



# Bifunctionality of a biofilm matrix protein controlled by redox state

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**Biofilms are communities of microbial cells that are encapsulated within a self-produced polymeric matrix. The matrix is critical to the success of biofilms in diverse habitats; however, many details of the composition, structure, and function remain enigmatic. Biofilms formed by the Gram-positive bacterium *Bacillus subtilis* depend on the production of the secreted film-forming protein BslA. Here, we show that a gradient of electron acceptor availability through the depth of the biofilm gives rise to two distinct functional roles for BslA and that these roles can be genetically separated through targeted amino acid substitutions. We establish that monomeric BslA is necessary and sufficient to give rise to complex biofilm architecture, whereas dimerization of BslA is required to render the community hydrophobic. Dimerization of BslA, mediated by disulfide bond formation, depends on two conserved cysteine residues located in the C-terminal region. Our findings demonstrate that bacteria have evolved multiple uses for limited elements in the matrix, allowing for alternative responses in a complex, changing environment.**

biofilm matrix | *Bacillus subtilis* | BslA | redox | hydrophobicity

**B**iofilms are assemblies of microbial cells that are attached to a surface or each other (1). Assembly is facilitated by manufacture of an extracellular matrix, which provides both structural and biochemical support to the biofilm community (2). After analysis of many diverse species, the biofilm matrix has been found to mainly comprise exopolysaccharides, extracellular DNA, and proteins, many of which form higher-order structures, such as fibers or filaments (3). Currently, there is increasing knowledge of the nature and form of the individual components in the matrix (3), but how the matrix components are deployed in the biofilm, how they interact with other elements in the matrix, and how the local physicochemical environment impacts the properties of the materials used in the biofilm are underexplored.

*Bacillus subtilis* is a Gram-positive, spore-forming bacterium, which is found in great abundance in the soil (4) and forms biofilms with a distinctive complex architecture (5). The biofilms display an unusual property: They are highly hydrophobic and remain as such after contact with water, inorganic or organic solvents, and commercial biocides (6). The hydrophobicity of the biofilm has been attributed to a small secreted protein named BslA (7, 8), which works alongside the fibrous protein TasA (9, 10) and the extracellular polysaccharide produced by the products of the *epsA-O* operon (5) to allow morphogenesis of the biofilm. Partial evidence of the mechanism used by BslA to confer hydrophobicity to the *B. subtilis* biofilm has been revealed (8, 11–13). Although transcription of *bslA* occurs uniformly within the population, secreted BslA is localized to the periphery of the biofilm, where it forms a hydrophobic layer at the air–biofilm surface (8, 12). Consistent with this finding, biophysical analysis of recombinant BslA in vitro demonstrated spontaneous formation of a stable elastic protein film at an air–water interface (12). Structural analysis revealed that BslA is an amphipathic

protein consisting of an immunoglobulin G-like scaffold appended with a hydrophobic “cap” (12) that can present in two forms: “cap in” and “cap out.” This property either shields (cap in) or reveals (cap out) the hydrophobic domain in response to the local environment (11). Thus, when BslA is in the aqueous environment of the matrix, it is likely that the hydrophobic cap is “hidden” toward the interior of the protein. In contrast, when BslA reaches a hydrophobic interface (e.g., air at the surface of the biofilm), it undergoes a limited conformational change to expose the hydrophobic cap. Nonetheless, the mechanism by which the BslA biofilm surface layer assembles in vivo is undefined.

*B. subtilis* encodes a monomeric BslA paralogue called YweA (14). Deletion of *yweA* does not impact the overall morphology or hydrophobicity of the biofilm (8); however, deletion in combination with removal of *bslA* exacerbates the biofilm defect of the single *bslA* deletion (8, 14). Contrary to the marginal contribution of YweA to biofilm formation, but consistent with the high level of amino acid sequence similarity, in vitro recombinant YweA undergoes the partial structural rearrangement at an interface to reveal the hydrophobic cap and forms an elastic protein film, albeit with limited stability (14). One notable difference between the primary amino acid sequences of BslA and YweA is that the BslA-like variants possess a short C-terminal extension that contains two conserved cysteine residues in a “CxC” configuration. Cysteine residues play an important role in the function of diverse proteins in a wide range of cellular processes (15), and therefore we

## Significance

**The biofilm matrix is a critical target in the hunt for novel strategies to destabilize or stabilize biofilms. Knowledge of the processes controlling matrix assembly is therefore an essential prerequisite to exploitation. Here, we highlight that the complexity of the biofilm matrix is even higher than anticipated, with one matrix component making two independent functional contributions to the community. The influence the protein exerts is dependent on the local environmental properties, providing another dimension to consider during analysis. These findings add to the evidence that bacteria can evolve multifunctional uses for the extracellular matrix components.**

