Structural Basis for Toughness and Flexibility in the C-terminal Passenger Domain of an Acinetobacter Trimeric Autotransporter Adhesin

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Running title: Structure of C-terminal passenger of an Acinetobacter TAA

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ABSTRACT

Trimeric autotransporter adhesins (TAAs) on the cell surface of Gram-negative pathogens mediate bacterial adhesion to host cells and extracellular matrix proteins. However, AtaA, a TAA in the nonpathogenic Acinetobacter sp. strain Tol 5, shows nonspecific, high adhesiveness to abiotic material surfaces as well as to biotic surfaces. It consists of a passenger domain (PSD) secreted by the C-terminal transmembrane anchor (TM), and the PSD comprises an N-terminal head, N-terminal stalk, C-terminal head (Chead), and C-terminal stalk (Cstalk). The Chead-Cstalk-TM fragment, which is conserved in many Acinetobacter TAAs, has by itself the head-stalk-anchor architecture of a complete TAA. Here, we show the crystal structure of the Chead-Cstalk fragment, AtaA_CPSD, providing the first view of several conserved TAA domains. The YadA-like head (Ylhead) of the fragment is capped by a unique structure (headCap), composed of three β-hairpins and a connector motif; it also contains a head insert motif (HIM1) before its last inner β-strand. The headCap, Ylhead, and HIM1 integrally form a stable Chead structure. Some of the major domains of the CPSD fragment are inherently flexible and provide bending sites for the fiber between segments whose toughness is ensured by topological chain exchange and hydrophobic core formation inside the trimer. Thus, although adherence assays using in-frame deletion mutants revealed that the characteristic adhesive sites of AtaA reside in its N-terminal part, the
flexibility and toughness of the CPSD part provide the resilience that enables the adhesive properties of the full-length fiber across a wide range of conditions.

In autotransporters, extracellular proteins in diverse Gram-negative bacteria, the transmembrane domain (TM) hosts the autotransport function, also called type V secretion, a process in which the passenger domain (PSD) is exported to the bacterial cell surface through a pore formed by the TM (1), with the assistance of periplasmic chaperones and the β-barrel assembly machinery (2). Trimeric autotransporter adhesins (TAA) belong to a subfamily of the autotransporters, form homotrimeric structures with a common N-terminus-head-stalk-membrane anchor-C-terminus architecture (3-5). The head-stalk domain, a PSD of TAAs, is secreted by a C-terminal TM that is formed by a 12-stranded β-barrel at the outer membrane (OM) (6). Although the TM is usually localized at the C-terminus and is homologous in all TAAs and, therefore, defines this family, there are amino acid sequence alterations in the PSDs of many TAAs. The PSDs have a variety of lengths and mosaically arranged multiple domain structures that are distributed in many TAAs, and the daTAA program was developed for the annotation of TAA domains (7).

TAAs adhere to host cells and/or extracellular matrix (ECM) proteins, such as collagen, fibronectin, and laminin, to mediate bacterial infection (3). Well-known TAAs include YadA of Yersinia enterocolitica (8), BadA of Bartonella henselae (9), UspA1 and A2 of Moraxella catarrhalis (10), NhhA and NadA of Neisseria meningitidis (11-13), HadA, Hia, and Hsf of Haemophilus influenzae (14-16), and BpaA of Burkholderia pseudomallei (17). Recently, many TAAs have been further identified, such as, BtaE of Brucella spp. (18), Apa of Actinobacillus pleuropneumoniae (19), and EibD of E. coli (20). Ata of Acinetobacter baumannii is involved in nosocomial infection (21). SadA in Salmonella promotes biofilm formation and host cell adherence (22). BimA in Burkholderia species drives actin-based motility in host cells by mimicking host actin polymerases (23).

The genus Acinetobacter is ubiquitously distributed in nature, such as in humans, animals, activated sludge, soil, water, and other environmental sources. For example, A. baumannii, A. Iwoffii, A. parvus, A. bereziniae, A. guillouiae, A. haemolyticus, A. johnsonii, A. pittii, and A. nosocomialis were isolated from clinical specimens (24). A. baumannii has especially attracted our attention because it has caused nosocomial infection worldwide and its multidrug resistant strains have spread globally (25). The toluene-degrading bacterium Acinetobacter sp. Tol 5, an environmentally isolated nonpathogenic strain, exhibits an autoagglutinating nature and nonspecific, high adhesiveness to both biotic, such as collagen, and abiotic material surfaces, from hydrophobic plastics to hydrophilic glass and stainless steel (26). This unique adhesive property is mediated by AtaA, the TAA of Tol 5 (27). Each polypeptide chain of AtaA comprises 3630 amino acid residues and the homotrimer of the signal peptide-eliminated polypeptide forms a common configuration that can be broadly divided into five regions: an N-terminal Ylhead domain (Nhead; 108-315 aa), an N-terminal stalk (Nstalk; 316-2904 aa), a C-terminal Ylhead domain (Chead; 2905-3169 aa), a C-terminal stalk (Cstalk; 3170-3561 aa), and a TM (3562-3630 aa) (Fig. 1A). Therefore, mature AtaA seems to be a short TAA comprising a complete set of domains, Chead–Cstalk–TM, to be fused with another set of PSD, Nhead–Nstalk. In other words, AtaA seems to have two sets of
PSDs that are tandemly fused: an N-terminal PSD (AtaA_NPSD, AtaA59-2904) and a C-terminal PSD (AtaA_CPSD, AtaA2905-3561).

In the present study, we crystallized new domains that are well conserved in TAAs, solved their crystal structures, and used them to determine the structure of AtaA_CPSD by modeling. In silico analysis revealed that AtaA_CPSD shares the common domain architecture of many Acinetobacter TAAs and that Ata of A. baumannii also has a similar domain architecture to Cstalk of AtaA in its C-terminal region. Therefore, functional analysis of this conserved region of AtaA was conducted to examine its contribution to the unique adhesive properties of AtaA and to expand our knowledge of the structural and functional features of Acinetobacter TAAs. In particular, we focused on the flexibility and toughness of the nanofibrous structure. Flexibility that allows TAA nanofibers to bend is considered important for exhibiting their adhesive function (10,20,22,28,29). However, if the nanofibers are too limp to extend to a target surface in an aqueous environment, TAAs cannot effectively interact with the surface. Resilience is required for fiber extension toward the target. Physical strength is also important for the AtaA nanofiber to resist shear stress and exhibit high adhesiveness without breaking of the fiber. In addition, the trimeric structure of TAAs has been shown to be so stable as to be resistant to boiling in an SDS solution, which is essential for full-level adhesive activity of TAAs (30-33). Here we show the structure of AtaA_CPSD exhibiting toughness, which is mainly brought by interchain interactions and provides AtaA nanofibers with the resilience and physical strength that are important to exert high adhesiveness through the highly stable trimeric structure.

**EXPERIMENTAL PROCEDURES**

**Construction of plasmids**

Primers used in this study are listed in Supplemental Table 1. To construct AtaA recombinant protein fragments for crystallization, encoding regions of DNA were subcloned into the E. coli expression vector pIBA-GCN4triHis (34). The YadA_head domain (YadA26-210) was subcloned from pYV-WA314 of Yersinia enterocolitica (type O:8) WA-314 between the Xba I and Bsa I sites of pIBA-GCN4triHis. pET28d was constructed to remove the thrombin-cleavage site and T7-Tag by inverse PCR using pET28d_f and _r as primers and pET28b (+) (Merck Millipore, Billerica, MA) as a template. To generate an anti-CheadCstalk antibody, a DNA fragment for AtaA3017-3542 (CheadCstalkX) was subcloned between the Bam HI and Xho I sites of pET28d. pIFD-CPSD was constructed by inverse PCR using a primer set, IFD-CPSD_f/r, and pAtaA (27) as a template. From pTA2-AtaA_Cter containing a DNA fragment for AtaA2842-3630, ∆CheadCstalk, ∆Chead, ∆Cstalk1, and ∆Cstalk2 were generated by inverse PCR using primers (Supplemental Table 1) for deleting DNA regions for AtaA2906-3475, AtaA2907-3172, AtaA3170-3475, and AtaA3177-3475, respectively. The truncated ataa fragments were subcloned into pAtaA between ataa-internal Kpn I and ataa-external Xba I sites. The DNA sequences of all of the constructs were confirmed by the Sanger method. A point mutation of Pro3061 to Gly was introduced by the commercial kit (KOD-plus-Mutagenesis kit, Toyobo, Osaka, Japan) using P3061G_f and _r as primers.

