Structural basis of the type IVb pilus-dependent transport for the colonization factor of *Vibrio cholerae*

Pathogenic bacteria utilize molecular machines called secretion systems to transport virulence-associated proteins to interact with and manipulate their host environment [1]. *Vibrio cholerae* secretes a cholera toxin (CT) through the type II secretion system (T2SS), leading to the development of severe diarrhea. The T2SS operates similarly to a piston, where a periplasmic proteinaceous filament, called endopilus (formerly known as a pseudopilus), extends and retracts repeatedly to expel the substrates from the cell. T2SS is evolutionally and structurally related to the type IV pilus (T4P) system. Some of T4P also function as a secretion system, transferring enzymes or colonization factors from the periplasm to the extracellular milieu. *V. cholerae* possesses a toxin-co-regulated pilus (TCP), which belongs to a subclass of T4P (T4bP) (Fig. 1(a)). This pilus is capable of transporting a soluble colonization factor, TcpF. TcpF is a bilobed protein comprising an N-terminal domain (NTD) and a C-terminal domain (CTD), and has an N-terminal flexible 25-residues extension [2]. Despite the important role of TcpF in the pathogenicity of *V. cholerae*, the mechanisms underlying TcpF secretion via T4bP remain unclear.

TCP consists of two structural components: the major pilin TcpA and the minor pilin TcpB (Figs. 1(b,c)). TcpA is arranged in a right-handed helical manner to form the main body of the TCP, whereas TcpB is located at the pilus tip and comprises three domains: pilin-like domain 1, beta-sheet domain 2, and β-sandwich-fold domain 3. TcpB forms a homotrimer by interacting with domains 2 and 3 and acts as the initiating complex for pilus formation [3]. Because TcpF is not secreted efficiently in a TCP-non-expressing strain lacking the *tcpA* gene, we hypothesized that TcpF interacts with one of the pilus subunits and is secreted upon pilus elongation. Isothermal titration calorimetry experiments showed that TcpF binds to TcpB, but not to TcpA. To clarify the interactions between TcpB and TcpF, we crystallized the complex and collected the diffraction data at SPring-8 **BL26B1**. The diffraction pattern of the TcpB–TcpF crystal was initially very poor at ~10.0 Å. However, the addition of sucrose to the reservoir solution and dehydration of the crystals resulted in a notable improvement in the resolution limit to ~4.0 Å. The initial phases were solved to a resolution of 4.05 Å by molecular replacement (MR) using the structures of TcpF and TcpB [4]. Three TcpF molecules were found above domain 3 of TcpB, forming a homotrimer shaped like a flower with three petals via interactions with the NTD (Fig. 1(d)). Overall, TcpB and TcpF formed a heterohexameric complex. Using native mass spectrometry and analytical ultracentrifugation experiments, we confirmed that this heterohexamer also exists in solution. Judging from the residual electron densities found at the clefts of the interface of each TcpB trimer, the N-terminal flexible extensions of TcpF molecules, rather than the NTDs and CTDs, are supposed to interact with the TcpB trimer. However, because of the low resolution of the data, it was not possible to model this interaction site.

To clarify the interaction between TcpB and the N-terminus of TcpF, we analyzed the structures of complexes formed between TcpB and the N-terminal TcpF (1–15) peptide. The diffraction data of these complexes at the highest resolution of 2.30 Å were obtained using BL26B1 [4]. We used the TcpB structure as an MR search model to solve the structures of the TcpB and TcpF (1–15) complexes. The N-terminal 11 residues of TcpF, from Phe1 to Val11, were responsible for its binding to the TcpB trimer (Fig. 2(a)). The N-terminal 5 residues of TcpF formed a type I β-turn structure like hook conformation stabilized by the pi–pi stacking of the Phe1–Tyr5 pair. Because Tyr5 is essential for TcpF secretion [2], this unique hook-like conformation seems to be important for its interaction with TcpB. We previously demonstrated that the N-terminal

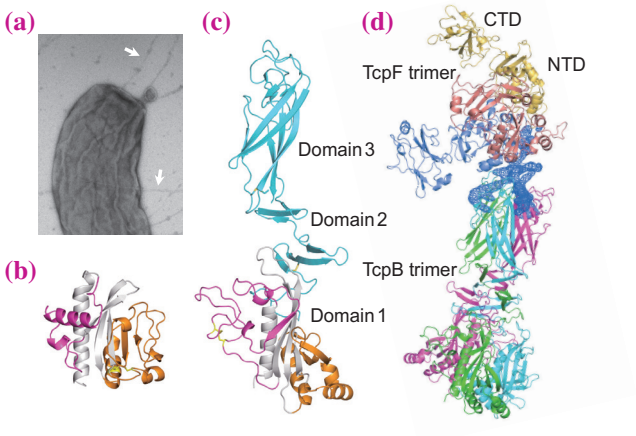

Fig. 1. The T4bP of *V. cholerae*. **(a)** Transmission electron micrograph of the pathogenic *V. cholerae* strain O395 forming TCP. **(b)** Crystal structure of the major pilin TcpA (PDB ID: 1oqv). **(c)** Crystal structure of the monomer of the minor pilin TcpB (PDB ID: 7w63). **(d)** Crystal structure of the TcpB–TcpF complex (PDB ID: 7w65). The omit map (blue, countered at 1.0σ) corresponding to the N-terminal portion of TcpF is depicted.

