ELSEVIER

FEMS Microbiology Reviews 24 (2000) 21–44

FEMS MICROBIOLOGY REVIEWS

www.fems-microbiology.org

Secretion and assembly of regular surface structures in Gram-negative bacteria

Luis Angel Fernández [b], José Berenguer [a,*]

[a] Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa, C.S.I.C.-U.A.M. Campus Cantoblanco, 28049 Madrid, Spain
[b] Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, C.S.I.C. Campus Cantoblanco, 28049 Madrid, Spain

Received 5 May 1999; received in revised form 12 August 1999; accepted 19 August 1999

Abstract

Bacteria synthesize large-sized surface structures through the ordered polymerization of protein subunits. This results in planar or tubular regular structures that have evolved to accomplish specific functions related to the particular environment in which these bacteria are found. Tubular assemblies known as flagella are the most complex structures known in bacteria and consist of a helical rigid filament, a torsion adapter or hook and a proton-fueled rotator known as the basal body. Pili or fimbriae are less complicated helical filaments, which consist of a major subunit and 3–5 minor subunits or pilins, whose main function is the attachment to specific surfaces. Planar structures known as S-layers are the simplest of these regular assemblies and are generally made up of a single subunit packed as a bidimensional crystal around the whole cell surface. Most of the components of these structures have to be secreted through the inner membrane (IM), the periplasm and the outer membrane (OM) before reaching their final destination. The so called general secretory pathway (GSP), or type II secretion system, appears to be implicated in this process to varying degrees, depending on the structure considered. A few S-layers and pili require GSP components but also need specific terminal branches, such as the well known chaperone-usher pathway. On the other hand, only two of the nearly 40 proteins involved in flagellar assembly are dependent on the GSP, while the external components are secreted through a specific pathway similar to the type III systems identified in some pathogens. Moreover, secretion of subunits of S-layers using dedicated type I machinery, without the involvement of any GSP component, has also been observed. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Secretion; Flagellum; Fimbria; Pilus; S-layer

Contents

1. Introduction 22
2. Flagella 22
   2.1. Structure of the flagella 22
   2.2. Genetics of the flagellar apparatus 23
   2.3. Flagellar assembly and secretion 24
3. Fimbriae (pili) 27
   3.1. Class I fimbriae 27
   3.2. Type IV pili 31
4. S-Layers 35
   4.1. Structure and function of S-layers 35
   4.2. Genetic regulation of S-layer expression 35
   4.3. Secretion and assembly of S-layers 36
5. Concluding remarks 38

* Corresponding author. Tel.: +34 (91) 3978099; Fax: +34 (91) 3978087; E-mail: jberenguer@cbm.uam.es

Abbreviations: OM, outer membrane; IM, inner membrane; PG, peptidoglycan; LPS, lipopolysaccharide; GSP, general secretion pathway; SP, signal peptide

0168-6445/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-6445(99)00026-1

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

Acknowledgements 39

References 39

1. Introduction

Protein secretion to the extracytoplasmic compartments is a basic aspect of the physiology of microorganisms. It serves to accomplish important biological processes like host tissue adhesion and invasion, the transfer of genetic information by conjugation, assembly of bacteriophages and cellular motility [1]. Extracellular enzymes, exotoxins, subunits of surface crystalline layers and appendages like flagella and pili must cross the two lipid bilayers of the cell envelope of Gram-negative bacteria to reach their final destination. In addition to the problem faced by a protein through its passage across a lipid membrane, the secretion and assembly of surface structures and organelles are complex phenomena requiring the coordinated synthesis of structural subunits, as well as 'auxiliary' proteins, which constitute the molecular machinery directly acting in the secretion and assembly processes. In this review, we attempt to summarize the current knowledge of the molecular events involved in the synthesis and assembly of surface structures found in Gram-negative bacteria, such us flagella, fimbriae (pili) and crystalline S-layers. Furthermore, since these molecular processes share important homologies with well known protein secretion pathways in bacteria [2], we have decided to discuss briefly those similarities within the context of each organelle in order to provide a more general understanding of the molecular mechanisms involved. Due to space limitations and the vast amount of knowledge accumulated in recent years, we chose to discuss only paradigm model systems which represent the best-characterized examples of each type of organelle and secretion/assembly mechanism. The reader will be referred to more specialized reviews within the different sections for a more detailed discussion of each topic.

2. Flagella

Motility in non-filamentous bacteria is generally provided by one or more helical rigid structures, or flagella, that protrude from the cells to push them like boat propellers by counter clockwise rotation (CCW). In a typical *Escherichia coli* wild-type strain, 6–8 of such flagella arise from random points on the surface. These flagella form a single bundle when coordinate CCW rotation is applied, propelling the cells at a rate of about 10 μm s^{-1}.

2.1. Structure of the flagella

The structure of the flagella has been a subject of research for many years and they have appeared to be one of the most complex proteinaceous structures known in bacteria. Flagella are generally described as composed of three different parts: a long and helical 'filament', a short curved structure known as the 'hook' and a complex 'basal body' made up of a central rod and a series of rings around and below it. Both the filament and the hook protrude from the cell surface, whereas the basal body spans the cell envelope and shows the lower rings protruding from the inner membrane (IM) into the cytoplasm.

The filament is by far the major component of the flagella in terms of mass. It is built up by the polymerization of around 20 000 copies of a single 50–60-kDa protein subunit, the flagellin [3]. The final assembly of such a protein results in a hollow tube of helical structure, with a constant diameter of around 20 nm and a variable length (5–10 μm) that depends on the number of flagellin subunits assembled. The specific role of the flagellar filament is to function as a propeller, thus demanding a low flexibility during CCW rotation to push the cell. Indeed, flagellar filaments are around two orders of magnitude stiffer than F-actin. In vitro self-assembled flagellin filaments also show this rigidity.

The sequence of flagellins can be roughly divided in three functional modules: the N- and C-terminal domains, which are responsible for the inter-subunits interactions and their polymerization, and a central domain protruding towards the filament surface that can be deleted without affecting assembly [4]. Mutations altering the macromolecular structure of the filaments have always mapped at the N- or C-terminal domains. The central domain, however, carries the main flagellar antigen and is post-translationally modified through *N*-methylation by FliB (see below) [5]. As mutants in FliB synthesize functional flagella, the role of such a methylation remains unclear.

The hook is a cylindrical structure similar to the filament but built up from a different subunit, named FlgE, which shows structural characteristics related to its role as a flexible coupling. Despite this flexibility, purified hooks are short and bent hollow tubes about half the length of their helical wavelength [6,7]. The hook is the result of the polymerization of $\sim$130 FlgE subunits and its length (50–60 nm) is well regulated through the addition of the junction proteins FlgK and FlgL [8] to the tip of the hook during flagellar assembly (see later). It has been proposed that these junction proteins allow for the transmission of the torque between the flexible hook and the stiff filament.

The basal body is a complex structure which spans the cell envelope and extends inwards to the cytoplasm. In Gram-negatives, the basal body in its simplest form con-

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

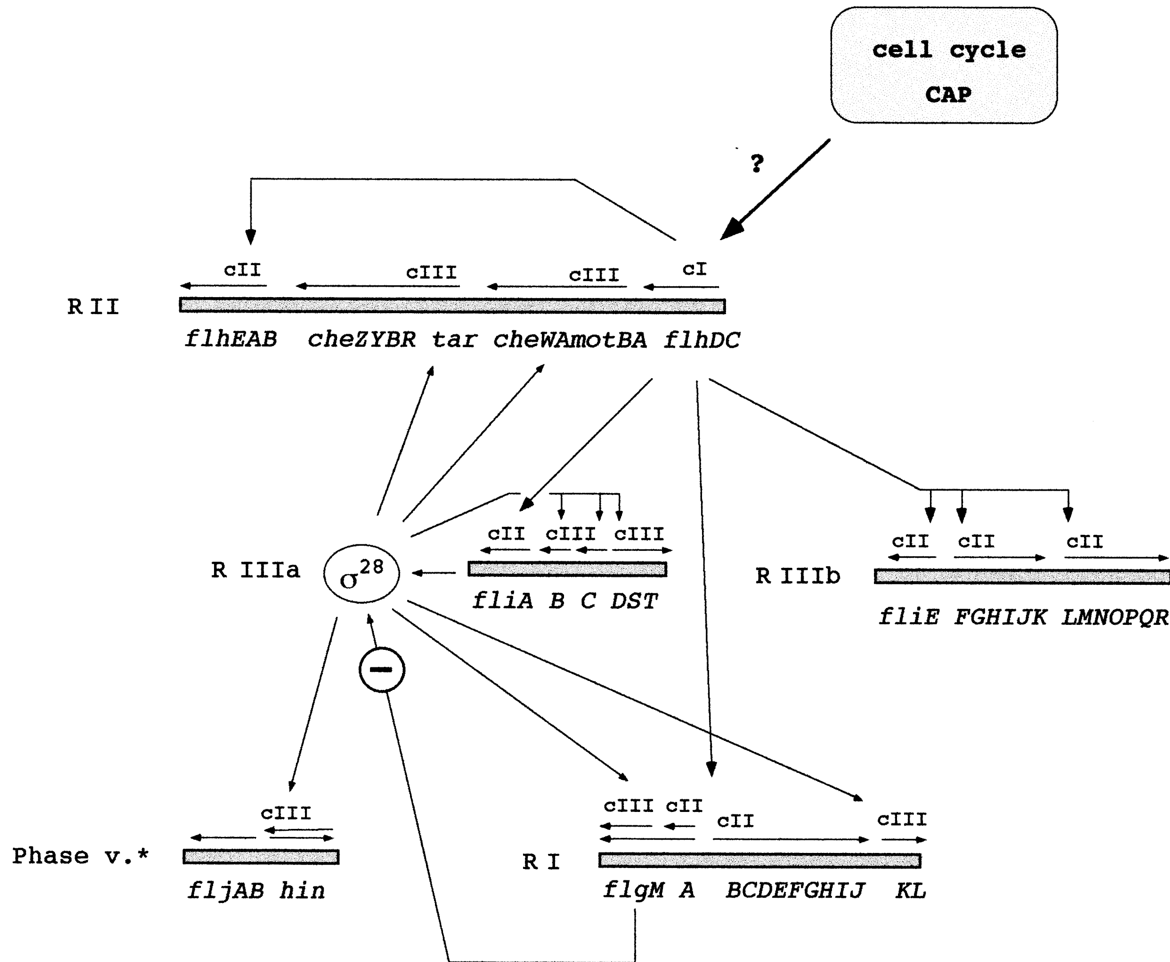


Fig. 1. Genetic control of flagellar expression. The genetic organization and hierarchy of expression of the five chromosomal regions (R) implicated in the synthesis of the flagellar apparatus and related genes from *S. typhimurium* are shown. Unknown signals related to the cell cycle (?, thick arrow) mediate the induction of the master or class I (cI) operon (*flhDC*), which results in the transcription of class II (cII) promoters (medium arrows). Among these, the *fliA* gene encodes a flagella-specific σ factor (σ^{28}) that recognizes class III (cIII) operons and promotes the transcription of the corresponding genes (thin arrows) until its reversible inactivation by the anti-σ factor FlgM. The *Salmonella*-specific region labelled phase v* allows for the expression of alternate flagellin genes at low frequencies.

sists of a central rod and three protein rings named L (for lipopolysaccharide (LPS)), P (for peptidoglycan (PG)) and MS (membrane/supramembrane). The L- and P-rings are embedded within the outer membrane (OM) and the PG layers, being apparently interconnected by a cylindrical wall, while the MS-ring seems to be inserted inside the IM. These rings are the result of the polymerization of ∼26 subunits of single proteins named FlgH, FlgI and FliF, for the L-, P- and MS-rings, respectively. On the other hand, the central rod is made up by four different proteins, three minor components (FlgB, FlgC and FlgF) and a major component, the distal rod protein FlgG, which is also present in ∼26 copies [9,10,5].

By electron microscopy and image analysis, it has been possible to detect the presence of an additional ring connected to the MS-ring, which protrudes from the IM into the cytoplasm [11,12]. The basal body, along with this additional structure named C-ring (from cytoplasmic), forms what is known as the extended basal body. Immunoassays with specific antibodies revealed that the C-ring contains the three motor/switch proteins FliG, FliM and FliN [13].

Two additional proteins, MotA and MotB, are required for the flagellar rotation, although not for its assembly. It has been proposed that the Mot proteins form a ring of 11 elements around the basal body, functioning not only as a PG anchor (MotB) [14], but also to form proton channels from the periplasm to the cytoplasm. This proton motion energizes the flagellar rotation, which could require up to several hundred protons per revolution.

2.2. Genetics of the flagellar apparatus

As suggested by its complex structure, the synthesis of flagella in *E. coli* requires a rather large genetic system, involving about 40 genes distributed among 15–17 operons

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

in four major clusters or regions [15]. Region I contains many of the structural genes, named *flgA-M*. Genes in region II encode proteins implicated in assembly and regulation (*flh* genes) and the Che and Mot proteins. Clusters IIIa and IIIb, which are separated by a short DNA fragment, encode the *fliA-T* genes. In the chromosome of *Salmonella typhimurium*, an additional region exists which encodes an alternative flagellin protein.

The flagellar operons are organized as a single regulon, in which each element is expressed in a specific order (Fig. 1). The highest level of this hierarchy corresponds to the *flhDC* operon, located within the cluster II at min 43 in the *E. coli* map. As both FlhD and FlhC are required for the expression of all the other flagellar genes, this is considered the master or class I operon. It was shown that cAMP activates the transcription of this master operon through the binding of a catabolite activator protein at a specific site on its promoter [16]. Moreover, it has been established that the formation of the flagella is coupled to the cell cycle through the transcription of this master operon, but the mechanism implicated in this process remains unknown [17,18].

Expression of the *flhDC* master operon results in the concomitant transcription of the so-called class II operons, which contain a flagellum-specific consensus at the -10 position of their promoters (GCCGATAA). FliA, a specialized σ factor (σ^{28}) encoded by class II operons, enhances by 3-fold the expression of class II promoters. However, in the absence of FliA, there is still significant transcription from class II promoters, suggesting a role for FlhD and FlhC in the modification of the specificity of the housekeeping σ^{70} factor [5].

The expression of class III promoters results from the specific binding of FliA (σ^{28}). These promoters contain a specific consensus at the -35 region (TAAA) in addition to the flagellum-specific -10 region common to class II promoters. The absence of FliA (σ^{28}) eliminates expression of class III genes, with the exception of the low transcription levels caused by readthrough of the preceding operon *flgMNK*. This operon encodes an anti-σ^{28} factor (FlgM), a hook-filament junction protein (FlgK) and a protein of unknown function [19,5]. Finally, the operon encoding the filament cap protein (FliD) is under the control of both class II and class III promoters.

A number of structural proteins encoded by class II operons are also required for the expression of class III operons. However, their action on transcription depends on the ability of FlgM to bind FliA (σ^{28}), acting as an anti-σ factor that prevents the binding of RNA polymerase σ^{28} to class III promoters [20]. FliA (σ^{28}) escapes from this inhibition by the secretion of FlgM from the cell once the hook has been assembled [21]. This extrusion is accomplished by the same export machinery used to secrete the flagellin and other distal components of the flagellar apparatus. After the completion of the filament, the extrusion of FlgM results blocked, leading to its cytoplasmic accumulation and binding to FliA [21].

The inhibition of FliA by FlgM is a paradigmatic example of transcriptional regulation by anti-σ factors. The mechanism by which FlgM inhibits the activity of FliA involves both FliA sequestration and destabilization of the existing FliA-RNAP complex [22]. FlgM was found to be mostly unfolded in solution, but its carboxy-terminal region became ordered upon interaction with FliA [23]. It is postulated also that the unfolded state of FlgM facilitates its passage through the narrow channel formed by the hook basal body structure. Observations in *S. typhimurium* suggest a tight control of FlgM expression. *flgM* transcription was found to be controlled by a FliA-dependent promoter and its translation by FliK, the hook filament junction protein that acts as a checkpoint for flagellar ring assembly [24].