**Production of recombinant proteins and antibodies**

Transformation of Tol 5 (∆ataA) was carried out as described in Ishikawa et al. (27). Production of His-tagged recombinant proteins in E. coli BL21
STAR (DE3) was induced by 100 µg/ml anhydrotetracycline or 0.3 mM isopropyl β-D-thiogalactopyranoside at 30 °C for 8 h. Cells were lysed in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl for AtaA recombinant proteins or 300 mM NaCl for YadA_head, 20 mM imidazole) at 1000 bar by a French press (SPX, Charlotte, NC) followed by sonication for 20 min (Pw, 7; duty, 30%; Branson Sonifier, Emerson Electric, Louis, MO). After centrifugation at 12,300 × g for 30 min, the supernatant was used as a soluble cell lysate sample. Proteins were purified by His-Nickel affinity chromatography (COSMOGEL His-Accept beads, Nacalai Tesque, Kyoto, Japan) and dialyzed in buffer solution (25 mM Tris-HCl, pH 9.0). The proteins were further purified by Q-anion-exchange chromatography (GE Healthcare, Little Chalfont, UK) with a NaCl gradient of 0–1 M, and size exclusion chromatography with HiLoad 26/600 Superdex 75 or 200 (GE Healthcare). Purification of the YadA_head was performed as described in Nummelin et al. (35). Proteins were concentrated by ultrafiltration using Vivaspin Turbo 15 (10 k MWCO for Chead1, Chead2, CstallK, CstallC1, and CstallC2, or 30 k MWCO for CheadCstall, CheadCstallX, and CstallFL; Sartorius, Goettingen, Germany). Circular dichroism (CD) spectroscopy (J-725, JASCO, Tokyo, Japan) was used to confirm the folding states of the purified proteins.

Rabbit polyclonal anti-Nhead, anti-CheadCstall, and anti-Chead antibodies were generated against synthesized polypeptide AtaA225-241 in Nhead, purified AtaA3017-3542 (CheadCstallX), and synthesized polypeptide AtaA3120-3136 in Chead, respectively. Anti-Nstallk antibody against AtaA999-1014 was generated previously (27). This antibody cross-reacts with AtaA103-1220 because of its repeat sequences. Rabbit anti-His-tag antibody, horseradish peroxidase (HRP)-conjugated antibody, Alexa Fluor 488-conjugated anti-rabbit IgG antibody, and colloidal gold (10 nm in diameter)-conjugated anti-rabbit IgG antibody were purchased from Medical & Biological Laboratories (Nagoya, Japan), GE Healthcare, Merck Millipore, and Abcam (Cambridge, UK), respectively.

Crystallization and X-ray crystallography

Proteins were first screened by a sitting drop vapor diffusion method at 20 °C in 768 conditions using Classics Suite, Classics II Suite, PEGs Suite, PEGs II Suite, PACT Suite, JCSG+ Suite, Protein Complex Suite, and Cryos Suite (Qiagen, Hilden, Germany). Protein and reservoir solutions (0.3 µl each) were mixed using a Honeybee 963 crystallization robot (Genomic Solutions, Ann Arbor, MI) and periodically observed for 2 months under a Rock Imager (Formulatrix, Bedford, MA). Crystallization conditions were optimized by a hanging drop vapor diffusion method. The final crystallization conditions are listed in Table 1.

Crystals were soaked in buffers containing cryo-protectants, which were prepared by mixing the reservoir solutions for crystallization and various concentrations of cryo-protectants (Table 1). Crystals were loop mounted and quenched in liquid nitrogen. Diffraction of the crystals was measured under cryo-conditions at 100 K at the synchrotron beamline X10SA (PXII) of the SLS (Paul Scherrer Institute, Villingen, Switzerland) by using a PILATUS 6M (DECTRIS, Baden, Switzerland), at the synchrotron beamline BL38B1 of the SPRing-8 (Japan Synchrotron Radiation Research Institute) by using a CCD Quantum315r (ADSC, Poway, CA), or at the X-ray beamline in Nagoya University by using an imaging plate detector R-AXIS VII (RIGAKU, Akishima, Japan). Diffraction images were processed and scaled by using the XDS program suite or HKL2000 (36,37). The CCP4 program suite was used for model
building and refinement (38). The phases of all structures were sequentially determined by molecular replacement using MOLREP (39). Chead1 and CstalkC1s were solved using UspA1 (PDB ID = 3NTN) and SadAK12 (PDB ID = 2YO2) as templates of molecular replacement, respectively. CstalkFL was solved using SadAK1 (PDB ID = 2YNY) for FGG_5, CstalkC1i for YDD and DALL3 domains, and BadA_head (PDB ID = 3D9X) for the GIN domain. CstalkN was solved using CstalkFL. After molecular replacement, novel structures of the headCap in the construct Chead1 and of the GANG domain in the construct CstalkFL could be traced and built automatically by ARP/wARP (40). All structures were manually modeled with Coot (41) and refined by REFMAC5 or PHENIX. (42,43). Crystallographic parameters are summarized in Table 2.

Computational analysis
daTAA (9) was used for the annotation of AtaA domains. Protein sequences were multiple-aligned by ClustalW2 (44) and edited by Jalview (45). The AtaA fiber length was calculated by the summation of the Nhead (9.1 nm estimated from UspA1’s Ylhead (PDB ID = 3PR7)), FGG-GANG-neck (10.5 nm estimated from CstalkN), Trp-ring-GIN-neck-short helix (3 × 9.8 nm and 2 × 9.0 nm estimated from BadA’s head (PDB ID = 3D9X)), GANG tandem (3 × 9.4 nm estimated from CstalkFL), Trp-ring-GIN-DALL1-neck-short helix (7 × 13.5 nm from the combination of BadA’s head and SadA’s K14 (PDB ID = 2YO3)), FGG-GANG-GIN-neck (2 × 11 nm estimated from CstalkFL), DALL1-neck (4.3 nm from SadA’s K14), and CheadCstalk (45 nm from the combination of Chead1, CstalkN, and CstalkFL). The volume of the domains from the PDB coordinates was calculated by the 3V web server (46).

Far-western blotting
ECMs (0.3 ng each) were separately applied onto all PVDF membrane sheets. As a positive control, one of the His-tagged recombinant proteins was applied onto each sheet. Once dried, 1 µl of 30 µM His-tagged recombinant proteins were applied onto the ECM spots and incubated for 30 min, followed by blocking with 5% skim milk. The bound proteins were immunodetected by a rabbit anti-His-tag antibody and a HRP-conjugated anti-rabbit Ig antibody.

Cell analysis
Electron microscopy, flow cytometry, adhesion assays, and autoagglutination assays were carried out as described previously (27) with slight modifications. For electron microscopy, cells were immunoreacted with the anti-Nhead antibody as first antibody and with colloidal gold (10 nm in diameter)-conjugated anti-rabbit IgG antibody as secondary antibody and observed under a transmission electron microscopy (TEM; H-7600 HITACHI, Tokyo, Japan). For flow cytometry, cells were immunoreacted with the rabbit anti-Nhead antibody as first antibody and Alexa Fluor 488-conjugated anti-rabbit Ig antibody as secondary antibody. For confocal laser scanning microscopy (CLSM), cells were fixed with 4% paraformaldehyde for 15 min on a slide glass coated with gelatin, washed with phosphate buffer saline (PBS), blocked with 2% BSA in PBS for 30 min, washed twice with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 5% Triton X-100; pH 7.6), and immunoreacted with the rabbit anti-Nstalk antibody for 10 min. After being washed twice with NET buffer, cells were immunoreacted with Alexa Fluor 488-conjugated anti-rabbit Ig antibody for 10 min. After further washing with NET buffer three times and deionized H2O once, cells were observed under a confocal laser scanning microscope (FV1000
RESULTS
Crystallization of domains of AtaA_CPSD
Using the daTAA program (7) and manual refinement (4,22), the domains of AtaA were reannotated, as shown in Fig. 1A. The Cstalk of AtaA was predicted to have FGG_4, GANG_10, GIN_15, YDD, DALL3, and FGG_5 from its N-terminus (domains were numbered from the N-terminus of AtaA) and eight coiled-coil segments (Fig. 2). The C-terminal Ylhead (Ylhead_2) was predicted to connect to a HANS motif and an unannotated region at its N-terminal end.