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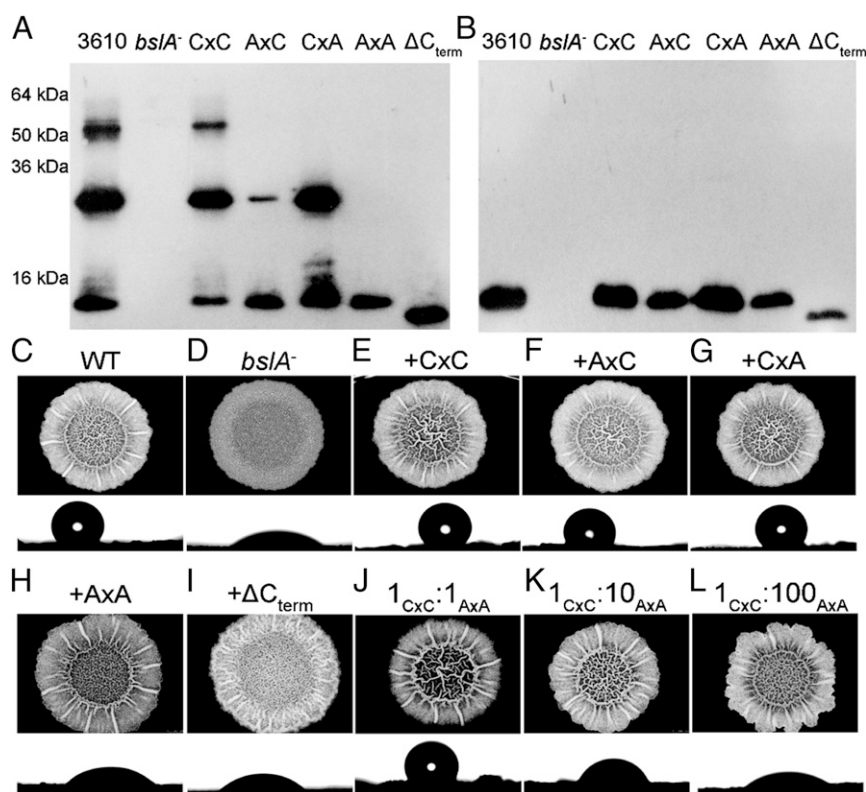
evaluated whether the BslA CxC motif played a significant biological role or was functionally redundant. Our analysis indicates that in addition to BslA having two structural forms (cap in/cap out) (11), it also has two functional forms mediated not by the hydrophobic cap region, but by the cysteines at the C terminus: Monomeric BslA dictates biofilm structure, whereas surface hydrophobicity requires at least dimeric protein. Thus, we show that the cysteine residues are crucial for full BslA function and surmise that, in the native biofilm, electron acceptor availability influences BslA oligomerization: In the oxygen-rich, surface-exposed region, the predominant form is a dimer, whereas the anoxic environment in the depths of the biofilm prevents BslA dimerization and allows for nutrient uptake. This work gives an example of a biofilm matrix protein with two functions that can be genetically separated and where the activity is controlled by redox state.

## Results

**BslA Disulfide Bond Formation.** Recombinant BslA<sub>42–181</sub> forms a mixture of predominantly monomeric and dimeric protein in vitro, with a small amount of tetramer observed (12) (Fig. S1). The requirement of the cysteine residues (C178 and C180) for oligomerization of BslA was assessed in vitro by using purified recombinant protein where the cysteine residue(s) were either replaced with alanine or where the C-terminal 10 amino acids were removed (Fig. S1). Replacement of either C178 or C180 with alanine abolished tetrameric BslA (Fig. S1 A and C), but dimers, which could be disrupted by the addition of  $\beta$ -mercaptoethanol, still formed (Fig. S1). In contrast, when both cysteines were replaced with alanine, or the C-terminal 10 amino acids were deleted, the protein was restricted to a monomeric form (Fig. S1).

Secondary structure analysis of the proteins by circular dichroism (CD) spectroscopy indicated that each of the BslA variants had the same overall fold as the wild-type protein, indicating that the inability to form dimers or tetramers was not due to a disruption of the protein structure (Fig. S24).

Next, we tested to see whether BslA formed dimers and/or tetramers in vivo, to explore the possibility that the conserved CxC motif at the C terminus of BslA plays an in vivo role in BslA oligomerization. Proteins were extracted from the wild-type biofilm either in the presence of Cu(II)-(*o*-phenanthroline)<sub>3</sub>, which cross-links disulfide bonds (16), or 10 mM DTT, a reducing agent. Western blotting revealed immunoreactive bands consistent with monomeric (~14 kDa), dimeric (~28 kDa), and tetrameric (~56 kDa) forms of BslA in the absence of reducing agent (Fig. 1A), whereas in the presence of 10 mM DTT, only monomeric BslA was detected (Fig. 1B). The *bslA* deletion strain (NRS2097) was used as a control for antibody specificity and did not reveal any interacting bands. We generated a series of strains to express the variant *bslA* coding regions in the *B. subtilis bslA* deletion strain (Table S1). Analysis of proteins extracted from the mature biofilms by immunoblot showed the same BslA monomer, dimer, and tetramer pattern in vivo as that observed in vitro for the recombinant proteins (compare Fig. S1 with Fig. 1A). Strains generating C178A (NRS5177) and C180A (NRS5178) variant BslA formed dimers, but not tetramers, whereas the double C178A, C180A mutant (NRS5179) and the BslA <sub>$\Delta$ 172–181</sub> (NRS2957) truncation variants were restricted to the monomeric state (Fig. 1A). In each case, only monomeric BslA was detected by Western blot analysis when 10 mM DTT was added during protein extraction (Fig. 1B). These



**Fig. 1.** BslA is a bifunctional protein. (A and B) Western blot analysis of BslA in a native (A) and reduced (B) state using proteins extracted from biofilms (SI Materials and Methods). (C–L) Architecture and hydrophobicity of biofilms. (C–H) Strains used were 3610 (wild-type; NCIB3610) (C), *bslA*<sup>-</sup> (NRS2097) (D), and the *bslA* mutant genetically complemented with the following variants: CxC (NRS2299) (E), AxC (NRS177) (F), CxA (NRS5178) (G), AxA (NRS5179) (H), and  $\Delta C_{term}$  (NRS2957) (I). Biofilms depicted in J–L are the result of coculturing strains NRS2299 and NRS5179. Under each biofilm is an image of a water droplet on the upper surface of the biofilm, and the values of the contact angle are in Table S2.

data demonstrate that BslA forms dimers and tetramers in vivo in a C178- and C180-dependent manner.

