How bacteria hack the matrix and dodge the bullets of immunity

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Bacterial pathogens adhere to human proteins in the lung to avoid being washed out. This can potentially be used as a strategy for therapeutical interventions. http://ow.ly/TW4P30kgGIB

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ABSTRACT *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* are common Gram-negative pathogens associated with an array of pulmonary diseases. All three species have multiple adhesins in their outer membrane, *i.e.* surface structures that confer the ability to bind to surrounding cells, proteins or tissues. This mini-review focuses on proteins with high affinity for the components of the extracellular matrix such as collagen, laminin, fibronectin and vitronectin. Adhesins are not structurally related and may be lipoproteins, transmembrane porins or large protruding trimeric auto-transporters. They enable bacteria to avoid being cleared together with mucus by attaching to patches of exposed extracellular matrix, or indirectly adhering to epithelial cells using matrix proteins as bridging molecules. As more adhesins are being unravelled, it is apparent that bacterial adhesion is a highly conserved mechanism, and that most adhesins target the same regions on the proteins of the extracellular matrix. The surface exposed adhesins are prime targets for new vaccines and the interactions between proteins are often possible to inhibit with interfering molecules, *e.g.* heparin. In conclusion, this highly interesting research field of microbiology has unravelled host–pathogen interactions with high therapeutic potential.

Introduction

40 years ago, Kuusela [1] reported that *Staphylococcus aureus* binds fibronectin to the bacterial cellular surface. During the following 40 years, research has aimed to discover vaccines and antimicrobial drug targets and has revealed intricate interactions between pathogens and the extracellular matrix (ECM).

Microbes enter the airway with inhaled air or by aspiration. Some bacteria reach the lower respiratory tract where the rich amount of nutrients, humidity and constant temperature fulfil most requirements for growth. The epithelium and mucus form the first host defence barrier against invading pathogens. Ciliated bronchial epithelial cells are covered by a thin fluid layer with the tips of the cilia reaching into the viscous mucus layer, propelling mucus and any trapped bacteria towards the oropharynx with their beats, where they are swallowed or expectorated [2]. Bacterial pathogens adhere to epithelial cells or any exposed ECM in order to prevent removal. Adherence is achieved by adhesins, which are bacterial surface structures that confer the ability to bind to surrounding cells, proteins or tissues. Both pili and flagella are adhesins, although adhesins may also be transmembrane proteins or lipoproteins. Bacterial adherence is a crucial step in bacterial colonisation and a prerequisite for subsequent invasion into host cells and further dissemination in the body.

The lungs of patients with chronic obstructive pulmonary disease (COPD) are commonly infected or in some cases colonised by Gram-positive *Streptococcus pneumoniae* or the Gram-negative *Haemophilus*

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influenzae, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* [3]. This mini-review focuses on the three Gram-negative bacterial species. *H. influenzae* and *M. catarrhalis* are human-specific pathogens that cause acute otitis media, sinusitis and bronchitis, and are frequent commensals in the human respiratory tract. Since the introduction of general immunisation programmes against *H. influenzae* type b (Hib), nontypeable *H. influenzae* (NTHi) has become an increasingly common pathogen [4]. In addition to infecting COPD patients during exacerbations, *P. aeruginosa* is feared for long-term colonisation of the lungs of cystic fibrosis patients [5].

The ECM is a dynamic meshwork of proteins surrounding cells in all mammalian tissues, with a composition that changes depending on external factors or injury. The main components are proteoglycans (*e.g.* heparan sulfate, perlecan and agrin), soluble glycoproteins (vitronectin and fibronectin) and fibrous proteins (collagen, elastin and laminin). These proteins cement cells together and organise them into tissues with different compositions and properties [6].

ECM proteins have multiple binding sites for cellular surface receptors and other ECM proteins. For instance, heparin-binding domains (HBDs) interact with cell-surface-bound heparan sulfate [7]. This domain is typically involved in ECM–bacterial interactions, suggesting that nebulised heparin theoretically may have a therapeutic role. Integrins act as cell surface receptors that anchor cells to the ECM and also link the ECM to intracellular signalling pathways. The integrins consist of heterodimers with one α and one β subunit that bind specifically to one or several protein domains. These surface molecules enable soluble glycoproteins to form links between the larger fibrils and cells, and to initiate cellular responses to changes in the surrounding environment.

