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Uptake of extracellular DNA: Competence induced pili in natural transformation of *Streptococcus pneumoniae*

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Transport of DNA across bacterial membranes involves complex DNA uptake systems. In Gram-positive bacteria, the DNA uptake machinery shares fundamental similarities with type IV pili and type II secretion systems. Although dedicated pilus structures, such as type IV pili in Gram-negative bacteria, are necessary for efficient DNA uptake, the role of similar structures in Gram-positive bacteria is just beginning to emerge. Recently two essentially very different pilus structures composed of the same major pilin protein ComGC were proposed to be involved in transformation of the Gram-positive bacterium *Streptococcus pneumoniae* – one is a long, thin, type IV pilus-like fiber with DNA binding capacity and the other one is a pilus structure that was thicker, much shorter and not able to bind DNA. Here we discuss how competence induced pili, either by pilus retraction or by a transient pilus-related opening in the cell wall, may mediate DNA uptake in *S. pneumoniae*.

Keywords:

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Introduction

Horizontal gene transfer is a major factor in the evolution of the bacterial kingdom. The ability of bacteria to internalize and incorporate DNA to alter their genomes allows them to adapt to different environments and hosts by acquisition of a variety of variable virulence and resistance traits.

In natural bacterial transformation, exogenous DNA released from donor bacteria is directly taken up from the environment by competent bacteria across their cell wall. The phenomenon of natural transformation was first described in the Gram-positive bacterium *Streptococcus pneumoniae* more than 80 years ago [1] and occurs in a number of different bacterial species including *Neisseria*, *Bacillus*, and *Staphylococcus* spp. [2, 3]. The ability of bacteria to take up DNA and to become transformed is referred to as competence. The competence state is highly regulated and reflects a window in which the proteins required for DNA binding, processing, and internalization are produced. While competence regulating circuits often are species specific, both Gram-positive and Gram-negative bacteria share fundamental similarities in the proteins required to constitute the transformation machinery. In most bacteria, these proteins are homologous of type II secretion (T2S) systems and type IV pili (T4P), suggesting that these systems have evolved from a common ancestor [4–6].

In Gram-negative bacteria, DNA has to pass three barriers: the outer membrane, the peptidoglycan cell wall and the inner membrane. In species such as *Neisseria* and *Haemophilus* DNA uptake is mediated by T4P, long filaments emanating from the surface of these bacteria. Although debated, it is widely believed that T4P bind to extracellular DNA and, by the action of a retraction ATPase, pull DNA into the periplasmic space. DNA then further translocates into the cytosol where homologous recombination can take place.

In Gram-positive bacteria a pseudopilus system was proposed as the responsible machinery for DNA uptake. The

pseudopilus was thought to act as a piston, moving up and down in the thick cell wall layer thereby binding to extracellular DNA and pulling it towards the cell membrane where DNA uptake proteins reside. In *Bacillus subtilis* biochemical data suggested that the major pseudopilin ComGC is able to assemble into a polymeric structure that is essential for transformation; however, no such structure had been visualized [7].

Recently, Laurenceau and co-workers described a type IV like pilus structure, with DNA binding capacity, on the surface of competent *S. pneumoniae* and proposed that this transformation pilus functions as primary DNA binding receptor [8]. Balaban et al. visualized a pilus structure that is rapidly released from the bacteria after induction of competence. It is much shorter than the previously described transformation pilus [8], and seems not to be involved in direct DNA binding [9]. Instead they demonstrated a correlation between transformation and pilus shedding, and proposed the name T2S system pilus.

In this review we discuss recent findings in the field of bacterial transformation in *S. pneumoniae* with specific focus on the two recently identified pilus structures and their role in the DNA uptake process of these bacteria. For reasons of simplicity, both pilus structures will be collectively referred to as competence induced pili. Based on common features between the pneumococcal DNA uptake machinery with T4P systems and T2S systems, we here address different models of DNA uptake with respect to existing concepts in *B. subtilis* transformation.

Common features of DNA uptake machines in Gram-positive bacteria

Competence systems in Gram-positive bacteria share many structural features with the closely related T4P systems and T2S systems [4, 10, 11] (Table 1). The key components in all of these systems are major and minor pilins, prepilin peptidase, and one or more ATPases. Although remarkably different in their functions, all three systems assemble filament structures composed of major pilin.

