Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1694 (2004) 207–217

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Review

Type III flagellar protein export and flagellar assembly

Robert M. Macnab ✠

Department of Molecular Biophysics and Biochemistry, Yale University, 0734, 266 Whitney Avenue, P.O. Box 208114, New Haven, CT 06520-8114, USA

Received 24 October 2003; accepted 29 April 2004
Available online 18 May 2004

Abstract

Bacterial flagella, unlike eukaryotic flagella, are largely external to the cell and therefore many of their subunits have to be exported. Export is ATP-driven. In *Salmonella*, the bacterium on which this chapter largely focuses, the apparatus responsible for flagellar protein export consists of six membrane components, three soluble components and several substrate-specific chaperones. Other flagellated eubacteria have similar systems. The membrane components of the export apparatus are housed within the flagellar basal body and deliver their substrates into a channel or lumen in the nascent structure from which point they diffuse to the far end and assemble. Both on the basis of sequence similarities of several components and structural similarities, the flagellar protein export systems clearly belong to the type III superfamily, whose other members are responsible for secretion of virulence factors by many species of pathogenic bacteria.

Keywords: Bacterial flagellum; Type III protein export; FliI ATPase; Morphogenesis; Chaperone

1. Introduction

Bacteria employ a number of different systems for transferring proteins from their cytoplasm to external structures, to the external medium, or into host cells. Each type of system has its own characteristics such as substrate recognition motifs, presence or absence of cleavable signal peptides, physical architecture, and export/secretion mechanism. Many of these systems are described in other chapters in this issue. This chapter discusses the process of flagellar protein export by a type III pathway. It uses *Salmonella* as its principal example but the same, or at least very similar, processes occur in all flagellated bacterial species. The chapter concludes with a brief comparison of the similarities and differences between type III flagellar protein export and type III secretion of virulence factors. The latter topic is dealt with in depth in the chapter by He.

1.1. The flagellum as motor organelle

The bacterial flagellum is an organelle for cell propulsion. Although it carries the same name as the eukaryotic flagellum, the two organelles are in fact quite distinct. The bacterial flagellum is powered by proton-motive force (with contributions from the electrical potential $\Delta\psi$ and the chemical potential ΔpH) or in some species sodium-motive force. The electrochemical energy is converted into torque, resulting in rotation of the flagellar motor, which is attached via the MS ring to the basal body rod, to the hook, and then to an extremely long thin helical structure, the flagellar filament (Fig. 1). Rotation of this helical filament converts torque into thrust and propels the cell. The system contains a switch that can generate either counterclockwise or clockwise rotation. The two directions have different consequences for the cell, forward motion and random reorientation. Chemical signals modulate the probabilities of the two rotational directions so as to produce the beneficial behavior called chemotaxis. For extensive reviews of motility and chemotaxis, see Refs. [1–4]. A recent review on flagellar assembly [5] includes a more extensive treatment of flagellar structure, motility and chemotaxis than that presented above.

1.2. The flagellar gene system

Throughout this chapter, explicit mention of the names of genes and their products will largely be restricted to those that are central to the export process. The flagellar/motility/

✠ Sadly, Professor Robert M. Macnab died on 7th September 2003.
E-mail address: may.macnab@yale.edu (M. Macnab).

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.04.005

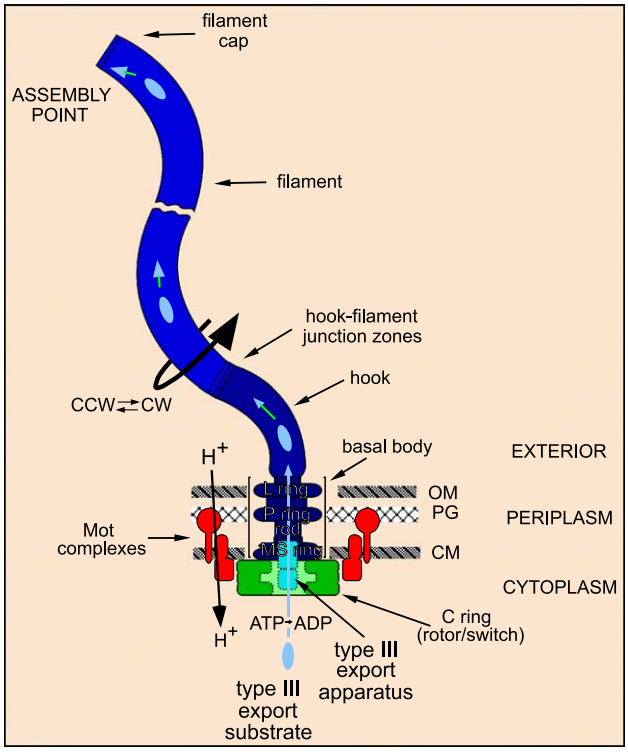

Fig. 1. Cartoon of the flagellum of *Salmonella*. Passive structural components such as the basal body, hook and filament are shown in dark blue. Motor components consist of the stator or Mot complexes (red) and the rotor/switch complex or C ring (green); the motor is rotary, reversible and uses protonmotive force as its energy source. The export apparatus (turquoise) consists of membrane components at the center of the basal-body MS ring and soluble or peripheral components including the ATPase that drives export. Exported proteins (light blue) enter a central channel in the nascent structure and assemble at the far end. CCW, counterclockwise rotation; CW, clockwise rotation; CM, cytoplasmic membrane; PG, peptidoglycan layer; OM, outer membrane. Modified from Ref. [25], with permission.

chemotaxis gene system constitutes a regulon, i.e, an integrated hierarchy of controlled expression of about 50 genes, excluding receptor genes of which there are perhaps 20 [6,7]. Regulation of gene expression plays a significant role in the processes of export and assembly in wild-type cells. At the top of the hierarchy (class 1) is the master operon, whose two genes must be expressed in order for any of the other genes in the regulon to be expressed. Class 2 operons contain primarily genes encoding flagellar structural proteins, a few regulatory proteins, and components of the type III export pathway. Class 3 operons (actually classes 3a and 3b) contain genes encoding flagellar structural proteins assembled late in the morphogenetic pathway (notably the filament protein, flagellin), the Mot proteins, and the chemotaxis proteins. The processes of genetic control and flagellar protein export intersect directly at one point when a protein, FlgM, which inhibits transcription of late genes by sequestering a flagellum-specific sigma factor, is itself inactivated by expulsion from the cell by the type III apparatus—surely one of the more unusual mechanisms of genetic regulation [8,9]. For a review of regulation of flagellar assembly, see Ref. [10].

1.3. Flagellar structure—the logistical problem

This chapter provides only enough structural information on the flagellum for the reader to understand what the architecture is and therefore what the assembly process entails. It does not provide detailed information on the function of the various components (see Refs. [1,4,5]).

The bacterial flagellum (Fig. 1) consists of several major elements. The passive (non-motor) structural elements are (i) the *MS ring*, *rod*, and *L* and *P rings* (collectively called the *basal body*), (ii) the *hook* and associated *hook–filament junction* zones, and (iii) the *filament* and its distal *cap*. The motor consists of (iv) a rotor/switch element or *C ring*, a peripheral membrane structure mounted on the cytoplasmic face of the MS ring, and (v) stator elements or *Mot complexes* that are embedded in the cell membrane and surround the MS and C rings. Finally, and central to the subject of this chapter, there is (vi) the *type III flagellar protein export apparatus*, which consists in part of integral membrane components located in the center of the MS ring, and soluble or peripheral components such as the ATPase that drives the export process.

