Physical interaction and assembly of *Bacillus subtilis* spore coat proteins CotE and CotZ studied by atomic force microscopy

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Abstract

The spore of *Bacillus subtilis*, a dormant type of cell, is surrounded by a complex multilayered protein structure known as the coat. It is composed of over 70 proteins and essential for the spore to withstand extreme environmental conditions and allow germination under favorable conditions. However, understanding how the properties of the coat arise from the interactions among all these proteins is an important challenge. Moreover, many specific protein–protein interactions among the coat proteins are crucial for coat assembly. In this study, atomic force microscopy (AFM) based single molecule force spectroscopy (SMFS) was applied to investigate the interaction as a dynamic process between two morphogenetic coat proteins, CotE and CotZ. The unbinding force and kinetic parameters characterizing the interaction between CotE and CotZ were obtained. It is found that there is a strong affinity between CotE and CotZ. Furthermore, the assembly behaviors of CotE and CotZ, individually or in combination, were studied by AFM at solid-liquid interfaces. Our results revealed that CotE–CotZ assembly is dependent on their molar ratios and the interaction between CotE and CotZ involves in the CotE–CotZ assembly.

Keywords

Atomic force microscopy, single molecule force spectroscopy, protein–protein interaction, self-assembly, CotE, CotZ

1. Introduction

The formation of *Bacillus subtilis* spores, a highly dormant cell type, is triggered by condition of nutrient limitation. Spores are able to withstand extremes of temperature, radiation, chemical assault and even the vacuum of outer space (Driks, 1999; Nicholson et al., 2000). The capability of the spores to withstand harsh environments is in large part due to the spore coat, a thick proteinaceous shell. The coat comprises no less than 70 individual proteins, organized into four main structural layers: a diffuse undercoat, a laminated lightly staining inner layer, a thick, highly electron dense outer layer and an outermost surface part of the coat called a crust (McKenney et al., 2013).

The assembly of the coat layers is controlled by a subset of coat proteins, known as the morphogenetic proteins (Henriques and Moran, 2007), including SpoIVA, SpoVID, SafA, CotE, and CotZ. They have no impact on coat protein gene expression but act post-translationally to direct the assembly of the various coat components around the forming spore (Kim et al., 2006). SpoIVA which assembles in the coat basement layer with the assistance of SpoVM, a small amphipathic α -helical peptide, plays a key role in localization of other coat protein components on the spore surface (Ramamurthi et al., 2006). SpoVID along with SpoVM are necessary for the spore encasement during which the spore becomes fully surrounded by coat proteins (de Francesco et al., 2012; Wang et al., 2009). SafA is the major inner coat morphogenetic protein and contributes to patterning of the coat structure (McKenney et al., 2010; Ozin et al., 2000; Ozin et al., 2001). The morphogenetic coat protein

CotE is localizing at the junction of the inner and outer coat layers, forming a ring around the forespore (Driks et al., 1994; Little and Driks, 2001). It regulates the assembly of a large set of coat proteins, most of the outer coat proteins, such as CotZ, CotA, CotB, CotC, CotH, CotM, CotU and CotG (Donovan et al., 1987; Zheng et al., 1988). A series of mutagenesis studies indicated that CotE has a modular structure with C-terminal region that directs various coat proteins assembly (Bauer et al., 1999). CotZ is acting as a morphogenetic protein and was identified to be the component of the outermost layer of the coat named the crust (Imamura et al., 2011; McKenney et al., 2010).

Coat assembly in *B. subtilis* is serving as an excellent tractable model for investigation of the self-assembly process of biological structures that has been studied for decades (Henriques and Moran, 2000; Piggot and Coote, 1976; Pogliano et al., 1995). These studies clarified the key role of morphogenetic proteins in formation of the coat structure. In addition, it was suggested that specific protein–protein interactions determine the manner how structural components of the coat are deposited within the coat (Costa et al., 2006; Muellerova et al., 2009). However, the interactions among the over 70 proteins making up the coat remain largely obscure.