Type IV pili in Gram-negative bacteria

T4P are membrane anchored fibers found on many Gram-negative bacteria [12] and some Gram-positive bacteria [13]. They are involved in a vast array of different functions, including DNA uptake, adhesion, twitching and gliding motility, biofilm formation, and immune escape and therefore constitute important virulence factors in many pathogenic bacteria [14–16].

T4P are very thin (5–8 nm), long (>1 μm), and flexible fibers, which appear smooth and featureless by electron microscopy. Each fiber is composed of thousands of copies of major pilin subunits and individual fibers often laterally interact to form bundles (Fig. 1).

Dependent on the bacterial species, type IV pilus assembly requires 10 to 18 proteins that are encoded in smaller clusters scattered throughout the genome (type IVa pili) or found within one operon (type IVb pili). Among those proteins are a major pilin and minor pilins, a prepilin peptidase, an assembly ATPase, an integral inner membrane protein, an outer membrane secretin as well as inner membrane accessory proteins. Once the prepilins are processed by prepilin peptidase, it is believed that the assembly ATPase drives pilus polymerization assisted by the minor pilins and inner membrane accessory proteins [12]. The pilus is anchored in the cytoplasmic membrane and extends through the outer membrane in Gram-negative bacteria via an oligomeric channel composed of secretins. T4P systems also encode a retraction ATPase, which mediates depolymerization of the pilus into pilin monomers, thereby facilitating pilus retraction [17, 18].

DNA uptake mediated by T4P in Gram-negative bacteria such as *Neisseria spp.* and *Haemophilus influenzae* is homotypic, and requires specific sequence motifs; DUS (DNA uptake sequence) in *Neisseria spp.* [18, 19] and USS (uptake signal sequence) in *H. influenzae* [20–22], which is suggestive of a specific DNA receptor in these bacteria. For a long time it was thought that the major pilin subunit PilE was the responsible element for DNA binding, but no type IV pilin

Table 1. Proteins involved in competence systems of Gram-positive bacteria and their homologs in T4P systems and T2S systems

	Gram-negative bacteria		Gram-positive bacteria
	T4P system[a]	T2S system[b]	Competence system[c]
Major pilins/ pseudopilins	PilE	PulG	ComGC
Minor pilins/ pseudopilins	PilV, PilX, ComP	PulH, PulI, PulJ, PulK	ComGD, ComGE, ComGF[f], ComGG
Prepilin peptidase	PilD	PulO	ComC
Assembly ATPase	PilF	PulE	ComGA
Retraction ATPase	PilT, PilU	–	–
Secretin	PilQ	PulD	–
IM protein[d]	PilG	PulF	ComGB
IM accessory proteins[e]	PilM, PilN, PilO, PilP	PulL, PulM	–

[a]*Neisseria meningitidis.*
[b]*Klebsiella oxytoca.*
[c]*Streptococcus pneumoniae, Bacillus subtilis.*
[d]Inner membrane protein.
[e]Inner membrane accessory proteins.
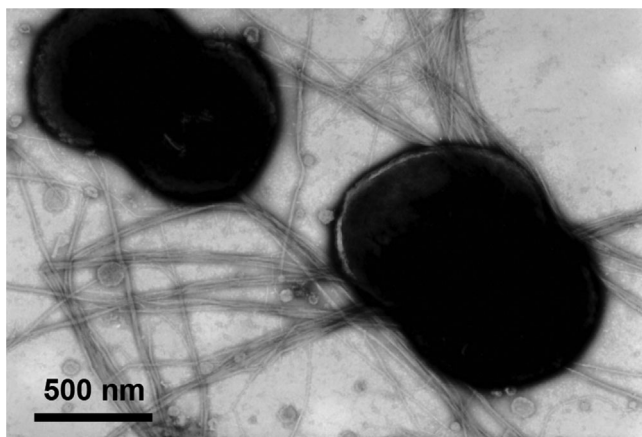[f]Not considered a minor pilin in *Bacillus subtilis.*

Figure 1. Type IV pili in *Neisseria meningitidis*. Negatively stained transmission electron micrograph showing the characteristic morphology of type IV pili (courtesy of V. Pelicic).

has ever been observed to directly bind DNA. Recently, it was shown in *Neisseria meningitidis* that the type IV pilus low abundance subunit protein ComP, belonging to the minor pilins, is responsible for DUS specific DNA binding. Through an electropositive stripe, presumably exposed on the surface of type IV pili, ComP can directly interact with DNA [23]. However, no ComP homologs were found in *H. influenzae*, hence arguing for a different DNA receptor in this bacterium.

