Chapter 6

Pili in Probiotic Bacteria

Vengadesan Krishnan, Priyanka Chaurasia and Abhiruchi Kant

Additional information is available at the end of the chapter

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Abstract

The ability to adhere to intestinal epithelial tissue and mucosal surfaces is a key criterion in selecting probiotics. Adhesion is considered to be a prerequisite for successful colonization and survival in the gastrointestinal tract to provide persistent beneficial effects to the host. Bacteria express a multitude of surface components that mediate adherence. Pili or fimbriae are surface adhesive components implicated in initiating bacterial adhesion and mediating interaction with the host. These nonflagellar proteinaceous fiber appendages were identified and explored over several decades in pathogenic bacteria, and many distinct types are known. However, the presence of pili in probiotics and/or commensalic bacteria has only recently been recognized. Thus knowledge about pili in probiotics is relatively limited, but structural and functional data have begun to emerge. Availability of these data in the future would enable us to understand the pili-mediated adhesion strategies of probiotics. This knowledge could be utilized to develop antiadhesion-based therapies against bacterial infections as well as probiotic designs for beneficial effects. This chapter will briefly summarize the current knowledge of pili in probiotics with emphasis on members of lactobacilli and bifidobacteria.

Keywords: Adhesion, Bifidobacteria, Lactobacilli, Pili, Probiotics

1. Introduction

Bacterial colonization of humans seems to commence at birth and evolves throughout life. It depends on several factors including mode of birth, age, geographical location, local environment, diet, stress, illness, medications, and antibiotic treatment. Bacteria colonize all parts of the human body that are exposed to external environment. Specifically, the gastrointestinal tract (GIT) harbors more than 1000 species, and this complex microbial community is referred to as

the "gut microbiota" [1, 2]. The gut microbiota are well recognized because of their impact on health and disease. However, knowledge on the precise mechanism(s) by which the microbiota exerts its influence remains largely unknown. *Lactobacillus* and *Bifidobacterium* species constitute a major part of the microbiota and are believed to play an essential role in modulating immune system, resisting pathogen colonization, metabolism, and energy balance [3, 4]. Some members of these two genera are also popular as probiotics. Though the specific contribution of these members to the beneficial effects is subject to investigation and speculation, it is widely accepted that their presence in the GIT often confers health benefits. The molecular mechanisms that allow these members to colonize the GIT have not yet been elucidated in detail, though their persistence was shown to be essential for the beneficial effects.

Most pathogenic bacteria are known to express multitude of surface components for establishing contacts and mediating interactions with the host for bacterial colonization. Among these, long, hair-like filamentous structures known as pili or fimbriae have been often implicated in adhesion processes and shown to be required for bacterial colonization on host tissues (for reviews, see [5–11]). Typically, these structures are made up of building blocks called pilins or fimbrilins. Genes for these pilins along with other genes required for the pilus assembly are located in the same place in the genome called pilus gene cluster or Pathogenicity Island. Distinct pilus structures (e.g., chaperone-mediated, type IV, Curli, and CS1) are known in Gram-negative pathogens. Their structure, function, and biogenesis have been well explored to some extent. The details of pili have begun to emerge for Gram-positive pathogens a decade ago (for reviews, see [8, 10–15]). The sortase-mediated pili seem to be conserved across the Gram-positive pathogens. Some of the pilus types (e.g., type IV) exist both in the Gram-negative and Gram-positive pathogens. The pilus types have been majorly categorized based on secretion systems, biogenesis, architecture, and function. The sortase-mediated pili differ from other known types by being a covalent polymer in which pilin subunits are covalently tethered to each other by sortase-mediated isopeptide bonds. The pili and their components in the pathogens are recognized as virulence factors as they play a key role in pathogenesis. Also, they are considered as potential vaccine candidates because of their immunogenic properties.

Although the focus is traditionally on pili in pathogenic bacteria for last few decades, they have been recently identified in many gut commensalic bacteria and often shown to be essential for their colonization and persistence in the the GIT and for immune modulation. Although the pili in pathogenic bacteria are regularly reviewed, this chapter attempts to give a brief overview of pili in beneficial bacteria, which is relatively recent.

2. Sortase-mediated pili

As demonstrated first in pathogen *Corynebacterium diptheriae* [15, 16], the sortase-mediated pilus (SpaA-type) model consists of three different types of pilins (one major pilin and two ancillary pilins). Typically, the loci for the pilins and at least one sortase are located together in the genome as a pilus operon or gene cluster (**Figure 1A**). Similar to microbial surface