#### Genetic Separation of Roles in Biofilm Architecture and Hydrophobicity.

We next tested whether there was a biological consequence of restricting BslA disulfide bond formation in vivo. Deletion of *bslA* results in a flat, featureless, wetting biofilm; our analysis revealed that production of the BslA<sub>C178A</sub>, BslA<sub>C180A</sub>, BslA<sub>C178A,C180A</sub>, and BslA<sub>Δ172–181</sub> variant proteins using the strains indicated could reinstate biofilm complexity of a *bslA* mutant to a level that was visually comparable to the wild-type strain (Fig. 1 C–I). We then assessed whether the biofilms formed were hydrophobic (8). As expected, the wild-type strain formed a nonwetting biofilm where the water droplet had a high contact angle ( $124.6 \pm 2.9^\circ$ ), whereas the *bslA* mutant was wetting ( $33.6 \pm 2.7^\circ$ ) (Table S2; refs. 8 and 12). Each of the single cysteine-to-alanine mutations, BslA<sub>C178A</sub> (NRS5177) and BslA<sub>C180A</sub> (NRS5178), displayed a nonwetting phenotype, although the contact angle calculated for the BslA<sub>C178A</sub> (NRS5177) strain was consistently lower than that measured for the wild-type strain ( $P < 0.01$ ; Student's *t* test) (Table S2). In sharp contrast, the architecturally complex biofilms formed in the presence of the monomeric BslA<sub>C178A,C180A</sub> (NRS5179) or BslA<sub>Δ172–181</sub> (NRS2957) variants were wetting (Fig. 1 H and I), with contact angles that were indistinguishable from the *bslA* mutant strain (Table S2). Thus, monomeric BslA seems to be associated with architectural complexity of the biofilm, whereas surface hydrophobicity requires at least the dimeric form of the protein. We note that the single point mutations, BslA<sub>C178A</sub> and BslA<sub>C180A</sub>, yielded only dimeric protein in addition to the monomer (Fig. 14), indicating that tetramer formation is not required. These results also reveal that the architectural complexity, which often makes a contribution to biological hydrophobicity (e.g., refs. 17 and 18), is not sufficient for hydrophobicity to manifest.

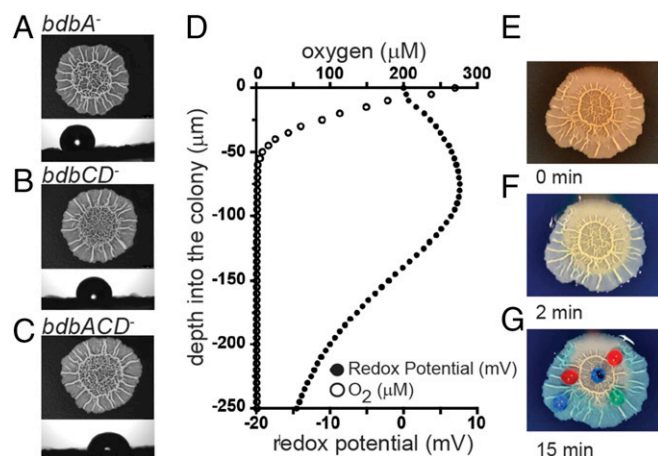
**Sharing BslA Molecules in the Biofilm.** Because hydrophobicity of the biofilms is at the macroscale, where BslA comprises a “common” or “public” good that can be shared by the population (19), we asked what minimum proportion of cells producing wild-type BslA was needed to achieve maximum nonwetting values. To determine this proportion, cells expressing either the wild-type *bslA* (NRS2299) or the *bslA*<sub>C178A,C180A</sub> (NRS5179) coding regions were cocultured over a range of ratios, and the hydrophobicity of the resulting mature biofilm was measured (Fig. 1 J–L). When the strains were mixed in equal proportions, a nonwetting biofilm surface could be sustained ( $116.2 \pm 2.8^\circ$ ). However, when 90% of the cells produced BslA<sub>C178A,C180A</sub>, the biofilm hydrophobicity remained above that of the *bslA* mutant, but there was a step change in the wettability of the surface ( $50.5 \pm 1.3^\circ$ ). Finally, wettability reached a value indistinguishable from that measured for the fully monomeric protein when 99% of the cells produced BslA<sub>C178A,C180A</sub> (Table S2). These findings demonstrate that noncontributing bacteria can be tolerated during formation of the hydrophobic layer, as long as they represent <50% of the population.

**Formation of the Hydrophobic Layer Depends on Thiol-Disulfide Oxidoreductases and Is Enhanced in an Oxidative Environment.** In the *B. subtilis* extracytoplasmic space, disulfide bond formation is catalyzed by one of two thiol-disulfide oxidoreductases (TDORs) named BdbA and BdbD (20–22). We predicted that if BslA was actively oligomerized, then disruption of either *bdbA* or *bdbD* (or both in the case of functional redundancy) would produce a structured, but wetting, biofilm similar to the phenotype displayed by the monomeric BslA variants. To test this hypothesis, we constructed *bdbA* (NRS5552), *bdbCD* (NRS5554) (a *bdbC bdbD* operon deletion), and *bdbA bdbCD* (NRS5553) deletion strains and assessed biofilm formation and surface hydrophobicity (Fig. 2 A–C and Table S1). Consistent with our hypothesis, each of the strains formed biofilms that were morphologically indistinguishable

from the parental strain NCIB3610 (Figs. 1C and 2 A–C), but measurement of surface wettability revealed that the double *bdbA bdbCD* mutant had a reduced contact angle of  $\sim 55^\circ$  compared with the wild-type strain (Table S2 and Fig. 2C). The single *bdbA* and *bdbCD* deletion strains produced surfaces with an intermediate level of hydrophobicity (Table S2 and Fig. 2 A and B).

