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DNA TRANSPORT DURING TRANSFORMATION

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1. ABSTRACT

The ability to take up exogenous DNA is a requisite for genetic competence and transformation. Here we review the process of DNA transport in competent bacteria. Two kinds of machineries have been described. In the first one, the components show similarities to the proteins involved in biogenesis of type IV pili and type II secretion systems. The second one is related to the type IV secretion system and conjugation apparatus.

2. INTRODUCTION

The term genetic transformation refers to the stable acquisition of exogenous DNA by an organism. This process was first described in pneumococci by Griffith (1), and his observations eventually led to the identification of DNA as a genetic material (2). Many bacterial species are naturally able to undergo transformation, and this ability is called competence. Competence has been most extensively studied in the Gram-positive organisms *Bacillus subtilis* and *Streptococcus pneumoniae*; in the Gram-negatives bacteria *Neisseria gonorrhoeae* and *N. meningitidis*, *Haemophilus influenzae* and, more recently, in *Helicobacter pylori.* Transformation can provide bacteria with new genetic information, and indeed high levels of horizontal transfer and recombination do occur within populations of naturally competent organisms, such as pathogenic *Neisseria* species (3, 4), *H. influenzae* (5), *H. pylori* (6) and *Campylobacter jejuni* (7).

The process of transformation comprises several events: the first step is binding of donor DNA to the surface of the bacteria, followed by fragmentation of the DNA molecule to create free ends. These two initial events generate the substrate for uptake, which is defined as the conversion of exogenous DNA to a DNase-resistant state. DNA uptake has distinct meanings for Gram-positive and Gram-negative organisms, due to the presence of an outer membrane in the latter. In Gram-positives, after binding and fragmentation, a single strand of DNA is translocated across the cytoplasmic membrane and achieves resistance to exogenously added DNase; in this review, this will be defined as “transport”. Uptake and transport are synonymous in Gram-positive systems. In Gram-negative organisms, on the other hand, double-stranded DNA crosses the outer membrane and achieves protection from DNase, which is operationally defined as uptake. After uptake, the DNA follows the Gram-positive path, being transported across the inner membrane as a single-stranded

molecule. In both systems, the non-transported strand is degraded to acid-soluble products, which are released into the medium or periplasm. Finally, once in the cytoplasm, the acquired DNA can integrate into the bacterial chromosome.

There is a considerable body of literature on DNA uptake/transport during genetic transformation. However, it pertains mainly to the identification of the factors involved in the process; much less is known about the specific roles they play and the biochemistry involved. Two kinds of machineries have been described: the first one is related to type II secretion systems and type IV pili, and is present in most known competent bacteria. The second has been recently described in *H. pylori* and it shows similarities to type IV secretion and conjugation machineries.

3. DNA UPTAKE MACHINERY RELATED TO TYPE II SECRETION PROTEINS AND TYPE IV PILI

This is the most studied and probably more widespread kind of DNA acquisition machinery, being present both in Gram-positive and Gram-negative organisms. It comprises a common set of elements involved in DNA uptake/transport, with significant sequence similarity, indicating the common evolutionary origin of the apparatus. The sequence conservation also allows the attribution of putative functions when new competence proteins are first identified. However, one must bear in mind that the competence systems from different organisms have diverged, and orthologs may have acquired distinct characteristics in the process.

Some of the competence proteins in this kind of system show similarities to proteins involved in the biogenesis or function of type IV pili and to components of the type II secretion system. These proteins have been termed PSTC proteins, for **P**ilus, **S**ecretion, **T**wiching motitlity and **C**ompetence (8). Type IV pili (Tfp) have been extensively studied in *P. aeruginosa* and *N. gonorrhoeae* (for reviews, see (9, 10)). They are long polar filaments that protrude from the surface of many Gram-negative bacteria, with a diameter of around 6nm. Tfp are involved in adherence to host cells and in twitching motility, and may also serve as bacteriophage receptors. The type II secretion system, also called the main terminal branch of the general secretion pathway (11), functions in the transport of folded substrates from the periplasm across the outer membrane of Gram-negative bacteria. The prototype is the pullulanase export system from *Klebsiella oxytoca*, and other well characterized examples are the extracellular enzyme and toxin secretion systems in *P. aeruginosa* and *Vibrio cholerae*, all of which have been reviewed elsewhere (12-15).

3.1. Tfp and DNA uptake/transport

The correlation between Tfp and competence was first observed in *N. gonorrhoeae*. Piliation in neisseriae is subjected to phase and antigenic variation, and it was observed that non-piliated variants were not competent (16). Furthermore, different variants of pilin, the structural subunit of Tfp, can influence competence levels (17, 18), and mutations in various genes involved in pilus assembly and biogenesis impair the ability to take up DNA (19-24). Piliation and competence are also correlated in *Dichelobacter nodosus* (25), *Synechocystis* sp. (26) and *P. stutzeri* (27).

Although the association of competence with piliation is well established, the relationship between these two phenotypes is not clearly understood. Does the type IV pilus itself participate in DNA uptake? Based on the structure of their respective pilins, models for the organization of the pilus fiber in *N. gonorrhoeae* and *P. aeruginosa* have been proposed (28, 29). In both cases, there is a helical fiber with 5 pilin subunits per turn, and the structure is held together by hydrophobic interactions among the N-terminal regions of the subunits, which form the core of the fiber. The model features a hole with 12Å of diameter in the middle of the filament, partially filled with the hydrophobic side chains from the residues in the pilin N-terminal domains (29). The dimension and the hydrophobic nature of this central cavity would make passage of DNA through it very unlikely.