Thus, everything beyond the MS ring has to be exported across the plane of the cell membrane, subunit by subunit, and delivered to its assembly destination at the tip of the growing structure. This is a logistical problem on a massive scale—hundreds of thousands of protein subunits per cell per generation over large distances (up to 10 times the body length of the cell). Success requires not only the export of subunits but also their ordered assembly, without the aid of anything other than the exported proteins themselves.

2. The morphogenetic pathway

How is the flagellum assembled from its component parts, i.e., what is the morphogenetic pathway? Morphogenesis proceeds in four main stages (Fig. 2) [11–13]. In the *first stage*, which we may call the pre-type III export stage, integral membrane proteins, including the basal body MS ring (FliF), the Mot proteins that constitute the stator element of the motor, and six components of the export apparatus (FlhA, FlhB, FliO, FliP, FliQ and FliR) are inserted into the membrane (presumably using the Sec pathway, as is true of most bacterial membrane proteins). The order of assembly of these proteins is not completely clear. The Mot proteins can be assembled after all other flagellar structure is complete [14,15], but in wild-type cells they may be assembled earlier, although control of gene expression (see above) probably affects the time of

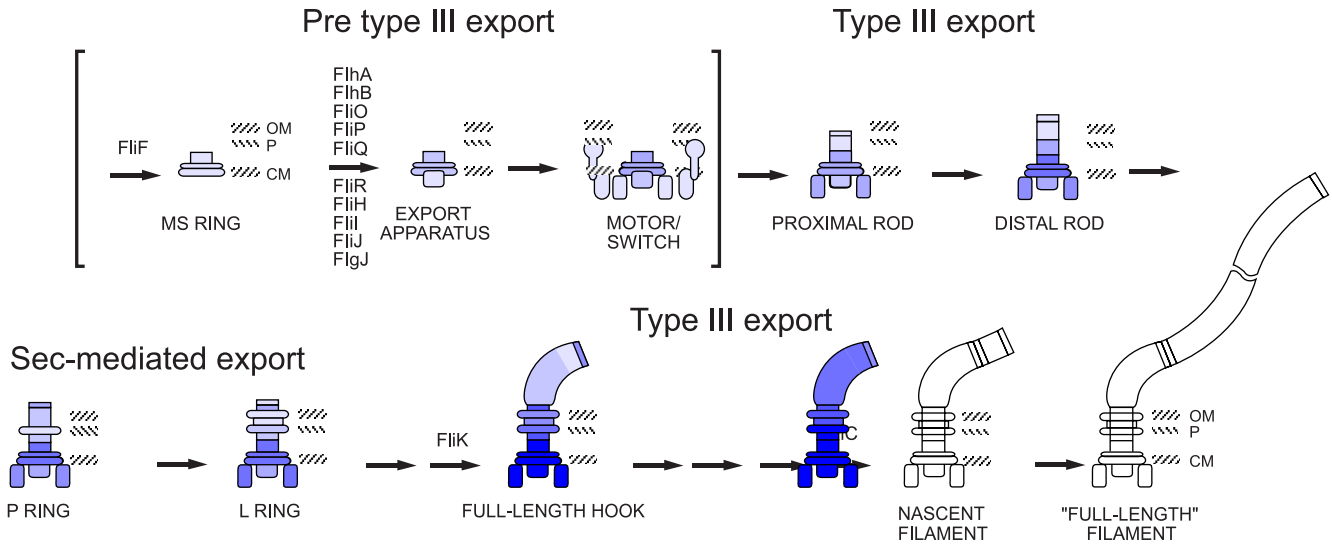

Fig. 2. Flagellar morphogenesis in *Salmonella*, emphasizing the type of pathway used at each stage. Substructures are shaded from dark blue (early) to light blue (late). Pre-type III assembly presumably involves Sec-mediated insertion for integral membrane structures, spontaneous assembly for peripheral structures, and transient association for soluble components; the order of assembly for this stage is not fully understood. Type III export is used for rod assembly, followed by Sec-mediated export with signal peptide cleavage for the P and L rings. Type III export resumes for the remainder of flagellar morphogenesis. Modified from Ref. [5], with permission.

assembly by affecting time of synthesis, since the *mot* genes belong to class 3. Also assembled at this stage is the C ring, a peripheral membrane structure that constitutes the rotor/switch element of the motor and presumably can self-assemble onto the MS ring. We also include in this stage the soluble components of the export apparatus, which are thought to be in dynamic association with the membrane components. In the *second stage* the rod is assembled from proteins that have been exported by the type III pathway. In the *third stage* the proteins of the periplasmic P ring and the outer membrane L ring are exported by the Sec pathway (see chapter by Economou in this issue) with signal peptide cleavage [16,17], and assemble as rings around the rod. [They may be exported before stage two is complete, but probably can only assemble when the rod is in place and can act as a nucleation center.] In the *fourth* (and final) *stage*, type III export resumes to assemble the hook protein, some junction and capping proteins, and the filament protein, FliC or flagellin. Thus, with the exception of the P and L rings, export and assembly proceeds in a linear fashion from more cell-proximal to more cell-distal structures. Also, at a more detailed level, assembly of subunits occurs distally within any given substructure [18,19]. The proteins exported by the type III pathway are often referred to as the *axial proteins* [20,21], to distinguish them from the proteins that constitute the various ring structures. The physical path by which the axial protein subunits reach their assembly point is a central ca. 3-nm channel in the nascent structure [22,23], as is indicated in Fig. 1. While this single-file diffusion is necessary for delivery of the subunits, it is not really part of the export process if that is defined narrowly as translocation across the plane of the cytoplasmic membrane.

3. Components of the export apparatus

In describing the export apparatus, it is important to distinguish between (i) components that house the export apparatus but also function in the flagellum as a motor organelle, and (ii) components whose sole function is in export. Included in the former category are structures like the MS and C rings, without which the export apparatus cannot assemble and function, but they do not in any direct sense function in export.

The proteins that function directly in export or assembly are listed in Table 1, which also serves later for a comparison between type III flagellar protein export and type III virulence factor secretion.

There are nine proteins (FliH, FliI, FliJ, FlhA, FlhB, FliO, FliP, FliQ and FliR) that are truly central to the flagellar export apparatus, in the sense that they participate in the export of all known substrates [24]. [Strictly speaking, only six of these components are essential when a broad range of flagellated species is considered; FliH, FliJ, and FliO are not always present.] Three of the proteins—FliH, FliI and FliJ—are soluble. The remaining six are integral membrane proteins and are believed to be located in a patch of specialized membrane within a central pore in the basal-body MS ring [25,26]. They range in size from 10 kDa (FliQ) to 75 kDa (FlhA), and vary in the predicted number of membrane spans from one (FliO) to eight (FlhA). Two of them (FlhA and FlhB) have

Table 1
Type III flagellar protein export components and a comparison of their homologs in virulence factor secretion systems

