CapP mediates the structural formation of biofilm-specific pili in the opportunistic human pathogen *Bacillus cereus*

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SUMMARY

Fibrillary proteins are structural scaffolds that diversify the functionality of the architectural bacterial extracellular matrix. Here, we report a previously uncharacterized bacterial factor called *bc1280* that is exclusive to *B. cereus* group and indispensable for the establishment of a biofilm lifestyle. We propose that BC1280 is an architectural conductor for the assembly of the amyloid platform, leading to the polymerization of heteropili with two functional amyloids, CalY and TasA. From its cellular localization in the cell membrane, aggregates of BC1280 nucleate the polymerization of CalY, which further incorporates TasA into nascent pili. Additionally, BC1280 modulates the expression of EPS via an uncharacterized pathway that is activated by a protease and an ECF-type sigma factor. The pilus biogenesis system described in this work partially mirrors curli system in *Escherichia coli*, unveiling a new paradigm in the structural biology of gram-positive bacteria and highlighting the complexity of extracellular matrix assembly in *B. cereus*.

KEYWORDS: fibrillary proteins, functional amyloids, pili, biofilm, extracellular matrix, *Bacillus cereus*

INTRODUCTION

Bacillus cereus, a bacterium commonly found in soil, is capable of colonizing the gastrointestinal tracts of mammals or arthropods; *B. cereus* is contracted through the ingestion of contaminated food, including fresh vegetables and ready-to-eat meals.[1-4] Consequently, *B. cereus* strains are frequently identified as the etiological agent of gastrointestinal diseases outbreaks, and the diseases can progress through emetic or diarrheal symptoms.[5,6] As approximately 70% of bacterial diseases in humans are reportedly associated with biofilms, much effort has been dedicated to understanding the genetic basis and structural components that, upon activation, lead to the formation of these microbial communities.[7] The transition from a planktonic to a sessile lifestyle is metabolically focused on the synthesis of proteins, polysaccharides and extracellular DNA (eDNA), which ultimately organize the extracellular matrix (ECM).[8,9] The ECM performs a wide range of functions, including adhesion to biotic and abiotic surfaces, regulation of nutrient and signaling molecule transport, defense against physical-chemical stress, and virulence, and provides support for biofilm architecture.[10-12] One of the most intriguing components of the ECM is the functional amyloid protein, which, like its pathogenic sibling in humans, polymerizes into fibers that are resistant to diverse environmental insults.[13-16] In bacteria, functional amyloids are frequently found as well-ordered macromolecular complexes within cell surfaces that serve as structural scaffolds, participate in surface adhesion, and contribute to the organization of the ECM.[17,18]

The developmental of *B. cereus* biofilms involves genetic pathways and structural components of the ECM identical to those of *B. subtilis*, a closely related species. However, differences arise and are predicted to have ecological and pathological

implications.[19,20] Complementary reports have described the role of flagella, matrix proteins, eDNA and exopolysaccharides in the formation of biofilms on *B. cereus.*[21-24] A previous study identified a specific genomic region in *B. cereus* that is orthologous to the corresponding region in *B. subtilis*, and it is expressed mainly in biofilm-associated cells.[25,26] This region encodes an ortholog of *sipW;* two orthologs of *tasA*, called *tasA* and *calY*; the main biofilm regulators *sinI* and *sinR*; and an uncharacterized locus called *bc1280*. Interestingly, no orthologs for *tapA* are present in *B. cereus*. SipW is the signal peptidase that processes TasA and TapA for efficient secretion, and both TasA and CalY of *B. cereus* conserve the signal peptide with the canonical cleavage site recognized by the cognate peptidase SipW.[27,28] The genes *sipW* and *tasA* constitute an operon, and *calY* is expressed independently. The deletion of *tasA* or *calY* resulted in differential defects in biofilm formation, suggesting that the product of each gene might be involved in cell-to-abiotic surface or cell-to-cell interactions, respectively. Structural studies have revealed the innate amyloid nature of TasA and CalY from *B. cereus*, as well as TasA and TapA from *B. subtilis*, and their ability to form fibers with canonical cross-β organization.[29-33] Nevertheless, El-Mammeri et al.[29] noted significant structural differences based on the secondary structure composition, which evidenced singularities in the "amyloid scaffold platforms" between these two phylogenetically related bacterial species.

In this study, we revealed that the *bc1280* product is indispensable for the growth of *B. cereus* biofilms and relies on two complementary functions. The first is a structural function, i.e., the promotion of CalY polymerization, which is related to the correct functioning of the amyloid platform. The second is a regulatory function in which the

expression of biofilm-related EPSs is controlled via an undescribed sigma-antisigma pathway. We demonstrated that the N-domain of BC1280 exhibits a canonical amyloid signature, with a core composed of at least two rigid regions predominantly in a β-sheet conformation and a more rigid C-domain, which likely mediates the interaction with CalY. From its localization in the cell membrane, BC1280 defines and triggers the polymerization of CalY, which in turn incorporates TasA into nascent heteropili. Based on these findings, BC1280 was renamed CapP, which is a CalY-assisting polymerization protein. Our study provides a new paradigm for the complex assembly of this proteinaceous appendage in *B. cereus* biofilms, identifies CapP as a structural orchestrator for efficient ECM development, and highlights CapP as a promising target for pioneering and precisely targeted antibiofilm therapies.

RESULTS

bc1280 is an essential locus in the developmental program of biofilm formation

First, a phylogenetic analysis of BC1280 was performed using the amino acid sequence of the type strain *B. cereus* ATCC14579 as a reference. The results revealed that BC1280 was present within the *B. cereus* group but not within the phylogenetically related species *B. subtilis* (Figure S1A). The *bc1280* product was annotated as a hypothetical protein with the following putative regions (Figure 1A): i) a signal peptide from position 1 to 39 predicted to be processed by a signal peptidase type I (SignalP5.0) (Figure S1B); ii) an N-terminal domain from position 40 to 189 (the N-domain from now on) that contains a highly conserved domain of unknown function (from I^{33} to E^{157}) annotated as DUF4047; and iii) a C-terminal domain (the C-domain from now on) from position 190 to the C-terminal end of the protein. This domain is characterized by a variable number of perfect repetitions of QKKV/AEE motifs, depending on the *B. cereus* strains.

A previous transcriptome analysis of the genetic features that distinguish planktonic and biofilm cells in *B. cereus* showed that *bc1280* is specifically expressed in biofilm-associated cells, and a complementary study revealed that the expression of *bc1280* comprised the regulon of the biofilm-specific master regulator SinR.[34] We confirmed the biofilm-specific expression of *bc1280* via RT–qPCR analysis of samples taken at 48 hours (Figure S1C) and determined that *bc1280* is transcribed as a monocistronic unit independent of the previously reported transcriptional units *sipW-tasA* or *calY*[25] (Figure S1D). The biofilm of the *B. cereus* wild-type strain ATCC14579 was

characterized by a white biomass ring that adhered to the plate wells; this ring was usually visible at 24 hours and reached maturity at 72 hours. A *bc1280* knockout mutant strain (*Δbc1280*) was completely arrested during biofilm formation, a phenotype that differed from the less adhesive or thinner biomass of *ΔtasA* or *ΔcalY,* respectively[25] (Figure 1B). According to the distribution and conservation observed in our phylogenetic analysis, deletion of *bc1280* in the emetic strain of *B. cereus* AH187, which belongs to a different *B. cereus* subgroup, resulted in the complete elimination of pellicle formation at the air–liquid interface compared to that in the wild-type strain (Figure S1E).

The wild-type phenotype for biofilm formation was completely restored through complementation of *Δbc1280*, which was achieved through ectopic integration of the *bc1280 locus* (*bc1280*::Δ*bc1280*) under the control of its own putative promoter at a neutral locus (Figure 1B). Moreover, these findings suggested that the promoter for the expression of *bc1280* must be contained within the noncoding sequence between the open reading frame of *tasA* and *bc1280*. BC1280 contains a distinctive C-domain with perfect tandem repetition. As previously described, these characteristic regions often have significant structural and functional importance for the stabilization of protein folding overall.[35] Therefore, we investigated whether the C-domain is involved in biofilm formation. The biofilm formation phenotype of *Δbc1280* was still rescued by version of BC1280 that lacked the C-domain from Q^{191}-E^{279} (Δ*C-domain*) (Figure 1B). Quantitative measurements at 575 nm of the biomass stained with crystal violet reinforced the visual differences in biofilm thickness (Figure 1C). No significant differences were observed between the strains at 24 hours, when initial cell attachment precedes biofilm maturation; however, differences became evident at 48 hours, coinciding with the initiation of ECM

assembly and the growth of biofilms. After cells are initially attached to surfaces, cell-to-cell interactions occur that facilitate the subsequent growth of the biofilm, a cellular phenomenon that can be monitored through auto-aggregation experiments. The wild-type and *ΔC-domain* strains exhibited sharp aggregation kinetics; in contrast, *Δbc1280* did not form sediments at the bottom of the tube at the end of the 24-hour experiment (Figure 1D). Taken together, these results indicate that the product encoded by *bc1280* is involved in the developmental stages of bacterial cell-to-cell adhesion and the subsequent assembly and maturation of the ECM, a function that may rely on the N-domain of the protein.

The biofilm-defective phenotype of *bc1280* is independent of the expression level of *tasA* or *calY*

The *Δbc1280* phenotype may result from changes in the expression level of ECM components or structural malfunctioning of the amyloid platform involving the polymerization of TasA and CalY; these two alternative explanations are not mutually exclusive. A whole-transcriptome analysis of the *Δbc1280* strain revealed 35 genes that were overexpressed and 4 genes that were repressed compared to those in the wild-type strain at 24 h. However, none of these genes were predicted to be associated with biofilm formation (Table S1). Notable transcriptional changes were observed at 48 hours, with 21 genes overexpressed and 28 genes repressed in *Δbc1280*, including *tasA* and *calY* (log2FC values of -3.13 and -3.14, respectively) (Table S2). This result was further supported by RT–qPCR analysis, which revealed lower expression levels for the genes located within the *eps1* and *eps2* genomic regions (Figure 2A). Our transcriptomic

analysis additionally revealed that the expression of *bc2793* and *bc2794* pairs (log2FC values of -8.11 and -8.43, respectively) was significant repressed. These uncharacterized loci are annotated as a putative Clp protease and an RNA polymerase ECF-type sigma factor, respectively. ECF sigma factors are known to transduce changes in the cell envelope into a bacterial genetic response[36,37]; thus, we hypothesized that under normal conditions, *bc2793* and *bc2794* positively regulate the gene expression of the main ECM components. The overexpression of *bc2793* and *bc2794* in *Δbc1280* and induction with a 10 μM solution of isopropyl β-D-thiogalactopyranoside (IPTG) significantly increased the biofilm biomass (Figure S2A and C). Considering that the putative protease (*bc2793*) could regulate the activity of *bc2794*, we solely overexpressed the ECF factor, which resulted in a twofold increase in crystal violet staining at 100 μM IPTG compared to that of *Δbc1280* (Figure S2B and D). In contrast to these findings, the deletion of *bc2794* or *bc2793-bc2794* did not result in a biofilm-impaired phenotype compared to that of *Δbc1280* (Figure S2E). RT–qPCR analysis revealed no significant changes in the expression levels of *tasA* or *calY* in *Δbc1280*-overexpressing *bc2794* or *bc2793-bc2794* compared to those in Δbc1280 (Figure S2F and G). However, the relative expression levels of *eps1* and *eps2* were significantly upregulated in response to both genetic combinations. Thus, the ECF factor and its anti-sigma factor were associated with the regulation of exopolysaccharide expression in *B. cereus*, rather than the biofilm-related genes *tasA* and *calY*.

In accordance with our hypothesis on the structural role of BC1280 in the polymerization of TasA or CalY, no reversion of phenotypes was observed in *Δbc1280* cells overexpressing *tasA, calY*, or both loci (Figure S2H). TEM analysis of negatively

stained samples and immunolabeled with anti-TasA or anti-CalY antibodies revealed (Figure 2B and C): i) the presence of fibers from both proteins on the surfaces of WT cells, ii) aggregates reactive to anti-TasA antibodies in *Δbc1280* cells overexpressing *tasA,* and iii) the absence of fibers in *Δbc1280* cells overexpressing *calY* but with an extracellular signal associated with the anti-CalY antibody. We next examined whether TasA or CalY, which were produced by heterologous expression in *E. coli* and added exogenously to *Δbc1280*, might rescue the wild-type biofilm phenotype. The monomeric conformation of TasA or CalY failed to rescue biofilm formation; however, the formation of ring biomass was partially restored when the proteins were added in their fibrillary form at a final concentration of 6 μM (Figure 2D and E). Furthermore, TEM analysis revealed that the cell surfaces were decorated with specific immunoreactive aggregates of TasA and CalY (Figure 2D and E). Taken together, these data suggest that BC1280 plays a functional role in the maturation of the ECM and likely participates in the polymerization of CalY or TasA.

Our previous findings indicated that the N-domain might partially fulfill the function of BC1280 in biofilm formation by specifically facilitating cell-to-cell interactions. Therefore, this domain may influence the final localization of TasA or CalY within the ECM. TEM analysis and immunolabeling of the *ΔC-domain* strain revealed that nanogold particles associated with TasA extracellular fibers were exposed on the cell surfaces; nevertheless, the morphologies of these fibers were different from those formed in the wild-type cells (Figure 2B and F). Nanogold particles corresponding to CalY monomers or oligomers, but not fibers, were observed in the extracellular matrix of the *ΔC-domain* strain (Figure 2B and F). Based on the alterations observed in the polymerization of TasA

and especially CalY in the *ΔC-domain* strain, the C-domain of BC1280 might be involved in the polymerization of these proteins or their initial association with the cell surface.

BC1280 is a membrane-associated protein that partially colocalizes with CalY in the ECM

To precisely determine how BC1280 contributes to the assembly of the ECM, we further studied the localization of the protein at the cellular level. Our preliminary data indicated that BC1280 is likely expressed at very low levels and is undetectable by immunocytochemistry assays. Therefore, we conducted these experiments using a derivative *Δbc1280* strain that expressed a version of BC1280 fused with a histidine tag at the carboxyl terminal domain. The biofilm formation of *Δbc1280* was rescued by plasmid leakage without IPTG induction or upon induction with a 10 µM IPTG solution (Figure S2I). Furthermore, fractionation and immunodetection experiments with an anti-histidine antibody confirmed that BC1280 was present in the extracellular matrix, cell wall, and membrane fractions (Figure 3A). TEM analysis and immunolabeling using anti-histidine antibodies revealed that foci of nanogold particles related to BC1280 were distributed regularly along fibers that emerged from the cell surfaces (Figure 3A). To better determine the localization of BC1280 in the cell wall, we conducted confocal laser scanning microscopy (CLSM) and immunocytochemistry using anti-histidine and secondary antibodies conjugated to Atto 488 and double staining with wheat germ agglutinin (WGA) and Hoechst to visualize the cell wall and DNA, respectively. According to the results of the fractionation experiments, a limited number of foci corresponding to BC1280 proteins fused to the histidine tag per cell were observed in association with the

cell surface (Figure 3C). Considering the relevance of the N-domain to the functionality of BC1280, we also studied its contribution to the cellular localization of whole BC1280. Based on crystal violet staining of the biomass that had adhered to the well, the amount of N-domain expressed without IPTG induction or after induction with 10 μM IPTG solution was sufficient to rescue the biofilm phenotype of *Δbc1280* (Figure S2I). Cellular fractionation and immunoblotting assays demonstrated a specific reactive band in the extracellular matrix and membrane fractions and an undetectable signal associated with the cell wall (Figure 3B). In addition, visualization via TEM confirmed the presence of N-domain-related foci along the fibers emerging from the cells, as previously observed for the entire protein, but no detectable fluorescent signals associated with the cell wall were observed via CLSM (Figure 3B and C). The disparities noted between the full-length and the truncated form of the protein suggest that the C-domain contributes to the retention of BC1280 in the bacterial cell wall, likely before BC1280 reaches the ECM.

