Specific DNA recognition mediated by a type IV pilin

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Natural transformation is a dominant force in bacterial evolution by promoting horizontal gene transfer. This process may have devastating consequences, such as the spread of antibiotic resistance or the emergence of highly virulent clones. However, uptake and recombination of foreign DNA are most often deleterious to competent species. Therefore, model naturally transformable Gram-negative bacteria, including the human pathogen *Neisseria meningitidis*, have evolved means to preferentially take up homotypic DNA containing short and genus-specific sequence motifs. Despite decades of intense investigations, the DNA uptake sequence receptor in *Neisseria* species has remained elusive. We show here, using a multidisciplinary approach combining biochemistry, molecular genetics, and structural biology, that meningococcal type IV pili bind DNA through the minor pilin ComP via an electropositive stripe that is predicted to be exposed on the filaments surface and that ComP displays an exquisite binding preference for DNA uptake sequence. Our findings illuminate the earliest step in natural transformation, reveal an unconventional mechanism for DNA binding, and suggest that selective DNA uptake is more widespread than previously thought.

DNA receptor | genetic competence

Natural transformation is the process by which competent bacteria take up free DNA that is abundant in many environments and incorporate it into their genomes through homologous recombination. This widespread property is shared by dozens of species (1), and was key to one of the most important discoveries in biology (that DNA carries genetic information) (2). Critically, transformation is a powerful mechanism for generating genetic diversity through horizontal transfer of genes. For example, the naturally competent *Neisseria meningitidis*, which is a human pathogen of global importance, displays an extensive genetic diversity, with as much as 10% of its gene content differing between isolates (3). This diversity is key to the success of the meningococcus in competing with our immune system and has dire practical consequences, such as the difficult design of vaccines providing universal protection against meningococcal disease (4). In other species, horizontal gene transfer may also have dramatic consequences, such as the unabated spread of antibiotic resistance or the emergence of highly virulent clones.

Our understanding of free DNA transport from the extracellular milieu into the bacterial cytoplasm during transformation remains fragmentary, but the following model is widely accepted (5). Competent bacteria first bind DNA at their surface before it is taken up by type IV pili (Tfp) that are long, hair-like filaments found in a myriad of species (6) or shorter, evolutionarily related competence pseudopili. In Gram-negative bacteria, DNA uptake is defined as the crossing of the outer membrane that is thought to occur through secretin channels, which are involved in the emergence of Tfp onto the bacterial surface. In Gram-positive species, DNA uptake consists in the crossing of the thick layer of peptidoglycan. The finding that PilT, which is a cytoplasmic ATPase powering the remarkable capacity of Tfp to retract and generate mechanical force (7), is necessary for DNA uptake (8) suggests that Tfp/pseudopili pull DNA through the outer membrane/peptidoglycan on retraction (5). DNA then interacts with ComE/ComEA

(9, 10) and is delivered to a conserved translocase machinery in the cytoplasmic membrane that further transports it into the cytoplasm. Finally, DNA is integrated in the bacterial chromosome in a RecA-dependent manner, but it can also be used as a template for the repair of DNA damage or a source of food (5).

How Tfp/pseudopili interact with DNA remains enigmatic for several reasons. (i) High nonspecific binding of DNA to whole bacteria (11, 12) has been a hinder to genetic studies. (ii) ELISA using purified filaments has revealed only weak DNA binding with biological significance that is unclear (13, 14). (iii) Various biochemical approaches have only identified DNA binding proteins that are involved at late stages during transformation (15, 16). Nevertheless, the fact that *Neisseria* species and *Pasteurellaceae* favor uptake of homotypic DNA by selectively recognizing genus-specific DNA uptake sequence (DUS) motifs [or uptake signal sequence (USS) as they are known in *Pasteurellaceae*] is clear evidence of the existence of specific surface-exposed DNA receptors (5, 17, 18). None of the known DNA binding proteins involved in transformation show preference for DNA containing DUS/USS, and the nature of the DUS/USS receptors has, thus, remained mysterious.