Once DNA is captured, it is transported across the outer membrane through the secretin channel into the periplasm. ComE, a periplasmic DNA-binding protein, then delivers the DNA to the cytoplasmic membrane channel ComA. Here dsDNA is unwound by a helicase, and one strand enters the cytosol while the other strand is degraded into nucleotides.

Type II secretion systems and pseudopili in Gram-negative bacteria

Many different Gram-negative bacteria such as *Klebsiella spp.*, *Pseudomonas aeruginosa* and *Yersinia enterolytica* employ T2S systems to translocate bacterial proteins from the periplasm through the outer membrane to the bacterial surface or the extracellular environment [24].

T2S systems represent major virulence determinants, as they facilitate secretion of various bacterial toxins and hydrolases [25]. T2S requires up to 15 different genes encoding the major pseudopilin, prepilin peptidase, a secretion ATPase, associated membrane proteins, minor pseudopilins, and outer-membrane secretin [26]. In contrast to T4P systems, no retraction ATPase has been identified in T2S systems. Central to this secretion process is a putative pilus structure, named pseudopilus, a short periplasmic fiber, unlike the long, flexible type IV pilus. Based on similarities between T4P and T2S systems, the "piston model" was proposed in which the growing pseudopilus acts as a piston to push proteins through the secretin channel [10].

Periplasmic pseudopili are short and unstable under physiological conditions or in liquid cultured bacteria [26],

hence their exact structure and composition is poorly understood. However, under overexpression conditions of major pseudopilin it is possible to obtain surface exposed fibers. Sauvonnet et al. have shown that PulG, the major pseudopilin in *Klebsiella oxytoca* can assemble surface exposed fibers when expressed from a high copy number plasmid in *Escherichia coli* [27]. Similar observations were made by overexpressing the major pilins of *P. aeruginosa* [28] and *E. coli* [29]. This feature seems to be restricted to major pseudopilins, because minor pseudopilins fail to form fibers even when overproduced [30]. Electron and fluorescent microscopy studies of these surface fibers revealed pseudopili that are approximately 6 nm in width; flexible structures with bundling propensity, very similar to T4P [28, 31].

The DNA uptake machinery in *S. pneumoniae*

Pneumococcal competence is induced by Competence Stimulating Peptide (CSP), a peptide pheromone secreted by the bacteria, and which renders all bacteria within a population competent [32, 33]. Additionally, antibiotic stress can also induce transformability in pneumococci [34]. The ability to take up DNA is a transient state, referred to as "competence", occurring at the beginning of exponential growth and rapidly lost after 30 min of competence [35].

Transformation in *S. pneumoniae* is a complex process that requires important competence regulators, the proteins to assemble the transformation machinery, as well as proteins required for homologous recombination [36, 37]. For a complete transformation event to happen extracellular DNA binds to the cell surface. Both dsDNA and ssDNA can be taken up although ssDNA displays ~200-fold reduced transforming activity compared to dsDNA [38]. In order to pass the cell wall and the cytoplasmic membrane a dedicated translocation system comprising ComEA, EndA, ComEC, and ComFA is required (Table 2). Once in the cytoplasm, ssDNA interacts with numerous soluble proteins including SsbB, DprA, RecA and DpnA (Table 2).

In addition, the genes of the *comG* operon are necessary for transformation [8, 9]. The *comG* operon is expressed only in competent bacteria [39], and consists of seven genes in *S. pneumoniae*, namely *comGA, comGB, comGC, comGD, comGE, comGF*, and *comGG*. The genetic organization and composition is highly similar to T2S systems and type IV pilus assembly systems in Gram-negative bacteria, suggesting that *S. pneumoniae* employs structural and functional homologs of both systems for DNA uptake [37]. In *B. subtilis*, the *comG* operon was demonstrated to be required for DNA binding and uptake [40, 41] and to assemble a pseudopilus, the structure and function of which remains unknown [7].