Over the past decades genetic and biochemical techniques has been employed for identification of non-covalent interactions among spore-coat proteins (Isticato et al., 2010; Krajcikova et al., 2009; Muellerova et al., 2009; Zhang et al., 1993; Zilhao et al., 2004). Although combining of these two basic approaches allows revealing a number of protein-protein interactions, the sensitivity and specificity of classical methods are

1 relatively low (Krajcikova et al., 2009). Recently atomic force microscopy (AFM) has
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3 become an extremely versatile tool to investigate morphological and mechanical
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5 properties of biological materials at the nanoscale in their near-native environment
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8 (Fotiadis et al., 2002; Hinterdorfer and Dufrene, 2006; Plomp et al., 2002; Shao et al.,
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10 1996). Furthermore, AFM based single molecule force spectroscopy (SMFS) allows
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12 the detection of unbinding forces and the dynamic processes of biological pairs at the
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14 single-molecule level (Cecchet et al., 2008; Chtcheglova et al., 2010; Dufrene and
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16 Hinterdorfer, 2008; Tang et al., 2009; Wollschlaeger et al., 2009; Zhu et al., 2010),
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18 owing to its capability of piconewton force sensitivity and nanometer positional
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20 accuracy. Therefore, the examination of the interactions among spore coat proteins by
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22 single molecule force spectroscopy is one of the most powerful approaches for
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24 visualizing the interactions (Liu et al., 2015; Qiao et al., 2012; Qiao et al., 2013).
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34 Previous work revealed that CotE could interact with most or all the outer coat
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36 components (Kim et al., 2006). In addition, pull-down assay indicated that a
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38 low-affinity interaction between CotE and CotZ exists (Krajcikova et al., 2009).
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40 Another indication of their interaction was acquired from a genetic approach (Kim et
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42 al., 2006; McKenney and Eichenberger, 2012; McKenney et al., 2010; Plomp et al.,
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44 2014). In this study, we investigate CotE and CotZ interactions and self-assembly
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46 characteristics of these proteins. The unbinding forces of the CotE–CotZ complex,
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48 which characterize the interaction strength between two proteins, were investigated by
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50 SMFS. Additionally, by measuring the unbinding force of the complex at different
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52 values of loading rate the binding kinetics was also studied. We further applied AFM
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1 imaging to characterize the assembly behaviors of CotE and CotZ, individually or in
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3 combination at solid–liquid interfaces. These studies could be extended to other spore
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5 coat proteins, which can provide insight into the complex network of interactions and
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7 assembly of the spore coat.
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10 11 **2. Experimental section**

12 13 **2.1. Materials**

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16 Silicon wafers were purchased from Institute of Microelectronics, Peking
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18 University. 3-aminopropyltriethoxysilane (APTES), triethylamine and ethanolamine
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20 were obtained from Sigma-Aldrich. NaCNBH₃ was obtained from J&K Scientific Ltd.
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22 NHS-PEG₁₈-aldehyde was obtained from Prof. Hermann J. Gruber (Johannes Kepler
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24 University, Austria). Other reagents used in all experiments were of analytical grade.
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26 Milli-Q-purified water (18.2 MΩ) was used for all solution preparations.
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33 34 **2.2. Protein expression and purification**

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36 *Escherichia coli* BL21(DE3) strain harboring the plasmids pEtcotE or
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38 pETDuetcotZ were used for protein production. Bacteria were grown at 37 °C and
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40 protein expression was induced by 1 mM IPTG. His-tagged recombinant proteins
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42 were isolated and purified exactly as described previously (Krajcikova et al., 2009)
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44 using metal affinity chromatography on Ni²⁺ column in Tris buffer containing 8 M
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53 54 **2.3. The dialysis procedure for CotE and CotZ**