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component recognizing adhesive matrix molecules (MSCRAMMs), the pilin precursors contain signal sequence at the N-terminal and sorting signal at the C-terminal. The C-terminal sorting signal is composed of a conserved LPXTG (Leu-Pro-any-Thr-Gly) motif, a hydrophobic domain, and a positively charged tail (**Figure 1B**). Multiple copies of major pilin form the pilus backbone like beads on a string (**Figure 1C**). Hence, they are also referred to as backbone or shaft pilins. The major pilins often contain a conserved YPKN (Tyr-Pro-Lys-Asn)-like motif close to the N-terminal (**Figure 1B**). The pilin-specific sortase, whose gene is located in the pilus gene cluster, generates the covalently cross-linked pilus shaft as follows. Prior to polymerization into pilus fibers, the prepilins or pilin precursors are exported across the membrane through the Sec apparatus. These precursors are then embedded into the membrane by their C-terminal hydrophobic domain and positively charged tail. The membrane-bound pilin-specific sortase forms acyl-enzyme intermediate by cleaving the LPXTG motif of major pilin between threonine and glycine, and creates a thioester bond between its catalytic cysteine residue and the nascent C-terminal threonine. This intermediate receives nucleophilic attack from the lysine residue of pilin motif of another major pilin that results in an amide bond formation between the cleaved threonine and lysine side chain. The repeated reaction promotes the growth of pilus structure on the cell surface (**Figure 1**). The ancillary pilins are incorporated into the pilus structure, presumably by similar transpeptidation reaction. Ancillary pilin 1, which is larger in size, is generally located at the pilus tip. This pilin, also known as tip pilin, often plays a role in adhesion to host. Ancillary pilin 2 or basal pilin is often observed at the base of pilus and smaller in size. These basal pilins are shown to contain a pilin-like motif for their incorporation into the pilus base [21]. A different transpeptidase known as housekeeping sortase, which is not part of the pilus gene cluster, anchors the assembled pilus structure on the cell wall. Similar to pilin-specific sortase transpeptidase reaction, the housekeeping sortase forms acyl-enzyme intermediate with basal pilin. This intermediate receives nucleophilic attack from the peptidoglycan cross-bridge that results in the formation of covalent link between the carboxyl threonine in the basal pilin and the free amino group of the cell wall lipid II precursors.

The pilins are commonly made up of two building blocks, which are variants of immunoglobulin fold known as CnaA [17] and CnaB [18], often with intradomain isopeptide bond [19] (for reviews, see [20–22]) (**Figure 2**). In addition, the tip pilins also contain adhesin modules such as von Willebrand factor type A domain (vWFA) with two inserted arms [23, 24] and thioester containing domains [25–27] (**Figure 2**). The pilus model of *C. diptheriae* appears to be conserved across the Gram-positive pathogenic strains (e.g., *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus parasanguinis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus cererus*, and *Actinomyces naeslundi*) with some variations in number of pilus gene clusters, number of pilins, number of pilin-specific sortases, and pilus architecture. They majorly participate in cellular adhesion and colonization processes. More than one sortase-mediated pilus gene cluster are often present in the same bacterial strains suggesting their different cellular targets and functions.

