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Review

Meningococcal Biofilm Formation: Let's Stick Together

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Extracellular DNA (eDNA) is an essential constituent of the extracellular matrix of biofilms of many microorganisms. In spite of many studies, it has long remained unclear how exactly eDNA exerts its role in biofilm formation. Here, we describe recent advances that have been made in understanding biofilm formation in the human pathogen *Neisseria meningitidis*. Several cell-surface-exposed proteins have been identified that bind DNA and other negatively charged polymers, such as heparin, by electrostatic interactions. By virtue of these proteins, eDNA can act as an adhesive that binds the bacteria together. We provide examples that indicate that the mechanism of binding eDNA via DNA/heparin-binding proteins is a conserved feature in biofilms of many different microorganisms, including fungi.

Biofilm Formation (BF) by *Neisseria meningitidis* (*Nm*)

Nm is a Gram-negative diplococcus and an obligate human pathogen. It resides asymptomatically in the nasopharynx in up to 30% of the population as part of the commensal microbiota. Only occasionally, the bacteria cross epithelial layers to reach the bloodstream and cause invasive meningococcal diseases, such as bacteremia and meningitis, with often fatal consequences. Humans develop antibody responses against carriage strains in the nasopharynx within 9–10 days after colonization [1,2]. Further, mechanical forces derived from mucus flow, coughing, or swallowing help to remove microorganisms. Such mechanisms are in contrast to the long-term carriage stage of *Nm* in the host. *Nm* has evolved various strategies to evade the host's immune system and persist for a long time, including microcolony formation [3]. Microcolonies resemble biofilms, which are defined as organized microbial communities enmeshed in a self-produced extracellular matrix (ECM) composed of polymeric substances [4]. The biofilm structure confers benefits to the members of the community, including enhanced resistance to changes in environmental conditions such as temperature and oxygen concentrations, and reduced accessibility to antimicrobials, antibodies, and predators [5].

Nm can adhere to epithelial layers and other surfaces and form highly ordered multicellular communities [6–11]. In the biofilm, the biomass and the spaces in between can be differently organized. The biomass can consist of aggregates of different sizes, single cells, or a combination of both [6–9]. Figure 1 illustrates the biofilm architecture of three different meningococcal strains grown under flow conditions (Box 1). The distribution of the biomass can generate well-defined channels which enable the exchange of molecules and resources within the community, as, for example, in strain HB-1 (Figure 1A). Biofilms of strain BB-1 contain intercellular spaces but they do not create defined channel-like structures (Figure 1B). Strain α14 forms biofilms consisting of patchy structures without obvious channel formation and, in contrast to HB-1 and BB-1, without connections between the biomass (Figure 1C). Thus, the biofilm architecture

Trends

Biofilms are bacterial communities embedded in a self-produced extracellular matrix (ECM). Extracellular DNA (eDNA) is a component of the ECM that is essential for initiation of biofilm formation in *Neisseria meningitidis* (*Nm*) and many other microorganisms.

Nm expresses several surface-exposed proteins that bind eDNA by electrostatic interactions, allowing eDNA to function as an adhesive between the bacteria.

The eDNA-binding proteins can be released from the cell surface by a protease, the expression of which is stochastically regulated. Expression of the protease allows for regulation of biofilm formation and dispersal.

The eDNA-binding proteins also bind other negatively charged polymers, such as heparin.

Examples suggest that eDNA-dependent formation of biofilms mediated by surface-exposed eDNA- and heparin-binding proteins is a widespread mechanism among microorganisms.

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Box 1. Methods Used to Study Biofilm Formation (BF)

BF has been studied using various methodologies each of which assesses particular aspects. The different methods and conditions may significantly affect BF and this must be carefully considered when different studies are compared.

A convenient way to study BF is to deposit bacterial suspensions in the wells of conventional microtiter plates and incubate the plates under static conditions. Biofilms will form on the plastic surfaces of the wells, and their biomass formed after a certain incubation period can easily be quantified by staining the biofilm with crystal violet and measuring the optical density after cell lysis. This method is rapid, cheap, and optimal for screening large sets of strains, treatments, or conditions. However, one has to consider that, under such conditions, oxygen and nutrients become limiting in time, resulting in increased cell death; this is especially relevant at later stages during biofilm maturation. Furthermore, acquisition and dispersal of members of the biofilm community are not controlled. Both disadvantages can be circumvented by growing biofilms in flow chambers. After bacterial attachment, fresh medium is constantly supplied to the flow chambers, and waste products and bacteria detached from the biofilm are simultaneously removed. The flow in the chambers may simulate mechanical forces as those found in natural conditions. A disadvantage of the method is that the number of strains that can be tested simultaneously is rather limited.

Plastic and glass are most commonly used as abiotic surfaces to study BF. Their use leads to controllable and standardized conditions allowing for comparison of different bacterial strains. BF, however, can also be studied on epithelial or endothelial cell layers, offering a close approximation of host–pathogen interactions in nature. In contrast to abiotic surfaces, the number of variables is less controllable. For example, bacterial attachment requires adhesins to bind to cognate receptors on eukaryotic cells, and any of these may or may not be expressed; thus, extrapolation of data is limited.