The wettability of the *bdbA bdbCD* mutant did not reach the level measured for the *bslA* deletion strain (Table S2). Therefore, unless there is a redundant enzymatic mechanism, we propose that a combination of catalytic and spontaneous disulfide bond formation drives dimerization of BslA and leads to the formation of the hydrophobic coat. Consistent with this hypothesis, measurement of the oxygen concentration through the biofilm by using a micro-sensor demonstrated a steep decrease in the available oxygen, with no oxygen measured after 50–60  $\mu\text{m}$ , as measured from the air-exposed surface, rendering the lower biofilm region anoxic (Fig. 2D). Furthermore, microelectrode-based assessment of the change in redox potential through the biofilm indicated that the upper region of the biofilm community was more oxidizing than the base (Fig. 2D). The lack of electron acceptors at the base of the biofilm suggests that BslA may be in a monomeric form in this region. Given the observation that surface hydrophobicity is associated with the ability to form dimeric protein (Fig. 1 C–I and Table S2), a predicted prevalence of monomeric protein at the base of the biofilm raises the possibility that this surface will be wetting. Two experimental findings supported this hypothesis: First, when pigmented water was flooded onto a Petri dish on which a biofilm had been grown, the dye was observed to move rapidly under the biofilm toward the center of the community (Fig. 2 E–G), indicating that it was not repelled by a hydrophobic layer. Second, when the mature wild-type biofilm was turned over, such that the base was facing up, an entirely wetting surface was revealed (Table S2). Together, these findings solve the conundrum (12) of how bacteria in the biofilm access nutrients, despite being surrounded by a layer of BslA.

**BslA Localization Depends on Disulfide Bond Formation.** We next addressed the mechanism by which blocking oligomerization of BslA impaired the formation of a nonwetting biofilm. It is not a



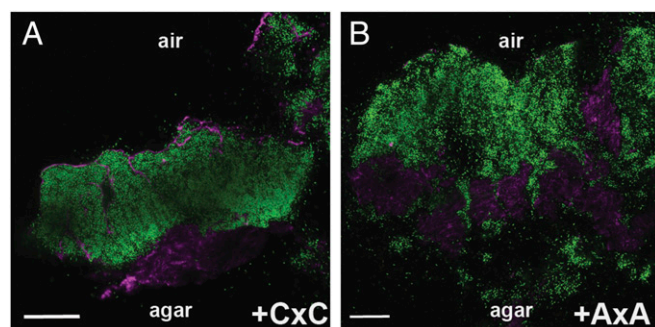
**Fig. 2.** The mechanism of disulfide bond formation. (A–C) Architecture and hydrophobicity of strains *bdbA*<sup>-</sup> (NRS5552) (A), *bdbCD*<sup>-</sup> (NRS5554) (B), and *bdbACD*<sup>-</sup> (NRS5553) (C). Under each biofilm is an image of a water droplet on the upper surface of the biofilm; contact angle values are in Table S2. (D) Measurement of the oxygen concentration ( $\mu\text{M}$ ) and redox potential (mV) as a function of the depth of the biofilm, with 0 mV being set at the biofilm surface (see SI Materials and Methods for details). (E–G) Time course of water uptake by a mature biofilm visualized by using pigmented water: before treatment, 0 min (E); 2 min after exposure (F); and 15 min after exposure (G). In G, 5-mL colored water droplets demonstrate retention of upper biofilm hydrophobicity.



consequence of protein misfolding or disruption of protein production in vivo, because CD spectroscopy demonstrated similar secondary structures in the variant proteins (Fig. S2A), and immunoblot analysis revealed comparable levels of the BslA cysteine mutants to the wild-type protein (Fig. 1A). Therefore, we considered two nonmutually exclusive hypotheses: (i) Mutation or deletion of the C-terminal cysteine residues impairs the innate ability of BslA to form a stable elastic film that depends on lateral interactions between the monomers (12), and consequentially the ability to render the biofilm nonwetting; and (ii) BslA<sub>C178A,C180A</sub> cannot form a dense layer over the surface of the biofilm as observed for the wild-type protein (12). The ability of recombinant BslA<sub>C178A</sub>, BslA<sub>C180A</sub>, BslA<sub>C178A,C180A</sub>, and BslA<sub>Δ172–181</sub> to form stable elastic protein films in vitro was assessed by using pendant drop analysis, coupled with quantification of the wrinkle relaxation speed (12). Each of the variant proteins formed a stable protein film at the oil–water interface with no significant differences compared with wild-type BslA (Fig. S2B). Furthermore, transmission electron microscopy (TEM) indicated that the ability of the variant proteins to form an ordered 2D lattice at an interface was not impeded (Fig. S2 C–E).

Next, in situ localization of BslA was assessed by using immunofluorescence staining of biofilm cross-sections coupled with confocal microscopy (12). The strains tested were the wild type, the *bslA* mutant, and the *bslA* mutant complemented with either the wild-type or *bslA*<sub>C178A,C180A</sub> coding region. In each case, the strains were modified to express the *gfp* coding region to allow detection of the cells by confocal microscopy (Table S1). As has been observed (12), both the wild-type strain (Fig. S3A) and the *bslA* mutant complemented with the wild-type *bslA* allele, showed BslA-associated fluorescence at both the air–cell and cell–agar interfaces, consistent with formation of the BslA integument (Fig. 3A and Fig. S3C). Specificity of the immunofluorescence staining was confirmed by analysis of the *bslA* mutant (Fig. S3B). In contrast, when the *bslA* mutant was complemented with the BslA<sub>C178A,C180A</sub> variant (NRS5136), BslA-linked fluorescence was not detected in high abundance at the cell–air interface and was only prevalent at the agar–cell junction (Fig. 3B and Fig. S3D). These findings indicate an inability of BslA<sub>C178A,C180A</sub> either to migrate to or accumulate at the air interface, which is consistent with the wetting phenotype of the biofilm formed when this variant of BslA is produced.

