FlhB Regulates Ordered Export of Flagellar Components via Autocleavage Mechanism*

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The bacterial flagellum is a predominantly cell-external super-macromolecular construction whose structural components are exported by a flagellum-specific export apparatus. One of the export apparatus proteins, FlhB, regulates the substrate specificity of the entire apparatus; i.e. it has a role in the ordered export of the two main groups of flagellar structural proteins such that the cell-proximal components (rod-/hook-type proteins) are exported before the cell-distal components (filament-type proteins). The controlled switch between these two export states is believed to be mediated by conformational changes in the structure of the C-terminal cytoplasmic domain of FlhB (FlhBC), which is consistently and specifically cleaved into two subdomains (FlhBCN and FlhBCC) that remain tightly associated with each other. The cleavage event has been shown to be physiologically significant for the switch. In this study, the mechanism of FlhB cleavage has been more directly analyzed. We demonstrate that cleavage occurs in a heterologous host, Saccharomyces cerevisiae, deficient in vacuolar proteases A and B. In addition, we find that cleavage of a slow-cleaving variant, FlhB(P270A), is stimulated in vitro at alkaline pH. We also show by analytical gel-filtration chromatography and analytical ultracentrifugation experiments that both FlhBC and FlhB(P270A) are monomeric in solution, and therefore self-proteolysis is unlikely. Finally, we provide evidence via peptide analysis and FlhB cleavage variants that the tertiary structure of FlhB plays a significant role in cleavage. Based on these results, we propose that FlhB cleavage is an autocatalytic process.

A large percentage of the bacterial flagellar structure lies outside of the cell envelope, thus requiring that the vast majority of the subunits that compose the flagellum be exported from the cytosol across both the inner and outer membranes. Salmonella employs a type III export pathway to accomplish this (1, 2). It is a Sec-independent pathway that utilizes a flagellum-specific export apparatus to transport flagellar components across the inner membrane. These exported proteins then travel the length of the developing flagellum within an interior channel prior to their incorporation at the structure’s cell-distal end (3–6) (the developing structure therefore facilitates export across the outer membrane). At least nine flagellar proteins are involved in the flagellum-specific export apparatus (7). Six are integral membrane components (FlhA, FlhB, FliO, FliP, FliQ, and FliR) postulated to be located within the basal body MS ring (8, 9), and three are soluble components: an ATPase (Flj) that drives export (10), a regulator of the ATPase (FliH) (11–13), and a general chaperone (FliJ) (14, 15).

One of the integral membrane proteins, FlhB, has been found to play a central role in export substrate-specificity switching; i.e. regulation of the order in which flagellar subunits are exported, such that proteins incorporated into the cell-proximal rod and hook structures (early export) are exported before proteins that polymerize to form the distal hook-filament junction and flagellum filament structures (late export) (16, 17). FlhB is a 42-kDa, 383-amino acid protein that has four putative transmembrane helices in its N-terminal domain (FlhBTM) and a sizable hydrophilic C-terminal domain (FlhBC) that is predicted to lie in the cytosol (17) (Fig. 1). The FlhBC domain itself is also divided into two subdomains: FlhBCN (amino acids 211–269) and FlhBCC (amino acids 270–383) connected via a proposed flexible hinge, based initially on the observation that overproduced soluble FlhBC was consistently and specifically cleaved within the hinge (N269↓P270) with a half-life of ~5 min, and the resulting subdomains remain tightly associated with each other so that they may be copurified (17). Site-specific mutagenesis of the highly conserved cleavage site sequence TN269↓P270 produced two variants, FlhBN269A and FlhBP270A, each having a significant effect on FlhB function (16). The FlhBN269A mutation completely inhibits cleavage, and the FlhBP270A mutation slows it down significantly. Both mutant proteins are severely defective in their ability to mediate export of the “late” flagellar protein, FliC, whereas early flagellar protein export is unchanged. Cleavage of FlhBC is thus necessary for proper FlhB function. Previous studies also indicate that the cleaved state of FlhBC, rather than the cleavage event itself, is necessary for substrate-specificity switching (16). When FlhBTM+CN and FlhBCC are expressed from two different plasmids (resulting in an “already cleaved” FlhB) they are able to successfully complete both rod/hook and filament formation, although not to wild-type levels (17). All these observations led to a proposal that the C-terminal domain of FlhB has two substrate-specificity states and that a conformational change, mediated by cleavage and the interaction between FlhBCN and FlhBCC, regulates the specificity-switching process.