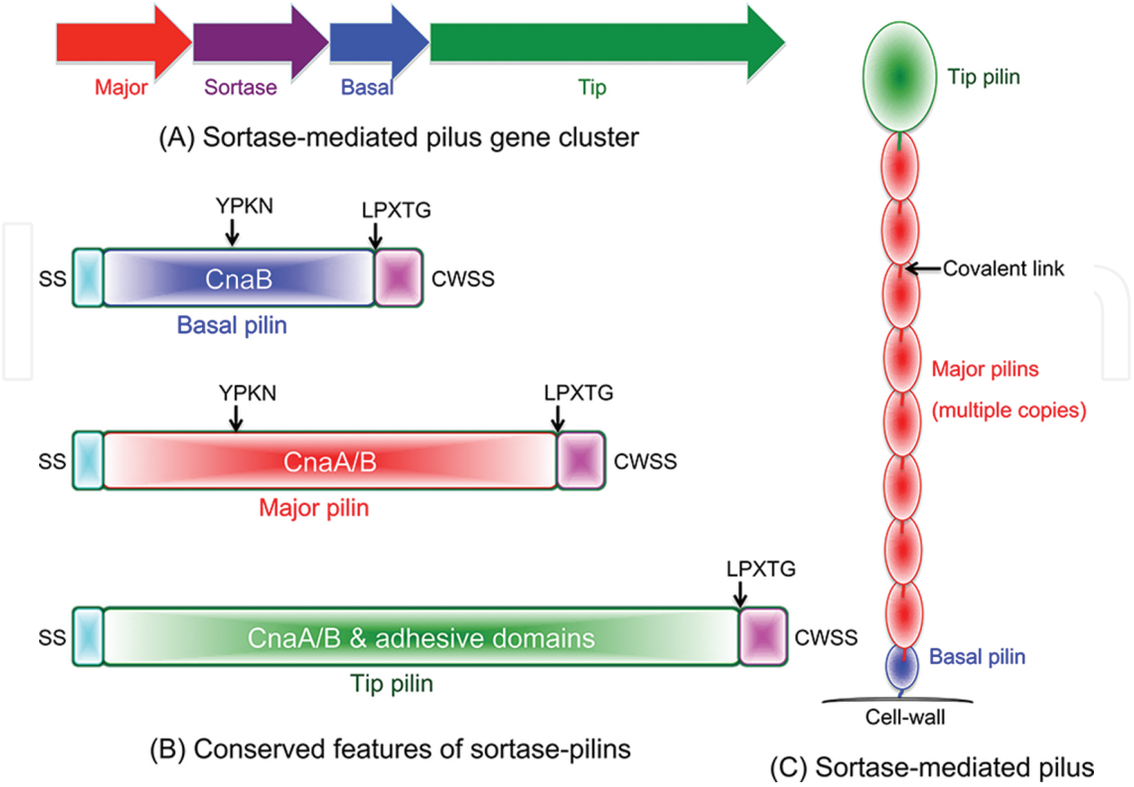

Figure 1. Schematic diagram of typical sortase-mediated pili. (A) Pilus gene cluster for sortase-mediated pilus assembly. It encodes genes for a major (red), basal (blue), tip (green) pilins, and a pilin-specific sortase (purple). More than one sortase (e.g., SpaD- and SpaH-pilus gene cluster in *C. diphtheriae*) and less than three pilins (e.g., type 1 and 2 pilus gene clusters in *A. oris*) have also been observed. In the pilus gene cluster, differences in the order of gene's arrangement and the presence of transposon elements in the vicinity are often observed. (B) Conserved features of sortase pilins. Signal sequence (SS) and LPXTG-containing cell wall sorting signal (CWSS) are at the N- and C-terminals of all the (basal, major, and tip) pilins. In addition, the basal and major pilins have pilin motif (YPKN) in the vicinity of N-terminals. A conserved element called E-box (LXET) has also been observed in the sortase pilins. The basal pilins consist of 1–3 CnaB domains (Figure 2A). The major pilins contain 2–4 CnaA/B domains. CnaB domains are often at the N- and C-terminals, and CnaA at the middle (Figure 2B). The tip plins have adhesive domains (vWFA/thioester containing domains) in addition to CnaA/B domains (Figure 2C). (C) Sortase-mediated pilus structure. The pilus is made up of three distinct types of pilins: basal (blue), major (red), and tip (green) pilin. In the pilus, the pilins are tethered to each other by sortase-mediated covalent links (see the text for details). Multiple copies of the major pilins form the pilus shaft in a head-to-tail fashion like beads on a string. The tip pilin is often located at tip projecting adhesive domain for favoring adhesion. The basal pilin is often located at the base of pilus shaft and helping for anchoring the polymerized pilus on the cell wall through the housekeeping sortase.

The sortase-mediated pili, which are being actively investigated in Gram-positive pathogens and considered as virulence factors, have been detected in several gut commensals as mentioned in the following sections. The pilus-like gene clusters were earlier noticed in probiotic *Lactobacillus johnsonii* NCC 533 [28], but first received attention through probiotic *Lactobacillus rhamnosus* GG in 2009 [29, 30]. Since then, it has been identified in several species and strains of probiotic and other commensal bacteria by genomic analysis and shown to be essential for their adherence and colonization in GIT. Their presence was further confirmed by imaging analysis in the *L. rhamnosus* GG [29, 31], genus of *Bifidobacterium* [32, 33], *Lacococcus lactis* IL1403 and TIL448 [34, 35], and recently in *Lactobacillus ruminis* ATCC 25644 [36]. Hence, the view of surface piliation has now been expanded to include its role also as a niche-adaptation factor.

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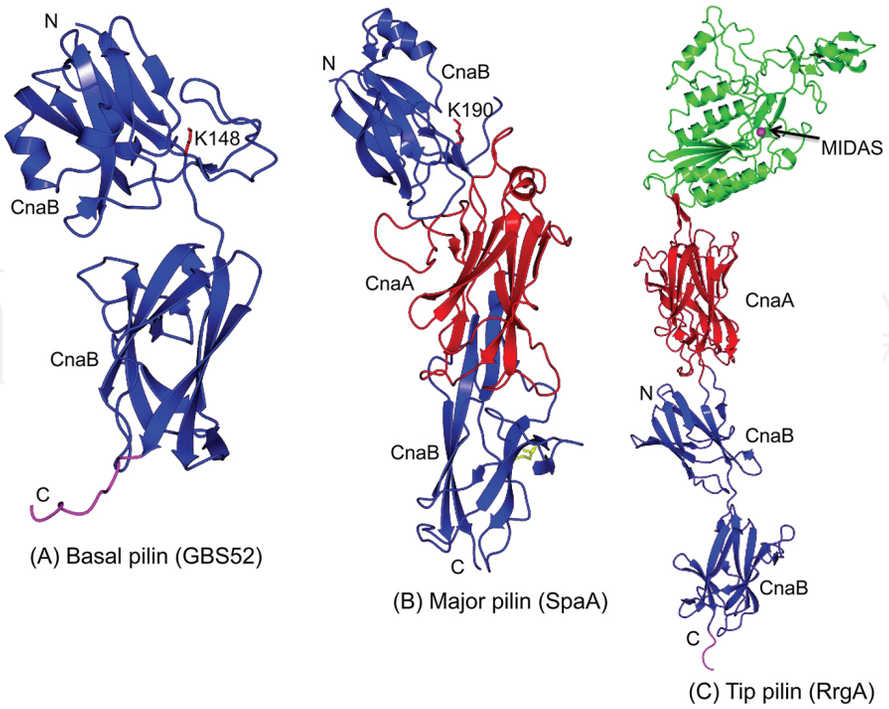