Flagellar protein[a]	Function/properties	Molecular mass (kDa)	Virulence protein[a]	Molecular mass (kDa)	*E* value[b]
FliH	Regulator of FliI activity	25.8	n.d.[c]	–	–
		25.8	YscL (*Yersinia enterocolitica*)	24.9	0.1
FliH (*Treponema denticola*)		35.2	YscL (*Yersinia enterocolitica*)	24.9	10^{-11}
FliI	Export ATPase	49.3	InvC	47.6	5×10^{-72}
FliJ	General chaperone	17.3	n.d.[c]	–	–
FliK	Hook-length control	41.8	InvJ	36.4	n.a.[d]
FlhA	Membrane component; interacts with soluble components	74.8	InvA	76.1	4×10^{-61}
FlhB	Membrane component; interacts with soluble components; specificity switching	42.4	SpaS	40.1	$2 \times ^{-13}$
FliO	Membrane component; function unknown	13.1	n.d.[c]	–	–
FliP	Membrane component; cleaved signal sequence	26.8	YscR	24.1	5×10^{-34}
FliQ	Membrane component; function unknown	9.6	SpaQ	9.4	0.006
FliR	Membrane component; function unknown	28.9	SpaR	28.5	6×10^{-7}
FliN	C ring component, involved in motor function as well as export	14.8	SpaO	33.8	0.002
FliF	MS ring protein; necessary for export; interacts with FlhA	61.1	HrpC (*Pseudomonas syringae*); presumed lipoprotein	29.1	0.009
FlgE	Hook protein	42.1	PrgI	8.9	n.a.[d]
FlgJ	Muramidase and putative rod capping protein	34.4	n.d.[c]	–	–

[a] Unless otherwise stated, the proteins are from *Salmonella*, but they exist in essentially all flagellated species and type III virulence species.
[b] A parameter generated by BLASTP [71] indicating the probability that the observed degree of sequence similarity would occur by chance.
[c] n.d., no homolog detected in *Salmonella.*
[d] n.a., not applicable; homology based on mutant phenotype or biochemical evidence; no significant sequence similarity.

large C-terminal cytosolic domains that interface with the soluble components (see below). The evidence for association with the MS ring is still fragmentary. FliP and FliR have been found in the basal body [27]; attempts to detect the others have been unsuccessful so far, perhaps because the detergent treatment used in preparation of basal bodies results in their dissociation. Genetic evidence (extragenic suppression) suggests an interaction between FlhA and the MS ring protein FliF [28]. Also, given the physical pathway by which exported subunits reach their destination, the core of the MS ring seems the only logical location for the export apparatus.

Although considerable effort has gone into studying the export components by biochemical and biophysical means, and many protein–protein interactions have been established (see below), their detailed mechanistic roles are for the most part not well understood. The best understood are the ATPase FliI [29], its regulator FliH [30–32], one of the membrane components, FlhB, which plays a role in determining export substrate specificity [33,34], and a putative general chaperone, FliJ [35,36]. For the remaining components (FlhA, FliO, FliP, FliQ, and FliR), all that can be said for now is that they are essential for flagellar protein export and that FlhA appears to be important for association of the export complex with the MS ring [28] and the ATPase [37].

In addition to the general components, there are at least three specific cytoplasmic chaperones, FlgN, FliS and FliT [38–42], which associate with their substrates, the hook–filament junction proteins, flagellin, and the filament capping protein, respectively. Whether or not they play a direct role in presenting their substrates to the export apparatus is not clear, but they do prevent substrate degradation [43]. There is also a specific periplasmic chaperone, FlgA, for the P-ring protein [44].

Finally, there are proteins that play important roles in various aspects of the assembly process, such as the hook-length control protein FliK [45–47], the muramidase/rod-capping protein FlgJ [48,49], the hook-capping protein FlgD [50], and the filament-capping protein FliD [51]. With the exception of FliD, these proteins are not present in the final flagellar structure.

4. Genomic organization of export components

The genomic organization of the genes for the nine general components of the export apparatus is given for a variety of genera in Fig. 3. The figure illustrates several points. (i) Gene clustering is evident in most species, a notable exception being *Campylobacter*; these clusters often include flagellar structural genes (not shown in Fig. 3; for a complete listing see, for example, Fig. 11 of Ref. [4]). (ii) There is a wide variation in organization with respect to degree of clustering, distance between clusters, order of clusters, and order of genes within clusters. (iii) Certain limited patterns of adjacency occur commonly: For example, *flhB* is often adjacent to either *flhA* or *fliR*, and *fliP* and *fliQ* are also often adjacent. (iv) Only *flhA*, *flhB*, *fliI*, *fliP*, *fliQ*, and *fliR* are found in all species whose genomes have been sequenced; *fliH*, *fliJ*, and *fliO* are often lacking (or have diverged beyond recognition). Since it is known that in *Salmonella* the absence of FliH (a regulator of the ATPase FliI) can be bypassed by FliI overproduction or by mutations in *flhA* or *flhB* [37], it is clear that FliH is not absolutely necessary and so its absence in some species is not too surprising. Likewise, the leaky phenotype associated with *fliJ* mutants indicates that the function of FliJ is not an absolutely essential one. However, *fliO* mutants appear to be totally nonmotile [13,24] (Jonathan McMurry, unpublished observation).

Some surprises have emerged from the survey of different species. One is the existence of fusion genes. *fliR*/*flhB* is found in *Clostridium acetylobutylicum* [52] and *Clostridium tetani* [53], and *fliO*/*fliP* is found in *Buchnera aphidicola* [54]. Another is the existence in several genera of a separate *flhB* fragment in addition to the intact gene (Matthew Wand, personal communication). This will be discussed later in terms of the function of FlhB in substrate-specificity switching.

5. Interactions among export components

Interactions among export components have been and continue to be an active subject of investigation. There are many such interactions, which probably occur in a dynamic fashion, at least as far as the soluble components are concerned [55,56]. Fig. 4 illustrates these interactions schematically. Only a few of the highlights are mentioned here. The ATPase FliI exists as a monomer in solution, or as a heterotrimer, FliI(FliH)$_2$, with its regulator FliH, which itself can exist as a homodimer (FliH)$_2$ [30,57]. The C-terminal