Smoking, inflammation, viral infections and mechanical ventilation disrupt the respiratory epithelium and expose patches of ECM that are recognised by bacterial adhesins [8]. The ECM composition is also altered by smoking or chronic lung diseases. The laminin layer in basal laminas of smokers is thicker, and patients with COPD have increased bronchial deposition of fibronectin, laminin and collagen [9, 10]. Moreover, sarcoidosis or interstitial lung diseases are associated with elevated vitronectin and fibronectin levels in bronchoalveolar lavage fluid [11, 12].

TABLE 1 A few examples of bacterial adhesins in Gram-negative bacteria

ECM components interacting with	NTHi adhesins		*M. catarrhalis* adhesins		*P. aeruginosa* adhesins	
	Name	Characteristics	Name	Characteristics	Name	Characteristics
Collagen	Hap	K_d 20 nM (ELISA), auto-transporter [14]	MID/ Hag	Auto-transporter [15]		
			UspA2	Trimeric auto-transporter [16, 17]		
Fibronectin	Hap	K_d 15 nM (ELISA), auto-transporter [14]	UspA1	Trimeric auto-transporter [16, 18]	OprQ	OprD-family porin [19]
	P4	K_d 10.2 nM (ELISA), lipoprotein [20]	UspA2	Trimeric auto-transporter [16, 18]		
Vitronectin	Protein E	K_d 400 nM (surface plasmon resonance), lipoprotein [21, 22]	UspA2	K_d 23 nM (surface plasmon resonance), trimeric auto-transporter [16, 23, 24]	OprD	K_d 3.6 nM (biolayer interferometry), OprD-family porin [25]
	Protein F	K_d 12.8 nM (ELISA), ABC transporter [26]			Lpd	Moonlighting protein [27]
	P4	K_d 16.5 nM (ELISA), lipoprotein [20]				
Laminin	Hap	K_d 35 nM (ELISA), auto-transporter [14]	UspA1	Trimeric auto-transporter [16, 28]	Paf	Protein F orthologue [29]
	Protein E	K_d 1.5 μM (surface plasmon resonance), lipoprotein [22, 30]	UspA2	Trimeric auto-transporter [16, 28]		
	Protein F	ABC transporter [31]	AfeA	Protein F orthologue [29]		
	P4	K_d 9.3 nM (ELISA), lipoprotein [20]				

ECM: extracellular matrix; NTHi: nontypeable *Haemophilus influenzae*; *M. catarrhalis*: *Moraxella catarrhalis*; *P. aeruginosa*: *Pseudomonas aeruginosa*; Hap: *Haemophilus* adhesion and penetration protein; K_d : dissociation constant for adhesin–ECM interaction; MID: *M. catarrhalis* IgD-binding protein; Usp: ubiquitous surface protein; Opr: outer membrane porin; ABC: ATP-binding cassette; Lpd: dihydrolipoamide dehydrogenase.

Three different interactions between respiratory tract bacteria and the ECM are known: 1) bacterial adhesion to the ECM, 2) degradation of the ECM by bacterial proteases, and 3) antibacterial action of fragments of ECM proteins that are formed as these proteins are cleaved [13]. This mini-review focuses on adhesion, and examples of known adhesins are listed in table 1. Most recent studies describe the nature of the interactions, phenotypes of mutated bacteria and in some cases structural data of the full or truncated proteins, which will only be briefly reported here.