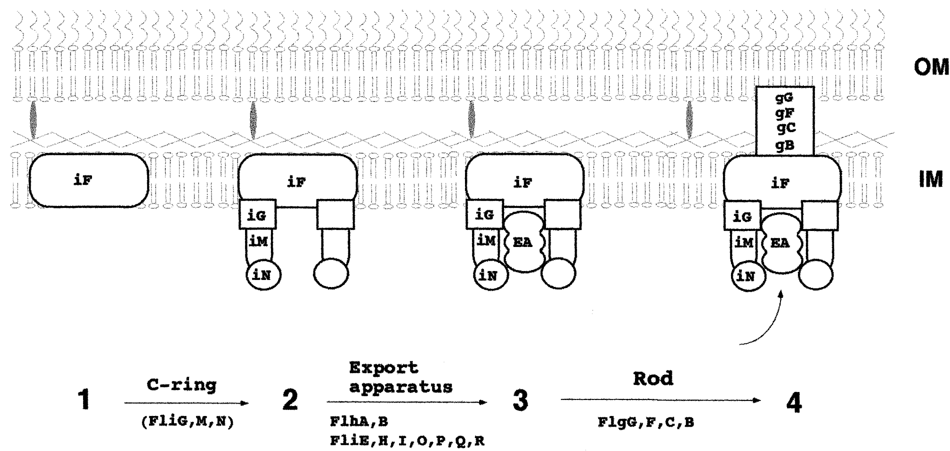
In addition to these hierarchical regulatory systems, in *S. typhimurium*, a specific mechanism known as phase variation allows for the change in the flagellin expressed by a given cell with a low frequency [25]. This is due to the presence of an operon in its genome encoding an additional flagellin (FljB) and a transcriptional repressor (FljA). These two genes are located inside an invertable element that also encodes a specific invertase (Hin) that recognizes both ends of the DNA fragment. When this element is in the correct orientation, the transcription from an external class III promoter allows for the expression of the flagellin FljB and the repressor FljA, which concomitantly blocks the expression of the flagellin FliC through binding to its class III promoter. When the element changes its orientation, transcription of *fljB* and *fljA* stops and FliC replaces FljB as the main filament component.

2.3. Flagellar assembly and secretion

Flagellar assembly has been analyzed using mutants impaired in specific steps of the process [5]. As depicted in Fig. 2, the overall order of synthesis of the various flagellar components is well known. Remarkably, the synthesis of flagella is different from other bacterial filamentous structures such as fimbriae (see later), since addition of the flagellar components occurs at the distal extreme of the structure [26,27]. This implicates an ordered process that can only be partially explained by the transcriptional control discussed above [5].

The first flagellar structure to be detected by electron microscopy is the MS-ring of the basal body, which is formed by self-assembly of FliF subunits (Fig. 2A, step 1). However, the mechanism used by the cell to select the location and number of the MS-rings synthesized, or the moment to initiate the flagellar assembly, remains unknown. Following MS-ring assembly, the peripheral structures that compose the cytoplasmic protruding C-ring

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024



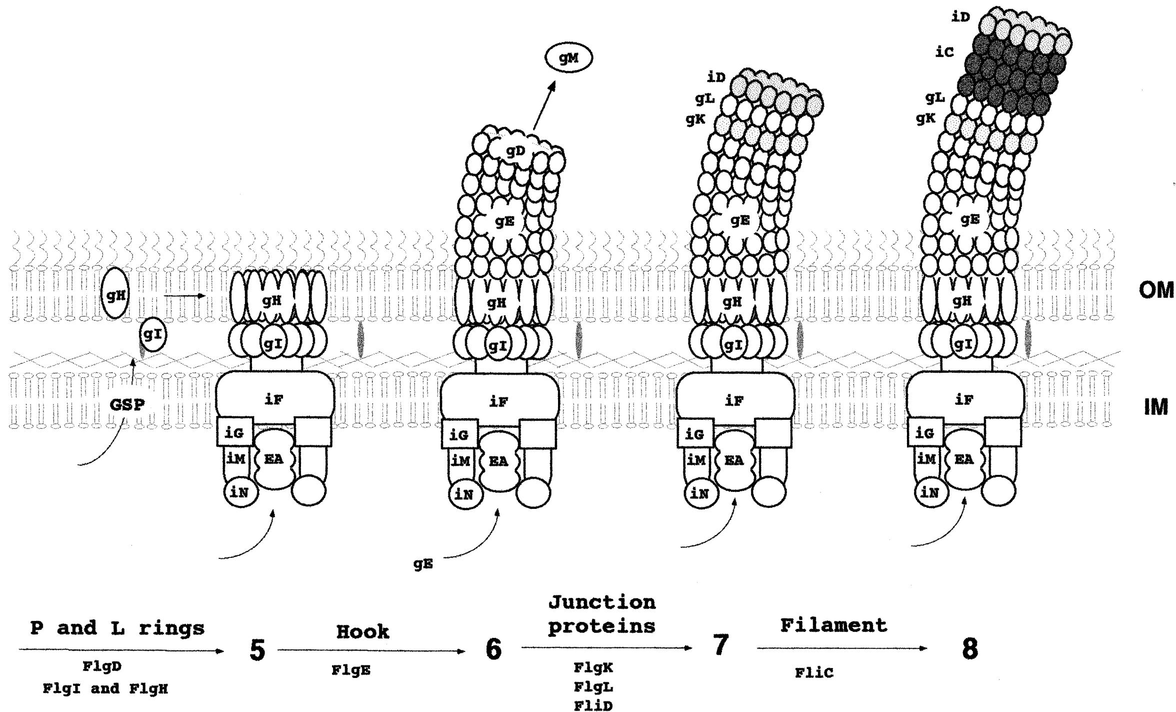


Fig. 2. Main steps in flagellar assembly. The pathway for the assembly of flagella is described in A and B in eighth steps. At first (1), around 26 FliF copies assemble into a ring structure within the IM, a process that is immediately followed by the assembly of FliG, FliM and FliN to form the C-ring (2). In (3), a group of proteins belonging to the flagellar EA are incorporated before the secretion of the rod components FlgB, FlgC, FlgF and FlgG (4). After this, the hook scaffolding protein, FlgD, is added to the tip of the rod and the P- and L-rings are subsequently formed once the corresponding components FlgI and FlgH have been secreted through the GSP (5). Once the L-ring is formed, the hook can grow crossing the OM by the incorporation of FlgE subunits, until an internal signal results in the secretion of the anti-σ factor FlgM (6). Finally, a series of annuli of the junction proteins FlgK, FlgL and the filament scaffolding protein FliD are added at the tip of the hook (7), allowing for the incorporation of the flagellin to form the filament (8). The last two letters of the corresponding genes have been used to identify each protein.

(FliG, FliM and FliN) are incorporated (Fig. 2A, step 2). Completion of these two rings marks the time for the assembly of the rod [28,29,30], as mutants in C-ring encoding genes fail to assemble the rod. Moreover, the proper assembly of the rod components requires the simultaneous expression of several other 'auxiliary' genes [26,5]. A recent study has shown that many of these components are required for the secretion of the rod and hook components, thus supporting their role as members of the export machinery and/or as specific chaperones required for the secretion [29]. In consequence, the poorly characterized components of this export apparatus (EA) have to

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

assemble following the formation of the C-ring (Fig. 2A, step 3). Only after that, secretion of the rod proteins (FlgB, FlgC, FlgF and FlgG) is thought to occur through the pore formed by the MS-ring (Fig. 2A, step 4).

Once the rod becomes mature, the FlgD protein is incorporated at its tip, acting as a scaffold, which allows for the hook protein (FlgE) to initiate its polymerization. However, the growth of the hook stops quickly due to the physical obstacle posed by the OM [26]. In order to cross the OM, the P- and L-rings must be added. A precursor of FlgI (P-ring protein) assembles and attaches roughly at the center of the rod, followed by FlgH (L-ring) which binds on top of it [31] (Fig. 2B, step 5). The assembly of P- and L-rings requires FlgA and the oxidation of two cysteines within FlgI by the periplasmic disulfide bond-forming system (Dsb) of the cell [32]. The secretion of FlgI (P-ring) and FlgH (L-ring) into the periplasm and OM, respectively, is dependent on the general secretion pathway (GSP) [1]. Accordingly, both proteins are synthesized with a *sec*-dependent signal peptide (SP) that is processed during their secretion. Although the assembly of the P- and L-rings is not fully understood, a requirement for the hook-capping protein FlgD has been demonstrated [29].

The number of FlgE subunits subsequently added to the hook (up to a size of 55 ± 6 nm) is apparently controlled by FliK, a protein whose location in the cell is not known. Mutants in *fliK* synthesize 'polyhook' structures of FlgE, unless an extragenic suppressor mutation in the integral membrane protein FlhB is present [33]. Apparently, FlhB is responsible for the selection of the type of protein secreted through the flagellar secretion apparatus (see below) [5,34,29].

Once the hook is completed, the anti-σ factor FlgM is secreted from the cell (Fig. 2B, step 6), allowing for the transcription of the class III operons by the FliA (σ^{28}), the hook-associated proteins FlgK, FlgL and FliD among them. The initial role of FlgK is to polymerize at the tip of the FlgE hook (the main hook component), replacing the scaffolding protein FlgD. This allows for the immediate polymerization of one or more annuli of FlgL on top of FlgK. Finally, FliD is added at the tip (Fig. 2B, step 7), acting as a new cap protein [7,35].

After addition of FliD, the flagellin subunits initiate their incorporation into the FlgL-FliD junction, leading to the extension of the filament with a variable length (Fig. 2B, step 8), which seems to depend on the amount of flagellin synthesized, as multiple copies of the flagellin gene lead to the synthesis of abnormally long filaments. An important problem for the growth of the filament is the energetic barrier that represents the movement of the flagellin subunits along its length to reach the tip of the filament (up to 10 μm). It has been proposed that FliI, an ATPase required for flagellar synthesis, could supply the energy required for the flagellin movement. Like many other proteins of the flagellar apparatus, FliI is homologous to components of type III secretion systems (see below) [36–38].

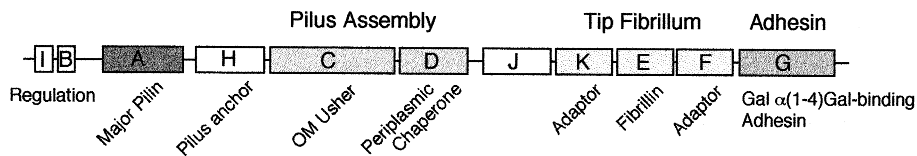
With the exception of the components of the P- and L-rings which are secreted by the GSP [39], the secretion of the other flagellar proteins requires a specific machinery, the EA, which is phylogenetically related to the type III secretion systems found in many bacterial pathogens [40,38]. Around 10 genes showing homology with genes belonging to the type III secretion apparatus have been identified in the flagellar clusters [38]. Some of these (encoding FlhA, FlhB, FliH, FliI, FliO, FliP, FliQ and FliR) might be integral components of this machinery since their mutation results in failure of the secretion of flagellar components (i.e. the hook protein FlgE and its capping protein FlgD) [29]. Moreover, some have also been located at the basal body (FliP, FliR and FlhA) [41], most probably in the 10-nm pore formed by the MS-ring, thus further supporting their involvement as components of the flagellar secretion apparatus [28].

The supramolecular structure of a type III system has recently been described [42] and shows an astonishing similarity to the flagellar basal body, including the presence of two close rings at its basis and two more associated to OM components. A rod, thinner than that of the flagella, passes between these two structures and projects outwards. This system acts as a molecular syringe directing the injection of several bacterial proteins inside the cytoplasm of the eukaryotic host, thus activating the host cell signaling pathways and leading to a variety of cellular responses that favor the phatogen invasion [38]. The structural similarity and sequence homology between type III systems and the flagellar secretion apparatus point to a common evolutionary origin for both structures.

Other cytoplasmic proteins play relevant roles in the biogenesis of the flagella. Some of them, like FliJ and FliS, could function as specific chaperones required for the secretion of particular components. In this sense, it has been demonstrated that FliJ is required for export of the hook components FlgD and FlgE, whereas FliS plays a similar role for the flagellin secretion [41,43,29].

One of the most intriguing aspects of the biosynthesis of flagella is how the secretion apparatus identifies which proteins must be secreted at each moment, even in the presence of more than one putative candidate. This is the case for the rod and hook components and it has been proposed that specific signal sequences within the proteins, like those found in the flagellin of *Caulobacter crescentus* [44], serve this recognition. It is known that FlhB plays a major role in the switching of substrate specificity, changing from a hook-type protein to a filament-type protein (FlgK, FlgL, FliC, FliD and the anti-σ factor FlgM) after the hook is completed [33,29]. Nevertheless, a more detailed recognition should exist to allow for the precise secretion of these proteins observed in vivo. Fur-

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024



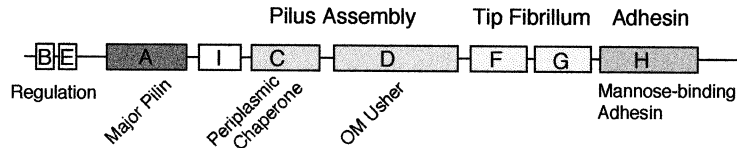


Fig. 3. Operons encoding type I pili in uropathogenic *E. coli* strains. A scheme is shown depicting the genes in the *pap* and *fim* operons of uropathogenic strains of *E. coli*, which encode the P and type I pili, respectively. These operons contain the genes encoding the structural subunits of the pilus shaft and tip fibrillum, including the adhesin, as well as their respective periplasmic chaperones and OM ushers. A similar gene organization can be observed between these two operons. This gene arrangement resembles the structural organization of the pili. Also notice that the gene encoding the major pilin is the first gene translated in the polycistronic mRNAs derived form these operons. This location is partially responsible for the production of higher levels of this structural subunit.

ther experiments are needed to unravel the mechanisms underlying this ordered secretion of flagellar components.

3. Fimbriae (pili)

Fimbriae, also known as pili, are hair-like appendages built by protein subunits called fimbrins or pilins and usually extend 1–2 μm from the bacterial surface with diameters ranging from 2 to 8 nm [45]. Fimbriae have been associated with host tissue adhesion properties of important pathogenic strains of bacteria [46], like class I pili of uropathogenic strains of *E. coli* and *Salmonella* and type IV pili of *Pseudomonas aeruginosa*, *Moraxella* spp., *Neisseria* spp., *Vibrio cholerae* and enteropathogenic and enterotoxigenic strains of *E. coli*. However, the specificity of the fimbriae adhesion is in some cases due to a minor component of the structure, like in type I and P pilus of *E. coli* [47–49] or in type IV pilus of *Neisseria gonorrhoeae* [50]. The actual contribution of pili to virulence has been demonstrated in vivo in a number of cases (see for example, [51–55]), but most of the evidence of their role in adhesion has been obtained by in vitro studies using organ and cell culture systems. However, since most pathogens express multiple types of fimbrial and/or afimbrial adhesins, the contribution of individual pili to virulence has been difficult to assess [46,56].

Pili can be classified on the basis of their physical properties, antigenic determinants, adhesion characteristics or similarities in the primary amino acid sequence between their major protein subunits [45]. Here, we will describe the class I and IV pili because they represent the two best-characterized model systems and have an ubiquitous presence among important Gram-negative pathogens. Furthermore, they show important differences in their molecular mechanisms of secretion and assembly.

3.1. Class I fimbriae

3.1.1. Structure and genetics

The best-studied examples of class I fimbriae are the P and type 1 pili of uropathogenic strains of *E. coli* [57–60]. These are composite assemblies consisting of a long rigid shaft of about 7 nm in diameter, which is made up by a few thousands copies of the major pilin subunit, and a short and thin flexible tip fibrillae, of about 2–3 nm in diameter, which is joined to the distal end of the pilus rod and composed mostly of minor pilin subunits [61,62]. The adhesin subunit of these pili is displayed at the fibrillar tip and so, it is distinct from the major fimbrial subunit [47,48,63–65]. In other pili belonging to class I, like the K88 and K99 fibrillae of *E. coli*, the major subunit contributes to the adhesive properties [66,67].