ComGA is an ATPase required for assembly of competence induced pili

The first gene in the *comG* operon encodes the putative secretion ATPase ComGA. ComGA belongs to the family of

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Table 2. Key components of DNA uptake systems in *S. pneumoniae* and homologs in *B. subtilis*

Protein	Function	Reference
ComEA	Membrane associated protein with helix loop helix DNA binding motif (dsDNA receptor)	[42–44]
ComEC	Membrane channel	[45, 46]
ComFA	ATP dependent translocase involved in ssDNA uptake	[47–50]
EndA	Endonuclease localizes to midcell and degrades dsDNA in *Streptococcus pneumoniae*, not identified in *Bacillus subtilis*	[51, 52]
SsbB	Single strand binding protein B protects and stabilizes internalized ssDNA	[39, 53–56]
DprA	DNA processing protein A mediates RecA loading onto internalized ssDNA	[38, 53, 57–60]
RecA	Recombinase A binds to ssDNA and mediates homologous recombination	[53, 57, 58]
DpnA	ssDNA methylase	[61–64]

AAA+ ATPases, which are powerful molecular engines involved in a broad range of different cellular processes including protein degradation, DNA replication, and cell division [65]. Homologs of ComGA function as assembly and retraction ATPase in type IV pilus assembly and are also found in T2S [12, 26].

In *B. subtilis*, ComGA is absolutely required for initial DNA binding to the cell surface as *comGA* point mutants are unable to bind to extracellular DNA [66]. Inactivation of *comGA* in *S. pneumoniae* also abolished DNA binding and further translocation [46], suggesting that ComGA is required early during transformation. In addition, ComGA is essential for the assembly of competence induced pili in *S. pneumoniae* [8, 9] and pseudopili in *B. subtilis* [7].

ComGB is a polytopic membrane protein

The second gene in the *comG* locus encodes the integral membrane protein, ComGB. In *K. oxytoca* in silico analysis suggests that polymerized pseudopilus is located at the inner membrane core protein pullulanase secretion protein PulF and is homologous to ComGB [67].

ComGC and visible structures of competence pili

Recently two studies have shown that *S. pneumoniae* can assemble competence induced pili, but very different structures were reported [8, 9] (Fig. 2). Laurenceau et al. showed that ComGC can form long type IV like pilus structures, 2–3 μm in length and 5 nm in width, that extend from the bacterial surface [8]. Using a ComGC-Flag expressing strain the authors confirmed by immunogold labelling and mass spectrometry that the observed filaments contain ComGC. In contrast, Balaban et al. have observed by electron microscopy that induction of competence leads to the formation of short, apparently plaited pseudopilus-like structures that are absent in a ComGC mutant strain. These structures are 8–10 nm in diameter and on average ~100 nm in length. They further demonstrated that *E. coli* encoding the T2S system structural elements of *K. oxytoca* can produce similar plaited structures when *pulE-K* was exchanged to respective homologs of the *comG* operon present in *S. pneumoniae* [9].

Despite lacking direct experimental proof that short, plaited structures are in fact composed of ComGC, no micrometer-long filaments were ever seen on the surface of competent bacteria. One reason for that might be that only a small proportion of bacteria show these long filaments and that these structures easily break into smaller fragments during sample preparation. Likewise, the plaited structures are fairly short, and might have been overlooked by others due to their low abundance. As different pneumococcal strains were used, in the respective studies, the abundance of each type of structure could differ. Both structures in their disparity might still be biologically relevant. Interestingly, competence pili of different lengths (ranging from a few hundred nm up to several μm) were visualized in *Vibrio cholerae* [68]. In the absence of more information it is difficult to speculate why both these structures could be assembled from the same *comG* operon.

It was further found that pneumococcal ComGC is present in the medium after competence induction that was not a result of bacterial lysis [9]. Furthermore, no active shearing forces were applied in the experimental procedure, suggesting that ComGC is released from the bacteria. In fact, when the amount of ComGC in supernatants from sheared and non-
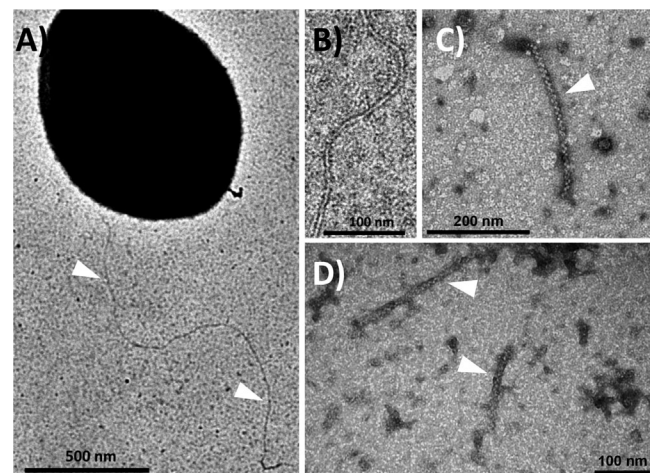

