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Murphy, Elizabeth; Janulczyk, Robert; Karlsson, Christofer; Mörgelin, Matthias; Frick, Inga-Maria

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Identification of pili on the surface of *Finegoldia magna* — A Gram-positive anaerobic cocci

Elizabeth C. Murphy [a,*], Robert Janulczyk [a,b], Christofer Karlsson [a], Matthias Mörgelin [a], Inga-Maria Frick [a]

[a] *Department of Clinical Sciences, Division of Infection Medicine, Lund University, SE-22184, Sweden*
[b] *Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy*

ABSTRACT

Pili have only been discovered in the major Gram-positive pathogens in the past decade and they have been found to play an important role in colonisation and virulence. Pili have been shown to have many important functions including attachment to host tissues, mediating bacterial aggregation, biofilm formation and binding to proteins in the extracellular matrix. In this study, sortase-dependent pili have been found to be expressed on the surface of *Finegoldia magna* ALB8. *F. magna* is a Gram-positive anaerobic coccus that, primarily, is a commensal of the skin and mucous membranes, but has also been isolated from various clinical infection sites and is associated with soft-tissue abscesses, wound infections and bone and prosthetic joint infections.

In this study, *F. magna* ALB8 was found to harbour three sortases at the pilus locus, two of which bear high similarity to class C sortases in *Streptococcus pneumoniae*. Two putative sortase-dependent pili proteins were found in the locus, with one being identified as the major pilus subunit, Fmp1 (*F. magna* pilus subunit 1), due to its high similarity to other major pilus proteins in prominent Gram-positive pathogens. The presence of sortase-dependent pili was confirmed experimentally through recombinant production of Fmp1 and production of antiserum. The Fmp1 antiserum was used in Western blot to show the presence of a high molecular weight protein ladder, characteristic of the presence of pili, in trypsin released cell wall surface proteins from *F. magna*. The presence of sortase-dependent pili was visually confirmed by transmission electron microscopy, which showed the binding of gold labelled anti-Fmp1 to individual pilus proteins along the pilus. Furthermore, pili could also be found to bind and interact with keratinocytes in the epidermal layer of human skin, suggesting an adhesive role for pili on *F. magna*.

Our work represents the first description of pilus structures in *F. magna*. This discovery further elucidates *F. magna* physiology and allows for additional analysis of host—bacterial interactions in future studies.

1. Introduction

Finegoldia magna (previously *Peptostreptococcus magnus*) is a commensal bacterium colonising human skin, mucous membranes and the gastrointestinal and urogenital tracts [1]. As well as being a commensal, it can also act as an opportunistic pathogen and is a member of the Gram-positive anaerobic cocci (GPAC). Among the Gram-positive anaerobic bacteria associated with clinical infections GPAC are the most prominent group, accounting for 25—30% of all anaerobic bacteria isolated from clinical specimens [1]. *F. magna* has the highest pathogenicity of the GPAC, being the most common GPAC isolated from clinical infection sites [1]. It is associated with soft tissue abscesses, bone and joint infections, wound infections and vaginosis [1—6]. *F. magna* is isolated from both mixed and pure infection sites, where approximately 70% of *F. magna* strains have been found to co-exist with other bacteria, such as group D streptococci, *Staphylococcus*, *Bacteroides* and *Fusobacterium* [7]. Problems in obtaining good quality anaerobic specimens and difficulties in culturing GPAC, mean that their abundance and importance, as both commensals and opportunistic pathogens, is probably highly underestimated.

* Corresponding author. Tel.: +46 46 222 81 68.
  E-mail addresses: Elizabeth.Murphy@med.lu.se (E.C. Murphy), janulczyk@yahoo.se (R. Janulczyk), Christofer.Karlsson@med.lu.se (C. Karlsson), Matthias.Morgelin@med.lu.se (M. Mörgelin), Inga-Maria.Frick@med.lu.se (I.-M. Frick).

Possible reasons for the prominence of *F. magna* in GPAC infections could be due to the expression of proteins that enhance virulence. Previously identified virulence factors in *F. magna* include the superantigen protein L, the albumin binding protein PAB, protein FAF (*F. magna* adhesion factor) and the subtilase-like enzyme SufA [8–12]. Other important pathogenicity factors of *F. magna* include capsule formation and production of the enzymes collagenase and gelatinase [13,14].

In this study, another potential virulence factor was identified — pili on the surface of *F. magna*. Pili are long filamentous structures extending from the cell surface and are present on most bacterial pathogens [15]. Pilus-like structures were first observed in Gram-negative pathogens in the early 1950s, but have only recently been identified in some of the more prominent Gram-positive pathogens [15]. In the past decade, pili have been characterised in the three most significant streptococcal pathogens — group A *streptococcus* (*Streptococcus pyogenes*) [16], group B *streptococcus* (*Streptococcus agalactiae*) [17,18] and *Streptococcus pneumoniae* [19]. Two types of pilus-like structures have thus far been identified in Gram-positive pathogens: short, thin rods or fibrils that extend between 70 and 500 nm from the bacterial surface and longer more flexible rods up to 3 μm long [15].