To determine the structure of AtaA_CPSD, we constructed seven redundant fragments that covered AtaA_CPSD as a series of recombinant proteins (Fig. 1A). Chead1 (2905-3168 aa), Chead2 (2777-3168 aa), CstalkN (3170-3332 aa), and CstalkC1 (3334-3474 aa) were designed to be connected to both N- and C-terminal trimeric GCN4 adaptors, GCN4pII, which were derived from a dimeric GCN4 leucine zipper (34). Such GCN4-fusions have been extensively used to stabilize coiled-coil domains of various proteins for structural studies (47). CheadCstalk (2905-3561 aa), CstalkC2 (3334-3561 aa), and CstalkFL (3170-3561 aa) carried the N-terminal GCN4 adaptor solely. We found that recombinant proteins of Chead1, Chead2, and CheadCstalk were partially degraded at Ylhead_2 (lanes WT in Fig. 1B and 1C), although native AtaA on the Tol 5 cell surface was not degraded at the Ylhead_2 during cultivation. A proline residue sometimes causes such a partial degradation. Therefore, we introduced a point mutation of Pro3061Gly into the recombinant proteins. This mutation prevented the recombinant proteins from partial degradation (lanes P3061G in Fig. 1B and 1C).

We successfully crystallized all of the recombinant constructs except for Chead2. Structures of Chead1 with a mutation of P3061G (PDB ID = 3WP8), CstalkFL (PDB ID = 3WPA), CstalkN (PDB ID = 3WPR), and CstalkC1 (PDB ID = 3WPO, 3WPP, 3WQA) were sequentially determined by molecular replacement. We did not solve the structures of CheadCstalk and CstalkC2. From the structures of Chead1, CstalkN, and CstalkFL, AtaA_CPSD (2905-3553 aa) was modeled (Fig. 1D). The last eight residues at the C-terminal coiled coil (3554-3561 aa) are not included in this model, as they were not traceable in the electron density.

From this model, the length of AtaA_CPSD was estimated to be ≥450 Å long. Chead1 and CstalkFL followed crystallographic three-fold symmetry and were therefore perfectly straight. The model suggested that HIM1 and FGG_4 are located close to each other. As seen in other TAAs (48), the coiled coils in Cstalk capture a Cl− ion and an unknown ion through Asn3404 and Asp3525, respectively. The right-handed coiled coil, which is often found in TAAs, was not observed in these structures of AtaA.

Unique structure of Chead: capped Ylhead
The crystal structure of the recombinant protein Chead1 revealed that its main domain, Ylhead, which is a trimer of left-handed β-helices composed of 9 repeats of the 14 residues comprising a set of an inner face and an outer face, is capped by three β-hairpins and HANS, which were en bloc named headCap (Fig. 3). In addition, Ylhead_2 is inserted by HIM1 before its last inner β-strand. The headCap, Ylhead_2, and HIM1 integrally form Chead.

The structures of the Ylhead domains from YadA, BpaA, UspA1, EibD, and SadA have been solved previously (10,17,20,49) and, of these, only the domain from BpaA is capped N-terminally,
being preceded by tightly connected FGG and HANS motifs. This cap structure contains a central coiled-coil core, which carries the FGG motif as an insertion and ends in the HANS motif. As in all determined structures, the FGG motif moves the path of the chain by 120° counter-clockwise (ccw) around the trimer axis, as viewed from the N-terminus. The Ylhead_2 of AtaA_CPSD is capped by a similar but architecturally more elaborate structure, the headCap, which has a number of unique features (Fig. 3). Like the BpaA cap, the headCap also consists of a helical core with coiled-coil character, which forms the central axis of the structure. It, however, carries three β-hairpin insertions, only the last one of which is homologous to the last hairpin of FGG. Like FGG, the previous two hairpins also move the path of the chain by 120° ccw, but, being more elaborate, they offer considerably more options for interchain contacts. The HANS motif is also substantially extended at its C-terminal end relative to the BpaA cap, looping over by 120° ccw to form a cleft with the first (outer) strand of the following Ylhead domain. Sequence searches revealed that C-terminally extended HANS motifs always correlate with a preceding FGG motif that is truncated, as seen in AtaA. The HANS works as a connector between the C-terminal α-helix of the truncated FGG and the first strand of the Ylhead domain. The structural elaboration of the AtaA headCap enables a large number of interchain contacts (Fig. 3C, 3D, and 3E), all of which are made to the preceding chain, 240° ccw around the trimer axis (Fig. 3B). Specifically, below, we review the interactions seen from the Ylhead domain toward the N-terminus, listing pairwise interactions representatively for the three-fold symmetric interactions along the fiber axis.

In the 3rd β-hairpin, the Gln2950 of chain A (A) is buried in a cleft formed by chain C (C) between the loop extending the HANS motif (2981C-2985C aa) and the 1st β-strand of Ylhead_2 (2989C-2993C aa) (Fig. 3E). Gln2950 forms a hydrogen-bond network consisting of its sidechain carbonyl group interacting with the backbone nitrogen of Leu2984C and its sidechain amino group interacting with the backbone carbonyl group of Asp2989C and with the sidechain carbonyl group of Asn3003C, whose sidechain amino group further anchors the sidechain carbonyl groups of Asp2983C and Asn3019C. This network is additionally strengthened by Lys2981C, which interacts with the backbone carbonyl groups of Gly2982C and Gly2985C to provide rigidity to the HANS motif extension. Sequence searches show that the conserved glutamine of the 3rd β-hairpin (Gln2950A) and HANS motif extension have coevolved and only occur jointly (Fig. 2).

The 3rd β-hairpin is capped by the 2nd β-hairpin of the same chain (2929A-2941A aa) (Fig. 3D). Specifically, Asn2930A of the 2nd β-hairpin interacts with the backbone nitrogen of Lys2932A and with the sidechain of Asp2954A and the backbone carbonyl group of Arg2955A in the 3rd β-hairpin. In addition, Asn2935A of the 2nd β-hairpin interacts with the backbone nitrogen of Asp2954A in the 3rd β-hairpin. The 3rd β-hairpin is anchored to the axis of the fiber at its N-terminal end by the bulky sidechain of Phe2942A and by a captured water molecule between the backbones of Asn2930A in the 2nd β-hairpin, Leu2956A in the 3rd β-hairpin, and Gly2917 of chain B (B) in the 1st β-hairpin.

The 2nd β-hairpin itself is capped by the 1st β-hairpin (Fig. 3C). The two hairpins form a joint hydrophobic core consisting of Val2914C, Val2922C, Ile2924C and Ile2925B, Val2928B, and Val2929B; as well as a hydrogen bond network spanned by the water molecule captured by Asn2930B, Leu2956B, and Gly2917C.
Although the FGG-like 3rd β-hairpin is found broadly among genera *Acinetobacter*, *Neisseria*, *Moraxella*, and *Haemophilus*, the 2nd β-hairpin is not often found among TAAs. Proteins that are homologous to the 2nd β-hairpin are only found in *Psychrobothrium aquaticus* and *Vitreoscilla stercoraria*, in addition to genus *Acinetobacter*.

HIM1 is longer than HIM2 and HIM3, containing an α-helix instead of the β-hairpin of HIM2 or the loop of HIM3 (Fig. 4). HIM1 is anchored to the following neck and the following coiled coil through hydrophobic interactions with Leu3117 and Val3119, forming a layer of a hydrophobic core for the coiled coil.