Although no type IV pilin has ever been reported to bind DNA, the following observations prompted us to address the question of whether ComP might be the elusive DUS receptor in *Neisseria* species. (i) ComP is one of three minor (low abundance) pilins in pathogenic *Neisseria* species (with PilV and PilX) that modulate Tfp-mediated properties without affecting Tfp biogenesis (19–22). ComP has a prominent role in competence (20, 21) at the level of DNA uptake (23). (ii) ComP displays unparalleled sequence conservation for a surface-exposed protein in *N. meningitidis*, because it was found to be virtually identical in a large collection of disease isolates (24). This finding is consistent with the hypothesis that sequence conservation of DUS should be matched by sequence conservation of its cognate receptor. To test the ability of ComP to bind DNA, we have, therefore, carried out and report here a multidisciplinary analysis combining biochemistry, molecular genetics, and structural biology.

Results

ComP Is Highly Conserved Throughout the Genus *Neisseria*. The 5'-atGCCGTCTGAA-3' DUS motif (less conserved nucleotides are in lowercase) (17, 25) is highly overrepresented within the genomes of all sequenced *Neisseria* species (on average, every kilobase), with single base variations in some of them (26). Therefore, we reasoned

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Data deposition: The NMR, atomic coordinates, chemical shifts, and restraints data have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2M3K).

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that, if ComP was the DUS receptor in *Neisseria* species, it must be conserved beyond *N. meningitidis*. Accordingly, ComP orthologs were found in all sequenced *Neisseria* species (Table S1). Strikingly, in the clade comprising *N. meningitidis*, *N. gonorrhoeae*, *N. lactamica*, and *N. polysaccharaea*, we found that ComP was virtually identical with a maximum of two nonconservative substitutions over the 149 residues of the preprotein (Fig. S1). Such 99% sequence identity is in line with that of key essential proteins, such as the components of the RNA polymerase (encoded by *rpo* genes) or the ribosome (encoded by *rps*, *rpl*, and *rpm* genes), and is consistent with a key cellular function for ComP.

ComP Is Required for Efficient DNA Binding by Purified Tfp. As the identification of the DUS receptor has been impossible by genetic means because of the high nonspecific binding of DNA to whole bacteria (11, 12), we first tested the ability of filaments purified from a WT strain and an isogenic *comP* mutant to bind DNA by ELISA. To use highly purified pili in this assay, we engineered the 8013-PilE_{His6} variant of *N. meningitidis* 8013, in which the last five residues in the major pilus subunit PilE were replaced by a poly-His tag, and designed a metal affinity chromatography methodology to purify Tfp (Fig. S2). We phenotypically characterized 8013-PilE_{His6} and found that it is piliated (slightly less than the parent 8013 strain) and displays all of the classical Tfp-linked phenotypes (Fig. S2). Critically, transformation frequencies in 8013-PilE_{His6} and its parent strain 8013 were comparable, showing that the poly-His tag does not interfere with Tfp-mediated DNA uptake. We, therefore, purified pili from 8013-PilE_{His6} (WT) and its isogenic *comP* mutant and loaded serial dilutions of equal amounts of fibers (as assessed by PilE immunoblotting) in the wells of streptavidin-coated microtiter plates, in which a biotinylated double-stranded (ds) 22-mer primer centered on DUS was previously immobilized. Quantification by ELISA showed that, although WT Tfp bound DNA in a concentration-dependent and saturable manner, filaments of a *comP* mutant were dramatically impaired for DNA binding (Fig. 1). This finding provided evidence linking ComP with DNA binding.

Fig. 1. *Neisseria* Tfp bind DNA through the minor pilin ComP. Tfp were purified from *N. meningitidis* 8013-PilE_{His6} (WT) or its isogenic *comP* mutant, and their DNA binding propensity was assessed by ELISA. Equal amounts of purified filaments, as assessed by PilE immunoblotting, were loaded in each well, into which a 5' biotin-labeled ds 22-mer DUS primer was immobilized. After 2 h of incubation at room temperature, bound Tfp were quantified spectrophotometrically using the 20D9 mouse monoclonal antibody that is specific for strain 8013 fibers and an anti-mouse HRP conjugate. Results are the means $\pm$ SDs of three replicates.