Bacterial interactions with collagen

Collagens are the most abundant proteins in the lung ECM [32]. They are the principal tensile element of tissues, shaped as elongated fibrils. Several α-chain monomers associate into a triple-helix form, which further associates to create various fibrillary or network arrangements [33]. The collagens are numbered with Roman digits according to their discovery and correspond to unique proteins composed of one, two or three gene products. Types I, II, III, IV and VI are of relevance in the respiratory tract. Type I collagen is present in all human tissues, whereas type II collagen associates with cartilage, including trachea. Type III collagen is present in connective tissues and the network-forming type IV is a major component of the

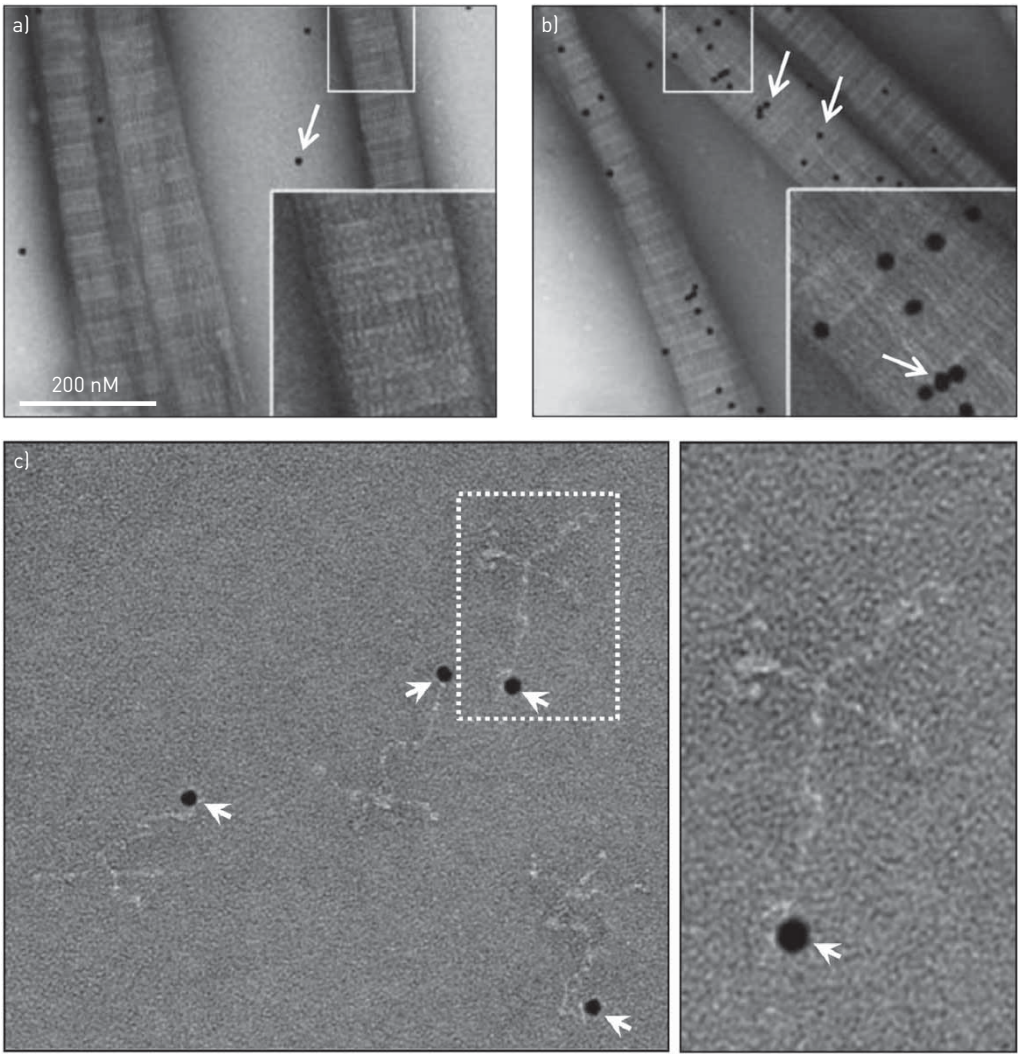

FIGURE 1 a and b) *Moraxella catarrhalis* ubiquitous surface protein (Usp) A2 binds collagen with high affinity. Transmission electron microscopy (TEM) demonstrates that a) gold-labelled recombinant UspA1 does not bind to collagen I fibrils, whereas b) UspA2 does. Reproduced and modified from [17] with permission. c) Bacterial adhesins target laminin globular domains at the base of the asymmetrical cross-shaped laminin. TEM image showing gold-labelled Protein F from nontypeable *Haemophilus influenzae* bound to laminin. Reproduced and modified from [31] with permission; these images are not included under the Creative Commons CC BY-NC 4.0 licence of the current article. In all panels, gold-labelled recombinant bacterial proteins are marked with white arrows.