To further define the connection between BC1280 and CalY or TasA at the cellular level, we studied their colocalization via immunocytochemical analysis of cells expressing pUTE657-*bc1280* fused to a histidine tag using specific primary antibodies. CLMS analysis revealed that CalY and BC1280 colocalized along the fibrils that compose the ECM (Pearson's coefficient of 0.58 and a p value of 0.0002) (Figures 3D and S2K). In most cases, the signal corresponding to CalY was homogeneously distributed, and BC1280 was detected as discrete foci dispersed along the fibers. However, no colocalization signal was detected for TasA or BC1280, with a Pearson's coefficient of 0.19 and a p value of 0.0002 (Figure S2J and L). These findings were supported by Mander's coefficient, which represents the percentage of overlapping pixels for each

fluorescent channel. For the BC1280-CalY pair, approximately 65% of the pixels overlapped in both channels; for BC1280-TasA, the overlap decreased to 26% for Atto 4'8 and 21% for Alexa Fluor 647 (Figure S2K and L). These observations provide solid support for the role of BC1280 in specifically assisting the polymerization of CalY into fibers.

BC1280 oligomerizes into macromolecular complexes driven by the N-domain

BC1280 and the truncated version comprising the N-terminal domain (from A^{39} to G^{190}) were purified by heterologous expression in *E. coli* Lemo21(DE3) and subjected to further structural analyses (Figure 4A). The two versions of the protein were purified under soluble conditions by affinity chromatography followed by size exclusion chromatography, and the purity was assessed by tandem mass spectrometry analyses (Figure S3A). The two fractions collected at different elution volumes in the size exclusion chromatogram suggested that in buffer containing 20 mM Tris and 50 mM NaCl at pH 7, BC1280 and N-domain$_{39\text{-}190}$ could assemble into two main molecular entities with distinct oligomeric states (Figure 4B and E). Dynamic light scattering (DLS) analysis showed that both protein assemblies exhibited a degree of polydispersity, with particles ranging in diameter from ~10 to 600 nm (Figure 4C-D and F-G for BC1280 and N-domain$_{39\text{-}190}$, respectively; for further details, see Table S3). Remarkable differences were observed in the second elution sample; specifically, the N-domain$_{39\text{-}190}$ exhibited a greater proportion (~53%) of small oligomers than did BC1280 (~18%) (Figure 4D and G). To complement the DLS results, each fraction was resolved in a native gel (Figure 4H). The sample corresponding to fraction 1 of each protein, which presumably contained larger aggregates, failed to

enter the gel, suggesting that the molecular complex was larger than 1000 kDa. The second elution fraction was resolved as a band at 480 or 240 kDa for BC1280 or N-domain$_{39-190}$, respectively, which corresponds to oligomers of approximately 15 subunits each. Large aggregates of different sizes were observed in the elution fractions, and these aggregates corresponded to fraction 1 of both proteins via TEM analysis (Figure 4I). The samples corresponding to the second elution fraction contained macromolecular complexes that were similar but smaller in diameter (Figure 4I). Taken together, these results indicate that the variability in the oligomerization of the full-length protein is preserved in the truncated version that contains only the N-terminal region.

To further delineate the dynamics of oligomerization, we focused on the second fraction, which was composed mainly of small oligomers and could promote the formation of larger molecular entities. The fluorescence signal emitted upon binding to thioflavin T (ThT)[38] showed a fast polymerization curve without a lag phase, aligning with the presence of oligomers under native conditions (Figure 4M). Interestingly, the slope of the exponential phase was more pronounced for the N-domain$_{39-190,}$ and the fluorescence intensity signal was twofold greater than that of the full-length protein after 150 hours of incubation. These results correspond with the higher percentage of small oligomers formed by the N-domain$_{39-190}$ (via DLS analysis), which suggested that the truncated protein seeds rapidly. The ThT kinetics of the aggregation of BC1280 and the N-domain$_{39-190}$ were similar to those of CalY amyloid fibrils,[29] reaching a plateau phase at ~150-180 hours. TEM analysis of negatively stained samples revealed macroscopic particles of variable sizes and annular morphology for both proteins, resembling the aggregates observed for CalY but not the fibrillar structures formed by TasA[29] (Figure 4N). These

results strongly indicate that the N-domain is involved in protein oligomerization and suggest that the absence of the C-domain in the truncated protein might facilitate a faster aggregation rate.

N-domain$_{39\text{-}190}$ displays the canonical cross-β fold

The amyloid nature of TasA and CalY, along with the association of BC1280 with these two proteins and its tendency to oligomerize, provided interconnected evidence that led us to propose an amyloid nature of BC1280. The N-domain retains the functionality of BC1280 and the ability to form oligomers; therefore, this version of the protein was considered for our initial structural studies. The X-ray diffraction pattern of the centrifuged pellet of the N-domain$_{39\text{-}190}$ assemblies displayed the typical features of the cross-β structure of amyloids, with strong signals at 4.7 Å and a slight signal at ~10-11 Å, corresponding to the inter-strand and inter-sheet spacings, respectively[39] (Figure 5A). In light of these findings, we explored the presence of potential amyloidogenic regions within the amino acid sequence of BC1280 using the bioinformatics tools AmylPred2,[40] FoldAmyloid,[41] MetAmyl,[42] PASTA 2.0[43] and TANGO.[44] At least two algorithms predicted the following amino acid stretches as putative amyloid-forming segments: i) L^{51}STAIIFP^{58}, ii) I^{98}S^{99}, iii) L^{115}QNVYT^{120} and iv) V^{144}SITVN^{149} (Figure S3B; letters in bold). Based on bioinformatics analysis using FoldUnfold,[45] the N-domain appears intrinsically folded but the C-domain is predicted to be unstructured, possibly due to the presence of repetitive amino acid sequences rich in Gln, Lys, and Glu, which areas disorder-promoter residues[35,46] (Figure S3B). As the propensity to form amyloid structures is also related to regions enriched in hydrophobic residues, we analyzed the hydrophobicity pattern of

BC1280 using ProtScale.[47] The peptide T^{50}LSTAIIFPKT^{60}, previously identified as a putative amyloidogenic segment, was found to be highly hydrophobic (Figure S3C). Finally, AlphaFold[48,49] was used to obtain a tentative secondary structure; however, the model primarily exhibited an α-helix conformation, contrary to the enrichment in β-sheet structural elements derived from ThT polymerization kinetics and the cross-β structure observed by X-ray diffraction (Figure S3D).

We further performed fine structural characterization of the molecular conformation of these protein assemblies using magic-angle spinning (MAS) SSNMR, which has been previously employed to study non-crystalline amyloids.[50-52] Uniform ^{13}C/^{15}N-labeled N-domain$_{39-190}$ assemblies were produced to improve NMR sensitivity and perform multidimensional MAS SSNMR studies. To investigate the presence of a rigid amyloid core within the assemblies, we used dipolar-based polarization transfer (cross-polarization) and two-dimensional ^{13}C-^{13}C spectroscopy. A 50 ms mixing time proton-driven spin-diffusion (PDSD) experiment was used to detect intra-residue ^{13}C-^{13}C correlations from the rigid core of the assembly. For the spectral analysis, we first attempted to exploit the secondary structure model predicted by AlphaFold,[48,49] and the Cα and Cβ chemical shifts were predicted using SPARTA.[53] Subsequently, the predicted spectrum was superimposed with the experimental 2D ^{13}C-^{13}C spectrum acquired for N-domain$_{39-190}$ (Figure S3E). No correlation was found between the experimental and predicted NMR spectral patterns, which is indicative of the inaccuracy of the AlphaFold model. These findings highlight that while bioinformatics tools can be useful in certain studies, they are often significantly limited by factors such as the nature of the sample, the buffer used, the intrinsic folding propensity of the protein or homomultimeric

associations. Thus, manually inspected the SSNMR cross-peaks and identified ~35-40 spin systems (i.e., corresponding to ~35-40 residues) (Figure 5B). Following well-established statistical data for Cα and Cβ chemical shifts according to their secondary structure,[54] we noticed that these residues are involved mainly in β-sheet secondary structure elements. This finding was illustrated by the isoleucine spectral pattern observed with Cα and Cβ chemical shift values of ~60 and 42 ppm, respectively, which are indicative of a β-sheet conformation. Approximately 35-40 out of the 151 amino acids comprising the N-domain$_{39\text{-}190}$ were detected within the rigid core by SSNMR, suggesting that the amyloid core encompasses only a portion of the N-domain$_{39\text{-}190}$ sequence. However, the presence of residues undetectable in dipolar-based SSNMR experiments is not surprising, given that this observation has been made for most pathological and functional amyloids to date.[55,56]

In light of these findings, we observed that the spectral resolution, as measured by the ^{13}C line width, is ~60-120 Hz (full width at half-height), which is indicative of a relatively well-ordered amyloid core. These observations are consistent with our previously reported ^{13}C line widths for *B. subtilis* TasA (~30-100 Hz), *B. subtilis* TapA (~200 Hz) and *B. cereus* CalY (~200 Hz), which, respectively form well-ordered amyloid fibrils (TasA) and polymorphic aggregates (TapA and CalY).[29] To gain more site-specific insights into the amyloid core of the N-domain$_{39\text{-}190}$, the protein was incubated with proteinase K, the bands corresponding to the rigid regions were resolved via SDS–PAGE, and the amino acid sequences were determined through tandem mass spectrometry analysis (Figure 5C and D). Interestingly, proteinase K digestion suggested that the following resistant regions were present: the highly hydrophobic region V^{47}-K^{59}, which contains the Ala-Ile-

Ile segment, and the region A^{88}-K^{134}. Further examination of the SSNMR data using an additional 200 ms PDSD experiment (Figure S4) led us to identify several rigid protein segments; this was achieved by considering the regions identified through proteinase K digestion analysis, namely, $K^{46}VEA^{49}$ and $E^{93}IKQAISV^{100}$. Notably, the total number of residues identified as resistant to proteinase K was slightly greater than the number of spin systems identified by SSNMR. This substantial difference suggests that compared to the regions identified as proteinase K resistant, the rigid and ordered amyloid core contains fewer residues; alternatively, the spectral resolution is insufficient to identify additional correlations. Based on these results, it can be inferred that the N-domain$_{39\text{-}190}$ contains a well-structured amyloid core that is characterized by the significant presence of a β-sheet conformation and rigid residues.

The tandem repetitions confer higher global rigidity to BC1280

As the full-length protein exhibits distinct polymerization kinetics from those of the N-domain$_{39\text{-}190}$, we conducted a comprehensive biophysical characterization of full-length BC1280 to identify putative differences from the truncated N-domain$_{39\text{-}190}$ in the context of macromolecular assemblies. The full-length protein sample underwent a similar treatment as the N-domain$_{39\text{-}190}$ sample, and upon ultracentrifugation, the following notable differences were observed: i) BC1280 assembled into a transparent gel-like pellet with high viscosity, while ii) the N-domain$_{39\text{-}190}$ pellet aggregated into a non-viscous white precipitate. Consistent with previous findings for the N-domain$_{39\text{-}190}$, the diffraction pattern of the BC1280 assemblies showed an intense signal at ~4.7 Å and a less pronounced peak at ~10 Å, indicating that the full-length assemblies exhibited the canonical cross-β

arrangement of amyloid fibrils (Figure 6A). The C-domain of BC1280 consists of fifteen tandem repeats that were predicted to be disordered; thus, we wondered whether this domain participates in the overall folding of the full-length protein assemblies. BC1280 was uniformly ^{13}C labeled, and the resulting aggregates were analyzed via MAS SSNMR. To compare the molecular conformation of N-domain$_{39\text{-}190}$ to that of full-length protein assemblies, we superimposed 2D ^{13}C-^{13}C SSNMR spectral fingerprints and observed significant overlap, suggesting that the rigid core of the N-domain is highly conserved in full-length protein assemblies (Figure 6B). As only a few additional NMR signals were observed in the full-length protein sample, we hypothesize that the C-domain is not a primary component of the rigid structural fold of the BC1280 assemblies.

To complement the SSNMR analysis of the rigid BC1280 core, we used dynamics-based spectral editing via J-coupling polarization transfer to detect dynamic protein segments[57] and gain insights into any dissimilarities between the two protein versions. The superimposition of 2D ^{1}H-^{13}C-insensitive nuclei was enhanced by polarization transfer (INEPT) of the BC1280 and N-domain$_{39\text{-}190}$ assemblies, and the results revealed a significant difference in the spectral fingerprint between the two samples (Figure 6C). More INEPT signals were observed in the N-domain$_{39\text{-}190}$ than the full-length protein, suggesting that the absence of tandem repetitions in the C-domain results in a more flexible conformation overall. To confirm this hypothesis, we estimated the degree of rigidity by comparing signal intensities from 1D ^{13}C-detected INEPT and CP experiments (Figure 6D), respectively, by probing mobile and rigid protein segments through the INEPT/CP intensity ratio. We observed a different behavior, with a decrease in mobile

signals in BC1280 (ratio ~0.1) compared to N-domain$_{39-190}$ (ratio ~0.25), suggesting fewer dynamic assemblies for the full-length protein.

Considering the differences in the overall rigidity between BC1280 and N-domain$_{39-190}$, we estimated the relative amount of secondary structural elements using attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) analysis. To identify the minimum peaks within the amide I range according to well-established assignments,[58] the second derivative was calculated from the raw data, and deconvolution was performed to determine the percentage of contribution for each secondary structure type (Figure 6E-G). The N-domain$_{39-190}$ exhibited 33% antiparallel β-sheets, and the full-length protein had 25% antiparallel β-sheets and a slightly greater proportion of parallel β-sheets. Both proteins displayed an equal overall percentage of secondary structure in the random coil conformation. Finally, there are slightly more α-helices and turns in BC1280 than in N-domain$_{39-190}$.

BC1280 interacts with CalY through a nucleation mechanism

Based on the *in vivo* colocalization of BC1280-CalY in the extracellular matrix and the potential involvement of BC1280 in CalY polymerization, we investigated protein–protein interactions via far-Western blot analysis. Samples corresponding to BC1280 and N-domain$_{39-190}$ were purified *in vitro*, resolved via SDS–PAGE, transferred to a nitrocellulose membrane, and incubated with CalY. The results obtained with a specific antibody against CalY showed the presence of specific immunoreactive bands in the presence of BC1280 (Figure 7A) and the absence of a specific signal when CalY was incubated with the N-domain$_{39-190}$. These findings, together with the failure of CalY to form fibers upon

overexpression in the *Δbc1280* strain, support the relevance of BC1280 in the polymerization of CalY (Figure S2H) and the participation of the C-domain in this functionality.

To determine the mechanism by which BC1280 catalyzes the polymerization of CalY, we determined the relative levels of BC1280 and CalY over time using an isobaric tag for relative and absolute quantification (iTRAQ) analysis (Figure 7B). The relative accumulation rate of CalY was approximately twofold greater than that of BC1280 after 72 h. This information was used to estimate the protein ratios for copolymerization assays of BC1280 or N-domain$_{39\text{-}190}$ with CalY using ThT fluorescence emission. The presence of low concentrations of BC1280 (2 and 10 μM) did not impact the polymerization kinetics of a 40 μM solution of CalY (Figure 7C). In contrast, the presence of the N-domain$_{39\text{-}190}$ significantly reduced the rate of polymerization kinetics of CalY, leading to a lower fluorescence intensity at the end of the experiment than that of CalY alone (Figure 7C). Interestingly, the negative impact on CalY polymerization was amplified in the presence of non-freshly purified or N-domain$_{39\text{-}190}$, which are enriched in preformed oligomers (Figure 7D and E). The samples corresponding to CalY incubated with a freshly purified 10 μM solution of BC1280 or N-domain$_{39\text{-}190}$ were visualized via TEM. Consistent with the ThT results, incubation of 10 μM BC1280 with CalY resulted in macromolecular aggregates similar to those formed by CalY (Figure 7F). However, the incubation of CalY with the N-domain$_{39\text{-}190}$ led to the accumulation of small oligomers (Figure 7F). These findings are consistent with previous *in vivo* results showing that the absence of the C-domain (*ΔC-domain*) arrested the formation of CalY fibers (Figure 2F).