Figure 2. Three-dimensional structures of sortase-pilins from pathogenic bacteria. (A) Basal pilin, GBS52 (PDB id: 3PHS), from *S. agalactiae*. It consists of two CnaB domains, and the lysine from the pilin motif is shown as stick (in red). A proline-rich C-terminal tail is shown in magenta. (B) Major pilin, SpaA (PDB id: 3HR6), from *C. diphtheriae* consists of three domains. CnaB domains (in blue) are at N- and C-terminals, and CnaA (in red) at the middle. Pilin motif lysine is shown in stick (red). (C) Tip pilin, RrgA (PDB id: 2WW8), from *S. pneumoniae* contains four domains. CnaB domains (in blue) are at the terminals and CnaA (in red) at the middle. Metal (pink)-ion-dependent adhesion site (MIDAS) containing vWFA domain with two inserted arms are shown in green.

2.1. Pili in *L. rhamnosus* GG

L. rhamnosus GG is one the of well-documented and widely used probiotic strains [37]. The pilus-like protrusions in *L. rhamnosus* GG were initially seen in 2009 [30]. *L. rhamnosus* GG contains two pilus gene clusters *SpaCBA* and *SpaFED* as shown by comparative genomic analysis [29] (**Figure 3A**). The *SpaCBA* encodes a major pilin SpaA, two ancillary pilins SpaB and SpaC, and a pilin-specific sortase (SrtC1). As further confirmed by western blotting and immunogold electron microscopy [29, 31], the SpaCBA pilus of *L. rhamnosus* GG has similar morphology to the three-pilins architecture model of *C. diptheriae* [15, 16]. The repeating SpaA makes the pilus backbone. The cell wall anchoring SpaB and adhesive SpaC ancillary pilins are found at the base and tip of the pilus, respectively (**Figure 3C**). However, in contrast to the pili from most Gram-positive pathogens, the tip pilin (SpaC) and, to a lesser extent, basal pilin (SpaB) are found sporadically throughout the SpaCBA pilus backbone. Such a distribution is thought to enhance adherence to the intestinal mucosa and epithelial layer and thereby then extend the relative longevity and transient colonization of *L. rhamnosus* GG cells in the gut. The SpaCBA pilus was demonstrated to be pivotal for efficient adherence to mucus [29, 38, 39], collagen [40], and Caco-2 intestinal epithelial cell line and biofilm formation [41]. The immunomodulation of SpaCBA pili includes toll-like receptor 2 (TLR2)-dependent activation

and dendritic cell cytokine production [42], dampening endogenous interleukin (IL)-8 mRNA levels [41], eliciting macrophage-mediated anti-inflammatory cytokine mRNA expression [43], inducing TLR-related gene expression in a human fetal intestine model [44], and stimulating cellular responses in intestinal epithelial cells [45]. Interestingly, the SpaC plays a role in most of the SpaCBA pili-triggered host cell immune responses. The surface piliation apparently provides a niche-specific fitness to *L. rhamnosus* GG cells for extending their transient colonization in the gut [46]. Presumably, this is an advantage over nonpiliated probiotic bacteria. For example, the non-SpaCBA piliated *L. rhamnosus* LC705, which is genetically similar to *L. rhamnosus* GG, shows decreased adherence to intestinal mucus in the comparative study [29]. More recently, the key role of *L. rhamnosus* GG pili in interaction with β-lactoglobulin has also been demonstrated [47].