Unspecific staining, for example, with crystal violet, is often used for quantification of the biomass of biofilms grown on abiotic surfaces. To determine the structure of biofilms, photographs of the biofilm are generated using confocal microscopy and structural parameters, such as biomass, thickness, and roughness of the biofilms, can be further analyzed with specific software, for example [87]. This requires fluorescent bacteria, generated either with specific dyes or by the endogenous production of fluorescent proteins.

of *Nm* is very diverse and strain dependent. The development of biofilms depends on cell-surface-exposed structures and the ECM that establish mutual contacts between bacteria and interactions with the substratum. Thus, variation in biofilm architecture must originate from variability in these structures. Recently, novel insight has been obtained in the role of the meningococcal **secretome** (Box 2 and Glossary) in direct and indirect associations between bacteria in a biofilm. This review focuses on the role of the ECM and the secretome in BF.

Composition and Generation of the ECM

In many bacteria, extracellular polysaccharides are prominent components of the ECM of biofilms, but *Nm* does not produce such polysaccharides. In most genetic lineages of *Nm*, extracellular DNA (eDNA) is a major constituent of the ECM required for initial attachment of cells to the substratum and for the development and stabilization of biofilms [9]. Treatment with DNase dramatically affects the initiation of BF in these strains. It is assumed that eDNA is released by bacterial lysis facilitated by the expression of autolysins, for example, the membrane-bound lytic transglycosylases (Mlt) A and B and *N*-acetylmuramyl-L-alanine amidase (AmpD) in initial biofilm stages and of the outer membrane phospholipase A (OMPLA) at later stages [9]. MltA and B and AmpD function in peptidoglycan turnover during cell growth and division and in maintenance of the cell wall. However, when uncoordinated, their activity leads to cell lysis and, therefore, to DNA release. *Neisseria gonorrhoeae* (*Ng*), a close relative of *Nm*, actively secretes single-stranded DNA via a type IV secretion system (T4SS) [12], and this DNA plays an important role in the initial phases of BF [13]. Some meningococcal strains also contain genes for the T4SS [14]; however, in most of these strains, these genes are disrupted by insertions and deletions, and those strains with a complete T4SS do not appear to exhibit increased DNA release. The amount of eDNA available for BF may further be controlled by the production of a DNA thermonuclease dubbed Nuc. Its effect on BF was experimentally proven in *Ng*, where a Nuc-deficient mutant had increased amounts of eDNA as well as increased biofilm production [15]. Nuc is produced with a signal sequence, but it was found in cell lysates and not in culture

Glossary

Clonal complex (cc): a group of bacterial strains that are genetically closely related based on their sharing of similar alleles at several phylogenetically relevant loci. Strains from the same cc are considered to be derived from a recent common ancestor.
Gene conversion: nonreciprocal genetic recombination process whereby a DNA sequence is replaced by a homologous sequence.
Outer membrane vesicles (OMVs): small spherical vesicles of 20–250 nm in diameter that pinch of from the cell. They are composed mostly of outer membrane with entrapped periplasmic compounds.
Phase variation: random switching of phenotype at frequencies higher than classical mutation rates. It involves stochastic genetic or epigenetic switches resulting in variation of protein expression. It generates heterogeneity in a population and allows for selection of the best adapted phenotypes under selective environmental pressure, created, for example, by the immune response.
Secretome: the totality of molecules secreted by a cell. However, usually, the term refers to the totality of secreted proteins. Here, we use this narrower definition and use it to refer to all proteins that are transported across the outer membrane in Gram-negative bacteria, independent of whether they remain attached to the bacterial cell surface or are released into the medium.
Slipped-strand mispairing: mechanism of phase variation that occurs during DNA replication at short nucleotide repeats. The nascent DNA strand can separate from the template strand and slip towards the front or the back along the template during re-annealing, resulting in a change in the repeat number in the newly synthesized strand. When this happens in a promoter sequence, it affects promoter strength. Within the coding sequence of a gene, it can change the reading frame resulting in on/off switching of gene expression.
Two-component regulatory systems: regulatory systems used by bacteria and other organisms to sense and respond to environmental changes. These systems are usually composed of a histidine kinase in the cytoplasmic membrane that senses

Box 2. Protein Secretion Systems in *Neisseria meningitidis* (*Nm*)

Gram-negative bacteria have developed several systems for the secretion of proteins across their cell envelope into the extracellular milieu or directly into the cytoplasm of eukaryotic target cells. Six of these systems, designated Types I–VI secretion systems (T1–6SS), are widely disseminated among Gram-negative bacteria. *Nm* contains only the T1SS and T5SS [48]. In the T1SS, an envelope-spanning machinery consisting of three proteins mediates the secretion of client proteins straight from the bacterial cytoplasm into the milieu. Substrates of T1SS in *Nm* are two RTX toxins, FrpA and FrpC, but a role in virulence could not be demonstrated [88].