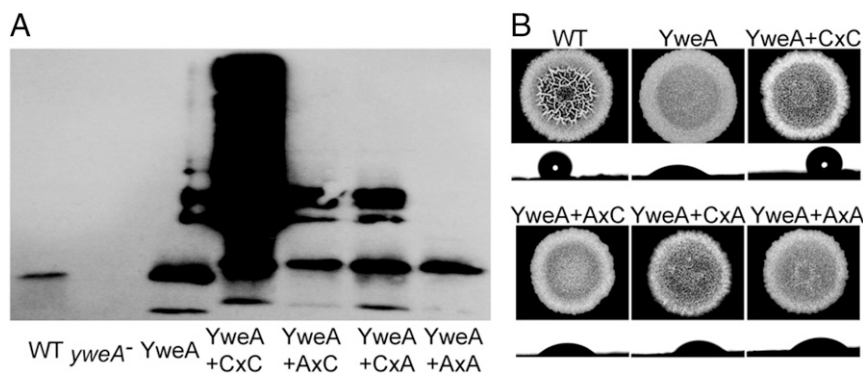
**Synthetic Dimerization of the BslA Parologue.** The strict requirement for dimeric BslA to mediate biofilm hydrophobicity led us to



**Fig. 3.** In situ analysis of BslA localization in the biofilm. Confocal scanning laser microscopy images of biofilm cross-sections through the *bslA* mutant complemented with either the wild-type variant of *bslA* (NRS5132) (A) or the gene encoding the BslA<sub>C178A,C180A</sub> variant (NRS5136) (B) strains are shown. Fluorescence from the GFP is shown in green, and the fluorescence associated with DyLight594, representing immunolabeled BslA staining, is in magenta. (Scale bars: 50  $\mu$ m.) The single-channel data and wild-type and *bslA* controls are in Fig. S3.

hypothesize that if the monomeric paralogue of BslA, YweA, could be engineered to dimerize in vivo, it may become capable of reinstating the nonwetting phenotype to the *bslA* mutant. This outcome is not the case when the wild-type *yweA* coding region is expressed from a heterologous location in the *bslA* mutant (14). To test this prediction, we synthesized a chimeric YweA allele (Fig. S4A), fusing the C-terminal 11 amino acids of BslA (containing C178 and C180) to the C terminus of YweA (hereafter YweA<sub>BslA171–181</sub>). We additionally generated chimeric constructs, where each of the cysteine residues in the BslA C-terminal region was individually, and in combination, mutated to alanine (Table S1). The wild-type YweA, chimeric YweA<sub>BslA171–181</sub>, and YweA<sub>BslA171–181,C178A,C180A</sub> recombinant proteins were purified from *Escherichia coli*, and the secondary structure was assessed by CD spectroscopy. This analysis revealed that the C-terminal extension did not significantly affect folding of the protein compared with the parental YweA protein (Fig. S5A). The oligomerization state of the YweA<sub>BslA171–181</sub> chimeric protein was assessed in vitro by SDS/PAGE (Fig. S4 B and C) and size-exclusion chromatography (SEC) (Fig. S4D), which showed that the chimeric YweA<sub>BslA171–181</sub> protein formed dimers that were reduced to the monomeric state by the addition of  $\beta$ -mercaptoethanol. Bands with the apparent molecular weight expected for dimers were also formed when either one of the two cysteine residues remained (YweA<sub>BslA171–181,C178A</sub> and YweA<sub>BslA171–181,C180A</sub>) (Fig. S4B), but only monomer was observed for the double cysteine-to-alanine chimeric protein (YweA<sub>BslA171–181,C178A,C180A</sub>) (Fig. S4B). Unlike BslA, we did not detect formation of tetramers for the YweA<sub>BslA171–181</sub> chimeric protein. Notably, fusion of the BslA C-terminal 11 amino acids to YweA did not alter the stability or organization of the protein film formed in vitro. Pendant drop analysis, coupled with quantification of wrinkle relaxation speed, revealed that YweA, YweA<sub>BslA171–181</sub>, and YweA<sub>BslA171–181,C178A,C180A</sub> each had an average relaxation time of  $\sim 25$  s (Fig. S5B), substantially less stable than the BslA elastic film ( $>600$  s; Fig. S5B). This result means that the proteins can form elastic films, but they are unstable under compression. Consistent with the film stability, TEM showed that each protein was able to form an organized lattice on a surface (Fig. S5 C–E). Together, these findings indicate that we can generate YweA oligomers in vitro by the addition of the BslA C-terminal 11 amino acids, but although they form an ordered 2D lattice, the chimeric proteins retain the fast film-relaxing properties of YweA (14).

**Oligomeric YweA Yields a Hydrophobic Biofilm.** To assess the impact of engineering YweA to form intermolecular disulfide bonds in vivo, we constructed a suite of plasmids designed to produce the YweA chimeric proteins in *B. subtilis*. The constructs were generated such that secretion through the Sec-system was directed by the BslA signal sequence (the variants are hereafter referred to as YweA<sub>BslA171–181</sub>, YweA<sub>BslA171–181,C178A</sub>, YweA<sub>BslA171–181,C180A</sub>, and YweA<sub>BslA171–181,C178A,C180A</sub>) (Fig. S4A). The plasmids carrying the required coding regions were introduced into the *bslA* mutant at the ectopic *amyE* locus (Table S1). Western blot analysis of proteins extracted from the biofilm, by using an anti-YweA antibody, revealed that only in the presence of both cysteine residues in the appended BslA C terminus could significant levels of disulfide bond-dependent oligomeric chimeric YweA protein be detected (Fig. 4A). As anticipated, the addition of a reducing agent during the extraction process rendered the protein fully monomeric (Fig. S4E). The ability of each of the YweA chimeric variants to reinstate biofilm structure and hydrophobicity to the *bslA* mutant was tested as before. As previously observed, induction of wild-type *yweA* expression did not alter the biofilm morphology or wetting phenotype, compared with the parental *bslA* mutant (Fig. 4B) (14). In sharp contrast, appending the BslA C-terminal 11 amino acids to YweA allowed the chimeric YweA<sub>BslA171–181</sub> protein to return hydrophobicity to the *bslA* mutant strain ( $114.8 \pm 1.1^\circ$ ) (Fig. 4B and Table S2). It should, however, be noted that the biofilm