Rod-like pili were first described in *Corynebacterium diphtheriae* and were found to consist of three covalently linked protein subunits, each consisting of a variant of the cell wall sorting sequence (canonically LPXTG where X denotes any amino acid), which is the target of sortase enzymes. Sortases act as important virulence factors, effectively helping Gram-positive bacteria interact with the host and environment by covalently attaching proteins to the cell wall or polymerising proteins in the biosynthesis of sortase-dependent pili [20–22]. They function as cysteine transpeptidases that connect proteins with a cell wall sorting signal (CWSS) to an amino group of peptidoglycan cross-bridges at the cell surface [23,24]. Variants of the cell wall sorting sequence, with their cognate sortases, lead to a diverging enzymatic reaction. For example, the prototypical reaction involves sortase class A targeting the substrate motif LPXTG of surface proteins and anchors the proteins to the cell wall envelope [25,26]. However, sortase class B targets the NP(Q/K)TN motif of haem transport factors and cross-links the anchored haem-containing products near membrane transporters [27–29]. Pilus proteins have the substrate motif (I/L)(P/A)XTG and are generally targeted by class C sortases, which catalyse a transpeptidation reaction forming covalent links between individual pilus proteins [27,30]. There is also a class D sortase, which targets the LPNTA motif of mother cell and endospore envelope proteins [24]. In order for pilus formation to occur, specific sortases catalyse the covalent binding of pilus subunits to each other by non-disulphide covalent linkages [31,32]. Usually, sortase-dependent pili consist of one main (backbone) protein and one or two ancillary proteins, which can occur at the tip of the pilus, along the side or at the bacterial surface, anchoring the pilus to the bacterial surface [15]. One of the most important functions of sortase-dependent pili is the adhesion and attachment to host cells during colonisation and invasion. Furthermore, pilus components from invasive streptococci have a significant homology to the microbial surface components recognizing the adhesive matrix molecules family of proteins (MSCRAMMs) [15,33]. Sortase-dependent pili have also been shown to mediate bacterial aggregation, which could contribute to the colonisation of tissues, increase the bacterial resistance to the host immune response and aid beneficial interactions between different bacterial species [15,34–36].

In this study, we describe the identification of sortase-dependent pili on the surface of *F. magna*, which sheds further light into its molecular mechanisms for both infection and commensalism and paves the way for further analysis.

2. Methods

2.1. Bacteria and growth conditions

F. magna strain ALB8 isolate was from Lund University Hospital, Lund, Sweden and has been described earlier [10]. *F. magna* ALB8, from which the virulence factors, SufA and FAF have been produced, was originally isolated from a scrotal abscess [11,12]. *F. magna* strain 505, which does not express FAF, but does express SufA, was isolated from a urethra infection site. *F. magna* strains 312, 1458, 1459, 1462, 1468, ATCC 29328, 17.30 and 2186 were also from Lund University Hospital, Lund, Sweden and were isolated from various clinical infection sites. All strains were a kind gift from Dr. Elisabet Holst at the Department of Clinical Microbiology, Lund University Hospital, Sweden and have been described before [11]. No ethical approval was required. *F. magna* was grown in Todd-Hewitt broth (TH) (Difco) supplemented with 0.5% Tween-80 at 37 °C under strict anaerobic conditions.

2.2. Proteins, antibodies and reagents

Antibodies against recombinant protein Fmp1 were raised in rabbits (Biogenes). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was purchased from Pierce.

2.3. Cloning, expression and purification of protein Fmp1

For the cloning and sequencing of *fmp1*, primers were designed based on the genome sequence of *F. magna* ALB8 (NCBI accession number: BA93_05025). Primers were designed to contain the *Bam*HI and *Xho*I restriction sites for later insertion into a pGEX_6p_1 vector. The 5′ forward primer 5′-CAGCAGGGATCC-GAACACAACTGGAATGCT-3′ and 3′ reverse primer 5′-CAGCAGCTC-GAGTGATTCAAGTTTTGCTTCGTA-3′ were used in PCR to amplify a 2209 bp fragment (bp 130-2339, removing the signal peptide and cell wall sorting signal) of *fmp1* from *F. magna* ALB8 chromosomal DNA. The gene was cloned into pGEX_6p_1 (Amersham Pharmacia) and positive clones were sequenced (Eurofins MWG Operon). The Fmp1 protein was expressed in *E. coli* BL21 (Life Technologies), fused to GST, by growing to an OD of 0.6 and induced to express Fmp1 by the addition of 0.5 mM IPTG for 3 h at 37 °C. Bacteria were lysed by the addition of 50 μg/ml lysozyme and freeze thawing of the cell pellet. Soluble proteins were clarified through centrifugation and analysed on a 12% SDS-PAGE gel. Fmp1 was later purified on glutathione Sepharose according to the manufacturer's instructions. The GST-tag was cleaved off using PreScission protease (Amersham Pharmacia).