It has been demonstrated that the presence of pili is not actually a requirement for competence in *N. gonorrhoeae*, although small amounts of pilin are necessary for transformation (19, 30). Finally, it should be considered that *H. influenzae, B. subtilis* and *S. pneumoniae* do not present type IV pili, although PTSC proteins are required for competence in these organisms. In fact, the evidence that proteins of this class are required for transformation was first obtained in *B. subtilis* (31). These observations suggest that the PTSC proteins are necessary for the formation of a structure involved in the translocation of DNA, that might show some structural resemblance to the pilus. This would be analogous to the proposed "pseudopilus" in type II secretion systems (11), which also involve proteins from the PTSC group.

There is no uniform nomenclature for the proteins involved in competence, particularly the PTSC proteins. To avoid confusion, we will adopt as prototypes the most studied organisms regarding competence, *B. subtilis* for the Gram-positives and *N. gonorrhoeae* for the Gram-negatives, but the other competent organisms will be considered as well. We will first present the features and components which are exclusive to the Gram-negative system, and then those which are common to both Gram-positive and Gram-negative bacteria.

3.2. DNA binding, fragmentation and uptake in Gram-negative organisms

3.2.1. Sequence specificity requirements

DNA uptake in *N. gonorrhoeae* and *H. influenzae* requires the presence of a short sequence motif (termed DUS, for DNA uptake sequence), in contrast to the Gram-positive organisms *B. subtilis* and *S. pneumoniae*, which can take up any kind of DNA. The neisserial DUS is 5'-GGCGTCTGAA-3' (32, 33), and the DUS of *H. influenzae* is 5'-AAGTGCGGTCA-3' (34, 35). *Actinobacillus actinomycetemcomitans*, the major causative agent of

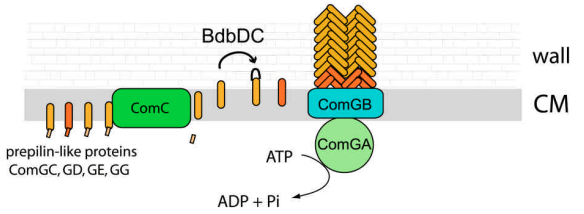

Figure 1. Model for the biogenesis of the DNA binding/transport machinery in *Bacillus subtilis*. Prepilin-like proteins ComGC, GD, GE and GG are processed by prepilin peptidase ComC, which cleaves the short leader peptides at their N-terminus. The mature ComGC is translocated across the membrane, and its two cysteine residues are oxidized by BdbDC, forming an intramolecular disulfide bridge. ComGC forms a polymeric complex which traverses the cell wall and allows the DNA-binding protein ComEA to access exogenous DNA. ComGA (a traffic NTPase), ComGB (a polytopic membrane protein) and the other pilin-like proteins are necessary for the complex formation, and it is possible that the minor pilin-like proteins may have structural roles. The major pilin-like protein (ComGC) is represented in orange and the minor pilin-like proteins in red.

periodontitis and a close relative of *H. influenzae*, requires the same DUS as the latter for DNA uptake (36). The genomes of *N. meningitidis* (very closely related to *N. gonorrhoeae*) and *H. influenzae* are each enriched in their respective DUS (5, 37), which explains the preferential uptake of homospecific DNA. The presence of DUS receptors on the surface of these organisms is postulated, since they should recognize, bind and effectively select the DNA molecule for uptake, but the identification of these receptors remains elusive. However, specific sequence requirement for uptake is not an absolute rule in Gram-negative organisms, since there is apparently no such specificity in *Acinetobacter* sp. (38), *Legionella pneumophila* (39), and probably *P. stutzeri* (27).

In *N. gonorrhoeae*, the initial binding of DNA to the cell surface was found to be non-specific (40), and various elements on the surface, including LPS and proteins, could contribute to this interaction. In fact, outer membrane opacity proteins (Opa) on the neisserial surface were shown to bind DNA, presumably by electrostatic interaction, and their presence can increase transformation frequencies (41). This non-specific binding should be followed by recognition of the DUS by its specific receptor, leading to DNA uptake.

Efficient DNA binding to the *H. influenzae* cell surface seems to require the presence of a DUS, although some degree of low affinity, non-specific binding of DNA could be observed in non-competent cells (reviewed in (42)). Another singular feature of this organism is the presence of membrane vesicles on the surface of competent cells, which have been associated with DNA uptake and termed “transformasomes” (43). DNase protection would be achieved by entry of double stranded DNA (either circular or linear molecules) into these structures. It should be noted that such structures have not been observed in other competent organisms, and there has been no follow-up work on this subject.

3.2.2. DNA fragmentation

Random cleavage of plasmid DNA occurs in *N. gonorrhoeae* (44), producing double-stranded linear molecules which serve as substrate for uptake. This observation suggests the presence of a non-specific endonuclease on the surface of the bacteria. Whether the endonucleolytic activity is dependent on previous recognition of the DUS is not known. In *H. influenzae*, there is a requirement of a free terminus in the DNA molecule for translocation into the cytosolic compartment (45).

3.2.3. Crossing the outer membrane: secretins

Secretins are outer membrane proteins involved in transformation, Tfp biogenesis, type II and type III secretion systems, and filamentous phage extrusion (46). Some secretins require the presence of an outer membrane lipoprotein, called the pilot protein, for their stability and correct insertion into the outer membrane (47, 23). In *N. gonorrhoeae*, the secretin PilQ and its pilot PilP are necessary both for piliation and for DNA uptake (20, 23). Secretins are also involved in piliation and DNA uptake in *Thermus thermophilus* (48) and *Synechocytis* sp. (26). There is a secretin ortholog necessary for transformation in *H. influenzae* (ComE) (49), though this organism lacks Tfp.

