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Localization of the flagellum-specific secretion signal in *Salmonella* flagellin ☆

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Abstract

The flagellum-specific export system is a specialized type III export machinery. Terminally truncated fragments of flagellin (FliC) were used to identify the secretion signal in the main component of flagellar filaments. The first 13 residues were not essential for export, but removal of 29 or more residues destroyed export ability. When an 8 kDa human protein domain was fused to various N-terminal fragments of FliC, the 26–47 sequence alone was sufficient to mediate secretion of this protein module through the flagellum specific export pathway. Neither half of this segment was enough to direct export of the attached protein domain. Our results demonstrate that the 22-residue long 26–47 segment within the disordered N-terminal region of *Salmonella* flagellin contains the recognition signal for the flagellar export machinery.

Keywords: Flagellum-specific export; Type III secretion; Export signal; Flagellin; *Salmonella typhimurium*

Flagella are the locomotion organelles of bacteria. These supramolecular surface structures extend from the cytoplasm to the cell exterior, and are composed of three major structural elements, the basal body, the hook, and the filament [1]. The major part of the flagellum is the helical filament, which works as a propeller rotated by a membrane-embedded molecular motor at its base part. Each filament may comprise as many as ~20000 flagellin subunits.

External flagellar proteins, lying beyond the cytoplasmic membrane, are synthesized in the cell and exported by the flagellum-specific export apparatus from the cytoplasm to the site of assembly at the distal end of the growing filament [2,3]. They are assumed to move through the narrow—25 to 30 Å wide—central channel of the flagellum as partially unfolded monomers. Since only the axial flagellar proteins and some controlling factors are transported through the central channel, some kind of gating apparatus has been thought to exist at the cytoplasmic face of the FliF ring complex of basal body to distinguish flagellar proteins from other cytoplasmic proteins and to facilitate their transportation. It is unclear how the axial proteins are recognized by the export system since they have no cleavable signal sequences or any common signals at the primary sequence level [4].

The flagellar export system shows close similarity to the family of type-III export systems [2,5]. The export substrates of type-III secretion systems do not contain cleavable signal sequences and they do not share common sequence motifs responsible for export. In the known cases they contain a segment of 15–30 amino acids in the N-terminal region which directs exports [6–8]. The recognition signal for flagellar export also seems to lie within the N-terminal region of export substrates. Previous experiments made in *Escherichia coli* have shown that a

☆ *Abbreviations*: CCP2, complement control protein 2 module of human complement C1r protein (C1r 375–447); FliC, flagellin.

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183-residue N-terminal fragment, which is just a little over one-third the size of the wild-type protein, can be recognized by the flagellum-specific export apparatus and exported from the cell [9]. Information essential for hook protein secretion is also located in the N-terminal portion of the molecule [10]. Nonetheless, comparison of the sequences of the exported flagellar proteins failed to identify candidates for the recognition signal at the primary sequence level, implying that recognition of a protein for export by the flagellum-specific system may involve recognition of a structure at higher levels [11].

Terminal disorder is a common structural feature of the axial proteins of the bacterial flagellum [12–14]. The disordered regions assemble into helical bundles upon polymerization of flagellar filaments [3] and belong to the most conserved part of these molecules. It has been speculated that this terminal disorder may serve as the recognition signal for the flagellum specific export apparatus [13]. The objective of this work was to localize the export signal in *Salmonella typhimurium* flagellin and to demonstrate the role of the disordered N-terminal region of the molecule in controlling recognition and translocation by the flagellum-specific export machinery.

Materials and methods

DNA constructs. Three N-terminally truncated flagellins, consisting of the amino acids 66–494 ($FliC_{66–494}$), 30–494 ($FliC_{30–494}$), and 14–494 ($FliC_{14–494}$), were made by mutagenesis using the Muta-Gene Phagemid kit from Bio-Rad (discontinued product). These three DNA constructs, which contained the natural promoter-operator and ribosome-binding sequence, were placed into the pBR322 vector.