2.4. Slot blot, SDS-PAGE and western blot analysis

SDS-PAGE was performed as described by Neville et al. [37]. Samples were prepared for boiling in sample buffer containing 2% SDS and 5% β-mercaptoethanol for 5 min and 12% SDS-PAGE were used to study Fmp1 expression. Separated proteins were visualised by PageBlue protein staining solution (Thermo Scientific). To examine for the presence of sortase-dependent pili, samples from trypsin digestion (see method below) were concentrated by trichloroacetic acid precipitation and resuspended in NuPAGE LDS sample buffer (Life Technologies). Concentrated protein samples were separated on 3–8% NuPAGE Tris-Acetate gels (Life Technologies). For Western Blot analysis, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Membranes were blocked in a phosphate buffered saline-Tween (PBS containing 0.1% Tween 20 (PBS-T)) solution containing 5% (wt/vol) skim milk powder at 37 °C for

30 min. The membranes were then incubated with Fmp1 (15 ng/μl) for 1 h at 37 °C in blocking buffer. Membranes were washed three times with PBS-T for 5 min followed by incubation with primary antibodies (rabbit anti-Fmp1 1:1000 dilution) in blocking buffer at 37 °C for 30 min. Membranes were washed three times with PBS-T for 5 min followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody (1:3000 dilution) in blocking buffer at 37 °C for 30 min. Following a repeat of the wash steps, bound antibodies were detected by chemiluminescence as described by Nesbitt et al. [38].

2.5. Release of F. magna cell surface proteins by trypsin digestion

Different strains of F. magna were grown to stationary phase and washed once with trypsin cleavage buffer (0.05 M KH_2PO_4, 0.005 M EDTA, pH 6.1) and adjusted to 2×10^9 bacteria/ml in the same buffer. One millilitre of each bacterial suspension was incubated with 20 μg trypsin (Sigma) for 60 min at 37 °C. The enzymatic reaction was terminated by the addition of 20 μg trypsin inhibitor (Sigma). Bacteria were removed by centrifugation at $3000 \times g$ for 15 min and the supernatant containing the trypsinised proteins was removed to a fresh tube. The supernatant was concentrated with 5% trichloroacetic acid precipitation and the entire precipitate was analysed for the presence of sortase-dependent pili by Western blotting (see method above).

2.6. Negative staining and transmission electron microscopy

F. magna ALB8 and 505 were grown to stationary phase and washed with Tris buffered saline (TBS) (50 mM Tris–Cl, 150 mM NaCl, pH 7.5) and adjusted to 2×10^9 bacteria/ml in TBS. As a control, 1 ml of a 2×10^9 bacteria/ml of F. magna ALB8 was treated with trypsin, as described above. Antiserum to Fmp1 was conjugated to 5 nm gold colloids, followed by incubation with 4×10^6 bacteria for 30 min at room temperature. Samples were prepared for negative staining by adsorption onto 400 mesh carbon-coated copper grids and staining with 0.75% (w/v) uranyl formate as described [39]. Samples were observed in an FEI Tecnai Spirit Bio-TWIN transmission electron microscope (North America NanoPort, Hillsboro, OR) operating at an accelerating voltage of 60 kV. Images were recorded using an Eagle™ CCD camera.

For transmission electron microscopy of skin sections, a stationary phase culture of F. magna ALB8 was washed twice in PBS and the concentration adjusted to 2×10^9 bacteria/ml in PBS. Skin specimens were obtained as excess healthy tissue from skin surgery, under protocols approved by the Ethics Committee at Lund University (permit No. LU 762-02). A 4 cm^2 section of a skin specimen was washed 3 times in PBS and 4 mm circular punches of epidermis were extracted. A measure of 2×10^9 bacteria was incubated with each skin biopsy for 1 h at 37 °C under anaerobic conditions. Following incubation, skin sections were removed to a 24 well plate, washed 3 times with PBS to remove unbound bacteria, followed by addition of 500 μl MEM (Life Technologies). Skin sections with bound bacteria were incubated for 72 h at 37 °C under anaerobic conditions. The skin specimen was then added to 1 ml of 2.5% glutaraldehyde in cacodylate buffer overnight at 4 °C. Samples were washed with cacodylate buffer and post-fixed for 1 h at room temperature in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded series of ethanol, and then embedded in Epon 812 (SPI Supplies) using acetone as an intermediate solvent. Specimens were sectioned with a diamond knife into 50–70 nm thick ultrathin sections on an LKB ultramicrotome. Immunolabelling was carried out with the primary antibody (rabbit anti Fmp1 antiserum, titre 1:50) followed by detection with secondary antibody conjugated with 5 nm gold (titre 1:10), and analyzed using a

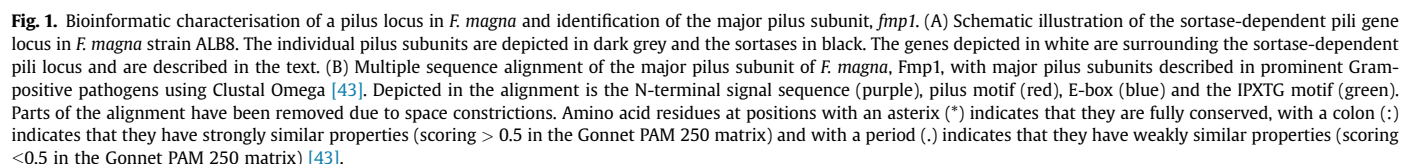JEOL JEM 1230 transmission electron microscope (JEOL, Peabody, MA) as previously described [40].