To determine the most likely interaction between the three proteins, we performed a multimer prediction using AlphaFold.[59] A portion of the N-terminal domain of BC1280 (I^{42}-S^{52}), previously identified as a rigid core by SSNMR and resistant to proteinase K digestion, was rearranged into a β-sheet conformation, contributing to the folding of CalY into nascent pili (Figure 7G). Subsequently, CalY seems to facilitate the incorporation of a TasA subunit into the polymer. Consistent with our results and the predicted structure, BC1280 might act as a chaperone in the chaperone-usher pathway by folding CalY monomers through the donation of its N-terminus via a nucleation mechanism. The function of the C-domain within the structural biology of BC1280 might involve controlling the polymerization rate of BC1280, preventing extensive self-aggregation through the N-domain and promoting interaction with CalY. According to our hypothesis regarding the potential role of BC1280 as a chaperone, circular dichroism analysis revealed significant structural changes in CalY in the presence of BC1280 in a concentration-dependent manner. After 16 hours of incubation under agitation, the presence of a 10 μM solution of BC1280 induced an increase in the β-sheet conformation of CalY compared to that of CalY alone or after incubation with 2 μM BC1280, which predominantly appeared as a random coil or a disordered conformation (Figure 7H).

DISCUSSION

Bacterial pili are fascinating polymers from structural and biological perspectives. The diversity of structures, sizes, functions and mechanisms of polymerization reflects the specificity of their functions and need for tight regulation. In this work, we pinpointed BC1280 as a specific biofilm orchestrator factor that is exclusive to the *B. cereus* group. In addition, we elucidated the intriguing molecular mechanism by which this protein drives the assembly of amyloid pili on the cell surface of this opportunistic human pathogen. The severe defective phenotype observed in *Δbc1280*, when contrasted with the phenotypes of *ΔtasA* and *ΔcalY*, substantiates the indispensable role of BC1280 as a nucleator for biofilm maturation.[25] We have profoundly demonstrated the presence of the following structurally and functionally distinct regions in BC1280: the N-terminal half, which is essentially sufficient to fulfill the functionality of BC1280 and is specifically involved in cell-to-cell interactions; and the C-terminal half, which appears to be dedicated to controlling the interaction of BC1280 with CalY. After establishing the association of BC1280 with the cell surface and its involvement in the proper polymerization of CalY, we proposed its role in facilitating the attachment of TasA-CalY pili to the bacterial cell surface through the following lines of evidence: i) the biofilm phenotype remains unchanged even after the overexpression of *tasA* and/or *calY* in the *Δbc1280* mutant strain; ii) fibers of CalY are not formed in the *ΔC-domain* strain; and iii) colocalization of BC1280 and CalY occurs *in vivo* and *in vitro.* Collectively, these findings prompted us to rename BC1280 to CapP, which is a CalY-assisted polymerization protein. In addition to its fundamental role in controlling pilus assembly, CapP functions as a checkpoint of biofilm maturation by regulating the

expression of EPS via an uncharacterized pair of Clp-proteases and cognate ECF-type sigma factors. However, this is a novel and specific biofilm regulatory pathway that deserves further investigation.

We determined the amyloid nature of CapP through our structural studies, complementing the previously described amyloid tandem TasA and CalY proteins and expanding our knowledge on the coordinated assembly of these pili in the extracellular matrix of *B. cereus*. Interestingly, CapP adds another level of structural complexity compared to TasA and CalY because it contains a C-domain region that is not involved in the rigid amyloid core but still mediates CalY polymerization. Moreover, CapP (Figure S3F), similar to TasA and CalY,[29] is disordered prior to aggregation, a notable difference from the TasA-TapA system of *B. subtilis*, which adopts a folded or partially folded conformation before assembly into amyloid fibrils.[29,31,60] These distinctions suggest that *B. cereus* depends on an external trigger, which could be chemical or biophysical, to initiate the assembly of functional amyloids. This additional requirement has been previously proposed for the *in vivo* polymerization of CalY, and moreover, CalY has been described to enhance the polymerization of TasA without perturbing the global filament architecture.[22,29] Based on these observations and our findings, the three amyloid-like proteins may work coordinately to properly assemble external pili and, consequently, organize the ECM. The biophysical studies performed in this work have provided valuable insights into the structural biology of CapP, highlighting the rigidity of the core of ~ 40 amino acids within the N-domain that confers canonical amyloid cross-β properties. Through SSNMR, it was determined at the atomic level that the amyloid core contains at least the following rigid regions: $K^{46}VEA^{49}$ and $E^{93}IKQAISV^{100}$. The role of each amino

acid stretch remains to be elucidated; nevertheless, we propose that the K^{46}VEATLSTAIIFP^{58} region significantly influences the amyloidogenicity of CapP, considering that i) the sequence is situated in the most hydrophobic region of the protein, ii) the residues within this region are highly conserved, and iii) the region was detected in the proteinase K-resistant fraction. Although the amyloid core is conserved in the full-length protein, the amyloid core exhibits slower polymerization kinetics and a greater polydispersity index than that of the truncated N-domain. In addition, a higher global rigidity is generated by the C-domain in CapP than by the truncated N-domain. Together, these findings strongly suggest that the entire protein has a more complex folding pattern in which tandem repetitions might be involved in the regulation of oligomer and amyloid pili assembly.

The diverse mechanisms that govern the assembly and attachment of non-flagellar proteinaceous filaments to bacterial cell surfaces[61] include various structures, such as chaperone-usher pili, type V pili, type IV pili, curli, fap fibers, conjugative, type IV secretion pili, e-pili in diderm bacteria, and sortase-mediated pili as well as type IV pili in monoderm bacteria.[62] In the monoderm *B. cereus*, BcpA-BcpB fimbriae are covalently bound to vegetative cells by sortase, while Ena appendages, exclusively found on spores, are assembled through subunit crosslinking via disulfide bonding.[63-65] *B. cereus* is phylogenetically related to *B. subtilis*; however, we suggest that the polymerization of CapP-CalY-TasA amyloid pili is more closely related to the amyloid curli system of *E. coli* and *Salmonella* spp. than to the TasA-TapA system of *B. subtilis*[66-71]. Building upon the novel insights unveiled in this study and the current knowledge on pili polymerization, we propose a revised model for the assembly of biofilm-specific pili in *B. cereus* (see

Graphical abstract), which involves the paralogous proteins TasA and CalY, as well as the biofilm-specific protein CapP. Once the switch from a motile to sessile lifestyle has occurred, further growth and maturation of the biofilm ECM rely upon several factors, including the products of the operon *sipW-tasA* and the loci *capP* and *calY*, which are expressed under the tight control of the master regulator SinR. TasA, CapP and CalY are processed by the cognate signal peptidase SipW and secreted via the Sec pathway in an unfolded conformation. In association with the cell membrane, CapP undergoes conformational changes that lead to the formation of aggregates with β-sheet conformations, which serve as nuclei for further pili growth. At this point, the C-domain of CapP interacts with unfolded CalY subunits via a nucleation mechanism, catalyzing the structural rearrangement necessary to initiate the growth of amyloid pili. Subsequently, CalY facilitates the incorporation of TasA monomers into growing pili. Finally, functional TasA-CalY heteropili serves as a scaffold within the ECM of *Bacillus cereus*, with the chemical and physical properties necessary for correct assembly of the biofilm.

Our findings contribute to knowledge on the pivotal role of the biofilm-specific protein CapP in orchestrating the formation and regulation of biofilms on *B. cereus* strains; in addition, the results provide insights into a new type of pilus assembly mechanism in monoderm bacteria. These remarkable advancements carry substantial implications for the clinical and food industries, in which innovative and effective strategies are urgently needed to address the critical challenge of *B. cereus* biofilm formation and its associated risks.

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AUTHOR CONTRIBUTIONS

D.R. conceived the study. D.R., A.L. and A.A.M. designed the experiments and method analysis. A.A.M. performed the main experimental work and analyzed the data, with the following exceptions: A.A.M. and M.B.A.S. analyzed the SS-NMR data; M.L.A.G. performed some microbiological experiments; A.A.M., M.B. and B.K. performed biophysical analysis; A.G., E.M., and A.L. performed NMR analysis. JCA contributed to setting the initial hypothesis and performing iTRAQ experiments. A.A.M., M.B.A.S., A.L. and D.R. prepared the figures. A.A.M., A.A.L. and D.R. wrote the manuscript. A.V. contributed critically to writing the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

FIGURE TITLES AND LEGENDS

Figure 1. *bc1280* is an essential locus in the developmental program of biofilm formation. A) Schematic domain composition of BC1280. The signal peptide (M1-A39) is shown in red; the domain of unknown function, DUF4047 (I33-E157), which is located within the N-domain, is highlighted in blue; and the C-domain containing the repetitive region (Q190-E 279) is shown in yellow. The amino acid alignment of DUF4047 among different strains of the *B. cereus* group revealed that it is highly conserved, with the residues that are 100% conserved in all the strains marked in color. The color pattern is determined by the chemical properties of each amino acid. **B)** Biomasses formed by the *B. cereus* ATCC14579 wild-type strain and the mutants that adhered to abiotic surfaces were evaluated at different time points. Biofilms were stained with crystal violet to enable visualization and subsequent quantitative comparison. **C)** The amount of biofilm mass for each strain was estimated. Three independent experiments were conducted, each with three replicates. Average values are shown, and the error bars represent the standard deviation for each strain. Multiple comparisons were performed at each time point, with the wild-type serving as the control, and ordinary one-way ANOVA was used (** p value < 0.01 and **** p value < 0.0001). **D)** The aggregation kinetics resulting from cell-to-cell interactions were studied for the wild-type and the *Δbc1280* and *ΔC-domain* strains. The optical density at 600 nm at the air-liquid interface of the cell suspensions was measured each hour. The initial optical density was set to 3 at the start of the experiment, and the samples were incubated without agitation at room temperature. The error bars indicate the SDs calculated from three biological replicates.

Figure 2. The biofilm-defective phenotype of *bc1280* is independent of the expression level of *tasA* or *calY.* A) Relative expression levels of *tasA*, *calY*, *eps2* and genes belonging to the *eps1* operon (*bc5263*, *bc5268*, *bc5277* and *bc5279*) obtained by RT–qPCR in *Δbc1280* planktonic cells compared to wild-type cells at 48 hours. The average values of three biological replicates are shown with error bars indicating the standard deviation. **B)** Transmission electron micrographs of the wild-type and *Δbc1280* strains overexpressing *tasA* immunolabeled with specific anti-TasA antibodies. **C)** Negative staining micrographs of wild-type and *Δbc1280* cells overexpressing *calY* immunolabeled with specific anti-CalY antibodies. **D)** and **E)** On the left: Extracellular complementation of *Δbc1280* after the addition of TasA (D) or CalY (E) at 6 μM in monomeric or fibrillar form. On the right: Phenotype reversion of *Δbc1280* upon the addition of 6 μM polymerized TasA (D) or CalY (E), visualized by immunolabeling with anti-TasA and anti-CalY antibodies, respectively. **F)** Negative-stained micrographs of TasA and CalY immunodetection in the *ΔC-domain* strain.

Figure 3. BC1280 is a membrane-associated protein that partially colocalizes with CalY in the ECM. A) and **B)** Left: Immunodetection by Western blotting was performed for BC1280 (A) and the N-domain (B) fused to a histidine tag in different cellular fractions of *Δbc1280*. This assay was conducted under conditions of overexpression using the vector pUTE657 and with the addition of 10 μM IPTG. Biofilm samples were grown for 48 hours, after which the resulting biomass was recovered for fractionation into EM (extracellular medium), CW (cell wall), and M (membrane) fractions. The immunoreactive bands were detected using an anti-His antibody (1:2500). The immunoblot images were edited by cropping and splicing for better illustration. Right: Negative stained images of

BC1280 (A) and the N-domain (B) in the ECM of the *Δbc1280* strain. The proteins were immunolabeled using an anti-His antibody (1:100) as the primary antibody and a goat-antirabbit secondary antibody conjugated with 20 nm nanogolds (1:100). **C)** Immunolocalization of BC1280 and the N-domain determined using CLSM. Biofilm samples were processed using immunochemistry, with anti-His antibody (1:100) serving as the primary antibody and a secondary antibody conjugated to Atto 488. The localization of BC1280 and the N-domain is shown in green. The cell wall is labeled in red using WGA, and the DNA is stained in blue using Hoechst. **D)** Immunolocalization images of BC1280 and CalY obtained through CLMS. An anti-His antibody (1:100) and a secondary antibody conjugated with Atto 488 (1:400) were used for the immunolabeling of BC1280. A specific primary anti-CalY antibody (1:100) and a secondary antibody labeled with Alexa Fluor 647 (1:400) were used for CalY detection. Additionally, DNA was stained with Hoechst (1:1000) to visualize the bacteria, which are shown in blue.

Figure 4. BC1280 oligomerizes into macromolecular complexes driven by the N-domain. A) Scheme illustrating the two constructs based on the full-length sequence of BC1280. Each construct was fused to a poly-histidine tag at the C-terminal domain, and the resulting two recombinant proteins were produced through recombinant expression in *E. coli*. **B)** Size-exclusion chromatogram obtained after BC1280 was purified under native conditions. **C)** and **D)** Size distribution histograms obtained via DLS showing the diameters of the aggregates formed by BC1280 for peaks 1 and 2, respectively, as determined by SEC. **E)** Size-exclusion chromatogram obtained after purifying N-domain$_{39\text{-}190}$ under native conditions. **F)** and **G)** Size distribution histograms obtained via DLS, illustrating the diameters of the aggregates formed by N-domain$_{39\text{-}190}$ for peaks 1

and 2, respectively, as determined by SEC. **H)** Native polyacrylamide gel showing the oligomers formed by BC1280 and N-domain$_{39\text{-}190}$ following native purification through heterologous expression in *E. coli*. The numbers on each line correspond to the first and second peaks, respectively, that were detected in the size-exclusion chromatograms of each sample. The gel lanes were cropped and spliced for illustrative purposes. All the lines superimposed on the image delineate the lanes and boundaries of the spliced images. **I)** Negative-staining micrographs of the first and second peaks detected by SEC for BC1280 and N-domain$_{39\text{-}190}$. **M)** Aggregation kinetics of BC1280 (black curve) and N-domain$_{39\text{-}190}$ (red curve) determined using ThT fluorescence. **N)** Transmission electron micrographs of BC1280 and N-domain$_{39\text{-}190}$ at the end of the polymerization experiment shown in M.

Figure 5. N-domain$_{39\text{-}190}$ displays the canonical cross-β fold. A) X-ray diffraction signals of N-domain$_{39\text{-}190}$ at 4.7 and 10 Å, indicating characteristic interstrand and intersheet spacings of the amyloid cross-β-arrangement. **B)** 2D ^{13}C-^{13}C PDSD experiments at 50 ms for the N-domain$_{39\text{-}190}$. The chemical shifts that correspond to isoleucine residues in the β-sheet conformation are highlighted. **C)** A 12% SDS–PAGE gel was obtained after the treatment of N-domain$_{39\text{-}190}$ aggregates with proteinase K for 45 minutes. The three bands marked were analyzed by tandem mass spectrometry. **D)** Determination of the rigid core of domain$_{39\text{-}190}$ by tandem mass spectrometry. MS data were obtained from the bands selected after digestion of N-domain$_{39\text{-}190}$ with proteinase K. The blue shading indicates the peptides obtained for N-domain$_{39\text{-}190}$ without incubation with proteinase K, and the amino acids marked in red correspond to the rigid regions identified for each band after treatment.