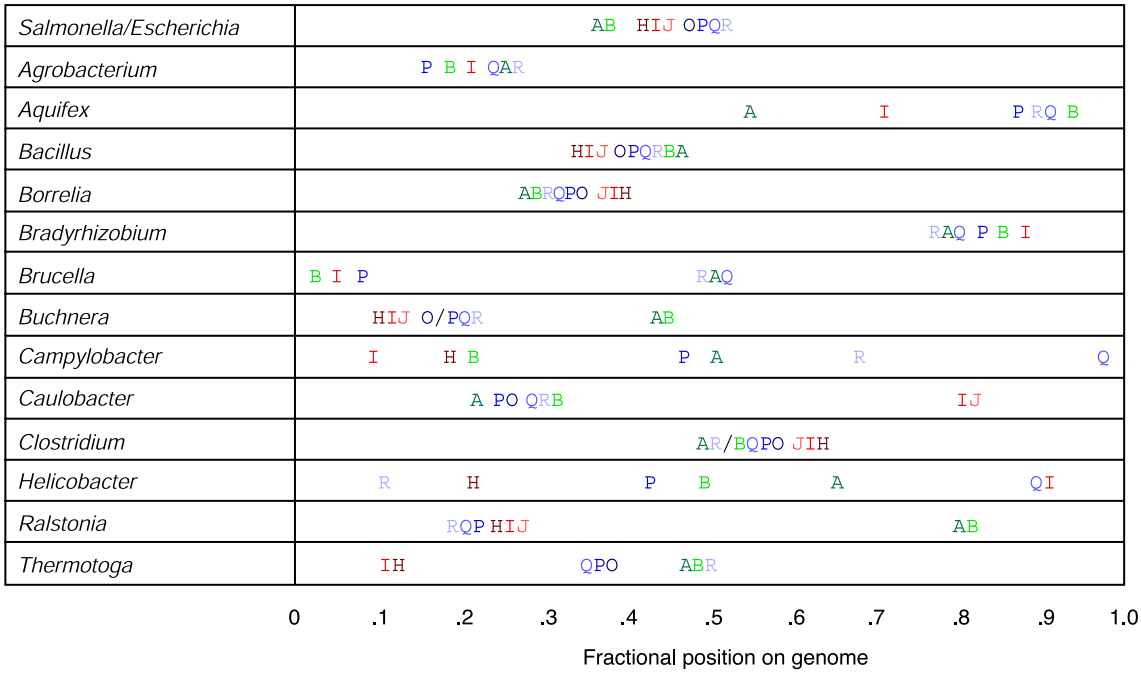

Fig. 3. Organization of the genes for the general components of the flagellar protein export apparatus. The data are taken from an arbitrary subset of the genomes that have been completely sequenced. Genome sizes, which vary widely, have been normalized. The genes are: A, *flhA*; B, *flhB*; H, *fliH*; I, *fliI*; J, *fliJ*; O, *fliO*; P, *fliP*; Q, *fliQ*; R, *fliR*. Genes shown without a space between them are immediately contiguous. Where spaces are shown, their extent indicates qualitatively the distance between the genes. *Salmonella* is taken as a reference point since it is the focus of this chapter; its three clusters are color-coded and the same color scheme is adopted for all other genera. The species used for the genera indicated are *Salmonella enterica* serovar *typhimurium* (commonly referred to simply as *Salmonella*), *Escherichia coli*, *Agrobacterium tumefaciens*, *Aquifex aeolicus*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Bradyrhizobium japonicum*, *Brucella suis* (chromosome I), *Buchnera aphidicola*, *Campylobacter jejuni*, *Caulobacter crescentus*, *Clostridium acetobutylicum*, *Helicobacter pylori*, *Ralstonia solanacearum* (megaplasmid), and *Thermotoga maritima*. Slashes (O/P of *Buchnera* and R/B of *Clostridium*) refer to natural fusion genes. All data were obtained using the ExPASy server and the SwissProt and TrEMBL protein databases.

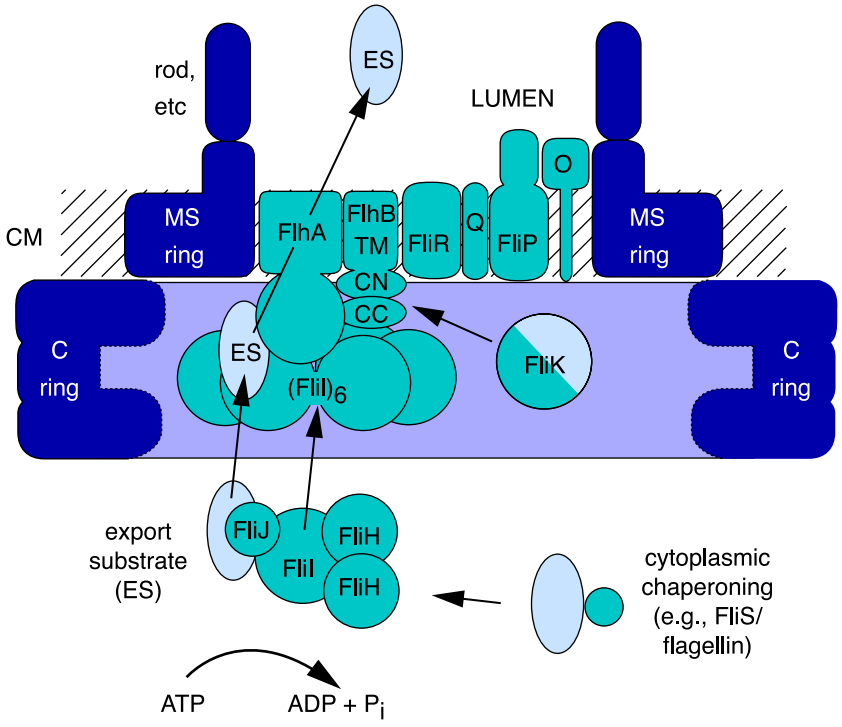

Fig. 4. Cartoon of a transverse section of the flagellar protein export apparatus. Its components (turquoise) are either membrane proteins housed within the cell-membrane (CM) basal-body MS ring (dark blue) or soluble proteins that dock with the cytosolic domains of those membrane components (FlhA and FlhB), which project into the C ring (dark blue and lilac). [The C ring also functions as the rotor/switch element of the flagellar motor.] No attempt has been made to indicate the stoichiometries of the membrane components. Protein interactions have been characterized by a variety of means. Interactions include (i) a heterotrimeric complex FliI(FliH)$_2$ between the ATPase FliI that drives export and its regulator FliH; (ii) a hexameric complex of FliI; (iii) interaction between the FliI(FliH)$_2$ complex and the general chaperone FliJ; (iv) interactions among the membrane components, such as FlhA–FlhA, FlhB–FlhB, FlhA–FlhB, FliR–FlhB, FliO–FliP, and FlhA with the MS-ring protein FliF; (v) interactions between soluble and membrane components, e.g., FliI–FlhA, FliI–FlhB, FliJ–FlhA. Export substrates (ES, pale blue) are in some instances protected from degradation by cytoplasmic chaperones. They are then postulated to be transferred to the FliHIJ complex. What happens next is unclear. The complex might associate directly with the membrane components, or it might undergo rearrangement to yield the hexameric form of FliI, as shown. It should be emphasized that there is no experimental evidence for this. FlhB has three domains: The N-terminal transmembrane domain (TM, containing four spans), and two cytosolic domains CN and CC. Cleavage between the CN and CC domains occurs spontaneously and is necessary for the export apparatus to switch the specificity of the substrates it will accept (see the text). FliK is also necessary for this switch; it is also exported and hence is shown in mixed turquoise/pale blue. Nothing is known about the actual substrate translocation event itself (except that both terminal regions, at least, are unfolded) or about the roles of FliO, FliP, FliQ or FliR. Nor is it known what the specific roles are of binding of ATP, hydrolysis or dissociation of the products. It is known that the products, following translocation, are placed in the lumen or channel of the nascent structure and make their way by diffusion to their assembly point at the distal end. The diameter of the lumen is greatly exaggerated in this representation; in reality, the lumen is so narrow that exported substrates must diffuse down it in single-file fashion.

domain of FliH interacts with the N-terminal domain of FliI; the catalytic C-terminal domain of FliI is not involved in the interaction. A short central region of FliH represents its dimerization domain. In the presence of ATP or analogs and also in the presence of phospholipids, FliI forms a hexamer [58]. We have already alluded to the fact that FliI interacts with FlhA and FlhB as judged by suppression studies [37] and biophysical measurements [56]. FlhA and FlhB interact with themselves and with each other. The C-terminal region of FliJ, a general chaperone, interacts with FliH; FliJ also interacts with the cytosolic domain of the integral membrane protein FlhA [36]. The association of FlhB with FliR, another membrane component, is strongly indicated by the existence of a natural FliR/FlhB fusion in *Clostridium* [52], and a similar inference regarding association of FliO and FliP may be made from the existence of a FliO/FliP fusion in *Buchnera* [54].