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56 The purified proteins (CotE and CotZ) at a concentration of 100 µg/mL were
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58 dialyzed against 3 litres of water for 3 hours at room temperature, during which the
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1 water was changed every hour. After dialysis, the proteins were centrifuged for 7
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3 minutes at 4 °C at 14000 rpm to remove protein aggregates and unsolved particles.
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5 Finally, the proteins were diluted to different concentration with phosphate-buffered
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7 saline (PBS) buffer (1.8 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 140 mM
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9 NaCl, pH 7.5).
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13 **2.4. Silicon substrates and AFM tips preparation**

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17 The silicon wafers were cut into 0.7 cm × 0.7 cm pieces. Prior to
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19 functionalization, the silicon pieces were cleaned with a freshly prepared piranha
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21 solution (98% H₂SO₄:30% H₂O₂ = 7:3, v/v, caution: piranha solution is extremely
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23 explosive and must be treated with great care), followed by extensive rinsing with
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25 large amounts of water. Then, the silicon pieces were ultrasonically cleaned with
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27 water (3×), ethanol (3×), and dried under a nitrogen flow.
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34 For SMFS, the general process for functionalization of the silicon pieces with
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36 CotE was similar to a previously reported procedure (Zhang et al., 2014). In brief, the
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38 cleaned silicon pieces were moved into an argon-gas-filled desiccator and incubated
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40 with 50 µL 3-aminopropyltriethoxysilane (APTES) and 15 µL triethylamine for 2
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42 hours using a vapor-phase deposition method (Ebner et al., 2007). This step was
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44 referred to as silylation, which generated the required amino group density on the
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46 surface of the silicon pieces. Then, attachment of a flexible heterobifunctional
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48 polyethylene glycol (PEG) linker, NHS-PEG₁₈-aldehyde, to the amino-functionalized
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50 silicon pieces via NHS reactive end was carried out by incubating the silicon pieces
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52 with PEG linker (2 mg/mL in chloroform containing 0.5% (v/v) triethylamine) for 2
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1 hours at room temperature. Following the reaction, the silicon pieces were cleaned
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3 thoroughly in chloroform to remove non-reacted PEG linkers. Subsequently, CotE
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5 was attached to the aldehyde end of the PEG linker via its lysine residues by mixing
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7 the activated silicon pieces with 100 µg/mL CotE protein solution containing freshly
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9 prepared 20 mM NaCNBH₃ for 1 hour at room temperature (Zhu et al., 2010). Finally,
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11 the unoccupied aldehyde groups on the silicon substrate were blocked by the addition
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13 of ethanolamine (final concentration of 25 mM, pH 9.6, preadjusted with NaOH) for
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15 30 minutes. After the reaction, the prepared silicon substrates were rinsed with PBS
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17 buffer and stored in the buffer at 4 °C until use.
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25 Silicon nitride AFM tips (MSCT, Veeco, USA) were used for force spectroscopy
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27 studies. Following the same steps as above, AFM tips were functionalized with CotZ.
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29 However, AFM tips for control experiments were modified only with a PEG linker
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31 whose terminal aldehyde group had been inactivated by incubating with 25 mM
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33 ethanolamine for 30 minutes.
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39 **2.5. AFM measurements**

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42 The AFM samples were prepared by applying 50 µL of proteins with various
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44 concentrations onto the cleaned silicon substrates following the incubation for 5 hours
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46 at room temperature and then stored overnight at 4 °C. AFM imaging was carried out
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48 on NanoScope Multimode 8 (Digital Instruments, Veeco, USA) in PBS buffer at room
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50 temperature. The images were captured in Tapping Mode, using commercial Si₃N₄
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52 tips (DNP-S10, 0.06 N/m, Veeco, USA). The scan rate for imaging was set at 1–2
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54 lines/s in the 512×512 pixel format. The acquired images were analyzed using
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NanoScope Analysis (Veeco, USA). A PicoSPM 5500 AFM (Agilent Technologies, Andover, USA) was used to perform all SMFS experiments in PBS buffer at room temperature. Force measurements were carried out with CotE-functionalized silicon substrates and CotZ-modified tips. More than 2000 force–distance curves were obtained at randomly chosen five to eight locations on silicon surface. The experiment was repeated three times with different CotZ-modified AFM tips. Data analysis of the selected thousands of curves was performed with Matlab Version 7 (MathWorks, Natick, MA) as previously described (Baumgartner et al., 2000b). Empirical probability density functions (pdf) calculated as described earlier was applied to present the force distributions of the unbinding events (Baumgartner et al., 2000a; Riener et al., 2003). The spring constant of every cantilever was calibrated after a given experiment by the thermal–noise mode in air (Butt and Jaschke, 1995; Hutter and Bechhoefer, 1993). The loading rate r , was determined by using the equation $r = \nu k_{\text{eff}}$, with ν being the cantilever retraction rate and k_{eff} being the effective spring constant (De Paris et al., 2000).