Figure 6. The tandem repetitions confer higher global rigidity to BC1280. A) X-ray diffraction signals of BC1280 within 0–15 Å. **B)** Superimposition of the 2D ^{13}C-^{13}C PDSD correlation spectra obtained by SSNMR for BC1280 (blue) and N-domain$_{39-190}$ (red) at a mixing time of 50 ms. **C)** Superimposition of the ^{1}H-^{13}C INEPT spectra of BC1280 (blue) and N-domain$_{39-190}$ (red), recorded at a 1H frequency of 600 MHz (276 K and 96 scans for BC1280; 300 K and 640 scans for N-domain$_{39-190}$) showing signals corresponding to the flexible part. **D)** Estimation of the global rigidity based on the INEPT:CP ratio of chemical shifts for each sample. **E)** and **F)** ATR-FTIR spectra of BC1280 and N-domain$_{39-190}$, respectively, in the amide I band. The contribution of each peak was determined through secondary derivative analysis of the raw data, followed by a deconvolution approach. **G)** Secondary structure content of the BC1280 and N-domain$_{39-190}$ aggregates probed via ATR-FTIR analysis.

Figure 7. BC1280 interacts with CalY through a nucleation mechanism. A) Left: BC1280, N-domain$_{39-190}$, BSA and CalY purified *in vitro* were loaded onto an SDS–PAGE gel and then stained with Coomassie blue. Right: The proteins shown on the left were transferred to a nitrocellulose membrane, and a far-Western blotting was conducted. After the proteins were renatured, CalY was used as bait, and a specific anti-CalY antibody (1:3000) was utilized for immunodetection. BSA was used as a negative control to prevent nonspecific interactions. The immunoblot images shown were cropped and spliced for illustrative purposes. **B)** Estimation of the relative levels of BC1280 and CalY in biofilm cells during biofilm formation using iTRAQ. **C)** Kinetics of ThT binding of CalY in the presence of BC1280 or the N-domain$_{39-190}$ at concentrations of 2 and 10 µM. Error bars represent the SEM. **D)** and **E)** Study of the effect of BC1280 and the N-domain$_{39-190}$

without recent purification on CalY polymerization. ThT binding kinetics of CalY in the presence of freshly and non-freshly purified BC1280 (D) or N-domain$_{39-190}$ (E) samples. Error bars indicate the standard error of the mean. **F)** Transmission electron micrographs of negatively stained samples of CalY aggregates in the presence of BC1280 or N-domain$_{39-190}$ at a concentration of 10 µM after 30 hours of incubation at 37°C. **G)** TasA-CalY-BC1280 multimer prediction using AlphaFold. **H)** Circular dichroism (CD) spectra of CalY incubated for 16 hours under agitation alone or in the presence of BC1280 at 2 and 10 µM.

Graphical abstract. During the process of biofilm formation in *B. cereus*, TasA, CapP, and CalY serve as the main components, in addition to other factors, ensuring that the extracellular matrix correctly assembles. The three proteins are produced and processed by the signal peptidase SipW and subsequently secreted through the Sec pathway in an unfolded conformation. CapP aggregates into macromolecular complexes that associate with the cell membrane. These complexes interact with unfolded subunits of CalY, facilitating CalY folding and forming the primary nuclei for fibril growth. CalY subsequently acts as a nucleator for the incorporation of TasA subunits into the pilus, ultimately leading to the formation of TasA-CalY heteropolymers with the canonical properties of amyloid proteins.

Materials and Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Diego Romero (diego_romero@uma.es).

Materials availability

Plasmids generated in this study are available at the BacBio Lab. Please contact Diego Romero (diego_romero@uma.es) for access. This study did not generate new unique reagents.

Data and code availability

RNaseq data have been deposited at GEO database and are publicly available as of the date of publication. Accession numbers are listed in the Key Resources Table. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010211. Microscopy data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDENT PARTICIPANT DETAILS

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in the Key Resources Table. For experiments conducted using *B. cereus* wild-type or mutant strains, cultures were routinely grown at 28°C from frozen stocks on Luria–Bertani (LB) plates containing 1%

tryptone (Oxoid), 0.5% yeast extract (Oxoid), 0.5% NaCl and 1.5% agar before the respective experiments were performed. Biofilm assays were performed in TY medium supplemented with 1% tryptone (Oxoid), 0.5% yeast extract (Oxoid), 0.5% NaCl, 10 mM $MgSO_4$ and 1 mM $MnSO_4$.

For cloning and plasmid replication, the *E. coli* DH5α strain was used. For recombinant protein expression and purification, *E. coli* BL21(DE3) pLysS (Merck, Kenilworth, NJ, USA) was used. All the strains were cultured in Luria–Bertani (LB) liquid media. For the agar-solid plates, LB media was supplemented with 1.5% bacteriological agar (Oxoid). The final antibiotic concentrations were 100 μg/ml ampicillin, 50 μg/ml kanamycin, 100 μg/ml spectinomycin, and 5 μg/ml erythromycin.

For the purification of $^{13}C/^{15}N$-labeled proteins, the cultures were grown in M9 minimal medium (the final concentration was 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 1 mM $MgSO_4$, 0.01 mM $ZnCl_2$, 0.001 mM $FeCl_3$, 0.1 mM $CaCl_2$ and 10 ml 100X minimum essential medium vitamin solution) supplemented with 1 g/L $^{15}NH_4Cl$ as nitrogen and 2 g/L $^{13}C_6$-D-glucose or 2-^{13}C glycerol as the carbon source.

METHOD DETAILS

Plasmid and strain construction

All the primers used to construct the strains used in this study are listed in Table S4.

B. cereus mutant strains were constructed via homologous recombination using the upstream and downstream regions of the gene of interest, which were subsequently cloned and inserted into the pMAD plasmid.[72] The constructs were designed using NEB Builder HiFi DNA Assembly Master Mix (New England Biolabs, MA, USA) following the manufacturer's instructions and utilizing specific primers. The pMAD vector was linearized

using the restriction enzyme SmaI (FastDigest, Thermo Fisher Scientific), and the digested vector was incubated at 50°C for 1 hour along with the respective upstream and downstream fragments. The combined total amount of fragments and plasmid was 0.2 pmol, and a proportion of 1:2 (vector: fragments) was maintained. The resulting reaction mixture was subsequently transformed into *E. coli* DH5α, and positive colonies were selected using ampicillin. Subsequently, the plasmids were purified, subjected to PCR amplification and digestion for verification, and sequenced.

For the overexpression experiments in *B. cereus,* the gene of interest was cloned and inserted into the plasmid pUTE657[73] under a promoter that was inducible by IPTG using the primers listed in Table S4. Following gene amplification and the inclusion of a specific RBS for *Bacillus*, the resulting PCR product was digested with SalI and SphI (FastDigest, Thermo Fisher Scientific) and subsequently cloned and inserted into the pUTE657 plasmid that was cut with the same restriction enzymes using T4 DNA ligase (Thermo Fisher Scientific). Subsequently, the resulting reaction was transformed into *E. coli* DH5α, and colonies were selected with 100 µg/ml ampicillin. After plasmid purification, all the vectors were subjected to PCR amplification, digestion with restriction enzymes, and sequencing.

The vectors were transformed into *B. cereus* by electroporation following previously described methods with some modifications. A single colony from a pure culture of *B. cereus* was inoculated in 5 ml of LB medium and incubated under shaking conditions at 30°C for 16 hours. This culture was subsequently used to inoculate a flask containing 100 ml of LB, which was incubated at 30°C with agitation until an OD_{600nm} of 0.3 was reached. The cell culture mixture was centrifuged (3000 rpm, 5 minutes, 4°C),

and the resulting pellet was resuspended in 12 ml of electroporation buffer (0.5 mM K_2HPO_4, 0.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, and 272 mM saccharose). The suspension was centrifuged again and then resuspended in 250 µl of electroporation buffer. The electrocompetent cells were mixed with 0.5–1 µg of plasmid and incubated on ice for 10 minutes. Next, the mixture was electroporated in a 0.2 cm cuvette using a voltage of 1.4 kV, a capacitance of 25 µF, and a resistance of 200 Ω. After electroporation, the suspension was recovered and incubated with 1.5 ml of LB for 5 hours at 30°C with agitation. The resulting culture was plated onto LB plates supplemented with the corresponding antibiotic to select the colonies that had been successfully transformed with the vector. For integration of the deletion, *B. cereus* transformants were incubated without antibiotics at 40°C, facilitating the integration of the mutation into the genome through homologous recombination. Finally, the culture was plated on LB agar plates, and the colonies were picked onto LB agar plates supplemented with erythromycin. Colonies that did not grow on LB supplemented with the antibiotic were selected, and the mutation was confirmed through PCR amplification.

For the heterologous expression of BC1280, the N-domain$_{39\text{-}190}$ and the C-domain$_{191\text{-}279}$ in *E. coli,* the open reading frames containing the *bc1280* gene, the region from amino acids 39 to 190, and the region from amino acids 191-279 were cloned and inserted into the plasmid pET24b without the signal peptide. The PCR products and the pET24b vector were digested with NdeI and XhoI (FastDigest, Thermo Fisher Scientific) and subsequently cloned using T4 DNA ligase (Thermo Fisher Scientific) following the instructions supplied by the manufacturer. The reaction mixtures were subsequently

transformed into *E. coli* DH5α, after which the colonies were successfully transformed with 50 µg/ml kanamycin.

Phylogenetic tree

The amino acid sequence of BC1280 was obtained from the KEGG database using *B. cereus* ATCC14579 as a reference. BLASTP analysis was performed, and the top 50 hits were aligned using Clustal Omega[74]. The phylogenetic tree was then constructed using neighbor joining distances.

RT–qPCR

Quantitative reverse transcription PCR was used to estimate the transcription levels of i) *bc1280* in planktonic and biofilm cells of the wild-type strain; ii) *tasA*, *calY*, *eps1* and *eps2* in *Δb1280* compared to those in the wild-type; and iii) *tasA*, *calY*, *eps1* and *eps2* in *Δb1280* overexpressing *bc2794* or *bc2793-2794* compared to those in the wild-type. RT–qPCR was performed using an iCycler-iQ system (Bio-Rad) and Power SYBR Green Master Mix (Thermo Fisher Scientific). Primer3 software (https://primer3.ut.ee/) was used for primer design, maintaining the default parameters.[75] First, 1 µg of total RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen) following instructions provided by the manufacturer. The reactions were performed in triplicate in 96-well plates with a total volume of 20 µl. The RT–qPCR cycle started with an initial denaturation at 95°C for 3 minutes, followed by 40 amplification cycles (95°C for 20 seconds, 56°C for 20 seconds, and 72°C for 30 seconds), and the final step was 95°C for 30 seconds. The target genes, including *bc1280*, which encodes the protein BC1280;

bc1279, which encodes TasA (also known as spore coat-associated protein N); *bc1281*, which encodes CalY (annotated as cell envelope-bound metalloprotease, camelysin); *bc5263*, which encodes UDP-glucose 4-epimerase; *bc5268*, which encodes secreted polysaccharide polymerase; *bc5277*, which encodes tyrosine-protein kinase; *bc5279*, which encodes tyrosine-protein kinase; and *bc1583*, which encodes O-acetyl transferase, were amplified using the primer pairs provided in Table S5. Primer efficiency and amplification product specificity were assessed as previously described.[76] The estimation of relative expression levels was calculated using the ΔΔCt threshold (Ct) method.[77] The housekeeping gene *rpoA* was used as a reference for data normalization. The relative expression value was calculated as the difference between the qPCR threshold cycle (Ct) of the gene of interest and the Ct obtained for *rpoA* ($\Delta Ct = Ct_{target\ gene} - Ct_{rpoA}$). The results obtained for the genes of interest were normalized to the values obtained for the wild-type strain. The RT–qPCR analyses were conducted three times, each with three independent biological replicates.

Determination of the transcriptional unit by PCR

To determine whether *bc1280* constitutes an operon with *tasA* or *calY*, primers were designed between the genes and inside each gene to amplify four specific fragments that were 974 bp, 572 bp, 1668 bp, and 560 bp in size (primers listed in Table S6). cDNA was used as the template, and PCR was performed using genomic DNA purified with the commercial JetFlex™ Genomic DNA Purification Kit (Thermo Fisher Scientific, Bremen, Germany) as a positive control. The fragments were amplified by PCR using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with the following program:

initial denaturation at 98°C for 30 seconds, followed by a 30-cycle amplification program (98°C for 10 seconds, 56°C for 30 seconds, and 72°C for 1 minute), and a final extension step at 72°C for 5 minutes. The samples were loaded onto an agarose gel and compared to the fragments amplified from genomic DNA.

Biofilm formation and extracellular complementation assays

To study the biofilm formation phenotype for each strain or condition, bacterial cultures were grown in 24-well plates as previously described.[25] The strains were subsequently grown on LB agar plates at 28°C for 24 hours. The bacterial mass was resuspended in 1 ml of TY medium, and the OD_{600} was adjusted to 1. Subsequently, 10 µl of the suspension was inoculated into each well containing 1 ml of TY medium. The plates were then incubated at 28°C without agitation for 72 hours.

For the extracellular complementation assay involving the addition of TasA or CalY, the proteins were previously purified *in vitro* from inclusion bodies through heterologous expression in *E. coli* following a protocol established by El-Mammeri et al.,[29] in 2019. Subsequently, the proteins were dialyzed against a 20 mM Tris and 50 mM NaCl solution at pH 7.4, after which the protein concentrations were measured. The assembly of filaments was promoted at 37°C under agitation at 200 rpm for one week. TasA or CalY protein, in both its monomeric and fibrillated forms, was added at a final concentration of 6 µM to 24-well plates inoculated with the *Δbc1280* strain. These plates were then incubated without agitation at 28°C for 72 hours, after which the resulting biomass was stained with crystal violet.

Crystal violet adhesion assay

Bacterial adhesion to the well plate surface was quantitatively measured using crystal violet staining, as previously described.[78] The biofilms were grown for 72 hours, after which the medium was removed. Then, 1 ml of 1% crystal violet was added to each well of the 24-well plate, and the cells were incubated for 15 minutes. The plates were washed multiple times with water and left to dry for 45 minutes. Next, the biomass that formed in each well was resuspended in 1 ml of 50% acetic acid, and the absorbance at 575 nm was measured for each sample using a plate reader (Tecan Infinite M1000 Pro; Tecan, Männedorf, Switzerland). The statistical significance was evaluated using one-way ANOVA with Dunnett´s multiple comparisons test. P values less than 0.05 were considered to indicate statistical significance.

Aggregation assay

The effect of *bc1280* and *C-domain* deletion on the aggregation rate of *B. cereus* was estimated using a kinetic method following a previously described protocol.[24] The strains were inoculated into a flask containing 20 ml of TY medium and incubated overnight at 28°C with agitation. The OD_{600} was adjusted to 3 for a final volume of 10 ml, after which the supernatant was centrifuged (5000 rpm, 10 minutes, 20°C) for dilution. The samples were then incubated in vertical tubes under static conditions at room temperature for 24 hours, after which the OD_{600} was measured every hour at the air–liquid interface.