Similarly to other member of this family, neisserial PilQ forms a ring-like structure, composed of 12 units, with a central cavity of 6.5nm, which could accommodate the pilus fiber (50). Studies on PulD, a secretin from the *K. oxytoca* pullulanase export system, show that the C-terminal domain, conserved among the secretin orthologs, is embedded in the outer membrane, while the N-terminal region seems to extend towards the periplasmic space and fold back into the cavity of the ring, occluding the channel (51, 52). The dimensions of the PulD multimeric ring would allow the complex to span the outer membrane, with the N-terminal region almost touching the cytoplasmic membrane, where it could interact with other components of the type II secretion apparatus (51).

It has been shown that protein pIV, the secretin of the f1 filamentous phage, forms an aqueous channel (53), which is indeed the conduit for the extrusion of the phage (54). Such a large channel must be gated to preserve outer membrane integrity, and substrates for export (the proteins secreted by type II secretion systems, the pilus fiber or the phage) must be recognized. It has been suggested that the protrusion force from the pilus fiber itself could be enough to open the PilQ channel, since *N. gonorrhoeae* mutants in both *pilQ* and *pilT* (see below) form ingrown pili in their periplasm that deform the outer membrane (55). Alternatively, the N-terminal region of PilQ that plugs the channel might recognize the substrate, causing the channel to open. In fact, there is some evidence implicating the N-terminal region of secretins in the determination of substrate specificity (56, 57).

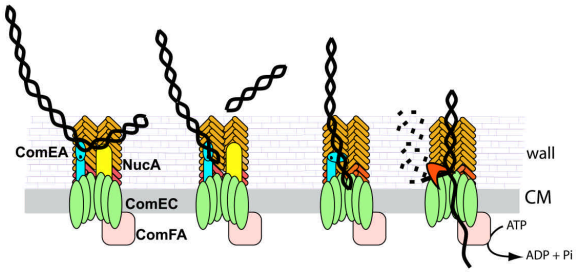

Figure 2. Model for DNA uptake in *B. subtilis*. Exogenous DNA binds to its receptor, ComEA, and perhaps to other protein(s) on the bacterial surface. The DNA undergoes endonucleolytic cleavage by NucA, leading to formation of new free termini. ComEA undergoes a conformational change, perhaps by bending at its hinge region, and delivers the DNA molecule to the channel formed by ComEC, which traverses the cytoplasmic membrane. The translocation of the DNA molecule to the cytosol as a single-stranded molecule is catalyzed by ComFA. The non-transported strand is degraded to acid soluble products by an unidentified nuclease. The major and minor pilin-like proteins are color coded as in Figure 1.

During uptake, double-stranded DNA may pass across the outer membrane through a channel formed by secretin. The diameter of the channel is certainly sufficient to accommodate the incoming DNA. However, nothing is known about DNA recognition and channel gating. There is no evidence that secretins involved in competence can bind DNA. Possibly, DNA binds to a receptor on the cell surface (the DUS receptors in *N. gonorrhoeae* and *H. influenzae*?), triggering the opening of the channel, either by direct interaction with the secretin or by signaling components within the periplasm, which in turn would open the channel from inside. A second DNA-binding protein could mediate passage through the secretin channel (see section 3.3). Until the DNA receptors on the surface are characterized, the gating mechanism will remain obscure.

3.3. DNA-binding: *Bacillus subtilis* ComEA and its orthologs

In competent *B. subtilis*, the proteins encoded by *comEA*, *comC* and the *comG* operon are necessary for DNA binding to the bacterial surface (58). ComEA has been shown to bind DNA (59), while ComC and the ComG proteins seem to be necessary for ComEA to gain access to exogenous DNA (see section 3.5). This led to the conclusion that ComEA is the DNA receptor for transformation in *B. subtilis*. ComEA is an integral membrane protein, with a transmembrane segment in its N-terminal region, while the C-terminal portion is located outside of the membrane (58). The DNA-binding activity was localized to its C-terminal domain (59), which contains a helix-hairpin-helix motif, predicted to bind DNA without sequence specificity (60).

ComEA seems to participate in DNA transport as well, since an in-frame deletion in the gene abolished uptake, but binding activity was retained (61). This deletion included a flexible stretch in the protein (QQGGGG), located immediately before the DNA-binding domain, which could bend and deliver the DNA to the entrance of the channel on the cytoplasmic membrane (see below and Figure 2).

Orthologs of *comEA* can be recognized in several bacterial species. In *S. pneumoniae*, the role of ComEA and other competence proteins in DNA-binding has been closely examined, and it has been suggested that there is an initial attachment of DNA to the surface, dependent on ComGA, which is followed by deep binding to ComEA (62). This model is not incompatible with the *B. subtilis* data, where residual binding can be observed in the absence of ComEA (61), suggesting that other DNA binding protein(s) may indeed be present on the surface of competent cells.