Figure 2. Competence induced pili in *S. pneumoniae*. **A**: EM of a long type IV like pilus (white triangle) emanating from the surface of competent *S. pneumoniae* R6 (adapted from [8], reprint from www.plospathogen.org). **B**: Transformation pilus at higher magnification (adapted from [8], reprint from www.plospathogen.org). **C**, **D**: Short, plaited pili (white triangle) released from competent pneumococci strain T4R.

sheared cultures was compared, no differences were observed by immunoblotting (unpublished). In the study by Laurenceau et al., mechanical pressure is applied in order to obtain pilus enriched supernatants [8]. In *B. subtilis*, the major pseudopilin ComGC can be found in membrane fractions or released in protoplast supernatants after lysozyme treatment [7, 40]. Notably, in *V. cholerae* many free-floating competence induced pili were observed and it was suggested that shearing occurs rapidly due to the fragility of the pilus [68]. It remains to be shown whether pneumococcal pilus shedding is indeed a form of active release or in fact a result of pilus fragility.

The function of competence pili and ComGC was also investigated in the two papers [8, 9]. Laurenceau et al. showed that the long type IV pilus structures bound directly to DNA [8]. As DNA and T4P are generally "sticky" molecules [69] it might need further investigation whether this binding is specific or a result of non-specific association of DNA with the pilus. While the pilus surface as a result of globular packing of major pilin monomers might possess some DNA binding capacity, individual monomers may not bind to DNA. Indeed, the soluble C-terminal part of pneumococcal ComGC, when recombinantly expressed and purified, was not found to bind to DNA in electromobility shift assays [9]. However, one also needs to be cautious in interpreting these results, as rapid dissociation during electrophoresis can prevent detection of complexes in these assays [70]. Clearly more experimental evidence is needed to address whether competence pili in fact are DNA-binding or not.

Minor pilins ComGD, ComGE, ComGF and ComGG are required for transformation

comGD, *comGE*, *comGF*, and *comGG* encode a set of minor pilin proteins, whose role is not well defined. Minor pilins are variable in size and overall hydrophobic. They are made as prepilins with short signal sequences which are recognized and cleaved by prepilin peptidase. All minor pilins share hydrophobic N-terminal domains, highly similar to major pilins, whereas their C-termini vary. In *S. pneumoniae*, mutants deficient in ComGD, ComGE, ComGF, and ComGG are non-transformable [9].

Evidence mainly coming from Gram-negative bacteria suggests that minor pilins are involved in priming pilus assembly [71] and in controlling fiber length [29, 30]. Studies of T4P in *P. aeruginosa* and *Neisseria gonorrhoeae* further demonstrate that minor pilins of these bacteria are incorporated into T4P as shown by immunoblotting and immuno-EM [72, 73]. However, it remains unclear whether minor pilins are part of competence induced pili in pneumococci. At least one minor pilin, pneumococcal comGG, was absent from pilus fractions when analyzed by immunoblotting [9]. Similarly, mass-spectrometry analysis of purified pneumococcal type IV like pili only revealed the presence of major pilin ComGC [8].

B. subtilis encodes three minor pilins, ComGD, ComGE, and ComGG. ComGF is not considered a classical minor pilin due to the lack of sequence similarity to other pilin-like proteins. All minor pilins are required for transformation and the assembly of pseudopili [7]. Recently, the minor pilins

ComGD, ComGE, and ComGG were shown to directly interact with each other and at least one minor pilin, comGG, requires direct association with other minor pilins for processing by prepilin peptidase [74].

Competence induced pili and possible mechanisms of DNA uptake in *S. pneumoniae*

Based on the recent findings and the observed differences in morphology and function of competence induced pili in *S. pneumoniae* [8, 9] two different models of DNA uptake have been proposed, which will be discussed below.

Retraction or trap model

Laurenceau and co-workers found that *S. pneumoniae* can assemble micrometer- long type IV like pilus structures capable of binding extracellular DNA, which led them to propose the retraction model [8]. In this model, the transformation pilus would directly capture DNA, and with the help of a retraction ATPase, pull the DNA through the cell wall to hand it over to ComEA in the cytoplasmic membrane (Fig. 3). While this is an appealing idea, no retraction ATPase has yet been identified in Gram-positive bacteria. So far, the secretion ATPase ComGA is the only protein known to drive competence pilus assembly and it seems unlikely that ComGA would function in both pilus polymerization and depolymerization, although this cannot be ruled out. In Gram-negative bacteria, there are examples of transformation systems that lack a PilT retraction ATPase or evidence of retraction, notably those of *H. influenzae* and non-typeable *H. influenzae* [75]. Interestingly, the former lacks visible pili while the latter is associated with T4P [76, 77]. Also, in *P. stutzeri* that utilize type IV pili and PilT, a transformation-deficient *pilT* suppressor mutant was isolated which enabled DNA-uptake when the major pilin PilA was expressed as 6x-His tagged protein. Hexahistidine tagged PilA does not form detectable pili but supports transformability independent of functional PilT [78].

