Molecular strategy for blocking isopeptide bond formation in nascent pilin proteins

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Edited by Yale E. Goldman, Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, PA, and approved August 3, 2018 (received for review May 4, 2018)

Bacteria anchor to their host cells through their adhesive pili, which must resist the large mechanical stresses induced by the host as it attempts to dislodge the pathogens. The pili of gram-positive bacteria are constructed as a single polypeptide made of hundreds of pilin repeats, which contain intramolecular isopeptide bonds strategically located in the structure to prevent their unfolding under force, protecting the pilus from degradation by extant proteases and oxygen radicals. Here, we demonstrate the design of a short peptide that blocks the formation of the isopeptide bond present in the pilin Spy0128 from the human pathogen Streptococcus pyogenes, resulting in mechanically labile pilin domains. We use a combination of protein engineering and atomic-force microscopy force spectroscopy to demonstrate that the peptide blocks the formation of the native isopeptide bond and compromises the mechanics of the domain. While an intact Spy0128 is inextensible at any force, peptide-modified Spy0128 pilins readily unfold at very low forces, marking the abrogation of the intramolecular isopeptide bond as well as the absence of a stable pilin fold. We propose that isopeptide-blocking peptides could be further developed as a type of highly specific antiadhesive antibiotics to treat gram-positive pathogens.

protein folding | isopeptide bond | protein mechanics | antibiotic peptide | single-molecule force spectroscopy

Bacterial infections are initiated by the attachment of bacteria to their host cells, which is mediated by specialized filamentous structures known as pili (1–3). As these adhesins are recognized virulence factors, pili have emerged as a target for the development of new vaccines and antiadhesives (4–9). In gram-positive bacteria, pili are assembled as a single polypeptide composed of up to hundreds of pilin protein repeats placed in tandem (10–12). Pili are known to be important factors in the virulence of gram-positive pathogens. Indeed, failure in the proper polymerization and assembly of pili leads to diminished virulence (13–15). This central role of the pili in bacterial virulence is largely related to their unique ability to resist the mechanical challenges caused by the drag forces of mucus flow, coughing, or sneezing (16–19) (Fig. 1A). These environmental perturbations can develop nanonewton-scale forces on a single pilus (16, 20), sufficient to unfold any protein domain stabilized through noncovalent bonds, such as networks of hydrogen bonds (21, 22). However, a key structural element of pilin proteins is the presence of intramolecular isopeptide bonds, which mechanically lock the protein structure and render it inextensible at any force (23–26). The pilus of the human pathogen Streptococcus pyogenes (27) is assembled as a long shaft of tandem repeats of the Spy0128 pilin protein, capped by a single adhesin Spy0125 protein. Such pilus can reach several micrometers in length (10, 28, 29).

The Spy0128 pilin is composed of two Ig-like domains arranged in tandem, each containing an isopeptide bond that deflects a stretching force away from the folded domain blocking their extension (Fig. 1A, Inset). For example, the C terminus Ig-like domain contains an isopeptide bond between the side chains of K179 (β-strand1) and N303 (β-strand11) (23). This intramolecular covalent bond accounts for the mechanical rigidity of the protein, as the carboxyl terminus of the C-terminal domain cross-links to the adjacent pilin subunit at K161, which leaves the majority of the N-terminal domain outside of the force pathway (23, 24) (Fig. 1A and SI Appendix, Fig. S1). Hence, the isopeptide bond is responsible for the resistance of bacterial adhesion to mechanical shocks, since mechanically labile pilin proteins that extend under force are vulnerable to degradative processes (30–33). In this work, we demonstrate the design of short peptides that, by blocking the formation of the critical intramolecular isopeptide bonds, readily disrupt the mechanical properties of the Spy0128 pilin. We targeted the C-terminal domain of the Spy0128 pilin protein with a peptide—isopeptide blocker—designed to mimic the β-strand11 of the pilin domain. Our strategy was simple: by presenting an exogenously added peptide that mimics β-strand11, we forced emerging Spy0128 pilin proteins to form intramolecular isopeptide bonds between K179 (β-strand1) and N14 in the isopeptide blocker (Fig. 1B-D). Timing of the intervention is critical. The intramolecular isopeptide bond between the Spy0128 pilin and the peptide must take place before the native β-strand11 of the emerging pilin can complete the fold and trigger the formation of the native isopeptide bond, after which intervention is futile. We demonstrate the crucial nature of this timing by expressing the isopeptide blocker and its target Spy0128 pilins in sequence. Blockage occurs only if the isopeptide blocker is expressed before Spy0128, resembling an antibiotic peptide that would interfere with the shaft protein before its