There is an ortholog of *B. subtilis* ComEA in *N. gonorrhoeae*, named ComE, which was found to be necessary for DNA uptake (63). However, neisserial ComE corresponds to a truncated version of *B. subtilis* ComEA, containing solely the DNA-binding domain in the C-terminus of the latter. Furthermore, ComE has a cleavable signal peptide, localizes to the periplasm and binds DNA without sequence specificity. These data argue against the role of ComE in the initial binding of DNA to the cell surface in neisseriae or in DUS recognition, and suggest that ComE may bind to DNA which has already been selected for DUS presence and committed to uptake. The exact role of ComE in DNA uptake remains to be characterized, but one could speculate that ComE would have the same function as ComEA in *B. subtilis*: to bind DNA (without sequence specificity) and direct it to the cytoplasmic membrane channel. The fact that ComE is soluble, in contrast to the membrane localization of ComEA, may indicate that the former protein shuttles between the secretin and the inner membrane channels. There is a similar ORF in *H. influenzae* genome (hypothetical protein HI1008), which also contains only the DNA-binding domain, but its role in competence has not yet been verified.

A ComEA ortholog required for competence was identified in the gram-negative *Thermus thermophilus* (64). The protein has a molecular size equivalent to *B. subtilis* ComEA, with a predicted transmembrane domain. This thermophilic organism has other distinctive features in its DNA transformation machinery, which could reflect the characteristics of its cell envelope and murein layer, with similarities to both Gram-positive and Gram-negative bacteria (48).

3.4. DNA fragmentation in Gram-positive bacteria

In *B. subtilis*, after binding to the cell surface, donor DNA undergoes limited double-stranded breakage (65, 66). NucA, an integral membrane protein with endonuclease activity, cleaves DNA bound on the surface of competent *B. subtilis* (67). Although NucA is not absolutely required for transformation, its presence increases the rate at which DNA is internalized, which is compatible with the notion that DNA with a free end is the substrate for transport. The delay in transport noted in a *nucA* mutant is presumably due to the time required for rare

termini to diffuse to the transport site. In fact, the *nucA* phenotype could be overcome by using shorter fragments of DNA for transformation, which would make DNA termini more abundant (67).

3.5. Pilins, pilin-like and associated proteins

In *N. gonorrhoeae*, several of the proteins involved in pilus biogenesis and function are necessary for competence (19, 21, 22, 24). In *B. subtilis*, the *comG* operon contains 7 ORFs, each of which is required for DNA binding to the surface of competent cells (68). ComGA and ComGB are similar to proteins associated with Tfp and type II secretion system. Four proteins (ComGC, GD, GE and GG) have similarities to type IV pilins. ComGF is predicted to be a membrane protein, with no clear ortholog in the type IV pilus system.

3.5.1. Traffic NTPases: pilus assembly and retraction/twitching motility

Members of the large family of NTP-binding proteins, called traffic NTPases, are involved in DNA uptake and transport, as well as Tfp biogenesis and both type II and type IV secretion systems. They contain four main conserved motifs: Walker boxes A and B, the Asp box and the His box (12). Although the predicted NTP-binding site is known to be essential for function in many cases (69-71), NTP-binding and NTP hydrolase activities have been demonstrated for few of these proteins, with varying nucleotide requirements observed (72, 73). Some members of this family form hexameric ring-like structures (74), and it has been suggested that these rings constitute channels facing the cytoplasmic membrane (74) or pores in the inner membrane itself (75), involved in translocation of proteins through the inner membrane.

B. subtilis ComGA is a cytoplasmic protein that can be found associated with the membrane (76); it contains a predicted NTP-binding site essential for DNA binding to competent cells (77). As noted above, all the *comG* proteins appear to play a role in permitting access of incoming DNA to the ComEA receptor. ComGA is also involved in growth and replication arrest during competence, and this function is not dependent on the ATP-binding site (77). In *N. gonorrhoeae*, PilF is the traffic NTPase ortholog involved in pilus assembly and DNA uptake (21).

ComGA and the traffic NTPases involved in Tfp assembly reside in the cytoplasmic face of the inner membrane; it is likely that they provide energy or function as chaperones for the assembly of pilins or pilin-like proteins into Tfp, the pseudopilus or the transformation apparatus. In this connection, we have shown that ComGA is needed for the assembly of a complex containing a multimer of the pilin-like protein ComGC in *B. subtilis* (I. Chen and D. Dubnau, unpublished).

In some organisms, there is a second class of traffic NTPases necessary for competence, represented by PilT. PilT is involved in twitching motility in *P. aeruginosa* and *N. gonorrhoeae*, which is a kind of movement powered by retraction of the pilus (78, 79). *P. aeruginosa pilT* mutants are hyperpiliated, but their pili are not functional in either twitching motility or phage entry (reviewed in (80)). PilT belongs to the same family of traffic NTPases as *N. gonorrhoeae* PilF, but they seem to have antagonistic actions: whereas PilF is necessary for pilus assembly, PilT contributes to pilus disassembly and its consequent retraction (55). Thus, besides its role in twitching motility, PilT has a negative regulatory function in *N. gonorrhoeae* pilus assembly: when this process is perturbed or blocked, such as by mutation of PilC (a tip adhesin, thought to stabilize the pilus) or PilQ (the secretin, necessary for protrusion of the pilus across the outer membrane), PilT causes depolymerization of the pilus fiber (81, 55).