ComP Is the only Pilus Component Capable of Binding DNA. To determine whether ComP has intrinsic DNA binding activity, we purified this protein and the other three known pilin components of *N. meningitidis* pili (PilE, PilV, and PilX). Using a strategy previously validated during the structural characterization of PilX (19), the soluble portion of these proteins in strain 8013, without their highly conserved and hydrophobic N-terminal α -helices (27), was fused to maltose binding protein (MBP). The fusion proteins are directed to the periplasm, which is important for disulfide bond formation, a characteristic feature of type IV pilins (27). After a two-step purification that yielded pure proteins, we tested the ability of these four pilins to interact with DNA using agarose EMSAs (10). As a target, we used the pSY6 plasmid that contains one DUS, which has been widely used to measure transformation efficiencies in *Neisseria* species (13). These experiments showed that ComP is the only pilus component capable of binding DNA, because no shift was seen with the other pilins with as much as 10 μ M pure protein (Fig. 2A). The ladder pattern observed with increasing concentrations of ComP most likely indicates that this protein was able to bind to multiple sites in the target plasmid. We further characterized ComP's DNA binding activity by using a more sensitive acrylamide EMSA and a ds 22-mer DUS primer labeled with biotin as a target (Table S2). This assay confirmed that ComP is capable of binding DNA (Fig. 2B). A shift was observed in a concentration-dependent manner with as little as 0.2 μ M protein, indicating a significant affinity of ComP for DNA.

ComP Interacts Better with DUS. We next used a range of approaches to determine whether ComP has a higher affinity for DUS. Using acrylamide EMSA, we performed competition assays by assessing the effect of an excess of unlabeled ds primer on the formation of ComP–DUS complex obtained with 0.4 μ M protein. Although unlabeled DUS efficiently competed with biotinylated DUS in a dose-dependent manner (Fig. 3A), which was demonstrated by the gradual disappearance of the ComP–DUS complex, a scrambled primer (SUD; in which every second base of DUS was changed) was incapable of competing, even at very high concentration. Similar results were obtained with another scrambled primer (SDU), showing that ComP has a marked preference for DUS.

The affinity of ComP for DUS, SUD, and SDU was then estimated in real time using surface plasmon resonance (SPR), a method widely used for analysis of protein–DNA interactions (28). A streptavidin-coated sensor chip was used for immobilizing equivalent amounts of biotinylated DUS, SUD, and SDU ds primers before increasing concentrations of ComP were injected. Although binding to DUS was dose-dependent and saturable (Fig. 3B), with a dissociation constant (K_d) of 29 ± 8 μ M, saturation was not reached for binding to SUD or SDU with up to 100 μ M ComP, after which the protein started to precipitate. Although K_d cannot be measured for SUD and SDU, a comparison of response values obtained with equivalent amounts of DUS, SUD, or SDU and a 1- to 30- μ M range of ComP clearly shows that ComP has a much higher affinity for DUS (Fig. 3B).

ComP interaction with DNA was further characterized by a multidimensional NMR analysis. On DUS titration (Fig. 4A), large chemical shifts perturbations were observed in the 2D ^1H - ^{15}N TROSY spectrum of ComP, leading to the disappearance and re-appearance of multiple peaks. At saturation (reached at approximately 1:1 stoichiometry), several new resonances appeared, arising from areas of ComP experiencing conformational exchange in the free form. Critically, on SUD binding (Fig. 4B), although chemical shifts perturbations were detected, they were clearly distinct, and no additional resonances were observed at saturation. Taken together, these findings indicate that ComP interacts better with DUS, with the formation of a single homogeneous complex in which conformational heterogeneity is removed.