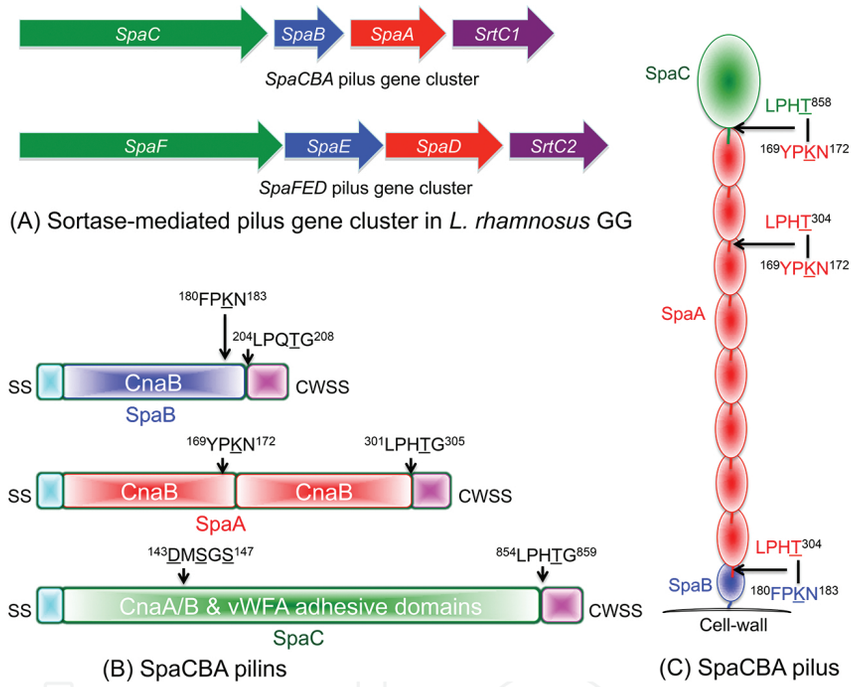

Figure 3. Schematic diagram of sortase-mediated pili in *L. rhamnosus* GG. (A) *SpaCBA* and *SpaFED* pilus gene clusters identified in *L. rhamnosus* GG. Each cluster encodes a tip pilin (SpaC/SpaF), major pilin (SpaA/SpaD), basal pilin (SpaB/SpaE), and pilin-specific sortase (SrtC1/SrtC2). (B) Predicted elements required for the pilus assembly in the SpaCBA pilins. The basal pilin SpaB contains a single CnaB domain with FPKN pilin motif and LPQTG-containing CWSS at C-terminal. Residue numbers and positions were labeled and marked by arrow. The major pilin SpaA contain two CnaB domains, and its pilin and sorting motif are marked. The tip pilin SpaC contains a vWFA domain and its MIDAS (DMSGS) motif is marked. (C) The SpaCBA pilus model consists of SpaA, SpaB, and SpaC. The possible sortase-mediated intercovalent link is marked by arrow with details of residues involved. A possible mode of association for SpaC and SpaB along the pilus shaft other than at the tip and base of the pilus needs to be further shown by a high-resolution imaging technique or structural studies.

Similar to *SpaCBA*, the *SpaFED* operon encodes the pilus backbone (SpaD), the pilus tip (SpaF) and the base (SpaE) pilins, as well as a putative sortase enzyme (SrtC2) required for pilus assembly (**Figure 3A**). Though the recombinant SpaF has been shown to bind intestinal mucus [39], the genes associated with the spaFED pilus gene cluster are not constitutively expressed

in the tested laboratory conditions [31]. Thus, the native form of the SpaFED pilus remains hypothetical, not only in *L. rhamnosus* GG, but also in other strains carrying the spaFED operon (e.g., *L. rhamnosus* LC705) [31, 46]. However, *L. rhamnosus* GG SpaFED pili can be readily produced as an assembled structure in recombinant *L. lactis* [48].

Obtaining three-dimensional structural insights into pilus assembly and adhesion mechanisms through the structural biology techniques has been instrumental for Gram-negative pathogens in the past (for reviews, see [5, 8, 49, 50]), and it was begun much later for Gram-positive pathogens in 2007 ([19, 51], for reviews, see [11, 20–22]). The structures of individual major as well as ancillary pilins from several pathogenic strains have been determined (for recent review, see [21]) (**Figure 2**). A Cryo-EM study on *S. pneumoniae* pili has also supported the sortase-mediated three pilins architectural model [52]. According to current structural knowledge, the basal pilins consist of 1–3 CnaB domains often with intradomain isopeptide bonds (**Figure 2A**). Conserved proline-rich C-terminal tails in the known basal pilins suggest their likely role in pilus anchoring via housekeeping sortase. The presence of a pilin-like motif with a lysine in the basal pilin indicates that they could be incorporated into the pilus base by sortase (**Figure 2**). The major pilins are made of 2–4 CnaB/A domains (**Figure 2**). The CnaB domains are at the N- and C-terminals, whereas the CnaA domain is in the middle. The pilin motif is present at the C-terminal region of N-terminal CnaB domain (**Figure 2B**). The N-terminal domain in many pilins seems to be flexible with no or slow forming internal isopeptide bond. In some crystal structure studies, a fiber-like pilus arrangement in the crystal packing has been observed though the sortase-mediated intermolecular amide bond between the backbone pilins was absent. The tip pilins contain adhesive domains at the tip in addition to CnaA and CnaB domains that form a stalk and connect adhesive domains to the pilus shaft (**Figure 2C**). These adhesive domains are often a modified vWFA domain with two inserted arms [23, 24], and thioester containing domain [25]. The complicated domains arrangement and folding in tip pilins makes difficult to predict them from their sequence.

Detailed structural knowledge is yet to emerge for pili and related components for probiotic bacteria. However, preliminary crystallographic data are available for some of the pilins (SpaA [53], SpaD [54], and SpaC [55]) in *L. rhamnosus* GG. Our initial analysis of ongoing structural investigations on pilus constituents of *L. rhamnosus* GG and comparison with their counterparts in pathogens suggest that SpaA may consist of two CnaB domains (**Figure 3B**), and SpaD contains three domain with CnaB domains at the terminals and CnaA domain in the middle. Though it is yet to be validated, it is tempting us to describe Lys171 from the pilin motif SpaA as the possible linking lysine that could involve in the SpaA–SpaA and SpaA–SpaC pilins covalent association during SpaCBA pilus shaft polymerization by pilin-specific SrtC1 (**Figure 3B** and **C**). Similarly, Lys182 in SpaB seems a likely candidate for its incorporation into the pilus (**Figure 3B** and **C**). Such a linking lysine is yet to be predicted for SpaC for its incorporation other than at the pilus tip. In contrast to known pathogenic tip pilins (e.g., GBS104 [24] and RrgA [23]), but similar to eukaryotic proteins (e.g., integrins, complement C2a, and Fb), the vWFA domain predicted in SpaC [55] seems not to have the two inserted arms, suggesting both possible differences and similarities in

binding mechanism via a metal-ion-containing vWFA adhesin domain. Certainly, knowledge generated from our ongoing structural investigations would provide new insights into pilus assembly and adhesion mechanisms in *L. rhamnosus* GG, and serves as a model for probiotics.