In T5SS, client proteins are first transported across the inner membrane via the general export pathway, mediated by the Sec system, after which the periplasmic intermediate is transported across the outer membrane (OM). Based on differences in the latter step, five subsystems are discriminated: T5a–eSS [89]. In *Nm*, T5aSS, T5bSS, and T5cSS are present. The T5aSS encompasses classical autotransporters, which are synthesized as precursors consisting of three domains: an N-terminal signal sequence, which is required for targeting the Sec machinery, a passenger domain, and a C-terminal translocator domain. The translocator domain is inserted as a 12-stranded β-barrel into the OM via the general OM-protein assembly machinery, the BAM complex [90,91], and/or the alternative TAM complex [92]. During this process, the passenger is translocated to the cell surface where it can remain associated via the translocator domain, or be released by one of several proteolytic mechanisms. *Nm* can produce six classical autotransporters [48], four proteases, that is, IgA protease, NalP, App, and AusI/MspA, which are released into the extracellular medium, and AutA and AutB.

The T5cSS encompasses trimeric autotransporters, which are similar to classical autotransporters, but it requires three subunits, which each contribute four β-strands, to form a similar 12-stranded β-barrel as in the T5aSS. *Nm* produces two trimeric autotransporters, NadA and NhhA [48].

In T5bSS, or two-partner secretion systems, a large β-helical protein, generically designated TpsA, is translocated across the OM via a protein designated TpsB [93]. TpsB is a 16-stranded β-barrel with two N-terminal POTRA domains that extend into the periplasm. Recognition and subsequent secretion require interaction between an N-terminal domain in TpsA and the POTRA domains of TpsB. The number of T5bSS in *Nm* varies: up to five TpsAs and up to three TpsBs can be identified in a single genome sequence [48].

environmental signals and a response regulator in the cytoplasm that generates an appropriate response, usually by affecting the expression of specific sets of genes.

Type IV pili (Tfp): extracellular filamentous organelles, of 6–8 nm wide and several micrometres in length, that extend from the bacterial surface into the medium. They are composed of pilins and the adhesin PilC. The minor (PilH, I, J, K, V, and X) and major (PilE) pilins multimerize in a helical array at the inner membrane, and the pilus structure is extruded by a complex machinery through a pore in the outer membrane formed by a protein called secretin. The pili can be retracted at the expense of ATP, generating considerable forces of ~100 nN per filament.

supernatants, suggesting that it resides in the periplasm and might control DNA release rather than degrading eDNA present in the ECM [15]. However upon cell lysis, Nuc may be released and mediate dispersal of the biofilm.

Initiation of BF in autolysis-deficient *Nm* mutants could be restored with crude DNA obtained from a wild-type strain after autolysis but not with highly purified DNA or proteinase-treated crude DNA [9]. Thus, initial attachment of the bacteria to the substratum is not mediated by DNA alone, but it requires complexes of DNA with proteins that are also released during lysis or that are actively being secreted into the milieu. **Outer membrane vesicles** (**OMVs**), which pinch off from the bacteria during growth, are also prominent components of the ECM. Their importance for BF was demonstrated in *Ng* using an *msbB* (*lpxL*) mutant, which was found to be defective in OMV production as well as in BF [16].

In some genetic lineages of *Nm*, that is, the **clonal complexes** (cc) 8 and 11, initiation of BF does not require eDNA as it is insensitive to DNase [9]. Strains of these cc do not express OMPLA [9,17]. Although this could explain the lack of use of eDNA during biofilm development, these strains do express MltA and B and AmpD, indicating that eDNA should be available during initiation of BF. Furthermore, addition of exogenous DNA did not stimulate BF, suggesting that these strains cannot use eDNA to form biofilms even if it is available [9]. Biofilms of cc8 and cc11 strains have an architecture that is different from those of other strains (e.g., compare Figure 1A and B), and they are more fragile as demonstrated by their higher sensitivity to shear forces [9]. It is noteworthy that strains from cc8 and cc11 are usually isolated from patients with meningococcal disease and hardly from healthy individuals, while other lineages are isolated both from patients and from carriers. This observation resulted in the proposal that the species *Nm* includes spreader and settler strains with different phenotypes [18]. Members of cc8 and 11 have a spreader phenotype characterized by poor colonization of the nasopharynx but high