FliC fragments corresponding to the amino acids 1–192 ($FliC_{1–192}$), 14–192 ($FliC_{14–192}$), 14–99 ($FliC_{14–99}$), 14–65 ($FliC_{14–65}$), 26–47 ($FliC_{26–47}$), 26–36 ($FliC_{26–36}$), and 37–47 ($FliC_{37–47}$) of *S. typhimurium* FliC were amplified by PCR using the proofreading advantage 2 DNA polymerase enzyme (BD Bioscience Clontech) and the full length *fliC* gene template. The PCR products were gel-purified, digested with *Hin*dIII and *Bam*HI enzymes, and were cloned into the pGFP expression vector (BD Bioscience Clontech) under the control of the *lac* promoter. The resulting constructs had the N-terminus of MTMITPSL followed by the flagellin sequence. $FliC_{1–192}$ and $FliC_{14–192}$ were made in two versions one set had a stop codon after the flagellin sequence, the other set, just like the other constructs, allowed to create fusion proteins using the *Bam*HI site. The gene encoding CCP2 (human C1r 375–447) [15] was inserted in-frame after each of the flagellin fragments. The CCP2 forward primers incorporated a *Bam*HI restriction site while the reverse primers incorporated a stop codon and a *Kpn*I site. Each resulting PCR product was gel-purified, digested with *Bam*HI/*Kpn*I, and ligated into the same sites of the flagellin-pGFP plasmid. The resulting fusion constructs contained a 6 bp sequence between the flagellin part and the CCP2 domain. All plasmids were sequenced to confirm the desired DNA sequence and the correct reading frame.

Electroporation, protein expression. The DNA used for electroporation was prepared by the alkaline lysis procedure. About 1 μg of circular DNA fragment was electroporated into the flagellin-deficient *S. typhimurium* strain SJW2536 [16]. The transformants were selected on Luria–Bertani medium plates containing ampicillin. For each protein construct one selected colony was grown at 37 °C in LB containing ampicillin to the late log phase. The supernatant was concentrated on 5 kDa cut-off spin concentrators and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting.

In order to check potential cell lysis or leakage, we carried out Western blot analysis of cellular fraction and growth medium supernatant using monoclonal antibodies against the periplasmic maltose binding protein.

SDS–polyacrylamide gel electrophoresis and immunoblotting. The samples were separated on 15% polyacrylamide gels, and the proteins were electrophoretically transferred to a nitrocellulose membrane. The membranes were treated with 5% nonfat dried milk in Tris-buffered saline and incubated with polyconal anti-flagellin antibody raised in rabbit, or anti-human C1r antibody raised in goat provided by Bio-Rad (C1r CCP2 containing samples). Signals were developed with alkaline-phosphatase-labeled anti-rabbit/goat immunoglobulin G (Sigma).

Results

Secretion of truncated flagellin fragments

To identify sequences essential for secretion of the *S. typhimurium* flagellin protein (FliC) across the cell envelope, we constructed various deletion mutants of flagellin. The role of the deleted segments in secretion was analyzed by testing for the extracellular accumulation of the truncated flagellins by Western blot assay of the culture medium.

Salmonella typhimurium flagellin is a 51 kDa protein with 494 amino acid residues. The terminal regions of the flagellin chain (about 66 N-terminal and 44 C-terminal residues) are unfolded in the monomeric form in solution [12]. In order to explore the contribution of the disordered N-terminal region of flagellin to the export mechanism we constructed several deletion mutants designated as $FliC_{66–494}$, $FliC_{30–494}$, and $FliC_{14–494}$ (Fig. 1A) which lacked 65, 29, and 13 N-terminal residues, respectively. All three fragments had an intact C-terminus. These DNA constructs which contained the natural flagellin promoter were introduced into flagellin deficient *Salmonella* SJW2536 cells, and protein expression was monitored. We detected the recombinant proteins by immunoblotting using polyclonal anti-flagellin antibody. Both the cellular and the cell culture supernatant fractions were analyzed. All three N-terminal deletion mutant flagellins could be detected inside the *Salmonella* cells, while only the $FliC_{14–494}$ mutant appeared in the cell culture supernatant (Fig. 2A). This means that all three mutant proteins were synthesized inside the cells but only $FliC_{14–494}$ contained the necessary signal for secretion. Truncation of 29 residues, or more, destroyed secretion ability.