RNA extraction

For RNA extraction, a previously described protocol was followed with several modifications.[26] Bacterial strains were cultivated in 24-well plates to retrieve the desired population—either planktonic or biofilm cells—at a specific incubation time. For the biofilm cells, the supernatant was removed from each well, followed by three wash steps, and the biomass was subsequently resuspended in 1 ml of PBS. To minimize variability between replicates, each sample was prepared by extracting 500 µl from 8 distinct wells. The bacterial suspensions were then centrifuged at 12000 × g for 5 minutes, and the resulting pellets were frozen at -80°C for 30 minutes or until needed. The pellets were resuspended in 900 µl of TRI-Reagent (Merck), and the cells were disrupted with 0.1 mm beads using a TissueLyser (QIAGEN) for 3 minutes, followed by incubation at 55°C for 3 minutes. Then, 200 µl of chloroform was added to each tube, which was vortexed for 10 seconds and incubated at room temperature for 3 minutes. Finally, the samples were centrifuged at 12000 × g for 10 minutes at 4°C. The resulting aqueous phase was transferred to a new tube containing 500 µl of ice-cold isopropyl alcohol. The tubes were inverted a few times, incubated for 10 minutes at room temperature, and then centrifuged at 12000 × g for 10 minutes at 4°C. The supernatants were subsequently removed, and the pellets were washed with 1 ml of ice-cold 75% ethanol, followed by centrifugation (12000 × g, 5 minutes, 4°C). Briefly, the RNA pellets were dried and then resuspended in 50 µl of DEPC-treated water. The residual DNA was eliminated through treatment with rDNAse, which is included in the Nucleo-Spin RNA Plant Kit (Macherey-Nagel), following the instructions provided by the manufacturer. The quality and integrity of the total RNA were assessed using an Agilent 2100 bioanalyzer (Agilent Technologies) and gel electrophoresis.

955

956 **Whole-transcriptome analysis**

957 A RNA-Seq analysis was performed on *Δbc1280* planktonic cells in comparison to the
958 wild-type after 24 and 48 hours of incubation at 28°C. RNA extraction was performed
959 following a previously described protocol, and each sample corresponding to a specific
960 condition was collected in duplicate. RNA sequencing was performed by the Omics Unit
961 of Supercomputing and Bioinnovation Center (SCBI, University of Malaga, Spain). rRNA
962 removal was achieved using the RiboZero rRNA Depletion Kit (Illumina, CA, USA), and
963 subsequently, 100-bp single-end read libraries were prepared using the TruSeq Stranded
964 Total RNA Kit (Illumina). Next, the libraries were sequenced using the NextSeq550
965 instrument (Illumina). To eliminate regions of low quality, ambiguity, and low complexity,
966 as well as potential contamination, the raw reads were preprocessed using SeqTrimNext
967 with the specific configuration parameters used for NGS technology.[79] Subsequently, the
968 clean reads were aligned and annotated using the *B. cereus* ATCC14579 genome
969 (NC_004722.1) as the reference. This alignment was conducted with Bowtie2, resulting
970 in BAM files that were subsequently sorted and indexed using the SAMtools v1.484
971 program.[80,81] To calculate the read count for each gene, the script Sam2counts
972 (https://github.com/vsbuffalo/sam2counts) was used to determine the number of uniquely
973 mapped reads. The analysis of differentially expressed genes (DEGs) between *Δbc1280*
974 and the wild-type strain was conducted using the R script DEgenes Hunter. This script
975 employs a combined p value, utilizing Fisher´s method to consider the nominal p values
976 obtained from edgeR and DEseq2.[82,83] The combined p value was adjusted using the
977 Benjamini–Hochberg (BH) test, which employs the false discovery rate approach. The

adjusted p value was subsequently used to rank all the DEGs obtained. A p value < 0.05 and a log2FoldChange < -1 or > 1 were considered to indicate statistical significance.

Transmission electron microscopy and immunochemistry studies

To investigate the location of the proteins within the ECM using transmission electron microscopy, we followed a protocol that was previously established and described, with several modifications.[24,29,84] For TasA and CalY immunodetection, the strains were cultured in multi-well plates at 28°C for 72 hours. Depending on the specific experiment, samples of either biofilm or planktonic cells were collected for subsequent analysis. Alternatively, for the localization of BC1280 or the N-domain within *B. cereus* cells, the strains corresponding to *Δbc1280* containing the plasmids pUTE657:*bc1280-6xHis* or pUTE657:*N-domain-6xHis* were grown in multiwell plates with TY medium supplemented with a 10 µM IPTG solution. The biofilm mass was subsequently harvested in 1 ml of PBS after 48 hours.

Then, 20 µl of each sample was applied to a copper grid and incubated for 2 hours. The grids were subsequently washed with PBS and incubated for 5 minutes to remove any excess bacterial cells. The cells that adhered to the grid were fixed using a 2% paraformaldehyde solution diluted in PBS and incubated for 10 minutes. The grids were washed again with PBS for 5 minutes and then blocked for 30 minutes using Pierce™ Protein-Free T20 (TBS) Blocking buffer (Thermo Scientific). Next, the samples were incubated for 2 hours with the primary antibody at a concentration of 1:100, which was diluted in blocking buffer. Specific primary antibodies against TasA and CalY were used to detect TasA and CalY, respectively. For the immunolocation of BC1280-His and N-

domain-His, we employed the primary antibody anti-6-His produced in rabbits (Merck). Subsequently, the grids were washed twice with TBS-T, each time with a 5-minute incubation. The samples were incubated for 1 hour with a goat anti-rabbit 20-nm gold-conjugated secondary antibody (Ted Pella, Redding, CA, USA) at a dilution of 1:100 in blocking buffer. Next, the grids were subjected to two additional washes with TBS-T, each lasting 5 minutes. The samples were fixed with 2.5% glutaraldehyde for 10 minutes. The samples were washed with Milli-Q water for 5 minutes and subsequently negatively stained with a 1% uranyl acetate solution. Finally, the grids were rinsed with a single drop of water and dried under dark conditions.

The samples were examined using a Thermo Fisher Scientific Tecnai G^2 20 TWIN transmission electron microscope at an accelerating voltage of 120 kV. The final images were taken using an Olympus Veleta side-mounted CCD with a resolution of 2k × 2k Mpx.

Cellular fractionation

For protein immunodetection by Western blotting, the samples were fractionated into the extracellular medium, proteins associated with the cell wall, and membrane and cytoplasmic contents. Preliminary studies of BC1280 and the N-domain were conducted through cellular fractionation using the *Δbc1280* strain harboring the plasmid pUTE657:*bc1280*-6xHis or pUTE657:N-*domain*-6xHis, respectively. The strains were subsequently grown in TY media supplemented with agitation at 28°C. When the cultures reached an OD_{600nm} of 0.4, 10 µM IPTG was added to each sample, and the cultures were incubated for 4 hours.

All the samples were subjected to the following protocol. The samples were centrifuged at 6000 × g for 5 minutes, after which the supernatant was passed through a 0.45 µm PES filter and retained as the extracellular medium fraction. The pellets were resuspended in 10 ml of PBS containing 100 µg/ml lysozyme and incubated for 2 hours at 37°C. Subsequently, the samples were centrifuged at 9000 × g for 20 min at 4°C, after which the resulting supernatant and the pellet were retained as the cell wall and cellular fraction, respectively. The fraction corresponding to the cellular content was resuspended in 1 ml of PBS and then ultracentrifuged at 100,000 × g for 1 hour at 4°C. The supernatant that contained the cytosolic proteins was retained, while the resulting pellet, which contained the membrane proteins, was resuspended in a small volume of PBS. Finally, all the samples were precipitated with trichloroacetic acid (TCA) to a final concentration of 10%. The mixture was then incubated on ice for 1 hour. Next, the precipitated proteins were centrifuged at 13000 × g for 20 minutes at 4°C, and the resulting pellets were washed twice with 1 ml of acetone. Finally, the samples were dried at 37°C for 5 minutes, resuspended in 1x Laemmli buffer (Bio-Rad), and loaded onto a 12% SDS–PAGE gel for the immunodetection assay.

Polyacrylamide gels and western blotting

The samples were diluted in 1x Laemmli buffer (Bio-Rad) and then heated at 100 °C for 5 minutes. The proteins were separated by 12% SDS–PAGE with the Spectra™ molecular weight marker (Thermo Fisher Scientific) and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V for 30 minutes. Next, the membrane was blocked for 1 hour

using 5% nonfat milk diluted in 50 mM Tris-HCl (150 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBS-T). The membrane was then incubated with the primary antibody in a solution of 3% nonfat milk in TBS-T. The membrane was washed three times with TBS-T, with 10 minutes of incubation between each wash. Next, the membrane was incubated with the secondary antibody against rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) for 2 hours at a concentration of 1:3000 and diluted in TBS-T. Following the incubation, the membrane was washed twice with TBS-T and once with TBS. Finally, for immunodetection, the membranes were exposed to Pierce ECL Western blotting Substrate (Thermo Fisher Scientific).

For the immunodetection of BC1280-His or N-domain-His, we used an anti-6-His (Merck) primary antibody produced in rabbits at a dilution of 1:2500. TasA and CalY were detected using specific monoclonal antibodies, namely, anti-TasA (targeting the peptide KGISAGKSDKFK) and anti-CalY (targeting the peptide QSEPVYETTLAD), both at dilutions of 1:5000 (NovoPro; Vivitek, Hoofdorp, The Netherlands).

Immunolocalization studies by fluorescence microscopy

For the immunofluorescence experiments, the samples were grown as previously described for transmission electron microscopy studies. Then, 150 µl of each sample was added to well slides coated with 0.1% poly-L-lysine (Sigma–Aldrich) and incubated for 2 hours to facilitate bacterial adhesion to the substrate. The sample was then removed, and the bacteria that had attached to the slide surface were fixed with fixation buffer (3% paraformaldehyde and 0.1% glutaraldehyde diluted in PBS) for 10 minutes. Briefly, the wells were washed twice with PBS, and the samples were incubated for 1 hour with

blocking buffer (3% m/v bovine serum albumin (BSA) and 0.2% v/v Triton X-100 in PBS). Then, the buffer was removed, and the wells were incubated for 3 hours with the primary antibody at a concentration of 1:100 diluted in blocking buffer. The primary antibodies used for visualization by TEM were also used for this experiment. The wells were then rinsed three times with washing buffer (0.2% m/v BSA and 0.05% v/v Triton X-100 in PBS), and each incubation lasted for 5 minutes. Next, the wells were incubated for 2 hours with the secondary antibody goat anti-rabbit IgG-Atto488 (manually labeled) at a dilution of 1:400 in blocking buffer. The samples were washed once with washing buffer and twice with PBS, for an incubation period of 5 minutes each. The immunostainings were fixed for 5 minutes using fixation buffer, and the wells were subsequently rinsed with PBS three times. To detect the cell wall, the slide was incubated with wheat germ agglutinin labeled with Alexa Fluor 647 (WGA, Thermo Fisher Scientific) for 20 minutes at a dilution of 1:100 in PBS. Finally, the bacterial DNA was stained for 20 minutes using Hoechst at a dilution of 1:1000. As a negative control, immunostaining was performed without incubation with the primary antibody.

The immunostaining was visualized using confocal laser scanning microscopy. For the fluorescence corresponding to Atto-488 visualization, we employed an excitation wavelength of 488 nm and detected the emission between 497 and 572 nm. The signal corresponding to Alexa Fluor 647 was visualized with an excitation wavelength of 561 nm, and emission was detected between 576 and 686 nm. The Hoescht signal was visualized independently using a specific dichroic filter.

For colocalization studies of BC1280-CalY/TasA, the same immunostaining protocol was followed with a few modifications. First, BC1280-6xHis was labeled using

the anti-6-His antibody produced in rabbits (Merck), followed by goat anti-rabbit antibody conjugated to Atto-488 as the primary and secondary antibody, respectively. After three washes were performed with washing buffer, the samples were treated with quenching solution (0.1 M glycine in PBS) for 10 minutes. Next, TasA or CalY were detected using the following specific primary antibodies: anti-TasA and anti-CalY, which were produced in rabbit, followed by anti-rabbit conjugated to Alexa-Fluor 647 produced in goat as the secondary antibody. Following the final steps, the samples were fixed, stained with Hoechst, and finally visualized via CLSM, as described previously.

The colocalization images were analyzed using the software ImageJ and the colocalization threshold plugin. The region of interest (ROI) was defined for the region corresponding to Alexa Fluor 647, and both Pearson´s and Mander´s coefficients were calculated.[85] For each sample, a minimum of 5 different images were analyzed, and statistical significance was determined for Pearson´s coefficient using an unpaired t test with Welch´s correlation, with a p value < 0.005.

Protein expression and purification

BC1280 and N-domain$_{39\text{-}190}$ were purified under native conditions. For the heterologous expression of BC1280, the plasmid pET24b-*BC1280* was transformed into *E. coli* BL21 (DE3) (Merck, Kenilworth, NJ, USA), and colonies were selected with 50 µg/ml kanamycin. The plasmid pET24-*N-domain$_{39\text{-}190}$* was subsequently transformed into *E. coli* Lemo21 (DE3)pLyss (New England Biolabs, USA) for N-domain$_{39\text{-}190}$ expression, after which colonies were selected with 50 µg/ml kanamycin and 5 µg/ml chloramphenicol. The following steps were performed for both proteins. A single fresh colony was picked and

cultured in 10 ml of LB supplemented with the corresponding antibiotics mentioned previously. The preculture was incubated under shaking conditions for 6 hours at 37°C. A total of 10% (v/v) of the precultures were inoculated into 1 L of LB supplemented with the corresponding antibiotics. The culture was incubated at 37°C with agitation until the optical density reached 0.6. For BC1280, the culture was induced with 400 µM IPTG and incubated for 16 hours at 28°C. Otherwise, the culture corresponding to the overexpression of N-domain$_{39\text{-}190}$ was induced with 400 µM IPTG and 100 µM rhamnose and incubated for 3 hours at 28°C. Then, the cells were harvested (6000 × g, 30 minutes at 4°C, JLA 8.1 rotor, Beckman Coulter, Brea, CA, USA), and the pellets were frozen at -80°C until use.

The pellets were resuspended in 18 ml of buffer A (20 mM Na_2PO_4, 500 mM NaCl and 20 mM imidazole, pH 8) with cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) and CelLytic^{TM} B-Cell Lysis reagent (Merck). The lysates were incubated for 30 min with agitation at room temperature. The samples were sonicated using a Branson 450 digital sonifier on ice with 3 pulses for 45 seconds at 40% amplitude and then centrifuged at 15000 × g for 60 minutes at 4°C (F34-6-38 rotor; Eppendorf, Hamburg, Germany). The supernatant was loaded onto a HisTrap HP 5 ml column (GE Healthcare) and purified using an AKTA Start FPLC system (GE Healthcare). The column was preequilibrated with buffer A, and after sample loading, it was washed with the same buffer. Next, the proteins were eluted using a linear gradient of buffer B (20 mM Na_2PO_4, 500 mM NaCl and 500 mM imidazole, pH 8). Since the elution mixture was not completely pure, the sample was loaded onto a HiLoad 16/600 Superdex 200 pg column (Cytiva), and the pure protein was subsequently eluted in buffer containing 20 mM Tris and 50 mM

NaCl, pH 7.5. The elution fractions were loaded onto a 12% Tris-Tricine SDS–PAGE gel, and the samples containing the purified protein were concentrated using Amicon Ultra15 centrifugal filter units (Millipore) with a 3 kDa cutoff. Subsequently, the purified fractions were separated via 12% SDS–PAGE to assess their purity, which was determined through Coomassie blue staining. Finally, the gel bands were analyzed using tandem mass spectrometry.