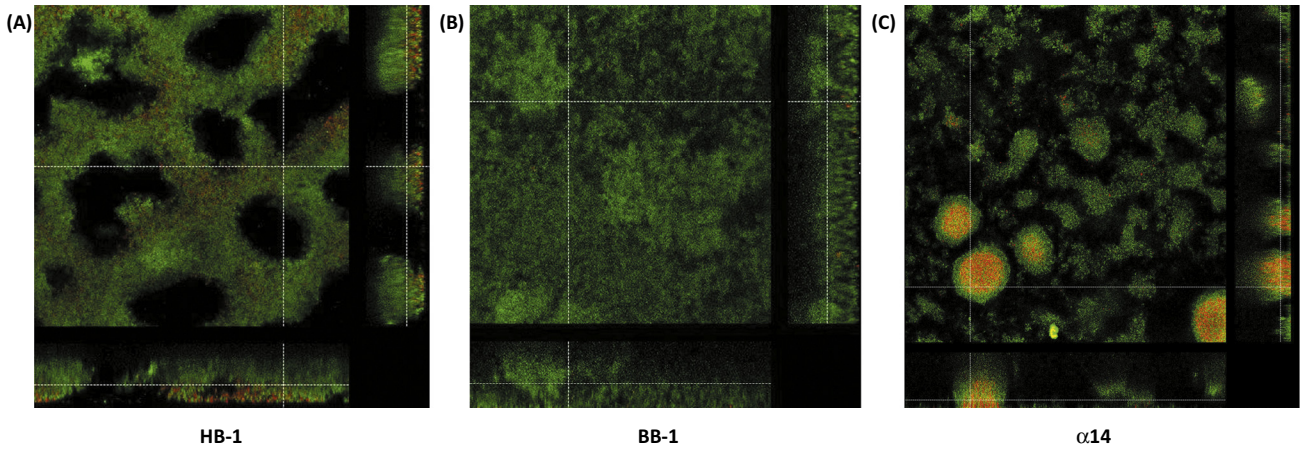

Figure 1. Variable Architecture of Meningococcal Biofilms. Biofilms of three different meningococcal strains, that is, cc32 strain HB-1 (A), cc11 strain BB-1 (B), and cc53 strain α14 (C), were formed under flow conditions on abiotic surfaces. Strains HB-1 and BB-1 are unencapsulated derivatives of isolates H44/76 and B16B6, respectively, whilst α14 is an unencapsulated isolate. The 14-hour-old biofilms were stained with the LIVE/DEAD Baclight bacterial viability stain (where red cells are dead and green cells are living) and observed by confocal microscopy. Reproduced with permission from [6,7].

transmission and invasion rates. In contrast, the settlers are characterized by a higher colonization and persistence in the nasopharynx of the host, probably as a consequence of the high DNA-binding ability and the formation of sticky biofilms.

Cell-Surface Organelles

Nm cells can be surrounded by a capsule that is made of polysaccharides, and long proteinaceous filamentous fibers, known as pili or fimbriae, can protrude from their surface. The expression of both types of organelles affects BF.

The Capsule as an Inhibitor of BF

Capsular polysaccharides of *Nm* can have various chemical compositions; this forms the basis for the classification of *Nm* into 12 serogroups. The capsule provides resistance against immune defenses [19], such as phagocytosis, the complement system, and defensins [20], and may increase survival during transmission to a new host by protecting against desiccation and irradiation damage. Because of its role in immune escape, the vast majority of strains isolated from patients are encapsulated. However, isolates from carriers are often unencapsulated [21]. The capsule inhibits the interaction of many adhesins with their cognate receptors on eukaryotic cells through steric hindrance. This is particularly the case for integral outer-membrane proteins, such as the Opa/Opc proteins, which have only short exposed loops as interaction surfaces. However, very long structures, such as type IV pili (Tfp) and, possibly, some substrates of the type V secretion system (T5SS), may extend beyond the capsule. Thus, although the capsule is important during pathogenesis and transmission, its absence is beneficial during colonization of the nasopharynx. Accordingly, expression of the *sia* genes, which are involved in the synthesis of capsular polysaccharides, is downregulated upon cell contact through a mechanism involving the transcription factor CrgA [22]. However, it is remarkable that *sia* promoter activity did not respond to the presence in the medium of α-methylene-γ-butyrolactone, an inducer of CrgA activity [23]. Other regulatory mechanisms include the **two-component regulatory system** MisS/MisR [24] and the presence of an RNA thermosensor, which reduces capsule expression in the nasopharynx where the ambient temperature is considerably lower than in the bloodstream [25]. Capsule expression is also prone to **phase variation** through **slipped-strand mispairing** or through the reversible insertion of an IS*1301* element [26]. In addition, carrier

isolates often contain various irreversible disruptions of genes involved in capsule biosynthesis [27]. Several studies concluded that capsule expression inhibits BF on abiotic substrates [8,11] and that disease isolates produce less biofilm than carriage isolates [11]. However, BF of encapsulated strains on human bronchial epithelial (HBE) cells was only slightly less efficient than that of their unencapsulated derivatives [10]. These findings demonstrate that the substratum is relevant for meningococcal BF. Probably, the hydrophilic capsule inhibits binding of the bacteria to abiotic surfaces [11], whereas the Tfp, which extend beyond the capsule, mediate initial contact with epithelial cells.