A fragment containing only the N-terminal 183 residues of the 497-residue long wild-type *E. coli* flagellin had been reported to be exported by the flagellar export apparatus [9]. We constructed a similar deletion mutant of *Salmonella* flagellin, the $FliC_{1–192}$ fragment, which comprised the first 192 amino acid residues, and also the $FliC_{14–192}$ fragment which lacked additional 14 N-terminal residues (Fig. 1A). The corresponding gene constructs were inserted into the pGFP plasmid under the control of *lac* promoter and the proteins were expressed in SJW2536 cells. In these and subsequent experiments we used the non-natural *lac* promoter to see whether the secretion signal lies in the N-terminal region of the polypeptide chain or the 5′ untranslated

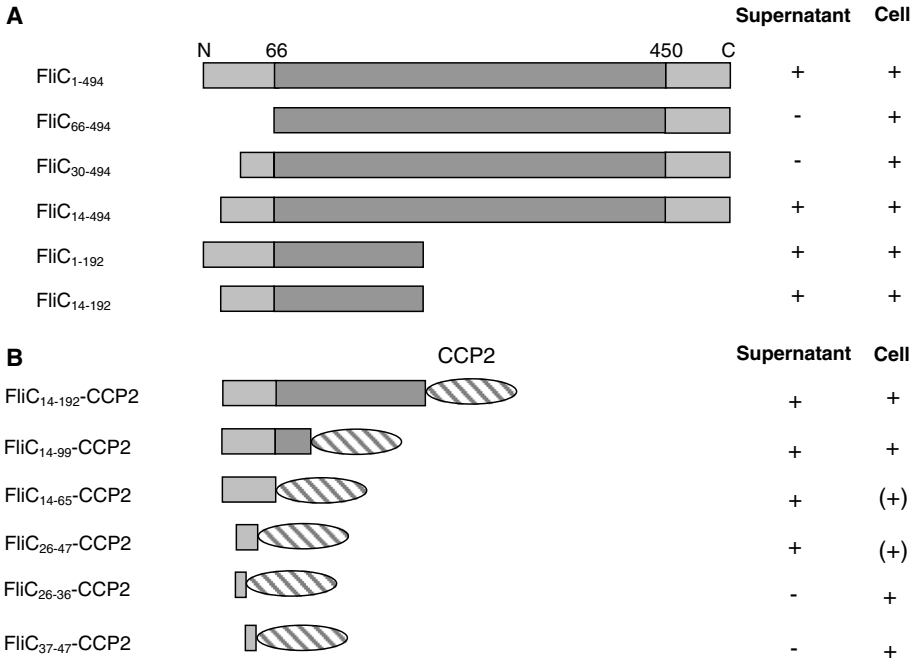

Fig. 1. Schematic representation of the gene constructs expressed in *Salmonella typhimurium* (SJW2536), and the localization of the resulting polypeptides in the cellular and supernatant fractions. The disordered terminal regions of FliC are indicated with light grey color. (A) Terminally truncated FliC fragments. (B) Flagellin fragments fused to the CCP2 domain of human complement protein C1r. (+) Below detection level.

region of *fliC* mRNA is required to promote export, as has been suggested by Majander et al. [17]. Both the $FliC_{1–192}$ and $FliC_{14–192}$ mutants appeared inside the cells and also in the cell culture supernatant (Fig. 2B). From the experiments with truncated FliC fragments we can draw the conclusion that the export signal in *Salmonella* flagellin resides between Thr14 and Ala192.