6. Mechanism of export

We are still far from a complete understanding of the mechanism of export, but a number of individual pieces of data are slowly building up to a consistent picture. Fig. 4, which as described above illustrates the geometrical arrangement of the apparatus and some of its protein–protein interactions, also illustrates some hypotheses regarding export mechanism.

Surprisingly, one of the most elusive parts of the process is the recognition and recruiting of the substrates. The recognition signal within the substrate lies within its N-terminal region but is not obvious at the primary structural level [59]. There is no cleavable signal peptide in type III systems. The substrate-specific chaperones in the flagellar export system bind the C-terminal regions of their substrates in the cytoplasm and protect them from degradation [43],

but there is as yet no evidence that they participate in export per se. It is unclear why some export substrates have chaperones while others do not. Those that do have chaperones all belong to the "late" export category (see Section 7.2), and so may have to spend more time in the cytoplasm and hence require chaperone protection. FliJ prevents aggregation of substrates, presumably by interacting with them to prevent mis-folding [35]. It may be that specific chaperones transfer their substrates to FliJ at some point, either before or after formation of the FliHIJ complex.

There are several lines of evidence that the FliHIJ complex, or some subset of it, interacts with the large cytoplasmic C-terminal domains of FlhA and FlhB ($FlhA_C$ and $FlhB_C$). FliH and FliI have been found to interact with both $FlhA_C$ and $FlhB_C$, and FliJ has been found to interact with $FlhA_C$ [36,56]. FliI can be induced to form a hexamer by the presence of ATP or phospholipids (Ref. [58] and H. Suzuki, T. Minamino, K. Namba, personal communication). The fact that hexamer formation enhances ATPase activity suggests that this event may be physiologically significant, and might occur in the final stages of assembling the translocation competent state of the export apparatus. However, the hexamer measurements were made in vitro and there is as yet no direct evidence regarding the in vivo situation. It is interesting in this regard that the ATPases of type IV secretion systems (specifically, the VirB11 ATPases of *Helicobacter*; see chapter by Christie in this issue) form hexamers in the presence of nucleotide; the structure of these hexamers in various nucleotide binding states has been solved at atomic resolution [60], with results that suggest a physiologically significant gating function. Also, the catalytic domain of FliI shows significant sequence similarity to the catalytic β subunit of the F_0F_1 ATPase, whose enzymology has been studied extensively and which functions with β and the regulatory α subunit as an $(\alpha\beta)_3$ hexamer.

Essentially nothing is known about the translocation event itself. Isolation or reconstitution of a translocation system seems essential before any real understanding can be reached. Affinity blotting measurements indicate that the soluble domains of FlhA and FlhB interact with substrates [55]; in the case of FlhB, it seems to be the C-terminal subdomain (see below) that is primarily responsible for the interaction [33]. These interactions probably reflect the initial encounter between the substrates and the membrane components of the export apparatus, and do not necessarily imply that the transmembrane domains of FlhA and FlhB constitute part of the physical translocation pathway; they could merely be anchors for the soluble domains. Whether FliP, FliQ and FliR contribute to the physical pathway is not known. FliO may not, since it is not present in all species (Fig. 3).

What is the state of the substrate as it is being translocated? Monomeric substrates in solution have largely disordered N- and C-terminal regions [61,62] and a stably folded central region. This is probably the state of these substrates in the cytoplasm, but the central region may become unfolded during the translocation process. If it does not, the flagellar export system would have at least some features in common with the Twin Arginine Translocation (TAT) pathway which exports proteins in their fully folded form (see chapter by Robinson in this issue).

7. Assembly of substructures

From in vitro and other studies (e.g., Refs. [26,63,64]) it is evident that many of the substructures (the hook, junction proteins and filament, the MS ring, and the MS plus C ring) are capable of self-assembly, although in cases like the filament either seed, ammonium sulfate or extremely high concentrations of flagellin are needed for the process to occur at an appreciable rate. Once assembled, they are very stable. However, in vivo assembly of the exported substrates (the axial proteins) that form filamentous structures such as the rod, hook and filament apparently requires assistance.

7.1. The role of the capping proteins in assembly

In vivo the filament, the hook, and probably the rod employ a capping structure at the point of monomer assembly (the distal end). The best studied example is the filament. It was originally thought that the filament cap (made of FliD) functioned simply as a kinetic trap to give flagellin time to go from its pre-folded state (in which the terminal regions are in an unstable flickering α-helical state) to the state where it has undergone folding and quaternary interactions with its neighbors. However, a recent study indicates that the role of the cap, which has pentameric annular symmetry, is more akin to that of a chaperone, creating a chamber that actively assists the folding process and promotes the assembly by directing flagellin to its appropriate binding position [51]. It is likely that the same will hold true for the hook cap (FlgD) and rod cap (FlgJ). The rod cap has an interesting additional feature, namely, that its C-terminal domain has muramidase activity [48]; mutants defective in this domain assemble as far as the rod and P ring but can go no further [49]. It is hypothesized that the muramidase domain is responsible for chewing a local hole in the peptidoglycan layer, thus permitting penetration by the rod and continuation of flagellar assembly.

7.2. Hook-length control and substrate-specificity switching

FliK (a soluble protein) and FlhB (a membrane protein) function together in the process by which, in wild-type cells, the hook elongates to a fairly closely defined length of about 55 nm, or about 130 subunits [47]. At that point the export system switches from recognition of "early" substrates (rod cap, rod, hook cap and hook proteins) to

"late" substrates (hook–filament junction, flagellin, and filament cap proteins) [65]. Thus, the demarcation between hook elongation and filament elongation is a quite sharp one. *fliK* mutants exhibit polyhook phenotype [45], with hook elongation continuing indefinitely and the specificity switch failing to occur. FliK, long thought to be located exclusively in the cytoplasm, is now known to be exported as an early substrate [66], although it has not been detected in the flagellar structure. Presumably after the switch has occurred it accumulates in the cytoplasm, but whether this plays any role in the switching process is not known. As with other exported flagellar proteins, the recognition region for export lies towards the N terminus [66], whereas the C terminus is absolutely required for length control and specificity switching [46].

FlhB has some unusual properties. Its N-terminal transmembrane region is predicted to have four spans, with the remainder of the sequence residing in the cytoplasm. It was known from earlier studies that *fliK* mutations (even null ones) could be suppressed by second-site mutations in *flhB* [46], and that all of these suppressors lay from approximately halfway into the *flhB* cytoplasmic sequence to the C terminus; none were found in the first half of the cytoplasmic sequence, suggesting the existence of two subdomains, which have since been termed $FlhB_{CN}$ and $FlhB_{CC}$. Detailed biochemical analysis revealed that a cleavage event occurs at precisely the predicted subdomain boundary, with the cleavage site being between Asn269 and Pro270 [33], and the cleavage products being (i) the transmembrane domain $FlhB_{TM}$ plus $FlhB_{CN}$ and (ii) $FlhB_{CC}$. The cleaved $FlhB_{CC}$ fragment remains associated with the rest of the protein, although presumably the interface has been altered. The event is physiologically significant, since a non-cleavable mutant FlhB (Asn269 → Ala) cannot support specificity switching, and so polyhook phenotype results [34]. The available evidence suggests this may be a self-cleaving event such as occurs in intein/extein splicing [67]. An equivalent cleavage phenomenon occurs with the FlhB homolog, YscU, in the *Yersinia* type III virulence secretion system [68].

