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# Calcium-mediated Protein Folding and Stabilisation of Salmonella Biofilm-associated Protein A

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## Abstract

Biofilm-associated proteins (BAPs) are important for early biofilm formation (adhesion) by bacteria and are also found in mature biofilms. BapA from *Salmonella* is a ~386 kDa surface protein, comprised of 27 tandem repeats predicted to be bacterial Ig-like (Blg) domains. Such tandem repeats are conserved for BAPs across different bacterial species, but the function of these domains is not completely understood. In this work, we report the first study of the mechanical stability of the BapA protein. Using magnetic tweezers, we show that the folding of BapA Blg domains requires calcium-binding and the folded domains have differential mechanical stabilities. Importantly, we identify that >100 nM concentration of calcium is needed for folding of the Blg domains, and the stability of the folded Blg domains is regulated by calcium over a wide concentration range from sub-micromolar ( $\mu\text{M}$ ) to millimolar (mM). Only at mM calcium concentrations, as found in the extracellular environment, do the Blg domains have the saturated mechanical stability. BapA has been suggested to be involved in *Salmonella* invasion, and it is likely a crucial mechanical component of biofilms. Therefore, our results provide new insights into the potential roles of BapA as a structural maintenance component of *Salmonella* biofilm and also *Salmonella* invasion.

**Keywords:** Magnetic tweezers; Calcium-binding protein; Mechanical stability of proteins; Biofilm-associated protein, *Salmonella*.

## Introduction

Bacteria have an amazing capability to adapt and survive a wide range of environmental conditions. A bacterial cell can live on its own, the planktonic lifestyle, or be a part of a multicellular structured community, the biofilm [1, 2]. Biofilm is formed by adhesion of communities of bacteria to various surfaces and to each other, which involves surface-sensing and molecular transmission events that alter their genetic program [3]. Biofilm formation is promoted under various mechanical, osmotic and chemical stresses [1, 4], and is beneficial for bacterial survival. For example, bacterial biofilms result in increased resistance towards antibiotic treatment [1, 5] and disinfectants [6].

Most bacterial species have the capacity to form biofilms [7]. The major components of these biofilms are water, bacteria cells and extracellular polymeric substances (EPS). EPS are mainly polysaccharides, extracellular nucleic acids, proteins and lipids. Biofilm associated proteins were first reported in *Staphylococcus aureus* and proteins that have homology to these are termed biofilm-associated proteins (BAPs). Biofilm-associated proteins are conserved across the bacterial kingdom, in proteins such as Mus-20 (*Pseudomonas putida*), BapA (*Salmonella enteritidis*), Bap (*Burkholderia cepacia*), Espfm (*Enterococcus faecium*), Esp (*Enterococcus faecalis*), and LapA (*Pseudomonas fluorescens*) [8]. Although the function of these proteins is not fully understood, they have been suggested to be involved in adherence to external substrates, other bacterial cells, and host cells [9, 10]. Bioinformatics suggests that they contain Ig-like domains called bacterial immunoglobulin-like (BIg) domains. The BIg domains are part of the E-set

clade of the Ig-like fold superfamily [11, 12].

*Salmonella enterica* serovar Typhimurium is a gram-negative bacterium, having a rod-shaped structure. It can infect humans causing foodborne illness, mainly typhoid fever. BapA from *Salmonella enterica* serovar Typhimurium can be divided into 3 regions [10]: regions A (aa 1 to 158), B (aa 159 to 3003) and C (aa 3004 to 3824) (Fig. 1a). Region A contains a largely unstructured region and likely positions BapA away from the cell wall. Region C contains three Vibrio-Colwellia-Bradyrhizobium-Shewanella (VCBS) domains (3117-3214, 3371-3466, and 3626-3724), suggesting a potential role in cell adhesion (TIGRFAMs database). Region C also has a secretion domain (3741-3821) bearing a signalling peptide, which is recognized by the ABC transporter and allows type-1 (T1SS) secretion [10].

Region B is comprised of ~27 Blg repeats. Importantly, all the domains in region B contain RTX motifs [10]. Such RTX motifs contain glycine-aspartate rich sequences that have been shown to be calcium sensitive [13, 14]. Interestingly, many BAPs such as SiiE from *Salmonella* [15], Bap from *S. aureus* [16], LapF from *Pseudomonas putida* [17], LapA from *Pseudomonas fluorescens* [8], Vibrio cholerae FrhA [18], Shewanella oneidensis BpfA [19], Legionella pneumophila RtxA [20] Lig protein in *Leptospira* [21] and many more [22] share RTX homology in their Blg domains. Since RTX motifs are calcium binding sites; therefore, the stability of the RTX domains is calcium dependent.

A recent study showed that LapA from *Pseudomonas fluorescens* Pf01, is secreted via a two-step mechanism, involving an intermediate step where the

secretion substrate is tethered to the bacterial surface [23]. This example suggests that the secreted BAPs have two fractions, one remains attached to the cell surface and one is released from the cell surface. Although the function of the largest region (region B) of BapA is unclear, it contains multiple protein domains that may act as a spacer between the cell and neighbouring cells or other extracellular surfaces. Considering that the biofilm may be subject to complex mechanical perturbations, we reason that region B may also serve as a mechanical element to regulate cell adhesion and the biofilm mechanical property.