3. Results

3.1. The F. magna ALB8 genome contains a pilus locus

The recent publication describing the complete genome sequence of F. magna ATCC 29328 highlighted the presence of a large number of sortases, four encoded on the chromosome and seven encoded on plasmid pPEP1 [41]. The potential presence of sortase-dependent pili in F. magna led us to investigate the recently sequenced ALB8 strain of F. magna. Using the RAST server [42], four sortases were identified on the chromosome of F. magna ALB8. One of these was predicted to be a housekeeping sortase (SrtA) and the remaining three were found clustered together in a gene locus, adjacent to genes encoding proteins with predicted cell wall attachment signals. By analogy with previously described pilus-encoding loci, we hypothesized that these genes encode two putative pilus subunits, see Fig. 1(A). The three putative sortases have been designated srt959, srt960 and srt961 (NCBI accession numbers BA93_05035, BA93_05040 and BA93_05045 respectively) and the pilus subunits fmp1 (F. magna pilus subunit 1) and fmp2 (NCBI accession numbers BA93_05025 and BA93_05030 respectively). Downstream of the pilus locus are two genes − a putative membrane protein haemolysin III homologue and a hypothetical protein. Upstream of the pilus locus is a very large protein, of 3787 amino acid residues, with 26.53% identity (Clustal Omega) to a fibronectin binding protein in the same position on a pilus locus in Streptococcus pyogenes [43]. It also contains many Cna protein B-type domains (BlastP), which are found in the collagen binding surface protein of Staphylococcus aureus [44]. This protein could possibly be located on the cell surface and mediate binding to host proteins such as collagen or fibronectin. Examination of the protein sequences of both pilus subunits identified a characteristic pilus motif in Fmp1. The pilus motif is thought to be important for pilus polymerisation as it contains a lysine residue that provides a nucleophile through its ε-amino group for the nucleophilic attack of the bond linking the pilus subunit and the sortase [15]. The typical pilus motif found in the majority of pilus subunits, which have been characterised is VYPK [45]. Fmp1 was also found to contain an E-box, which contains a highly conserved glutamic residue and is thought to determine the specificity of the pilus subunits for the sortases [46]. It also contains a characteristic IPXTG amino-acid motif (where X denotes any amino acid), which is targeted by sortase enzymes [15]. The pilus motif and E-box were not found in Fmp2, so this is likely to be an accessory pilus subunit rather than a major pilus subunit. To investigate the similarity between the major pilus subunit of F. magna and other characterised pilus subunits from prominent Gram-positive pathogens (S. pyogenes, Streptococcus agalactiae and Streptococcus pneumoniae), an alignment was made using Clustal Omega, see Fig. 1(B) [43]. The high sequence identity seen between the four proteins, in particular at the pilus motif, strongly suggests that Fmp1 is a major pilus protein.

3.2. Classification of sortases in F. magna

Bioinformatics was employed to classify the sortases. An earlier work [47] proposes a classification scheme and provided a downloadable hidden markov model (HMM) to test new sortases [48]. The HMM was applied on the sortases in the pilus locus and the putative SrtA. Srt960 and Srt959 were designated as members of sortase family 3, with scores clearly exceeding the original criteria described (threshold for sortase family 3 is 150, while Srt960 = 487

A

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                                  srt
              fmp1        fmp2   959  960  961
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B

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S.pyogenes_MGAS10750   MKT--KIFSRLAAVLLVLGSLLPTVVAVA-----EAESSHKTDVVIHKIKMTSLKGWPKE    53
F.magna_ALB8           MKK--RFLSL----MLVLAMMVGVFTPLIAN---AADAEHKTKVNIHKILMKEH-NWNAK    50
S.agalactiae_2603V     MKRINKYFAMFSALLLTLTSLLSVAPAFADE-------ATTNTVTLHKILQ---------    44
S.pneumoniae_SP18      MKSINKFLTMLAALLLTASSLFSAATVFAADNVSTAPDAVTKTLTIHKLLL---------    51
                       **    : ::     :*.  :. . .       .. : :**:

                                  ............174 residues............

S.pyogenes_MGAS10750   ADGYYWFVENSGS---NIANGETLSSSAAVPFGLELPVYKADGST----ITELHVYPKNT   210
F.magna_ALB8           KDGEYRIVEDKAKSTYKGENGETLTGMKAVPFDLVLPIGKPDGSGDYSEKDPLNVYPKNT   284
S.agalactiae_2603V     LKGEFKIVEVKSKSTYN-NNGSLLAASKAVPVNITLPLVNEDGVV-----ADAHVYPKNT   204
S.pneumoniae_SP18      LKGVYRIREDRTKTTYVGPNGQVLTGSKAVPALVTLPLVNNNGTV-----IDAHVFPKNS   186
                       .* : :  *    .      **. *:.  *** : **: : :*    .*:***:

S.pyogenes_MGAS10750   TTKPKIDKNFSKDEKDA-------ALAGGANYDYYQKDKGYVSRIIGSEVSYQIKTEIPA   263
F.magna_ALB8           EKKVKFDKNFAKDNGLEKITDPNKLKDVGAVFDNYEKEKANAEAEIGKEIKYEAKAFLPK   344
S.agalactiae_2603V     EEKPEIDKNFAKTNDLTALTDVNRLLTAGANYGNYARDKATATAEIGKVVPYVEVKIKHH   264
S.pneumoniae_SP18      YNKPVVDKRIADTLNYN--------------------DQNGLSIGTKIPYVVNTTIPS    224
                       *  .**.::.                          **. : * :: :

                                  ............336 residues............