In an attempt to better understand the role of FlhB cleavage in substrate-specificity switching, we addressed the fundamental question:

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2 The abbreviations used are: FlhBC, cytoplasmic domain of FlhB; FlhBP270A, slow-cleaving variant of FlhB; FlhBN269A, non-cleaving variant of FlhBC; FlhBCC, N-terminal subdomain of FlhBC; FlhBCN, C-terminal subdomain of FlhBC; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight.
how does FlhB cleavage occur? We proposed and tested three possible causes of cleavage: 1) protease; 2) self-proteolysis; and 3) autocleavage. To do this, we assayed for FlhB cleavage in a heterologous host, characterized cleavage in vitro, assayed for FlhB domain interactions, tested an autocleavage mechanism, and further analyzed the cleavage site. The results support autocleavage via a succinimide pathway in which the cleavage site itself is the catalytic domain, although active only when FlhB is in the appropriate conformation. The implications of this on current models of export substrate-specificity switching are discussed.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Media**—Strains and plasmids used are listed in TABLE ONE. Luria medium and plates were prepared as described previously (18). Ampicillin was added to growth media as needed at a final concentration of 100 μg/mL. T4 DNA ligase, and all restriction enzymes were purchased from New England Biolabs (Beverly, MA). Primers were purchased from Integrated DNA Technologies (Dallas, TX). DNA sequencing was performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University.

**Overproduction and Purification of FlhB**—Tagged proteins were overproduced and purified from BL21(DE3)plyS carrying PET-based plasmids encoding N-terminally His- and FLAG-tagged FlhB and variants, as described in the BD TALON™ Metal Affinity Resins User Manual (BD Biosciences). Cell lysates were treated with the protease inhibitor mixture, Complete, Mini-EDTA free (Roche Diagnostics, Mannheim, Germany), prior to column purification. Eluates were separated by 15% SDS-PAGE, and proteins were either detected by staining with Coomassie Brilliant Blue or transferred to nitrocellulose for Western blotting.

**Immunoblotting**—Immunoblotting with anti-Flag and anti-His was carried out using an ECL Plus immunoblotting detection kit (Amersham Biosciences).

Detection of Exported Proteins in Culture Supernatants—Isolated second-site flhB suppressor strain cell cultures were grown at 37 °C to mid-log phase (A600 = 0.6–0.8) and then harvested by low speed centrifugation. Proteins in the culture supernatants were precipitated by 10% trichloroacetic acid, incubated at 4 °C overnight and centrifuged at 27,720 × g, 4 °C, for 30 min. The pellets were washed with 1 ml of acetone, centrifuged as before, resuspended in SDS-PAGE loading buffer, and boiled for 10 min before SDS-PAGE and immunoblotting with polyclonal antibody against either FlgD or FliC.