Several aspects related to the BAPs secretion and potential functions still remain unclear. To name a few: 1) whether the intracellular RTX Blg domains indeed exist in an unfolded conformation is unclear; 2) the mechanical stability of the Blg domains at physiologically relevant loading rate has yet to be quantified; 3) how the stability of the Blg domains depends on calcium concentrations largely remains unknown. In this study, we addressed these questions at the single-molecule level using an in-house constructed magnetic tweezers setup [24, 25]. We show that: 1) The intracellular calcium concentration is sufficient to fold the majority of the Blg domains in region B, 2) The calcium binding sites on BapA Blg domains interact with calcium over a wide range of concentration of sub- $\mu\text{M}$  – mM, and 3) Increasing calcium concentration over this range results in increased mechanical stability of the Blg domains. Together, the differential calcium sensing capability of the calcium binding sites on the BapA Blg domains ensures that BapA can respond to changes in its environmental calcium level. We propose that it is only when the BapA protein is exposed to the elevated calcium concentration

of the extracellular environment does the formation of the highly stable, rigid, conformation of the BapA protein occur. At intracellular calcium concentration the BapA domains are folded but more flexible relative to each other, facilitating the secretion of the entire protein.

## Results

### Purified BapA segments in a calcium free buffer solution

Simple Modular Architecture Research Tool (SMART database) sequence analysis predicts that region B of BapA contains 27 repeats of bacterial immunoglobulin (B<sub>Ig</sub>)-like domains (Fig. 1a). The sequence similarities and conserved domains are shown in figure. S1. Due to the challenge of expressing the full-length region B, we divided the 27 tandem repeats into three segments (Fig. 1a). The first segment contains domains 2 to 5 (BapA<sub>2-5</sub>), the second contains domains 6 to 17 (BapA<sub>6-17</sub>) and the third contains domains 18 to 26 (BapA<sub>18-26</sub>), each of these three segments expressed well as recombinant proteins.

The purified segments were covalently attached to a coverslip surface coated with Halo-tag ligand using Halo-tag chemistry [26-28]. Following attachment, streptavidin coated 2.8- $\mu$ m-diameter super-paramagnetic beads were introduced to bind the C-term AviTag and form protein tethers between the bead and the surface (see materials and methods section) (Fig. 1b). A pair of magnets was used to apply force to the bead, and the amount of force applied was adjusted by changing the distance between the magnets and the bead. Unfolding of the B<sub>Ig</sub> domains will cause extension change of the tether,



resulting in a change of the bead height from the surface which is monitored in real time with nanometer resolution (Fig. 1c). During force change, the bead height change is affected by both bead rotation and extension change of the molecule, while at the same force, the bead height change equals the extension change [25]. In all the mechanical unfolding experiments in this work, the force was increased from  $<1$  pN up to 120 pN at a constant loading rate of  $1.8 \pm 0.2$  pN/s. The technical details of the force control and the detection resolution of our magnetic tweezers can be found in our recent review [25].

Figure 2a shows three representative force-height curves for each segment recorded during the force-increase process. Stepwise extension changes were observed for each segment indicating mechanical unfolding of the Blg domains. During each stepwise extension change, the force remains the same before and after the transition; therefore, the step size is the extension change of the molecule. The maximum number of such unfolding steps were 4, 12 and 9 for BapA<sub>2-5</sub> (4 domains), BapA<sub>6-17</sub> (12 domains) and BapA<sub>18-26</sub> (9 domains), respectively. These numbers are consistent with the numbers of domains in the respective segments predicted in the SMART database, confirming the domain structure and revealing that all the domains in these constructs are folded. Here note that all experiments in figure 2 were done with purified protein in calcium free buffer. In addition, no calcium was added during the purification process (see material and methods section). Therefore, these results suggest that either the intracellular level of calcium is sufficient to fold the domains, or the folding of the domains does not depend on calcium.

In 100 mM KCl, at our loading rate of  $1.8 \pm 0.2$  pN/s, the domains in the segments unfolded over a wide range of forces (Fig. 2b). The majority of unfolding forces for BapA<sub>2-5</sub> are distributed over a range of 5-50 pN, with a rather flattened distribution between 5-40 pN. The unfolding forces of BapA<sub>6-17</sub> and BapA<sub>18-26</sub> domains exhibit a peaked distribution in the range of 5-80 pN. These results show that the BapA Blg domains have differential mechanical stabilities.

Unfolding of protein domains results in an unfolded polypeptide chain under force. It is known that the force extension curve of a polypeptide chain can be described by the worm-like chain polymer model, with a persistence length of  $\sim 0.8$  nm [28-30]. From the force-extension curve calculated using the Marko-Siggia's formula [31], the unfolding step sizes can be converted into contour lengths released during unfolding. The histograms of the contour lengths exhibit a Gaussian-like distribution over a range of 20-40 nm (Fig. S2 red colour histogram and red colour Gaussian-like distribution), peaked at  $\sim 30$  nm spread over a range from  $\sim 22$  nm to  $\sim 38$  nm (full width at half maximum range (FWHM)  $\sim 16$  nm). The range of peptide contour lengths corresponds to unfolding of 58-100 amino acids, the domain size range predicted from the SMART database is 66-82 amino acids, which is in good agreement with our experimentally defined size.

## Calcium-binding sites in BapA

To obtain further insights of how calcium binds to the BapA domains, we performed server-based protein structure and function prediction for BapA<sub>2-5</sub> using RaptorX server (<http://raptorx.uchicago.edu>) [32]. As expected the

server predicted Ig-like domain structures for Blg domains in all segments (Fig. 3 shows the predicted domain structures for BapA<sub>2-5</sub> as an example). A prediction by Griessl et al. suggested calcium-binding sites throughout the Blg domains of BapA based on sequence homology between Blg domains of SiiE and BapA [15] (Fig. S3), which is consistent with the calcium-binding prediction done by the RaptorX server (Fig. 3). Both the predicted structures, and the crystal structure of the triple Blg<sub>50:52</sub> segment from SiiE [16], show that calcium may bind at the interface between two Blg domains inter-locking the  $\beta$ -strands (Fig. 3 and Fig. S3) or in folded Blg domains via RTX-motif interactions. As a test of the structure prediction software we also performed the prediction for filamin A (FLNa) rod domain 1-8 and titin I27 that have known structures, and found the server correctly predicted their structures. In addition, no calcium-binding sites were predicted for FLNa and I27 (Table. S1 and Fig. S8). These results suggest that the calcium binding sites on the BapA Blg domains are differentially exposed to calcium binding, which are likely associated with different calcium binding affinities.