Fig. 2. ComP has intrinsic DNA binding activity. (A) DNA binding propensity of four purified pilins (ComP, PilE, PilX, and PilV) as assessed by agarose EMSA. A standard amount (500 ng) of the DUS-containing pSY6 plasmid was incubated for 25 min at room temperature with increasing concentrations of purified ComP, PilE, PilX, and PilV and resolved by electrophoresis on an agarose gel. (B) Titration of ComP's DNA binding activity by acrylamide EMSA. Increasing concentrations of purified MBP-ComP were incubated for 25 min with 5' biotin-labeled ds DUS primer (5 fmol). DNA was resolved by electrophoresis on an acrylamide native gel, transferred to a positive nylon membrane, UV cross-linked, and detected using a streptavidin-HRP conjugate. Similar results were obtained with purified ComP, showing that the MBP moiety had no effect on DNA binding.

ComP Interacts with DNA Through an Electropositive Stripe Predicted to Be Exposed on the Surface of Tfp. NMR assignment of the majority of backbone and side-chain resonances could be obtained for ComP in the presence of DUS (Table S3). A family of solution structures was successfully calculated (Fig. S3), revealing the typical α/β -roll of type IV pilins (27) with the $\alpha 1C$ helix cradled against a four-stranded antiparallel β -sheet (Fig. 5). Mapping the residues of ComP showing DNA-induced chemical shift perturbations (Fig. S4) on this structure revealed that they form an electropositive stripe on the surface of ComP (Fig. 5). This stripe is flanked underneath by a loop delimited by two disulfide bonds, an unusual feature in type IV pilins that usually contain one C-terminal disulfide bond (27). This loop is crucial for DNA binding, which was revealed by the absence of shift when EMSAs were performed in the presence of the reducing agent DTT and suggests that it might serve as a docking platform for DNA, positioning it correctly for interaction with the electropositive stripe of ComP. Importantly, this DNA interaction motif is on the opposite side of the $\alpha 1C$ helix and thus, is predicted to be exposed on the surface of the filaments according to the structural model for *N. gonorrhoeae* Tfp (29).

Finally, to probe the functional significance of this electropositive stripe in DNA binding, we generated ComP variants in which three positively charged residues (Fig. 5) were individually replaced by site-directed mutagenesis by the small hydrophobic residue Ala

(Comp_{R78A}, Comp_{K94A}, and Comp_{K108A}). These variants were fused to MBP, purified as above, and shown to be correctly folded by NMR (Fig. S5). We first tested their ability to interact with biotin-labeled ds DUS primers using acrylamide EMSA and compared it to Comp_{WT} (Fig. 6A). These experiments revealed that Comp_{K94A} and Comp_{K108A} were dramatically affected for DNA binding, because protein–DNA complexes were either undetectable (Comp_{K108A}) or seen at much higher concentration (Comp_{K94A}) of protein. Then, we measured the DNA binding propensity of these variants using SPR (Fig. 6B), which confirmed that each variant displayed impaired DNA binding with reductions ranging between 1.8- (Comp_{R78A}) and 12-fold (Comp_{K108A}) compared with Comp_{WT}. To determine the impact of these defects in vivo, we constructed strains in which the corresponding mutant alleles placed under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG) -inducible promoter were integrated ectopically in the genome of a *comP* mutant, and we quantified their competence. As shown earlier (21), complementation with the WT allele fully restored competence. Comp_{R78A} displayed an impaired stability in vivo, and the corresponding *comP/comP_{R78A}* strain was not analyzed further. Mirroring the in vitro DNA binding results, competence for DNA transformation of *comP/comP_{K94A}* was only slightly affected while *comP/comP_{K108A}* was 40-fold less transformable than the WT (Fig. 6C). In conclusion, the DNA binding motif that we have identified in Comp plays a crucial role

Fig. 3. ComP has a higher affinity for DUS. (A) Effect on the ComP–DUS complex of the addition of increasing amounts of unlabeled competitor as assessed by acrylamide EMSA. ComP (0.4 μ M) and biotin-labeled DUS (5 fmol) were incubated with increasing concentrations of unlabeled ds primers DUS or SUD (in which every second base of DUS was changed). DNA was then resolved by electrophoresis on acrylamide native gels and detected as in Fig. 2B. (B) SPR analysis of ComP's binding to DUS, SUD, or SDU (another primer in which every second base of DUS was changed). A streptavidin-coated sensor chip SA was used for immobilization of similar amounts of biotinylated DUS, SUD, and SDU ds primers before increasing concentrations of purified ComP were injected. Response values shown are an average of six measurements taken from two repeats each of three separate dilution series.