Significance

At the onset of an infection, gram-positive bacteria adhere to host cells through their pilum, filamentous structures built by hundreds of repeats of pilin proteins. These proteins can withstand large mechanical challenges without unfolding, remaining anchored to the host and resisting cleavage by proteases and oxygen radicals present in the targeted tissues. The key structural component that gives pilins mechanical resilience are internal isopeptide bonds, strategically placed so that pilins become inextensible structures. We target this bond by designing a blocking peptide that interferes with its formation during folding. We demonstrate that peptide-modified pilins lack mechanical stability and extend at low forces. We propose this strategy as a rational design of mechanical anti-biotics, targeting the Achilles heel of bacterial adhesion.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1807689115/-/DCSupplemental.

Published online August 27, 2018.
Using fluorescent HaloTag ligands, we can readily identify modified Spy0128 pilin proteins in SDS/PAGE gels. Moreover, we can combine the anchoring capabilities of the HaloTag together with paramagnetic beads to specifically enrich the modified fraction of Spy0128 pilin proteins recovered after their coexpression with the isopeptide-blocker peptide.

Fig. 2 shows the constructs used in our protein expression experiments: the 39-kDa Halo-tagged isopeptide blocker (Fig. 2A) and the 96-kDa polyprotein I91-Spy0128-I91-Spy0128-I91 (Fig. 2B). Since the isopeptide-blocker peptide must compete with the native β-strand11 before the folding and formation of the native isopeptide bond, a high concentration of the isopeptide blocker is required before the Spy0128 polyprotein folds. Thus, we coexpress selectively each construct using two different inducible expression vectors within the same bacteria (Materials and Methods). The purified proteins are then labeled with the fluorescent Alexa488 Halo ligand. Fig. 2C shows an SDS/PAGE gel of the purified proteins demonstrating the labeling of the Spy0128 polyprotein by Alexa488, and its corresponding Coomassie blue on the right. Inducing the Spy0128 polyprotein alone or the polyprotein before the peptide does not result in detectable Alexa488 labeling of the Spy0128 polyprotein (Fig. 2C, I and IA bands). However, when the peptide is expressed before folding and the assembly of the pilus in the periplasm. The isopeptide blocker is Halo-tagged to label and purify modified pilin molecules, the mechanics of which are thereafter investigated through atomic-force microscopy (AFM) force spectroscopy. We find that isopeptide-blocked Spy0128 pilin is extensible under force and shows reduced mechanical stability, becoming a potential target of degradation by oxidative modifications or proteases.

We propose the targeting of the isopeptide bond of gram-positive pilin molecules as a rational design for peptide antibiotics.

**Results**

**Blocking Intramolecular Isopeptide Bond Formation.** We designed a 19-residue-long peptide that imitates the β-strand11 of the pilin C-terminal domain (Fig. 1B). This strand contains the asparagine residue (N303) that forms the intramolecular isopeptide bond with K179 in the N-terminal β-strand (SI Appendix, Fig. S1); hence, our peptide is designed to compete with the formation of the native isopeptide bond (Fig. 1 B–D). The isopeptide-blocker peptide is expressed as a HaloTag fusion protein, which allows for stable expression of the peptide and for labeling and purifying modified Spy0128 proteins (Fig. 1D) using HaloTag ligands. By
the Spy0128, a fraction of the Spy0128 polyproteins are modified by the isopeptide blocker, resulting in two high-molecular-weight bands labeled with Alexa488 (Fig. 2C, 150 kDa > AI bands < 250 kDa). The Coomassie blue for total staining indicates that all three expression strategies yield comparably high levels of expression of the intact Spy0128 construct with a molecular weight of 96 kDa (SI Appendix, Fig. S2). The HaloTag isopeptide blocker should appear as a band at 39 kDa. However, given that we did not include a His-Tag in the isopeptide-blocker construct, the free form of this peptide was not purified together with the Spy0128 polyprotein. This was an important modification given that due to its high levels of expression, the free form of the isopeptide-blocker tends to overwhelm the Alexa488 fluorescent gels shown in Fig. 2C. This is the reason why the 39 kDa band of the free isopeptide-blocker construct appears only faintly in these gels. The molecular weight of the fluorescent bands that appear in the gel of Fig. 2 C, i–iii and SI Appendix, Fig. S2 can be approximated by using the bands of the molecular weight standards. A doubly decorated Spy0128 polyprotein should migrate at 174 kDa, and a singly decorated polyprotein at 135 kDa. Using the standards (Materials and Methods), we estimate that the high-molecular-weight bands labeled with Alexa488 correspond to 184 (Fig. 2C, i) and 157 kDa (Fig. 2C, ii, iii). The branched structure of the protein constructs that result from the HaloTagged peptidation strategy is consistent with intact free migration to the same molecular weights that do not exactly match the predicted values, which may explain these discrepancies. We also find two extra bands below and above the 75-kDa marker that do not correspond to any expected molecular weight. We speculate that they arise from the proteolytic degradation of modified Spy0128 polyproteins, which our protease inhibitors were not able to prevent during the purification. Similar proteolytic susceptibility has been demonstrated in the E258A Spy0128 protein, a mutant protein in which the isopeptide of the C-terminal does not form (24, 30). In our case, this situation is expected since we did not include a His-Tag in the isopeptide blocker and modified Spy0128 polyproteins are likely to be far more susceptible to proteolysis due to the presence of the isopeptide blocker.