3. Results and discussion

3.1. Single molecule force spectroscopy study of the CotE–CotZ interaction

AFM based SMFS was used to investigate the interaction between CotE and CotZ at the single-molecule level. To perform this experiment, CotZ was covalently coupled to the amino-functionalized AFM tip and CotE was immobilized onto the amino-modified silicon substrate through a flexible heterobifunctional PEG linker (Fig. 1a). Attributed to the nonlinear stretching elasticity of the PEG linkers, it is easy

to discriminate between specific and nonspecific unbinding events in the force-distance cycles (Hinterdorfer and Dufrene, 2006). The force-distance curves were obtained by approaching and subsequently retracing the AFM tip with CotZ from the silicon substrate, on which CotE was immobilized. A typical force-distance curve with a single molecular recognition event between CotE and CotZ is depicted in Fig. 1b. The peak in the retracting curve, which emerged about 13 nm away from the separation of the tip and substrate, represents the specific unbinding event between CotE and CotZ. The characteristic nonlinear force signal in the retracting curve originates from the stretching elasticity of the PEG linker (Wildling et al., 2012). In order to confirm that CotE could specifically recognize CotZ, a control experiment was carried out by using an AFM tip functionalized only with the PEG linker. As shown in Fig. 1b, inset, the specific recognition event disappeared in the retracting curve. The probability density function (pdf) of the unbinding forces obtained from about 2000 force-distance curves shows the distribution of unbinding forces between CotE and CotZ (Fig. 1c). The maximum of the distribution was 40.9 ± 0.1 pN at the loading rate of 1.12×10^3 pN/s, reflecting the most probable unbinding force (f_u) of CotE-CotZ complex. The binding probability (probability of detecting specific recognition events in all force-distance cycles) was 13.6%. However, in the control experiment, the binding probability remarkably decreased to 3.4%. These data clearly prove that CotE immobilized on the silicon substrates could be specifically detected at the single-molecule level by CotZ attached to AFM tips and that this interaction is specific.

The unbinding forces of the receptor–ligand complex rely not only on the interaction of molecules but also on the loading rate of the AFM tip (Merkel et al., 1999). According to the Bell–Evans model (Bell, 1978) there is a linear relationship between the individual unbinding force f_u and the logarithm of the loading rate r following Equation (1):

$$f_u = \left(\frac{k_B T}{x_\beta} \right) \ln \left(\frac{r x_\beta}{k_B T k_{\text{off}}} \right) \quad (1)$$

where x_β is the difference of the energy barrier to the minimum energy along the separation path, k_{off} is the dissociation rate constant at zero force, k_B is the Boltzmann constant, T is the thermodynamic temperature.

The dynamic force measurement of the CotE–CotZ complex was carried out at different loading rates from 3.5×10^2 to 4.9×10^3 pN/s. As illustrated in Fig. 2, the unbinding forces of the CotE–CotZ complex varied in the range of 38–102 pN and increased linearly with increasing logarithm of the loading rate. Based on the slope and the intercept of the fitting curves (Fig. 2), the separation of energy barrier from the dissociated state to bound state of the CotE–CotZ complex x_β was $0.836 \pm 0.006 \text{ \AA}$, the kinetic off-rate constant k_{off} and bond-survival time $\tau (= 1/k_{\text{off}})$ were $0.122 \pm 0.001 \text{ s}^{-1}$ and $8.19 \pm 0.05 \text{ s}$, respectively. The interaction forces between these proteins and kinetic rate constants obtained from SMFS characterize the interaction strength of protein pairs. Based on the comparisons of the interaction forces and the dissociation rates, the stability of the complexes could be deduced. It was found that at the same loading rate, dissociation of CotZ from CotE requires higher unbinding forces than