basal lamina that anchors epithelial cells to the mesenchyme. Finally, collagen type VI is abundantly present in the ECM of the respiratory tract and, like collagens I and III (in addition to laminin), is upregulated in COPD [17]. During epithelial damage, collagens are exposed in the airway mucosa and accessible to intruding bacteria [10]. However, only a few pathogens have been described to bind collagen directly. The majority use surface-bound fibronectin as a bridging molecule [34].

Clinical *H. influenzae* isolates are known to bind collagen I and IV [35]. NTHi binds to collagen using *Haemophilus* adhesion and penetration protein (Hap), an adhesin that belongs to the auto-transporters. Hap promotes adhesion to epithelial cells and causes bacterial aggregation, which facilitates colonisation of the respiratory mucosa. It also mediates bacterial invasion, leading to an intracellular bacterial reservoir that may be responsible for recurrent infections seen in COPD [36]. Hap has a β-barrel domain anchoring it in the outer membrane and a protruding passenger domain with protease activity. It binds multiple ECM components simultaneously and binds to laminin and fibronectin using different domains [14, 37].

All tested *M. catarrhalis* clinical isolates bind collagens I, II, III, IV and VI, but the binding capacity varies between isolates. *M. catarrhalis* IgD-binding protein (MID, also known as Hag) is structurally similar to Hap and has been reported to bind collagen [15, 17]. MID also confers adherence to type II alveolar cells and is highly immunogenic. However, recent evidence suggests that the ubiquitous surface protein (Usp) A2 and the related UspA2H are more important for collagen binding (figure 1a and b) [17]. The UspAs are trimeric auto-transporter adhesins with a lollipop structure. A membrane anchor is embedded in the outer membrane, and the stalk, neck and head domains extend outwards 800 Å from the cell, where they form a densely packed molecular coating [16]. The UspAs bind several components of the ECM and are crucial for the virulence of *M. catarrhalis* (figures 2 and 3). Mice with smoke-induced COPD are