**Fig. 4.** Forcing dimerization of YweA can rehabilitate biofilm hydrophobicity in a *bsIA* mutant. (A) Western blot analysis of YweA in a native state detected from biofilm protein extracts (*SI Materials and Methods*). Strains used were 3610 (WT; NCIB3610), *yweA*<sup>-</sup> (NRS2405), and the *bsIA* mutant engineered to produce the following YweA variants: YweA (NRS5551), YweA<sub>BsIA171-181</sub> (NRS4834), YweA<sub>BsIA171-181 C178A</sub> (NRS5515), YweA<sub>BsIA171-181 C180A</sub> (NRS5516), and YweA<sub>BsIA171-181 C178A C180A</sub> (NRS5210). (B) Biofilms using strains detailed in A: Under each biofilm is an image of a water droplet on the upper surface of the biofilm: values of the contact angle are in Table S2 and strains are as in A.

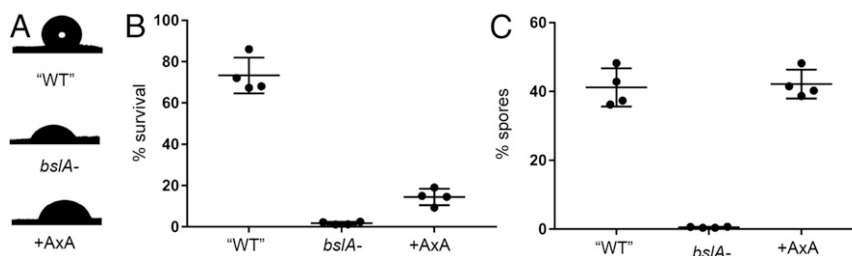
structure retained more architectural similarities with the *bsIA* mutant, rather than the wild-type NCIB3610 strain (Fig. 4B). Expression of YweA<sub>BsIA171-181</sub> thus results in an unstructured, yet nonwetting, phenotype. The slightly lower contact angle measured ( $114.8 \pm 1.1^\circ$  vs.  $124.6 \pm 2.9^\circ$  for the structured, wild-type biofilms) may indicate that biofilm architecture makes some contribution to overall hydrophobicity, as has been suggested elsewhere (18). In contrast, when the C-terminal extension was mutated such that one (NRS5515 and NRS5516) or both (NRS5210) cysteine residues were replaced with alanine, yielding monomeric BslA *in vivo*, the colonies formed retained both the wetting and unstructured phenotypes exhibited by the *bsIA* mutant (compare Fig. 1D with Fig. 4B). Thus, dimeric chimeric YweA containing the two cysteine residues is able to reinstate biofilm hydrophobicity, whereas monomeric chimeric YweA is unable to reinstate biofilm architectural complexity.

**Hydrophobicity and Architecture Play a Role in Resistance to Chemical Attack.** The ability to genetically separate biofilm structure from hydrophobicity allowed us to assess whether protection from external insult is conferred to the bacteria by blocking access of the chemical and/or mediated by the architectural complexity. *B. subtilis* biofilms are nonwetting when challenged with selected commercial biocides (6), with chlorhexidine gluconate being the reactive agent of a number of such biocides. Analysis revealed that 1% (vol/vol) chlorhexidine gluconate is nonwetting on “wild-type” biofilms (Fig. 5A) and, in contrast, is wetting on both the *bsIA* mutant and the variant carrying the monomeric BslA<sub>C178A,C180A</sub> (Fig. 5A and Table S2), providing an ideal test agent to test the question posed above. The number of surviving cells after treatment for 5 min with 1% (vol/vol) chlorhexidine

gluconate was calculated relative to a comparable sample exposed to saline solution. We noted a clear role for BslA in mediating cell survival: In the absence of *bsIA*, exposure to 1% (vol/vol) chlorhexidine gluconate resulted in ~38-fold fewer surviving cells compared with when BslA was produced (Fig. 5B). In contrast, when BslA<sub>C178A,C180A</sub> was present, resulting in a structured, but wetting, biofilm, limited cell survival was measured. Cell survival was approximately fivefold lower than that measured for the wild-type strain (Fig. 5B). Because BslA is known to have a role in sporulation, specifically during biofilm formation (19), we calculated the level of heat-resistant spores in each biofilm at the time point used to test chemical resistance. As established previously, the level of sporulation was low in the *bsIA* mutant (Fig. 5C); however, the number of spores formed in the presence of BslA<sub>C178A,C180A</sub> was the same as when wild-type BslA was produced (Fig. 5C). In toto, these data demonstrate that the protection received by the cells in the biofilm results from a combination of the hydrophobicity and structure conferred by BslA, a phenomenon only revealed by the ability to genetically separate the two functional contributions.

## Discussion

The mechanism revealed here for controlling protein function through oxidation allows us to classify BslA as a bifunctional protein with genetically separable roles in biofilm formation, mediated by cysteine residues in the C terminus. BslA is a key component of the *B. subtilis* biofilm and plays a role in both biofilm architecture and hydrophobicity (8, 12, 23). Dimerization of BslA directly facilitates the development of the nonwetting layer and relies on a cysteine motif (CxC) at the C terminus.