S.pyogenes_MGAS10750   --KPNSQAIEKAKKARDDAFKKARTAYEWVSDKQKAVKFTSNSKGQFEVTGLAAGTYYLE   623
F.magna_ALB8           TKDKALAKVAGLQKTRDEAFVTANLNYTWVDKQEDATQFFTNKDGQFEVYGIAYGNYRAV   740
S.agalactiae_2603V     KGITAKELIKTKQADYDAAFIEARTAYEWITDKARAITYTSNDGQFEVTGLADGTYNLE   620
S.pneumoniae_SP18      DKQAAQALVDQAQQEYNVAYKEAKFGYVEVAGKDEAMVLTSNTDGQFQISGLAAGTYKLE   558
                       :    :  : *:  *.  *  :  *    :* .***:: *:* *.*

S.pyogenes_MGAS10750   EVAAPTGFAKL--QEKVEFTLGFNSYNGHKDQSGQSLEGHTQYEKGTPDFGYGQRVINKK   681
F.magna_ALB8           ERKAAPGFALDTNKENFKFVVNKGSYAGNNTEYKYE----AKL-EKDQKQTYGKRIDNKK   795
S.agalactiae_2603V     ETLAPAGFAKL--AGNIKFVVNQGSYITGG-NIDYV---------ANSNQKDATRVENKK   668
S.pneumoniae_SP18      EIKAPEGFAKI--D-DVEFVVGAGSWNQ-G-EFNYL---------KDVQKNDATKVVNKK   604
                       *  * ***     ..:*.:  *:        *       .  . :: ***

S.pyogenes_MGAS10750   ITIPQTGGGIGTVIFTVVGLAIMTVTGLMMIRRNKNDKSE-   720
F.magna_ALB8           VTIPETGGGIGTIIFTAIGLAIMASAVIAIKKRQATEAR--   833
S.agalactiae_2603V     VTIPQTGGGIGTILFTIIGLSIMLGAVVIMKRRQSKEA---   705
S.pneumoniae_SP18      ITIPQTGGGIGTIIFAVAGAAIMGIAVYAYVKNNKDEDQLA   644
                       :***:*******:;*:  * :** :    :.: :
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Fig. 1. Bioinformatic characterisation of a pilus locus in *F. magna* and identification of the major pilus subunit, *fmp1*. (A) Schematic illustration of the sortase-dependent pili gene locus in *F. magna* strain ALB8. The individual pilus subunits are depicted in dark grey and the sortases in black. The genes depicted in white are surrounding the sortase-dependent pili locus and are described in the text. (B) Multiple sequence alignment of the major pilus subunit of *F. magna*, Fmp1, with major pilus subunits described in prominent Gram-positive pathogens using Clustal Omega [43]. Depicted in the alignment is the N-terminal signal sequence (purple), pilus motif (red), E-box (blue) and the IPXTG motif (green). Parts of the alignment have been removed due to space constrictions. Amino acid residues at positions with an asterix (*) indicates that they are fully conserved, with a colon (:) indicates that they have strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix) and with a period (.) indicates that they have weakly similar properties (scoring ≤0.5 in the Gonnet PAM 250 matrix) [43].

and Srt959 = 482). This family is typical for previously described pilus-associated sortases in *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae*. Srt961 and SrtA were unclassified when using the strict threshold of 150. However, both proteins showed borderline scores for a single family (Srt961 = 121 family 3, and SrtA = 136 SrtA, all other families scored less than 55), suggesting that Srt961 and SrtA belong to family 3 and SrtA, respectively. Further classification of the *F. magna* sortases was carried out by examining the percentage identity between all of the sortases in *F. magna* strain ALB8 and the sortases in *S. pneumoniae* strain TIGR4. Sortase classification facilitates the allocation of sortases into putative classes. Of all the Gram-positive bacteria, which have had sortase-dependent pili described, *S. pneumoniae* is the closest relation to *F. magna*. In addition, the *S. pneumoniae* TIGR4 sortases have already been characterised [49]. A percentage identity matrix was constructed, using Clustal Omega, to measure identity between the amino acid sequences of the sortases of *F. magna* ALB8 and *S. pneumoniae* TIGR4, allowing predicted functions to be assigned to the ALB8 sortases, see Table 1 [43]. The *S. pneumoniae* sortases are labelled SrtA (NCBI accession number: NP_345684) and SrtC-1, SrtC-2 and SrtC-3 (NCBI accession number: NP_344986, NP_344987 and NP_344988 respectively). As the name suggests, SrtA has already been assigned as a class A sortase [49]. SrtC-1, SrtC-2 and SrtC-3 have all been assigned to the class C sortase group [49]. As shown in Table 1, SrtA has highest identity (34.26%) with Srt562, suggesting Srt562 is most likely the house-keeping class A sortase,

SrtA of *F. magna* ALB8. SrtC-1 has a very high identity with Srt959 of *F. magna* ALB8 (61.85%), higher than even its identity with its neighbouring sortase, SrtC-2 (51.34%). SrtC-2 has highest percentage identity with Srt960 (54.37%). SrtC-1, SrtC-2, Srt959 and Srt960 all have percentage identities of between 50.19% and 61.85%, suggesting they are all very similar in structure and function. Both SrtC-3 and Srt961 do not share such a high identity either to each other (27.14%) or the other sortases. LeMieux and co-workers found that none of the class C sortases in *S. pneumoniae* were essential on their own for polymerisation of the pilus backbone subunit RrgB, as

Table 1
Percentage identity matrix of sortases in *S. pneumoniae* TIGR4 vs *F. magna* ALB8.