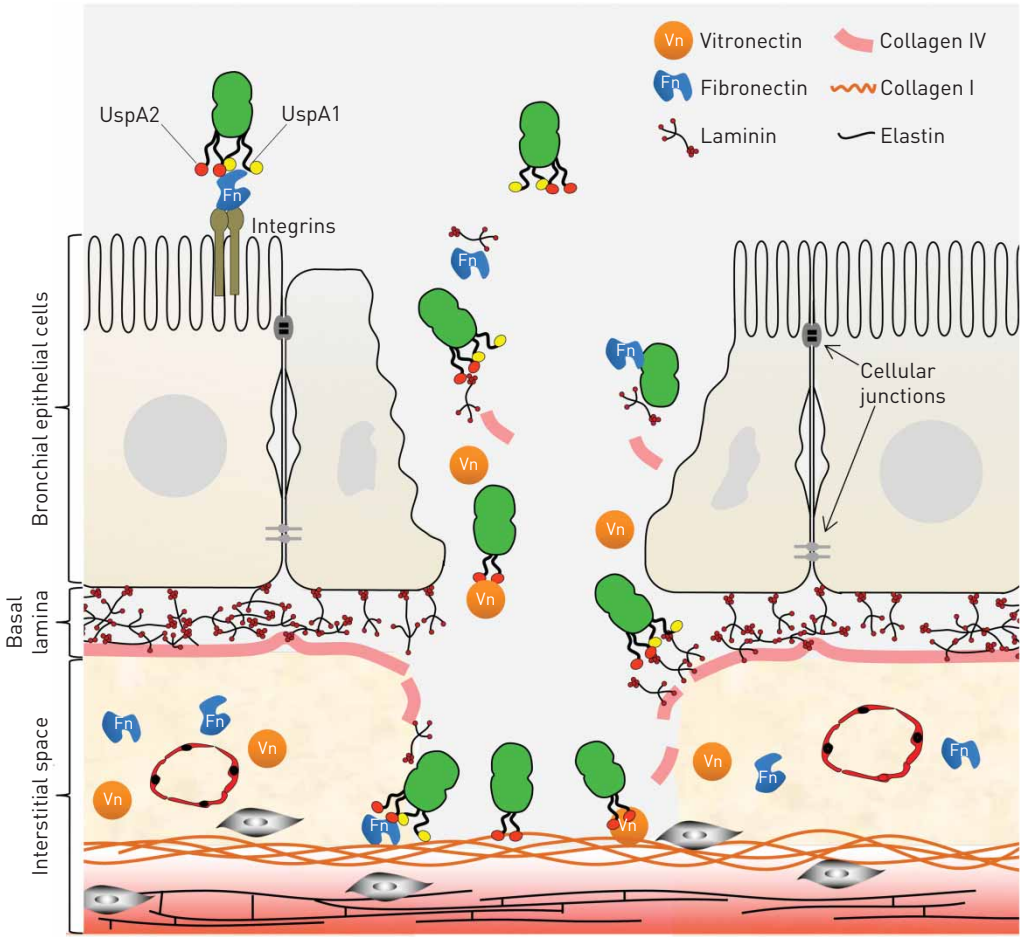

FIGURE 2 *Moraxella catarrhalis* adheres to the extracellular matrix in the respiratory tract. Cartoon that illustrates a disrupted bronchial epithelium and how *M. catarrhalis* colonises the human lung by using ubiquitous surface protein (Usp) A1 and UspA2 to adhere to laminin, collagen, fibronectin (Fn) and vitronectin (Vn). For simplicity, MID and AfeA were omitted from this cartoon. Reproduced and modified from [17] with permission.

colonised about four times less efficiently by mutated bacteria lacking UspA2 [17]. Most patients with COPD have high titres of antibodies directed both against the adhesive domain of MID and the ECM-binding domains of UspA1/A2 [38]. However, the binding regions of *M. catarrhalis* UspAs are highly diverse; thus, the bacterium evades recognition by IgG and complement-mediated attacks [39].

P. aeruginosa adheres to collagen I, tracheal collagen II and collagen IV in basal lamina, but surface receptors are yet to be described. *P. aeruginosa* collagen binding is inhibited by heparin, suggesting binding to a conserved heparin-binding domain of the collagen molecule [40]. During long-term *P. aeruginosa* colonisation, adherence to sessile ECM or cells may be of less importance than "hiding" in the self-produced biofilm and binding to mucin, for which it has high affinity [41]. Exposing adhesins may in this setting be counterproductive, as maintaining adhesion needs to be balanced against detection by the immune system.

Fibronectin as a bacterial target

Fibronectin is a soluble multi-adhesive matrix protein. Its primary role is to attach cells to other components of the ECM, especially collagen. Most bacterial pathogens have evolved strategies to utilise fibronectin for adhesion to cells or to collagen by using fibronectin as a bridging molecule. Fibronectin attaches to the cell surface as fibrils that are continuously replaced or endocytosed. Bacteria with fibronectin on the surface exploit the same mechanism to become internalised by human cells [42]. However, macrophages phagocytose fibronectin-covered bacteria and internalisation poses a threat to the bacteria, although, for example, NTHi is able to replicate and attract nutrients intracellularly [43].

NTHi binds fibronectin using Hap and lipoprotein P4, although the initial reports indicated that a pilus was responsible for the binding [14, 20]. P4 is a lipoprotein that is involved in nicotinamide adenine dinucleotide uptake and haemin utilisation, both essential nutrients for this species. P4 binds ECM components, including laminin and vitronectin, using a central α-helix. In addition, P4-dependent NTHi adhesion is observed to type II alveolar cells and bronchial epithelium. P4 is necessary for long-term survival in mouse lung and mutant strains devoid of P4 are attenuated in the otitis media *Junbo* mouse model [20, 44].