Fig. 4. NMR analysis of DNA binding by ComP. (A) Region of the ^1H - ^{15}N TROSY spectrum of ComP showing differences in amide shift changes and appearance of new resonances in the presence of saturating amounts of DUS (red). Free ComP spectrum is in black. (B) Differences in the ^1H - ^{15}N TROSY spectrum of ComP in the presence of saturating amounts of DUS (red) or SUD (black).

in the interaction of this protein with DNA during natural transformation.

Discussion

Natural transformation is an important biological process that has been studied for almost a century. Competent bacteria can take up free DNA through the use of widely conserved Tfp/pseudopili, incorporate it into their genomes through homologous recombination, and hence, be genetically altered (or transformed) (5). In many species, any DNA can be taken up effectively, but the ability of foreign DNA to be stably inherited must be limited to preserve species structure. Usually, transformation by foreign DNA is limited either by its inability to recombine within the genome because of extensive sequence divergence or through degradation by restriction enzymes (30). However, bacteria belonging to the genus *Neisseria* and the family *Pasteurellaceae*, including *N. gonorrhoeae* and *Haemophilus influenzae*, two of the species in which natural transformation has been most

studied, manage to circumvent this problem by preferentially taking up homotypic DNA (5). It was discovered in the 1980s that this selectivity was dependent on short sequences motifs that are required for efficient uptake: DUS in *Neisseria* and the 5'-AAGTGC GGT-3' USS in *Haemophilus* (17, 18). Consistently, uptake sequences represent 1% of the genomes of these bacteria and are 1,000-fold more common than statistically expected. In the following three decades, the quest for specific DUS/USS receptors has been unsuccessful, leaving the molecular basis for selective DNA uptake mysterious.

Because pilus/pseudopilus retraction powers DNA uptake, these organelles must interact with DNA at an early stage. However, the biological significance of the weak interactions previously reported between Tfp and DNA remains unclear (13, 14), and no interaction with DNA has ever been reported for a purified pilus component. Our finding that the minor pilin ComP displays intrinsic DNA binding activity is, thus, highly significant, because it sheds light on the earliest step in DNA transformation. This finding suggests that competent bacteria are likely to initiate uptake by binding DNA through a pilus component. However, it remains to be determined whether the DNA binding motif that we have identified in ComP will be conserved in other pilin DNA receptors. It is possible that the receptor might be a major pilin in species not exhibiting the frequent antigenic variation seen with PilE in pathogenic *Neisseria* (24, 31). In *Neisseria* species, a highly conserved minor pilin has to be used, because the surface of the PilE filaments is far too variable (27). It can be predicted that, in the absence of such a receptor (when the corresponding gene is mutated), DNA will not interact with Tfp and be pulled through the outer membrane on Tfp retraction and that the transformation will be dramatically affected. Accordingly, as shown here, the fibers produced by a *comP* mutant display a dramatically reduced ability to bind DNA, and as shown previously, *comP* mutants are defective for DNA uptake (23) and noncompetent (20, 21). It is important to note that *comP* mutants are otherwise indistinguishable from WT for piliation levels or any other Tfp-linked property (20, 21), underlining the exclusive role of ComP in competence. Consistent with this scenario, ComP overexpression enhances transformation frequencies by increasing both DNA binding and uptake (21, 23).

The second major finding in this study is that ComP, although it can bind any DNA, displays a marked preference for DUS as assessed by several methods and thus, might be directly responsible for selective DNA uptake in *Neisseria* species. Although the DNA binding surface in ComP has been identified by NMR and site-directed mutagenesis, the preference for DUS remains to be

Fig. 5. Structural analysis of ComP. Ribbon representation of ComP (Left) with residues experiencing significant DNA-induced chemical shift perturbations in the presence of DUS colored from red for large perturbations to pink for small perturbations (yellow is used for residues for which NMR assignment could not be performed). Residues that were mutated for functional studies (R78, K94, and K108) and the two disulfide bonds (C76-C125 and C118-C139) are highlighted as sticks. Surface charge representation in the same orientation (Right) shows that these residues form an electropositive stripe on the surface of ComP. This patch is flanked underneath by a docking platform for DNA, which consists of a loop delimited by the two disulfide bonds.