Directed export of CCP-fusions

Precise localization of the export signal required investigation of the secretion ability of small N-terminal fragments of flagellin. To avoid difficulties accompanying detection and identification of small fragments, we fused them to the 74-amino-acid long complement control protein 2 (CCP2) module of human complement protein C1r [15]. This non-bacterial fusion partner is unlikely to interfere with the bacterial secretion systems. The fusion constructs were produced at the DNA level, expressed in SJW2536 cells, and the secretion ability of the fusion proteins was studied. Immunoblotting was applied to identify the recombinant proteins in the culture medium or in the cell fraction using antibodies raised against the CCP2 domain. The following constructs were designed and produced: $FliC_{14–192}$-CCP2, $FliC_{14–99}$-CCP2, and $FliC_{14–65}$-CCP2 (Fig. 1B). The latter fusion contained a segment located exclusively within the disordered N-terminal part of flagellin. DNA constructs encoding the fusion proteins were inserted into the pGFP expression vector, and protein expression experiments were carried out. As shown in Fig. 1B and 3, all of these constructs were secreted into the culture medium. Secretion efficiency of fusion proteins was similar to that of wild-type FliC, producing protein yields of 10–15 mg/l as estimated from Coomassie stained SDS–PAGE of concentrated cell culture supernatants. In all cases, the periplasmic maltose binding protein could not be detected in the same culture supernatant preparations, showing that the presence of fusion polypeptides in this fraction was not a result of a bacterial cell lysis or non-specific leakage (data not shown). Our observations clearly indicate that the export signal is situated in the disordered N-terminal region of flagellin.

Comparison of the N-terminal part of flagellin sequences from various bacteria shows (Fig. 4) that the 26–47 segment is highly conserved which implies important structural or functional roles. We checked whether the export signal is contained within this conserved region. The fusion protein $FliC_{26–47}$-CCP2 was constructed and found to be secreted into the culture medium with high efficiency (Fig. 3C). The concentration of secreted $FliC_{26–47}$-CCP2 was 10–12 mg/l in the culture supernatant. When the first or second half of the highly conserved 26–47 segment was used to create the $FliC_{26–36}$-CCP2 or $FliC_{37–47}$-CCP2 constructs, the fusion proteins remained inside the cell (Fig. 3C). These experiments demonstrate that the 26–47 segment is required for flagellar secretion.

Discussion

The most elusive part of flagellar export is the recognition of export substrates. We attempted here to localize the export signal in *S. typhimurium* flagellin. Using N-terminal

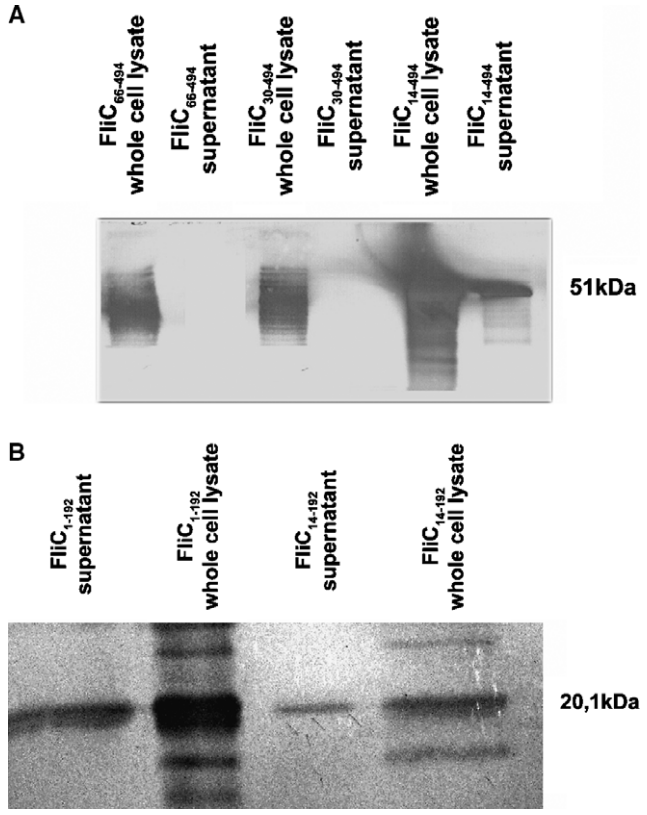

Fig. 2. Secretion analysis of (A) N-terminal deletion mutant flagellins which include natural promoter-operator and ribosome-binding sequences, and (B) flagellin fragments containing large C-terminal deletions under the control of *lac* promoter. Flagellin fragments were expressed in flagellin-deficient *Salmonella* SJW2536 cells. Whole-cell lysates and culture supernatants were examined by Western blotting using a polyclonal anti-flagellin antibody. The antibody developed against the full-length flagellin recognizes these heavily truncated fragments only weakly. Longer development is required resulting in the appearance of nonspecific bands.