In *N. gonorrhoeae* and *P. stutzeri*, PilT is necessary for DNA uptake (24, 82). It is tempting to speculate that pilus retraction itself plays a role in the uptake of DNA across the outer membrane and/or periplasm, perhaps by pulling in bound DNA. Nevertheless, one should consider that competent organisms which do not possess Tfp (*B. subtilis*, *S. pneumoniae* and *H. influenzae*) have only one member of the traffic NTPase family involved in competence, which is more similar to those involved in pilus assembly, and that these organisms therefore lack a PilT ortholog. It is conceivable that ComGA and its orthologs are capable of performing both functions (assembly/disassembly). In this context, it is interesting to note that the NTPases involved in pilus assembly and type II secretion systems usually contain two close CXXC motifs, resembling a zinc-binding motif; mutation of these cysteine residues in *K. oxytoca* PulE reduced pullulanase secretion (83). In contrast, in the orthologs involved only in transformation, the two CXXC motifs are not conserved: *B. subtilis* ComGA and *H. influenzae* PilB contain only one of the motifs, whereas *S. pneumoniae* ComGA and *S. gordonii* ComYA do not have them at all.

As an alternative explanation, PilT may be necessary for DNA uptake by piliated bacteria because of its negative regulatory function: since the same components are apparently utilized for assembly of a transformation apparatus and of the Tfp, PilT may contribute to a balance between the two kinds of structures, and avoid depletion of common subunits by Tfp formation. A recent finding may support this hypothesis: *P. stutzeri* major pilin, PilAI, has been substituted by a modified version with C-terminal hexahistidine tag replacing the last six residues. Interestingly, this modified pilin could support transformation but not pilus assembly, and its expression suppressed the transformation deficiency caused by *pilT* mutation (82). Since the modified pilin cannot be assembled into a pilus, but can still form the transformation apparatus, there would be no competition between the two systems, making PilT unnecessary for competence.

3.5.2. Cytoplasmic membrane protein involved in assembly

B. subtilis ComGB is a polytopic membrane protein, with orthologs involved in the Tfp and type II secretion systems. In the Tfp system from *P. aeruginosa*, the ComGB ortholog, PilC, is required for pilus formation

and seems to be necessary for the membrane-associated localization of PilB, which is the ComGA ortholog (11). It is not known whether ComGB interacts with ComGA. The ortholog in *N. gonorrhoeae* is PilG, a protein necessary for pilus assembly, but its exact role in the process is not known.

3.5.3. Prepilin peptidases

Type IV pilins are made as precursors (prepilins) which are processed by a prepilin peptidase. This bifunctional enzyme has been mostly characterized in *P. aeruginosa* (reviewed in (84)). It belongs to a family of aspartic acid proteases (85), and catalyzes the proteolytic cleavage of a short leader peptide from prepilins, as well as the *N*-methylation of the mature protein. The biological significance of pilin processing has been discussed recently (14).

The prepilin peptidase ComC from *B. subtilis* participates in the cleavage of the leader peptide of the prepilin-like proteins ComGC, GD, GE and GG (86, 76). At least for ComGC, processing by ComC is necessary for its translocation across the membrane (86). It is not known whether the processed proteins are *N*-methylated in *B. subtilis*.

3.5.4. Pilins and pilin-like proteins

Pilins are the structural subunits of Tfp, but as noted above they also participate in the type II secretion systems and in competence. They are small proteins (usually less than 20kDa), with a short N-terminal leader peptide, which is recognized and processed by a prepilin peptidase (see section 3.5.3), followed by a conserved hydrophobic stretch; the C-terminal regions show no sequence conservation among the members of this family.

Tfp have been most studied in *P. aeruginosa* (reviewed in (9), (80)). In this organism, PilA is the major pilin, but several so called minor pilins are also necessary for pilus assembly. PilA also participates in the Xcp type II secretion system, with a different set of pilin-like proteins (87, 12). The major pilins from *N. gonorrhoeae* and *P. stutzeri* which assemble into Tfp are also necessary for competence (19, 30, 27). Another example of pilin versatility is the Tfp subunit FimA from *Dichelobacter nodosus*, which participates in three different cellular processes: Tfp formation, secretion and competence (25).

In the pullulanase export system from *K. oxytoca*, there are 5 pilin-like proteins involved, of which PulG is the most abundant. It has been hypothesized that these pilin-like proteins would form some kind of supramolecular complex, the pseudopilus, which would participate in the export of protein substrates (11). In fact, PulG is capable of forming pilus-like structures when the *pul* operon was overexpressed in *Escherichia coli* (88), and formation of this structure was dependent on the other components of the secreton. The relation of this structure to the proposed pseudopilus is unclear.

In *B. subtilis*, there are four pilin-like ComG proteins (ComGC, GD, GE and GG), which are processed by the prepilin peptidase ComC (76, 86). They are necessary, together with the other ComG proteins, for ComEA to access exogenous DNA (68). ComGC seems to be the most abundant of them. It is found as an integral membrane protein as well as in a peripheral membrane location, outside the membrane, and a portion of the cellular ComGC can be released from the bacteria upon lysozyme treatment, suggesting a cell wall localization (76. 86). It is not known if ComGC translocation occurs spontaneously or if other ComG proteins participate in the process. The other pilin-like ComG proteins show similar localization patterns (76). The protein ComGC contains 2 cysteine residues that form an intramolecular disulfide bond (see below); this feature is shared by other pilins or pilin-like proteins (89-91).

As mentioned in section 3.1, the pilus is not required for competence in *N. gonorrhoeae*, but the pilin protein PilE is necessary. In this organism, at least one pilin-like protein is necessary for competence: mutants in *comP* form fully functional Tfp, but have impaired ability to take up DNA, and overexpression of the gene actually increases DNA uptake and transformation frequencies, suggesting ComP may be limiting under normal conditions (63). A similar situation is observed in *Acinetobacter sp*., whose pilin-like proteins ComP, ComB, ComE and ComF are dispensable for Tfp but necessary for DNA uptake (92-94).