N. meningitidis strains used in this study are derivatives of the sequenced and systematically mutagenized serogroup C clinical isolate 8013 (35, 36). The *comp* mutants have been previously described (21). The 8013-PilE_{His6} derivative

has been constructed by splicing PCR as described previously (37). To create a *comP* mutant in this background, 8013-PilE_{His6} was transformed with chromosomal DNA extracted from a *comP::aadA* mutant (21). To test the functionality of the above site-directed mutants in vivo, we subcloned the mutant *comP* alleles under the control of an IPTG-inducible promoter in the complementing pGCC4 vector as described previously (21). The resulting vectors were first transformed into 8013, where they integrated ectopically by homologous recombination, before the resulting strains were transformed with chromosomal DNA extracted from a *comP* mutant. Competence assays were performed at IPTG concentrations leading to levels of ComP comparable with levels in the WT as assessed by immunoblotting, which was not possible for the strain expressing ComP_{R78A}, which showed an apparently impaired stability in vivo.

Phenotypic Characterization of Meningococcal Strains. Piliation and all classical Tfp-linked phenotypes were assessed using previously published protocols (21, 37).

Affinity Purification of Native Tfp from 8013-PilE_{His6} Derivatives. Tfp, recovered from several agar plates heavily inoculated with 8013-PilE_{His6} derivatives, were sheared by vigorously vortexing. The shear fraction was recovered after centrifugation, treated with DNase I (New England Biolabs), pelleted by ultracentrifugation, and purified using Ni-NTA slurry (Qiagen) according to the manufacturer's instructions.

Protein Purification. MBP fusions were purified mainly as previously described for PilX (19). For NMR analysis of ComP, bacteria were grown in minimal medium containing ¹⁵NH₄Cl, [¹³C]-D-glucose, and vitamin supplements to produce uniformly ¹⁵N/¹³C-enriched protein.

DNA Binding Assays. For DNA binding assays with purified Tfp, biotin-labeled ds primers (Table S2) were bound to the wells of Reacti-Bind plates that are

precoated with streptavidin (Thermo Scientific) and incubated with filaments. Bound Tfp were detected by ELISA using the 20D9 monoclonal antibody as previously described (21).

Agarose EMSAs were performed essentially as described elsewhere (10). Acrylamide EMSAs were performed as follows. Purified proteins and biotin-labeled ds primers were incubated, separated by electrophoresis in native acrylamide gels (containing no SDS), and transferred to positively charged Hybond N+ membrane (Amersham). DNA was detected using a stabilized streptavidin-HRP conjugate and the LightShift chemiluminescent EMSA kit (Pierce) as suggested by the manufacturer. For competition assays, an excess of unlabeled ds primer was added in the reaction before the addition of biotin-labeled ds DUS primer.

SPR was performed on a Biacore 3000 instrument at 25 °C. Equivalent amounts of biotinylated DUS, SUD, and SDU ds primers were coupled to streptavidin on the sensor surface of SA chips (GE Healthcare). ComP proteins were then flowed over the chip surface, and resonance units were measured.

NMR Spectroscopy and Structure Calculation. NMR spectra were recorded on a Bruker Avance III 600 spectrometer equipped with a TCI cryoprobe or a Bruker Avance II 800 spectrometer equipped with a TXI cryoprobe. Structure calculations were carried out using standard methods.

SDS/PAGE and Immunoblotting. Separation of proteins by SDS/PAGE was done using standard molecular biology techniques (38). SDS/PAGE gels were stained using either Bio-Safe Coomassie or Silver Stain Plus (Bio-Rad). Immunoblotting was done as described previously (21).

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