The fact that several species have a gene encoding $FlhB_{CC}$ in addition to one encoding FlhB (Matthew Wand, personal communication) suggests that concentration effects of $FlhB_{CC}$ may be important in the control of switching. For example, free $FlhB_{CC}$ could sequester FliK (it is known that they interact in vitro [33]).

8. Comparison with type III secretion of virulence factors

We conclude with a brief comparison of type III export of flagellar proteins and type III secretion of virulence factors (cf. chapter by He). Where complete genomes are available, it appears that type III virulence systems only exist in flagellated Gram-negative species. In contrast, a number of nonpathogenic but flagellated species (e.g., *Caulobacter crescentus*), not surprisingly, lack type III virulence systems.

[An interesting intermediate case is the obligate aphid symbiont *B. aphidicola*, which has a degenerate gene system in which most of the flagellar genes are present but the flagellin gene is absent [54]; thus, it is nonmotile, consistent with its life style (F. Silva, personal communication). It does not seem to have a type III virulence system. Why the basal body, hook and export genes have survived is a puzzle.]

In this author's opinion, the difference between the terms *export* and *secretion* is a semantic one, which is not particularly important. The important differences between the flagellar and virulence systems are functional ones. In the case of the flagellar system, with a few exceptions (the anti-σ factor FlgM and the hook-length control protein FliK), the export substrates are incorporated into flagellar structure, in some cases temporarily as scaffolds such as the hook cap but most often permanently. In the case of the virulence factor system, the early substrates are incorporated into bacterial structure (the needle complex), but the rest are either incorporated into host structure (e.g., the pore complex in the host plasma membrane) or as soluble factors that function either in the extracellular milieu or in some compartment of the host cell.

A number of the structural components of the needle complex appear to be functionally homologous to those of the flagellar hook–basal body complex, and in at least two cases (the flagellar MS-ring protein FliF and the FliN component of its C ring) there is significant sequence similarity (Table 1). However, the case of FliN represents something of a puzzle, since there does not appear to be any equivalent of the C ring in the needle complex. However, it must be acknowledged that the role of FliN in motor rotation and switching is still rather mysterious.

The existence of the needle complex (which as far as is known has no role other than in secretion and virulence) confirms the presumption that the hook–basal body complex is properly to be viewed as part of the flagellar export apparatus as well as part of the flagellum as a motor organelle. This applies not only to the MS ring, but also to the C ring (the rotor/switch element of the motor), since severe mutations in any of its three component proteins prevent flagellar assembly. The rod and L ring are also essential for total export to the external medium.

When it comes to the components directly involved in the export and secretion processes themselves, the degree of sequence similarity is striking in almost all cases (Table 1). Interestingly, the probability values (*E* values) that this is a chance event can vary widely from species to species, and the similarity is not necessarily highest for a comparison within the same species. This is illustrated in Table 1 for the case of FliH, where a comparison between FliH (*Treponema denticola*) and YscL (*Yersinia enterocolitica*) shows a far higher degree of similarity

than between FliH (*Salmonella*) and YscL (*Y. enterocolitica*), and no match at all is found between FliH (*Salmonella*) and any *Salmonella* virulence factor secretion protein.

Not surprisingly, there is no similarity between the virulence factors (effectors) and the exported flagellar proteins, since they perform entirely different functions.

It has been suggested that the type III virulence factor secretion system evolved from the type III flagellar protein export system, since flagella are far more ancient (existing in very diverse genera) than the organisms that are targets for type III virulence systems [4]. However, it is possible that the original targets were other bacteria. Also, the possibility of lateral gene transfer cannot be ruled out. Finally, one could argue that evolution from a less complex structure (the needle complex) to a more complex one (the flagellum) is more probable than the other way around.

A comparison of the genomic organizations of the flagellar export system (discussed earlier) and of the type III virulence systems reveals that the gene order of homologs is often preserved. [Fig. 9 of Ref. [69] gives an excellent representation of the organization of virulence genes in a variety of species]. As examples, we may cite *Chlamydia lcrD–YscU* (homologous to *flhB–flhA*) or *Salmonella spaP–spaQ–spaR* (homologous to *fliP–fliQ–fliR*). This provides strong support (in addition to the amino acid similarities) for the idea that the two systems are evolutionarily related.

There is a striking difference in the degree of clustering of the genes in the two systems. Although flagellar export genes are clustered to varying degrees (Fig. 3), virulence genes are very highly clustered, often to the point where all of the secretion apparatus genes (as well as many effector genes) exist as a single large cluster (often referred to as a pathogenicity island); for example, *Salmonella* pathogenicity island 1 (SPI-1) contains 30 genes. These islands are often flanked by direct repeats and the genes often have a higher GC content than the rest of the chromosome. All of these facts suggest lateral gene transfer from a heterologous source [69]. If the flagellar system is the ancestral one, the issue of clustering raises a puzzling question, because how do several separated clusters give rise to a single one? A single recombination event seems impossible, at least in some cases. To illustrate this point, consider *Ralstonia*, where the distance between the end of the *flhA–flhB* cluster and the beginning of the *fliR–fliQ–fliP* cluster is 815 kb! The corresponding virulence gene system, in contrast, consists of a single cluster of 19 genes.

This brings us back to the question of the evolutionary relationship between the flagellar export system and the virulence factor secretion system. Comparative phylogenetic analyses have been carried out on the two systems [70]. This study concludes that there is no intermingling, i.e., the two systems evolved separately. Thus, all FlhA proteins lie on one major branch from the root and all LcrD (the homologous or, more strictly, paralogous) proteins on the other. However, it is not easy to explain the substantial preservation of gene organization on this theory. Also, although the two systems are obviously related functionally, they are not identical, and it is perhaps reasonable to expect that orthologs within one system would resemble each other more closely than they resemble their paralogs in the other system.

As the above discussion indicates, there is much about the evolution of type III systems that remains mysterious.

Acknowledgements

Thanks are due to the following colleagues for helpful suggestions regarding this chapter: Hedda Ferris, Bertha González-Pedrajo, May Kihara, Jonathan McMurry, Tohru Minamino, and Keiichi Namba. Work in the author's laboratory has been supported by USPHS Grant AI12202. Collaboration with the laboratory of Keiichi Namba has been under the auspices of the ICORP program of the Japan Science and Technology Corporation.