**Detailed structures of the domains in Cstalk**

- **FGG domain**

The structure and amino acid sequence of the region containing the 3rd β-hairpin of the AtaA_headCap (2942-2968 aa) are similar to those of the C-terminal half of the FGG domain of BpaA (16). The region (2912-2940 aa) of the AtaA_headCap corresponding to the N-terminal half of the FGG domain of BpaA is quite different and is no longer an FGG domain (Fig. 5A and 5B). Therefore, we call it ΔN-FGG. AtaA_CPSD contains two FGG domains in Cstalk and a ΔN-FGG in Chead. FGG_4 and FGG_5 are quite similar to the SadA FGG domain (22). LGG, the most frequent motif in this domain (4), was conserved in FGG_4 and FGG_5 of AtaA. Their structures are composed of an N-terminal α-helix (αN), two β-hairpins, and a C-terminal α-helix (αC), and the chains are topologically exchanged 120° ccw around the trimer axis. FGG domains have a characteristic and conserved aromatic residue, Tyr3204 in FGG_4, Tyr3504 in FGG_5, and Phe2942 in ΔN-FGG, which forms a hydrophobic interaction at a transition of the chains (Fig. 5A, 5B, and 5C). As shown in Fig. 5C, the hydrophobic cluster at the N-terminal half of FGG_5 is formed by Val3482, Val3483, and Leu3486 in the coiled coil of chain A; Thr3478, Ala3481, and Tyr3485 in the coiled coil of chain B; and Tyr3492, Phe3499, and Pro3502 in a β-hairpin of chain A. The hydrophobic cluster at the C-terminal half of FGG_5 is formed by Val3514, Ala3517, and Val3521 in the coiled coil of chain A; Val3514 and I3518 in the coiled coil of chain C; and Tyr3504 and Val3506 in a β-hairpin of chain A. The N-terminal and C-terminal hydrophobic cores are continued by the hydrophobicity of Pro3502, Val3514, and Tyr3504.

FGG_4 and FGG_5 of AtaA_CPSD contact with their N-terminally neighboring domains HIM1 and the neck following DALL3, respectively, resulting in a continuous enlarged hydrophobic core (Fig. 5D and 5E). Although the model of CheadCstalk shows that HIM1 and FGG_4 are situated closely to each other, no interaction was observed between them. However, the enlarged hydrophobic core of HIM1 continues due to the following FGG_4, which also forms a layer of a hydrophobic core for the coiled coil (Fig. 5D).

- **GANG domain**

The crystal structure of the GANG domain was first determined in this study. The GANG domain is topologically similar to the Trp-ring domain except for the absence of the 1st and 2nd β-strands of the Trp-ring (β1’ and β2’ in Fig. 6): a short variant of the Trp-ring, that is, a truncated interleaved head (4). Therefore, the path of the chain of the GANG domain by topological crossover around its trimer axis is 40° ccw as viewed from the N-terminus, compared with 120° ccw of that of Trp-ring domain. Although the characteristic motif Gly/Asn-Ala-Asp/Asn-Gly of the GANG domain is often Gly-Ala-Asn-Gly (GANG), it is NADG in AtaA_CPSD. This motif
is positioned in the 2nd loop between the 2nd and 3rd β-strands and is exposed outside of a trimer (Fig. 6B), suggesting that the loop interacts with substance surfaces. The GANG domain captures four water molecules around the connection between the N-terminal α-helix and the 1st β-strand of the GANG domain, suggesting that the GANG domain is more flexible than other TAA domains. Crystal structures showed high B-factors at the connection between FGG_4 and GANG_10 and at the NADG loop. The N-terminal α-helix does not interact with the GANG loop, probably also contributing to the flexibility of the loop and bending between the FGG and GANG domains. A phenylalanine residue in a Trp-ring of HiaBD1 stabilizes trimerization of the Trp-ring (50). The Trp-ring of the BadA head also contains phenylalanine. The conserved Tyr in the GANG domain, which corresponds to the phenylalanine in the Trp-ring, may stabilize trimerization of the GANG domain (Fig. 6E).

-DALL domains-
We determined the structures of two novel DALL variant domains that are conserved in TAAs, that is, YDD and DALL3 in AtaA_CPSD. The DALL domain is composed of N-terminal α-helix and β-strands. In the structures of CstalkFL and CstalkC1, YDD is now seen to represent a divergent form of DALL2. The YDD domain does not have inserted loops containing an additional β-strand (β" in Fig. 8A), resulting in as simple structure as the DALL2 domain (Fig. 8B). However, YDD has a tyrosine that is the conserved aromatic amino acid residue in the DALL1 domain (Fig. 8A, asterisk), whereas the tryptophan in the DALL2 domain of SadA at this position forms a π-π interaction with histidine across the chains (41st position in Fig. 8A) (22). Neither YDD nor DALL1 have the histidine residues, resulting in the absence of the π-π interaction in these domains in AtaA. In DALL3, only its C-terminal half is equivalent to DALL1 and DALL2/YDD; DALL3 contains two additional N-terminal strands. The N-terminal β-strand with GTVG forms a short β-strand on another β-strand with LVQQQA (Fig. 8A and 8C). Because of the additional N-terminal strands, the DALL3 domain transits a chain to axis different from other DALL domains (Fig. 8C). These additional β-strands enclosed 13 water molecules in a trimer (Fig. 8D), which may make the N-terminal end of DALL3 more flexible than other DALL domains. Both YDD and DALL3 in AtaA_CPSD are followed by a neck (Fig. 8B and 8C), forming a globular and rigid domain that is topologically akin to a head domain, as usually seen in all DALL variants (4).

Bending of TAAs with DALL domains
B-factors at the connections between the N-
terminal α-helix and first β-strand in the DALL variant domains were shown to be high. Further, we solved crystal structures of CstalkC1 in various crystal forms, showing different bending angles at the YDD and DALL3 domains. CstalkC1ii was found to mostly bend: the trimer of CstalkC1ii bent 5° with the trimer axis of the DALL3-neck (Fig. 9A and 9C). Because of this flexibility, the structure of the C-terminus of CstalkC1iii was disordered, even in a crystal (PDB ID = 3WPP). As well as CstalkC1, the overall structure of CstalkN bent compared with that of CstalkFL (Fig. 1D). The trimer of CstalkN bent 1° at the root of the GANG domain (Fig. 9B and 9C). Because of this flexibility, the N-terminus of CstalkFL was disordered in a crystal (Fig. 1D). These structural flexibilities are considered to be caused by the absence of interactions between the C-terminus of the α-helix and the first β-layer in the DALL3 and YDD domains and between the C-terminus of the α-helix of FGG and the GANG loop in the GANG domain. On the basis of these structures, we constructed a bending model for CheadCstalk (Fig. 9D). This model shows that AtaA_CPSD bends a total of 6° from the C-terminus.