To purify the C-domain$_{191\text{-}279}$, the plasmid pET-C-domain$_{191\text{-}279}$ was transformed into *E. coli* BL21(DE3) pLyss, and colonies were selected using 50 μg/ml kanamycin. Then, a single colony was picked and cultured in 10 ml of LB medium supplemented with antibiotics and incubated with shaking for 6 hours at 37°C. A 10% (v/v) preculture was inoculated into 1 L of LB medium supplemented with 50 μg/ml kanamycin, and the culture was agitated at 37°C until an optical density of 0.6 was reached. The culture was induced with 400 μM IPTG and 500 μM rhamnose, followed by incubation for 16 hours at 28°C. Subsequently, the cells were harvested (6000 × g, 30 minutes at 4°C, JLA 8.1 rotor, Beckman Coulter, Brea, CA, USA), and the resulting pellet was frozen at -80°C until use. The pellet was resuspended in buffer A (50 mM Tris, 150 mM NaCl, pH 8) and subjected to sonication on ice for 3 minutes with pulses of 30 seconds at an amplitude of 40% (Bandelin sonopuls vs. 70T probe; Bandelin, Germany). Next, the lysate was centrifuged at 15000 × g for 60 minutes at 4°C (F34-6-38 rotor; Eppendorf, Hamburg, Germany). The resulting pellet was then resuspended in denaturing buffer (50 mM Tris, 500 mM NaCl, and 6 M guanidine hydrochloride) and incubated at 60°C overnight with agitation. The lysate was sonicated under the same conditions, and the sample was ultracentrifuged at 42000 rpm for 1 hour at 23°C (50.2 Ti rotor; Beckman Coulter). The supernatant was

loaded onto a HisTrap HP 5-ml column, and the protein was purified by affinity chromatography. Equilibration and washing steps were performed using buffer C1 (50 mM Tris, 0.5 M NaCl, 20 mM imidazole, 8 M urea, pH 8), while elution was achieved using buffer C2 (50 mM Tris, 0.5 M NaCl, 500 mM imidazole, 8 M urea, pH 8). Finally, the protein was dialyzed against a solution containing 20 mM Tris and 50 mM NaCl, pH 7.5. To assess purity, the purified protein was separated via 15% SDS–PAGE and subjected to Coomassie blue staining. The gel band was analyzed by tandem mass spectrometry.

CalY was purified via heterologous expression in *E. coli* BL21(DE3) pLyss and from inclusion bodies under denaturing conditions following the protocol established by El Mammeri et al.,[29] with modifications specified in the Materials and Methods section of Chapter IV.

Tandem mass spectrometry analysis of protein bands

The SDS–PAGE gel bands that corresponded to the protein purification and proteinase K digestion experiments were subjected to analysis via tandem mass spectrometry using a nanoion trap system [HPLC–electrospray ionization–tandem mass spectrometry (MS)]. The bands were cut and destained using a mixture of 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate. Then, the samples were dehydrated and dried using ACN. Disulfide bridges were reduced with 10 mM dithiothreitol (DTT) diluted in 50 mM ammonium bicarbonate, followed by incubation at 56°C for 30 minutes. Subsequently, the excess DTT was removed, and the cysteine residues were carbamidomethylated using 55 mM iodoacetamide diluted in 50 mM ammonium bicarbonate for 20 minutes at room temperature in the dark. The gel bands were then dehydrated again, and the

proteins were digested using 10 ng/µl trypsin (Promega) at 30°C overnight. To extract the peptides from the gel, the samples were incubated with a solution of 0.1% ACN/formic acid (FA) for 30 minutes at room temperature. To eliminate ACN and ammonium bicarbonate, the samples were dried using a SpeedVacTM. Next, the samples were resuspended in a solution containing 0.1% FA, ultrasonicated for 3 minutes, and centrifuged at 13,000 × g for 5 minutes. Subsequently, the samples were purified and concentrated using C18 ZipTip® (Merck) following the instructions supplied by the manufacturer. Finally, the samples were injected into an Easy nLC 1200 UHPLC system, which was coupled to a Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The software versions used for acquisition and analysis were Tune 2.9 and Xcalibur 4.1.31.9. The mobile phases used in the HPLC consisted of i) buffer A, which contained 0.1% FA dissolved in water, and ii) buffer B, which contained 0.1% FA dissolved in 80% ACN. Peptides were loaded onto a precolumn (Acclaim PepMap 100, 75 µm × 2 cm; C18, 3 µm; 100 A; Thermo Fisher Scientific) at a flow rate of 20 µl/min, while elution was performed using a 50 cm analytical column (PepMap RSLC C18, 2 µm; 100 A, 75 µm × 50 cm; Thermo Fisher Scientific). Elution was carried out through a gradient concentration over 60 minutes, transitioning from 5% to 20% buffer B. This was followed by a 5-minute gradient from 20% to 32% buffer B, resulting in a 10-minute elution with 95% buffer B. Then, the column was equilibrated with 5% buffer B using a constant flow rate of 300 µl/min. Before sample analysis, an external calibration was performed using LTQ Velos ESI Positive Ion Calibration Solution (Pierce, IL, USA), along with internal calibration using the polysiloxane ion signal at m/z 445.120024 obtained from ambient air. The MS1 scans were conducted within a m/z range of 375-

1.600 at a resolution of 120.000. Employing a data-dependent acquisition strategy, the 15 most intense precursor ions with a charge ranging from +2 to +5 within a window of 1.2 m/z were selected from fragmentation to generate the corresponding MS2 spectra. The fragmentation ions were generated through high-energy collision-induced dissociation with an initial mass set at 110 m/z and subsequently detected using a mass-analyzed Orbitrap at a resolution of 30.000. The dynamic exclusion for the selected ions was set to 30 seconds, and the maximum accumulation times for MS1 and MS2 were 50 ms and 70 ms, respectively. Finally, for protein sequence identification, the raw data were analyzed using Proteome Discoverer 2.4 (Thermo Fisher Scientific) with the Sequest HT search tool with mass tolerance parameters of 10 ppm and 0.02 Da for precursor and fragment ions, respectively.

Dynamic light scattering experiments

The samples corresponding to the elution peaks obtained by size exclusion chromatography for BC1280 and N-domain$_{39\text{-}190}$ were filtered through a 0.46-µm syringe filter. The size measurements were performed using a Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, United Kingdom) with a laser wavelength of 632.8 nm as the excitation source and a 1 cm pathway polystyrene cuvette as the sample holder. A total volume of 1 ml of the protein solution was transferred to the cuvette, and the dynamic light scattering signal was measured with a count rate ranging from 100 to 230 kcps, depending on the sample. Data collection and subsequent analysis were conducted using Zetasizer software v.6.34 (Malvern Panalytical).

Polyacrylamide gels and Western blotting

The samples were diluted in 1x Laemmli buffer (Bio-Rad) and then heated at 100 °C for 5 minutes. The proteins were separated via 12% SDS–PAGE with a Spectra™ molecular weight ladder (Thermo Fisher Scientific) as a reference marker. Subsequently, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V for 30 minutes. The membrane was subsequently blocked for 1 hour using a solution of 5% nonfat milk diluted in 50 mM Tris-HCl and 150 mM NaCl (pH 7.5) supplemented with 0.1% Tween-20 (TBS-T). The membrane was incubated with the primary antibody in 3% nonfat milk with TBS-T. Next, the membrane was washed three times with TBS-T, with 10-minute incubations between each wash. Then, the secondary antibody, anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) produced in goat, was incubated with the membrane for 2 hours at a concentration of 1:3000 and diluted in TBS-T. After the incubation, the membrane was washed twice with TBS-T and once with TBS. Finally, for immunodetection, the membranes were exposed to Pierce ECL Western blotting Substrate (Thermo Fisher Scientific).

For immunodetection, CalY was detected using a specific monoclonal anti-CalY antibody produced in rabbits (NovoPro; Vivitek, Hoofddorp, The Netherlands) at a dilution of 1:5000 against the peptide QSEPVYETTLAD.

To study the molecular weight of the oligomers formed by BC1280 and N-domain$_{39-190}$ under native conditions or from inclusion bodies, the proteins were diluted in native sample buffer (Bio-Rad) and loaded onto polyacrylamide gels (Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad). The native gels were run in buffer

containing 25 mM Tris and 192 mM glycine at a constant voltage of 200 V. To visualize the proteins, the gels were stained with Coomassie brilliant blue.

Transmission electron microscopy

To study the morphology of BC1280, N-domain$_{39\text{-}191}$ and C-domain$_{191\text{-}279}$ purified *in vitro*, the samples were subjected to 2-fold serial dilutions and incubated on copper grids for 2 minutes. Then, the samples were stained with a 2% uranyl acetate solution for 1 minute and dried under dark conditions.

The samples were examined using a Thermo Fisher Scientific Tecnai G^2 20 TWIN transmission electron microscope at an accelerating voltage of 120 kV. The final images were captured using an Olympus Veleta side-mounted CCD with a resolution of 2k × 2k Mpx.

Thioflavin T (ThT) assays

The polymerization kinetics were studied using thioflavin T staining. The experiment was conducted in a 96-well plate, and 40 µM ThT was added to each well at a specific concentration, after which the mixture was diluted in buffer containing 20 mM Tris and 50 mM NaCl at pH 7.4. The polymerization kinetics of CalY were assayed at 40 µM using low concentrations of BC1280 and N-domain$_{39\text{-}190}$ (2 and 10 µM).

The plates were incubated at 37°C in a plate reader and shaken at 100 rpm before each measurement (Tecan Infinite M1000 Pro; Tecan, Männedorf, Switzerland). The fluorescence intensity was monitored every 30 minutes through the bottom of the plate

using an excitation wavelength of 440 nm and an emission wavelength of 480 nm. All measurements were recorded in triplicate.

Sequence analysis by different bioinformatics tools

The primary sequence of BC1280 was obtained from UniProt (https://www.uniprot.org/) with the accession number Q81GC7. SignalP5.0 was used to predict the cleavage site for the signal peptidase.[86] The hydrophobicity was analyzed using the ProtScale tool provided by the ExPASy server, and the prediction of disordered regions was conducted using FoldUnfold with default parameters.[45,47] Putative amyloidogenic regions were predicted employing the following bioinformatics tools: AmylPred2, FoldAmyloid, MetAmyl, PASTA2.0 and TANGO.[40-44]

AlphaFold prediction

The secondary structure model of BC1280 was predicted by AlphaFold using the sequence available in UniProt with the accession number Q81GC7 and default parameters.[48] The pdb model obtained was subsequently used for predicting chemical shifts via SPARTA.[87] Finally, the predicted correlations overlapped with the experimental data obtained by SSNMR.

***In vitro* assembly of oligomers**

BC1280 and N-domain$_{39\text{-}190}$ were diluted to a final concentration of 50 µM in buffer containing 20 mM Tris and 50 mM NaCl at pH 7.5. The oligomers self-assembled under agitation for 2 weeks at 37°C and a speed of 200 rpm. For the oligomerization of the C-

domain$_{191-279}$, the sample was agitated at 200 rpm in the same buffer at room temperature for 16 hours. The aggregates were then ultracentrifuged (40k rpm speed, 5 hours, 18°C, TLA 120.1 rotor; Beckman Coulter), and the resulting pellets were analyzed using X-ray diffraction, ATR-FTIR, and SSNMR.

X-ray diffraction measurements

Fiber diffraction patterns were obtained at 4°C using a Rigaku FR-X rotating anode X-ray generator (Rigaku, Tokyo, Japan) equipped with an EIGER 1 M hybrid pixel detector (Dectris, Baden, Switzerland) at the copper wavelength. The concentrated hydrated samples were mounted in MicroLoops from Mitegen (Ithaca, NY, USA) on a goniometer head under cold nitrogen flow. Each diffraction pattern represents a 360° rotation along the φ axis, with an exposure time of 720 s. No correction, such as smooth filtering or baseline correction, was applied to the data. WinPLOTR (https://cdifx.univ-rennes1.fr/winplotr/winplotr.htm) was used for converting q values to reticular distance (d) values before plotting.

Proteinase K (PK) digestion

A 22.5 µg solution of aggregated N-domain$_{39-190}$ was treated for 45 minutes at 37°C with 3 µg/ml proteinase K in 20 mM Tris and 50 mM NaCl at pH 7.5. The proteinase activity was stopped using 5 mM PMSF. The samples were mixed with an equal volume of Laemmli buffer (Bio-Rad) and heated at 100°C for 5 minutes. Subsequently, the samples were separated using 12% SDS–PAGE, and the bands were visualized by Coomassie

blue staining. The bands of interest were excised and subjected to analysis by tandem mass spectrometry.

Attenuated total reflection FTIR spectroscopy

The samples were treated under the same conditions as those used for the X-ray studies. Infrared spectra for BC1280 and N-domain$_{39\text{-}190}$ were recorded with an FT-IR spectrophotometer (model Vertex70; Bruker) in attenuated total reflectance (ATR) mode utilizing a Golden Gate Single Reflection Diamond ATR System accessory (Specac). No sample preparation was needed, and spectra were recorded with an air background and 64 scans for both the background and sample measurements. The spectral resolution was set at 4 cm-1". The raw data within the spectral range of the amide I region were used for estimating the total amount of secondary structure. To increase the resolution of the minimal absorbance peaks, a second derivative was calculated. Next, a deconvolution approach was employed using PeakFit software to determine the contribution percentage for each type of secondary structure: parallel β-sheet (1634 cm^{-1}), antiparallel β-sheet (1620 cm^{-1} and 1690 cm^{-1}), α-helix (1658 cm^{-1}), turns (1675 cm^{-1}) and random coil (1646 cm^{-1}), in accordance with the well-established assignments.[58]

Proteomic analysis by the isobaric tag for relative and absolute quantification (iTRAQ) method

To estimate the relative levels of BC1280 and CalY during biofilm formation in the wild-type strain *B. cereus* ATCC14579, we conducted sample preprocessing and subsequent analysis following the protocols described by Caro-Astorga et al.,[26] in 2020.

Far-Western blotting

The protein–protein interaction between BC1280 and CalY was detected using far Western blotting.[88] Samples corresponding to BC1280 and N-domain$_{39\text{-}190}$ were considered the prey, while CalY served as the bait protein. BSA was used as a negative control to replace the prey protein. Twenty micrograms of each protein was loaded onto a 12% SDS–PAGE gel and transferred to a PVDF membrane using a Trans-Blot Turbo transfer system (Bio-Rad). To denature and renature the proteins, the membrane was incubated in AC buffer (100 mM NaCl, 20 mM Tris (pH 7.5), 0.5 mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skin milk power and 1 mM DTT) with decreasing concentrations of guanidine HCl (6, 3, 1, 0.1, and 0 M). Subsequently, the membrane was blocked with 5% milk in PBST for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with the purified bait protein in AC buffer without guanidine HCl. The membrane was subsequently washed three times for 10 minutes each with PBST to remove the unbound protein. The blot was incubated overnight at 4°C with the specific anti-CalY primary antibody produced in rabbits (NovoPro; Vivitek, Hoofdorp, The Netherlands) at a dilution of 1:3000 in 3% milk in PBST buffer. The membrane was washed three times for 10 minutes each with PBST buffer and then incubated with the secondary antibody, anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), for 2 hours at room temperature. Next, the blot was washed three times with PBST for 10 minutes and then once with PBS for 5 minutes. Chemiluminescent detection was performed by incubating the membrane with Pierce ECL Western blotting Substrate (Thermo Fisher Scientific).

NMR spectroscopy

Solid-state NMR experiments were performed on a 600 MHz spectrometer (Bruker Biospin, Germany) equipped with a triple resonance 4mm probe. Experiments were performed at 11 kHz MAS frequency at a sample temperature of 283K. Chemical shifts were referenced with DSS. For 2D ^{13}C-^{13}C PDSD spectra (mixing time of 50 and 200 ms), an initial cross-polarization step of 0.5 ms was used and high-power SPINAL-64 decoupling was applied during acquisition times. 2D ^{13}C-^{13}C PDSD (50 ms) and ^{1}H-^{13}C INEPT spectra were recorded for 1-3 days, and 6 days for the ^{13}C-^{13}C PDSD spectrum (200 ms).