References

[1] S.-I. Aizawa, in: M. Sussman (Ed.), Medical Microbiology, Academic Press, New York, NY, 2002, pp. 155–175.
[2] R.B. Bourret, A.M. Stock, Molecular information processing: lessons from bacterial chemotaxis, J. Biol. Chem. 277 (2002) 9625–9628.
[3] J.B. Stock, M.G. Surette, Chemotaxis, in: F.C. Neidhardt, et al, (Eds.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, vol. 1, ASM Press, Washington, D.C., 1996, pp. 1103–1129.
[4] R.M. Macnab, in: F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger (Eds.), Flagella and Motility. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd ed., ASM Press, Washington, DC, 1996, pp. 123–145.
[5] R.M. Macnab, How bacteria assemble flagella, Annu. Rev. Microbiol. 57 (2003) 77–100.
[6] K. Kutsukake, Y. Ohya, T. Iino, Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*, J. Bacteriol. 172 (1990) 741–747.
[7] R.M. Macnab, Genetics and biogenesis of bacterial flagella, Annu. Rev. Genet. 26 (1992) 129–156.
[8] K.T. Hughes, K.L. Gillen, M.J. Semon, J.E. Karlinsey, Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator, Science 262 (1993) 1277–1280.
[9] K. Kutsukake, Excretion of the anti-sigma factor through a flagellar structure couples flagellar gene expression with flagellar assembly in *Salmonella typhimurium*, Mol. Gen. Genet. 243 (1994) 605–612.
[10] P. Aldridge, K.T. Hughes, Regulation of flagellar assembly, Curr. Opin. Microbiol. 5 (2002) 160–165.
[11] T. Kubori, N. Shimamoto, S. Yamaguchi, K. Namba, S.-I. Aizawa, Morphological pathway of flagellar assembly in *Salmonella typhimurium*, J. Mol. Biol. 226 (1992) 433–446.
[12] C.J. Jones, R.M. Macnab, Flagellar assembly in *Salmonella typhimurium*: analysis with temperature-sensitive mutants, J. Bacteriol. 172 (1990) 1327–1339.

[13] T. Suzuki, Y. Komeda, Incomplete flagellar structures in *Escherichia coli* mutants, J. Bacteriol. 145 (1981) 1036–1041.
[14] D.F. Blair, H.C. Berg, Restoration of torque in defective flagellar motors, Science 242 (1988) 1678–1681.
[15] S.M. Block, H.C. Berg, Successive incorporation of force-generating units in the bacterial rotary motor, Nature (London) 309 (1984) 470–472.
[16] C.J. Jones, M. Homma, R.M. Macnab, L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: gene sequences and deduced protein sequences, J. Bacteriol. 171 (1989) 3890–3900.
[17] M. Homma, Y. Komeda, T. Iino, R.M. Macnab, The *flaFIX* gene product of *Salmonella typhimurium* is a flagellar basal body component with a signal peptide for export, J. Bacteriol. 169 (1987) 1493–1498.
[18] S.U. Emerson, K. Tokuyasu, M.I. Simon, Bacterial flagella: polarity of elongation, Science 169 (1970) 190–192.
[19] T. Iino, Polarity of flagellar growth in *Salmonella*, J. Gen. Microbiol. 56 (1969) 227–239.
[20] M. Homma, D.J. DeRosier, R.M. Macnab, Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum, J. Mol. Biol. 213 (1990) 819–832.
[21] M. Homma, K. Kutsukake, M. Hasebe, T. Iino, R.M. Macnab, FlgB, FlgC, FlgF and FlgG. A family of structurally related proteins in the flagellar basal body of *Salmonella typhimurium*, J. Mol. Biol. 211 (1990) 465–477.
[22] Y. Mimori, I. Yamashita, K. Murata, Y. Fujiyoshi, K. Yonekura, C. Toyoshima, K. Namba, The structure of the R-type straight flagellar filament of *Salmonella* at 9 Å resolution by electron cryomicroscopy, J. Mol. Biol. 249 (1995) 69–87.
[23] D.G. Morgan, R.M. Macnab, N.R. Francis, D.J. DeRosier, Domain organization of the subunit of the *Salmonella typhimurium* flagellar hook, J. Mol. Biol. 229 (1993) 79–84.
[24] T. Minamino, R.M. Macnab, Components of the *Salmonella* flagellar export apparatus and classification of export substrates, J. Bacteriol. 181 (1999) 1388–1394.
[25] R.M. Macnab, The bacterial flagellum: reversible rotary propellor and type III export apparatus, J. Bacteriol. 181 (1999) 7149–7153.
[26] H. Suzuki, K. Yonekura, K. Murata, T. Hirai, K. Oosawa, K. Namba, A structural feature in the central channel of the bacterial flagellar FliF ring complex is implicated in Type III protein export, J. Struct. Biol. 124 (1998) 104–114.
[27] F. Fan, K. Ohnishi, N.R. Francis, R.M. Macnab, The FliP and FliR proteins of *Salmonella typhimurium*, putative components of the type III flagellar export apparatus, are located in the flagellar basal body, Mol. Microbiol. 26 (1997) 1035–1046.
[28] M. Kihara, T. Minamino, S. Yamaguchi, R.M. Macnab, Intergenic suppression between the flagellar MS ring protein FliF of *Salmonella* and FlhA, a membrane component of its export apparatus, J. Bacteriol. 183 (2001) 1655–1662.
[29] F. Fan, R.M. Macnab, Enzymatic characterization of FliI: an ATPase involved in flagellar assembly in *Salmonella typhimurium*, J. Biol. Chem. 271 (1996) 31981–31988.
[30] B. González-Pedrajo, G.M. Fraser, T. Minamino, R.M. Macnab, Molecular dissection of *Salmonella* FliH, a regulator of the ATPase FliI and the type III flagellar protein export pathway, Mol. Microbiol. 45 (2002) 967–982.
[31] T. Minamino, J.R.H. Tame, K. Namba, R.M. Macnab, Proteolytic analysis of the FliH/FliI complex, the ATPase component of the type III flagellar export apparatus of *Salmonella*, J. Mol. Biol. 312 (2001) 1027–1036.
[32] T. Minamino, B. González-Pedrajo, K. Oosawa, K. Namba, R.M. Macnab, Structural properties of FliH, an ATPase regulatory component of the *Salmonella* type III flagellar export apparatus, J. Mol. Biol. 322 (2002) 281–290.
[33] T. Minamino, R.M. Macnab, Domain structure of *Salmonella* FlhB, a flagellar export component responsible for substrate-specificity switching, J. Bacteriol. 182 (2000) 4906–4914.
[34] G.M. Fraser, T. Hirano, H.U. Ferris, L.L. Devgan, M. Kihara, R.M. Macnab, Substrate specificity of type III flagellar protein export in *Salmonella* is controlled by subdomain interactions in FlhB, Mol. Microbiol. 48 (2003) 1043–1057.
[35] T. Minamino, R. Chu, S. Yamaguchi, R.M. Macnab, Role of FliJ in flagellar protein export in *Salmonella*, J. Bacteriol. 182 (2000) 4207–4215.
[36] G.M. Fraser, G.M. González-Pedrajo, J.R.H. Tame, R.M. Macnab, Interactions of FliJ with the *Salmonella* type III flagellar export apparatus, J. Bacteriol. 185 (2003) 3983–3988.
[37] T. Minamino, B. González-Pedrajo, M. Kihara, K. Namba, R.M. Macnab, The ATPase FliI can interact with the type III flagellar protein export apparatus in the absence of its regulator, FliH, J. Bacteriol. 185 (2003) 5546–5554.
[38] G.M. Fraser, J.C.Q. Bennett, C. Hughes, Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly, Mol. Microbiol. 32 (1999) 569–580.
[39] J.C.Q. Bennett, C. Hughes, From flagellum assembly to virulence: the extended family of type III export chaperones, Trends Microbiol. 8 (2000) 202–204.
[40] J.C.Q. Bennett, J. Thomas, G.M. Fraser, C. Hughes, Substrate complexes and domain organization of the *Salmonella* flagellar export chaperones FlgN and FliT, Mol. Microbiol. 39 (2001) 781–791.
[41] F. Auvray, J. Thomas, G.M. Fraser, C. Hughes, Flagellin polymerisation control by a cytosolic export chaperone, J. Mol. Biol. 308 (2001) 221–229.
[42] T. Yokoseki, K. Kutsukake, K. Ohnishi, T. Iino, Functional analysis of the flagellar genes in the *fliD* operon of *Salmonella typhimurium*, Microbiology 141 (1995) 1715–1722.
[43] A.J. Ozin, L. Claret, F. Auvray, C. Hughes, The FliS chaperone selectively binds the disordered flagellin C-terminal D0 domain central to polymerisation, FEMS Microbiol. Lett. 219 (2003) 219–224.
[44] T. Nambu, K. Kutsukake, The *Salmonella* FlgA protein, a putative periplasmic chaperone essential for flagellar P ring formation, Microbiology 146 (2000) 1171–1178.
[45] J. Patterson-Delafield, R.J. Martinez, B.A.D. Stocker, S. Yamaguchi, A new *fla* gene in *Salmonella typhimurium—flaR*—and its mutant phenotype—superhooks, Arch. Mikrobiol. 90 (1973) 107–120.
[46] A.W. Williams, S. Yamaguchi, F. Togashi, S.-I. Aizawa, I. Kawagishi, R.M. Macnab, Mutations in *fliK* and *flhB* affecting flagellar hook and filament assembly in *Salmonella typhimurium*, J. Bacteriol. 178 (1996) 2960–2970.
[47] T. Hirano, S. Yamaguchi, K. Oosawa, S.-I. Aizawa, Roles of FliK and FlhB in determination of flagellar hook length in *Salmonella typhimurium*, J. Bacteriol. 176 (1994) 5439–5449.
[48] T. Nambu, T. Minamino, R.M. Macnab, K. Kutsukake, Peptidoglycan-hydrolyzing activity of the FlgJ protein, essential for flagellar rod formation in *Salmonella typhimurium*, J. Bacteriol. 181 (1999) 1555–1561.
[49] T. Hirano, T. Minamino, R.M. Macnab, The role in flagellar rod assembly of the N-terminal domain of *Salmonella* FlgJ, a flagellum-specific muramidase, J. Mol. Biol. 312 (2001) 359–369.
[50] K. Ohnishi, Y. Ohto, S.-I. Aizawa, R.M. Macnab, T. Iino, FlgD is a scaffolding protein needed for flagellar hook assembly in *Salmonella typhimurium*, J. Bacteriol. 176 (1994) 2272–2281.
[51] K. Yonekura, S. Maki, D.G. Morgan, D.J. DeRosier, F. Vonderviszt, K. Imada, K. Namba, The bacterial flagellar cap as the rotary promoter of flagellin self-assembly, Science 290 (2000) 2148–2152.
[52] J. Nölling, G. Breton, M.V. Omelchenko, K.S. Makarova, Q. Zeng, R. Gibson, H.M. Lee, J. Dubois, D. Qiu, J. Hitti, Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*, J. Bacteriol. 183 (2001) 4823–4838.
[53] H. Brüggemann, S. Bäumer, W.F. Fricke, A. Wiezer, H. Liesegang, I. Decker, C. Herzberg, R. Martínez-Arias, R. Merkl, A. Henne, G. Gottschalk, The genome sequence of *Clostridium tetani*, the causative