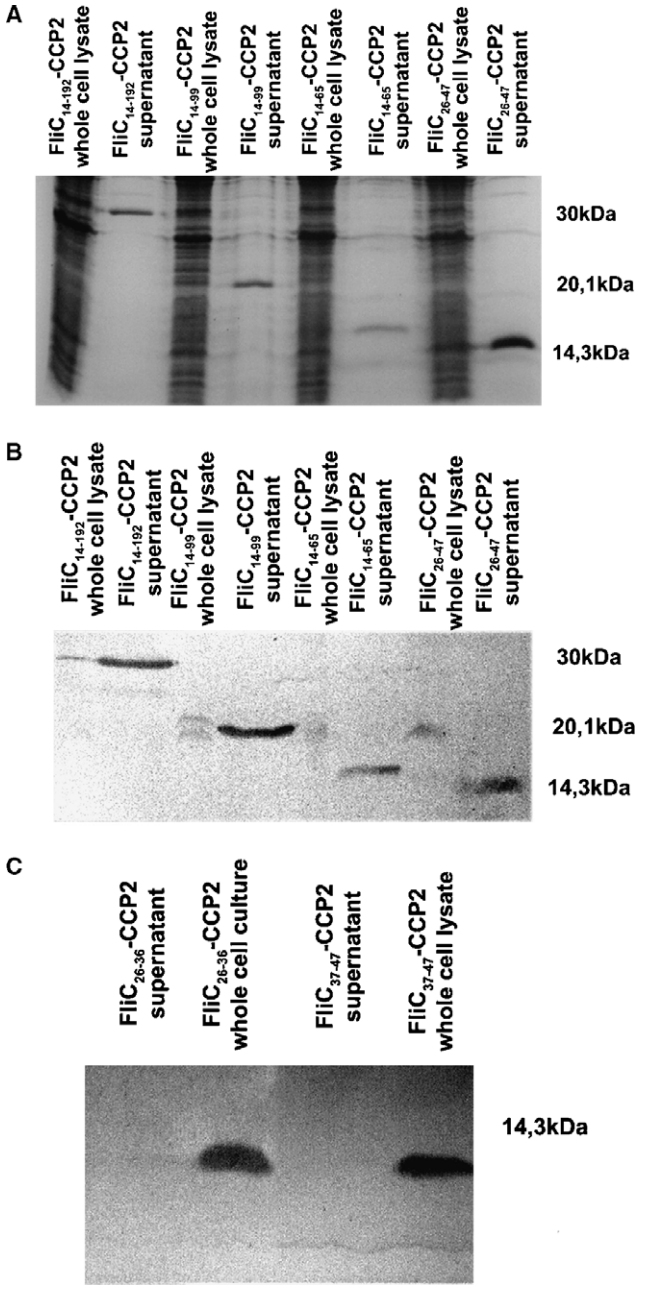

Fig. 3. The heterologous CCP2 domain from human complement C1r protein was attached to the C-terminus of various FliC fragments. Fusion constructs were expressed under the control of *lac* promoter in flagellin-deficient *Salmonella* SJW2536 cells. Whole-cell lysates and culture supernatants were examined by SDS–PAGE (A) and Western blotting (B,C) using a polyclonal anti-C1r antibody. (A) Coomassie-stained gel and (B) Western blot of $FliC_{14–192}$-CCP2, $FliC_{14–99}$-CCP2, $FliC_{14–65}$-CCP2, and $FliC_{26–47}$-CCP2 fusion constructs. (C) Western blot of $FliC_{26–36}$-CCP2 and $FliC_{37–47}$-CCP2.