The genes encoding the P pili, or pyelonephritis-associated pili, are clustered in the *pap* operon, located in the chromosome of up to 90% of the *E. coli* strains isolated from the urinary tract of children and adults with acute pyelonephritis [68,69,60]. The *pap* operon contains 11 genes, encoding the structural components of the pilus rod and fibrillum (*papAH* and *papKEFG*), the pilus assembly machinery (*papCDJ*) and some regulatory proteins (*papIB*) [70,60] (see Fig. 3). The major subunit of the pilus rod is PapA, whereas PapE is the major component of the tip fibrillum [61]. PapG is the adhesin which specifically binds to Galα(1-4)Gal moieties present in the globoseries of glycolipids on epithelial cells from the urinary tract, the kidney and other tissues [48,71]. PapH is a minor component involved in anchoring of the pilus to the cell membrane and probably also functions as a signal to switch off

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

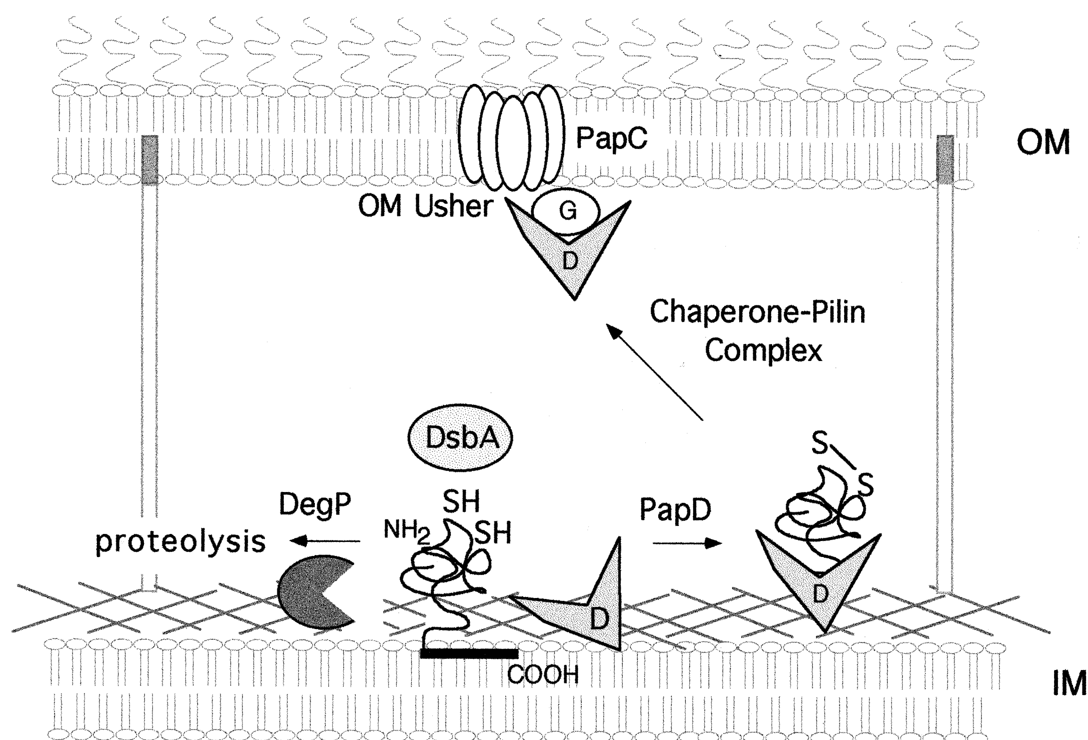


Fig. 4. Periplasmic chaperones involved in folding of type I pilins. Once type I pilin subunits have been transported into the periplasm in a *sec*-dependent manner, they remain associated with the IM by their C-terminal motif. The periplasmic oxidoreductase DsbA catalyzes the formation of disulfide bridges within cysteine residues of pilins, and also those of the PapD chaperone, allowing for a stabilization of their tertiary structure. The periplasmic chaperone (PapD) binds to pilins by forming a β-zipper with their C-domains, thus facilitating the release of pilins from the IM. PapC, the OM component known as the usher, recognizes the PapD-pilin complexes in the periplasmic side of the OM. In the absence of the PapD chaperone, the pilins are degraded by the periplasmic protease DegP.

the biogenesis of the pilus rod [72]. Some pilin minor subunits (PapK and PapF) serve as adaptors linking the pilus fibrillum to the rod and the adhesin, respectively [73]. Two other proteins encoded by the *pap* operon, PapC and PapD, are essential for pilus biogenesis but they are not structural components of the pilus. PapD is a periplasmic protein that functions as a chaperone for proper folding and assembly of the pilin subunits in the periplasm [74,75]. PapD forms stable complexes with the pilins in the periplasm and presents these subunits to PapC, an integral OM protein located at the assembly sites of the pilus. Also known as the usher, the PapC protein allows for the ordered uncapping of the PapD-pilin complexes and the assembly and secretion of the pilin subunits to form the pilus organelle [76–79].

Other well characterized type I fimbriae are also encoded by operons that resemble the *pap* operon [45]. For example, type 1 fimbriae of *E. coli* are encoded by the *fim* operon [80], in which functional and structural homologues to proteins of the *pap* operon can be found: FimA is the major subunit of the pilus rod, FimH is the adhesin recognizing β-D-mannosides [65] and FimC/FimD represent the periplasmic chaperone and OM usher components of the assembly machinery [81,60]. Importantly, more than 25 homologues to PapD/PapC have been identified among Gram-negatives, constituting an important family of proteins involved in the assembly of both pili and afimbrial adhesins [82,83,60].

3.1.2. *Type I pilins: secretion and interaction with periplasmic chaperones*

The products of all nine *pap* genes directly involved in P pilus biogenesis are synthesized as precursors containing classical N-terminal SPs which direct them to the periplasmic space using the Sec-machinery (GSP) to cross the cytoplasmic membrane [1]. Early work demonstrated the role of SecA protein and LepB signal peptidase in the secretion of type I fimbriae [84,85].

The pilin subunits of P pilus can be purified as complexes with the PapD chaperone from the periplasm of *E. coli* [86,87,60]. For example, the PapG adhesin was isolated from the periplasm of *E. coli* by affinity chromatography using a matrix containing Galα(1-4)Gal in 1:1 complexes with PapD [86]. The expression of PapG in the absence of PapD resulted in its proteolytic cleavage. Similarly, the expression of the type 1 pilus adhesin FimH was shown to be susceptible to proteolytic cleavage when expressed in the absence of FimC, the corresponding periplasmic chaperone [81,62]. These results, which were corroborated by the expression of other pilin subunits [88], suggested that PapD-like periplasmic chaperones form a complex with pilins which stabilizes them in the periplasm.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

Since resistance to proteolysis is considered to be a marker of a folded conformation [89], PapD-like chaperones may maintain pilin subunits in a native-like and assembly-competent state. This conclusion was also supported by the fact that the adhesin was able to bind its target molecule in the chaperone complex [86,75].

Although the mechanism of action of PapD-like chaperones seems different than that of cytoplasmic chaperones, like SecB and DnaK which bind and maintain proteins in a partially unfolded state [90], their ultimate function is probably identical: the prevention of non-productive (aggregative) interactions during protein folding and protein complex assembly [91].

PapD chaperone is also involved in the targeting of pilin subunits to the periplasm, facilitating their release from the IM [88] (Fig. 4). This conclusion was derived from studies in which pilin subunits, expressed in the absence of PapD, were found to accumulate in the IM of an *E. coli* strain defective for the major periplasmic protease DegP. Since DegP substrates are partially unfolded periplasmic proteins [92], these experiments provide additional support for a role of PapD in the folding of pilins.

Most pilins have a low molecular mass (15–20 kDa), except for PapG adhesin which has roughly twice this size, and share some structural features which are important in the assembly of pili (Fig. 5). For example, pilins contain a conserved disulfide bridge that needs to be oxidized by the periplasmic oxido-reductase DsbA to allow for their proper folding and incorporation into growing pili [93,32]. Functional DsbA is also needed for the formation of a disulfide bridge in the PapD chaperone [94].

Especially relevant is the presence of a C-terminal motif in the pilins with a common pattern of alternating hydrophobic amino acids flanked by a glycine and a conserved aromatic amino acid (Phe or Tyr; Fig. 5). This C-motif, located within the last 15 amino acids, is essential for the binding of pilins to the PapD chaperone and for pili polymerization. Its deletion in PapGΔC avoids the interaction with PapD chaperone and renders PapGΔC unstable in the periplasm [86]. In addition, PapD chaperone binds to synthetic peptides containing the sequence of the C-terminus of pilins [95,91]. Furthermore, this C-motif of pilins is also involved in the transient insertion of pilins into the IM, before binding to PapD in the periplasm [88]. Expression of the truncated PapGΔC in an *E. coli degP papD* mutant led to its accumulation in the periplasm, but not to its association with the IM. Recently, it has been demonstrated that this C-motif is also required for inter-subunit

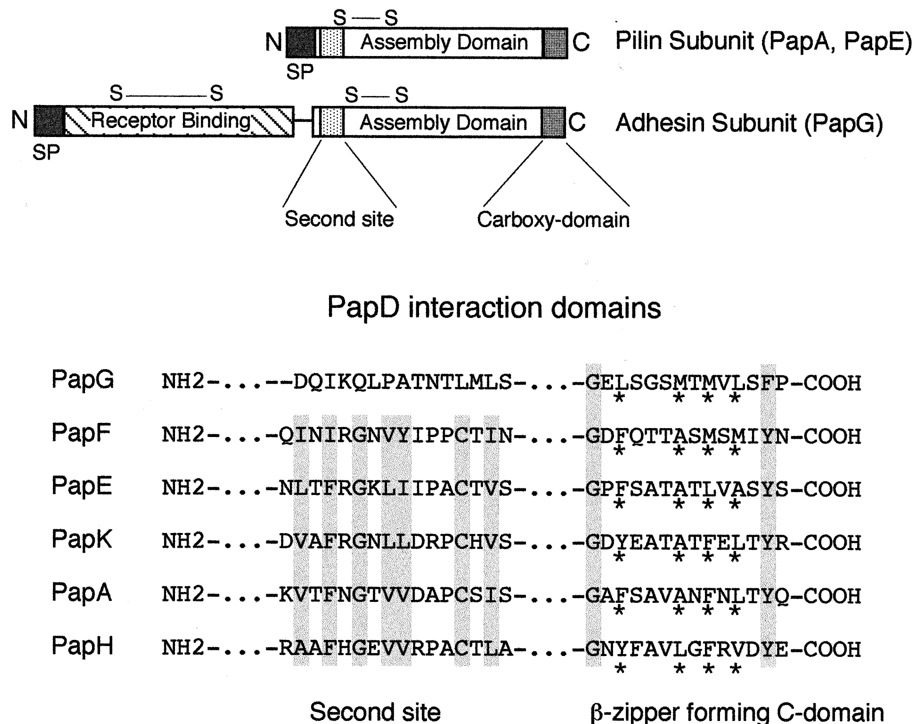


Fig. 5. Structural motifs within type I pilins. A cartoon is presented showing distinct elements of the pilins of P pili (top). As indicated, the structural pilins (like PapA and PapE) are small polypeptides in which most of their sequence is thought to be involved in the inter-subunit interactions required for assembly of the pili (assembly domain). Apart from this, two short sequences within the pilins (the carboxy domain and the second site, near the N-terminus of the mature pilin) are implicated in binding to the periplasmic chaperone (PapD). In contrast, the adhesin subunit (PapG) has two distinct protein modules. The N-terminal module has the receptor binding specificity (i.e. it recognizes the Galα(1-4)Gal moiety of glycolipids), whereas the C-module contains the assembly and PapD interaction motifs (i.e. the second site and the carboxy domain). Both the structural pilins and the adhesin have N-terminal *sec*-dependent SPs and internal disulfide-bonded cysteine resides (S-S). A detail of the amino acid sequence of the PapD interaction domains of pilins is shown at the bottom. The conserved residues are within boxes. The asterisks (*) mark the alternating hydrophobic amino acids in the β-zipper-forming C-domain.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

quaternary interactions in the pilus [91]. Thus, a primary role of PapD chaperone would be to cap this interactive surface in order to prevent premature oligomerization of pilus subunits [77,91].

Another highly conserved domain of pilin is a short region located near their N-termini [60]. Mutations within this N-region have been shown to abolish the formation of stable PapD chaperone-pilin complexes and thus, it was postulated to be a 'second site' for PapD binding [91] (Fig. 5). This N-terminal motif is not found in PapG adhesin, where an internal region was found to be a 'second site' for the binding of the chaperone [96]. PapG adhesin is also unique among pilins because it is a two domain protein, containing a N-terminal domain involved in Galα(1-4)Gal binding and a structurally independent C-terminal domain, which has all the determinants for PapG assembly into pili [86,60].

3.1.3. Structure of the periplasmic PapD chaperone

The three-dimensional structure of PapD periplasmic chaperone has been solved, both as an apo-protein [97] and in complexes with peptides comprising the C-terminal sequences of PapG [95] and PapK [91]. PapD has a boomerang-like V-shape consisting of two domains. Each domain has a β-barrel structure formed by antiparallel β-pleated sheets, in a folding similar to the immunoglobulin (Ig) fold [98,99]. The structure of the N-terminal domain of PapD chaperone has the highest degree of homology to the variable domain of an Ig, whereas the C-terminal domain has a topology analogous to domain 2 of the human CD4 surface molecule [97,82,60]. The PapD C-terminal domain contains an essential disulfide bond interconnecting two β-strands at the end of the domain [94].

Other members of the PapD-like periplasmic chaperone superfamily probably have a similar structure since they share a high sequence similarity [83]. Conserved residues are concentrated in the cleft region of the V-shaped PapD structure, which are directly involved in maintaining the Ig fold and orienting the two PapD domains.

PapD chaperone binds pilins recognizing their C-motif of alternating hydrophobic amino acids (see above). The obtention of a co-crystal between PapG-derived peptide and PapD revealed the specific way by which PapD recognizes the pilins [95]. More recently, the structure of a co-crystal of a PapK C-terminal peptide and PapD has also been obtained [91]. Despite significant sequence differences between PapG and PapK C-termini, both peptides bound PapD chaperone in an identical manner, with an extended β-strand conformation. Their carboxy-terminus was anchored to two invariant positively charged residues of the PapD cleft, whereas the amide bonds of the peptides hydrogen-bonded to the amide bonds of a β-strand of the N-domain of PapD, thus continuing the β-sheet structure of this domain. In addition, the hydrophobic side-chain groups of the peptides interlocked with side-chain groups of the PapD β-strand, further stabilizing the complex by Van der Waals contacts. This interaction between two β-strands of different polypeptides has been called a 'β-zipper'. The low sequence-dependence of these β-zippering interactions explains why the PapD chaperone is able to bind different pilus subunits which only share modest sequence identity (Fig. 5).

3.1.4. Assembly of type I pili: the role of the OM usher

The basis for the coordinated assembly of type I pili is the recognition of the periplasmic pilin-chaperone complexes by the OM PapC usher in an ordered fashion (Fig. 6) [76]. In the absence of PapC, pilin-chaperone complexes accumulate in the periplasm but cannot translocate across the OM [100,101].

Partially purified PapC was shown to bind in vitro PapD-pilin complexes with different affinities. Chaperone complexes formed with more distal pilus subunits (PapG, PapF and PapE) efficiently bound to PapC. In contrast, PapC did not bind in vitro to PapD-PapA, PapD-PapK or PapD alone. This characteristic ensures the ordered incorporation of pilin subunits and avoids the assembly of the pilus rods in the absence of tip fibrillae [76]. It was suggested that PapA- and PapK-chaperone complexes would bind PapC once associated with tip fibrillae [60].

In *E. coli* P and type 1 pili, adhesin-chaperone complexes show the highest affinity for the OM usher in vitro [76,78]. Therefore, it is likely that the PapG-PapD complex would be the first to bind to the usher in vivo. Once PapG adhesin is bound to PapC, PapF- and PapE-chaperone complexes can incorporate into the growing tip of the fibrillae. Thus, a critical step in type I pilus biogenesis is the initial binding of the adhesin-chaperone complex to the periplasmic side of the OM usher (PapC, FimD). Indeed, some natural isolates that do not produce pili contain mutations in the gene encoding the adhesin subunit [53,102].