How do pilins participate in the process of DNA uptake? As discussed previously (section 3.1), Tfp are thin filaments, without a large central cavity, so it is difficult to envision their role as conduits for folded proteins in type II secretion systems or as channels for DNA passage during uptake. Thus, we believe the pilins involved in competence form a different structure, a transformation apparatus devoted to uptake/transport of DNA. In *B. subtilis*, this complex would modify the cell wall and allow exogenous DNA to bind to the membrane-anchored receptor ComEA. The ComGC multimer mentioned in section 3.5.1 may correspond to this proposed complex. In *N. gonorrhoeae*, the complex might interact with the secretin channel, perhaps keeping it in an open configuration so DNA can pass through and bind to ComE. The complex might also facilitate passage of DNA through the periplasm and across the peptidoglycan layer.

The minor pilins may play a role in determining whether the structure formed by the major pilin will be a pilus or a transformation apparatus. For instance, overexpression of the pilin-like protein ComP in *N. gonorrhoeae* leads to increased DNA uptake and transformation frequencies. On the other hand, the pilin-like PilAII from *P. stutzeri* has the opposite effect: knock-out mutants display increased competence, whereas its overexpression inhibits transformation (95). These observations are consistent with the suggestion that the competence pseudopilus and the Tfp are distinct structures and that their formation may actually be competitive processes. It is conceivable that the minor pilins, together with the biogenesis proteins, form a complex close to the cytoplasmic membrane which would prime the appropriate structure to be assembled above it.

3.6. Disulfide bond oxidoreductases

In *B. subtilis*, a pair of protein disulfide bond oxidoreductases encoded by the operon *bdbDC* was recently found to be essential for efficient transformation (96). These proteins are necessary for the stability of ComGC, the major pilin-like protein in this organism, which contains an intramolecular disulfide bond (76). The role of disulfide bond oxidoreductases in the stability of pilins or pilin-like proteins has been reported for Tfp (90) as well as for type II secretion system (91). Por, a periplasmic protein disulfide oxidoreductase in *H. influenzae*, is required for DNA binding and uptake (97). Mutation of *por* seems to affect the redistribution of some inner membrane proteins that occurs during competence, but these proteins were not identified. *B. subtilis* BdbDC acts on competence proteins other than ComGC in *B. subtilis* (I. Draskovic and D. Dubnau, unpublished), and the expression of the *bdbDC* operon is up-regulated during competence development (96), suggesting that this oxidoreductase pair is somewhat competence specific.

3.7. A channel across the cytoplasmic membrane

In *B. subtilis*, ComEC is a polytopic membrane protein, with 7-9 predicted transmembrane domains. In its absence, DNA uptake is completely abrogated, but the bacteria can still bind DNA (98). In fact, *comEC* mutants tend to accumulate DNA on their surface (67). It has been suggested that ComEC forms a channel spanning the cytoplasmic membrane (98), through which one strand of donor DNA would enter the cytosol, while the other strand is degraded. Mutants in *comEC* do not release acid-soluble degradation products into the medium, suggesting that DNA transport and degradation are concomitant (67). In contrast, *comEC* mutants in *S. pneumoniae* can degrade DNA to nearly the same extent as the wild-type strain, but are also deficient in DNA transport (62).

A similar gene was characterized in *N. gonorrhoeae*, and termed *comA* (99). Mutants in this gene are still able to take up DNA, but the molecule cannot access the cytoplasm, being presumably blocked in the periplasm (100). Nucleolytic processing of DNA after uptake was also abolished in *comA* mutants (100), supporting the notion that transport and degradation of DNA are functionally linked. However, formation of single-stranded DNA molecules was observed in *comA* mutants, albeit at low levels (101).

Although the molecular details of these systems apparently differ, it is likely that ComEC and its orthologs form all or part of a gated channel across the inner membrane. It is a large polytopic integral membrane and it is absolutely required for DNA transport in all the systems in which it has been studied.

3.8. The translocase

B. subtilis ComFA is a membrane protein which is required for efficient DNA transport during transformation, but not for DNA binding (102, 103). It contains a predicted ATP-binding site, and mutants in this site had a *comFA* null phenotype (103). The protein shows resemblance to members of the DEAD family of helicases as well as to the ATP-driven DNA translocase PriA from *E. coli*, suggesting that it may function as a DNA translocase (104).

An ortholog of ComFA is required for transport but not for binding in *S. pneumoniae* (62). No clear ortholog could be identified in the complete genome sequences from *N. gonorrhoeae* or *H. influenzae*.

3.9. Degradation of the non-transforming strand

The membrane bound nuclease EndA is required for transformation in *S. pneumoniae* (105-107). This enzyme has endonucleolytic activity, and in its absence, transformation is impaired, but not completely abolished (107). Mutants in *endA* can bind DNA, but there is little degradation or transport into the cytoplasm. In the absence of the DNA-binding protein ComEA, there was residual binding but no degradation, which led to the suggestion that ComEA binds DNA and presents it to EndA. On the other hand, mutants in the proteins involved in transport, ComFA and ComEC, were still able to degrade DNA (62).

The fact that EndA activity is not dependent on DNA transport in *S. pneumoniae* is in contrast with *B. subtilis*, where no degradation is observed in *comFA* or *comEC* mutants (67). No EndA ortholog was identified in *B. subtilis* or in the other competent bacteria. These organisms may have recruited some other nuclease(s) to perform this function, which could account for the differences outlined above.