Tfp

Various types of pili can protrude from the surface of bacteria. Tfp are remarkable in being retractile [28]. A variety of functions required for BF has been attributed to meningococcal Tfp, including adhesion to epithelial and endothelial cells, interbacterial interaction, and twitching motility [28–30]. In addition, Tfp contribute to transformation competence. Tfp consist of multiple copies of the major subunit PilE and several minor subunits. *Nm* can express either one of two structurally different classes of major pilins, class I and class II [31]. In contrast to class I pilins, class II pilins do not show antigenic variability through **gene conversion** and they are more heavily glycosylated [32]. It is noteworthy that strains of cc8 and cc11 express class II pilins, whereas strains from most other cc express class I pilins [33,34]. Perhaps, this is related to the eDNA-independent initiation of BF in cc8 and cc11 strains (see above), a hypothesis that warrants further investigation.

PilC1 was reported to be located at the pilus tip and to function as an adhesin [35]. It has been proposed that CD46 is the receptor of Tfp, since CHO cells transfected to express CD46 were found to bind piliated, but not nonpiliated, gonococci, and this binding was blocked with purified CD46 [36]. However, this notion was later disputed as CD46 resides on the basolateral side of epithelial layers, and binding of *Ng* or purified PilC to epithelial cells was not affected by downregulation of CD46 expression by RNA interference [37]. The minor pilin PilX was reported to be responsible for interbacterial interactions [30]. However, although the protein was previously demonstrated to be localized in pilus filaments [38], recent evidence indicates that it is localized in the periplasm, which would be incompatible with a direct role in interbacterial interactions [39]. An indirect role is feasible as a *pilX* mutant showed decreased piliation and was affected in twitching motility [8]. Twitching motility is mediated by the attachment of the tip of extended pili to a surface and subsequent pilus retraction resulting in bacterial movement over the surface. Tfp are not required for BF on abiotic surfaces but their expression affects biofilm architecture [8,11]. Tfp-expressing strains formed biofilms composed of microcolonies, while deletion of *pilE* or *pilX* generated flat biofilms. This difference could be correlated with a defect in twitching motility of the mutants rather than to a defect in interbacterial interactions mediated by pili [8]. Hence, microcolony formation could be explained by the existence of two types of mechanical forces: (i) bacterial movement mediated by Tfp, and (ii) interbacterial interactions mediated by protein–protein and/or protein–ECM interactions. Variations in both forces may explain the differences in biofilm architecture between different meningococcal strains (Figure 1).

Whilst Tfp are essential for initial contact of encapsulated strains with epithelial or endothelial cells, they are lost during the establishment of intimate contact [40]. The transcriptional regulator CrgA, which is also involved in the downregulation of capsule synthesis (see above), appears to be involved in the repression of *pilE* expression under these conditions [22].

Role of the Secretome in BF

The secretome of *Nm* consists of several secreted and cell-surface-exposed proteins (Box 2) with various attributed roles in BF.

Role in Binding eDNA

A first indication for the role of the secretome in BF was the observation that deletion of the gene for the autotransporter NalP resulted in enhanced BF and alteration of biofilm architecture [6]. This effect was observed independent of whether the strain examined followed an eDNA-dependent or -independent strategy of BF. Interestingly, also in the latter case, the enhanced BF was dependent on eDNA as it was abrogated after DNase treatment of the cells. NalP is a protease that cleaves the complement factor C3, thereby facilitating survival of *Nm* in serum [41]. It also releases itself from the bacterial surface by autoproteolytic cleavage [42]. However, it remains temporarily anchored to the outer membrane by virtue of an N-terminal lipid moiety [43]. In this position, it can proteolytically release other surface-exposed proteins [42–44], including proteins important for BF. One of the targets of NalP is *Neisseria* heparin-binding antigen (NHBA). NHBA is a surface-exposed lipoprotein with an arginine-rich segment responsible for binding heparin [45]. Proteolytic cleavage by NalP releases the C-terminal half, including this positively charged segment, of a portion of the NHBA molecules from the cell surface [45]. As heparin, similar to DNA, is a highly negatively charged polymer, NHBA might also bind eDNA and, in this way, contribute to BF. This hypothesis was confirmed as purified NHBA was demonstrated to bind DNA and deletion of the *nhbA* gene impaired BF [6].