PapC-like ushers are β-stranded proteins that form oligomeric complexes in the OM of *E. coli*. The majority of PapC was found in a hexameric complex, although other oligomerization states (from dimers to dodecamers) were also detected [79]. The PapC complex has an O-ring shape and displays a central pore of 2–3 nm in diameter. This pore size is large enough to allow for the passage of the tip fibrillae, made up of a linear array of pilus subunits, but too narrow for the translocation of the mature pilus rod of about 7 nm. To explain this discrepancy, it has been proposed that the pilus rods unravel to a thin 2–3-nm fiber during their passage through the usher and later fold in the extracellular medium to an helical structure [79]. This model has found experimental support since unraveled pilus rods, caused by unwinding of the helix into linear fibers, has been obtained in vitro in P and type 1 pili [103,79]. Thus, the present model for type I pilus assembly

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

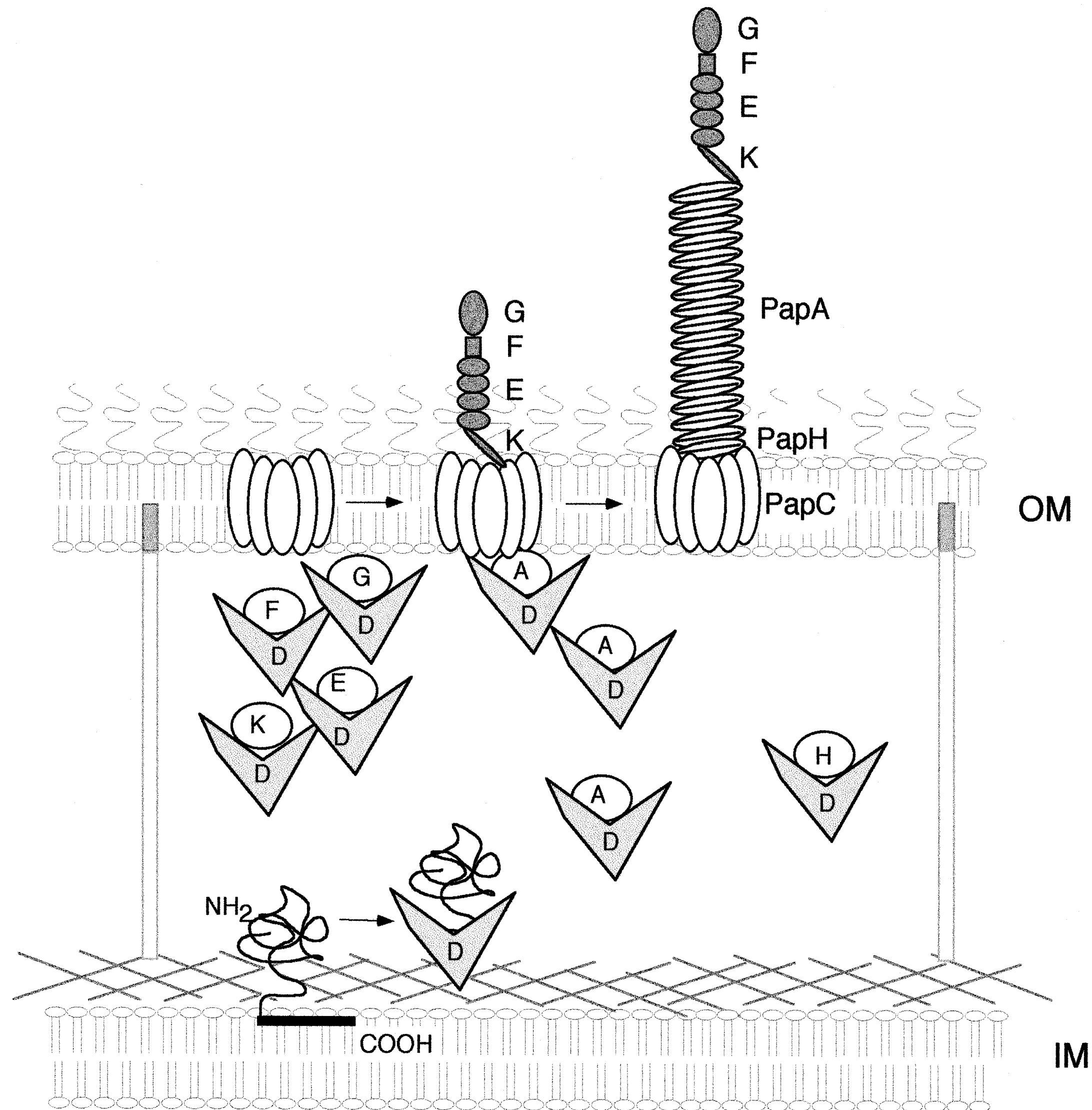


Fig. 6. Assembly of type I pili. In the periplasm of *E. coli* cells producing P pili, there is an accumulation of pilin-PapD complexes. Apart from its function in folding of the pilins (see Fig. 4), PapD interaction impedes the premature aggregation of pilins in the periplasm. Pilin-chaperone complexes are bound in order to the OM usher PapC, from those containing a pilin of the tip fibrillae (PapG, E, F, K) to those having the pilin, which forms the shaft of the fimbria (PapA). Finally, the incorporation of PapH ends the growth of the pilus and anchors it to the OM.

proposes that an 'immature' structure is translocated across the OM through the central pore of the usher as a 3-nm fiber, which folds into its final quaternary structure in the extracellular medium. Newly incorporated subunits are taken directly from chaperone complexes in the periplasm and added to the base of the growing pilus. It is not clear whether pilus folding in the extracellular medium or another energy source drives the elongation process. Finally, the pilus is terminated and anchored to the OM through PapH [72]. As we will discuss in the following section, type IV pilus biogenesis also requires the presence of OM proteins, which form oligomeric structures containing large central pores (secretins) through which the pilus is expelled.

3.2. Type IV pili

3.2.1. General characteristics

The pili from a broad spectrum of Gram-negative bacteria are grouped as type IV on the basis of amino acid sequence similarities among their major pilin component. The homology between different type IV pilins is highest at their amino-terminus, but also extends to some areas of the C-terminus, where other conserved features, like a pair of cysteine residues that form a disulfide bridge in the mature protein, can be identified. This group of pili has been extensively studied since they mediate adhesion to host tissues in many important pathogens, like *V. cholerae*, *P. aeruginosa*, enteropathogenic and enterotoxigenic *E. coli*

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

(EPEC and ETEC), *N. gonorrhoeae*, *Neisseria meningitidis* and *Moraxella bovis* among others [104].

All type IV pili use a similar secretion and assembly machinery, made up of at least a dozen different polypeptides, which are functionally and evolutionary related to components of the main terminal branch (MTB) of GSP, or the type II secretion system from Gram-negative bacteria [1,105], and to components of filamentous phage assembly and DNA uptake systems [106–108,2]. In addition, type IV pili are involved in a form of surface non-flagellar movement called 'twitching motility', which is presumably mediated by reversible depolymerization and assembly of the filament [109,104,110]. A related process, the gliding motility in *Myxococcus xantus*, is also mediated by type IV pili [111,112].

Immunologic as well as crystallographic evidence has provided a model for the pilin structure in type IV pili. In this model, the pilin C-terminus is exposed at the surface of the filament, whereas the hydrophobic N-terminal domain forms the pilus inner core. These two domains are interconnected by a α-helical region [113,114]. Most type IV pili are flexible rod-like filaments of about 5–6 nm in diameter and 1–2 μm in length, in which the pilin subunits are arranged in a helical manner. The toxic co-regulated pili (TCP) of *V. cholerae* [55], the bundle-forming pili (BFP) of EPEC [115] and the *Longus* pili of ETEC [116] have some particular characteristics among type IV pili [104]. Remarkably, TcpA and BfpA pilins assemble into straight fibers of variable length that have a strong tendency to aggregate laterally, both in vivo and in vitro, and which mediate the formation of 'microcolonies' important for the virulence of these strains [51].

The genes responsible for the biogenesis of type IV pili are located at various regions in the bacterial chromosome [104]. Important exceptions to this rule exist, however, like in *V. cholerae*, where the *tcp* genes are clustered in a single region of the chromosome [117], or in EPEC, where the *bfp* genes are present in a 80-kbp virulence plasmid [115,118,119].

At present, only two tip adhesins have been identified among type IV pili: PilC of *N. gonorrhoeae* and *N. meningitidis* [50,120]. There is a homologue of *Neisseria pilC* in *P. aeruginosa* (*pilY*) [121]. However, the actual role of PilY is unclear since the main adhesion determinant of *P. aeruginosa* type IV pili is found in their major pilin subunit [122,123]. Thus, although tip adhesins may generally exist in type IV pilus, it seems clear that the major pilins can also participate directly in adhesion to host tissues.

PilC tip adhesin was initially identified as a 110-kDa protein present in the OM of *N. gonorrhoeae* [124] and later recognized as a component of the pilus tip involved in adhesion to host tissues [50]. Unlike type IV major pilins (see later), PilC contains a *sec*-dependent classical N-terminal SP and from this point of view, it is thus more closely related to a substrate of a type II secretion system. The gene encoding PilC is present in two copies (*pilC1* and *pilC2*) in the *N. gonorrhoeae* MS11 chromosome [124,125]. *PilC1* was initially found to be inactive in the MS11 strain analyzed, due to an out of frame mutation in a homopolymeric G-run located in the region

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

Fig. 7. Structure of the N-terminal type IV pre-pilin SP. The amino acid sequence of the N-terminus of OmpA, a classical *sec*-dependent SP, and that of the N-terminus of PilA, the SP of the major type IV pilin of *P. aeruginosa*, are shown for comparison. As indicated, the amino acid sequences of both proteins share some overall similarities, like the presence of positives residues at the N-terminus and a stretch of hydrophobic amino acids with the propensiy to form an α-helix once it spans a lipid bilayer. These N-terminal SPs are processed by distinct endopeptidases located in the IM. The *sec*-dependent SPs are cleaved after the hydrophobic region by the major signal peptidase (LepB in *E. coli*), which has its catalytic site in the periplasmic face of the IM. By contrast, the pre-pilin SPs are cleaved in front of their hydrophobic region by the pre-pilin peptidase, which has its catalytic site in the cytoplasmic face of the IM. The amino group of the residue located immediately after the cleavage site of the pre-pilin peptidase (usually phenylalanine) is methylated by the same enzyme. The amino acid sequence of the N-terminal part of type IV pilins, including the SP, is highly conserved, so that it can be used as a protein motif for recognition of type IV pilins.

encoding the SP of PilC. An identical G-run, but in-frame with the PilC reading frame, is present in the active *pilC2* [124]. Mutations affecting the length of these *pilC* G-runs are frequent in *Neisseria* isolates. Out of frame mutations in *pilC* lead to non-piliated strains (P^-), although the synthesis of the major pilin (PilE) is not affected. On the other hand, restoration of the *pilC* reading frame reverts the gonococci to a piliated phenotype (P^+). These on/off changes in piliation, known as phase variation, represent a way for the microorganism to evade the immune response.

In addition to changes in PilC expression, *N. gonorrhoeae* phase variation is also mediated by changes in the gene encoding the major pilin [126,127]. One or two different loci are used for the expression of the major pilin encoding gene (*pilE*), whereas various silent genes (*pilS*) are spread throughout the chromosome. Recombination between *pilS* and *pilE* copies leads to changes in the pilin coding sequence and subsequently to the expression of antigenically different pili. A similar mechanism has been described for the antigenic variation of the S-layer from *Campylobacter fetus* (see Section 4.2).

3.2.2. The pre-pilin SP: relations between type IV pili and the GSP

The pilins belonging to the type IV pili are synthesized as precursor proteins of around 18 kDa. These precursors contain a short and basic amino-terminal SP, usually consisting of seven amino acids, that is cleaved after a conserved glycine residue by a specialized family of IM endopeptidases [128,129] (Fig. 7). These pre-pilin peptidases are bifunctional enzymes that also *N*-methylate the first amino acid of the mature pilin polypeptide (Phe or Met) [130], although mutations abolishing this methylating activity do not interfere with the assembly of functional pili in vivo [131].

Besides major pilin itself, other proteins with pre-pilin-like leader sequences participate in type IV pilus biogenesis, probably as components of the secretion apparatus [132,133]. Interestingly, some polypeptides of the GSP machinery contain pre-pilin-like leader sequences and share homology with the N-terminal moiety of the pilins [2]. Although it has been speculated that these GSP subunits may form a pilus-like fiber in vivo, experimental evidence proving the actual existence of such a structure has not

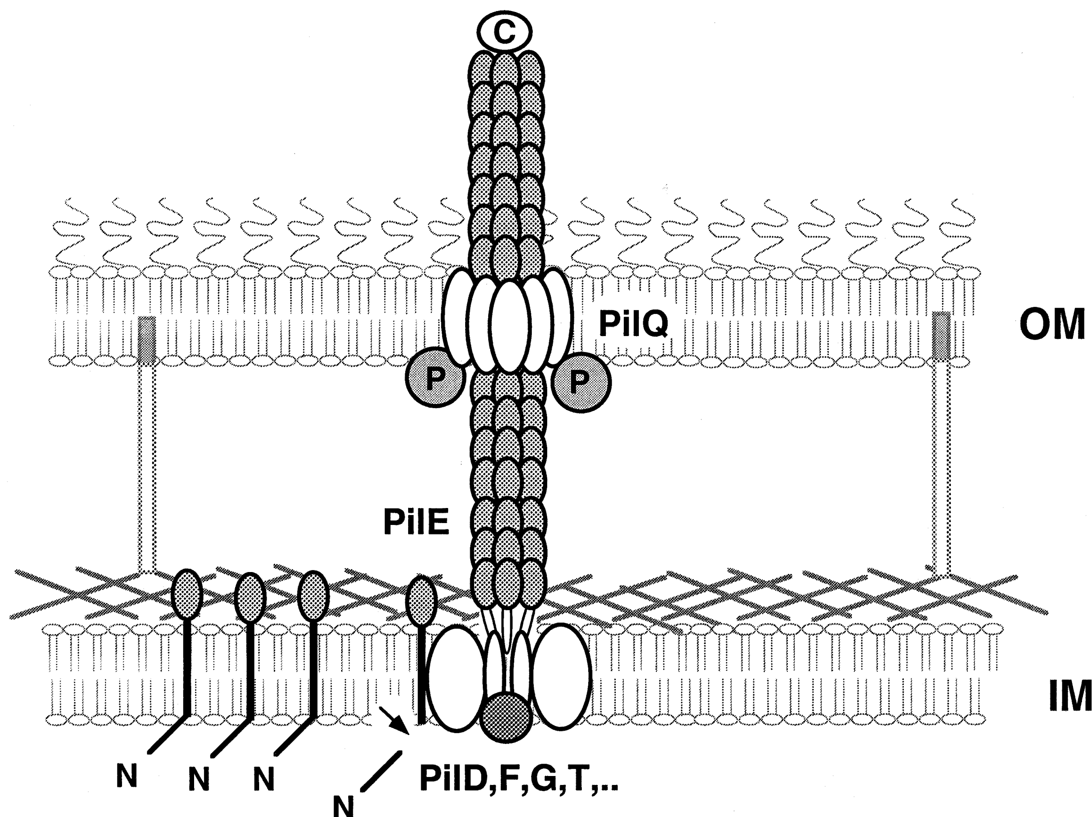


Fig. 8. Assembly of type IV pili. A model for the assembly of type IV pili from *N. gonorrhoeae* is shown. The major pilin (PilE) is translocated into the periplasmic side of the IM but remains anchored to the membrane by a hydrophobic N-sequence. A short and basic N-terminal SP is processed in the cytoplasmic side of the IM by the pre-pilin peptidase (PilD). PilD probably forms a complex with about 10 different proteins located in the cytoplasm and IM, some of which contain pre-pilin-like N-sequences or ATP binding motifs (PilF, PilT). Assembly of the pili occurs at this protein complex by expelling the pilins from the IM. A secretin component (PilQ), stabilized by an OM lipoprotein (PilP), forms a large pore in the OM through which the pili is secreted to the extracellular medium. During the first steps of pilin synthesis, the adhesin (PilC) is incorporated at the tip of the filament. In both type I and type IV pili biogenesis, periplasmic DsbA oxidoreductase is required for disulfide bond formation within pilins or among other components of the secretion apparatus.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

been obtained. However, some interactions have been detected between type IV pilin and pilin-like subunits of the GSP of *P. aeruginosa*, which may be assembly intermediates of the secretion apparatus [134]. Interestingly, *P. aeruginosa* pre-pilin peptidase, PilD (XcpA), is also involved in the maturation of pre-pilin-like components of the type II secretion machinery and is required for type II-dependent secretion [135–138]. This finding provided the strongest evidence that there exists a close relationship between the molecular apparatus for the biogenesis of type IV pili and type II secretion in *P. aeruginosa*. More recently, it has been proved that the *P. aeruginosa* pilin, PilA, is required for an efficient secretion of type II substrates [134]. In the same study, protein-protein complexes containing PilA and pre-pilin-like components of the type II secretion system were also identified by in vivo crosslinking. However, in spite of these relationships, mutations in other genes encoding specific components of the type IV pilus or type II secretion machinery have led to specific phenotypes related to piliation or protein secretion, clearly demonstrating that both systems operate separately [139].

3.2.3. Secretion of type IV pilin

Prior to its polymerization into pili, the pilin polypeptide is transitory anchored into the IM by a conserved hydrophobic domain located at the N-terminus, immediately after the pilin SP (Fig. 8). Certain mutations in genes encoding components of the pilus assembly machinery, like *pilB* and *pilC* in *P. aeruginosa*, induce the absence of pili on the bacterial surface and cause accumulation of the processed pilin in the IM [140]. Similarly, mutations in the pre-pilin peptidase lead to a pilus-deficient phenotype that accumulates pilin in the IM, but in an unprocessed form [128,141].

The insertion of the pre-pilin precursor into the IM can occur independently of the *sec* genes [141]. By contrast, the substrates of the GSP are exported into the periplasm in a *sec*-dependent fashion using classical SP cleaved by the major signal peptidase (LepB). The periplasmic intermediates of these proteins are the substrates for the type II secretion apparatus, which possibly recognize structural elements at different parts of the polypeptide chain [142]. Similarly, the pilin subunits expose the majority of the protein to the periplasmic space, but remain anchored into the IM by their hydrophobic N-domain.