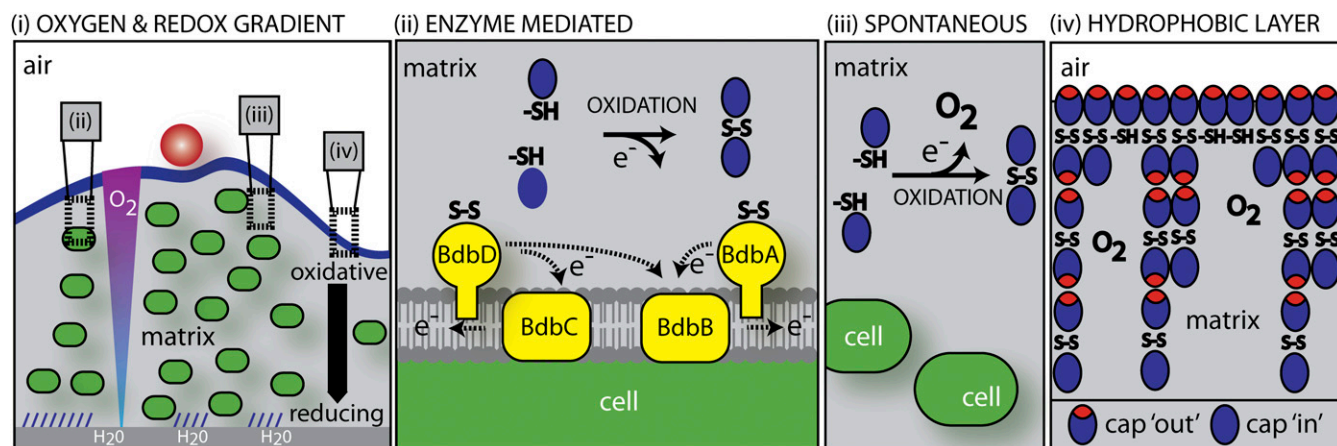
**Fig. 5.** Cell survival after exposure to chlorhexidine gluconate. (A) An image of a 1% (vol/vol) chlorhexidine gluconate droplet on the upper surface of the biofilm for strain WT (NRS5132), *bsIA* mutant (NRS5131), and the *bsIA* mutant complemented with BslA<sub>C178A C180A</sub> +AxA (NRS5136); the values of the contact angle are in Table S2. (B) The strains described above were exposed to 1% (vol/vol) chlorhexidine gluconate, and the percentage survival was calculated. (C) Percentage sporulation was calculated for the strains detailed in A. The error bars represent the SD from the mean.

Monomeric variants of BslA yield biofilms that are architecturally complex, but lack hydrophobicity, whereas, conversely, transplantation of the BslA C terminus onto the normally monomeric nonfunctional paralogue YweA allows the chimeric protein to dimerize and consequentially rehabilitate hydrophobicity, but not architecture, to a *bslA* mutant. These findings are consistent with the accumulation of BslA at the air–biofilm interface only when it can dimerize, as observed by high-resolution in situ immunofluorescence microscopy (Fig. 3). The BslA layer is thus shared by the entire community and can form over and shield nonproducing bacteria (Fig. 1 *J–L*). By virtue of being able to separate the role of BslA in biofilm architecture from hydrophobicity, we have shown that both architectural complexity and the hydrophobic layer contribute to protecting the residents from biocides, thus providing an evolutionary advantage to the resident cells. Finally, we have demonstrated that an effective hydrophobic barrier can be generated by a subpopulation of the biofilm, and this finding raises the question of why the whole population has evolved to produce BslA (12) when other biofilm matrix molecules are produced in a bimodal manner (24).

A model can be postulated that explains formation of a BslA layer that is more than one molecule (or dimer) deep at the air interface (Fig. 6). It is known that BslA dimers are orientated longitudinally (tail-to-tail), with only one cap of each dimer able to interact with the air interface at a time (11). The orientation of tetrameric forms of the protein is unclear, however; because dimeric protein is sufficient to give rise to the observed effects, it is not considered further here. Based on the premise that the hydrophobic cap of one dimer can serve as a hydrophobic interface for another dimer, it leads to a scenario where the intertonguing of the biofilm could comprise the dimeric proteins layered against each other (Fig. 6). Furthermore, because monomeric and dimeric BslA are able to coexist in vitro in a protein film, the mature in vivo hydrophobic layer has the potential to be generated from a mixture of monomeric and dimeric protein. We have confirmed that stable in vitro film formation is not needed to confer hydrophobicity; the chimeric YweA<sub>BslA171–181</sub> variant is able to render the community hydrophobic, but forms a protein film that relaxes quickly under compression (Fig. 4*B* and Fig. 5*SB*). These findings are consistent with a recent analysis of

BslA orthologs, where a *Bacillus pumilus* variant lacked stability in the elastic film, but could substitute for the *B. subtilis bslA* gene in vivo (14). Together, these data indicate that stability in the lateral interactions between the BslA molecules is independent of the ability to confer hydrophobicity to the community, but may determine architectural complexity. However, details of the lateral interaction sites between the protein molecules are currently unknown.

BslA is therefore multifunctional across three different axes: (i) Dimerization (and/or tetramerization) contributes to hydrophobicity, whereas monomeric protein is sufficient for biofilm complex architecture; (ii) the cap-out form of the protein renders a surface layer hydrophobic, whereas we can infer that a cap-in form is present in the wetting protein layer at the base of the biofilm where the protein is exposed to water (11, 13); and (iii) stable lateral interactions between BslA molecules, which can be measured in vitro, appear to be required for architectural complexity, whereas unstable lateral interactions (reflected in unstable film formation in vitro) nonetheless are sufficient to give rise to hydrophobicity if dimeric protein is present. We have previously elucidated a link between the behavior of the cap region and the strength of lateral interactions (13), such that these two functionalities appear to influence each other. Conversely, the behavior of the cap region of BslA is functionally independent from the protein oligomerization state, because both monomeric and dimeric protein are able to flip between cap-in and -out conformations. Thus, the bottom of the biofilm is wetting not just because the protein is monomeric, but because the cap region is also exposed to water; likewise, the top of the biofilm is hydrophobic, not just because BslA forms dimers and tetramers, but because the cap region is also exposed to air. The mechanism(s) by which monomeric BslA gives rise to an architecturally complex biofilm, and the reasons why dimeric protein (at least) is required to give rise to a hydrophobic coat, remain to be elucidated. We have previously observed a correlation between architectural complexity of the biofilm and the ability of BslA variants to form a stable elastic film (14); these previous results are supported by our current findings that chimeric YweA, which forms an unstable film in vitro, can reinstate biofilm hydrophobicity, but not complex architecture. We speculate



