to this scaffold is appended a highly hydrophobic 'cap' containing leucine and isoleucine residues. For 8 of the monomers in the decameric asymmetric unit the side chains of these residues pointed out into the external environment, making up a large, surface-exposed hydrophobic patch. This is unusual since in most proteins hydrophobic groups are sequestered away from the aqueous environment, or are buried in contact interfaces with other proteins. Further in-depth biophysical analysis revealed that BslA actually exists in two forms: the one in which the hydrophobic cap is exposed, and a second in which it is safely sequestered away from water (figure 1(d)). Cleverly, this allows BslA to undergo a limited structural metamorphosis in an environmentally responsive manner: it is soluble in aqueous solution, while at an air/water, oil/water or solid interface it transforms to reveal the structured hydrophobic 'cap' region which drives the formation of a robust viscoelastic interfacial protein film (Bromley *et al* 2015).

BslA shares functional properties with fungal hydrophobins (Hobley *et al* 2013) and therefore, in homage, we coined BslA a 'bacterial hydrophobin'. The fungal hydrophobins have been the subject of substantial industrial interest for their interface-stabilising properties. However it is important to be clear that the primary amino acid sequence, structure and mode of forming an elastic film by BslA is fundamentally different to the mechanism of action used by the well-characterised fungal hydrophobins and is, perhaps, unique. It is still not clear to us what BslA contributes to biofilm architecture other than hydrophobicity, but it is clear that the analysis of the biophysical and molecular mechanisms underpinning the assembly of matrix components such as BslA have implications for understanding how to disrupt or engineer bacterial biofilms.

It was the capability of BslA to interact with oil/water and air/water interfaces as well as with solid surfaces that led us to investigate the applicability of BslA in applied settings. One of the test scenarios was ice cream, which at the basic level is a mixture of air, fat, milk proteins, sugar and water (at least in part in the form of ice crystals). *B. subtilis* is already used in food production: for example *B. subtilis* subspecies *natto* is used to make the fermented Japanese breakfast food "nattō" and other *B. subtilis* isolates are core constituents of the microbial consortium used to make 'meju', a component for many Korean dishes. Therefore BslA is fundamentally already found in the food chain. Through laboratory testing we have shown that during ice cream production BslA binds the air, fat and water together, creating a super-smooth consistency (figure 1(e)). This is because BslA is capable of adhering to both fat droplets (figure 1(f)) and air bubbles making the mixture more stable. It is the stability of the ice cream that theoretically offers significant advantages for ice cream makers by, for example, allowing ice creams to keep frozen for longer in hot weather. BslA also slows down the growth of ice crystals, ensuring a fine, smooth texture is maintained for longer. The development could also allow products to be manufactured with lower levels of saturated fat—and thus fewer calories—than is possible at present. From the manufacturers perspective, BslA could also provide benefit from a reduced need to deep freeze their product, as the ingredient would keep ice cream frozen for longer, and the supply chain would also be eased by a reduced need to keep the product very cold throughout delivery and merchandising.

Thus our research takes us from a fundamental understanding of bacterial biofilm architecture, through the possibility of engineering or disrupting biofilm formation in a wide variety of contexts, to the formation of new versions of familiar foodstuffs. And BslA is not alone in a diverse family of structurally and evolutionarily unrelated proteins that have evolved various strategies for the controlled stabilisation of interfaces: along with the fungal hydrophobins mentioned above, there are the chaplins produced by filamentous bacteria (Elliot and Talbot 2004), latherin that is a component of horse sweat (Beeley *et al* 1986), and ranaspumin which is component of frog foam nests (Mackenzie *et al* 2009). More no doubt remain to be discovered, and all offer the potential for stabilisation and intelligent engineering of future formulations.

When we reflect in this way we find that we are pleased with the synergy that has been generated by our interdisciplinary working that is underpinned by a genuine desire to tackle a shared problem. Now, only time will tell if the translational off-shoots of our

fundamental research bear fruit (or should that be ice cream?!).

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