Solution NMR ^{1}H,^{15}N SOFAST-HMQC experiment was carried out at 298 K on a protein sample containing 200 µM BC1280, 20mM Tris, 50mM NaCl, pH7.5, on a 700 MHz spectrometer (Bruker Biospin, Germany) equipped with a 5mm TXI ^{1}H/^{13}C/^{15}N/^{2}H probe. The NMR data were processed using the TOPSPIN 4.0.6 software and analyzed by CCPNMR.[89]

Circular dichroism

CalY (40 µM) alone or in combination with BC1280 (at 2 or 10 µM) was incubated in 10 mM sodium phosphate buffer at pH 8 for 16 hours at 37ºC under agitation. Circular dichroism spectra were recorded using a JASCO J-815 spectrometer, using a 0.1 cm path-length cuvette in the range of 180-260 nm, with a 0.5 nm step and a 1 s collection time per step. The scan rate was 50 nm/min. Final spectra were obtained as the average of six scans after a blank correction. The spectrometer was continuously purged with dry N2 gas.CD spectra were buffer subtracted, and the results were expressed as mean residue ellipticity.

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Figure 1

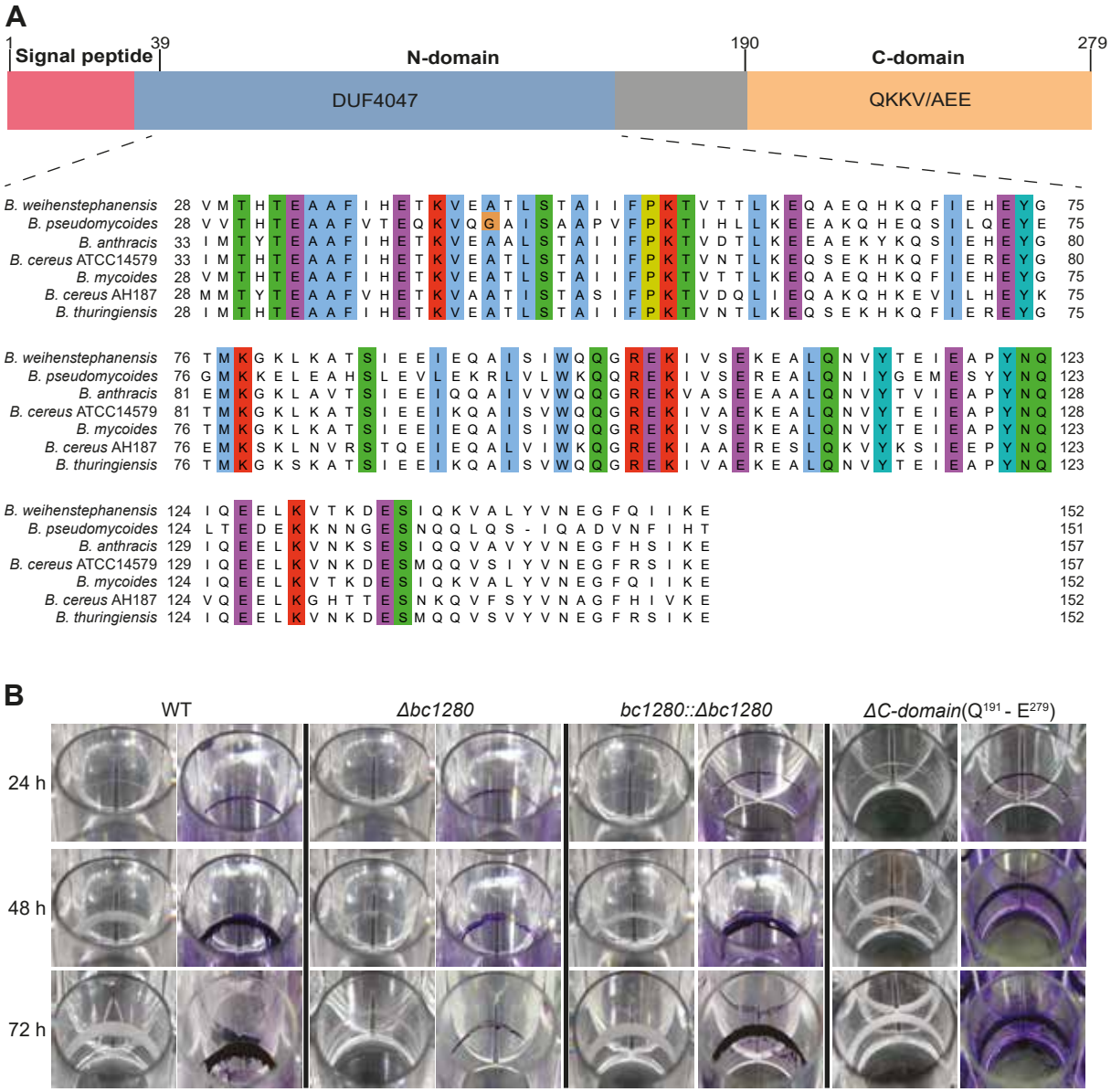

A
1
39
190
279
Signal peptide
N-domain
C-domain
DUF4047
QKKV/AEE
B. weihenstephanensis
B. pseudomycoides
B. anthracis
B. cereus ATCC14579
B. mycoides
B. cereus AH187
B. thuringiensis
B
WT
Δbc1280
bc1280::Δbc1280
ΔC-domain(Q191 - E279)
24 h
48 h
72 h
C
Abs 575 nm
WT
Δbc1280
bc1280::Δbc1280
ΔC-domain
ns
**
****
Time (hours)
D
OD 600nm
WT
Δbc1280
ΔC-domain
Time (hours)

Figure 2

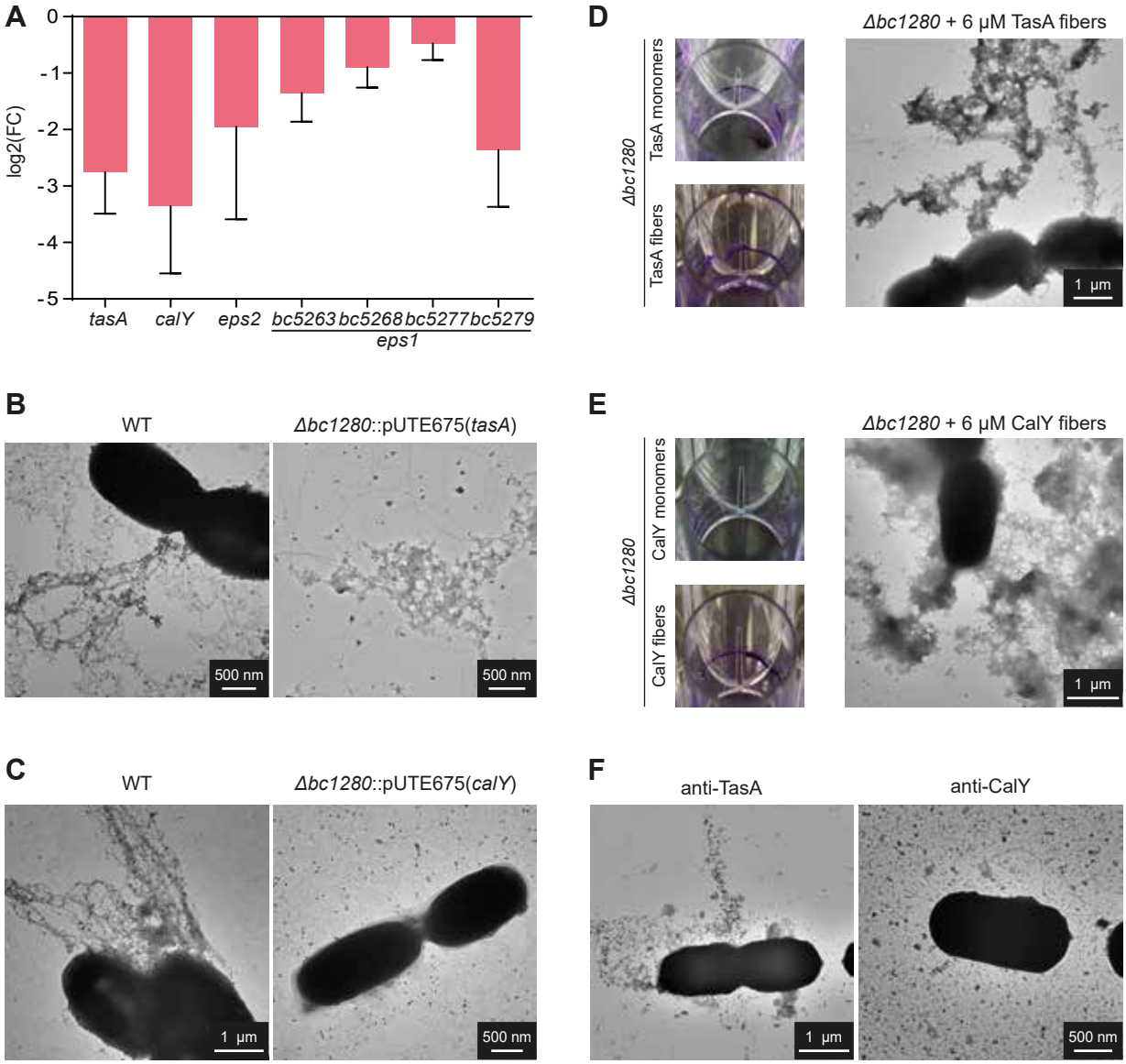

A
0
-1
-2
-3
-4
-5
log2(FC)
tasA
calY
eps2
bc5263
bc5268
bc5277
bc5279
eps1
B
WT
Δbc1280::pUTE675(tasA)
500 nm
500 nm
C
WT
Δbc1280::pUTE675(calY)
1 µm
500 nm
D
Δbc1280 + 6 µM TasA fibers
Δbc1280
TasA monomers
TasA fibers
1 µm
E
Δbc1280 + 6 µM CalY fibers
Δbc1280
CalY monomers
CalY fibers
1 µm
F
anti-TasA
anti-CalY
1 µm
500 nm

Figure 3

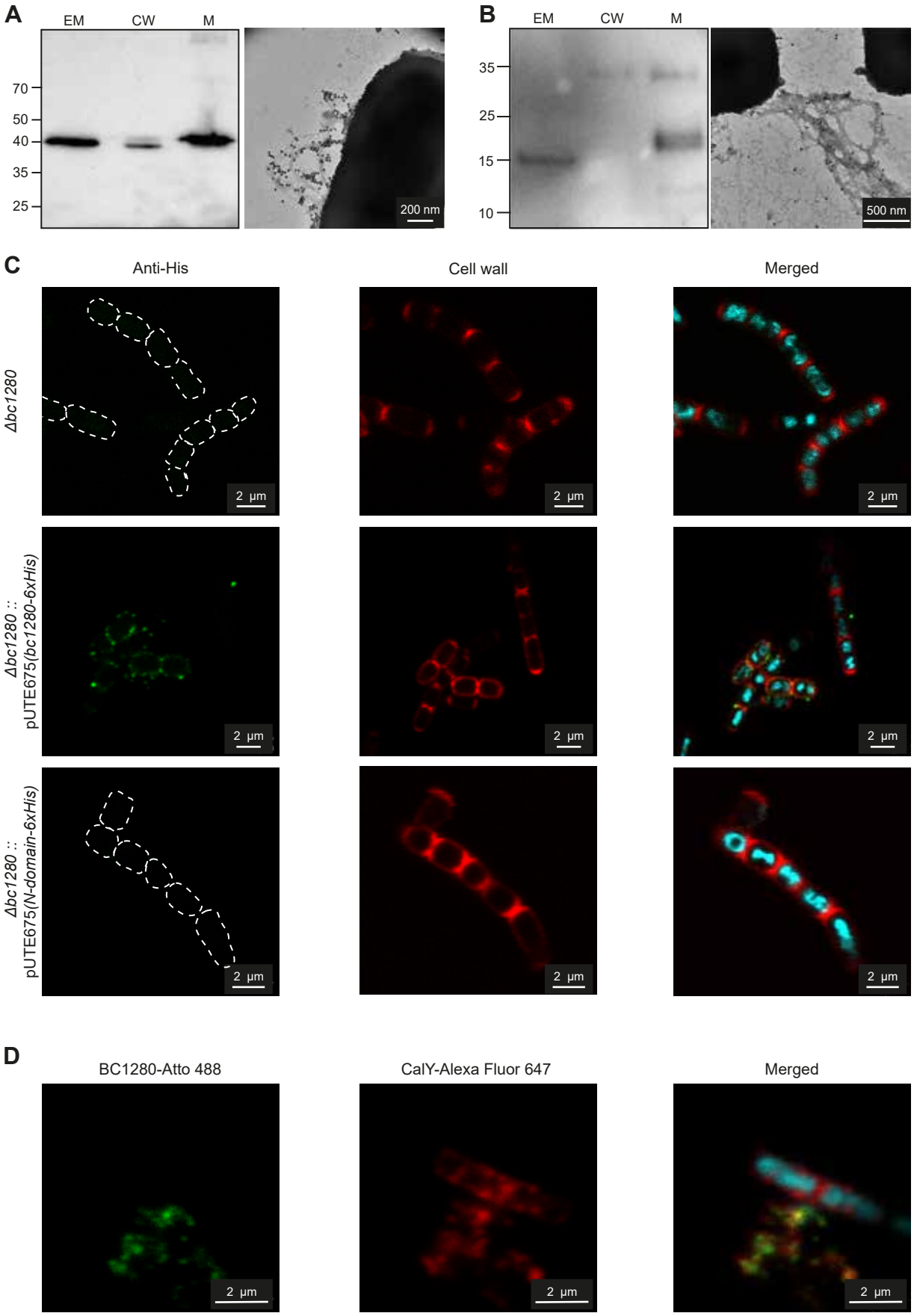

A
EM
CW
M
70
50
40
35
25
200 nm
B
EM
CW
M
35
25
15
10
500 nm
C
Anti-His
Cell wall
Merged
Δbc1280
Δbc1280 :: pUTE675(bc1280-6xHis)
Δbc1280 :: pUTE675(N-domain-6xHis)
2 µm
D
BC1280-Atto 488
CalY-Alexa Fluor 647
Merged
2 µm