2.2. Pili in *L. ruminis*

L. ruminis, one of the dominating *Lactobacillus* species in the mammalian intestines, is routinely isolated from the feces of human, cattle, and pigs. It is one of the few motile members known in lactobacilli. It is also recognized as an autochthonous microbiota in the GIT. The pilus gene identified in the human-derived intestinal isolate *L. rumini* ATCC 25644 has been named as *lrpCBA* (*L. r*umini *p*ilus) [36] since they appear to be different from the known lactobacillar pilus types (SpaCBA and SpaFED) at the primary structural level. The *LrpCBA* pilus operon encodes tip (lrpC), basal (lrpB), and major (lrpA) pilins and a pilin-specific sortase (SrtC). Sequence of *L. ruminis* pilins displays the common pilin features such as LPXTG-like motifs, E-box motif, and pilin motifs (in major and basal pilins) [36] (**Figure 2**). The expression and surface localization of *lrpCBA* pilus gene product have further confirmed by immunoblot analysis and immune-electron microscopic visualization (for details, see [36]). Interestingly, the pilus genes have also been detected in *L. ruminis* ATCC 27782 from bovine gut origin [56], but the microarray analysis showed that the corresponding genes were upregulated in human strain compared with the bovine isolate. The ability of LrpCBA pilus to adhere to gut epithelial cells and extracellular matrix (ECM) proteins, and immune-modulation activities has been demonstrated using recombinant-piliated lactococci (for details, see [36]). Interestingly, the tip pilin LrpC supports *L. ruminis* binding to ECM-related substrates but not to the mucosal surfaces.

2.3. Pili in other *Lactobacillus* species

The presence of sortase-mediated pilus gene clusters has been reported in many strains of *Lactobacillus casei* [57–60] and *Lactobacillus paracasei* [61], which are members of the normal human gut microbiota and used extensively as probiotics and in the food industries. Although the pilus expression and function are yet to be studied in detail, the most analyzed strains in the *L. casei* and *L. paracasei* group show that they contain *SpaCBA* and *SpaFED* pilus gene clusters. In contrast, only few strains in *L. rhamnosus* group have *SpaCBA* cluster (e.g., *L. rhamnosus GG* and LMS2-1 strain). However, several *L. paracasei* strains including COM0101 are shown to have truncated SpaC gene [60]. The transposon genes, which are present in the vicinity of the *SpaCBA* cluster in *L. rhamnosus*, seem to be absent in the *L. casei* suggesting that L. *rhamnosus* GG and LMS2-1 could have acquired the *SpaCBA* pilus gene cluster through horizontal gene transfer (HGT) from *L. casei* [57, 62]. This is further evidenced by the presence of high nucleotide sequence identity in spaCBA cluster of *L. rhamnosus* and *L. casei* [57, 62].

2.4. Pili in *L. lactis*

L. lactis is another widely used species as starter in dairy fermentation and best characterized strain in lactic acid bacteria (LAB). They seem to present in nutrient-rich ecological niches (gut mucus, milk, and plants). A functional pilus operon (*pil*) has been shown to present in *L. lactis* IL1403 [34, 63]. It encodes tip (YhgD), major (YhgE), and basal (YhhB) pilins and a pilin-specific sortase (SrtC). The presence of pilus structures has been confirmed by immunogold electron microscopy and atomic force microscopy (AFM) analyses. The major YhgE and basal YhhB pilins display typical LPXTG motifs and pilin motifs. Additionally, the YhgE has an E-box. The pili were also shown to promote biofilm formation by confocal laser scanning microscopy (CLSM). The occurrence of pili in few other *L. lactis* isolates from clinical and vegetal environments was also visualized by by transmission electron microscopy (TEM) analysis [34]. Later, a proteomic analysis study has also detected pilus genes (YhgE2, YhhB2, ORF4, and SrtC2) in a vegetal isolate *L. lactis* subsp. *lactis* TIL448 [35]. The YhgE2 was shown to play a major role in intestinal epithelial Caco-2 cells adhesion. The pilus biogenesis and morphology were further analyzed by immunoblot, electron micrograph, transcriptional, and AFM experiments [35, 64].