**Fig. 6.** Model of BslA function. (i) Schematic of a biofilm cross-section depicting the oxygen and redox gradient through the depth of the structure. The blue layer at the air interface represents the BslA hydrophobic coat with a water droplet on top (red circle), and the hatched lines at the base represent BslA in a form that allows water and nutrient uptake into the community. The bacteria are green ovals, and the agar surface is the gray zone at the base of the biofilm. (ii) Oligomerization of BslA is mediated by thiol-oxidoreductases that reside in the membrane and extracytoplasmic space. The electrons ( $e^-$ ) released by oxidation upon formation of the disulfide bond flow into the respiratory chain. BslA is shown as a blue oval in the biofilm matrix (gray). For simplicity, only one disulfide bond has been depicted (S–S). The reduced form of the protein is represented by the (–SH) annotation. (iii) Oligomerization of BslA is also likely to occur using molecular oxygen as the electron acceptor. (iv) Depicted is a model for how the BslA coat might present in the biofilm as a mixture of oligomers. The ability of BslA to present in a cap-in and -out configuration is represented, where the cap-out form is adopted by the molecules at a hydrophobic interface.



that the stable elastic film formed by monomeric BslA facilitates an association with another component(s) of the biofilm matrix—for example, the exopolysaccharide. Moreover, consistent with this proposal, an association between different molecular components of the *B. subtilis* biofilm matrix can be presumed, because loss of any individual element leads to impaired structure (19).

**Bifunctionality Through Dimerization.** Cysteine residues, possessing a thiol group (15), have specialized roles in a wide range of cellular processes and control protein folding and stability, multimerization, and function through disulfide bond formation (25). It is clear from our secondary structural analysis (Fig. S2) that the role for disulfide bond formation in modulating BslA activity is not linked with either protein stability or folding, but is instead associated with imparting new function through multimerization. This function is in contrast to the fungal hydrophobins, where disulfide bonds stabilize the structure of the protein (26), and is more analogous to the mechanism used to control activity of the von Willebrand factor during blood clotting (25). Previous bioinformatic analyses revealed that Firmicutes, including *B. subtilis*, limit the number of proteins that contain cysteine residues (27). Consistent with this conclusion, there are no essential components that require disulfide bond formation in the *B. subtilis* biofilm, because deletion of the known extracytoplasmic thiol oxidoreductases, *bdbA* and *bdbD*, does not lead to pleiotropic defects. It is only when the integrity of hydrophobicity is assessed that differences in the surface wettability are uncovered (Table S2). It has been proposed that in Firmicutes, proteins that contain disulfide bonds have roles in “niche” functions that are not linked with essential cellular processes—for example, genetic competence that allows the uptake of exogenous DNA (21, 28) and production of the S-linked glycopeptide sublancin that has antimicrobial properties (23, 29, 30). Arguably, biofilm hydrophobicity would fit within this category, and, consistent with genetic competence and sublancin production, successful formation of the hydrophobic layer imparts a fitness advantage by, in this case, excluding chemicals from entering the biofilm.

**Heterogeneity in Electron Acceptor Availability Drives BslA Function.** In addition to catalysis-driven disulfide bond formation by extracytoplasmic thiol-oxidoreductases, spontaneous disulfide bond formation using molecular oxygen (or via a redox active molecule) as the direct electron acceptor enhances BslA dimerization at the air–biofilm surface interface. This process is driven in the top layer of the biofilm by an oxidative, oxygen-rich zone that is conducive to disulfide bond formation (Fig. 6) (31).

It is well established that oxygen gradients stratify natural, mixed-species biofilms by driving distribution of microorganisms with different respiratory requirements (32–34). Furthermore, the availability of electron acceptors, including oxygen, can drive structuring of biofilm morphology, as has been shown for *Pseudomonas aeruginosa* (35, 36). Therefore, these findings expand the role that oxygen gradients can play in biofilm formation by highlighting an ability to modulate protein function. The ability to respond to localized oxygen heterogeneity in this manner provides an efficient mechanism for bacteria to maximize resource utilization by generating bifunctionality through redox sensitivity. Furthermore, BslA dimerization in response to the oxygen gradient solves the conundrum of how *B. subtilis* obtains nutrients when it is surrounded by a layer of BslA (12) [i.e., the anoxic base of the biofilm is hydrophilic despite the abundance of BslA (Fig. 6)]. We predict that bifunctionality of matrix components will emerge as a common theme across the species. Consistent with this proposal, the *Vibrio cholerae* biofilm matrix protein, RmbA, is proteolytically cleaved to a form that promotes recruitment of cells to the biofilm in an exopolysaccharide-independent manner; effectively, the proteolytic event changes the function of RmbA (37). Additionally, flagella synthesized by *E. coli* are used, not only for motility, but also for imparting structural rigidity to the community by entwining the bacterial cells (38). Control of bifunctionality for each of these examples is distinct and raises the question of how many different mechanisms have evolved to maximize use of a limited number of components.

## Materials and Methods

Details of all methods used are provided in full in *SI Materials and Methods*.

**Growth Conditions.** The *B. subtilis* strains used and constructed in this study are detailed in Table S1. The full details of growth conditions are provided in *SI Materials and Methods*. Biofilm colonies were grown on MSgg medium (5) solidified with 1.5% Select Agar (Invitrogen) at 30 °C for 48 h.

**Strain Construction.** All strains, plasmids, and primers used are presented in Tables S1, S3, and S4 and were constructed by using standard techniques. See *SI Materials and Methods* for full details.

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