**Yeast Methods**—The yeast-bacterial shuttle vector, pCu415CUP1 (20), with a Cu2+ -inducible promoter as well as a selectable Leu marker, was used for cloning and expression of His-FLAG-FlhB and His-FLAG-FlhB(N269A), and His-FLAG-FlhB(P270A). This shuttle vector replicates well in Escherichia coli and yeast and expresses well in yeast. Rich (YPD) and minimal (SD) media were prepared as described (21). Wild-type Saccharomyces cerevisiae (MHY5000) (22) and protease-deficient (MHY1060) (23) yeast were transformed with plasmids encoding the wild-type or variant flhB gene or the empty vector. Total cell extracts were prepared from logarithmically growing yeast cultures (2.5 A600). The cells were subsequently lysed in a protocol similar to the 20% trichloroacetic acid method of Fiorni et al. (24). Briefly, the cells were washed with 20% trichloroacetic acid and then resuspended in 400 μL of 20% trichloroacetic acid. Cells were disrupted by vortexing for 5 min in the presence of glass beads. The lysates were drained from the beads and centrifuged for 10 min at 16,000 × g in a tabletop microcentrifuge. The pellets were washed once with 2% trichloroacetic acid and then resuspended in 200 μL of 1× Laemmli buffer, neutralized by adding 30 μL of 1 M Tris base, boiled for 5 min, and clarified by centrifugation. Cell lysates were loaded onto an SDS-PAGE gel and analyzed by Western blotting.

**Analytical Ultracentrifugation**—Sedimentation equilibrium analytical ultracentrifugation was performed using a Beckman Optima XL-A analytical ultracentrifuge with an An60Ti rotor (Beckman). Prior to ultracentrifugation the protein samples were extensively dialyzed against TNE buffer (50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, and 1 mM EDTA), which was also used as the blank. Measurements were made at 20 °C at speeds of 26,000, 29,000, and 32,000 rpm on wild-type FlhB (A600 of 0.36) and FlhB(P270A) (A600 of 0.46) using a charcoal-filled Epon and quartz windows. Concentration profiles of the samples were monitored by absorbance at a wavelength of 280 nm and recorded at a spacing of 0.001 cm in the step mode, with 20 averages per step, for 10, 16, and 22 h after each rotor speed was reached. Equilibrium data were analyzed using Beckman Optima™ XL-A/XL-D data analysis software, version 4.0, provided as an add-on to Origin Version 4.1 (MicroCal Inc.). The partial specific volume, 0.729 ml/g, for wild-type FlhB and for FlhB(P270A), was based on the amino acid composition of each protein.

**Analytical Gel-filtration Chromatography**—Analytical gel-filtration chromatography of wild-type FlhB and FlhB(P270A) was performed with a Superdex™ 75 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) were used as size markers. All fractions were monitored by SDS-PAGE.

**Peptide Synthesis**—Small scale, N-(9-fluorenyl) methoxycarbonyl (Fmoc) peptide synthesis was carried out on a Rainin Symphony instrument (Rainin Instrument, Woburn, MA) that provides on-instrument (Fmoc) peptide synthesis was carried out on a Rainin Symphony instrument (Rainin Instrument, Woburn, MA). The partial specific volume, 0.729 ml/g, for wild-type FlhB and for FlhB(P270A), was based on the amino acid composition of each protein.

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**Peptide Synthesis**—Small scale, N-(9-fluorenyl) methoxycarbonyl (Fmoc) peptide synthesis was carried out on a Rainin Symphony instrument (Rainin Instrument, Woburn, MA) that provides on-instrument cleavage of the peptide from the resin, by the Keck Foundation Biotechnology Resource Laboratory at Yale University. The peptide synthesis service included an analytical reverse-phase high-performance liquid chromatography profile and a MALDI-TOF mass spectrum of each peptide synthesized.
FlhB Autocleavage