### **Calcium-binding is needed for stable Blg domain folding**

Whilst the results described in figure 2 were obtained from purified protein in calcium free buffer solution, the Blg domains were expressed and purified from *E.coli* where the intracellular calcium concentration is ~100 nM [33]. There is a possibility that the folding of the Blg domains was mediated by this level of calcium concentration in cells, and that bound calcium ions remained bound in calcium free buffer solution. To explore this possibility, we repeated the experiments in the presence of 10 mM EGTA in order to remove any

potential bound calcium ions (Fig. 4; N.B. The experimental design throughout figure 4 is shown schematically in the inset). Figure 4a shows the representative force-height curves of BapA<sub>18-26</sub> in 100 mM KCl before introducing EGTA (force cycles C-1 and C-2, equivalent to Fig. 2a) and after introduction of 10 mM EGTA (force cycles C-3 and C-4). These experiments reveal a striking result that after addition of EGTA (force cycles C-3 and C-4), the Blg domains are no longer able to refold into stable structures upon release of force, indicated by the disappearance of unfolding steps at forces above 10 pN and a much longer extension at forces below 40 pN compared to the data from force cycles in C1 and C2. This is markedly different behaviour to what was seen in figure 2, where repeated cycles of unfolding-refolding gave similar unfolding profiles. Some steps are observed below 10 pN, which indicates that the BapA rod domains may exist in a partially folded unstable conformation.

Following removal of EGTA, the force-bead height curves (force cycles C-5 and C-6) remained similar to those in force cycles C-3 and C-4 in the presence of EGTA, indicating that the loss of the stabilization of the Blg domains is not caused by any potential interaction between EGTA and the unfolded polypeptide. These results suggest that 1) calcium-binding is needed for the stabilization of the Blg domains, and 2) these calcium ions can be rapidly removed by the chelating agent EGTA in the unfolded conformation of the Blg domains (as shown in Fig. 4b). Thus, in the absence of calcium, the Blg domains exist in a partially folded conformation or disordered peptide conformation. Since calcium is needed for stable folding of the Blg domains, the fact that we observed stably folded domains for purified BapA rod

segments in calcium-free buffer solution implies that the purified proteins have calcium ions bound to the Blg domains, which are retained during series of unfolding and folding cycles (Fig. 2).

Figure 4b shows that the loss of domain refolding in BapA<sub>18-26</sub> after treatment of EGTA could be rescued by reintroducing calcium (force cycles C-9 to C-13), but not magnesium (force cycles C-7 and C-8). This result suggests that the domain stabilization may specifically depend on calcium ions. At a low concentration (1  $\mu$ M) of CaCl<sub>2</sub> (force cycles C-9, and C-10), the refolded domains unfold at comparable forces to that obtained from purified protein in calcium free buffer solution (force cycles C-1 and C-2). Following this recapitulation of the unfolding response of the Blg domains by addition of calcium, we tested what would be the effect of adding additional calcium. Strikingly, at higher calcium concentration (10 mM CaCl<sub>2</sub>), the mechanical stability of the domains is significantly enhanced, the same number of unfolding steps are present but they unfold at much higher forces (force cycles C-11 to C-13). Similar effects of calcium were also observed for BapA<sub>2-5</sub> and BapA<sub>6-17</sub> (Fig. S4). Notably, we found that the folding of the Blg domains could be folded at concentrations as low as 100 nM (Fig. S5). These results reveal that the calcium binding sites on the Blg domains have a wide range of affinities to calcium, allowing them to respond to different level of calcium concentrations.

### **Bound calcium is retained during unfolding/refolding cycles in calcium-free buffer solution**

We have shown that calcium-binding is needed for stable Blg domain folding,

based on which we have inferred that the purified BapA rod segments have calcium ions bound to the Blg domains which are retained during series of unfolding and folding cycles. To directly show that calcium ions can be retained after domain unfolding, after the force cycle C-13, free  $\text{CaCl}_2$  in solution was removed by washing the channel (volume < 100  $\mu\text{l}$ ) with a large volume (500  $\mu\text{l}$ ) of 100 mM KCl solution. In the absence of free calcium, the Blg domains could still refold in multiple unfolding/refolding force cycles (data from the first two cycles, C-14 and C-15, are shown in Fig. 4c). The unfolding forces (C-14 and C-15) of the refolded domains become smaller than in 10 mM  $\text{CaCl}_2$  (C-11 to C-13) but higher than the purified proteins in calcium free buffer solution containing 100 mM KCl (C-1 and C-2). This result confirms that Blg domains can retain calcium through multiple rounds of unfolding and refolding cycles after free calcium is removed from solution.

Overall, these results indicate that there are multiple calcium-mediated structural processes involved in the Blg domain folding and stabilization. Some of the high-affinity calcium binding sites are required for Blg domain folding at low calcium concentrations, while some lower-affinity sites bind calcium at high calcium concentration that significantly increases the mechanical stability of the domains. Consistently, circular dichroism (CD) of BapA<sub>2-5</sub> confirms that the purified protein is already folded (Fig. S6), and addition of 3 mM  $\text{CaCl}_2$  has little effect on domain folding as no additional secondary structure was observed. As the same number of unfolding steps are present (and no additional increase of secondary structure is seen via CD), we suggest that the additional increase in mechanical stability arises from binding of calcium ions to some lower-affinity calcium-binding sites.