Interestingly, in *N. gonorrhoeae* the speed of type IV pilus retraction (1–2 μm/second) is much faster than the speed of DNA uptake measured in *B. subtilis* (80 bp/sec which corresponds to 48 nm/second), suggesting that DNA uptake in Gram-positive bacteria is likely not dependent on pilus retraction [79–81]. In the absence of a retraction ATPase in *S. pneumoniae* the flexible transformation pilus may simply function as an excellent DNA trap to bring DNA close to cell surface receptors, as recently proposed by the Dubnau laboratory [74].

Hole in the wall model

Recent work published by our group presented a visualization of short, plaited pilus structures formed on the bacterial cell surface during competence, and rapidly released from the bacteria [9]. Pilus shedding directly correlated with
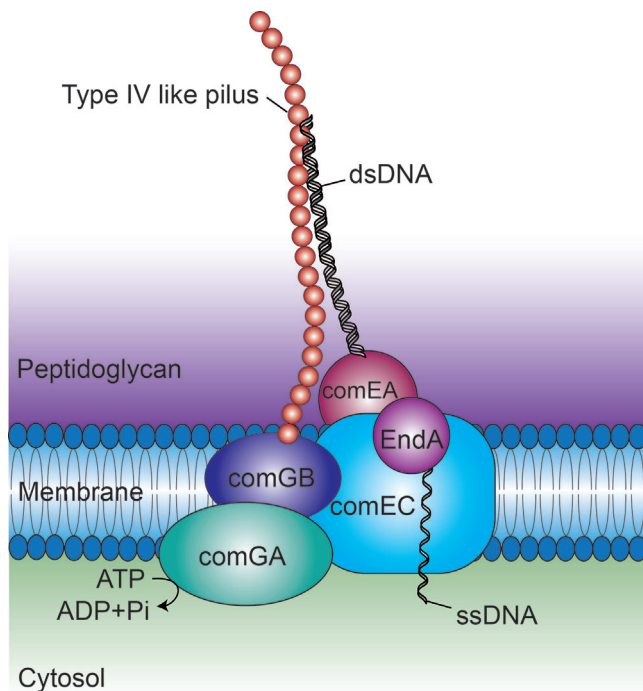
Figure 3. Retraction or trap model showing key components of the DNA uptake system in *S. pneumoniae*. A long type IV like transformation pilus is assembled on the surface of bacteria and directly binds exogenous DNA. Captured DNA is pulled through the cell wall by an unknown retraction ATPase (retraction model) to facilitate access of DNA to the DNA receptor ComEA and the transmembrane channel protein ComEC. Alternatively, the flexible pilus directly brings captured DNA in close proximity to cell surface receptors independent of a retraction ATPase (trap model). DsDNA is then cleaved by the EndA nuclease and ssDNA enters the cytoplasm through the ComEC pore.

transformability, which led to the proposal of the "Hole in the wall" model (Fig. 4). According to this model, the pilus functions as a molecular drill to create a transient opening in the cell wall, which would allow DNA to access ComEA and ComEC. The observation that pilus release correlates with transformability suggests that these two events may be linked. Indeed, *comGC* point mutants, with normal expression and processing of ComGC, were found that exhibited no release of ComGC into the supernatant. Such mutants were non-transformable. Also, experiments using different clinical isolates showed that shedding and transformation are tightly linked, although different dynamics were observed among the different strains tested.

Enriched short pili as well as recombinant purified ComGC lacking the first 39 amino acids showed no DNA binding activity in an electromobility shift assay. This limited experimental evidence, which needs to be substantiated, suggests that DNA uptake may not involve direct pilus binding to DNA. If so, pilus retraction is not a requirement for DNA uptake, which could explain the lack of a retraction ATPase in Gram-positive bacteria.