agent of tetanus disease, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 1316–1321.
[54] R.C.H.J. van Ham, J. Kamerbeek, C. Palacios, C. Rausell, F. Abascal, U. Bastolla, J.M. Fernández, L. Jiménez, M. Postigo, F.J. Silva, J. Tamames, E. Viguera, A. Latorre, A. Valencia, F. Morán, A. Moya, Reductive genome evolution in *Buchnera aphidicola*, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 581–586.
[55] T. Minamino, R.M. Macnab, Interactions among components of the *Salmonella* flagellar export apparatus and its substrates, Mol. Microbiol. 35 (2000) 1052–1064.
[56] K. Zhu, B. González-Pedrajo, R.M. Macnab, Interactions among membrane and soluble components of the flagellar export apparatus of *Salmonella*, Biochemistry 41 (2002) 9516–9524.
[57] T. Minamino, R.M. Macnab, FliH, a soluble component of the type III flagellar export apparatus of *Salmonella*, forms a complex with FliI and inhibits its ATPase activity, Mol. Microbiol. 37 (2000) 1494–1503.
[58] L. Claret, S.R. Calder, M. Higgins, C. Hughes, Oligomerization and activation of the FliI ATPase central to bacterial flagellum assembly, Mol. Microbiol. 48 (2003) 1349–1355.
[59] M.G. Kornacker, A. Newton, Information essential for cell-cycle-dependent secretion of the 591-residue *Caulobacter* hook protein is confined to a 21-amino-acid sequence near the N-terminus, Mol. Microbiol. 14 (1994) 73–85.
[60] S.N. Savvides, H.-J. Yeo, M.R. Beck, F. Blaesing, R. Lurz, E. Lanka, R. Buhrdorf, W. Fischer, R. Haas, G. Waksman, VirB11 ATPases are dynamic hexameric assemblies: new insights into bacterial type IV secretion, EMBO J. 22 (2003) 1969–1980.
[61] F. Vonderviszt, R. Ishima, K. Akasaka, S.-I. Aizawa, Terminal disorder: a common structural feature of the axial proteins of bacterial flagellum? J. Mol. Biol. 226 (1992) 575–579.
[62] F. Vonderviszt, S. Kanto, S.-I. Aizawa, K. Namba, Terminal regions of flagellin are disordered in solution, J. Mol. Biol. 209 (1989) 127–133.
[63] T. Ikeda, S. Asakura, R. Kamiya, Total reconstitution of *Salmonella* flagellar filaments from hook and purified flagellin and hook-associated proteins in vitro, J. Mol. Biol. 209 (1989) 109–114.
[64] R. Lux, N. Kar, S. Khan, Overproduced *Salmonella typhimurium* flagellar motor switch complexes, J. Mol. Biol. 298 (2000) 577–583.
[65] T. Hirano, T. Minamino, K. Namba, R.M. Macnab, Substrate specificity classes and the recognition signal for *Salmonella* type III flagellar export, J. Bacteriol. 185 (2003) 2485–2492.
[66] T. Minamino, B. González-Pedrajo, K. Yamaguchi, S.-I. Aizawa, R.M. Macnab, FliK, the protein responsible for flagellar hook length control in *Salmonella*, is exported during hook assembly, Mol. Microbiol. 34 (1999) 295–304.
[67] H. Paulus, Protein splicing and related forms of protein autoprocessing, Annu. Rev. Biochem. 69 (2000) 447–496.
[68] M. Lavander, L. Sundberg, P.J. Edqvist, S.A. Lloyd, H. Wolf-Watz, Å. Forsberg, Proteolytic cleavage of the FlhB homologue YscU of *Yersinia pseudotuberculosis* is essential for bacterial survival but not for type III secretion, J. Bacteriol. 184 (2002) 4500–4509.
[69] C.J. Hueck, Type III protein secretion systems in bacterial pathogens of animals and plants, Microbiol. Mol. Biol. Rev. 62 (1998) 379–433.
[70] L. Nguyen, I.T. Paulsen, J. Tchieu, C.J. Hueck, M.H.J. Saier, Phylogenetic analyses of the constituents of type III protein secretion systems, J. Mol. Microbiol. Biotechnol. 2 (2000) 125–144.
[71] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.