TABLE ONE

| Strains and plasmids used in this study | Relevant characteristics | Source or reference |
|----------------------------------------|--------------------------|---------------------|
| Strain/plasmid                          |                          |                     |
| E. coli                                |                          |                     |
| Nova Blue                              | Recipient for cloning experiments | Novagen            |
| BL21(DE3)pLysS                         | For overproduction of proteins from pET-based plasmids | Novagen            |
| S. cerevisiae                          |                          |                     |
| SJW1103                                | Wild-type for motility and chemotaxis | (43)               |
| MMB2701                                | Isolated flhB extragenic suppressor of fliK polyhook mutant | (28)/This study    |
| MMB2714                                | Isolated flhB extragenic suppressor of fliK polyhook mutant | (28)/This study    |
| MMB3018                                | Isolated flhB extragenic suppressor of fliK polyhook mutant | (28)/This study    |
| MMB3201                                | Isolated flhB extragenic suppressor of fliK polyhook mutant | (28)/This study    |
| MMB3519                                | Isolated flhB extragenic suppressor of fliK polyhook mutant | (28)/This study    |
| S. cerevisiae                          |                          |                     |
| MHY500                                 | Wild-type                | (22)                |
| MHY1060                                | Proteinase A- and B-deficient | (23)                |
| Plasmids                                |                          |                     |
| pTrc99A-FF4                            | Modified pTrc expression vector | (44)               |
| pET19bF                                | pT7 expression vector, N-terminal His and FLAG tags | (10)               |
| pCU415CUP1                             | Yeast-bacterial shuttle vector, Cu^{+2}-inducible promoter | (20)               |
| pMM4                                   | pET19bF flhBc           | (17)                |
| pHFFBc(P270A)                          | pET19bF flhBc(P270A)    | This study          |
| pHF201                                 | pTrc99A-FF4 flhBc(N269A) | This study          |
| pHF500                                 | pCu415CUP1 N-terminal His and FLAG tags flhBC | This study          |
| pHF501                                 | pCu415CUP1 N-terminal His and FLAG tags flhBC(P270A) | This study          |
| pHF502                                 | pCu415CUP1 N-terminal His and FLAG tags flhBC(N269A) | This study          |

RESULTS

Analysis of FlhBc Cleavage in a Heterologous Host—As reported previously, cleavage of overexpressed FlhBc occurs in mutants defective in the flhDC master operon (16), suggesting that should a protease be involved, it is highly unlikely to be a flagellar-specific protein. Attempts to identify a candidate protease in E. coli and Salmonella genome databases were unsuccessful. Also, querying a peptidase database against the full consensus TNPTH and the limited consensus NP of the FlhB cleavage site failed to identify any candidates (16). We thus decided to assay for cleavage when FlhBc is overexpressed in S. cerevisiae, based on the assumption that it is highly unlikely that this heterologous host would have a protease that would consistently and specifically cleave a bacterial type III flagellar export component. For convenience the assays were conducted with the soluble domain of FlhB (FlhBc), because both overexpressed full-length FlhB and FlhBc are cleaved. His-FLAG-FlhBc, His-FLAG-FlhBc(N269A), and His-FLAG-FlhBc(P270A) were expressed in two different yeast strains: MHY500 (wild-type) and MHY1060 (deficient in the vacuolar proteinases A and B, which affect non-specific proteolysis of proteins produced in the cell). Whole cell lysates were analyzed by SDS-PAGE followed by Coomassie staining or of yeast whole cell lysates monitored by immunoblotting with a monoclonal anti-FLAG antibody. MHY500 is wild-type S. cerevisiae. MHY1060 is a proteinase A- and B-deficient strain. As described previously, cleavage products of N-terminally tagged wild-type FlhBc run as an apparent singlet on SDS-polyacrylamide gels (17). Due to the fact that the anti-FlhBc antibody recognizes flagella proteins produced in both yeast strains. His-FLAG-FlhBc is detected in a completely cleaved state, His-FLAG-FlhBc(N269A) in a non-cleaved state, and His-FLAG-FlhBc(P270A) also in a non-cleaved state. Similarly, the blot probed with anti-FlhBc shows the secondary cleavage site products observed previously (16). A second set of aliquots from the same cultures used for these analyses was prepared in parallel but using a different lysis method (NaOH/SDS). Interestingly, the cleavage states of His-FLAG-FlhBc and His-FLAG-FlhBc(N269A) were identical to that shown in Fig. 2, but His-FLAG-FlhBc(P270A) was detected in a completely cleaved state (data not shown).