## High calcium concentration enhances the mechanical stability of the BapA rod domains

Our data show that BapA segments contain calcium-dependent folded domains that retain calcium throughout the purification process and, even following unfolding of the tethered protein. Furthermore, as seen in figure 4b, further mechanical stabilization occurs upon exposure to high, millimolar, concentrations of calcium, indicated by much greater forces needed to unfold the domains compared to the same domains at  $\mu\text{M}$  calcium concentrations. The RaptorX structure and binding sites prediction server predicted different types of calcium-binding sites (Fig. 3). Since the mechanical stability of the Blg domains is dramatically enhanced when the calcium concentration increases from  $\mu\text{M}$  to  $\text{mM}$ , we reason that the lower-affinity calcium-binding sites are responsible for this enhanced mechanical.

To further explore this stabilisation effect at high calcium levels we tested the effect of 3  $\text{mM}$  calcium on the three BapA segments. Representative force-bead height curves of BapA<sub>2-5</sub>, BapA<sub>6-17</sub> and BapA<sub>18-26</sub> segments before (red) and after adding 3  $\text{mM}$   $\text{CaCl}_2$  (blue) are shown in Figure 5a. For the BapA<sub>2-5</sub>, BapA<sub>6-17</sub> and BapA<sub>18-26</sub> segments, 4, 12 and 9 unfolding steps were observed respectively. The addition of millimolar calcium to the chamber results in a marked mechanical stabilisation of the Blg domains in all three segments. For BapA<sub>2-5</sub> the unfolding forces are shifted from 5-50  $\text{pN}$  to 40-90  $\text{pN}$ , whereas for BapA<sub>6-17</sub> and BapA<sub>18-26</sub> the unfolding forces are shifted from 40-80  $\text{pN}$  to 80-120  $\text{pN}$ . In all cases, the unfolding forces significantly shift to higher range after adding calcium. Strikingly, no additional unfolding steps are observed

and there is no change of contour length after addition of 3mM CaCl<sub>2</sub> (Fig S4.)

## Discussion

Bacterial cells adhere to surfaces, and to cells, and are subject to mechanical constraints (e.g. shear force applied by body fluid or shear force by the environment). In these conditions, when attached, bacterial adhesion proteins will be under mechanical force. Therefore, understanding the mechanical properties of BAPs is important to understand their adhesive functions.

It has been suggested that BapA and similar surface proteins are involved in bacterial adhesion [8]. BapA is classified as a non-fimbrial adhesion protein secreted by the type 1-secretion systems (T1SS) in *Salmonella* [10]. It has been shown that disruption of a similar surface protein Bap (*S. aureus*) by proteinase can impede the initial bacterial adhesion, as well as biofilm dispersal [34]. Over-expression of BapA (*S. Enteritidis*) causes pellicle formation, and disruption of BapA causes inability to form biofilm in LB medium [10].

Several interesting findings were revealed from this study, namely, 1) calcium is needed for the folding of the Blg domains, 2) the intracellular calcium level is sufficient for folding of the Blg domains, 3) increasing concentration of the calcium enhances the mechanical stability of the Blg domains with millimolar calcium concentrations being required for maximal stability. Unexpectedly, bound calcium can be retained during cycles of unfolding and refolding transitions. These findings collectively underscore the importance of calcium



in regulating the folding/stability of BapA Blg domains. Since the BAP domains share strong sequence identity and similarity, the calcium dependent folding and stabilization may be a shared property across the bacterial kingdom.

The finding that the BapA Blg domains requires sub- $\mu\text{M}$  calcium concentration for folding and higher concentration for enhanced stabilization implies the existence of multiple types of calcium binding sites with different functions - the higher-affinity ones mediate structural folding of the domains and the lower-affinity sites regulate the stability.

Our results suggest that the BapA Blg domains have a wide range of mechanical stabilities. Although the Blg domains are predicted to have similar ternary structures, they still have significant differences in sequence – they share only about 30% of sequence identity (Fig. S1). They also differ in the number of residues in the domains. These factors could affect the exact unfolding transition pathways and the unfolding energy barrier, resulting in a variety of mechanical stability of the domains.

Our data suggest that the BapA Blg domains are likely folded intracellularly. It was previously suggested that the intracellular calcium concentration is  $\sim 100$  nM [13, 35, 36]. Consistently, we found that calcium at concentrations as low as 100 nM is sufficient to fold the Blg domains (Fig. S5). Such calcium mediated protein-folding and mechanical stabilization is not observed for magnesium (Fig. S7), indicating selectivity of calcium over magnesium and perhaps other divalent cations.

Interestingly, a recent publication reported that the ice-binding surface protein mpAFP from *Marinomonas primoryensis* contains calcium-binding sites and that calcium-binding is needed for mechanical stability of the mpAFP rod domain [37, 38]. In contrast to the BapA rod domain studied in this work that contains heterogeneous tandem repeats of Ig-like structures, the mpAFP rod domain consists of identical repeats of 104 amino acids that form a continuous  $\beta$ -solenoid structure. In addition, previous studies on the rod domains of SiiE from *Salmonella* [15] and Bap from *S. aureus* [16] also revealed the importance of the calcium-binding on the stability of the domains. Together with these previous studies, our results suggest that calcium-assisted stabilization of secreted bacterial surface protein could be a generic phenomenon.