Figure 4

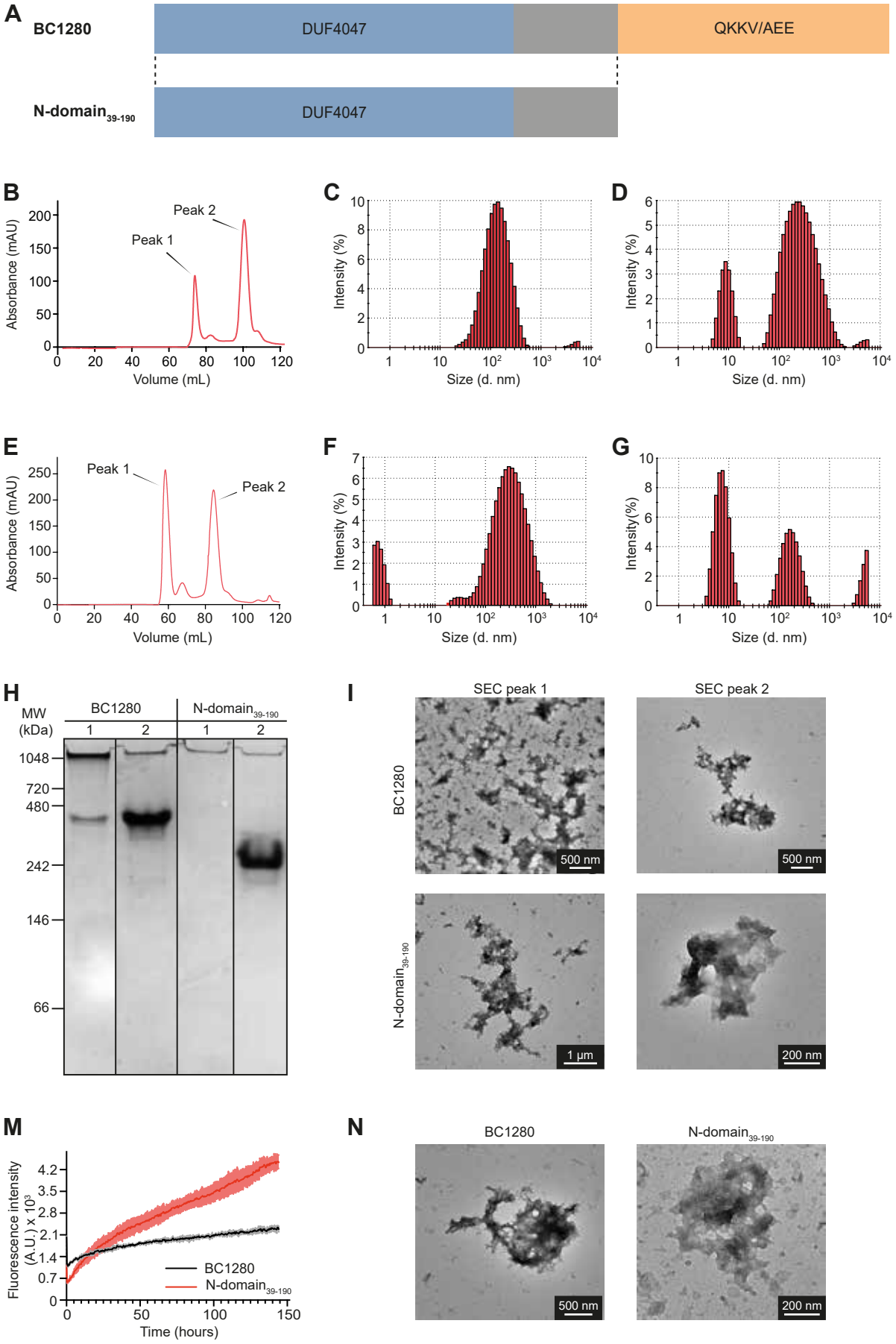
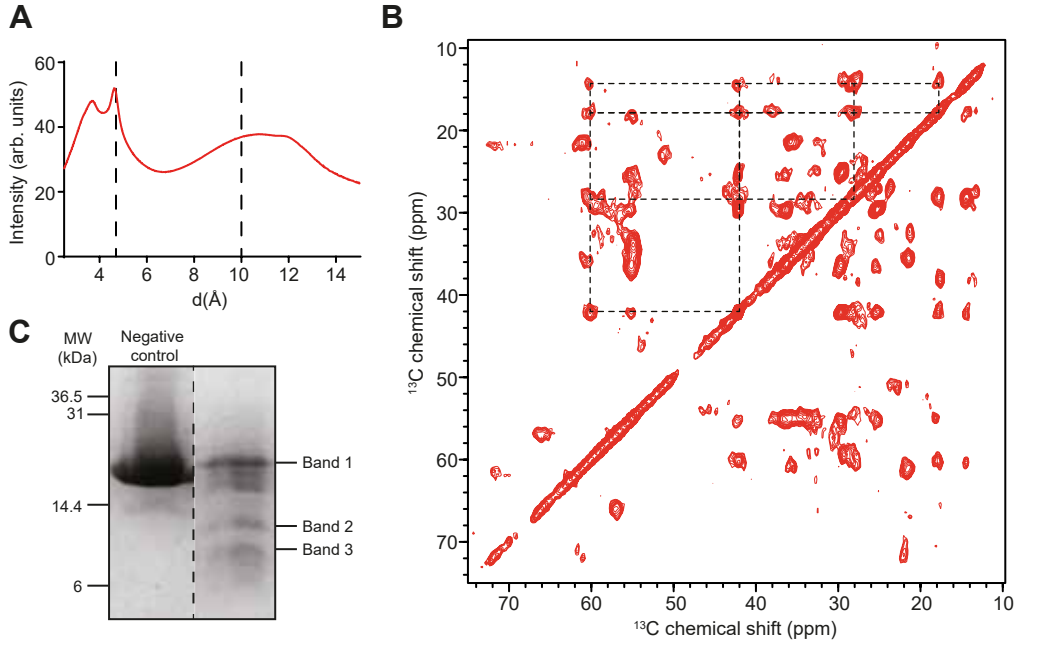

A
BC1280
DUF4047
QKKV/AEE
N-domain39-190
DUF4047
B
Absorbance (mAU)
Peak 1
Peak 2
Volume (mL)
C
Intensity (%)
Size (d. nm)
D
Intensity (%)
Size (d. nm)
E
Absorbance (mAU)
Peak 1
Peak 2
Volume (mL)
F
Intensity (%)
Size (d. nm)
G
Intensity(%)
Size (d. nm)
H
MW (kDa)
BC1280
N-domain39-190
1048
720
480
242
146
66
I
SEC peak 1
SEC peak 2
BC1280
N-domain39-190
500 nm
500 nm
1 µm
200 nm
M
Fluorescence intensity (A.U.) x 10³
BC1280
N-domain39-190
Time (hours)
N
BC1280
N-domain39-190
500 nm
200 nm

Figure 5



D

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Control  39 MAFIHETKVEATLSTAIIFPKTVNTLKEQSEKHKQFIEREYGTMKGKLKATSIEEIKQAISVWQQGREKIVAEKEALQNVYTEIEAPY 126
Band 1      MAFIHETKVEATLSTAIIFPKTVNTLKEQSEKHKQFIEREYGTMKGKLKATSIEEIKQAISVWQQGREKIVAEKEALQNVYTEIEAPY
Band 2      MAFIHETKVEATLSTAIIFPKTVNTLKEQSEKHKQFIEREYGTMKGKLKATSIEEIKQAISVWQQGREKIVAEKEALQNVYTEIEAPY
Band 3      MAFIHETKVEATLSTAIIFPKTVNTLKEQSEKHKQFIEREYGTMKGKLKATSIEEIKQAISVWQQGREKIVAEKEALQNVYTEIEAPY

Control 127 NQIQEELKVNKDESMQQVSIYVNEGFRSIKEKRDYIEKEISLKAIDEQIQALQQQLNVAIEAEG 190
Band 1      NQIQEELKVNKDESMQQVSIYVNEGFRSIKEKRDYIEKEISLKAIDEQIQALQQQLNVAIEAEG
Band 2      NQIQEELKVNKDESMQQVSIYVNEGFRSIKEKRDYIEKEISLKAIDEQIQALQQQLNVAIEAEG
Band 3      NQIQEELKVNKDESMQQVSIYVNEGFRSIKEKRDYIEKEISLKAIDEQIQALQQQLNVAIEAEG
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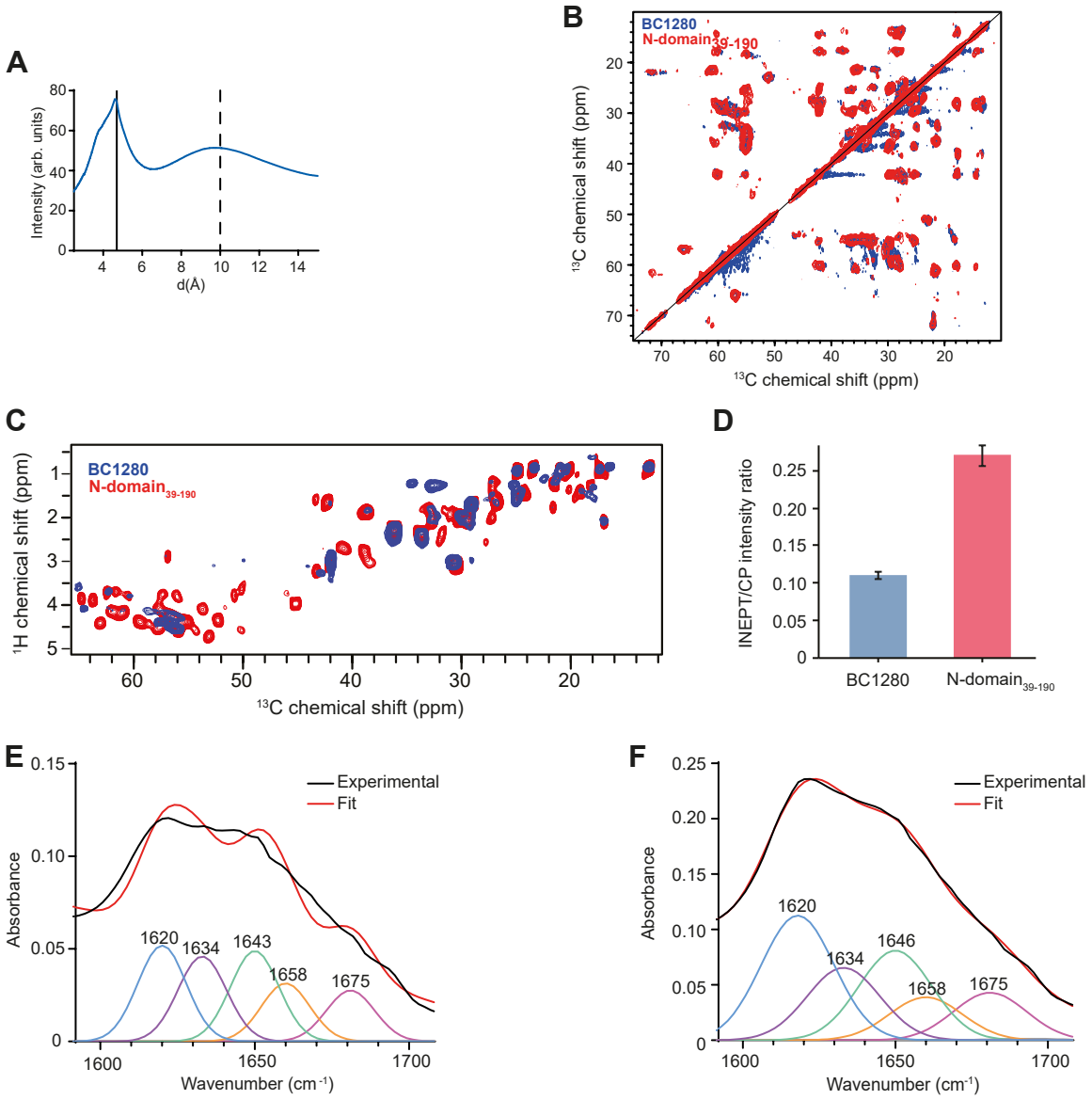
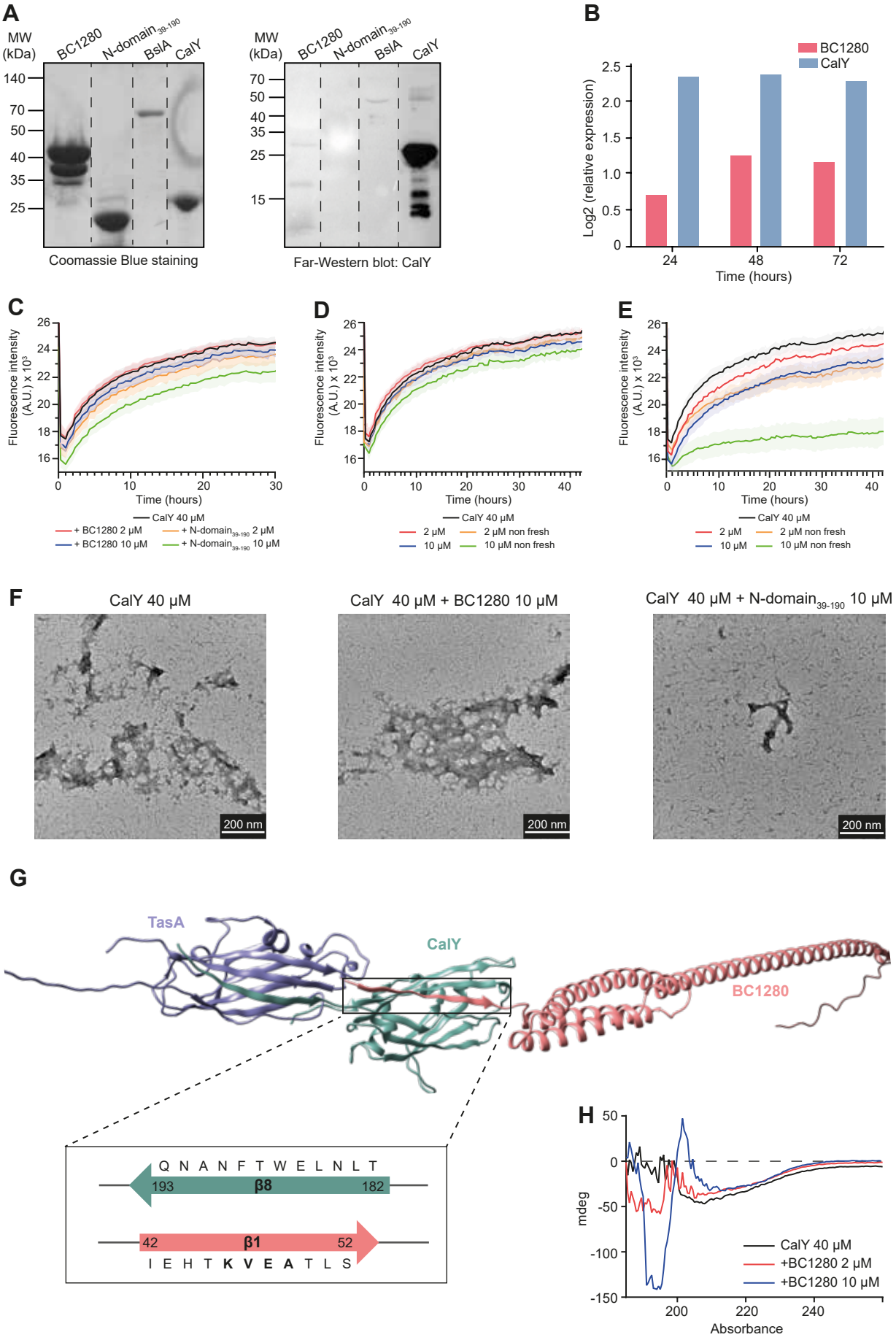
Figure 6



G

		Percentage of structural elements (%)	
Secondary structure element	**Wavenumbers (cm^{-1})**	**BC1280**	**N-domain$_{39-190}$**
β-sheet antiparallel	1620,1690	25%	33%
β-sheet parallel	1634	22%	19%
Random coil	1646	24%	24%
α-helix	1658	15%	12%
Turn	1675	14%	12%

Figure 7


A
MW (kDa)
BC1280
N-domain39-190
BslA
CalY
140
70
50
40
35
25
Coomassie Blue staining
MW (kDa)
BC1280
N-domain39-190
BslA
CalY
70
50
40
35
25
15
Far-Western blot: CalY
B
BC1280
CalY
Log2 (relative expression)
2.5
2.0
1.5
1.0
0.5
0
24
48
72
Time (hours)
C
Fluorescence intensity (A.U.) x 10³
Time (hours)
CalY 40 µM
+ BC1280 2 µM
+ BC1280 10 µM
+ N-domain39-190 2 µM
+ N-domain39-190 10 µM
D
Fluorescence intensity (A.U.) x 10³
Time (hours)
CalY 40 µM
2 µM
10 µM
2 µM non fresh
10 µM non fresh
E
Fluorescence intensity (A.U.) x 10³
Time (hours)
CalY 40 µM
2 µM
10 µM
2 µM non fresh
10 µM non fresh
F
CalY 40 µM
CalY 40 µM + BC1280 10 µM
CalY 40 µM + N-domain39-190 10 µM
200 nm
200 nm
200 nm
G
TasA
CalY
BC1280
Q N A N F T W E L N L T
193
β8
182
42
β1
52
I E H T K V E A T L S
H
mdeg
50
0
-50
-100
-150
200
220
240
Absorbance
CalY 40 µM
+BC1280 2 µM
+BC1280 10 µM