To demonstrate the generality of our strategy to block native isopeptide bonds, we designed two additional peptides to interfere with the structural pilin protein of a different organism, Actinomyces oris. In this bacterium, the shaft protein FimA shows analogous structural features with Spy0128 (34). Although FimA is a pilin protein structured as three β-sandwich domains, only the last two operate under force (34). We designed isopeptide blockers to target both the CnaA (domain N2) and the CnaB domain (domain N3) (SI Appendix, Fig. S3). To target the domain N2, we mimicked the β-strand1 of the CnaA domain (Isopeptide-N), whereas, for the domain N3, we mimicked the β-strandA of the CnaB domain (Isopeptide-K). We followed the same protein expression protocol used for the Spy0128 polyprotein, expressing the isopeptide blocker before the induction of the pilin protein. SI Appendix, Fig. S3, shows that both the isopeptide-N and the isopeptide-K blockers successfully decorate the FimA protein, as suggested by the bands located at 70 kDa, since the molecular weight of the undecorated FimA is 32 kDa. These experiments confirm that our isopeptide-blocker strategy can be translated to shaft pilins from other gram-positive bacteria.

### Isopeptide Blocker Prevents the Folding of Spy0128

We carried out AFM force spectroscopy experiments to probe the mechanics of the isopeptide-blocker–modified Spy0128 protein and to simulate the putative conditions that pilus proteins experience upon bacteria attachment. Specifically, we compared the unfolding mechanisms of the modified pilus proteins with the intact Spy0128 and the mutant E258A, which lacks the isopeptide bond, as previously demonstrated (24). As shown in Fig. 2B, our polyprotein constructs have two Spy0128 proteins flanked by titin I91 domains, used as fingerprints to discard spurious traces (24, 35). When pulling intact Spy0128 constructs, only the three I91 domains unfold, which are identified as unfolding events with a contour length increment of 29 nm, as determined by fits to the worm-like chain model (36), and unfolding forces of ~200 pN (Fig. 3A). In contrast, the mutant E258A is extensible under force, and unfolds after the I91 domains with a contour length increment of 50 nm, and unfolding forces of ~300 pN.

However, the isopeptide-blocker–modified Spy0128 polyproteins show very different unfolding patterns, which reveal a radical change in the mechanical properties of Spy0128. While in the former two constructs the initial extension (LΔ) never exceeds 50 nm—defined as the extension before the unfolding of the first I91 domain (35)—modified Spy0128 proteins show initial extensions between 100 and 150 nm, which typically include several low-stability events that are hard to interpret systematically (Fig. 3C). This indicates that the isopeptide blocker not only converts Spy0128 into an extensible protein, but also lessens its mechanical stability, compared with the mutant E258A (Fig. 3B and C).

We can take advantage of the AFM sawtooth patterns and use L0 as a quantitative proxy to estimate the total fraction of protein decorated with the Halo-tagged isopeptide blocker. Fig. 3D shows a histogram of initial extensions calculated from sawtooth patterns containing at least two I91 unfolding events (fingerprint). We distinguish three populations, centered at 41, 80, and 130 nm, respectively, which can be associated with intact Spy0128, singly and doubly decorated, given the 50-nm contour length increment that each Spy0128 renders. This allows us to estimate that 32.5% of the traces have at least one modified Spy0128, which translates into 21.1% of blocked Spy0128.