2.5. Pili in bifidobacteria

Bifidobacteria are the common components of the gut microbiota of a broad range of hosts [65]. Several members of bifidobacteria are typical inhabitants of the infant intestine [66], which is thought to be sterile at birth. Identification of many bifidobacterial strains in the stools of healthy infants suggests that they could be the first colonizers in the GIT subsequent to birth. Genomic analysis has revealed pilus genes cluster in several bifidobacterial strains [67]. Interestingly, many pilus gene clusters are flanked by transposon elements indicating their acquisition by HGT. The presence of pilus structures was further examined by AFM and transcription analysis in *Bifidobacterium bifidum*, *Bifidobacterium dentium*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium adolescentis*, and *Bifidobacterium animalis* subsp. *lactis* [67]. The pilus gene clusters often found to contain one major pilin (FimA or FimP) and one or two ancillary pilins (FimB or FimQ) with a pilin-specific sortase. Many of these pilus genes are similar to the (two-pilins) pilus gene clusters identified in Gram-positive pathogens such as *Actinomyces oris* [68, 69] and *Bacillus cereus* [70], which lack basal pilus genes differing from the three-pilins architectural model of *C. diphtheriae* [15, 16]. *A. oris* encodes two different fimbriae (types 1 and 2). Type 1 fimbria, which mediates the interaction of actinomyces to tooth enamel, consists of the major pilin (FimP) and tip pilin (FimQ). Whereas, the type 2 fimbria that mediate interaction with oral streptococci and host cell for causing dental plaque is made of major pilin (FimA) and tip pilin (FimB). Similarly, *B. cereus* pili is composed of major pilin BcpA and the tip pilin (BcpB). In the two-pilin sortase-mediated pili model, the last major pilin may function as the pilus base. The three-dimensional structures for major pilins for *A. oris* are available while they are yet to be elucidated for tip pilins. The major pilins of bifidobacteria have typical pilin motif and LPXTG motif required for pilus polymerization [67]. The role of pili in adherence, immunomodulation, and bacterial aggregations was further extensively explored in *B. bifidum* PRL2010, which contains three different pilus gene clusters (*pil1*, *pil2*, and *pil3*)

[71]. Apart from sortase-mediated pili, the presence of type IV pili has also been reported in bifidobacteria (e.g., *Bifidobacterium breve* UCC2003 [32]), which is described below.

3. Tad pili

The Tad (tight adherence) pili, which was first described in *Aggregatibacter (Actinobacillus) actinomycetemcomitans* [72], is a specialized subtype of type IV pili (for reviews, see [5, 7, 8, 73, 74]). Tad pili in this bacterium were shown to mediate adhesion to surfaces and essential for colonization and pathogenesis. Apart from adhesion, the type IV pili have been implicated in several functions such as aggregation, biofilm formation, twitching motility, DNA uptake, and electron transfer. Type IV pili are found to be present in Gram-negative (e.g., enteropathogenic *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Neisseria meningitides*, and *Vibrio cholerae*) as well as Gram-positive bacteria (*Clostridium perfringens*, *Mycobacterium tuberculosis*, and *Ruminococcus albus*). Type IV pili are typically 6–8 nm in diameter and several micrometers long. The type IV pilus is comprised of homopolymers of a single (major) pilin subunit (**Figure 4**). The major pilins in Tad pili are relatively smaller in size (~7 kDa) compared with other known pilus types in type IV. The flexible homopolymer filaments in type IV often have tendency to form characteristic helical bundles by lateral interactions. Some pili possess an adhesive or ancillary pilins at the pilus tip or can be decorated with pseudopilins along the pilus.

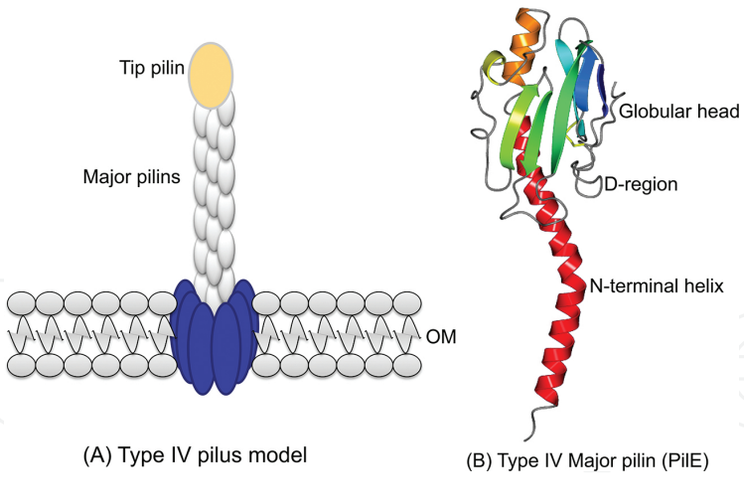

Figure 4. Schematic diagram of type IV pilus structure. (A) Type IV (gonococcal) pilus model. Major pilins form the filament majorly by hydrophobic interactions between their N-terminal helices in the filament core. The globular head of major pilins pack on the filament surface. (B) Type IV major pilin, PilE (PDB id: 1AY2), from *N. gonorrhoeae* showing N-terminal helix (in red) and globular head with D-region.