Another target of NalP is the autotransporter IgA protease [42], which cleaves, amongst others, IgA molecules and the lysosomal marker Lamp-1 [46,47]. The passenger of IgA protease contains two subdomains, the serine protease domain and the α-peptide [46,48]. The α-peptide is variable in length (~20–40 kDa) and sequence, and it contains either one or four positively charged regions [49], which function as nuclear localization signals. In the nucleus of a eukaryotic cell, IgA protease can cleave the transcription activator NF-κB, thereby preventing immune activation [50]. After transport to the bacterial cell surface, the entire passenger of IgA protease can be released into the milieu by NalP-mediated cleavage [42]. After uptake by a eukaryotic cell, this form of IgA protease can be targeted to the nucleus. When NalP is not expressed, the protease domain without the α-peptide can be released by autocatalytic processing [42], and the α-peptide remains at the cell surface covalently connected to the translocator domain, although there is some variation between strains in this respect [49]. The purified α-peptide was shown to bind DNA and heparin [6,49] presumably by virtue of its positively charged nuclear localization signals. Thus, the α-peptide that remains attached to the bacterial surface when NalP is not expressed could contribute to BF by binding eDNA. Indeed, BF was reduced when the gene for IgA protease was knocked out in a *nalP* mutant and further abrogated when the *nhbA* gene was also eliminated [6]. It is important to note that expression of NalP is prone to phase variation due to slipped-strand mispairing [42]. Examination of a large collection of isolates indicated that the gene was in the phase-off state in ~50% of isolates [51]. Thus, when NalP is not expressed, the surface-exposed NHBA and α-peptide mediate binding of the bacteria to eDNA, thereby increasing the ability to constitute a biofilm, while switching to NalP expression leads to release of NHBA and the α-peptide, which may promote biofilm dispersal.

Two other autotransporters, App and AusI, are released from bacterial cell surface with an attached α-peptide when NalP is expressed [42,52]. Both proteases were demonstrated to be targeted to the nucleus of eukaryotic cells, presumably by virtue of the attached α-peptides, where they were shown to cleave histone H3 and to promote apoptosis [53]. The α-peptide of App contains two positively charged arginine-rich segments similar to the nuclear localization signals in the α-peptide of IgA protease that may bind DNA. As in the case of IgA protease, the α-peptide of App remains associated with the cell surface in the absence of NalP [49] where it could potentially contribute to BF by binding eDNA. However, such a role has, so far, not been supported experimentally, possibly because of the low expression level of App relative to that of IgA protease [6]. The α-peptide of AusI does not contain obvious stretches of positively charged

residues that could function as nuclear localization signals, and no role for AusI in BF on abiotic surfaces could be demonstrated [6].

The gene for the autotransporter AutA is disrupted in ca. 75% of *Nm* strains, whilst its expression is prone to phase variation in the other strains [7]. When expressed, AutA is exposed at the surface but not released into the milieu. Its expression induces autoaggregation, which has drastic consequences on biofilm architecture. The biomass is organized in separate microcolonies without interconnections (Figure 1C). Aggregation is caused by interaction of AutA molecules on neighboring cells. In addition, AutA binds DNA, which may contribute to the autoaggregative properties depending on the strain background [7].

The data summarized above indicate that the bacteria stick together by binding eDNA via positively charged polypeptides that are exposed at the cell surface. Besides NHBA and the α-peptide of IgA protease, other proteins may contribute to eDNA binding under specific conditions. T and B cell-stimulating protein B (TspB) is encoded by prophages, several copies of which can be present in a genome. Expression of the genes is induced in the presence of human serum, and the protein was detected at the bacterial cell surface by flow cytometry [54]. How the protein is transported to the cell surface and into the medium is unclear; it is not part of released bacteriophages. The protein binds immunoglobulins with a preference for the Fc portion of human IgG2 [54]. It was shown to facilitate survival of *Nm* in the presence of normal human serum by inhibiting IgM-mediated activation of the classical complement pathway [55]. Besides immunoglobulins, the protein also binds eDNA, thus creating biofilm-like structures consisting of bacterial aggregates embedded in an ECM of eDNA, TspB and immunoglobulins. A similar matrix was formed by purified TspB, IgG, and DNA even in the absence of bacteria [55]. Thus, a variety of proteins facilitate binding of *Nm* to eDNA, but, since expression of TspB is induced by human serum, it is unlikely that it contributes to BF in the nasopharynx.

Other Functions

Several components of the secretome are implicated in BF by mediating adhesion to eukaryotic cells or interbacterial interactions. Although no role for App and AusI in BF on abiotic surfaces could be demonstrated, they were reported to function as adhesins. When expressed in *Escherichia coli*, both proteins mediated adherence to epithelial and endothelial cells [56,57]. Their function as adhesin was confirmed even in encapsulated *Nm*, suggesting that these large proteins (>1400 amino acid residues) extend beyond the capsule. App, but not AusI, also mediated bacterial aggregation on cell layers, suggesting that App promotes interbacterial interactions [56,57].

The surface-exposed trimeric autotransporters NhhA and NadA have been demonstrated to promote binding to various eukaryotic cells lines [58–60]. NhhA, but not NadA, also mediates binding to extracellular matrix proteins of the host [58,60]. Both proteins have additional functions; for example, NadA was shown to promote invasion of host cells [58], whilst NhhA protects against complement activation by binding activated vitronectin [61].