The sawtooth patterns in Fig. 3C indicate indisputably that the mechanical stability of Spy0128 is altered, which suggests that the introduction of an extra β-strand compromises the acquisition of the folded structure of the Spy0128 C-terminal domain. Since the experiments in Fig. 3 were carried out with the purified protein, and thus the peptide contains the HaloTag, we must discard the notion that the decreased mechanical stability is not due to steric interactions of the entire domain N2. The end-capped Spy0128 protein is not due to the presence of the HaloTag, but rather to the presence of the peptide. The histogram of initial extensions (Fig. 4D) shows an increase in the population of modified Spy0128 to 60%, characterized by initial extensions of 86 and 135 nm, compatible with those described in Fig. 3. However, we still find a fraction of intact molecules, likely coming from contamination during the magnetic separation.

To characterize the mechanics of the modified Spy0128, we measured comparatively the unfolding forces (F0) and contour length increments (ΔL0) of the mutant E258A and the unfolding events observed in the traces of the TEV-digested Spy0128 polyprotein. A contour plot of F0 versus ΔL0 for the isopeptide-blocked Spy0128 polyprotein shows scattered unfolding events with forces below 100 pN and contour length increments ranging from 7 to 50 nm. This suggests a lack of well-defined unfolding pathways, as each modified Spy0128 shows a different unfolding pattern (Fig. 4E). Interestingly, 14% (40 traces) of the modified Spy0128 showed large initial extensions, without any unfolding peak (ΔL0 = 0 nm and F0 = 0 pN), suggesting that the peptide can turn the Spy0128 into an unstructured polymer. In contrast, the mutant Spy0128 (Fig. 4F) shows structured unfolding pathways: most times it unfolds through a unique step with ΔL0 = 50 ± 1 nm and F0 = 293 ± 64 pN, while in some occasions it unfolds through an intermediate ΔL0 = 18 ± 1 nm and F0 = 182 ± 41 pN followed by a second of ΔL0 = 32 ± 1 nm and
FU2 = 111 ± 15 pN (24) (Fig. 4F). From a structural point of view, we can hypothesize that the introduction of the isopeptide blocker not only abducts the native isopeptide bond, but also competes with the natural β-strand11 for the noncovalent interactions that conform the mechanical clamp, responsible for the mechanical stability of the mutant E258A. The displacement of this strand would likely give rise to steric interaction that would destabilize the fold. Remarkably, the absence of recurrent unfolding patterns in Fig. 4E suggests that the modified Spy0128 is unable to acquire a definitive fold, but rather populates an ensemble of mechanically weak structures that result in the multipeaked unfolding patterns.

**Discussion**

Gram-positive bacteria have evolved isopeptide bonds in their adhesive pili as a molecular strategy to withstand the large mechanical perturbations that they experience when colonizing a host (23). These intramolecular covalent bonds clamp the shaft domains strategically to render a molecule that does not exceed under nanonewton-level forces. In this environment, an extensible
protein would be an immediate target for molecular degradation, such as protease digestion or oxidative cleavage, which would compromise bacterial adhesion (31, 32). Thus, isopeptide bonds are the Achilles heel of pilus mechanical integrity.

We have reported a molecular strategy to disrupt the formation of the intramolecular isopeptide bond in Spy0128, the shaft protein from *S. pyogenes*, which results in an extensible protein that unfolds under forces of a few piconewtons. Our strategy is inspired by the split-protein technique developed by Howarth and coworkers (37–39), which takes advantage of the isopeptide bond in pilin proteins to assemble molecular constructs in vitro. However, we target the native isopeptide bond with an engineered peptide, which must compete for its formation with the native β-strand before the folding of the pilin domain.

We suggest the design of isopeptide-blocker peptides as a strategy for the rational design of antibiotic peptides that target the mechanics of pilin proteins and disrupt the early stages of bacterial infection. As we have demonstrated, the timing of our strategy is a key aspect of its success, which actually mimics the physiological process. Due to the presence of isopeptide and disulfide bonds, gram-positive pilin proteins cannot fold in the cytoplasm, and they must translocate in the unfolded state to the periplasm (40, 41). Once in the periplasm, pilin proteins fold, form the internal covalent bonds, and polymerize with the assistance of sortases to form micron-long pilis that anchor to the cell wall (11). Thus, as shown in the sequential expression of the peptide polyprotein, nascent translocating Spy0128 would interact with the blocker peptides, forming an irreversible isopeptide bond before the protein folds, thereby displacing the native β-strand. Regarding potential clinical use, mechanical antibiotics would offer an enormous specificity, given that they imitate the sequence of a β-strand of the pilin protein and thus are exclusive for each particular pathogen. In this sense, our strategy allows combining different designs, given the specific structure of each pilin domain. While our isopeptide blocker was the only feasible design for Spy0128—given that the N-terminal domain does not experience force and that β-strand 1 emerges the last—FimA offered at least two targeting candidates, the combined use of which would increase the fraction of extensible pilins and increase the potential effectiveness of the antibiotic peptide. However, it must be noted that our inhibition strategy takes advantage of the tandem architecture of the pilis—assisted from hundreds of shaft subunits—so that disrupting only a few pilin proteins would render a vulnerable anchor, the degradation of which would compromise the adhesion of the entire bacterium.