Type IV pilus assembly is a complex process, which requires protein products from multiple genes (~14) including minor pilins, prepilin peptidase, ATPase, inner membrane core proteins, and accessory proteins. Many of the core genes are conserved across different bacterial species. Tad pili seem to differ from other type IV pilus types by lacking four core homologous minor

pilins. The type IV pilins are synthesized as precursors with a leader peptide and transported across the inner membrane into the periplasmic space, where they are retained in the inner membrane through their N-terminal hydrophobic segments. The globular domain is folded with stabilizing intramolecular disulfide bonds. A dedicated prepilin peptidase cleaves the positively charged leader sequence and methylates the N-terminal amine to generate the mature pilin. The methylated, positively charged N-terminal residue is thought to attract negatively charged glutamate (at fifth position) of adjacent major pilin in the growing pilus fiber. This results in vertical displacement between one pilin and the next. The assembly ATPase associated with the cytoplasmic part of the inner membrane protein undergoes conformational change during ATP hydrolysis and pushes the pilus filament out of the membrane, providing a gap for the next major pilin. Type IV pili is further complicated by divergence and divided into two classes (types IVa and IVb) based on the length of leader peptides and mature pilins. The pilins of type IVa are typically 150–160 residues long with a short leader peptide (<10 residues), whereas the pilins of type IVb are either long (180–200 residues) or short (40–50 residues) with longer leader peptides (~15–30 residues). The Tad pili are monophyletic subclass of type IVb pili [73]. The pilins of Tad pili are short with 40–50 residues long.

Though the sequence and structural diversity are associated with the pilins in type IV, they share a common lollipop-like architecture consisting of an extended N-terminal helical stick followed by a globular head containing a β-sheet with 4–7 strands [74] (**Figure 4B**). The N-terminal half of the helix is hydrophobic and multifunctional regulatory domain. It protrudes from the globular head and forms the central hydrophobic core of the growing filament during the pilus assembly. Prior to assembly, it acts as transmembrane segment to retain individual pilin in the cytoplasmic membrane. The C-terminal half of the helix is amphipathic and embedded in the globular head. For many pili, a hypervariable C-terminal loop known as D-region or disulfide-bonded loop (DSL) performs an essential role in surface adherence (**Figure 4B**). The conserved disulfide bridge in the D-region observed in several Gram-negative major pilins appears to be off in Gram-positive pilins (e.g., PilA1 in *Chlostrodium difficle* [75]). The Tad genes are also widespread in the genomes of Gram-positive species (*C. diphtheriae*, *Thermobifida fusca*, and *Streptomyces coelicolor*). Recently, they have been identified in probiotic *B. breve.*

3.1. Tad pili in *B. breve*

Apart from sortase-dependent pili, *B. breve* UCC2003 was recently shown to contain the type IVb or Tad pilus gene cluster named tad_{2003} [32]. The presence of pili was further confirmed by immunogold transmission electron microscopy and shown to be essential for efficient gut colonization in a murine model by mutational analysis [32]. Specifically, the Tad locus is highly conserved among all sequenced bifidobacterial strains supporting a ubiquitous pilus-mediated host colonization and persistence mechanism for intestinal bifidobacteria. The structural data are yet to come for pilins of Tad pilus from beneficial bacteria for shedding light on their structure and function.

4. Future perspectives

Adhesion of bacteria to host surfaces is a prerequisite and crucial step for bacterial colonization, which may result in pathogenic or commensal relationship. The pili have been often implicated in initiating adhesion and mediating interaction with host. Understanding pilus structure and function, and their mediated interactions with the host has been achieved to a certain extent in pathogenic strains. The pili and their components are recognized as virulence factors in pathogenic strains, and also considered as potential vaccine candidates in combating bacterial infection. Recent identification of such surface organelles in probiotic or commensal bacteria gives a new perspective as a niche-adaption factor as well. The sortase-mediated pili initially discovered in Gram-positive pathogens appear to be widespread among commensals. The Tad pili, which are known to present in both Gram-negative and Gram-positive pathogens, have also been detected in some commensal strains. It may not be a surprise if additional pilus type comes in the future from the fast-growing technology and genomes for gut microbiota. Available preliminary data suggest that the pili from pathogenic and beneficial bacteria share several sequence and structural features. The presence of transposable element in several pilus gene clusters indicates that the pathogenic and commensal bacteria may be acquired from each other during the evolution. The challenge is now to understand the differences between the (enemy) pathogenic and (friendly) beneficial bacteria in their pili-mediated adhesion strategies and interactions with the host. This knowledge is crucial in optimizing probiotics and targeting adhesion-based therapies for human health. The journey of pilus research in probiotics has begun with the prototype SpaCBA pili in *L. rhamnosus* GG. The ongoing and future research hopefully would shed light in this area.

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Author details

Vengadesan Krishnan[1*], Priyanka Chaurasia[1,2] and Abhiruchi Kant[1,2]

*Address all correspondence to: kvengadesan@rcb.res.in

1 Regional Centre for Biotechnology, NCR Biotech Science Cluster, Faridabad, India

2 Department of Biotechnology, Manipal University, Manipal, Karnataka, India

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