Our results indicate that the purified Blg domains retain calcium from *E. coli* that maintain the folded domains. Consistently, we found that the BapA Blg domains could be folded at intracellular calcium concentrations of  $\sim 100$  nM [33]. Strikingly, our results reveal a dramatic increase in mechanical stability of the domains when the calcium concentration is further increased to 3 mM, levels that resemble the conditions of the extracellular environment. This result suggests that on a partially translocated BapA, the extracellular Blg domains are more stable than the intracellular Blg domains. This also suggests that maximal stability, and likely rigidity, of the BapA protein is only achieved extracellularly. Therefore, translocation of the BapA could result in reduction of the free energy, driving the directional translocation of the BapA protein. Interestingly, BapA contains a signalling peptide at the C-terminal that can be recognized by the ABC transporter [10], an ATPase associated with

the T1SS system that is known able to transport long multidomain peptides. Therefore, it is possible that the translocation of the protein domains may also be powered by the energy released from the ATP hydrolysis of ABC transporter.

We observed that calcium ions bound on the Blg domains could remain bound for a long time in solution without free calcium. Our experiments in calcium free buffer (Fig. 2a) demonstrate that Blg domains retain calcium from expression in *E. coli*. Even following unfolding by mechanical force these domains can retain the calcium ions to facilitate refolding. While the slow dissociation of calcium ions in calcium-free buffer solution seems surprising, it is consistent with a series of recent studies revealing that the dissociation rate of a bound ligand on a substrate in the absence of free competitive ligand/substrate in solution can be a few orders of magnitude slower than that in the presence of competition [39]. The mechanisms underlying these observations are still unclear, and are currently a hot topic of theoretical study [40-42].

Adhered bacterial cells are likely subject to external mechanical perturbation and as a result large conformational deformation. Our results show that under force, the BapA domains will undergo unfolding transitions. BapA is a large protein, comprised of 3824 amino acids, and a fully unfolded BapA could release a contour length of  $\sim 1.3 \mu\text{m}$ . A recent study has suggested that any force-transmitting protein containing an array of modular domains has the capacity to act as a molecular mechanical absorber, buffering force within a certain range over a large range of strain change [43, 44]. The large multi-

domain composition of BapA suggests that it may also serve as a mechanical absorber. This may provide flexibility within the biofilm and at the interface between *Salmonella* and substrates, which may be beneficial for biofilm to resist mechanical perturbations [43].

Interestingly, force has been reported to play a crucial role in the formation and maintenance of biofilm. For example, force increases the bacterial adhesion to surfaces [45] and to cells [46, 47]. These results suggest that components in biofilms can sense and convert mechanical cues to cellular responses. It remains unclear which biofilm components are responsible for biofilm mechano-sensing. We reason that BapA and similar surface proteins with Ig-like domains may take a part in this process, as Ig domains often provide binding sites for other partners. The conformational change of BapA induced by mechanical perturbation observed in our experiments suggests possible force-dependent interactions between BapA and other binding partners.

## **Materials and methods**

### **Protein purification**

Plasmid pFN18A (Promega) was used to construct the vectors and co-expression with biotin holoenzyme synthetase plasmid BirA. Briefly the relevant domain fragments were amplified from *Salmonella enterica* serovar Typhimurium (str. 14028S) using PCR. An AviTag and 6x His-tag coding sequence was added at the C-terminal of the protein segments. The primers used to construct these segments are listed in Appendix Table S2. pFN18A

plasmid was cut at PmeI and SgfI restriction sites followed by ligation with PCR products of BapA subdomains. DH5 $\alpha$  cells were used for cloning of BapA<sub>2-5</sub> and BL21(DE3) competent cells were used for expression. Single step competent cells KRX (Promega) were used for cloning and expression for BapA<sub>6-17</sub> and BapA<sub>18-26</sub>. Bacteria were grown in LB medium at 37 °C in presence of ampicillin (100  $\mu$ g/ml) with orbital shaking. When the OD<sub>600</sub> of the culture reached 0.5, the temperature was reduced to 21 °C. The culture was supplemented with addition of biotin (final concentration 50  $\mu$ g/ml). The cells were induced overnight by the addition of 0.5 mM isopropyl-1-thio- $\beta$ -galactopyranoside (IPTG). Harvested bacteria were resuspended in 10 mM Tris pH 7.4, 250 mM KCl, 0.5 mM PMSF and disrupted by French press. Protein purification was done with Ni<sup>2+</sup>-NTA column followed by gel filtration. The quality and purity of the protein were confirmed by 4-12% SDS-PAGE and mass spectrometry.

### **Force calibration**

Force calibration was carried out using the method detailed in our previous publication [24], and has an intrinsic relative error of <15% (due to heterogeneous bead size). The loading rate control was achieved by programming the movement of the magnet in such a way that the loading rate is a constant. Briefly, in magnetic tweezers experiments, force is a function  $F(d)$  of bead-magnet distance  $d$ . Therefore, one can change the force by changing  $d$ . A time varying force can be applied by changing  $d$  with time. In a loading rate experiment, the force is changed linearly with time with a slope of  $r$ , which is called the loading rate. In a typical loading rate experiment, force

was increased from an initial value  $F_0$  through a time trajectory  $g(t) = F_0 + r t$ . In order to achieve loading rate control, one can correspondingly program the movement of the magnets through a trajectory of  $d(t) = F^{-1}(g(t))$ , where  $F^{-1}$  is the inverse function of  $F(d)$ . In our instrument,  $F(d)$  has been calibrated (see Chen *et al.* 2011 for details), based on which the constant loading rate control was achieved using LabVIEW program to move the magnets attached on a linear translational motor.