truncated fragments we demonstrated that the first 13 residues are not essential for export, but removal of 29 or more residues destroys export ability. When various N-terminal fragments of flagellin were fused to the CCP2 domain of human complement protein C1r [15], the $FliC_{26–47}$ fragment was found to be the smallest segment of flagellin which was sufficient to cause secretion of the CCP2 module through the flagellum-specific export pathway. Our results demonstrate that the 22-residue long $FliC_{26–47}$ segment contains the recognition signal for the flagellar export machinery. Neither half of this segment was enough to direct export of the attached CCP2 domain, indicating that secretion of $FliC_{26–47}$-CCP2 is not a result of overloading the flagellar export apparatus with a protein product overexpressed by a high copy number artificial plasmid.

Recently Majander et al. [17] reported that N-terminal FliC polypeptides lacking the C-terminus were efficiently secreted when the corresponding gene fragments were expressed from the *fliC* promoter, but remained within the cell when non-natural promoters were used. They concluded that the 5′ untranslated region (UTR) of *fliC* mRNA mediates secretion of flagellin in *E. coli*. However, our results clearly show that the 5′ UTR of *Salmonella* flagellin gene is not essential for export and the polypeptide signal contains enough information to direct secretion of flagellin or other fusion proteins. In our secretion experiments with CCP2 fusions, the non-flagellar *lac* promoter was used without any natural upstream

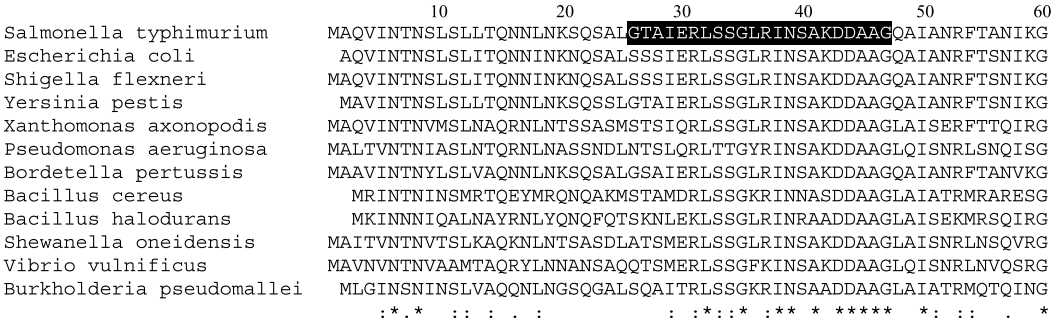

Fig. 4. Comparison of the N-terminal amino acid sequences of flagellins from various bacterial species. The export signal identified in *Salmonella* flagellin is indicated by a black box. FliC sequences were aligned by the ClustalW program [19].

or downstream DNA sequences of the *fliC* gene, but the secretion efficiency of fusion proteins was very similar to that reported in Ref. [17]. Moreover, truncated flagellin molecules having large N-terminal deletions ($FliC_{66–494}$ and $FliC_{30–494}$) were not secreted even under the control of their natural promoter (Fig. 2B) and remained inside the cells.

Comparison of the amino acid sequences of flagellins from various bacterial sources revealed that the segment containing the export signal corresponds to the most conserved part of the disordered N-terminal region (Fig. 4). On the other hand, this segment does not show significant sequence homology to the previously identified flagellar export signals of hook protein [10] or the anti-sigma factor FlgM [18]. However, there are common features: all of the known signal sequences were mapped to the disordered N-terminal portions of the export substrates. It is worth noting that disordered terminal regions of all flagellar axial proteins as well as FlgM show high propensity for formation of amphipathical helical structure [11,12,18]. It is tempting to speculate that the recognition process by the flagellum-specific export apparatus is accompanied by inducing a stable amphipathic helical conformation in the largely different sequences of flagellar export signals.

Acknowledgments

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