CotY from CotE, which reveals that the CotE–CotZ interaction is stronger than CotE–CotY. Previous studies showed that the interactions of CotE with CotY could be regarded as the strong one (Liu et al., 2016). Thus, it can be concluded that CotE displays a high binding affinity to CotZ to form a stable complex. Moreover, the CotE–CotZ complex has similar dissociation rate and lifetime to CotE–CotY complex, indicating that the stability of CotE–CotZ complex is high. All these results indicate that there is a strong affinity between CotE and CotZ. These AFM measurements also reflect that the localization of CotZ on the spore surface is directly controlled by CotE (Kim et al., 2006).

3.2. Assembly of CotE and CotZ on silicon substrate in vitro

The study of CotE and CotZ assembly behavior in vitro might help understand a small fraction of complex biological process of formation highly organized spore coat. To obtain some basic information about their assembly, we first performed AFM to characterize the morphology of CotE and CotZ individually assembling on silicon substrate. The representative AFM topographic images at different scan sizes of CotE and CotZ are presented in Fig. 3. It can be observed that CotE self-assembled into extended net-like structures (Fig. 3a₁, white arrows). At higher magnification, the mesh sizes (Fig. 3a₂, white arrows) were measured in a range from 40 to 90 nm ($n = 20$). These results are in good agreement with electron micrographs of typical CotE aggregates, displaying extended net-like structures (Jiang et al., 2015). However, the average repeat spacing was about 15 nm, although the mesh size was highly variable. When CotZ assembled on silicon substrate, ordered strip structures were visualized

(Fig. 3b₁, black arrows). To observe more details, a portion of Fig. 3b₁ was magnified as shown in Fig. 3b₂. The strips we observed were arranged in parallel to each other and also made contacts with adjacent strips. The average width of parallel strips is 15–35 nm ($n = 20$).

Since CotE, one of the most important morphogenetic proteins, could direct the assembly of most of the outer coat proteins, we then investigated the assembly behaviors of CotE in complex with CotZ at different CotE/CotZ ratios (3:1, 2:1, 1:2 and 1:3). Fig. 4 shows typical AFM images of the complex of CotE with CotZ formed at different molar ratios, which were different from the morphologies when individual CotE and CotZ proteins were applied. We found out that there is extensive network of filaments in cases of CotE/CotZ ratio 3:1 and 2:1 (Fig. 4a₁ and b₁). The primary difference between these two images is the size of the mesh. The mesh sizes (white arrows) in Fig. 4a₂ distribute in the range of 15–40 nm ($n = 30$), which are bigger than that in Fig. 4b₂. However, when the CotE/CotZ ratio is decreased from 2:1 to 1:2, the complex assembled from initial mesh-like structure to parallel strips (black arrows) with an average width of 5–20 nm ($n = 20$) and some also stuck together (Fig. 4c₁ and 4c₂). The width of most strips became greater than 40 ± 5 nm (Fig. 4d₁ and 4d₂, $n = 15$) when the ratio of CotE/CotZ was kept at 1:3. The morphology investigation indicates that CotE-CotZ assembly is dependent on their molar ratios. That is, at high CotE/CotZ ratio, the complex prefers to form extensive mesh-like structure, and a low ratio of CotE to CotZ facilitates the formation of parallel strips.