### Single-molecule manipulation

Experimental setup was as described elsewhere [48]. Briefly, coverslips are cleaned with detergent, acetone and isopropanol for 30 min each followed by plasma treatment. Coverslips are then incubated with 1% APTES in methanol and acetone solution for 20 min, after washing, the coverslips are cured at 110°C for 1 hr. Channels were prepared as described previously and have a volume  $\sim 40 \mu\text{l}$  [48]. Flow channels are treated with 0.1% glutaraldehyde for 1 hr followed by incubation with 3  $\mu\text{m}$  polyamine beads and then halo-tag ligand (Promega). The channels were blocked with 25 mM Tris pH 7.4, 100 mM KCl, 1% BSA and 0.02% T-20 (control buffer). Protein segments were introduced into the flow channel in control buffer and incubated for 15 min. Free protein was washed from the channel and then 2.8  $\mu\text{m}$  streptavidin-coated superparamagnetic beads (M-270, Invitrogen) were added into the channel. Force was calibrated for each tether before the experiment. A constant loading rate of  $\sim 1.8 \text{ pN/s}$  was applied in all experiments [24]. Note: The magnetic tweezers detect the height change of bead, which has an offset to the actual extension change of the molecule. In experiments, we set the extension to be

zero at forces below 1 pN. The uncertainty of the offset doesn't affect the measurement of protein unfolding.

1  $\mu$ M, 3 mM or 10 mM  $\text{CaCl}_2$  was added to control buffer for calcium experiments as required. For buffer switch experiments (Fig. 4), protein was held at a force  $\sim$ 12 pN and buffer of 300-500  $\mu$ l was applied depending on buffer condition and concentration.

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### Author contributions

J.Y., P.S.D., and Y.L., designed the research. D.G., M.Y. and K.B performed the experiments. J.Y. P.S.D., B.T.G and D.G interpreted and analysed the data. P.S.D, B.T.G., D.G, and J.Y wrote the paper.

### Conflict of interest

The authors declare no competing financial interests.

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## Figure Legends

**Fig. 1. Experimental design.** (A) Schematic structure of BapA. The 27 Blg domains (green), the three VCBS domains (magenta) and the Type 1 secretion sequence (T1SS) domain (red) are shown. (B) Schematic representation of magnetic tweezers set up. The protein is tethered via its two termini between a coverslip and a super-paramagnetic bead. (C) Schematic representation of unfolding of protein domains that can be detected by the extension change.

**Fig. 2. Mechanical unfolding of BapA domains.** Top, middle and bottom panels show BapA<sub>2-5</sub>, BapA<sub>6-17</sub> and BapA<sub>18-26</sub> protein segments respectively. (A) Representative force-bead height curves during force-increase scans at a loading rate of  $1.8 \pm 0.2$  pN/s. Data in different colours were obtained from different cycles of the same protein tether (see inset schematic of the experiment). Unfolding steps are labelled with numbers. (B) The unfolding force histogram. Top, middle and bottom panels show corresponding histograms of forces at which unfolding steps were observed. Where  $n$  is the number of independent protein tethers and  $N$  is the number of cycles.

**Fig. 3. Calcium-binding site prediction.** Representative BapA<sub>2-5</sub> segment predicted structure by RaptorX server (<http://raptorx.uchicago.edu>). Red colour spheres are the calcium ions, which are bound to the protein.

**Fig. 4. Calcium-dependent BapA<sub>18-26</sub> domain folding.** All data in this Figure are taken from the same molecule, subjected to a series of different buffer exchanges. Inset: The experimental design shown as a schematic. Force-bead height curves are sequentially labelled C-1, C-2, ... C15. (A) Domain folding is calcium dependent. Data obtained first in control buffer (black, C1

and C2), then switched to 10 mM EGTA (red, C-3 and C-4) and then back to control buffer (cyan, C-5 and C-6). The protein shows an unfolding profile in C-1 and C-2 but loses its unfolding steps following addition of 10 mM EGTA (C-3,4) and remains unfolded when subsequently returned to control buffer (C-5,6). (B) Magnesium does not enable protein refolding. 10 mM  $\text{MgCl}_2$  buffer (green, C-7 and C-8) as compared with control buffer (black, C-1 and C-2). Calcium mediated protein folding going from green to magenta (1  $\mu\text{M}$   $\text{CaCl}_2$ , C-9 and C-10) and further stabilised by addition of 10 mM  $\text{CaCl}_2$  (blue, C-11 to C-13). (C) Calcium ions stay associated with the protein after washing with calcium-free control buffer (data shown in wine colour (C-14 and C-15) as compared with data in black colour.)

**Fig. 5. The effect of calcium on the mechanical stability of the BapA domains.** Top, middle and bottom panels show BapA<sub>2-5</sub>, BapA<sub>6-17</sub> and BapA<sub>18-26</sub> protein segments respectively. (A) representative force-bead height curves before (red) and after (blue) introduction of 3 mM  $\text{CaCl}_2$ . (B) Probability distribution of unfolding forces (i.e., histograms of unfolding forces normalized by the total number of unfolding events), before (red) and after (blue) introduction of 3 mM  $\text{CaCl}_2$ . Where  $n$  is the number of independent protein tether and  $N$  is the number of cycles.

**Fig. 6. BapA protein secretion through the membrane.** (A) Cartoon representation of the three states of BapA, (left) in the absence of calcium BapA is unstructured, (middle) At ~100 nM calcium concentration the Blg domains fold, but are joined by flexible linkers, (right) At 3 mM calcium concentration the domains interact to form the rigid, mechanically strong, rod

like structure. (B) BapA exists as a series of domains like beads on a string in the cytosol or less stable folded domains, where the calcium ion concentration is lower than  $\leq 100$  nM. Following translocation across the membrane into the extracellular environment, where the calcium concentration is  $\geq 3$  mM, the BapA adopts its mechanically stable conformation either tethered to the bacteria or, following cleavage or complete secretion, as a surface detached protein that forms part of the biofilm matrix.

**Research highlights:**

- 1) Diverse mechanical stability of Salmonella biofilm-associated protein (BapA).
- 2) Identified differential calcium-binding sites with different functionality at different concentrations.
- 3) Calcium-binding is needed for proper folding of BapA.
- 4) Calcium-binding increases the mechanical stability of the BapA domains.