In Vitro Cleavage Assays of the Slow-cleaving Variant of FlhBc, FlhBc(P270A)—A straightforward experiment addressing the possibility that FlhBc may be capable of self-cleaving, either via a proteolytic

![FIGURE 2. Cleavage states of FLAG-tagged wild-type FlhBc, a slow-cleaving variant, FlhBc(P270A), and a non-cleaving variant, FlhBc(N269A), A, SDS-polyacrylamide gel of yeast whole cell lysates monitored by immunoblotting with a polyclonal anti-FLAG M2 antibody; B, monitored with a polyclonal anti-FlhBc antibody. MHY500 is wild-type S. cerevisiae. MHY1060 is a proteinase A- and B-deficient strain. As described previously, cleavage products of N-terminally tagged wild-type FlhBc run as an apparent singlet on SDS-polyacrylamide gels (17). Due to the fact that the anti-FlhBc antibody recognizes flagella proteins produced in both yeast strains. His-FLAG-FlhBc is detected in a completely cleaved state, His-FLAG-FlhBc(N269A) in a non-cleaved state, and His-FLAG-FlhBc(P270A) also in a non-cleaved state. Similarly, the blot probed with anti-FlhBc shows the secondary cleavage site products observed previously (16). A second set of aliquots from the same cultures used for these analyses was prepared in parallel but using a different lysis method (NaOH/SDS). Interestingly, the cleavage states of His-FLAG-FlhBc and His-FLAG-FlhBc(N269A) were identical to that shown in Fig. 2, but His-FLAG-FlhBc(P270A) was detected in a completely cleaved state (data not shown).]
domain or an autocleavage mechanism, was to test if, and under what experimental conditions, purified FlhBc cleavage occurs in vitro. Due to the fact that wild-type FlhBc is cleaved very rapidly, the slow-cleaving variant, FlhBc(P270A), was used to follow cleavage under different conditions. His-FLAG-FlhBc(P270A), was overexpressed in E. coli cells and purified under native conditions via a cobalt-affinity column in the presence of a protease inhibitor mixture. Those elution fractions containing purified protein was divided into three aliquots, and each was incubated under different pH conditions for 90 min at 25 °C. Concentrated hydrochloric acid was added to achieve a pH of 13.6. All samples were neutralized prior to analysis by SDS-PAGE. The pH value of 8.6 is the unmodified pH of the protein in column elution buffer. Cleavage products of N-terminally tagged wild-type FlhBc run as an apparent singlet on SDS-polyacrylamide gels (17). C, FlhBc; CC, FlhBc CC, FlhBc CC.