The process of expelling pilin subunits from the IM and into a growing pilus filament resembles the assembly of filamentous phages in *E. coli*, where the precursor of the major coat protein (pVIII) is inserted, in a *sec*-independent manner, into the bacterial IM and later expelled to a growing coat of the viral particle [143]. Interestingly, an additional link between type IV pilus, type II secretion systems and filamentous phage assembly has been provided by studies on cholera toxin secretion [144]. The *ctxAB* genes were found in a lysogenic filamentous phage (CTX) that uses type IV pili (Tcp) as a receptor to infect the bacteria. Moreover, the cholera toxin polypeptides are assembled in the periplasm and secreted using a type II system [145].

3.2.4. Biogenesis of type IV pili

In *P. aeruginosa*, about 30 genes have already been identified as part of the type IV pilus biosynthetic apparatus [146]. Most of these genes, located in six chromosomal clusters, encode proteins directly involved in the export and assembly machinery or are components of regulatory networks that control type IV pili production. Four of these clusters contain genes directly involved in fimbrial assembly. The *pilA-D* locus encodes the major pilin (PilA) and three ancillary proteins: PilB, a cytoplasmic protein possessing an ATP binding motif; PilC, an IM protein, and the pre-pilin peptidase PilD (see above). Located at 25 kbp from *pilA-D*, the genes *fimT*, *fimU*, *pilV* and *pilE* encode products that contain pre-pilin-like N-terminal hydrophobic domains. Other pre-pilin-like encoding genes are found in other areas of the *P. aeruginosa* chromosome [133].

The operon *pilMNOPQ* encodes components of pilus assembly located at the IM and OM. PilQ appears to be the sole protein of the assembly system that is an integral OM component [147] and it is capable of oligomerizing to form a doughnut-shaped channel with a central cavity of about 55 Å [148] (Fig. 8). The size of the central pore formed by PilQ oligomers is in perfect agreement with the calculated outer diameter (52 Å) of the type IV pilus cylinder [149]. These characteristics make PilQ the ideal candidate for assembly of the channel through which the pilus rod is expelled to the outer medium. This situation contrasts with that of the OM usher of type I pilus systems (see above).

Homologues to the OM component (PilQ) of *P. aeruginosa* are found in the assembly machineries of other type IV pili [150], filamentous phages [151,152], type II secretion systems [153–156,148], systems for DNA uptake [157] and type III contact-dependent secretion systems [158]. This family of pore-forming OM proteins has been named secretins. The secondary structure of these secretins is predicted to be β-sheets, like that of the trimeric OM porins [159,105]. They all probably form oligomers, which are composed of 10–14 monomers, highly resistant to detergent denaturation and frequently stabilized by an OM lipoprotein, like PilP in *N. gonorrhoeae* [150] (Fig. 8).

Apart from the OM secretins and their cognate lipoproteins, the specific components of type IV pilus and type II secretion systems, about 10–12 other polypeptides, are IM or cytoplasmic proteins. The only periplasmic protein recognized to play a role in these secretion systems is the oxidoreductase DsbA [32]. DsbA is required for pilin stability and its assembly into BFP pili in EPECs [160]. In *V. cholerae*, a gene homologue to *dsbA* (*tcpG*) was found to be necessary for secretion of cholera toxin and the assembly of toxin-co-regulated pili [161,162].

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

The participation of so many cytoplasmic/IM polypeptides in a specialized secretion process across the OM is a puzzling observation. Among all these components, the only one with an assigned certain function is the pre-pilin peptidase. Another essential component is PilB, a putative ATPase that may energize the secretion process and whose mutation in the ATP binding motif abolishes pilus biogenesis [163]. However, the putative ATPase activity of these polypeptides has not yet been demonstrated, although an auto-kinase activity was found in EspE of *V. cholerae* [164]. In the case of filamentous phages, both ATP hydrolysis and the proton motive force (pmf) are required for their assembly [165]. Thus, it is tempting to speculate that some of the IM components of these systems may be required for coupling the pmf to the secretion process.

Since functional knowledge of the different secretion systems of Gram-negative bacteria continues to accumulate, it will soon be possible to understand how these complex machineries couple the cellular energy to the secretion of a pilus filament or a folded polypeptide.

4. S-Layers

The presence of crystalline protein layers (S-layers) that completely surround the cells has been described in around 350 different species belonging to most of the main branches of the Bacteria and Archaea domains [166]. The phylogenetic dispersion of this character, the presence of additional amorphous layers masking their regular architecture [167] and the frequent isolation of S-layer defective mutants in laboratory-grown bacteria [168] suggest that S-layers have a much wider distribution in natural environments than previously supposed. As an exception, we will include in this section some comments on Gram-positive bacteria, because most of the work on the S-layer field has been developed with this group of bacteria.

4.1. Structure and function of S-layers

All the S-layers studied so far are made up of a single (glyco)protein that polymerizes in vitro to form bidimensional paracrystalline structures indistinguishable from those found surrounding the cells in vivo. This simplicity has led to the suggestion that S-layers are ancestral forms of the cell wall that have further evolved towards the present day matrix proteins and porins [166,169]. In favor of this proposal is the fact that the oldest branches of bacterial evolution (i.e. *Thermotoga, Aquifex, Thermus*) possess S-layers with a morphogenetic function [170,171].

The molecular mass of different S-layer proteins (S-proteins) ranges from 50 to 169 kDa, with some species showing different-sized S-proteins, depending on the strain analyzed and the growth conditions used [166,172]. Additional differences might be found as a consequence of post-translational modifications such as glycosylation, addition of phosphate groups or proteolytic processing at different regions of the proteins. Despite these differences, most S-proteins show a similar overall composition, with a low content or even absence of cysteine and methionine and a high content in acidic residues [166]. With a few exceptions, which include S-proteins from Gram-positive *Lactobacilli* and the archaea *Methanococcus* [172], this results in a weakly acidic isoelectric point.

Comparison of S-protein sequences from phylogenetically unrelated organisms does not reveal homologous regions among them, suggesting a strong selective pressure against their conservation (i.e. proteases, phages and immune system). The main exception to this rule is the presence of one or more copies of the so-called S-layer homology (SLH) motif within the S-layer sequences of some Gram-positives and the *Thermus* group [173]. Tandemly organized SLH motifs have also been identified in several extracellular enzymes from Gram-positives [174], which are responsible for their binding to the PG [175,176].

The S-proteins from Gram-negative bacteria interact with the sugar moiety of the LPS component of the OM. In *C. crescentus*, the S-layer is anchored to the cell surface via a non-covalent interaction between the N-terminus of the protein and a specific smooth LPS in the OM [177]. Similarly, a variant strain of *Aeromonas salmonicida* defective in its smooth LPS secretes the S-protein into the medium [178], supporting the proposed interaction between the S-layer and this LPS moiety. In *C. fetus*, two serotypes (A and B) exist, based upon the type of LPS expressed, and each of these LPSs are able to interact with a specific S-protein (also named A and B) through a N-terminal domain [179].

4.2. Genetic regulation of S-layer expression

S-proteins constitute between 5–10% of the total protein content of the cell. Thus, the production of a S-layer is a process requiring high amounts of energy for the synthesis and incorporation of enough subunits to completely cover the cell surface. It has been estimated that $\sim 5\times10^5$ S-proteins are required to surround an average-sized bacilli [171]. This means that about 500 molecules of S-protein s^{-1} need to be synthesized, secreted and incorporated into the S-layer by each single cell growing with a generation time of 20 min. Keeping in mind that S-proteins are expressed from single copy genes, both their transcription and translation must be highly efficient. Analysis of the codon usage and ribosome binding sequences of S-layer genes confirms their adaptation to a high translation efficiency [172].

At the transcriptional level, most S-layer genes are monocistronic units that are expressed from one or more strong promoters. In most cases, the transcripts contain an untranslated 5′ region (ranging from 33 to 358 bases long) which can fold into stable secondary structures. The ex-

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

tended half-life of the S-layer mRNA (from 10 to 22 min) could be a consequence of this folding [172]. Such an extended half-life contributes to maintaining high levels of S-layer mRNA, but could be deleterious for the cell when an abrupt change in the environmental conditions blocks the growth. This suggests the existence of overlapping translational controls (see below).

Little is known about the transcriptional regulation of S-layer genes. More than one promoter are frequently found upstream of the S-layer genes in both Gram-positives [180–182] and Gram-negatives [183]. Such a control scheme could be implicated in the adaptation of their transcriptional level to specific environmental conditions. As an example, the *A. salmonicida* S-layer gene (*vapA*) is controlled by two promoters, one of which is activated by the transcription factor AbcA during the exponential growth phase [183].

In *Thermus thermophilus* HB8, the S-layer gene (*slpA*) is expressed from a single promoter that contains a 17-bp inverted repeat immediately after the +1 mRNA start site. Two proteins, named SlrA and SlpM, were identified and shown to bind this promoter region both in vitro as well as in vivo in a heterologous *E. coli* system [184]. Mutant *T. thermophilus* strains carrying insertions in *slrA* and *slpM* showed increased and decreased S-layer gene transcription, respectively, supporting a model in which SlrA functions as a repressor and SlpM as an activator of the S-layer gene transcription in vivo [184]. Furthermore, SlpM was independently identified as a protein overexpressed and secreted in S-layer defective *slpA* mutants, in which it forms an additional crystalline layer detached from the cell [185]. Thus, it appears that feedback transcriptional control between *slpA* and *slpM* regulates their coordinated expression in vivo. Besides their effect on *slpA* transcription, neither *slrA* nor *slpM* mutants showed special differences in the amount of the S-protein expressed, thus claiming the existence of post-transcriptional mechanisms of control.

As commented above, most S-layer genes are transcribed with long-lived mRNAs that could generate problems if a sudden change in cell growth is required. Consequently, transcriptional regulation could be envisioned as a means of long-term control, but overlapping post-transcriptional mechanisms could be responsible for fine tuning the S-layer expression in the short-term. In *T. thermophilus* HB8, a C-terminal fragment of the S-protein itself was shown to bind the 5′ untranslated leader region of its mRNA [184]. Thus, it was proposed that accumulation of S-protein fragments in the cytoplasm (as a consequence of a blockage in secretion) could stop its translation. In fact, soluble C-terminal fragments of the S-protein have been detected in *T. thermophilus* after a sudden stop in cellular growth (our unpublished results). Further investigations will confirm whether a similar mechanism could be applied to other S-layer genes.

Mechanisms of S-protein phase variation have also been described which allow for the alternate expression of different S-layer genes in a single strain. In *C. fetus* serotype A cells, there are up to eight different S-layer gene homologues (*sap*) [186] that can be expressed at different times during an infection process, allowing for the bacteria to change its surface in a dynamic way to evade the immune response. The mechanisms responsible for this phase variation depend on an invertable 6.2-kbp DNA fragment that contains a single promoter at one of its ends. Inversion occurs by recombination at a region of sequence identity found in the 5′ part of each S-layer gene [179,187]. In addition, this element contains four genes (*sapCDEF*), encoding a type I secretion apparatus (*sapDEF*) and a 40-kDa cytoplasmic protein (*sapC*) unknown function [188]. More complex chromosomal rearrangement could explain the expression of up to six more silent S-layer genes that are present in the neighboring 90-kbp DNA region [179,187].

4.3. Secretion and assembly of S-layers

S-proteins from Gram-negatives have to be transported across the IM, the periplasm and the OM before reaching their final destination on the cell surface. Most S-proteins contain N-terminal SP that allows for their secretion by the *sec*-dependent GSP. These signal sequences, with a length ranging from 21 (*A. salmonicida*) to 34 amino acids (*Haloferax volcanii*), are generally processed by signal peptidases, resulting in a mature S-protein slightly smaller than its corresponding precursor [172]. In some cases, such as the S-layers from *Deinococcus radiodurans* [189] and *T. thermophilus* HB8 [190], the N-terminus of the protein seems to be chemically modified. Further processing also occurs in the S-protein of *Rickettsia prowazekii* [191].

A. salmonicida and *Aeromonas hydrophila* are the best-studied models of SP-containing S-proteins. Secretion of the *A. salmonicida* S-protein (VapA) through its OM implicates a substrate-specific MTB of the GSP [192]. In fact, mutations in two genes, *spsP* and *apsE*, result in periplasmic accumulation of the S-protein without affecting the secretion of other extracellular enzymes. The *spsP* gene encodes a homologue of PulD, a secretin protein implicated in the formation of channels in the OM (see Section 3; [1]). On the other hand, ApsE shows homology with PulE, an ATP binding protein that could supply the energy required for pullulanase secretion, and with PilB, a cytoplasmic protein implicated in P pilus assembly in *P. aeruginosa* [172]. These observations therefore support that both proteins are part of an S-layer-specific MTB of the GSP. As shown in Fig. 9, it can be postulated that SpsP functions as a secretin that could form a pore in the OM, through which a partially unfolded S-protein could be secreted. On the other hand, the role of ApsE could be to act as a periplasmic S-protein-specific chaperone implicated in the presentation of VapA to SpsP (like in type I

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

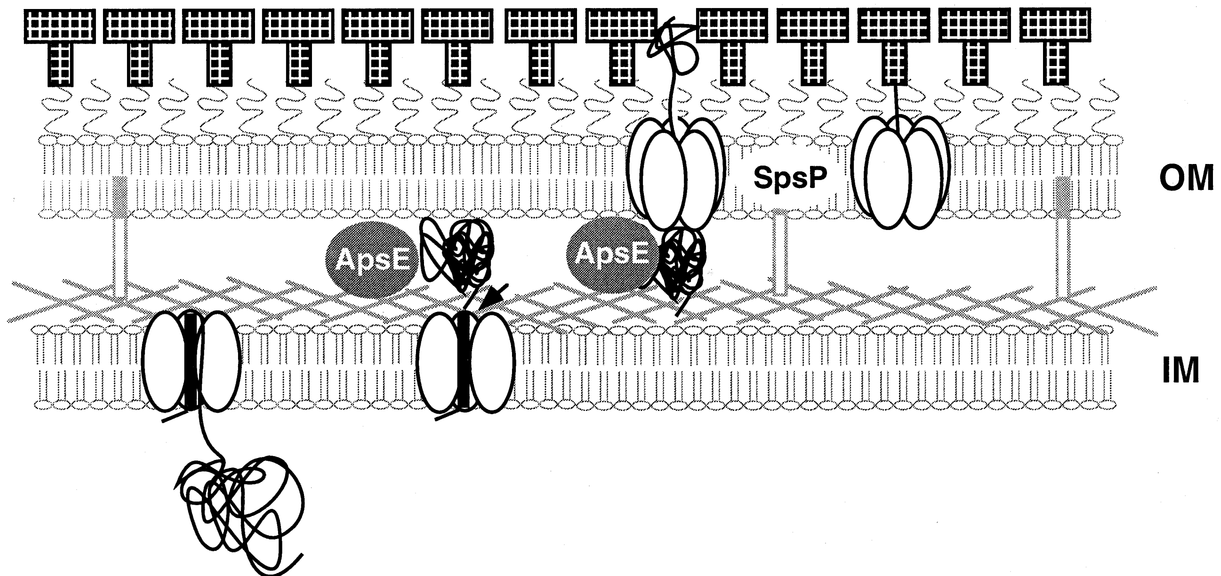


Fig. 9. Putative pathway for the secretion of *A. salmonicida* S-protein VapA. The VapA protein is secreted to the periplasm by the *sec*-dependent GSP, where a hypothetical chaperone (ApsE?) could be required to avoid its premature self-assembly and to present it to the OM pore-forming secretin (SpsP). The steps leading to integration of the S-proteins inside a crystalline layer and their binding to LPS are not known.

pili) or, alternatively, in supplying the energy required for the S-protein translocation across the IM. Further experimental support is required to demonstrate the actual role of AspE and SpsP in the secretion and assembly of the S-protein from *A. salmonicida*.