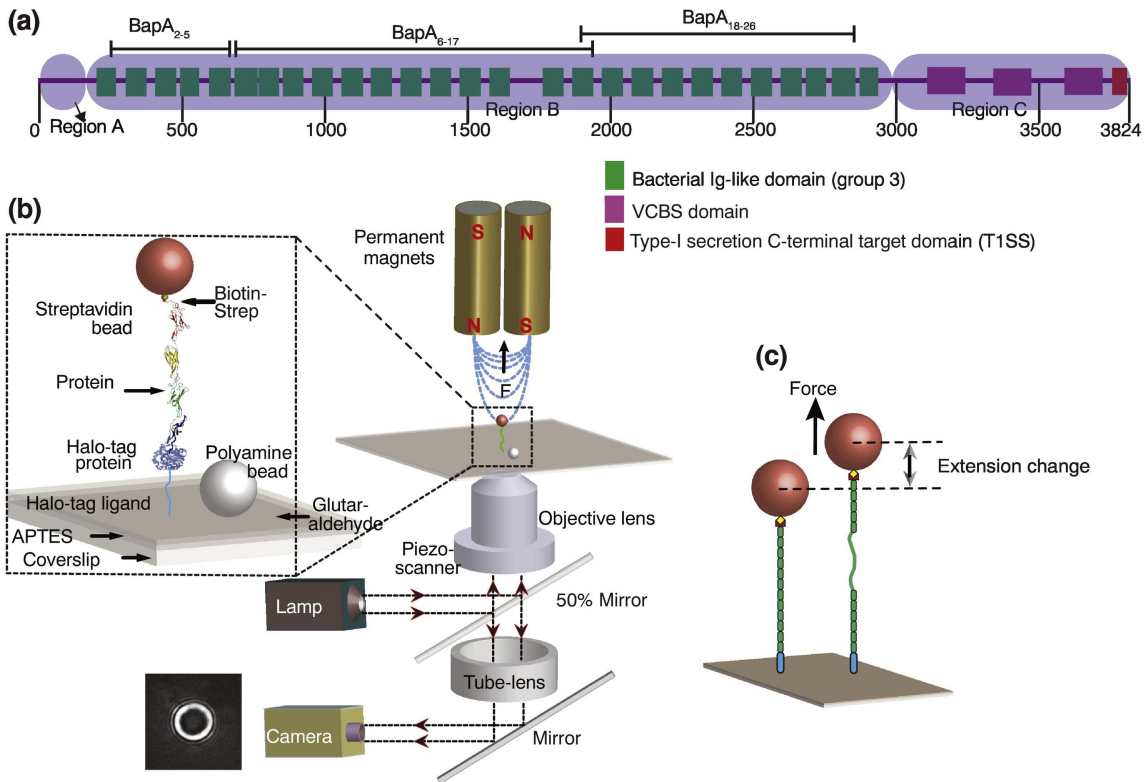


Figure 1

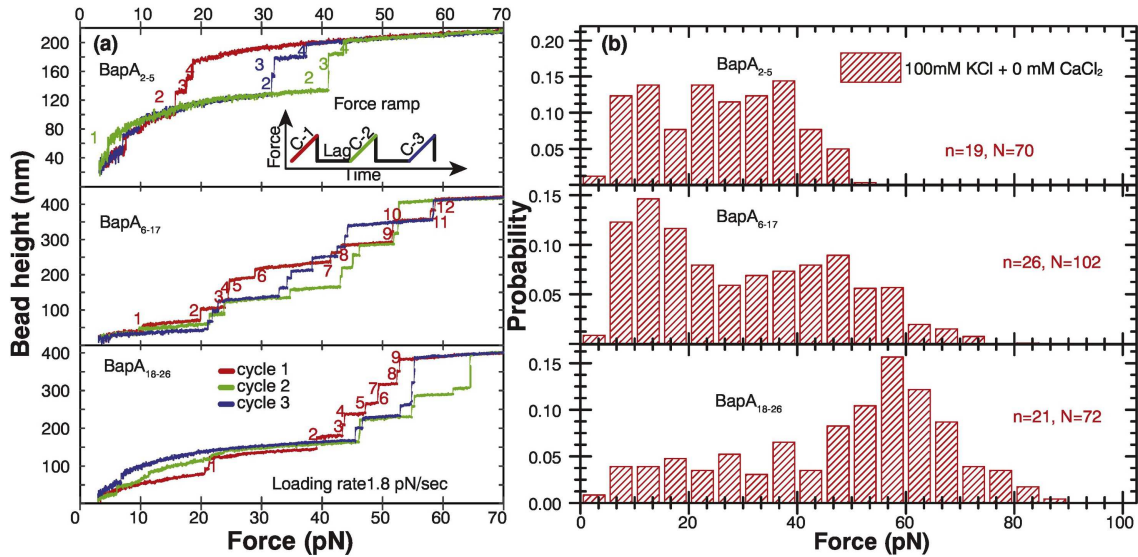


Figure 2

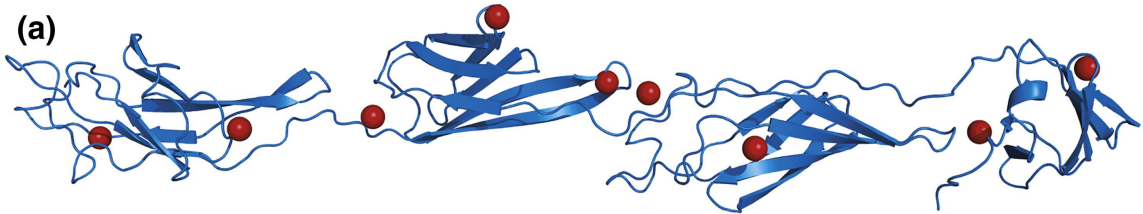


Figure 3