Hydrodynamic Properties of Wild-type and Slow-cleaving Variants of FlhBc—Oligomerization analysis was performed to assess whether FlhBc might be able to act as a protease on another FlhBc. To accomplish this, we first carried out analytical gel-filtration chromatography of His-FLAG-FlhBc and His-FLAG-FlhBc(P270A) with a Superdex 75 HR 10/30 column (Fig. 4). Wild-type FlhBc and FlhBc(P270A) were found to elute at volumes of 12.5 and 12.3 ml, respectively, yielding apparent molecular masses of ~26 kDa and 27 kDa, respectively, indicating that they are monomeric in solution. To further test the oligomeric states of His-FLAG-FlhBc and His-FLAG-FlhBc(P270A), we performed sedimentation equilibrium analytical ultracentrifugation, a technique used to determine the molecular mass of particles independent of their shape (Fig. 5). The data were collected and analyzed using the manufacturer’s software. A single species model produced the best fit for both samples in terms of low residuals and calculated molecular masses of 19.5 kDa for His-FLAG-FlhBc and 21.1 kDa for His-FLAG-FlhBc(P270A), which agree well with each of their deduced molecular masses. Therefore, according to both techniques, FlhBc and FlhBc(P270A) are monomeric in solution, suggesting that cleavage is internal and not due to persistent dimerization interactions. Cleavage as a result of transient interactions seems unlikely as there was no observed change in FlhBc(P270A) cleavage when incubated with other FlhBc variants. Further emphasizing the role of internal conformation, although previous studies showed that a construct expressing FlhB(P270A) and an additional wild-type FlhBc subdomain had previously been shown to be unnecessary for cleavage to occur (16, 17). Thus,
the possibility that the cleavage site itself might constitute the "catalytic domain" was considered. Precedent for such a non-enzymatic autocleavage mechanism is found in the intein excision pathway (26), as well as in the aging process of the optical lens protein, α-crystallin (27). Both events involve asparagine cyclization to a succinimide intermediate resulting in deamidation or peptide bond cleavage. Deamidation is impossible with wild-type FlhB because of the presence of a proline residue downstream of the cleavage site, and thus we tested for the presence of the succinimide ring at the C terminus of the FlhBC, using high-performance liquid chromatography, and was of the expected amino acid sequence; i.e., no deviation that might be attributed to the presence of a succinimide ring was detected, suggesting that a local conformation that is present in FlhB is necessary for cleavage to occur. In parallel, three 33-residue oligopeptides composed of the cleavage region (and its two variants) and an 82-residue oligopeptide with the wild-type cleavage site, all bearing N-terminal His and C-terminal FLAG tags, were produced in vitro, and no cleavage was detected by Coomassie staining or by immunoblotting using anti-His and anti-FLAG antibodies.3 Also, the oligopeptides were produced in wild-type Salmonella and tested for effects on motility, considering the possibility of titration of a possible protease away from wild-type FlhB, and no change in swimming behavior was observed (data not shown).

**Flagellar Export Assays of FlhB Cleavage Variants**—The FlhB export substrate-specificity switch is also regulated by interactions between FlhB and another flagellar protein, FliK (17, 28). FliK is the flagellar hook-length control protein responsible for maintaining the well-defined hook length of ~55 nm observed in wild-type flagella (29). The typical fliK mutant phenotype is the appearance of extended hooks without filaments called "polyhooks" (30). Interestingly, all fliK intergeneric suppressor mutations found, i.e., mutations that cause random but belated switching of export substrate-specificity and thus restore filament formation, lie within the FlhBC subdomain, the earliest being S274F (28), just four residues after the FlhBCN-FlhBCC boundary. In these strains, FlhB has the capacity to switch specificity state autonomously, but in a manner that is unlinked from correct completion of the hook, so that the polyhook filament phenotype is generated. In addition, these strains are substantially resistant to the Asn-269/Pro-270 cleavage process, indicating that the conformation of the protein has been changed in a manner that affects the hinge region. Based on this latter observation we made a closer inspection of the literature and determined that there exists an approximate correlation between cleavage efficiency and motility. The reported motility abilities, as measured by motility assay on soft agar tryptone plates (28), of the isolated second site fliB* suppressor strains (with a wild-type copy of fliK present) approximately correlate to cleavage abilities, as determined by pulse-chase labeling (17) and subsequent analysis of the autoradiogram using ImageJ densitometry software (National Institutes of Health, this study). In an attempt to clarify this correlation further, we carried out export assays of the isolated second site fliB* suppressor strains and determined that the amount of FlhC (late export substrate) present in culture supernatants also approximately correlates with the published motility abilities of the suppressor strains and thus the cleavage abilities of the FlhB variants, i.e., those strains that swim well (e.g. MMB2701), cleave efficiently and export FliC close to wild-type levels, whereas those strains that swim poorly (e.g. MMB2714), cleave poorly and export FliC at low levels (Fig. 6). Because the levels of FlgD (early export substrate) exported are also diminished relative to wild-type, it remains unclear whether an altered substrate recognition or inefficient cleavage is responsible for the change in export ability. It should be recalled that preventing cleavage, as in FlhB(N269A), does not prevent export altogether, rather only the export of filament-type substrates is blocked.