1 The interaction forces and dynamic data of CotE–CotZ complex demonstrate that
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3 CotE has a strong affinity for CotZ to form a stable complex. The changes in
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5 morphology of CotE–CotZ assembly may be attributed to the conformational changes
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7 of CotE and CotZ when they interact with each other. Importantly, it is tempting to
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9 hypothesize that the two-dimensional nets formed by CotE encapsulate the forespore
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11 and serve as a platform for the assembly of CotZ. Our study provides further evidence
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13 that CotE can directly interact with CotZ and this can contribute to the attachment of
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15 CotZ onto the spore surface allowing the crust firmly adhering to the spore. Therefore,
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17 this work has an important contribution to better understand the relationship between
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19 the assembly and interaction of the spore coat proteins.
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28 **4. Conclusion**

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30 The spore coat of *B. subtilis* represents a unique example of macromolecular
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32 assemblies. Like many other examples of morphogenesis in biology, the coat
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34 assembly is a dynamic and complex multistep process. The protein interactions
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36 between the coat proteins, especially morphogenetic proteins are pinpointing factor
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38 which determines the coat assembly process. Thus, the specific interaction between
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40 CotE and CotZ studied here refines our perception about the principles which govern
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42 the morphogenetic proteins to create a basic scaffold where other coat proteins are
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44 attached within the process of spore coat development. Using the AFM based SMFS
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46 the unbinding force and kinetic data characterizing the interaction of CotE and CotZ
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48 was obtained. We demonstrated that the morphogenetic protein CotE could interact
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50 directly and specifically with CotZ forming a stable complex. Moreover, AFM
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1 imaging was performed to study the assembly behaviors of CotE and CotZ at
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3 solid-liquid interfaces. Our results showed that at high CotE/CotZ ratio, the complex
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5 prefers to form extensive mesh-like structure, and a low ratio of CotE to CotZ
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7 facilitates the formation of parallel strips. Together with our previous results obtained
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9 using this method we gradually are getting the more accurate perception of the
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11 assembly and properties of the spore coat. This work clearly shows unique advantages
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13 of the SMFS technique for future characterization of other coat proteins.
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32 analyzing the data.
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Figure Legends

Fig. 1. (a) Schematic representation of AFM force measurements with a CotZ-attached tip and the CotE-attached silicon substrate. (b) Typical force-distance cycle for a recognition event between CotE and CotZ. Recognition signal disappears in the control experiment, which uses an AFM tip functionalized only with the PEG linker (inset). (c) Distribution of the unbinding forces for the interaction of CotE and CotZ (black, solid line) and the PEG linker functionalized AFM tip and CotE in the control experiment (red, dashed line).

Fig. 2. The unbinding forces for the interaction between CotE and CotZ were plotted against the logarithm of the loading rate.

Fig. 3. AFM height images of CotE (a_1) and CotZ (b_1) individually assembling on the silicon substrates. The height scales are from -7.5 to 7.5 nm and -5.0 to 5.0 nm for panels (a_1) and (b_1), respectively. (a_2) Higher-magnification view of CotE from a portion of panel (a_1), with a height scale of -7.6 to 7.6 nm. (b_2) Higher-magnification view of CotZ from a portion of panel (b_1), with a height scale of -4.3 to 4.3 nm. Cross-sections obtained along the white lines. White and black arrows indicated meshes and filaments formed by the proteins, respectively.

Fig. 4. Typical AFM images of the complex of CotE with CotZ formed at different molar ratios: (a_1) CotE:CotZ 3:1, with a height scale of -4.0 to 4.0 nm, (b_1) CotE:CotZ

1 2:1, with a height scale of -2.0 to 2.0 nm, (c₁) CotE:CotZ 1:2, with a height scale of
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3 -4.0 to 4.0 nm and (d₁) CotE:CotZ 1:3, with a height scale of -7.5 to 7.5 nm. Right
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5 panels (a₂, b₂, c₂ and d₂) are the corresponding magnification views of a portion of left
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7 panels, with the height scales of -5.1 to 5.1 nm, -2.0 to 2.0 nm, -3.5 to 3.5 nm and -7.0
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9 to 7.0 nm, respectively. Cross-sections obtained along the white lines. White and
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11 black arrows indicated meshes and filaments formed by the proteins, respectively.
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Figure 1

Figure 2

Figure 3

Figure 4