	SrtC-3	*Srt961*	SrtC-2	*Srt960*	*Srt959*	SrtC-1	*Srt562*	SrtA
SrtC-3	100	27.14	24.37	25.10	23.86	26.42	18.45	17.39
Srt961	27.14	100.00	24.80	29.32	26.42	25.59	20.10	13.79
SrtC-2	24.37	24.80	100.00	54.37	50.19	51.34	16.49	24.35
Srt960	25.10	29.32	54.37	100.00	57.40	54.62	18.40	21.05
Srt959	23.86	26.42	50.19	57.40	100.00	61.85	18.78	24.35
SrtC-1	26.42	25.59	51.34	54.62	61.85	100.00	17.82	23.48
Srt562	18.45	20.10	16.49	18.40	18.78	17.82	100.00	34.26
SrtA	17.39	13.79	24.35	21.05	24.35	23.48	34.26	100.00

The sortases in *S. pneumoniae* TIGR4 are SrtA, SrtC-1, SrtC-2 and SrtC-3 (unitalicised) and the sortases in *F. magna* ALB8 are Srt562, Srt959, Srt960 and Srt961 (italicised). The identity matrix was constructed by Clustal Omega using a multiple protein sequence alignment [43].

mutants produced of each sortase, still produced polymerised sortase-dependent pili [50]. Possibly, due to their high similarity, SrtC-1 and SrtC-2 could act as a back-up for each other in pilus assembly. Both SrtC-1 and SrtC-3 were found to be responsible for incorporating the accessory subunits, RrgA and RrgC on the pilus, but the mechanism is not yet fully understood. Upon analysis of the amino acid sequences of Srt959, 960 and 961, both Srt959 and Srt960 were found to contain the conserved catalytic site TLXTC, with a conserved proline residue located two residues towards the C-terminus, see Fig. 2. This is characteristic of class C sortases, and along with their high percentage similarity to SrtC-1 and SrtC-2, they are most likely also class C sortases. The results of these identity scores are in agreement with the classification used above, which looked at hidden markov models, confirming the presence of one sortase A and three sortase C sortases in the *F. magna* ALB8 genome. Note that sortase class C is directly corresponding to sortase subfamily 3 [49]. Thus, both Srt959 and Srt960 may be involved in the incorporation of the backbone pilus subunit, Fmp1 into the pilus. On the other hand, Srt961, in contrast with SrtC-3, was not found to contain a conserved catalytic site, see Fig. 2. As *F. magna* ALB8 contains only two pilus subunits, compared to *S. pneumoniae* TIGR4, which has three pilus subunits, it may not be necessary for *F. magna* to produce all three sortases at the sortase-dependent pili locus. It may only need Srt959 and Srt960 for incorporation of the major and minor pilus subunit and expression of the third sortase, Srt961 may be unnecessary.

3.3. Cloning and expression of recombinant Fmp1 and purification using glutathione sepharose

To further characterise the pilus of *F. magna*, the backbone pilus protein, Fmp1, was recombinantly produced. The PCR product was purified, cloned and sequenced as described in the Materials and Methods section. Expression of Fmp1 was carried out in *E. coli* BL21, see Fig. 3(A). In Lane 2, over-expression of a protein band close to 130 kDa can be seen. This band is close to the predicted molecular weight of the recombinant protein, including the GST tag, of 108 kDa. The protein band can also be seen in the soluble fraction in Lane 3 of the gel, showing that Fmp1 is being produced in the cytoplasm of *E. coli* in soluble form. The fusion protein was recombinantly expressed and purified by affinity purification, see Fig. 3(B). After removal of the GST tag, the recombinant Fmp1 migrated close to the predicted molecular weight of 81.3 kDa (ProtParam) [51]. The low level of smaller contaminating bands is probably due to protein degradation and self-aggregation as they could not be removed by further purification methods. Following purification, antiserum to recombinant Fmp1 protein was raised in rabbits.

3.4. Trypsinised F. magna ALB8 cell wall extracts produce a characteristic high molecular weight ladder when probed with antiserum to Fmp1

Cell wall extracts of *F. magna* ALB8 were produced using trypsin, see Fig. 4(A). These protein extracts were separated on 3–8% gradient gels and immunoblotted using antiserum specific for the Fmp1 protein, see Fig. 4(B). This blot shows a high molecular weight protein ladder in lane 2 that starts at just below the 250 kDa marker and extends upwards to beyond the resolution of the gel. Furthermore, as can be seen from the positive control, the antiserum also binds to the monomeric protein, Fmp1, which migrates slightly higher than its predicted molecular weight of 81.3 kDa. This abnormal migration of pilus monomeric units has also been seen for SpaA in *C. diphtheriae*, which migrates at 68 kDa instead of its predicted molecular mass of 50 kDa [32]. The high molecular weight protein ladder that interacts with the Fmp1 antiserum, represents covalently polymerised major pilus subunits of various lengths that have been released from the surface of *F. magna* through trypsin cleavage and has been seen in many other pilated Gram-positive bacteria [22]. The results from this western blot confirm the bioinformatic analysis already carried out, that *F. magna* ALB8 expresses sortase-dependent pili on its surface. Several other *F. magna* strains including 505, 312, 1458, 1459, 1462, 1468, ATCC 29328, 17.30 and 2186, were also strained for the