3. 10. Competence proteins in *N. gonorrhoeae* that are absent from *B. subtilis*

A few proteins are necessary for transformation in *N. gonorrhoeae* but are absent from the *B. subtilis* system. PilC is a protein involved in adhesion to epithelial cells which can be found on the pilus tip as well as on the outer membrane (108, 19). It is also related to pilus biogenesis, but not indispensable, since assembly of intact pili can occur in the absence of PilC (109). It is thought that PilC interacts with PilE and helps stabilize the pilus fiber (24, 55), but its role in competence is not known. Competence can be restored in piliated *pilC* mutants by the addition of purified PilC to the cells, and it was suggested that PilC interacts with components from the pilus assembly complex on the bacterial surface and participates in DNA binding and/or uptake (19). An ortholog of PilC was identified in *Acinetobacter sp* and named ComC; this protein is not involved in pilus assembly but is required for DNA binding and uptake during transformation (110).

Some competence proteins from *N. gonorrhoeae* are apparently unrelated to Tfp formation or function. ComL is a lipoprotein that associates with the peptidoglycan layer; most *comL* mutants are lethal, but one particular mutation led to bacteria of smaller size than the wild-type and impaired competence (111). Tetrapac (Tpc) is probably a murein hydrolase, and *tpc* mutants show a tetracoccal shape and rough colony morphology(112). Neither ComL nor Tpc are required for DNA uptake, and their effect in competence may be due to a role in cell wall metabolism

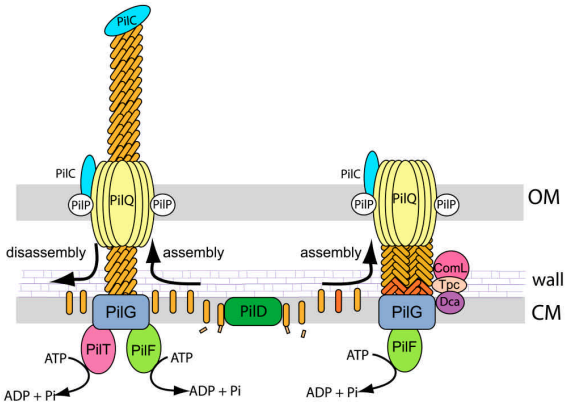

Figure 3. Model for biogenesis of the type IV pilus and the DNA uptake and transport machinery in *N. gonorrhoeae*. The prepilin (PilE, shown in orange) and prepilin-like proteins (ComP and perhaps others, represented in red) are processed by PilD. The mature pilin PilE is assembled to form the pilus fiber; PilF (a traffic NTPase) and PilG (a cytoplasmic membrane protein) participate in this process. It is not known whether there are minor pilins involved in pilus assembly in *N. gonorrhoeae*, as it occurs in *P. aeruginosa*. The protein PilC is an adhesin, found associated to the pilus tip amd to the outer membrane, and it is thought to stabilize the pilus fiber. PilT, another traffic NTPase, is involved in the disassembly and consequent retraction of the pilus. The same components are involved in the assembly of the DNA transport machinery: PilD, PilF and PilG participate in the formation of a pilin complex, with PilE in an arrangement distinct from the pilus fiber organization. PilC may participate in the stabilization of the complex, whereas the minor pilin ComP may have a chaperone function or a structural role together with PilE. PilQ is the secretin which forms a channel across the outer membrane which is necessary for protrusion of the pilus fiber, and PilP is the pilot lipoprotein which acts as a chaperone for PilQ. The secretin channel could be used for DNA translocation across the outer membrane.

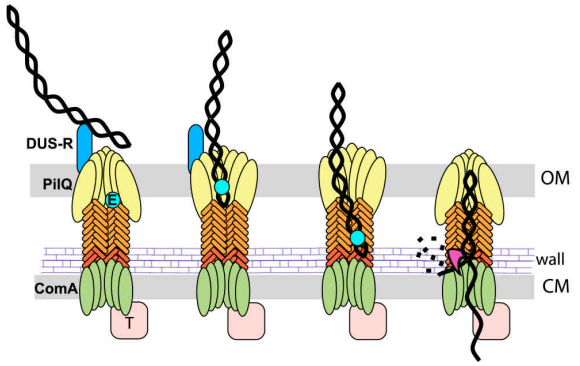

Figure 4. Model for DNA uptake in *N. gonorrhoeae.* Exogenous DNA binds to the bacterial surface; DNA containing the specific DUS is recognized the DUS receptor (DUS-R), which signals the opening of the secretin channel (PilQ). The selected DNA binds to the periplasmic protein ComE, and traverses the periplasmic space and the peptidoglycan layer through the putative pilin complex, reaching a channel across the cytoplasmic membrane formed by ComA. The translocation of a single strand to the cytosol ensues, catalyzed by an unknown translocase (T), while the non-transported strand is degraded to acid soluble products. PilE and ComP are color coded as in Figure 3.

which allows a DNA transport complex to assemble into the peptidoglycan layer (100). Finally, the gene *dca* is required for *N. gonorrhoeae* transformation; it encodes a predicted polytopic inner membrane protein which may assist the transport of DNA across the cytoplasmic membrane (113).