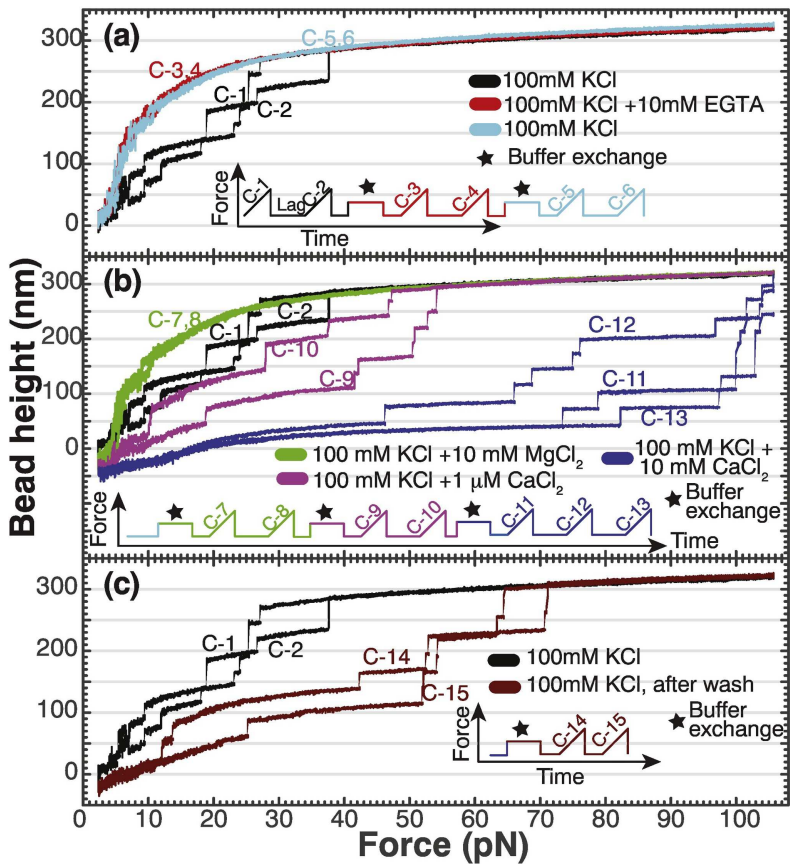


Figure 4

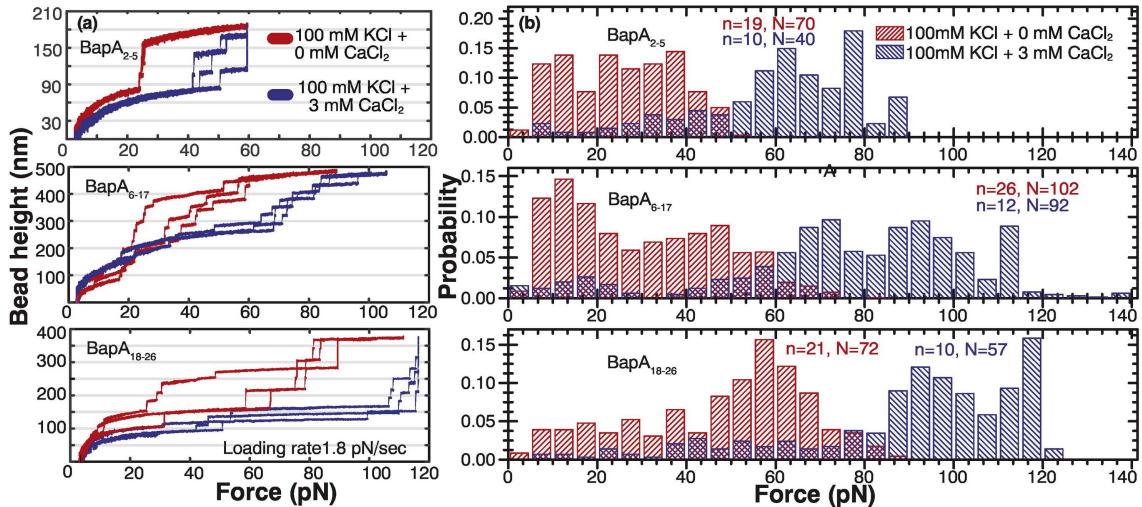


Figure 5

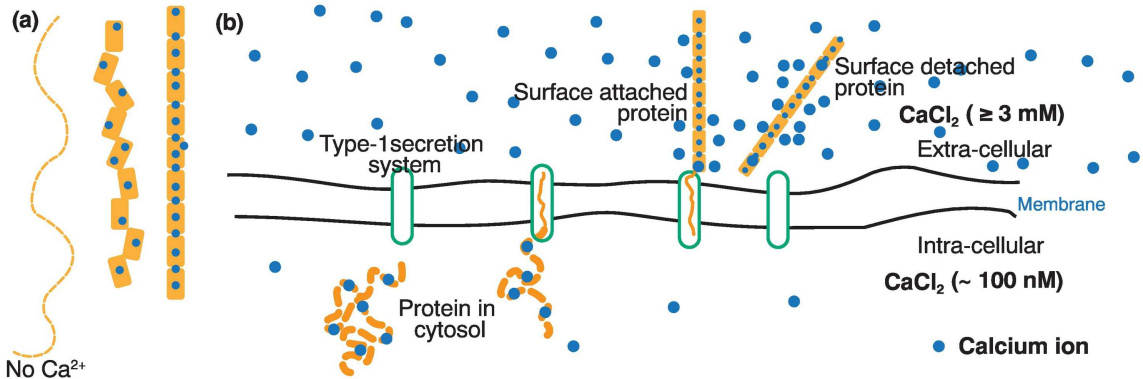


Figure 6