**Functional analyses of AtaA_CPSD**

To examine the contribution of AtaA_CPSD to the nonspecific, high adhesiveness of AtaA, we first constructed three in-frame deletion (IFD) mutants by deleting Chead and/or Cstalk from the full-length AtaA; they were IFD-∆CheadCstalk (deletion from 2906 to 3475 aa), IFD-∆Chead (deletion from 2902 to 3167 aa), and IFD-∆Cstalk1 (deletion from 3170 to 3475 aa) (Fig. 10A). These constructs (IFD-AtaAs) were designed to connect the neck to the α-helix without breaking the secondary structure on the basis of the domain annotation on the amino acid sequence of AtaA (Fig. 11). After confirmation of the cell surface display of IFD-AtaAs by flow cytometry and CLSM (Fig. 10E), the states and appearances of their fibers extending from the cell surface were observed using immunoelectron microscopy (Fig. 10D). On IFD-∆CheadCstalk- and IFD-∆Chead-expressing cells, many extended fibers of the respective IFD-AtaAs were observed. The distal ends of these fibers, labeled with colloidal gold-conjugated anti-Nhead antibody, were observed approximately 200 nm apart from the cell surface. However, on IFD-∆Cstalk1, the distal ends of many fibers were observed at the margin of the cells, in spite of the presence of a few extended fibers, suggesting that most of the fibers formed fell down or hardly extended or stood on the cells. The IFD-AtaA-expressing mutant cells were subjected to adherence assays for polystyrene (PS) and collagen surfaces and to autoagglutination assays. We found that IFD-∆CheadCstalk- and IFD-∆Chead-expressing cells showed adhesiveness to PS and collagen surfaces and autoagglutination that were as high as those of WT cells (Fig. 10G and 10H). In contrast, IFD-∆Cstalk1-expressing cells showed greatly decreased adhesiveness and autoagglutination, probably due to the abnormally formed IFD-AtaA fibers mentioned above. In spite of the decreased adhesiveness of this mutant, flow cytometry and CLSM showed that immunolabelling against Nhead was more intensive on IFD-∆Cstalk1 than on WT AtaA (Fig. 10E). This could be explained by the increased reactivity of the antibody against this IFD construct in a partially unfolded state. After determination of the crystal structures of recombinant constructs from AtaA_CPSD, structure models of the new domain connection arising from the deletions in the IFDs were simulated (Fig. 10B and 10C). This suggested that loops of HIM1 and FGG_5 slightly clash in IFD-∆Cstalk1 because the intervening coiled coil was shortened too far, which would
cause the formation of abnormal IFD-AtaA fibers. Therefore, we designed a new IFD, IFD-ΔCstalk2 (deletion from 3177-3475 aa), in which the coiled coil was extended by one heptad. In this new construct, the collision of the loops was simulated to be avoided (Fig. 10C). The resultant cells displayed IFD-ΔCstalk2 on the cell surface in the level similar to original AtaA on WT cells (Fig. 10E). The observed IFD-AtaA fibers normally extended: their distal ends were approximately 200 nm distant from the cell surface (Fig. 10D). Cells of this IFD mutant exhibited adhesiveness to PS and collagen surfaces and autoagglutination that were as high as those of WT cells (Fig. 10G and 10H). Thus, deletion of Chead and/or Cstalk had no effect on the adhesion or autoagglutination of Tol 5 cells. Then, we constructed IFD-CPSD, in which the region of AtaA 108-2966 corresponding to most of the Nhead and Nstalk was deleted from the full-length AtaA and the N-terminal repeats of Ylhead_1 and Ylhead_2 were connected to each other, so as to generate truncated AtaA fibers mostly consisting of AtaA_CPSD. We confirmed that many short truncated AtaA fibers grew from IFD-CPSD cells by normal electron microscopy after negatively staining cells (Fig. 10F). However, IFD-CPSD cells lost adhesiveness to not only PS and collagen surfaces, but also to fibronectin and laminin at a similar level as the ΔataA mutant (Fig. 10I). Finally, recombinant proteins whose crystal structure could be solved were subjected to a far-western blotting assay against ECM proteins (Fig. 10J). Chead, CstalkN, and CstalkC2 did not bind to fibronectin, type I collagen, or laminin, whereas the head domain of YadA used as a control bound to collagen and laminin. From all of the results of the adhesion assays, we concluded that the sites responsible for the nonspecific, high adhesiveness of Tol 5 cells do not reside in AtaA_CPSD.

**Domain architecture of Acinetobacter TAAs**

Proteins homologous to the C-terminal domains of AtaA (residues 2905-3630 or 3476-3630) were identified by BLASTP. Of 54 Acinetobacter species characterized so far, 23 species have a region homologous to AtaA_CPSD (Fig. 12). These TAAs can be separated into two types based on the presence or absence of Chead. In the former, the Chead does not reside at the N-terminus of the protein and there is an Nstalk at its N-terminal side as well as a Cstalk at its C-terminal side. In addition, these Cstalks have the same domain architecture as that of AtaA. Therefore, many Acinetobacter species have TAAs with a C-terminal region sharing common domain architecture with AtaA_CPSD. Among them, a hypothetical TAA of A. bereziniae has a CPSD that shows the highest homology with AtaA_CPSD; their amino acid sequences differ from each other at only three residues. The other types of TAAs have no Chead but many of them have a C-terminal region with domain architecture similar to the Cstalk of AtaA. Ata of A. baumannii also belongs to this type and only GANG_10 in the Cstalk of AtaA is substituted by the Trp-ring in Ata at its corresponding site.

**DISCUSSION**

In the current study, we determined, for the first time, the crystal structures of the HIM, GANG, YDD, and DALL3 domains, which are well conserved in TAAs. In addition, we newly identified headCap and determined its unique structure. These structures add information to the 'dictionary approach' to TAAs, based on which whole TAA fibers can be modeled on the basis of known fragments (22). Many Acinetobacter species have TAAs. In the present study, it was revealed that AtaA_CPSD does not include a binding site responsible for high adhesiveness to various abiotic surfaces of full-length AtaA. This is
reasonable because none of the other TAAs that have the same domain architecture as AtaA_CPSD exhibit similar adhesive properties to AtaA. The characteristic binding sites of AtaA are considered to reside in the unique AtaA_NPSD consisting of Nhead and Nstalk.

In the CPSD, however, we can see structurally important features that probably contribute to high adhesiveness, that is, flexibility and toughness. For strong adhesion to targets that are distant from the cell surface, AtaA fibers must project their binding sites on Nhead and/or Nstalk sufficiently far from the cell surface. In fact, IFD-ΔCstalk1 cells could not exhibit high adhesiveness because they formed fibers that hardly extended (Fig. 10). If the AtaA_CPSD at the base of the fiber is too limp to support the long fiber on the cell for extending beyond the cell surface, the interaction between the binding sites and target surfaces might be inefficient. Therefore, sufficient toughness to support the fiber and permit its extension is essential for TAAs to function as adhesins.

The toughness of TAAs largely depends on the interchain interactions of the three polypeptides forming the trimer. Hydrophobic core formation by residues on the inside of the trimer and topological chain exchange are mainly responsible for TAA toughness. GIN, FGG, and Ylhead domains significantly enlarge the hydrophobic core around the trimer axis. GIN and Ylhead domains are quite stable because of stacking of β-strands. FGG domains exert hydrophobic interactions between their β-hairpins and the coiled coil (Fig. 5C), contributing to toughness against horizontal shear stress. In addition, at the FGG domains, chain exchange occurs accurately 120° ccw around the trimer axis (Fig. 5C, 5D, and 5E), contributing to toughness against vertical shear stress. There are many other chain interactions in AtaA_CPSD. In Chead and at the transitions of the FGG-GANG, GANG-GIN-neck, YDD-neck, and DALL3-neck, polypeptide chains twist 120° or 240° ccw around the trimer central axis, resulting in complex chain exchange along the parallel line to the fiber axis. Of these, the headCap forms a markedly complex interaction network by frequent topological chain transitions in a short range of amino acid residues.

Ylhead_2 of AtaA is strengthened by the headCap and HIM1, integrally forming the Chead that exhibits structural toughness. Chead is the most bulky domain in the AtaA_CPSD (the volume per length of the trimer axis of Chead is 1043 Å³/Å, that of GANG-GIN-neck is 306 Å³/Å, that of YDD-neck is 394 Å³/Å, and that of DALL3-neck is 394 Å³/Å) (Fig. 1D). The structure of Chead is reasonable to sustain this bulky domain on AtaA fibers. In addition to headCap strengthening Chead from the top, HIM1 and FGG_4 sustain it from below the standing fiber by making a large hydrophobic core. FGG domains also fill a gap in the thickness between the bulky domains (Ylhead and DALL-neck) and slim coiled coil. (Fig. 5F), contributing to toughness against vertical shear stress. A sharp shift in thickness usually creates a point of stress concentration like a notch. In particular, β-hairpins in FGG_4 cover and hydrophobically stabilize the coiled coil neighboring the most bulky domain, Ylhead_2, continuously with HIM1 so as not to cause stress concentration at this point. The coiled coil at this region is elongated by one heptad insertion to avoid the collision between the HIM1 loop and the FGG loop. Slight collision of the loops would not be allowed to form the normal extended fibers in the IFD-ΔCstalk1, demonstrating the exquisite design of AtaA in nature.

On the other hand, the importance of flexibility for adhesion has also been pinpointed for different TAAs (10,20,22,28,29). Bending of TAA fibers allows them to face and angle their binding sites to...
target surfaces, especially to large receptors, matrix proteins, or abiotic surfaces with a large radius of curvature, for effective interaction. The angle of bending is estimated at about 90° by the summation of DALL1 (8 × 10° from SadA K14), FGG-GANG (4 × 1° from CstalkN), and a combination of YDD and DALL3 (1 × 5° from CstalkCii) (Fig. 9E). In fact, we observed that AtaA bent flexibly on the cell surface (Fig. 9F).