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Srt961    ------MRS--SRKIGY-VILFVGIGILTFCLSNLSYKIFSQDKKMDDFLRSQGEMST-E    50
Srt959    MRKVIVMWKKIKHNFVFILIFLLGFGVMMYPVISRLYYRVDSANQVKEFDKAVKGLSKEE    60
Srt960    -----MKKSKLKENLKYVLIFLVGLAVAMYPLISRLYYRIEANSEVHDFDTTAKKMDKKE    55
                 .  ..:: : :*:::*:.:  : : .  *   ..  .:::..:*  :   :..  *

Srt961    LSERIEKY-NSNVNVDETGLMDPFNSEDIE---LEGSKVIDENGIFGYLDIPKINFKKPI   106
Srt959    LAKRLALAKGYNDSLNNVVSKDPYDKKNQEEGRKAYARMLEVKEKIGHVEIPKINQDLPI   120
Srt960    IDRRIRLAQIYNKTLDPSKLADPYSEKEKKNAISEYARMLEVKEKIGHVEIPKINQDLPI   115
          : .*:     *  .::    **:..:: :   :::::  : :*::***** . **

Srt961    YLGASYNHLAKGVGQLDGTSIPVGGKGRRSVLAGHRGWYGDIMLLKVGQLYSNDVVYVHH   166
Srt959    YAGTSEDVLQIGVGHLEGTSLPIGGNNTHAVLTAHSGLPTAKLFTDLNKMKIGDKFYVHN   180
Srt960    YAGTSEEVLQKGVGHLEGTSLPVGGKSTHTVLTAHRGLPTAKLFTDLNKMKKGDVFFIHN   175
          * *:*: : *  ***:*:***:*:**:  ::**:.* *     :: .: :   *  .::*.

Srt961    HGKTLTYKVVGRQYISPSDWDMLKPVEGKDML<span style="color:red">TILSC</span><span style="color:green">D</span>PMYPPFTRRILINCVRVDENSG   226
Srt959    IFGTLAYQVDQIKIVEPSNFNDLLIVKGHDYC<span style="color:red">TLLTC</span><span style="color:green">T</span>PIMINSH-RLLVRGHRVPYVPA   239
Srt960    IKEVLAYKVIDIFVVEPSDFDKVLVVEGKDYC<span style="color:red">TLLTC</span><span style="color:green">T</span>PYMINSH-RLLVRAERTEYNPA   234
          .*:*:*    :.**::: : *:*:* *:*:* *     *:*:. *.  .

Srt961    KTGVDARSDKKNTTQLNTKNDSSSKTVDYVL---LAFTILAWIVLIVNIRKFV-LDIRKD   282
Srt959    VDEALIA-----------DNKASYIYRYLFYVSLVIIAILLYLIYRQRKARKKLRELDVE   288
Srt960    AVEKDLV-----------SQVT-GKYKIYLIISLIIIAILLWYYIRQRRRKKN-NEIK--   279
                    .:: :       .   : ::**  :    .  :     ::