Interestingly, S-proteins from other Gram-negative bacteria do not contain a N-terminal SP. So far, three examples are known of SP-less S-proteins: those from *C. crescentus* [193], *Serratia marcescens* [194] and *C. fetus* [188]. Secretion of these S-proteins involves specific type I secretion systems [195,196]. As depicted in Fig. 10, type I systems are composed of three different components: an integral OM protein and two other proteins located at the IM. These systems recognize their substrate proteins on the cytoplasmic side of the IM and allow for their direct secretion to the extracellular medium without the formation of any periplasmic intermediate [196,197]. The energy for this process is obtained from the hydrolysis of ATP by one of the two IM proteins in this complex containing an ATP binding cassette (ABC) [198]. The second IM component belongs to a membrane fusion protein (MFP) family and has been implicated in maintaining a specific inter-

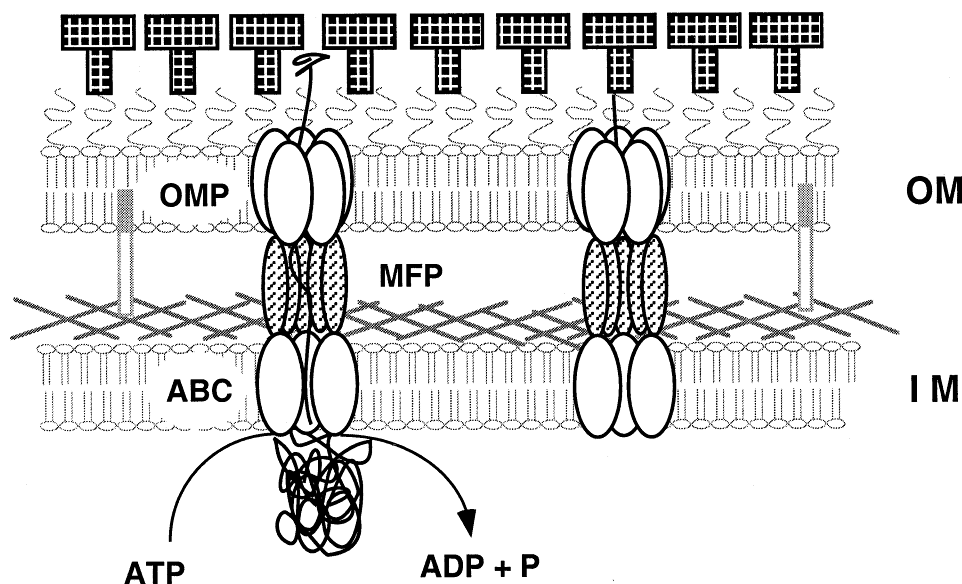


Fig. 10. Assembly of type I secreted S-layers. The ABC component of the secretion apparatus, which supplies the energy through the hydrolysis of ATP, recognizes partially unfolded S-proteins in the cytoplasmic face of the IM. The S-protein is then directly secreted through the hypothetical pore formed by the MFP and the outer membrane protein (OMP). As in the case of *A. salmonicida*, the steps leading to the integration the S-proteins inside a crystalline layer and their binding to LPS are not known.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

action between the IM and OM during the secretion process [197]. Finally, the OM component forms a pore through which the secreted protein cross this envelope (a special class of secretin).

In general, it is not understood which sequence elements are involved in substrate recognition by type I transporter components. Moreover, in cases in which the same type I system secretes different proteins, no obvious sequence homology exists between them. This is the case for the S-protein of *S. marcescens*, which is secreted by the LipBCD type I exporter. This exporter was previously known to secrete a lipase (LipA) and a metalloprotease (PrtA) which have no apparent sequence homology [194]. This common use of a single secretion system by more than one protein strongly supports the existence of domains in these proteins that are specifically recognized by the ABC-like component of the secretion machinery. The sequence responsible for this recognition has been identified using hybrid proteins and in most cases, it is near the C-terminus of the secreted proteins. In the case of *E. coli* hemolysin (HlyA), so far the best-characterized model of type I secretion, the IM components (HlyBD) specifically recognize the C-terminal segment of HlyA [196]. This is also the case for the S-layer of *C. crescentus* (RsaA), whose C-terminal domain is sufficient to direct the secretion of heterologous reporter proteins [199]. The secretion signal of RsaA might be a series of repeats of acidic amino acids followed by groups of glycines, which may also play a role as a calcium binding motif [193].

With the exception of *Caulobacter*, in which the OM pore protein is located at a different locus [193], the genes encoding the three proteins of the corresponding type I secretion system are located within a single cluster immediately upstream or downstream of the S-layer gene. In *C. fetus*, the three components of the secretion apparatus (SapDEF) are clustered within the invertable 6.2-kbp DNA segment implicated in S-layer phase variation (see above).

Apart from their secretion by type I or type II systems, little is known about the incorporation of S-proteins into a growing S-layer. The S-layer rigidity makes unlikely a mechanism of horizontal diffusion of S-proteins similar to that proposed for the synthesis of LPS. Indeed, the S-protein secretion model requires a site-specific mechanism of subunit incorporation that has not been demonstrated yet. The earlier proposal of S-protein addition at places of crystal dislocation [200] requires further experimental support.

There are some studies aimed at the identification of specific insertion points of S-proteins, most of them conducted in Gram-positives. In *Bacillus sphaericus* and *Bacillus stearothermophilus*, the highest incorporation signal overlapped with the septation sites, while the cell poles remained as inert structures. In addition, the longitudinal growth of the S-layer cell cylinder took place in several bands organized in a helical manner near the cell division site [201,202]. By contrast, the incorporation of S-proteins in the Gram-negative *C. crescentus* resembled a diffuse pattern on the whole cell body surface, with a higher rate around the septum and the stalk [203]. The absence of similar studies in other Gram-negatives (*Caulobacter* is a special case that does not divide by bipartition) impedes generalization of the conclusions derived from this study. However, the absence of non-incorporated S-protein stocks in any cellular compartment of Gram-negative bacteria implies that a tight coordination should exist between the synthesis and secretion of the S-protein and that of other cell wall components, like PG and LPS, to which the S-layer is bound.

5. Concluding remarks

Regular surface structures are common in most Gram-negative bacteria. They can be as simple as a two-dimensional crystal built by the polymerization of a single protein or highly complex tubular multimers requiring the ordered expression, secretion and polymerization of tens of proteins, as in the case of the flagellar apparatus.

In all instances, their single or diverse components must cross the double-layered envelope of Gram-negatives before reaching their final destination outside the cell, and to this end, diverse secretory pathways have evolved. Moreover, this diversity can be extended even to functionally equivalent structures that are secreted by different mechanisms depending on the bacteria, as in the case of S-layers from *A. salmonicida* and *C. fetus* or in type I and type IV pili from *E. coli* and *N. gonorrhoeae*.

Biosynthesis and secretion of S-layers, pili and flagella uses homologues to the three main secretion pathways: the type I, type II and type III systems, respectively. In addition, special terminal branches of the GSP exist, like the chaperone usher pathway involved in type I pili biogenesis. Besides, there are common aspects among these apparently unrelated systems, such as the use of specific chaperones to avoid a premature self-assembly of the regular structure, inside the cell or within the periplasm, or to catalyze the formation of disulfide bonds within the subunits. These elements have been clearly identified in type I pilus assembly and putative candidates exist in the flagellar biosynthetic apparatus.

Despite the work already done, protein secretion in Gram-negatives still has many important unknown aspects. The signals that identify the protein to be secreted and assembled into a growing structure are poorly characterized in various systems (e.g. in the secretion of flagella, S-layers and type IV pili). Likewise, the studies of chaperone-like proteins (either periplasmic or cytoplasmic) and their role in the folding and targeting of the structural subunits are still in a very early stage in most systems. In fact, in many instances, the elements composing the secretion machineries have not even been identified yet (e.g.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

S-layers). Another question that is pending is the kind of cellular energy that drives the secretion of the subunits across the OM. In some cases, there are components of the secretion machineries with an ATPase activity, but it is even uncertain whether ATP is the sole energy source used by these systems. Indeed, the coupling of the proton motive force (generated in the IM) to the translocation of subunits across the OM has been pointed out as an alternative energy source, but little is known about the subunits of the secretion machineries involved in this coupling. Polypeptides located in the IM, but spanning the periplasm to contact the OM, are good candidates for such a role. At last, it has also been suggested that the assembly of the subunits into the final structure may participate in providing the energy for secretion.

Other unsolved questions of physiological importance are related to the selection of the location and timing for the synthesis of the surface structure. How does the cell define the number and location of their flagella? How is the motility-related type IV pili in Myxobacteria polymerized differentially at the cell poles? What mechanisms allow for the insertion of S-layer subunits within a pre-existing bidimensional crystal? Future work is required to answer these questions, but meanwhile, what must be appreciated is the astonishing diversity, complexity and precision of the regular pseudo-organelles developed by bacteria during their evolution to adapt to specific environments.

Acknowledgements

We thank Dr Steve Van Dien for his comments and reading of the manuscript.

References

[1] Pugsley, A.P. (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. 57, 50–108.
[2] Lory, S. (1998) Secretion of proteins and assembly of bacterial surface organelles: shared pathways of extracellular protein targeting. Curr. Opin. Microbiol. 1, 27–35.
[3] Kondoh, H. and Hotani, H. (1974) Flagellin from *E. coli* K12 polymerization and molecular weight in comparisson with *Salmonella* flagellins. Biochim. Biophys. Acta 336, 117–139.
[4] Kuwajima, G. (1988) Construction of a miniums-size functional flagellin of *Escherichia coli*. J. Bacteriol. 170, 3305–3309.
[5] Macnab, R.M. (1996) Flagella and motility. In: *Escherichia coli* and *Salmonella*, Cellular and Molecular Biology 1 (Neidhardts, F.C., Ed.), pp. 123–145. ASM Press, Washington, DC.
[6] Iino, T. (1974) Assembly of *Salmonella* flagellin in vitro and in vivo. J. Supramol. Struct. 2, 372–384.
[7] Ikeda, T., Asakura, S. and Kamiya, R. (1987) Total reconstitution of *Salmonella* flagellar filaments froM hook and purified flagellin and hook-associated proteins in vitro. J. Mol. Biol. 209, 109–114.
[8] Homma, M., DeRosier, D.J. and Macnab, R.M. (1990) Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. J. Mol. Biol. 213, 819–832.
[9] Jones, C.J., Macnab, R.M., Okino, H. and Aizawa, S.-I. (1990) Stoichiometric analysis of the flagelar hook-(basal-body) complex of *Salmonella typhimurium*. J. Mol. Biol. 212, 377–387.
[10] Sosinsky, G.E., Francis, N.R., DeRosier, D.J., Wall, J.S., Simon, M.N. and Hainfeld, J. (1992) Mass determination and estimation of subunit stoichiometry of the bacterial hook-basal body flagellar complex of *Salmonella typhimurium* by scanning transmission electron microscopy. Proc. Natl. Acad. Sci. USA 89, 4801–4805.
[11] Francis, N.R., Irikura, V.M., Yamaguchi, S., DeRosier, D.J. and Macnab, R.M. (1992) Localization of the *Salmonella typhimurium* flagellar swictch protein FliG to the cytoplasmic M-ring face of the basal body. Proc. Natl. Acad. Sci. USA 89, 6304–6308.
[12] Francis, N.R., Sosisnsky, G.E., Thomas, D. and DeRosier, D.J. (1994) Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. J. Mol. Biol. 235, 1261–1270.
[13] Oosawa, K., Ueno, T. and Aizawa, S.-I. (1994) Overproduction of the flagellar switch proteins and their interactions with the MS ring complex in vitro. J. Bacteriol. 176, 3683–3691.
[14] DeMot, R. and Vanderleyden, J. (1994) The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both Garm-positive and Gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. Mol. Microbiol. 12, 233–334.
[15] Kutsukake, K., Ohya, Y., Yamaguchi, S. and Iino, T. (1988) Operon structure of flagellar genes in *Salmonella typhimurium*. Mol. Gen. Genet. 214, 11–15.
[16] Komeda, Y., Suzuki, H., Ishidsu, J.-I. and Iino, T. (1975) The role of cAMP in flagellation of *Salmonella typhimurium*. Mol. Gen. Genet. 142, 289–298.
[17] Nishimura, A. and Hirota, Y. (1989) A cell division regulatory mechanism controls the flagellar regulon in *Escherichia coli*. Mol. Gen. Genet. 216, 340–346.
[18] Pruss, B.M. and Matsumura, P. (1997) Cell cycle regulation of flagellar genes. J. Bacteriol. 179, 5602–5604.
[19] Gillen, K.L. and Hughes, K.T. (1993) Transcription from two promoters and auto-regulation contribute to the control of the expression of the *Salmonella typhimurium* flagellar regulatory gene *flg*M. J. Bacteriol. 175, 7006–7015.
[20] Kutsusake, K., Iyoda, S., Ohnishi, K. and Iino, T. (1994) Genetic and molecular analysis of the interaction between the flagellum specific sigma and anti-sigma factors in *Salmonella typhimurium*. EMBO J. 13, 4568–4576.
[21] Kutsukake, K. (1994) Excretion of the anti-sigma factor through a flagellar structure couples flagellar gene expression with flagellar assembly in *Salmonella thyphimurium*. Mol. Gen. Genet. 243, 605–612.
[22] Chadsey, M.S., Karlinsey, J.E. and Hughes, K.T. (1998) The flagellar anti-sigma factor FlgM actively dissociates *Salmonella typhimurium* sigma 28 RNA polymerase holoenzyme. Genes Dev. 12, 3123–3136.
[23] Daughdrill, G.W., Chadsey, M.S., Karlinsey, J.E., Hughes, K.T. and Dahlquist, F.W. (1997) The C-terminal half of the antisigma factor, FlgM, becomes structured when bound to its target, sigma 28. Nat. Struct. Biol. 4, 285–291.
[24] Karlinsey, J.E., Tsui, H.C.T., Winkler, M.E. and Hughes, K.T. (1998) Flk couples *flgM* translation to flagellar ring assembly in *Salmonella typhimurium*. J. Bacteriol. 180, 5384–5397.
[25] Silverman, M. and Simon, M. (1980) Phase variation: genetic analysis of switching mutants. Cell 19, 845–854.
[26] Kubori, T., Shimamoto, N., Yamaguchi, S., Namba, K. and Aizawa, S.-I. (1992) Morphological pathway of flagellar assembly in *Salmonella typhimurium*. J. Mol. Biol. 226, 433–446.
[27] Aizawa, S.-I. (1996) Flagellar assembly in Salmonella typhimurium. Mol. Microbiol. 19, 1–5.
[28] Katayama, E., Shiraishi, T., Oosawa, K., Baba, N. and Aizawa, S.-I.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

(1996) Geometry of the flagellar motor in the cytoplasmic membrane of *Salmonella typhimurium* as determined by stereo-photogrammetry of quick-freeze deep-etch replica images. J. Mol. Biol. 255, 458–475.

[29] Minamino, T. and Macnab, R.M. (1999) Components of the Salmonella flagellar export apparatus and classification of export substrates. J. Bacteriol. 181, 1388–1394.

[30] Kubori, T., Yamaguchi, S. and Aizawa, S.-I. (1997) Assembly of the switch complex onto the MS ring complex of *Salmonella typhimurium* does not require any other flagellar proteins. J. Bacteriol. 179, 813–817.

[31] Akiba, T., Yoshimura, H. and Namba, K. (1991) Monolayer crystallization of flagellar L-P rings by sequential addition and depletion of lipid. Science 252, 1544–1546.

[32] Raina, S. and Missiakas, D. (1997) Making and breaking disulfide bonds. Annu. Rev. Microbiol. 51, 179–202.

[33] Willians, A.W., Yamaguchi, S., Togashi, F., Aizawa, S.-I., Kawagishi, I. and Macnab, R.M. (1996) Mutations in *flik* and *flhB* affecting flagellar hook and filament assembly in *Salmonella typhimurium*. J. Bacteriol. 178, 2960–2970.

[34] Muramoto, K., Makishima, S., Aizawa, S.-I. and Macnab, R.M. (1998) Effect of cellular level of FliK on flagellar hook and filament assembly in *Salmonella typhimurium*. J. Mol. Biol. 10, 871–882.

[35] Ikeda, T., Oosawa, K. and Hotani, H. (1996) Self-assembly of the filament capping protein, FliD, of bacterial flagella into an annular structure. Mol. Biol. 259, 679–686.

[36] Dreyfus, G., Willians, A.G., Kawagishi, I. and Macnab, R.M. (1993) Genetic and biochemical analysis of Salmonella typhimurium FliI, a flagellar protein related to the catalytic domain of F_0F_1 ATPase and to virulence proteins of mammalian and plant pathogens. J. Bacteriol. 175, 3131–3138.

[37] Fan, F. and Macnab, R.M. (1996) Enzymatic characterization of FliI. An ATPase involved in flagellar assembly in *Salmonella typhimurium*. J. Biol. Chem. 271, 31981–31988.

[38] Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens. Microbiol. Mol. Biol. Rev. 62, 379–433.

[39] Jones, C.J., Homma, M. and Macnab, R.M. (1989) L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: gene sequences and deduced protein sequences. J. Bacteriol. 171, 3890–3900.

[40] Galan, J.E. and Bliska, J.B. (1996) Cross-talk between bacterial pathogens and their host cells. Annu. Rev. Cell Dev. Biol. 12, 221–255.

[41] Fan, F., Ohnishi, K., Francis, N.R. and Macnab, R.M. (1997) The FliP and FliR proteins of *Salmonella typhimurium*, putative componentsof the type III flagellar export apparatus, are located in the flagellar basal-body. Mol. Microbiol. 26, 1035–1046.

[42] Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J.E. and Aizawa, S.-I. (1998) Supramolecular structure of the *Salmonella typhimurium* Type III protein secretion system. Science 280, 602–605.