Ions are often captured by hydrophilic residues, one of which is often asparagine at position d of the heptad coiled coil of TAAs and is therefore called the N@d motif (48). Cstalk has Asn3404 at position d and Asp3525 at position a to capture Cl− and an unknown ion, respectively. The latter was not included in the final model but we suspected it to be Mg2+ because of its coordination geometry. These ions may also give Cstalk flexibility by breaking the hydrophobic core.

In DALL domains, flexibility and toughness are compatibly exhibited; the former comes from the connection between N-terminal α-helix and the first β-strand and the latter from topological chain exchange at the connection to the neck and a hydrophobic core. Note that DALL-necks are often found in relatively long TAAs with over 1000 residues. TAAs may have evolved themselves to achieve flexibility and toughness, which appear opposite features, by scattering DALL-neck insertions along coiled-coil fibers, such as in AtaA, which has 10 repeats of this structure.

Although AtaA_CPSD is not essential for the nonspecific, high adhesiveness of Tol 5 cells, its structure is exquisite for exhibiting both flexibility and toughness, which provide the resilience needed by full-length AtaA fibers to exert their adhesive properties across a wide range of conditions.

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Author Contributions: KK, ANL, and KH designed the study and wrote the paper. ANL annotated the domains of AtaA. KK, MDH, DL, and ANL designed and constructed vectors for expression of recombinant proteins. ANL and KH designed, and KH constructed and characterized IFD mutants. KK and MDH purified and crystallized the proteins and determined their structures. All authors analyzed the results and approved the final version of the manuscript.
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FIGURE LEGENDS

FIGURE 1. Structure of the C-terminal part of the AtaA passenger domain.
(A) Schematic representations of AtaA and of recombinant constructs from its C-terminal part (AtaA_CPSD): CheadCstalk (2905-3561 aa), Chead1 (2905-3168 aa), Chead2 (2777-3168 aa), CstalkFL (3170-3561 aa), CstalkN (3170-3332 aa), CstalkC1 (3334-3474 aa), and CstalkC2 (3334-3561 aa). All constructs were connected to GCN4-tags (white boxes). The Ylhead, FGG, GANG, Trp-ring, DALL1, and GIN domains are labeled and numbered from the N-terminus of full-length AtaA. Neck domains are not labeled. Pro3061 of the CheadCstalk and Chead1 was mutated to Gly. The numbers above the structures indicate amino acid residues. Signal peptide, YadA-like head, and transmembrane anchor, which are annotated by the daTAA program, are abbreviated as SP, Ylhead, and TM, respectively. The headCap was newly annotated from the solved crystal structure in this study and consists of \( \beta \)-hairpins, an N-terminally truncated FGG motif (\( \Delta \)N-FGG), and a HANS motif.
(B) SDS-PAGE analyses of recombinant CheadCstalk proteins. CheadCstalk samples (WT and P3061G) purified by Ni-affinity chromatography were fractionated by SDS-PAGE and detected by Coomassie blue staining (CBB). Only WT was sensitive to proteolysis, being degraded into 55-kDa and 23-kDa fragments (WT frag. 55 and WT frag. 23, respectively), whereas the P3061G mutant was protease resistant.
(C) Western blotting analyses of purified CheadCstalk samples using an anti-His-tag antibody (IB). The recombinant proteins carried the His-tag at the C-terminus and only the 55-kDa fragment of the WT protein was detected. This confirms that cleavage had occurred in the region of P3061 and that proline at this position represents a folding obstacle, presumably due to a necessary cis-trans isomerization step that overloads the endogenous prolyl isomerase during overexpression.
(D) 3-D structure of the AtaA_CPSD. The structures of Chead1, CstalkFL, CstalkN, and CstalkC1 were determined experimentally. CheadCstalk is a model constructed from these crystal structures. Of the three CstalkC1 structures (CstalkC1i, PDB ID = 3WPO; CstalkC1ii, PDB ID = 3WQA; CstalkC1iii, PDB ID = 3WPP), CstalkC1i is shown. In each structure, the three polypeptide chains forming a homotrimer are colored brown, green, and yellow, respectively; GCN4-tags are colored gray. Ions in CstalkC1i, CstalkFL, and CheadCstalk are shown as spheres: blue – chloride and black – unidentified. Neck domains occur following the HIM1, GIN_15, YDD, and DALL3 domains. PDB IDs are indicated below the construct names.

FIGURE 2. Domain structure of the C-terminal region of AtaA (AtaA_CPSD) marked on a sequence alignment with the same region of other Acinetobacter TAAs.
Well-characterized Acinetobacter TAAs with the Chead and A. baumannii Ata were aligned by ClustalW2. A. baumannii Ata has Trp-ring and DALL1 domains instead of GANG and YDD, respectively. Tol 5 AtaA is highly similar to a hypothetical TAA of A. bereziniae and the amino acids differing between these TAAs are marked by asterisks. Pro3061 is marked by an “at” mark. Coiled coils with hendecad periodicity are indicated by bold lines. The domains of AtaA are numbered from the N-
terminus. Circles colored in blue and black indicate residues capturing chloride and unknown ions, respectively. Hydrophobic residues are shaded gray.

FIGURE 3. The C-terminal head domain contains a unique headCap structure.
(A) Overall structure of the AtaA Chead1 construct, consisting of headCap, Ylhead, HIM1, and neck domains. The headCap is composed of a helical core with three peripheral β-hairpin insertions, the third of which is a truncated FGG domain; it ends with a HANS motif that makes the transition to the Ylhead. Colors are as in Figure 1. The characteristic residues Asn2930 of the 2nd β-hairpin and Gln2950 of the 3rd β-hairpin are shown in stick representation. P3061G in each chain is indicated by an asterisk.
(B) Topological diagram of the headCap, with loops as lines, α-helixes as cylinders, and β-strands as arrows. The hairpins are colored as in panel A. A β-strand with HANS motif is indicated by HANS β. The residues Asn2930 of the 2nd β-hairpin and Gln2950 of the 3rd β-hairpin are shown in stick representation.
(C, D, E) Enlarged views of the headCap β-hairpins, as marked by squares in panel A, highlighting the extensive stabilizing interactions that each chain makes in this region with the chain preceding it by 120° counter-clockwise around the trimer axis, as viewed from the N-terminus. All views are in stick representation; dotted lines indicate hydrogen bonds. Colors are as in panel A. Sidechains that do not contribute specific interactions are not shown. Orange and blue atoms indicate oxygen and nitrogen, respectively. Gray spheres indicate captured water molecules. (C) Interchain interactions between the 1st and 2nd β-hairpins via a shared hydrophobic core and a hydrogen bond network mediated by a structural water molecule. (D) Intrachain interactions between the 2nd and 3rd β-hairpins. (E) Interchain interactions among the 3rd-hairpin, a C-terminal extension of the HANS motif, and the following N-terminal strand of the Ylhead domain.

FIGURE 4. The head insert motif 1 (HIM1) of AtaA_CPSD.
(A) Sequence alignment of HIMs. Amino acid sequences of AtaA HIM1, BpaA HIM2, UspA1 HIM2, and SadA HIM3 were aligned with ClustalW2. Of the three head insert motif variants, only HIM1 contains an α-helix (α). Hydrophobic residues are shaded gray. The conserved hydrophobic Gly-Gly sequence of FGG motifs is indicated by asterisks. A conserved aromatic residue, tyrosine or phenylalanine, is indicated by a caret (^). The β-strands of the domains are indicated
by straight arrows. N-terminal and C-terminal \(\alpha\)-helixes in FGG domains are indicated by waved arrows labeled \(\alpha_N\) and \(\alpha_C\), respectively. \(\alpha_N\) has a hendecad periodicity.