M. catarrhalis uses both UspA1 and UspA2 for fibronectin binding [18]. Intriguingly, during "cold shock" (26°C), the UspAs are upregulated. This facilitates bacterial colonisation by enhancing the adhesive properties of *M. catarrhalis* at physiologically relevant temperatures in the upper respiratory tract [45].

The genome of *P. aeruginosa* is almost four times larger than that of NTHi or *M. catarrhalis*, causing a redundancy of adhesins. Already by 1993 it was discovered that *P. aeruginosa* uses fibronectin for adherence to collagen. In 2002, at least six fibronectin-binding proteins were suggested. Eventually, OprQ (outer membrane porin Q), a porin belonging to the OprD superfamily, was identified as a fibronectin-binding adhesin [19]. The OprDs are β-barrel-shaped transmembrane proteins that transport

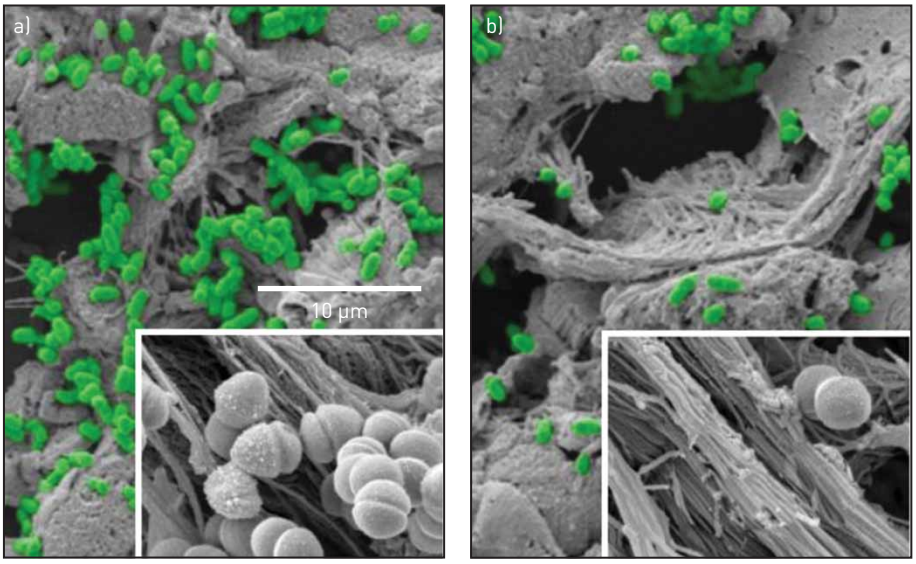

FIGURE 3 Scanning electron micrographs showing that a) *Moraxella catarrhalis* bacteria (in green pseudocolour) adhere to human tracheal specimens, whereas b) UspA2-deficient mutants do not. Reproduced and modified from [17] with permission.

nutrients into the cell. Imipenem enters the periplasm through OprD, which is frequently mutated in carbapenem-resistant strains [25].

Vitronectin is used by respiratory pathogens in various ways

The glycoprotein vitronectin is mainly produced in the liver, although bacterial stimulation triggers local production in the respiratory tract by alveolar epithelial cells [46]. More than a "cell glue", vitronectin plays homeostatic roles and regulates the terminal pathway of the complement cascade, where it inhibits formation of the cytolytic membrane attack complex (MAC). Most Gram-negative bacteria are sensitive to lysis by MAC and are killed by it unless complement regulators are recruited at the bacterial surface, *i.e.* they are serum resistant. Escaping complement by recruiting complement regulators is thus a key characteristic for respiratory pathogens. The majority of tested pathogenic bacteria recruit vitronectin to the cellular surface using the same C-terminal HBD3, which is inhibited by low concentrations of heparin [47].

Similarly to fibronectin, vitronectin is used as a bridging molecule between bacteria and human cells. NTHi adheres to bronchial epithelial cells *via* vitronectin using Protein E [48]. This adhesin is a conserved lipoprotein in the *Pasteurellaceae* family and other unrelated species (*e.g. Enterobacter cloacae* and *Listeria monocytogenes*) [22]. Although Protein E is a high-affinity adhesin, mutants devoid of Protein E have residual vitronectin-binding capacity. Protein F and P4 have since been identified as additional vitronectin receptors in NTHi [20, 21, 26].