[43] Ohnishi, K., Fan, F., Schoenhals, G.J., Kihara, M. and Macnab, R.M. (1997) The FliO, FliP, FliQ, and FliR proteins of *Salmonella typhimurium*: putative components for flagellar assembly. J. Bacteriol. 179, 6092–6099.

[44] Kornacker, M.G. and Newton, A. (1994) 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, 73–85.

[45] Low, D., Braaten, B., van der Woude, M. (1996) Fimbriae. In: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidhardts, F.C., Ed.), pp. 146–157. ASM Press, Washington, DC.

[46] Finlay, B.B. and Falkow, S. (1997) Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61, 136–169.

[47] Hanson, M.S., Hempel, J. and BrintonJr, C.C. (1988) Identification and characterization of *E. coli* type-1 pilus adhesion protein. Nature 332, 265–268.

[48] Lund, B., Lindberg, F., Marklund, B.I. and Normark, S. (1987) The PapG protein is the α-D-galactopyranosyl-(1-4)-β-D-galactopyranose-binding adhesin of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA 84, 5898–5902.

[49] Hoschutzky, H., Lottspeich, F. and Jann, K. (1989) Isolation and characterization of the α-galactosyl-1,4-β-galactosyl-specific adhesin (P adhesin) from fimbriated *Escherichia coli*. Infect. Immun. 57, 76–81.

[50] Rudel, T., Scheurerpflug, I. and Meyer, T.F. (1995) Neisseria PilC protein identified as type-4 pilus tip-located adhesin. Nature 373, 357–359.

[51] Bieber, D., Ramer, S.W., Wu, C.-Y., Murray, W.J., Tobe, T., Fernandez, R. and Schoolnik, G.K. (1998) Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. Science 280, 2114–2118.

[52] Langermann, S. et al. (1996) Prevention of *Escherichia coli* infection by FimH-adhesin-based systematic vaccination. Science 276, 607–611.

[53] Connell, I., Agace, W., Klemm, P., Schembri, M., Marild, S. and Svanborg, C. (1996) Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. Proc. Natl. Acad. Sci. USA 93, 9827–9832.

[54] Sato, H., Okinaga, K. and Saito, H. (1988) Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection. Microbiol. Immunol. 32, 131–139.

[55] Taylor, R.K., Miller, V.L., Furlong, D.B. and Melakanos, J.J. (1987) Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA 84, 2833–2837.

[56] Soto, G.E. and Hultgren, S.J. (1999) Bacterial adhesins: Common themes and variation in architecture and assembly. J. Bacteriol. 181, 1059–1071.

[57] Hull, R.A., Gill, R.E., Hsu, P., Minshaw, B.H. and Falkow, S. (1981) Construction and expression of recombinant plasmids encoding type 1 and D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. 33, 933–938.

[58] Clegg, S. and Gerlach, G.F. (1987) Enterobacterial fimbriae. J. Bacteriol. 169, 934–938.

[59] Hull, R.A. and Hull, S.I. (1994) Adherence mechanisms in urinary tract infections. In: Molecular Genetics of Bacterial Pathogenesis (Miller, V.L., et al., Eds.), ASM Press, Washington, DC.

[60] Hultgren, S.J., Jones, C.H. and Normark, S. (1996) Bacterial adhesins and their assembly. In: *Escherichia coli* and *Salmonell*a: Cellular and Molecular Biology (Neidhardts, F.C., Ed.), pp. 2730–2756. ASM Press, Washington, DC.

[61] Kuehn, M.J., Heuser, J., Normark, S. and Hultgren, S.J. (1992) P pili in uropathogenic *E. coli* are composite fibres with distinct fibrillar adhesive tips. Nature 356, 252–255.

[62] Jones, C.H., Pinkner, J.S., Roth, R., Heuser, J., Nicholes, A.V., Abraham, S.N. and Hultgren, S.J. (1995) FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. Proc. Natl. Acad. Sci. USA 92, 2081–2085.

[63] Maurer, L. and Orndorff, P.E. (1985) A new locus, *pilE*, required for the binding of type 1 piliated *Escherichia coli* to erythrocytes. FEMS Microbiol. Lett. 30, 59–66.

[64] Minion, F.C., Abraham, S.N., Beachey, E.H. and Goguen, J.D. (1989) The genetic determinat of adhesive function in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding the fimbrial subunit. J. Bacteriol. 165, 1033–1036.

[65] Krogfelt, K.A., Bergmans, H. and Klemm, P. (1990) Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. Infect. Immun. 58, 1995–1998.

[66] Bakker, D., Willemsen, P.T.J., Simons, L.H., Zijderveld, F.G.v. and Graaf, F.K.d. (1992) Characterization of the antigenic and adhesive properties of FaeG, the major subunit of K88 fimbriae. Mol. Microbiol. 6, 247–255.

[67] Willemsen, P.T.J. and Graaf, F.K.d. (1993) Multivalent binding of

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

K99 fimbriae to the N-glycolyl-GM3 ganglioside receptor. Infect. Immun. 61, 4518–4522.

[68] Kallenius, G., Svenson, S.B., Hulberg, H., Molby, R., Helin, I., Cedergen, B. and windberg, J. (1981) Ocurrence of P fimbriated *Escherichia coli* in urinary tract infection. Lancet ii, 1369–1372.

[69] Latham, R.H. and Stamm, W.E. (1984) Role of fimbriated *Escherichia coli* in urinary tract infections in adult women: correlation with localization studies. J. Infect. Dis. 149, 835–840.

[70] Kuehn, M.J., Jacob-Dubuisson, F., Dodson, K., Slonim, L., Striker, R. and Hultgren, S.J. (1994) Genetic, biochemical, and structural studies of biogenesis of adhesive pili in bacteria. Methods Enzymol. 236, 282–306.

[71] Lindberg, F., Lund, B., Johansson, L. and Normark, S. (1987) Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. Nature 328, 84–87.

[72] Baga, M., Norgren, M. and Normark, S. (1987) Biogenesis of *E. coli* Pap pili PapH, a minor pilin subunit involved in cell anchoring and length modulation. Cell 49, 241–251.

[73] Jacob-Dubuisson, F., Heuser, J., Dodson, K., Normark, S. and Hultgren, S.J. (1993) Initiation of assembly and association of structural elements of a bacterial pilus depend on two specialized tip proteins. EMBO J. 12, 837–847.

[74] Lindberg, F., Tennent, J.M., Hultgren, S.J., Lund, B. and Normark, S. (1989) PapD, a periplasmic transport protein in P-pilus biogenesis. J. Bacteriol. 171, 6052–6058.

[75] Kuehn, M.J. et al. (1991) Immunoglobulin-like PapD chaperone caps and uncaps interactive surfaces of nascently translocated pilus subunits. Proc. Natl. Acad. Sci. USA 88, 10586–10590.

[76] Dodson, K.W., Jacob-Dubuisson, F., Striker, R.T. and Hultgren, S.J. (1993) Outer membrane PapC usher discriminately recognizes periplasmic chaperone-pilus subunit complexes, Proc. Natl. Acad. Sci. USA 90.

[77] Bullit, E., Jones, C.H., Striker, R., Soto, G., Jacob-Dubuisson, F., Pinkner, J., Wick, M.-J., Makowski, L. and Hultgren, S.J. (1996) Development of the pilus organelle subassemblies in vitro depends on chaperone uncapping of a β zipper. Proc. Natl. Acad. Sci. USA 93, 12890–12895.

[78] Saulino, E.T., Thanassi, D.G., Pinkner, J. and Hultgren, S.J. (1998) Ramifications of kinetic partitioning on usher-mediated pilus biogenesis. EMBO J. 17, 2177–2185.

[79] Thanassi, D.G. (1998) The PapC usher forms an oligomeric channel: implications for pilus biogenesis across the outer membrane. Proc. Natl. Acad. Sci. USA 95, 3146–3151.

[80] Orndorff, P.E. and Falkow, S. (1984) Organization and expression of genes responsible for type 1 piliation in *Escherichia coli*. J. Bacteriol. 159, 736–744.

[81] Jones, C.H., Pinkner, J.S., Nicholes, A.V., Slonim, L.N., Abraham, S.N. and Hultgren, S.J. (1993) FimC is a periplasmic PapD-like chaperone that directs assembly of type 1 pili in bacteria. Proc. Natl. Acad. Sci. USA 90, 8397–8401.

[82] Holmgren, A., Kuehn, M.J., Brädén, C.I. and Hultgren, S.J. (1992) Conserved immunoglobulin-like features in a family of perisplasmic pilus chaperones. EMBO J. 11, 1617–1622.

[83] Hung, D.L., Knight, S.D., Woods, R.M., Pinkner, J.S. and Hultgren, S.J. (1996) Molecular basis of two subfamilies of immunoglobulin-like chaperons. EMBO J. 15, 3792–3805.

[84] Dood, D.C., Bassford, P.J.J. and Eisentein, B.I. (1984) Dependence of secretion and assembly of type 1 fimbrial subunits of *Escherichia coli* on normal protein export. J. Bacteriol. 159, 1077–1079.

[85] Roosendaal, E., Jacobs, A.A.C., Rathman, P., Sondermeyer, C., Stegehuis, F., Oudega, B. and Graaf, F.K. (1987) Primary structure and subcellular localization of two fimbrial subunits involved in the biogenesis of K99 fimbrillae. Mol. Microbiol. 1, 211–217.

[86] Hultgren, S.J., Lindberg, F., Magnusson, G., Kihlberg, J., Tennent, J.M. and Normark, S. (1989) The PapG adhesin of uropathogenic *E. coli* contains separate regions for receptor binding and for the incorporation into the pilus. Proc. Natl. Acad. Sci. USA 86, 4357–4361.

[87] Hultgren, S.J., Normark, S. and Abraham, S.N. (1991) Chaperone-assisted assembly and molecular architecture of adhesive pili. Annu. Rev. Microbiol. 45, 383–415.

[88] Jones, C.H., Danase, P.N., Pinkner, J.S., Silhavy, T.J. and Hultgren, S.J. (1997) The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J. 16, 6394–6406.

[89] Hardy, S.J. and Randall, L.L. (1991) A kinetic partitioning model of selective binding od nonnative proteins by the bacterial chaperone SecB. Science 251, 439–443.

[90] Lecker, S., Lill, R., Ziegelhoffer, T., Georgepoulus, C., Bassford, P.J., Kumamoto, C.A. and Wickner, W. (1989) Three pure chaperone proteins of *Escherichia coli* -SecB, trigger factor and GroEL-form soluble complexes with precursor proteins in vitro. EMBO J. 8, 2703–2709.

[91] Soto, G.E., Dodson, K.W., Ogg, D., Liu, C., Heuser, J., Knight, S., Kihlberg, J., Jones, C.H. and Hultgren, S.J. (1998) Periplasmic chaperone recognition motif of subunits mediates quaternary interactions in the pilus. EMBO J. 17, 6155–6167.

[92] Kolmar, H., Waller, P.R.H. and Sauer, R.T. (1996) The DegP and DegQ periplasmic endoproteases of *Escherichia coli*: specificity for cleavage sites and substrate conformation. J. Bacteriol. 178, 5925–5929.

[93] Bardwell, J.C.A. (1994) Building bridges: disulphide bond formation in the cell. Mol. Microbiol. 14, 199–205.

[94] Jacob-Dubuisson, F., Pinkner, J., Xu, Z., Striker, R., Padmanhaban, A. and Hultgren, S.J. (1994) PapD chaperone function in pilus biogenesis depends on oxidant chaperone-like activities of DsbA. Proc. Natl. Acad. Sci. USA 91, 11552–11556.

[95] Kuehn, M.J., Ogg, D.J., Kihlberg, J., Slonim, L.N., Flemmer, K., Bergfors, T. and Hultgren, S.J. (1993) Structural basis of pilus subunit recognition by PapD chaperone. Science 262, 1234–1241.

[96] Xu, Z., Jones, C.H., Haslem, D., Pinkner, J.S., Dodson, K., Kihlberg, J. and Hultgren, S.J. (1995) Molecular dissection of PapD interaction with PapG reveals two chaperone-binding sites. Mol. Microbiol. 16, 1011–1020.

[97] Holmgren, A. and Brändén, C.I. (1989) Crystal structure of chaperone protein PapD reveals an immunoglobulin fold. Nature 342, 248–251.

[98] Williams, A.F. and Barclay, A.N. (1988) The immunoglobulin superfamily domains for cell surface recognition. Annu. Rev. Immunol. 6, 381–405.

[99] Hultgren, S.J., Jacob-Dubuisson, F., Jones, C.H. and Brändén, C.I. (1993) PapD and superfamily of periplasmic immunoglobulin-like pilus chaperones. Adv. Protein Chem. 44, 99–123.

[100] Norgren, M., Baga, M., Tennent, J.M. and Normark, S. (1987) Nucleotide sequence, regulation and functional analysis of the *papC* gene required for cell surface localization of Pap pili of uropathogenic *Escherichia coli*. Mol. Microbiol. 1, 169–178.

[101] Jacob-Dubuisson, F., Striker, R. and Hultgren, S.J. (1994) Chaperone-assisted self-assembly of pili independet of cellular energy. J. Biol. Chem. 269, 12447–12455.

[102] Langermann, S. et al. (1997) Prevention of mucusal *Escherichia coli* infection by FimH-adhesin-based systemic vaccination. Science 276, 607–611.

[103] Abraham, S.N., Land, M., Ponniah, S., Endres, R., Hasty, D.L. and Babu, J.P. (1992) Glycerol-induced unraveling of the tight helical conformation of *Escherichia coli* type 1 fimbriae. J. Bacteriol. 174, 5145–5148.

[104] Strom, M. and Lory, S. (1993) Structure-function and biogenesis of type IV pili. Annu. Rev. Microbiol. 47, 565–596.

[105] Russel, M. (1998) Macromolecular assembly and secretion across the bacterial cell envelope: type II secretion systems. J. Mol. Biol. 279, 485–499.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

[106] Hoobs, M. and Mattick, J.S. (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. 10, 233–243.

[107] Russel, M. (1995) Moving through the membrane with filamentous phages. Trends Microbiol. 3, 223–228.

[108] Russel, M., Linderoth, N.A. and Sali, A. (1997) Filamentous phage assembly: variation on a protein export theme. Gene 192, 23–32.

[109] Henrichsen, J. (1983) Twitching motility. Annu. Rev. Microbiol. 37, 81–93.

[110] Watson, A.A., Mattick, J.S. and Alm, R.A. (1996) Functional expression of heterologous type 4 fimbriae in *Pseudomonas aeruginosa*. Gene 175, 143–150.

[111] Wu, S.S. and Kaiser, D. (1995) Genetic and functional evidence that Type IV pili are required for social gliding motility in *Mixococcus xanthus*. Mol. Microbiol. 18, 547–558.

[112] Wall, D. and Kaiser, D. (1999) Type IV pili and cell motility. Mol. Microbiol. 32, 1–10.

[113] Forest, K.T. and Tainer, J.A. (1997) Type-4 pilus structure: outside to inside and top to bottom- a minireview. Gene 192, 165–169.

[114] Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D. and Tainer, J.A. (1995) Structure of the fiber forming protein pilin at 2.6 Å resolution. Nature 378, 32–38.

[115] Girón, J.A., Ho, A.S. and Schoolnik, G.K. (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 254, 710–713.

[116] Girón, J.A., Levine, M.M. and Kaper, J.B. (1994) Longus: a long pilus structure produced by human enterotoxigenic *Escherichia coli*. Mol. Microbiol. 12, 71–82.

[117] Kaufman, M.R., Shaw, C.E., Jones, I.D. and Taylor, R.K. (1993) Biogenesis and regulation of the *Vibrio cholera* toxin-coregulated pilus: analogies to other virulence factor secretory systems. Gene 126, 43–49.

[118] Sohel, I., Puente, J.L., Ramer, S.W., Bieber, D., Wu, C.Y. and Schoolnik, G.K. (1996) Enteropathogenic *Escherichia coli*: identification of a cluster coding for bundle-forming pilus morphogenesis. J. Bacteriol. 178, 2613–2628.

[119] Stone, K.D., Zhang, H.-K., Carlson, L.K. and Donnenberg, M.S. (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the biogenesis of a type IV pilus. Mol. Microbiol. 20, 325–337.

[120] Ryll, R.R., Rudel, T., Scheuerpflug, I., Barten, R. and Meyer, T.F. (1997) PilC of Neisseria meningitidis is involved in class II pilus formation and restores pilus assembly, natural transformation competence and adherence to epithelial cells in PilC-deficient gonococci. Mol. Microbiol. 23, 879–892.

[121] Alm, R.A., Hallinan, J.P., Watson, A.A. and Mattick, J.S. (1996) Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein secretion systems and *pilY1* encodes a gonococcal PilC homologue. Mol. Microbiol. 22, 161–173.

[122] Farinha, M.A., Conway, B.D., Ellert, L.M.G., Irvin, N.W., Sherburne, R. and Paranchych, W. (1994) Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte and decreased virulence in mice. Infect. Immun. 62, 4118–4123.