(B) Comparison of FGG structures. The structures were superimposed using the C-terminal helix (residues 2958-2968 of the AtaA headCap, brown; residues 3214-3224 of AtaA FGG_4, green; residues 3514-3524 of AtaA FGG_5, yellow; residues 292-302 of SadA, gray; residues 2335-2345 of BpaA, black). The conserved aromatic residue indicated by a caret in panel A is shown in stick representation.

(C) The hydrophobic interaction between the \(\beta\)-hairpins of FGG_5 and the cognate coiled coil with hendecad periodicity. Sidechains of residues contributing to the hydrophobic interaction between the coiled coil and \(\beta\)-hairpins are shown in stick representation. The residues on the \(\beta\)-hairpins are shown by bold italics. Colors are as in Figure 1. The tyrosine residue (Tyr3504) indicated by a caret is the conserved residue in panel A.

(D) Contact between HIM1 and FGG_4. Colors are as in Figure 1. Both HIM1 and \(\beta\)-hairpins of FGG_4 project to outside at positions rotated 60° around the trimer axis. Both HIM1 and FGG_4 have high B-factors of \(\text{C}^\alpha\) in the crystal structures of Chead1 and CstalkN, suggesting that HIM1 and FGG_4 can form different conformations to contact each other.

(E) Contact between the neck following DALL3 and FGG_5. Colors are as in Figure 1. The neck following the DALL3 and \(\beta\)-hairpins of FGG_5 project to the outside at 120° and 60° around the trimer axis, respectively.

(F) Surface of models of AtaA_CheadCstalk, FGG (+), and a truncation of \(\beta\)-hairpins in FGG_4 and FGG_5 of CheadCstalk, FGG (-). The surface is colored by distance from the trimer axis in a gradient from red to white and from white to green as shown by a scale bar.

**FIGURE 6. The GANG domain is a truncated variant of the Trp-ring domain**

(A) Sequence alignment of the 10 GANG domains of AtaA with two Trp-ring domains, one from *A. baumannii* Ata, which substitutes in that protein for GANG_10, and the other from *B. henselae* BadA. Asterisks (*, bold) and carets (^, bold) indicate the conserved GANG and Tyr-polar-Val motifs, respectively. The characteristic tryptophan of Trp-ring domains is highlighted in yellow. Residues forming the hydrophobic core are shaded gray. The \(\beta\)-strands of the domains are indicated by arrows, above the alignment for GANG and beneath for Trp-ring.

(B) Overall structure of GANG_10. Colors are as in Figure 1. The GANG motif (NADG in this domain) is colored blue and shown in stick representation, as is the conserved tyrosine, Tyr3256. Water molecules are shown as spheres; a water molecule at the trimer axis is colored red, the others gray.

(C) Overall structure of the Trp-ring domain, exemplified by the domain of *B. henselae* BadA (PDB ID = 3D9X). The conserved tryptophan and phenylalanine residues of the Trp-ring domain are shown in stick representation. The phenylalanine is at the equivalent position to the conserved tyrosine of GANG domains.

(D) Topological diagram of GANG and Trp-ring domains, with loops as lines and \(\beta\)-strands as arrows. One chain of the trimer is colored, yellow for the part common between the GANG and Trp-ring domains and black for the two additional \(\beta\)-strands of Trp-ring domains (\(\beta'1\) and \(\beta'2\)).

(E) Superimposition of single monomers from AtaA GANG_10 (brown) and BadA Trp-ring (gray) obtained by structural alignment of the following GIN domains.
FIGURE 7. The GIN domain of AtaA_CPSD.
(A) Sequence alignment of the GIN domains of AtaA and BadA. Asterisks (*, bold) indicate the conserved GIN motif. Residues forming the hydrophobic core are shaded gray. The β-strands of the domains are indicated by arrows, above the alignment for the GIN domain.
(B) Superimposition of the GANG-GIN and Trp-ring-GIN structures. The structures of GANG_10–GIN_15 from CstalkFL (colored in red (helix), yellow (strand), and green (loop)) and Trp-ring–GIN from the BadA head (PDB ID = 3D9X, blue) are superimposed using their C-terminal neck and helix.
(C, D) Hydrophobic residues of the GIN domain, colored gray and shown in stick representation. The monomer (C) and trimer (D) of CstalkFL are shown, colored as in Figure 1. Ile3315, Val3318, and Ala3321 are in a neck domain.

FIGURE 8. Structure of DALL domain variants
(A) Sequence alignment of DALL domain variants from AtaA and SadA. AtaA has eight DALL1 domains in its Nstall region (see Figure 1) and single YDD and DALL3 domains in the Cstalk. A histidine residue highly conserved in DALL2 domains is highlighted in yellow. A key aromatic residue of DALL1, DALL2, and YDD is indicated by an asterisk. DALL1 differs from DALL2/YDD by an extended β1–β2 hairpin; in β2, the extension is separated from the rest of the β-strand by a β-bulge at a conserved tyrosine residue corresponding to Tyr1325 in SadA, indicated by a caret and marked as β” in the figure. The N-terminal half of DALL3, shown in lowercase letters, is topologically different from DALL1 and DALL2/YDD, containing two additional β-strands (β1’ and β2’) and orienting β1 in the opposite direction. Residues forming the hydrophobic core are shaded gray.
(B) Structures of DALL domain variants and the following necks. DALL1 and DALL2 are from SadA (PDB ID = 2YO3 and 2YNZ, respectively); YDD and DALL3 are from CstalkFL. In each structure, the main chain of one subunit forming the DALL domain is shown in color and stick representation. Water molecules interacting with this subunit are indicated by spheres of the same color. In DALL3, water molecules interacting with the other subunits are colored black. Water molecules along the three-fold axes of DALL1, DALL2, and DALL3 are colored red. YDD alone does not contain an axial water molecule, allowing the central water molecule of the following neck to be visible (colored in white).
(C) Superimposition of DALL domain monomers, obtained by structural alignment of the following neck domains. The residues of the two additional β-strands in DALL3 are labeled.
(D) Hydrogen bond network of water molecules in DALL3, showing the 13 water molecules of a trimer in the same colors as in panel B. Hydrogen bonds are indicated by dotted lines.

FIGURE 9. Bending of the AtaA fiber at GANG, YDD, and DALL3.
(A) Superimposition of monomers from CstalkC1ii (gray) and CstalkFL (red), obtained by structural alignment of the neck–coiled coil tandem following DALL3. All three monomers of the asymmetric CstalkC1ii structure are shown.
(B) Superimposition of monomers from CstalkN (gray) and CstalkFL (red), obtained by structural alignment of the GIN_15–neck–coiled coil segment. All three monomers of the asymmetric CstalkN
structure are shown. The N-terminus of CstalkFL was not traceable in the electron density.

(C) Gallery of the domains causing bending in the AtaA fiber. Extended (red) and bent (gray) structures for GANG, YDD, and DALL3, respectively, were superimposed using their C-terminally adjacent domains.

(D) Model of bending in the CheadCstalk structure. The bent conformation (gray) was modeled from the structures of the entire Chead1 and of CstalkN for FGG_4 and GANG_10; CstalkFL for GIN_15, coiled coil, and FGG_5; and CstalkC1ii for YDD and DALL3. The extended conformation (red) is as in Figure 1. The two models were superimposed using FGG_5 and the C-terminal coiled coil.

(E) Schematic representation of bending in the full-length AtaA fiber. Models colored in gray differ by the number of DALL1 domains assumed to be bent (none, two, and four).

(F) Bent AtaA fibers on the cell surface. Tol 5 4140/pAtaA cells were imaged by transmission electron microscopy (TEM) using negative staining. Arrows indicate bent fibers. The right panel is the same image as the left panel, with the bent AtaA fibers traced in black. The scale bar indicates 100 nm.

FIGURE 10. Construction, cell surface display, and functional analyses of in-frame deletion mutants of AtaA.

(A) Schematic representation of in-frame deletion (IFD) mutants of AtaA. WT, IFD-ΔCheadCstalk (truncation of 2906-3475 aa), IFD-ΔChead (truncation of 2902-3157 aa), IFD-ΔCstalk1 (truncation of 3170-3475 aa), IFD-ΔCstalk2 (truncation of 3177-3475 aa), and IFD-ΔPSD (truncation of 108-2996 aa) were inserted into pARP3 plasmids for expression in Tol 5 4140 (ΔataA). Details of these IFD mutants are shown in Fig. 11.