The main vitronectin receptor in *M. catarrhalis* is the lollipop-shaped UspA2 [16, 23]. Mutated bacteria lacking UspA2 are highly sensitive to killing by human serum [23]. The interaction between UspA2 and vitronectin is independent of ionic strength and heparin inhibition [24]. A recent study comparing vitronectin-binding capacity of several bacterial species reported that *M. catarrhalis* and group A streptococci have higher vitronectin-binding capacity than other species tested [45].

P. aeruginosa also utilises vitronectin for adhesion to mammalian cells and to evade complement-mediated attack. Two vitronectin receptors for *P. aeruginosa* are known, OprD and dihydrolipoamide dehydrogenase (Lpd) [25, 27]. Lpd is involved in pyruvate metabolism in the cytoplasm, but is also exposed on the bacterial surface where it binds multiple complement regulators and components of the ECM.

Laminin is an important molecule for bacterial attachment

Laminins are large ECM proteins (~800 kDa) and major components of the basal lamina. They are composed of three polypeptide chains (α, β and γ), which together form an asymmetrical cross containing five globular domains and a coiled-coil region. The globular domains interact with a range of integrins, heparin sulfate proteoglycans and other receptors, contributing to cell–ECM attachment but also to cell shape and differentiation, maintenance of tissue phenotypes and to the stability and integrity of the basal lamina. There are several laminin isoforms. Embryonic laminin-111 (α1, β1 and γ1) is the isoform used in all cited papers. Based on tissue-specific localisation, laminin α5 is, however, more relevant to include in future studies since this isoform appears in the lung [32].

To date, NTHi has four known laminin-binding adhesins: Hap, Protein E, Protein F and P4 [14, 20, 30, 31]. All of these are multifunctional ECM-binding proteins but share no sequence similarity with each other, although both Protein E and Protein F bind to the laminin globular domains 4–5 (figure 1c) [31]. These globular domains contain the binding sites for heparin; hence, the interaction with these bacterial adhesins competes with heparin.

M. catarrhalis binds laminin using UspA1 and UspA2. While the "head" of the UspA molecules interacts with laminin, the stalks interact with fibronectin, both contributing to adhesion [28]. In addition, *M. catarrhalis*, *P. aeruginosa* and several other species have Protein F orthologues (*e.g.* AfeA in *M. catarrhalis* and Paf in *P. aeruginosa*) that interact with the globular domains [29].

Concluding remarks

Respiratory bacteria interact with the ECM through a multitude of adhesins with overlapping functions. Adherence to the ECM is a conserved mechanism among pathogenic bacteria and is most likely to be important during colonisation of the respiratory tract.

The prospect of developing adhesin-based vaccines has motivated the research field. There are examples from other bacterial species where surface proteins have been successfully used in vaccinology, *e.g.* factor H-binding protein in *Neisseria meningitidis* serogroup B vaccine. NTHi has received more attention after the introduction of a Hib vaccine, and several of the adhesins listed in table 1 have been or are currently being evaluated for their immunogenic and protective properties. Considering the urgency for a vaccine against *P. aeruginosa*, it is surprising that less is known about adhesins in that species than in *M.*

catarrhalis. There have, however, been clinical trials on vaccines against *P. aeruginosa*, but they have hitherto been unsuccessful.

As most bacterial adhesins target HBDs in the ECM proteins, the use of nebulised heparin for patients with high risk of pulmonary infections is theoretically tempting. In a recent meta-analysis, three out of four studies reported increased bacterial clearance after nebulised heparin was administered [49]. Unfortunately, this did not protect against ventilator-associated pneumonia or hasten recovery from pneumonia in patients receiving mechanical ventilation [50].

In summary, much effort has been made in exploring the interactions between the ECM and respiratory bacteria and several possible vaccine targets and targetable host–pathogen interactions have been suggested. The next few years will tell if the therapeutic potential can be translated into clinically useful vaccines or antimicrobial drugs.

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