[123] Lee, K.K., Sheth, H.B., Wong, W.Y., Sherburne, R., Paranchych, W., Hodges, R.S., Lingwood, C.A., Krivan, H. and Irvin, R.T. (1994) The binding *of Pseudomonas aeruginosa* pili to glycoshingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. Mol. Microbiol. 11, 705–713.

[124] Jonsson, A., Nyberg, G. and Normark, S. (1991) Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. EMBO J. 10, 477–488.

[125] Jonsson, A.B., Rhaman, M. and Normark, S. (1995) Pilus biogenesis gene, *pilC*, of *Neisseria gonorrhoeae*: *pilC1* and *pilC2* are each part of a larger duplication of gonococcal genome and share upstream and downstream homologous sequences with *opa* and *pil* loci. Microbiology 141, 2367–2377.

[126] Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A. and So, M. (1993) Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. Mol. Microbiol. 8, 719–725.

[127] Seifert, H.S. (1996) Questions about gonococcal pilus phase and antigenic variation. Mol. Microbiol. 21, 433–440.

[128] Nunn, D. and Lory, S. (1991) Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc. Natl. Acad. Sci. USA 89, 47–51.

[129] Strom, M.S. and Lory, S. (1992) Kinetics and sequence specificity of processing of prepilin by PilD, the type IV leader peptidase of *Pseudomonas aeruginosa*. J. Bacteriol. 174, 7345–7351.

[130] Strom, M.S., Nunn, D.N. and Lory, S. (1993) A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc. Natl. Acad. Sci. USA 90, 2404–2408.

[131] Pepe, J.C. and Lory, S. (1998) Amino acid substitution in PilD, a bifunctional enzyme of *Pseudomonas aeruginosa*. Effect on leader peptidase and N-methyltransferase activities in vitro and in vivo. J. Biol. Chem. 273, 19120–19129.

[132] Alm, R.A. and Mattick, J.S. (1995) Identification of a gene, *pilV*, required for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*, whose product possesses a pre-pilin like leader sequence. Mol. Microbiol. 16, 485–496.

[133] Alm, R.A. and Mattick, J.S. (1996) Identification of two genes with prepilin-like leader sequences involved in type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. J. Bacteriol. 178, 3809–3817.

[134] Lu, H.-M., Motley, S.T. and Lory, S. (1997) Interactions of the components of the general secretion pathway: role of *Pseudomomas aeruginosa* type IV pilin subunits in complex formation and extracellular secretion. Mol. Microbiol. 25, 247–259.

[135] Strom, M.S., Nunn, D. and Lory, S. (1991) Multiple roles of the pilus biogenesis protein PilD: involvement of PilD in excretion of enzymes from *Pseudomonas aeruginosa*. J. Bacteriol. 173, 1175–1180.

[136] Bally, M., Ball, G., Badere, A. and Lazdunski, A. (1991) Protein secretion in *Pseudomonas aeruginosa*: the *xcpA* gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function PulO. J. Bacteriol. 173, 479–486.

[137] Nunn, D.N. and Lory, S. (1992) Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. USA 89, 47–51.

[138] Nunn, D.N. and Lory, S. (1993) Cleavage, methylation, and location of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. J. Bacteriol. 175, 4375–4382.

[139] Martin, P.R., Watson, A.A., McCaul, T.F. and Mattick, J.S. (1995) Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. 16, 497–508.

[140] Nunn, D., Bergman, S. and Lory, S. (1990) Products of the three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. 172, 2911–2919.

[141] Kaufman, M.R., Seyer, J.M. and Taylor, R.T. (1991) Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway for extracellular protein secretion by Gram-negative bacteria. Genes Dev. 5, 1834–1846.

[142] Sauvonnet, N. and Pugsley, A.P. (1996) Identification of two regions of Klebsiella oxytoca pullulanase that together are capable of promoting beta lactamase secretion by the general secretory pathway. Mol. Microbiol. 1996, 1–7.

[143] Silver, P., Watts, C. and Wickner, W. (1981) Membrane assembly from purified components. 1. Isolated M13 procoat does not require ribosomes or soluble proteins for processing by membranes. Cell 25, 341–345.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

[144] Waldor, M.K. and Mekalanos, J.J. (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272, 1910–1914.
[145] Sandkvist, M., Morales, V. and Bagdasarian, M. (1993) A protein required for secretion of cholera toxin through the outer membrane of *Vibrio cholera*. Gene 123, 81–86.
[146] Alm, R.A. and Mattick, J.S. (1997) Genes involved in the biogenesis and function of type-IV fimbriae in *Pseudomonas aeruginosa*. Gene 192, 89–98.
[147] Martin, P.R., Hobbs, M., Free, P.D., Jeske, Y. and Mattick, J.S. (1993) Characterization of pilQ, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. 9, 857–868.
[148] Bitter, W., Koster, M., Latijnhouwers, M., de Cock, H. and Tommanssen, J. (1998) Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. Mol. Microbiol. 27, 209–219.
[149] Folkhard, W., Marvin, D.A., Watts, T.H. and Parenchych, W. (1981) Structure of polar pili from *Pseudomonas aeruginosa* strain K and O. J. Mol. Biol. 149, 79–93.
[150] Drake, S.L., Sandstedt, S.A. and Koomey, M. (1997) PilP, a pilus biogenesis lipoprotein in *Neisseria gonorroheae*, affects expression of PilQ as a high-molecular-mas multimer. Mol. Microbiol. 23, 657–668.
[151] Kazmierczak, B.I., Mielke, D.L., Russel, M. and Model, P. (1994) pIV, a filamentous phage protein that mediated phage export across the bacterial cell envelope. J. Mol. Biol. 238, 187–198.
[152] Linderoth, N.A., Simon, M.N. and Russel, M. (1997) The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. Science 278, 1635–1638.
[153] d'Enfert, C., Reyss, I., Wandersman, C. and Pugsley, A.P. (1989) Protein secretion by Gram-negative bacteria. Characterization of two membrane proteins required for pullulanase secretion by *Escherichia coli* K-12. J. Biol. Chem. 264, 17462–17468.
[154] Hardie, K.R., Lory, S. and Pugsley, A.P. (1996) Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. EMBO J. 15, 978–988.
[155] Chen, L.-Y., Chen, D.-Y., Miaw, J. and Hu, N.-T. (1996) XspD, an outer membrane protein required for protein secretion by *Xanthomonas campestris* pv. *campestris*, forms a multimer. J. Biol. Chem. 271, 2703–2708.
[156] Shevchik, V.E., Robert-Badouy, J. and Condemine, G. (1997) Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins. EMBO J. 16, 3007–3016.
[157] Tomb, J.F., El-Hajj, H. and Smith, H.O. (1991) Nucleotide sequence of a cluster of genes involved in transformation of *Haemophilus influenza* Rd. Gene 104, 1–10.
[158] Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornellis, G.R. and Tommanssen, J. (1997) The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. Mol. Microbiol. 26, 789–797.
[159] Nikaido, H. (1994) Porins and specific diffusion channels in bacterial outer membranes. J. Biol. Chem. 269, 3905–3908.
[160] Zhang, H.Z. and Donnenberg, M.S. (1996) DsbA is required for stability of the type IV pilus of enteropathogenic *Escherichia coli*. Mol. Microbiol. 21, 787–797.
[161] Peek, J.A. and Taylor, R.K. (1992) Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA 89, 6210–6214.
[162] Yu, J., Weeb, H. and Hirst, T.R. (1992) A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for entertoxin biogenesis in *Vibrio cholerae*. Mol. Microbiol. 6, 1949–1958.
[163] Turner, L.R., Cano-Lara, J., Nunn, D.N. and Lory, S. (1993) Mutations in the ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J. Bacteriol. 175, 4962–4969.
[164] Sandkvist, M., Bagdasarian, M., Howard, S.P. and DiRita, V.J. (1995) Interaction between the autokinase EspE and EspL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. EMBO J. 14, 1664–1673.
[165] Feng, J.-N., Russel, M. and Modell, P. (1997) A permeabilized cell system that assembles filamentous bacteriophage. Proc. Natl. Acad. Sci. USA 94, 4068–4073.
[166] Messner, P. and Sleytr, U.B. (1992) Crystalline bacterial surface layers. Adv. Microbiol. Physiol. 33, 213–275.
[167] Castón, J., Carrascosa, J., de Pedro, M.A. and Berenguer, J. (1988) Identification of a crystalline layer on the cell envelope of the thermophilic eubacterium *Thermus thermophilus*. FEMS Lett. 51, 225–230.
[168] Ishiguro, E.E., Kay, W., Ainsworth, T., Chamberlain, J.B., Austin, R.A., Buckey, T.J. and Trust, T.J. (1981) Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. J. Bacteriol. 148, 333–340.
[169] Castón, J.R., Berenguer, J., Pedro, M.A.d. and Carrascosa, J.L. (1993) The S-layer protein from *Thermus thermophilus* HB8 assembles into porin-like structures. Mol. Microbiol. 9, 65–75.
[170] Lasa, I., Castón, J.R., Fernandez-Herrero, L.A., Pedro, M.A. and Berenguer, J. (1992) Insertional mutagenesis in the extreme thermophilic eubacteria *Thermus thermophilus*. Mol. Microbiol. 11, 1555–1564.
[171] Sleytr, U.B. (1997) Basic and applied S-layer research: an overview. FEMS Microbiol. Rev. 20, 5–12.
[172] Boot, H.J. and Pouwels, P.H. (1996) Expression, secretion and antigenic variation of bacterial S-layer proteins. Mol. Microbiol. 21, 1117–1123.
[173] Lupas, A., Engelhardt, H., Peters, J., Santarius, U., Volker, S. and Baumeister, W. (1994) Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. J. Bacteriol. 176, 1224–1233.
[174] Leibovitz, E. et al. (1997) Ocurrence and function of a common domain in S-layer and other exocellular proteins. FEMS Microbiol. Rev. 20, 127–133.
[175] Olabarría, G., Carrascosa, J.L., de Pedro, M.A. and Berenguer, J. (1996) A conserved motif in S-layer proteins is involved in peptidioglycan binding in *Thermus thermophilus*. J. Bacteriol. 178, 4765–4772.
[176] Engelhardt, H. and Peters, J. (1998) Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. J. Struct. Biol. 124, 276–302.
[177] Walker, S.G., Karunaratne, D.N., Ravenscroft, N. and Smit, J. (1994) Characterization of mutants of *Caulobacter crescentus* defective in surface attachment of the paracrystalline surface layer protein. J. Bacteriol. 176, 6312–6323.
[178] Griffiths, S.G. and Lunch, W.H. (1990) Characterization of *Aeromonas salmonicida* variants with altered cell surfaces and their use in studying surface protein assembly. Arch. Microbiol. 154, 308–312.
[179] Tummuru, M.K.R. and Blaser, M.J. (1993) Rearrangements of *sapA* homologs with conserved and variable regions in *Campylobacter fetus*. Proc. Natl. Acad. Sci. USA 90, 7265–7269.
[180] Adachi, T., Yamagata, H., Tsukagoshi, N. and Udaka, S. (1989) Multiple and tandemly arranged promoters of cell wall protein gene operon in *Bacillus brevis* 47. J. Bacteriol. 171, 1010–1016.
[181] Vidgrén, G., Palva, I., Pakkanen, R., Lounatnaa, K. and Palva, A. (1992) S-layer protein form *Bacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. J. Bacteriol. 174, 7419–7427.
[182] Pouwels, P.H., Kolen, C.P. and Boot, H.J. (1997) S-layer protein genes from *Lactobacillus*. FEMS Microbiol. Rev. 20, 78–82.
[183] Noonan, B. and Trust, T.J. (1995) The leucine zipper of *Aeromonas salmonicida* AbcA is required for the transcriptional activation of

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024

the P2 promoter of the surface-layer structural gene, *vapA*, in *Escherichia coli*. Mol. Microbiol. 17, 379–386.

[184] Fernández-Herrero, L.A., Olabarría, G. and Berenguer, J. (1997) Surface proteins and a novel transcription factor regulate the expression of the S-layer gene in *Thermus thermophilus* HB8. Mol. Microbiol. 24, 61–72.

[185] Olabarría, G., Fernández-Herrero, L.A., Carrascosa, J.L. and Berenguer, J. (1996) *slpM*: a gen coding for an 'S-layer like' array overexpressed in S-layer mutants of *Thermus thermophilus* HB8. J. Bacteriol. 178, 357–365.

[186] Dworking, J., Tummuru, M.K.R. and Blaser, M. (1995) A lipopolysaccharide binding domain of the *Campylobacter fetus* S-layer protein resides within the conserved N-terminus of a family of silent and divergent homologs. J. Bacteriol. 177, 1734–1741.

[187] Dworking, J. and Blaser, M.J. (1997) Nested DNA inversion as a paradigm of programmed gene rearrangement. Proc. Natl. Acad. Sci. USA 94, 985–990.

[188] Thompson, S.A., Shedd, O.L., Ray, K.C., Beins, M.H., Jorgensen, J.P. and Blaser, M.J. (1998) *Campylobacter fetus* surface layer proteins are transported by a type I secretion system. J. Bacteriol. 180, 6450–6458.

[189] Peters, J., Peters, M., Lottspeich, F., Shafer, W. and Baumeister, W. (1987) Nucleotide sequence analysis of the gene encoding the *Deinococcus radiodurans* surface protein, derived amino acids sequence and complementary protein chemical studies. J. Bacteriol. 169, 5216–5223.

[190] Faraldo, M.M., Pedro, M.A.d. and Berenguer, J. (1992) Sequence of the S-layer gene of *Thermus thermophilus* HB8 and functionality of its promoter in *Escherichia coli*. J. Bacteriol. 174, 7458–7462.

[191] Carl, M., Bobson, M.E., Ching, W.M. and Dasch, G.A. (1990) Characterization of the gene encoding the protective paracrystalline-surface-layer protein of *Rickettsia prowazekii*: presence of truncated identical homology in *Rickettsia typhi*. Proc. Natl. Acad. Sci. USA 87, 8237–8241.

[192] Nooman, B. and Trust, T.J. (1995) Molecular analysis of an A-protein secretion mutant of *Aeromonas salmonicida* reveals a surface layer-specific protein secretion pathway. J. Mol. Biol. 248, 316–327.

[193] Awram, P. and Smit, J. (1998) The *Caulobacter crescentus* paracrystalline S-layer protein is secreted by an ABC transporter (type I) secretion apparatus. J. Bacteriol. 180, 3062–3069.

[194] Kawai, E., Akatsuka, H., Idei, A., Shibatani, T. and Omori, K. (1998) *Serratia marcescens* S-layer protein is secreted extracellularly via an ATP-binding cassette exporter, the Lip system. Mol. Microbiol. 27, 941–952.

[195] Wandersman, C. (1992) Secretion across the bacterial outer membrane. Trends Genet. 8, 317–321.

[196] Blight, M.A. and Holland, I.B. (1994) Heterologous protein secretion and the versatile *Escherichia coli* haemolysin translocator. TIBTECH 12, 450–455.

[197] Thanabalu, T., Koronakis, E.C.H. and Koronakis, V. (1998) Substrate-induced assembly of a contiguous channel for protein export from *E. coli* reversible bridging of an inner-membrane translocase to an outer membrane exit pore. EMBO J. 17, 6487–6496.

[198] Binet, R., Létoffé, S., Ghigo, J.M., Delepelaire, P. and Wandersman, C. (1997) Protein secretion by Gram-negative bacterial ABC exporters - a review. Gene 192, 7–11.

[199] Bingle, W.H., Nomellini, J.F. and Smit, J. (1997) Linker mutagenesis of the *Caulobacter crescentus* S-layer protein: toward a definition of an N-terminal anchoring region and a C-terminal secretion signal and the potential for heterologous protein secretion. J. Bacteriol. 179, 601–611.

[200] Pum, D., Messner, P. and Sleytr, U.B. (1991) Role of the S-layer in morphogenesis and cell division of the archaebacterium *Methanocorpusculum sinense*. J. Bacteriol. 173, 6865–6873.

[201] Howard, L.V., Dalton, D.D. and McCoubrey, W.K.J. (1982) Expansion of the tetragonally arrayed cell wall protein layer during growth of *Bacillus sphaericus*. J. Bacteriol. 149, 748–757.

[202] Gruber, K. and Sleytr, U.B. (1988) Localized insertion of new S-layer during growth of *Bacillus stearothermophilus* strains. Arch. Microbiol. 149, 485–491.

[203] Smit, J. and Agabian, N. (1992) Cell surface patterning and morphogenesis: biogenesis of a periodic surface array during *Caulobacer* development. J. Cell Biol. 95, 41–49.

Downloaded from https://academic.oup.com/femsre/article/24/1/21/525703 by guest on 23 April 2024