Two-partner secretion A (TpsA) proteins of *Nm* are implicated in BF [62]. Deletion of the *tpsA* gene resulted in a reduction in biofilm biomass formed on epithelial cells or collagen-coated coverslips in mature (48 h old) but not in early (6 h old) biofilms [62]. TpsA proteins of *Nm* are also involved in interbacterial competition [63] in a process known as contact-dependent growth inhibition (Box 3). TpsA–receptor and probably TpsA–TpsA interactions between neighboring cells [64] promote cell aggregation, and BF and may assist in kin recognition. Inhibiting the growth of other members in a biofilm via TpsA could be the basis for the generation of intercellular spaces and channels within biofilms and for the release of chromosomal DNA to

Box 3. Contact-Dependent Growth Inhibition

Contact-dependent growth inhibition (cdi) is mediated by a subclass of T5cSS (see Box 2). The secreted TpsA proteins with such function are often also called CdiA. They are produced in many different bacteria where they inhibit the growth of related bacteria usually of the same species [63,94]. These TpsA/CdiA proteins are large proteins (ca. 2000 amino acid residues) which display a small variable toxic domain of ca. 150 amino acid residues at the C terminus. TpsA displayed on the surface of a producer cell interacts with a receptor in the outer membrane of a target cell, which has been shown to be BamA, the central component of the outer-membrane-protein assembly machinery (the BAM complex) in one particular case in *Escherichia coli* [95]. The toxic domain is then cleaved off and imported into the target cells, where it can display, for example, DNase, RNase, or pore-forming activity. The producing cells are protected against the specific toxic activity by an immunity protein, called TpsI or CdiI, encoded by the gene immediately downstream of *tpsA*. The *tpsA* and *tpsI* loci are usually followed by a number of *tpsC* cassettes. These cassettes show homology with *tpsA* but they are shorter and lack a considerable part at the 5′ end encoding, amongst other entities, the signal sequence and the domain required for interaction with the translocator TpsB. In addition, these cassettes display very divergent 3′ ends, which encode different toxic domains. Recombination between *tpsA* and *tpsC* cassettes generates variation in the toxic domains displayed at the C terminus of TpsA [63]. Each *tpsC* is flanked by a cognate *tpsI* gene.

stimulate BF, but such putative roles still require experimental verification. Besides TpsA, another family of proteins with fratricide activities was recently described in *Nm*, that is, the MafB proteins [65,66]. Their possible role in BF has not yet been investigated.

Lessons from *Nm*: Valid for BF in Other microorganisms?

Research in *Nm* has shown that eDNA is a major component of the ECM of biofilms that functions as an adhesive to which the bacteria bind, mostly via cell-surface-exposed positively charged polypeptides. eDNA is also a major component of the ECM in many other bacteria, including Gram-negative [67–69], Gram-positive [70–72], and mycobacteria [73]. Possibly, these microorganisms use similar mechanisms for binding eDNA as *Nm* does. This could be the case, for example, for *Bordetella pertussis* [67], a Gram-negative bacterium that causes infections in the human upper respiratory tract. eDNA is an essential component of its biofilm matrix [67], and its filamentous haemagglutinin FHA is also essential for BF by mediating cell–cell and cell–substrate interactions [74]. FHA is secreted by the T5cSS (see Box 2) and it remains associated with the bacterial cell surface. Like NHBA and the α-peptide of IgA protease of *Nm*, FHA binds heparin [75], possibly via a conserved arginine-rich region (^{1069}RRARRALR^{1076}), and it is, therefore, likely to bind also eDNA. Interestingly, similar to NHBA and the IgA protease, FHA can be released from the cell surface by an autotransporter protease, SphB1 [76], which shows homology to NalP, thus allowing regulation of BF.

Listeria monocytogenes is an example of a Gram-positive bacterium that forms biofilms in an eDNA-dependent way [77]. DNase sensitivity of BF, however, is dependent on the ionic strength of the medium, suggesting that charge interactions are involved. ActA is a surface-exposed protein of *L. monocytogenes* that binds heparin and heparin sulfate, presumably via stretches of positively charged amino acids near the N terminus of the protein [78]. It seems likely that the protein also binds eDNA and thus contributes to BF under low ionic conditions. DNase-insensitive BF under high ionic conditions [77] may be explained by ActA–ActA interactions between neighboring cells, which have been shown to cause autoaggregation [79]. In another Gram-positive bacterium, *Streptococcus pneumoniae*, a positively charged domain of the surface-exposed protein PsrP was recently demonstrated to bind DNA and to promote eDNA-dependent cell aggregation and BF [80].

Mycobacterium tuberculosis also uses eDNA for BF [73] and the cell-wall-associated DNA- and heparin-binding protein MDP1 [81,82] and the heparin-binding haemagglutinin HBHA [83] could be candidates for binding the bacteria to the ECM. Not only bacteria, but also fungi, such as *Candida albicans*, require eDNA for BF [84]. Its virulence factor Int1 binds heparin through linear positively charged heparin-binding motifs, which could presumably also bind eDNA. Incubation of *C. albicans* with a specific antibody against such motifs prevented BF [85]. These examples

indicate that anchoring eDNA at the surface by DNA-binding proteins is a widespread mechanism for BF.