(B) Models of the wild-type and the new domain connections arising from the deletions in IFD-ΔCheadCstalk and IFD-ΔChead, obtained by homology modeling from the equivalent domains of CstalkN, Chead, and CstalkFL. Colors are as in Figure 1.

(C) Models of the wild-type and the new domain connections arising from the deletions in IFD-ΔCstalk1 and IFD-ΔCstalk2, obtained by homology modeling from the equivalent domains of Chead, CstalkN, and CstalkFL. Colors are as in Figure 1.

(D) Immunoelectron microscopy of Tol 5 cells displaying IFD mutants, imaged with an anti-Nhead antibody and colloidal gold-conjugated anti-rabbit IgG antibody. Scale bars indicate 200 nm.

(E) Confirmation of cell surface-displayed IFD-AtaA constructs by flow cytometry and CLSM (Inlets). Scale bars indicate 2 μm.

(F) TEM image of negatively stained IFD-PSD fibers on Tol 5 cells. The scale bar indicates 200 nm.

(G) Adherence assays of Tol 5 IFD mutants. Bacterial cells adhering to bare polystyrene (PS; blank bar) and to collagen-coated PS plates (filled bar) were quantified by a crystal violet staining method. Assays were performed in triplicate. Error bars indicate SEM.

(H) Autoagglutination assay of Tol 5 IFD mutants. The autoagglutination was quantified by a decrease in OD660 in tube-settling assays. Assays were performed in triplicate. Error bars indicate SEM.

(I) Adherence assay of the Tol 5 IFD-PSD mutant. Bacterial cells adhering to bare PS and to fibronectin-, laminin-, and collagen-coated PS plates were quantified by a crystal violet staining method. Assays were performed in triplicate. Error bars indicate SEM.

(J) Far-western blotting analysis of the recombinant proteins forming AtaA_CPSD (Chead, CstalkN, and
CstalkC2), compared with the YadA_head protein (26-210 aa) fused with a C-terminal GCN4-His-tag. Each of these His-tagged proteins was applied as input to membranes, along with three ECMs (fibronectin, collagen type I, and laminin); for the negative control, the input was His-tagged Chead1. The membranes were then subjected to a reaction with His-tagged proteins as listed on the right of the panels. After the reaction, the proteins were detected immunologically using an anti-His-tag antibody.

**FIGURE 11.** Domain configuration and amino acid sequence of AtaA_CPSD and in-frame deletion constructs.

Domain colors are the same in the schematic figure and the amino acid sequence, except for Ylhead_2, where only the inner β-strands are colored red. Trimeric coiled coils are underlined. In-frame deletion constructs (IFDs) of AtaA were truncated as indicated; IFDΔChead (red line), IFDΔCheadCstalk (narrow black line), IFDΔCstalk1 (dotted line), and IFDΔCstalk2 (bold black line). IFDΔChead and IFDΔCheadCstalk were designed to connect the neck to a coiled-coil segment. In order to avoid steric hindrance between HIM1 and FGG_5, IFDΔCstalk2 was designed to include seven additional residues (KAVGNQV) in the coiled coil separating the two motifs.

**FIGURE 12. Domain architecture of Acinetobacter TAAs (AcTAAs).**

Domains were predicted by daTAA and manually refined. Incomplete sequences are only shown for TAAs containing a Chead and were marked by dotted lines. The scale bar above the sequences indicates residue numbers. The domains are shown schematically as in Figure 1. Sequences are labeled with the species and strain name of the source organism, and the sequence ID in the NCBI nr database.
| Protein name | Crystal name | PDB ID | Protein concentration (mg/ml) | Formulation of reservoir solution | Cryo-protectant |
|--------------|--------------|--------|-------------------------------|-----------------------------------|-----------------|
| Chead1       | Chead1       | 3WP8   | 12                            | 3% PEG 6000, 1.0 M NaCl, 0.1 M Na-acetate, pH 3.6 | 22% PEG6000     |
| CstalkFL     | CstalkFL     | 3WPA   | 10.0                          | 1.26 M NaH2PO4, 0.14 M K2HPO4, pH 5.1 | 25% glycerol    |
| CstalkN      | CstalkN      | 3WPR   | 5.0                           | 10% PEG 4000, 0.1 M Na-citrate, 0.1 M Na-acetate, 0.4 M (NH4)2SO4, pH 5.5 | 20% PEG400      |
| CstalkC1     | CstalkC1i    | 3WPO   | 7.0                           | 15% MPD, 2% PEG 4000, 0.1 M Na-acetate, pH 5.0 | 20% PEG400      |
|              | CstalkC1ii   | 3WQA   | 7.0                           | 12% PEG 8000, 0.1 M NaH2PO4, pH 6.5 | 15% PEG400      |
|              | CstalkC1iii  | 3WPP   | 7.0                           | 1% PEG2000 MME, 1 M succinate, 0.1 M HEPES, pH 7.0 | 25% glycerol    |
| Structure | Chead1 | CstalkFL | CstalkN | CstalkC1i | CstalkC1ii | CstalkC1iii |
|-----------|--------|----------|---------|-----------|------------|-------------|
| PDB ID code | 3WP8   | 3WPA     | 3WPR    | 3WPO      | 3WQA       | 3WPP        |
| Monomer/AU | 1      | 1        | 3       | 3         | 3          | 1           |
| Space group | P63    | P321     | P21     | C2        | P21        | R32         |
| a, Å     | 54.4   | 46.7     | 71.4    | 159.7     | 86.0       | 43.5        |
| b, Å     | 54.4   | 46.7     | 43.0    | 44.1      | 43.6       | 43.5        |
| c, Å     | 210.7  | 406.1    | 118.1   | 95.7      | 95.5       | 825.4       |
| α, °     | 90     | 90       | 90      | 90        | 90         | 90          |
| β, °     | 90     | 90       | 101.01  | 108.1     | 111.9      | 90          |
| γ, °     | 120    | 120      | 90      | 90        | 90         | 120         |
| Resolution range, Å | 50.0-2.00 | 33.2-1.79 | 38.6-1.90 | 39.7-2.40 | 88.6-2.40 | 19.64-1.95 |
| Beamline | SPring-8 BL38B1 | SPring-8 BL38B1 | SLS X10SA | SLS X10SA | SLS X10SA | Nagoya Univ. |
| Wavelength, Å | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.54 |
| Completeness, % | 97.85 | 99.31 | 98.55 | 98.97 | 99.32 | 99.57 |
| Data processing | HKL2000 | HKL2000 | XDS | XDS | XDS | HKL2000 |
| Redundancy | 11.2 | 6.10 | 3.37 | 3.69 | 3.32 | 9.67 |
| I/σ(I) | 57.31 (6.19) | 19.3 (3.37) | 15.13 (1.85) | 9.77 (1.91) | 7.73 (1.83) | 15.63 (4.04) |
| Rmerge, % | 6.9 (42.1) | 11.2 (49.0) | 4.5 (69.4) | 10.7 (73.5) | 11.7 (64.1) | 10.0 (46.9) |
| Refinement | REFMAC5 | REFMAC5 | PHENIX | PHENIX | PHENIX | REFMAC5 |
| Template for molecular replacement | 3NTN | 2YNY | 3D9X, 3WP | 3WPP, 2YO2 | 3WPO | 2YO2 |
| Rwork/Rfree, % | 17.5/23.0 | 20.3/22.0 | 22.3/29.5 | 21.2/28.0 | 22.4/31.0 | 17.9/20.5 |
| Bond length/angle rmsd, Å/° | 0.018/1.764 | 0.006/1.056 | 0.004/0.689 | 0.009/1.139 | 0.008/1.089 | 0.012/1.38 |
| Ramachandran plot statistics, % | 96.19/3.49/0.32 | 98.16/1.84/0.0 | 97.52/2.48/0.0 | 95.83/3.13/1.04 | 95.73/4.10/0.17 | 100/0/0 |

(Preferred/Allowed/Outliers)
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Structural Basis for Toughness and Flexibility in the C-terminal Passenger Domain of Acinetobacter Trimeric Autotransporter Adhesin
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