**DISCUSSION**

The goal of this study was to determine how the flagellar export apparatus component FlhB undergoes cleavage, and to use this information to better understand the role that cleavage plays in export substrate-specificity switching. We found the most convincing explanation for FlhB cleavage to be that the region in the immediate vicinity of the cleavable bond is self-cleavable, i.e., that FlhB undergoes autocleavage. There are numerous precedents for this involving Asn and Asp cyclization to a succinimide intermediate (e.g. Ref. 31), although rates are generally much slower (hours to years) than the ones we observe (minutes). Cyclization can either involve the peptide nitrogen (with deamidation) or the amide nitrogen (with bond cleavage) (Fig. 7). In the former case, reopening of the ring results in reformation of the peptide bond. Frequently isomerization (Asp to Iso-Asp or Asn to Iso-Asn) is observed. In the case of Asn-Pro, as is present in wild-type FlhB, the peptide bond nitrogen cannot participate in cyclization and so cleavage is the only

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3 J. L. McMurray and H. U. Ferris, unpublished observation.
available pathway (32). In other words, deamination is prevented, because the Pro linkage to the peptide bond nitrogen prevents it from attaining the necessary charge density, through deprotonation, to displace the Asn side-chain amide (via a nucleophilic attack) (33).

There have been various studies to determine which factors influence the formation and resolution of succinimide rings, including the effects of pH. At alkaline pH, the Asn residue is quite labile, because its side chain is sterically suited to interact with the peptide nitrogen of the following residue to transiently form a succinimidyl derivative. Consistent with this, when purified FlhB_2 (P270A) was subjected to increasingly basic conditions, the cleavage rate was enhanced (Fig. 3B). This would explain why in previous studies a small percentage of full-length FlhB could be harvested from cell lysates prepared by boiling in SDS loading buffer, pH 6.8, whereas no full-length FlhB could be detected by column purification with an elution buffer of pH 8.0 or greater.

Although many of the succinimide studies have been performed using peptides, tertiary structure has been suggested to play a significant role in Asn degradation as well. It is thought that different conformations induced by changes in the side-chain of the residue following Asn make the peptide nitrogen more or less acidic (thus affecting the equilibrium between the protonated and the deprotonated states) (34). It is thus not unexpected that the replacement of the Pro residue by Ala in the FlhB(P270A) variant resulted in an altered cleavage behavior from that of wild-type FlhB. The reduced rate of cleavage of FlhB(P270A) might be due to a conformational difference, with the variant protein being in the proper conformation for succinimide ring formation only transiently. Yet, theoretically, Ala is not prohibited from deprotonation, like Pro is, and thus, the preferred deamination pathway should be available. We propose that FlhB(P270A) remains biased toward the cleavage pathway due to the influence of tertiary structure. The importance of structure is nicely illustrated with the flk suppressor FlhB variants, whose cleavage behavior varied significantly from that of the wild-type, after only a single amino acid substitution (from Gly to Val) 24 residues downstream from the cleavage site. Similarly, in the mechanism of autoproteolytic cleavage of the NoV viral coat protein proposed by Taylor et al. (35), two other amino acid residues, Glu-103 and Thr-246, are thought to promote Asn-570 side-chain acidity via hydrogen bonding (making it more amenable to nucleophilically attack the peptide-bond carbonyl carbon). The importance of tertiary structure in regards to cleavage also explains why both the synthetic peptides and the oligopeptides expressed in vivo were not cleaved; there was an insufficient amount of FlhB_2 domain present to facilitate succinimide ring formation, and hence cleavage.