Our findings provided a molecular proof of how these designed peptides target the mechanics of pilin proteins, but a potential application to clinical use would require further elaboration. Indeed, the rational design and delivery of antibiotic peptides is known to face a number of challenges, which can include proteolytic degradation, side effects, or low bioavailability (42). In this sense, the use of antimicrobial peptides is an emerging and active field, and the potential limitations are being investigated through different approaches, such as the use of nanocarriers for delivery, which has been proven to increase effectiveness and reduce side effects (43, 44). Our work demonstrates that isopeptide bonds in pilin proteins are an attractive and largely unexplored target for therapeutic treatment, and future work should be aimed for in vivo testing of our strategy. Single-cell force spectroscopy provides a natural next step to demonstrate if interfering with the mechanics of single-pilin proteins scales up to compromise the mechanics of single bacteria (45, 46). This would allow the proposal of more elaborated strategies toward a clinical implementation of isopeptide blockers as antimicrobial peptides.

Materials and Methods

**Protein Engineering and Isopeptide-Blocker Design.** We cloned and expressed the structural pilin protein from *S. pyogenes* (Spy0128) (23) and *A. oris* (FimA) (34). Whereas FimA was expressed as a monomer, which includes both CnaA and CnaB domains, for the Spy0128 protein, we used a previously engineered Spy0128 polyprotein (24). This polyprotein contains two Spy0128 proteins separated and flanked by three I91 titin domains, I91-Spy0128-I91-Spy0128-I91. Both constructs, FimA and Spy0128 polyprotein, were cloned in the pQE80L expression vector (Qiagen), which confers ampicillin resistance, and includes a promoter inducible by isopropyl β-D-thiogalactopyranoside (IPTG) and an N-terminal His-Tag. The isopeptide blocker was engineered by concatenating a short peptide sequence from the native β-strand region involved in the isopeptide bond formation from the Cna domain, followed by a TEV site, and the HaloTag sequence. For the Spy0128, we used the non-native sequence EFTDKMTITFT, which mimics the native TEVD (linker1) and isopeptide bond (linker2) of the periplasmic region and connects them with an N-terminal His-Tag. This peptide was followed by –EDIRS- (linker1) –EDLYFQDS- (TEV site) –DNTTPE- (linker2), and the HaloTag. In the case of the FimA, considering that both domains experience force during the bacterial attachment (34), we targeted both the CnaA (N2 domain) and CnaB domain (N3 domain). For the N2 domain, we used –EFARGATIONAQVSD–, which mimics the β-strand1, the penultimate β-strand of the CnaA domain, whereas for the N3 domain we used –EFWDGLLLKVVKVDNHOQG–, which mimics the β-strand2A, the first β-strand of the CnaB domain. Both peptides were followed by –HGVRS– (linker1), a TEV site, –DNTTPE– (linker2), and the HaloTag. The three isopeptide-blocker constructs were cloned in a custom-modified pBAD18 expression vector (ATCC), which confers kanamycin resistance, is inducible by arabinose, and lacks His-Tag.

**Protein Expression, Purification, and Labeling.** The coexpression of the pilin protein and the Halo-tagged isopeptide blocker was conducted in two stages. pQE80L and pBAD cotransformed in *Esherichia coli* BLRDE3 pLysS cells were grown at 37 °C in minimal M9CA broth (VWR) supplemented with 40 μg mL⁻¹ thymine, 2 mM magnesium sulfate, 0.1 mM calcium chloride, and 1.5% glycerol as carbon source until the culture reached an OD₆₀₀ of 0.5-0.6. First, the expression of the isopeptide blocker was induced with 0.2% of arabinose for 3 h at 37 °C. Second, the pilin proteins were expressed in LB broth for 3 additional hours at 37 °C with 1 mM IPTG. In the control, the protocol was inverted.