Concluding Remarks and Future Perspectives

It is well established that eDNA is an essential component of biofilms in many microorganisms. Research in *Nm* has revealed that eDNA functions as an adhesive to which bacteria bind via positively charged surface-exposed proteins. OMVs will also bind eDNA as they expose the same positively charged polypeptides, thus creating a complex network (Figure 2, Key Figure). BF and biofilm dispersal may be regulated by a surface-exposed protease that releases the DNA-binding proteins from the cell surface (Figure 2). Several examples described above suggest that anchoring microorganisms to eDNA via surface-exposed proteins is a widespread mechanism found not only in bacteria but even in fungi. This common mechanism may be of relevance in the generation of multispecies biofilms. Such biofilms can cause more severe infections as compared with monospecies biofilms, and they can be important in environmental

Outstanding Questions

Which structures and mechanisms are used for the eDNA-independent initiation of BF in cc8 and cc11 strains?

Do eDNA-binding proteins play a role in BF of capsulated strains on biotic surfaces?

How do contact-dependent growth-inhibition systems affect the formation and architecture of biofilms constituted of a single strain or of several strains of the same or different species?

Do heparin-binding proteins identified in other species also bind eDNA and are they involved in BF?

Do all eDNA-binding proteins in various species interact with eDNA via similar electrostatic interactions?

Which mechanisms regulate expression of eDNA-binding proteins in other microorganisms?

Are eDNA-binding proteins potential targets for the prevention of BF by novel antimicrobial compounds?

Key Figure

Model for Adhesive Properties of eDNA in *Neisseria meningitidis* (*Nm*) and Implication of NalP

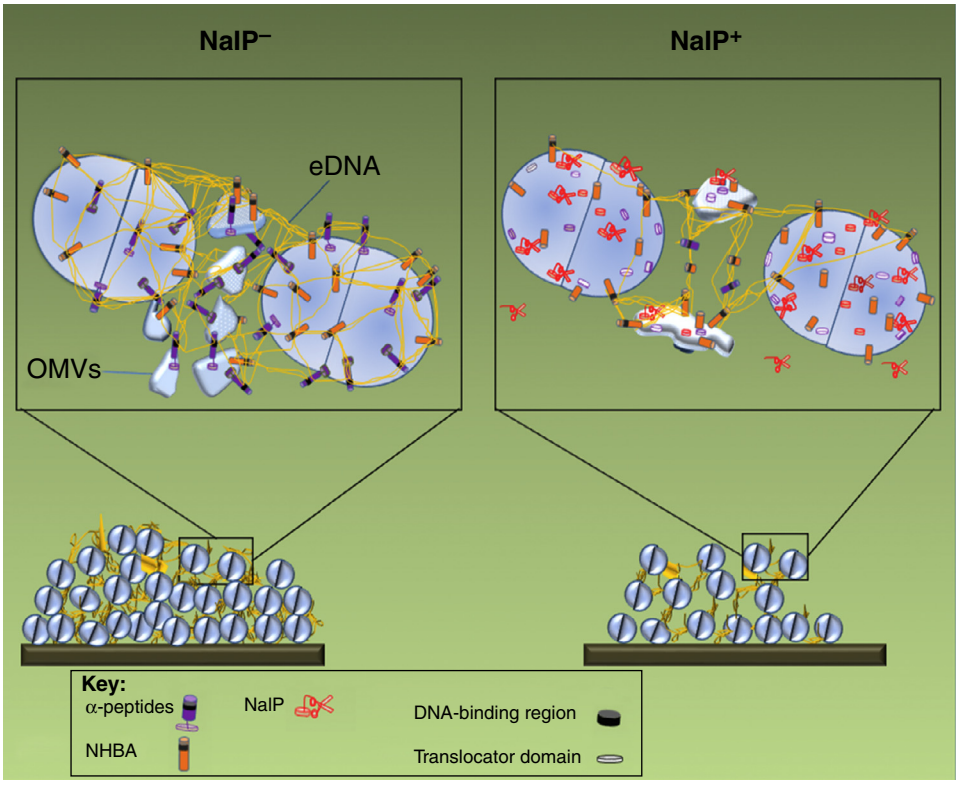

Figure 2. In the absence of NalP (left part), cell-surface-exposed, positively charged eDNA-binding proteins anchor entire cells and OMVs to eDNA (yellow), thus creating a complex network. When NalP is expressed (right part), eDNA-binding proteins are cleaved from the cell surface; thereby, the number of positive charges and interactions with eDNA are reduced, which allows for biofilm dispersal.

remediation [86]. The use of mechanisms to control anchorage by cleaving eDNA-binding proteins from the bacterial cell surface may also be more common than realized. Expression of these proteolytic enzymes may be controlled by regulatory or stochastic mechanisms, thus creating opportunities for bacteria to switch between biofilm and planktonic life styles. Hopefully, this review will stimulate researchers to explore the function of surface-exposed proteins with similar characteristics as those described for *Nm* on BF in other microorganisms (see Outstanding Questions).

Supplemental Information

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