Autocleavage, as the mechanism of FlhB cleavage, is even more plausible when we examine the evidence collected on the other two possible explanations for cleavage. We observed identical FlhB cleavage behavior in yeast as has been seen in Salmonella and E. coli, and this led us to reject the possibility of protease-mediated cleavage, based on the assumption that it is highly unlikely that the heterologous host would have a protease that would consistently and specifically cleave a bacterial type III flagellar export component. Furthermore, the cleavage site is clearly a single cut of the peptide bond and not the starting point for subsequent degradation. In addition, these observations in yeast confirm the already established ability of column-purified His-FLAG-

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**FlhB Autocleavage**

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**FIGURE 6.** Export into the culture supernatant of a rod/hook-type protein (FlgD) and a filament-type protein (FliC) by six isolated flhB* second site mutant strains with the wild-type as reference. Lane 1, wild-type Salmonella (SW1103); lane 2, MMB2701 (flhBA298V); lane 3, MMB2714 (flhBG293R); lane 4, MMB3016 (flhB frame-shifted (f-s), at residue 348 + 27 amino acids); lane 5, MMB3201 (flhBG293V); lane 6, MMB3501 (flhB 358 f-s + 5 amino acids); lane 7, MMB3519 (flhBW355stop). Proteins were detected by immunoblotting with polyclonal anti-FlgD and anti-FliC antibodies. Plus signs (+) indicate relative motilities of the strains.

**FIGURE 7.** A model of succinimide-mediated cleavage of FlhB. Two pathways of asparagine cyclization and reopening are illustrated. More commonly, succinimide ring formation occurs via the attack of the α-amino group of the carbonyl amino acid residue on the carbonyl carbon of the asparagine residue (I). Subsequent hydrolysis of the succinimide ring produces isomerized aspartyl (IV) and/or normal aspartyl (V) residues. Alternatively, the β-amide nitrogen can attack the peptide-bond carbonyl, causing cleavage and formation of a C-terminal succinimide ring (III). Subsequent hydrolysis of the succinimide ring yields C-terminal asparagines (VI) and isomerized asparagines (VII). The asterisk (III) marks the preferred hydrolysis of the succinimide ring between the peptide bond carbonyl and the β-amide nitrogen, resulting in C-terminal asparagines (VI). Only the cleavage pathway (III) is possible when the downstream residue is proline. (Modified from Ref. 27.)
FlhB Autocleavage

FlhB*(P270A) to undergo cleavage in vitro. Also, oligopeptides containing the cleavage site sequence, i.e. the possible "substrate site," have no effect on motility when produced in wild-type Salmonella. The further possibility of FlhB self-proteolysis was also abandoned based upon the oligomeric state of FlhB*. FlhB clearly is monomeric, as shown by analytical ultracentrifugation and size-exclusion chromatography, making the likelihood of one FlhB* acting as a protease on another FlhB* quite low. Furthermore, in vitro studies mixing variants of FlhB* had no significant effects on cleavage.

As mentioned before, the switch in export substrate-specificity is also regulated by another flagellar component, the hook-length control protein, FliK. It has been suggested that FliK might be directly responsible for the cleavage event. We showed in this study, that it is not the case. FlhB* expressed in yeast where no FliK is present, still undergoes specific cleavage within the hinge. Similarly, we find the proposal that FliK might sequester FlhB to delay the autocleavage event until the appropriate time (2) unlikely as Salmonella cells overexpressing both FlhB and FlhB did not show any difference in FlhB cleavage (data not shown).

Exactly how FliK and FlhB work together to switch export substrate-specificity remains to be elucidated. Yet the current evidence for autocleavage of FlhB adds an intriguing perspective to the role conformational changes in FlhB play in the specificity switch. Obviously, the apparent correlations we have discerned between FlhB autocleavage, motility, and substrate export deserve a more detailed study to determine causality in this relationship. However, our fresh analysis of data that has been published over the past decade combined with the results of our export assays add significantly to the mounting evidence that FlhB autocleavage is indeed physiologically relevant, and more importantly, directly associated with export substrate-specificity switching.

The implications of further pursuing a deeper understanding of the FliK-FlhB interaction as it relates to autocleavage and export substrate-specificity switching are particularly relevant as a related phenomenon of our export assays add significantly to the mounting evidence that FlhB autocleavage is indeed physiologically relevant, and more importantly, directly associated with export substrate-specificity switching.

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