3.11. A model for DNA transport in *Bacillus subtilis*

Exogenous DNA first binds to ComEA, which is not readily accessible on the bacterial surface, but a structure formed by the pilin-like proteins ComG modifies the cell wall, allowing ComEA to contact the DNA. All the ComG proteins, as well as ComC and the BdbDC proteins are required to form this structure, and it is conceivable that ComGA ATPase activity provides the energy necessary for assembly. The biogenesis of this complex is shown in Figure 1, and the DNA uptake pathway is represented in Figure 2. ComEA is the DNA receptor in *B. subtilis*, but the presence of other DNA-binding proteins cannot be ruled out. The bound DNA suffers double-stranded cleavage by NucA, creating free termini which are delivered, possibly by a conformational change in ComEA, to a channel formed by ComEC. At this point, one of the strands of the DNA molecule is pulled across the ComEC channel, with the energy provided by the ATPase activity of ComFA, gaining access to the cytosol, where it can function as substrate for homologous recombination. The non-transforming strand is degraded by a nuclease (EndA in *S. pneumoniae*, not yet identified in *B. subtilis*) and the degradation products are released into the medium.

3. 12. A model for DNA uptake/transport in *Neisseria gonorrhoeae*

DNA binds to the surface, in a non-specific manner. When the unknown DUS-receptor binds to DUS, it triggers the uptake of the DNA molecule, signaling the opening of the secretin channel. A structure formed by pilin and other pilin-like proteins (such as ComP), using the pilus biogenesis apparatus (including PilD, PilF, PilG), could participate in gating the secretin channel, in a manner analogous to the pilus itself. The assembly of this structure and its proposed competition with Tfp biogenesis is shown in Figure 3, and a schematic representation of DNA uptake and transport is displayed in Figure 4. The incoming DNA would pass through the open secretin channel, being summoned and effectively taken up, presumably by binding to ComE. From there, the scenario is quite similar to what happens in *B. subtilis*: ComE leads the DNA molecule through the structure formed by the pilins across the periplasm and the peptidoglycan layer, directing it to the channel traversing the cytoplasmic membrane formed by ComA. Upon passage through the ComA channel, the DNA would be converted to single-stranded form, which enters the cytosol, while the other strand is degraded and the nucleotides released into the periplasm.

This model would account for the requirement of the pilus biogenesis aparatus for competence, since it would also assemble the DNA transport structure, while pili *per se* do not participate in the process. The major pilin

PilE is necessary, though, since it would play a structural role in the DNA transport complex.

4. DNA UPTAKE MACHINERY RELATED TO THE TYPE IV SECRETION SYSTEM

The human gastric pathogen *H. pylori* is naturally able to take up exogenous DNA and undergo genetic transformation (114). There is conflicting evidence on the specificity of DNA uptake in this organism: homologous DNA competitively inhibits transformation, while heterologous DNA does not (115), similarly to what happens in *H. influenzae* and *N. gonorrhoeae*. However, no *Helicobacter* DUS has been identified, and genome searches did not detect any abundant sequence motifs that might function as DUS (116), although the significance of such analysis has been disputed (117).

The *H. pylori* genome does not contain clear orthologs of the genes involved in DNA transport system in other competent organisms. Transposon mutagenesis led to the identification of the *comB* locus, which is necessary for competence (118). The ORFs contained in this operon resemble genes of the Vir system from the plant pathogen bacterium *Agrobacterium tumefaciens*, and were designated *comB*7 to 10, following the Vir nomenclature. The gene *comB*4 encodes a putative ATPase with similarity to VirB4 from *A. tumefaciens* and was also implicated in competence (119). The Vir system from *A. tumefaciens* mediates the translocation of proteins and DNA from the bacterial cytoplasm into the plant cell; it is the prototype for type IV secretion systems, which are similar to conjugation machineries. In fact, certain pathogens seem to have adapted the ancestral conjugation apparatus for delivery of effector molecules into eukaryotic cells (120).

Thus, *H. pylori* takes up DNA by a novel mechanism, using components related to the type IV secretion system, which probably form a complex spanning the cell envelope (121). A similar complex may be present in the closely related bacterium *Campylobacter jejuni* (122). Some strains of *H. pylori* carry a pathogenicity island in their genome that encodes an additional type IV secretion system, which translocates the protein CagA into human gastric epithelial cells. The *cag* secretion system and the DNA transport system are not functionally related, since mutations in one system do not affect the other (115, 119).

A model of the *H. pylori* DNA transport machinery was recently proposed, based on the similarities to the Vir system (123). The existence of components with specific roles (such as a DNA receptor, an endonuclease, and a traffic NTPase) is also inferred, and candidate genes which could perform such functions were identified in this bacterium complete genome (123). It is interesting to note that type IV secretion systems mediate the translocation of both proteins and DNA, two very different macromolecules. Nevertheless, the single-stranded DNA molecules translocated by Vir and conjugative systems are bound to proteins, so the effective substrate for transport would be a nucleoprotein complex (120). It is not known whether the incoming DNA molecule during transformation is bound to proteins. Another distinction is that the direction of the transport is outwards in type IV secretion systems, whereas exogenous DNA gets internalized during transformation. Hence, considering these differences in the nature of the transport, the borrowed type IV secretion system components have presumably suffered substantial adaptations to form the DNA uptake system in *H. pylori.*

5. CONCLUDING REMARKS AND PERSPECTIVES

The study of DNA uptake during transformation offers us a view on how bacteria have made use of macromolecular structures (type IV pili and the conjugation apparatus), modifying their components to build complexes for different purposes, such as the export of proteins (type II or type IV secretion proteins) or the import of DNA. Further research is needed to understand the mechanism of these transport pathways. Information is sorely needed on protein-protein interactions, on the composition and structures of the proposed multicomponent complexes, on the interaction of DNA with these complexes and on the energetic requirements for DNA uptake and transport.

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