![Fig. 4. Mechanical properties of HaloTag-purified Spy0128 constructs. Using Promega paramagnetic beads, we enriched the fraction of proteins that have been modified by the blocking peptide. The proteins are subsequently detached from the beads using the TEV protease. From these purified proteins we obtained three types of sawtooth patterns: (A) Unmodified Spy0128 constructs with short initial extensions (L < 50 nm) and (B) showing a single modification (L = 50–100 nm) or (C) two modifications (L > 100 nm). (D) Histogram of initial extensions (n = 453). A Gaussian fit identifies three peaks at 34, 86, and 135 nm. (E) Two-dimensional histogram showing the density of unfolding forces (F₀) versus the contour length increments (ΔL₀) for the isopeptide-blocked Spy0128. Modified pilin proteins unfold at forces below 100 pN and do not show any well-defined unfolding pathway. Remarkably, 14% of the traces (n = 40) show long initial extensions without any unfolding event, suggesting that the modified Spy0128 extends as an unstructured polymer. (F) Mutant Spy0128 E258A unfolding through well-defined unfolding pathways with high mechanical stability, either through a single event with F₀ ~ 300 pN or through an intermediate with F₀ ~ 180 pN and F₀ ~ 110 pN. Data from ref. 24.](https://www.pnas.org/content/10.1073/pnas.1807689115)
The pilin proteins were purified using the protocol described previously (23, 25). Briefly, after the E. coli cells were lysed by French press in phosphate buffer (50 mM NaH₂PO₄, pH 7.0, 300 mM NaCl), the proteins were purified by Ni-affinity chromatography (Qiagen), followed by size exclusion chromatography (Superdex 200; GE Healthcare) in Heps buffer (10 mM Heps buffer, pH 7.2, 150 mM NaCl, 1 mM EDTA). The purified protein fractions were labeled with 1 mM of the fluorescent dye Alexa488-Halo-ligand (Promega) and analyzed by SDS/PAGE. The protein gels were imaged in a documentation gel station (G-box; Syngene) using epi illumination and a charge coupled device camera. The molecular weight of the intact pilin proteins, isopeptide blockers, and the decorated FimA and Spy0128 polyprotein was calculated with Gel-Pro Analyzer 3.1 (Media Cybernetics) using the molecular weight standard as reference (precision plus protein dual color; Bio-Rad).

Selected Spy0128 polyprotein fractions were concentrated to a volume of ∼50 μL and incubated with 50 μL of pre-equilibrated Magne-HaloTag-beads (Promega) for >4 h at 4 °C in a tube rotator. The intact polyproteins were washed three times in Heps buffer by centrifugation for 3 min at 1,000 × g at 4 °C. The immobilized proteins were eluted by incubation of the beads with 100 units of ProTEV Plus (Promega) at 4 °C in Heps buffer overnight in a tube rotator. Finally, we applied a magnet to separate the proteins from the Magne beads. TEV-treated fractions were stored at 4 °C for further use.

AFM Experiments. Polyproteins purified with or without the extra Magne-HaloTag-bead TEV step were incubated for 30 min in freshly nickel-chromium-gold-evaporated glass coverslides. The experiments were conducted at room temperature using a custom-built or a commercial AFM (Luigs and Neumann). Each of the silicon nitride cantilevers (MCLT) used in the pulling experiments was calibrated following the equipartition theorem (47), giving a spring constant of 10–20 pN nm⁻¹. The polyproteins were nonspecifically picked by pushing the cantilever at a force of 1–2 nN and retracting at a pulling velocity of 400 nm s⁻¹. All of the experiments were done in Heps buffer at room temperature.

Single-Molecule Data Analysis. We used the following criteria established for chimeric protein constructions: only traces containing two or three I91 unfolding events were considered for the analysis (24, 35). As the I91 domains are flanking the Spy0128, the traces included within the results should contain the unfolding of the pilin domain. The initial extension (L) of the polyprotein, the contour length increments of I91, and the pilin intermediates were analyzed using the worm-like chain model for polymer elasticity (36). Histograms were fitted using single and multiple Gaussian distributions implemented in Igor 7 (WaveMetrics).

ACKNOWLEDGMENTS. We thank all the members of the J.M.F. laboratory for their helpful comments on the project and Paulina Ramirez for her invaluable help in the expression of the different protein constructs. This work was supported by the National Institutes of Health Grants GM116122 and HL12228 (to J.M.F.). R.T.R. thanks Fundación Ramón Areces (Spain) for its financial support.