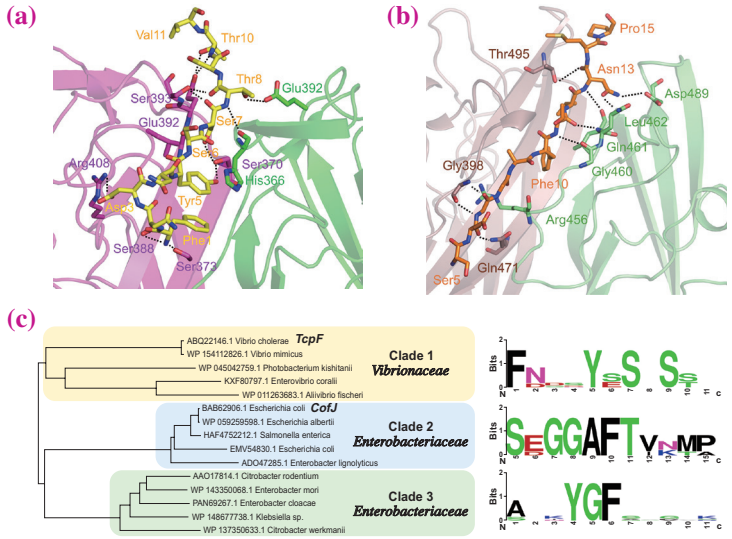

Fig. 2. Interaction between colonization factors and minor pilins in T4bP-expressing pathogenic bacteria. **(a)** Interactions between one TcpF (1–15) peptide (yellow) and two TcpB molecules (green and magenta) (PDB ID: 7w64). **(b)** Interactions between one CofJ (1–24) peptide (orange) and two CofB molecules (brown and lime green) (PDB ID: 5ypz). **(c)** Left panel: Phylogenetic tree of the colonization factor T4bP-SS in various pathogenic bacteria. Right panel: Sequence logo plots of the T4bP-SS in three clades.

region (Ser5–Pro15) of the secreted colonization factor CofJ binds to the minor pilin CofB of CFA/III and the T4bP of enterotoxigenic *Escherichia coli* (ETEC) (Fig. 2(b)) [5]. The aromatic residue Phe10 in the N-terminal region is essential for this interaction. Recognition of the aromatic amino acid in the colonization factor by the formation of a minor pilin trimer-dependent binding pocket is considered a common feature of the T4bP system. The colonization factor has an N-terminal segment containing approximately 10 residues, including important aromatic residues, named the T4bP secretion signal (T4bP-SS). We performed a phylogenetic analysis to compare the colonization factors of other pathogenic bacteria harboring T4bP and showed that T4bP-SS was divided into three clades, each with a characteristic motif containing aromatic residues (Fig. 2(c)). These results indicate that the minor pilins of each pathogenic bacterium may have evolved to recognize the T4bP-SS of its cognate colonization factor.

The structure of the TcpB–TcpF complex was refined using the TcpB–TcpF (1–15) structure to model the full length of TcpF, including T4bP-SS. We constructed an entire structural model of the TcpF–TCP complex, in which the TcpF trimer was found to be situated on the tip of TCP, using the TcpA filament model (Fig. 3) [4]. Based on these findings, we propose the hypothesis that the mechanism underlying TcpF secretion involves TCP elongation (Fig. 3) [4]. When TcpF binds to TcpB, TcpA assembles and the pilus elongates, simultaneously carrying TcpF through the TcpC secretin ring. Subsequently, TcpF separates from the TcpB trimer and is deposited near the bacterial cell. Alternatively, the TcpF trimer located at the TCP tip may bind to a putative receptor on intestinal epithelial cells.

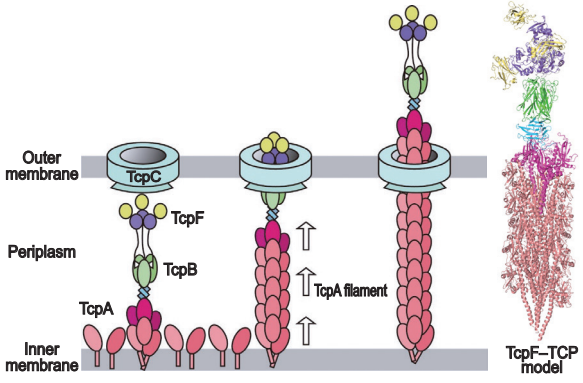

Fig. 3. A model depicting the transport of the colonization factor TcpF by TCP formation and elongation.

In recent years, there has been growing interest in anti-adhesive agents targeting bacterial adhesins and colonization factors. Because secreted colonization factors are critical for bacterial adhesion and colonization, the newly identified interactions between the minor pilin and the colonization factor T4bP-SS is an attractive target for the development of anti-adhesion agents against T4bP-expressing pathogenic bacteria, such as *V. cholerae* and ETEC.

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