Given the high degree of structural and functional relatedness of T4P and T2S systems with the pneumococcal *comG* operon, it seems not surprising that pneumococci can

assemble type IV like transformation pili. Interestingly, T2S systems also assemble surface exposed pili, similar to T4P, but only upon overproduction of the major pseudopilin [27–30]. Such structures are also referred to as hyperpseudopili (HPP), and are regarded as artificially extended periplasmic filaments [9, 82]. The exact nature of true periplasmic pseudopili and their structure is not known. As the unusual, plaited structures observed in the study by Balaban et al. shared no resemblance with long, smooth T4P, the term "T2S system pili" was proposed.

T4P and HPP are helical polymers made of major (pseudo) pilin subunits [82–84]. Notably, structural flexibility and heterogeneity has been reported in different pilus models, highlighting the possibility of different subunit arrangements and symmetries within the same fiber [82, 85]. Plaited competence pili have twice the width compared to the long type IV like competence pili, suggesting that if both structures are of similar helical orientation plaited competence pili may be differently packed compared with type IV like competence pili. Such a thicker helical structure would be much more robust when traversing the cell wall and creating a transient opening for DNA to enter.

In *B. subtilis* competence pili have never been visualized. Based on biochemical data estimated 40–100 ComGC monomers assemble into a pseudopilus, with each monomer contributing ~1 nm to the length of the fiber [7]. A structure of 40–100 nm would be sufficient to span the cell wall. Thus, competence pseudopili are believed to be much shorter compared to T4P in Gram-negative bacteria. The structures observed by Balaban et al. [9] are on average 100 nm, thus similar to the pseudopili described in *B. subtilis*.

The key question in this model is how DNA fragments of several hundred nanometers find their way through a 10 nm wide hole in the cell wall. One hypothesis is that electrostatic interactions of the peptidoglycan side chains create a charged environment that facilitates binding of DNA to the transient opening in the cell wall. Another hypothesis would be that exogenous DNA, by a yet to be identified protein(s), can be ushered through the peptidoglycan cell wall. In Gram-negative bacteria the pseudopilus mechanically pushes exotoxins through the cell wall and outer membrane, which thereby facilitates toxin secretion. The pneumococcal T2S system pilus behaves similar to the pseudopilus and perhaps a DNA binding protein is pushed to the cell surface in this way. A good candidate for such a protein would be a minor pilin that could be brought to the cell surface or perhaps line the transient channel through the peptidoglycan layers. Such a DNA binding protein may conceivably even be a non- pilin protein.

Competence induced pili assemble despite physical hindrance

In Gram-positive bacteria, DNA needs to pass through the thick cell wall (~70–80 nm), a physical barrier composed of many peptidoglycan layers. With its small pore size (2 nm) peptidoglycan is freely permeable to proteins up to 25 kDa [86]. However it is difficult to envision how
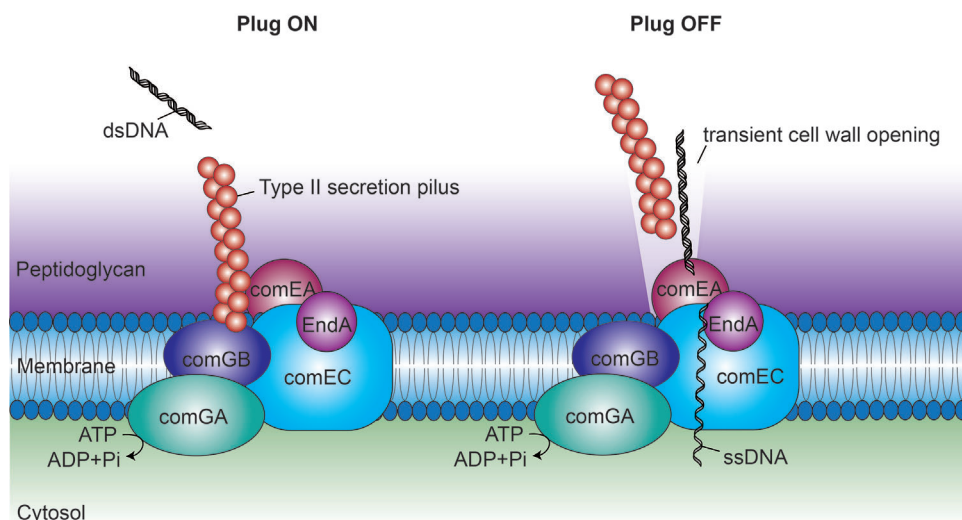
Figure 4. Hole in the wall model. Upon competence induction a short and rigid pilus structure is assembled at the membrane protein ComGB with energy provided by the ComGA ATPase (Plug ON). The pilus is then rapidly shed from the bacteria, creating an opening through the peptidoglycan cell wall (Plug OFF). This channel facilitates access of exogenous DNA to the DNA receptor ComEA and the transmembrane channel protein ComEC. DsDNA is then cleaved by the endonuclease EndA and ssDNA enters the cytoplasm through the comEC pore.

competence induced pili can assemble without local cell wall restructuring.

In Gram-negative bacteria, which do have a thinner peptidoglycan layer, many trans- envelope macromolecular structures such as type III secretion systems, T4P systems or flagellar systems, employ specialized peptidoglycan degrading enzymes in order to create sufficiently large gaps within the peptidoglycan layer to accommodate these structures [87, 88]. Among these enzymes are specialized lytic transglycosylases, amidases, or peptidases that modify the peptidoglycan meshwork to allow efficient assembly and anchoring of these multiprotein complexes in the cell envelope. In *S. pneumoniae* two lytic enzymes, namely LytA and CbpD, are specifically induced during competence [39]. LytA and CbpD, together with constitutively expressed LytC, a peptidoglycan hydrolase [89], were shown to contribute to competence induced fratricide by lysing non-competent sister cells [90, 91]. However, competent cells are immune against the action of these enzymes, by virtue of expressing ComM immunity protein [92]. Whether other cell wall hydrolytic enzymes are involved in local peptidoglycan rearrangements during pilus assembly remains to be investigated.

In pneumococci, transformation takes place at mid cell, during which EndA is specifically recruited to this area during competence [51]. At mid cell septal wall synthesis also takes place, and peptidoglycan integrity is maintained by the coordinated activities of lytic transglycosylases, N-acetyl-muramyl-L-alanine amidases and penicillin-binding proteins. Thus, this area may be particularly prone to structural rearrangements that would allow the assembly of competence induced pili. Interestingly, induction of competence in

S. pneumoniae leads to a transient growth arrest, which suggests a link between cell division and DNA uptake [55, 93]. By delaying cell division, bacteria may ensure efficient assembly and prevent premature disassembly of the DNA uptake machinery, thereby maximizing transformation efficiency.

In competent *B. subtilis* DNA replication is paused and cell division is inhibited [94–96]. However, transformation in *B. subtilis* takes place at cell poles, suggesting that perhaps other mechanisms are involved that govern pilus assembly through the peptidoglycan cell wall in Gram-positive bacteria. Indeed, also peptidoglycan binding proteins have been found to contribute to T4P assembly [97] and formation of T2S systems [98]. Recently TsaP, a peptidoglycan binding protein in *N. gonorrhoeae*, has been shown to anchor the secretin complex to peptidoglycan. Mutants deficient in TsaP produced T4P but were unable to pass the outer membrane suggesting that TsaP is required for efficient T4P assembly [99]. Similar proteins may be needed in *S. pneumoniae* transformation.

Conclusion and outlook

The work by Laurenceau and co-workers [8] and Balaban et al. [9] started to shed light on the possible mechanisms that govern DNA uptake in pneumococci. Both authors describe a polymeric structure composed of ComGC, albeit with different morphology and function. Laurenceau et al. suggest that type IV pilus-like structures directly mediate DNA uptake in pneumococci; Balaban et al. propose that DNA uptake is independent of DNA binding to the competence pilus. Instead, the pneumococcal competence pilus is suggested to generate a transient opening in the thick cell wall to give way to incoming DNA. Although not easily reconcilable into a single schema, both models might prove to be biologically relevant.

Clearly, further studies are needed to resolve the observed differences. Essential next steps involve more detailed biochemical characterizations of both polymeric structures. In particular, unambiguous confirmation that short pili are composed of ComGC is needed. Furthermore, the presence of

one, or both observed structures needs to be explored in different pneumococcal strains. Similarly to what has been done for many T4P and HPP, determining the structure of ComGC by X-ray crystallography and/or nuclear magnetic resonance will also help in understanding its function. Structural information on individual ComGC monomers might yield evidence for different subunit arrangements that could explain the different pilus fibers observed.

Moreover, little is known about the function of pneumococcal minor pilins and their presumed role in the initiation of pilus assembly. All minor pilins in *S. pneumoniae* are required for transformation, and investigating their functions will be pivotal to understand how DNA can translocate through the cell wall layers in competent bacteria.

In conclusion, further unravelling the molecular mechanisms that lead to pilus retraction and/or a transient opening in the cell wall will provide important fundamental insights into how DNA uptake in pneumococci takes place.

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