Srt961    RK----    284
Srt959    DKHEEV    294
Srt960    ------    279
```

Fig. 2. Multiple sequence alignment of the three class C sortases of *F. magna* ALB8. Depicted in the alignment is the TLXTC conserved catalytic site (red) and the conserved proline residue (green). Amino acid residues at positions with an asterix (*) indicates that they are fully conserved, with a colon (:) indicates that they have strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix) and with a period (.) indicates that they have weakly similar properties (scoring ≤0.5 in the Gonnet PAM 250 matrix) [43].
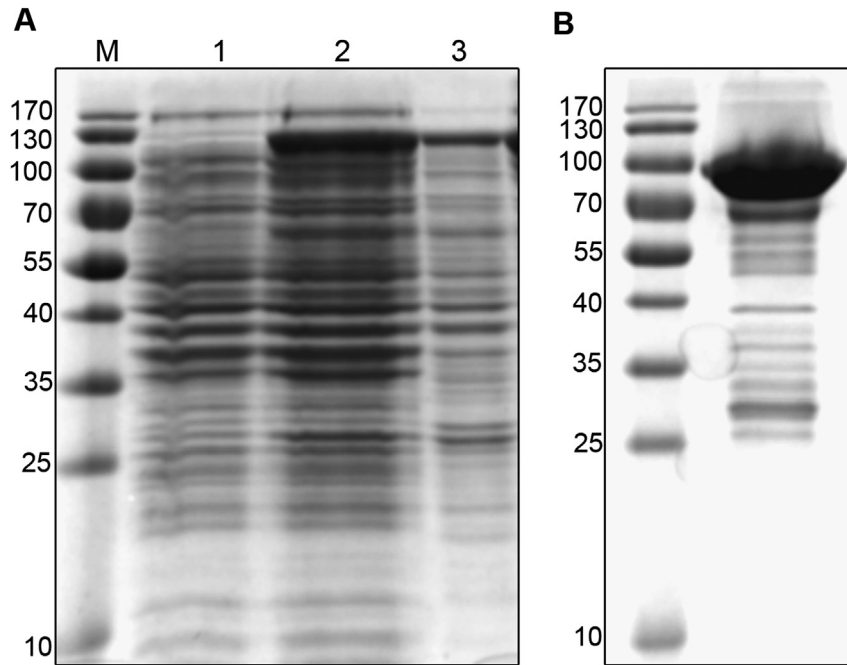
Fig. 3. Expression and purification of the recombinant Fmp1 protein. (A) Analysis of Fmp1 expression in *E. coli* BL21 by 12% SDS-PAGE. M: PageRuler Plus Prestained Protein Ladder (Thermo Scientific); Lane 1: Uninduced *E. coli* BL21; Lane 2: Induced *E. coli* BL21; Lane 3: Soluble fraction *E. coli* BL21. (B) Purification of Fmp1 by glutathione sepharose and removal of the GST tag by PreScission Protease. The purified protein was analysed on a 12% SDS-PAGE gel. M: PageRuler Plus Prestained Protein Ladder (Thermo Scientific); Lane 1: 7.6 μg purified Fmp1.

presence of sortase-dependent pili in their trypsinised cell wall extracts. However, none were found to contain Fmp1 pili backbone homologues on their surface. *F. magna* 505 is shown in Fig. 4(B), Lane 4 as a representative of these findings. This could signify that

these strains contain no sortase-dependent pili, or that the pilus backbone protein has a different DNA sequence. Future genome sequencing projects will shed light on this.

3.5. Gold labelled antiserum to Fmp1 identifies pilus structures on the surface of F. magna ALB8 and pilus interaction with keratinocytes of human skin

In order to visualise pilus structures on the surface of *F. magna*, immunogold transmission electron microscopy was carried out using gold labelled Fmp1 antiserum, see Fig. 5. As a negative control for this experiment, *F. magna* strain 505 was included, as trypsinised cell surface extracts of this strain were found to be unresponsive to the Fmp1 antiserum, suggesting that it does not express Fmp1. In addition, another control in the form of ALB8 pre-treated with trypsin was included to show that trypsin cleaves pilus structures from the bacterial surface, as seen in the Western blot in Fig. 4(B). As shown in the electron micrograph in Fig. 5(B), sortase-dependent pili structures can be visualised on the surface of ALB8. Significantly, the gold labelled antibody can be seen binding to monomeric subunits of the major pilus protein, Fmp1, throughout the length of the pilus. This result confirms our bioinformatic analysis that Fmp1 is, in fact, the major pilus subunit and is incorporated into the pilus in multiple copies. In the absence of the gold labelled antiserum in Fig. 5(A), faint traces of sortase-dependent pili structures can be seen, but these are much more apparent in (B). No Fmp1 containing sortase-dependent pili structures could be visualised for *F. magna* 505 or trypsin treated *F. magna* ALB8, and only some low-level background binding can be seen in the presence of the gold labelled Fmp1 antiserum. The low level binding seen in (F) could be due to the presence of Fmp1 monomers released from the cell wall by trypsin. However, the background binding seen in (D) may not be due to the presence of Fmp1 monomers, as western blot analysis of *F. magna* strain 505 trypsin cell wall extracts, found no Fmp1 sortase-dependent pili
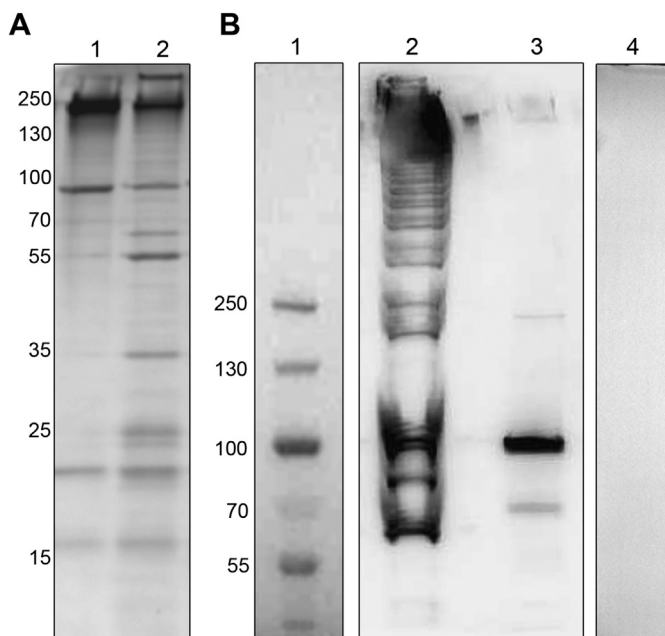

Fig. 4. Presence of sortase-dependent pili on the surface of *F. magna* is confirmed by immunoblotting. (A) Trypsinised cell wall extracts of *F. magna* were analysed by electrophoresis on a 12% SDS-PAGE gel. Lane 1: *F. magna* 505; Lane 2: *F. magna* ALB8. (B) Trypsinised cell wall extracts were concentrated, separated on a 3–8% gradient gel and probed with Fmp1 antiserum on a Western blot. Lane 1: PageRuler Plus Prestained Protein Ladder (Thermo Scientific); Lane 2: Concentrated *F. magna* ALB8 cell wall protein extract; Lane 3: recombinant Fmp1 positive control (0.5 μg); Lane 4: Concentrated *F. magna* 505 cell wall protein extract.
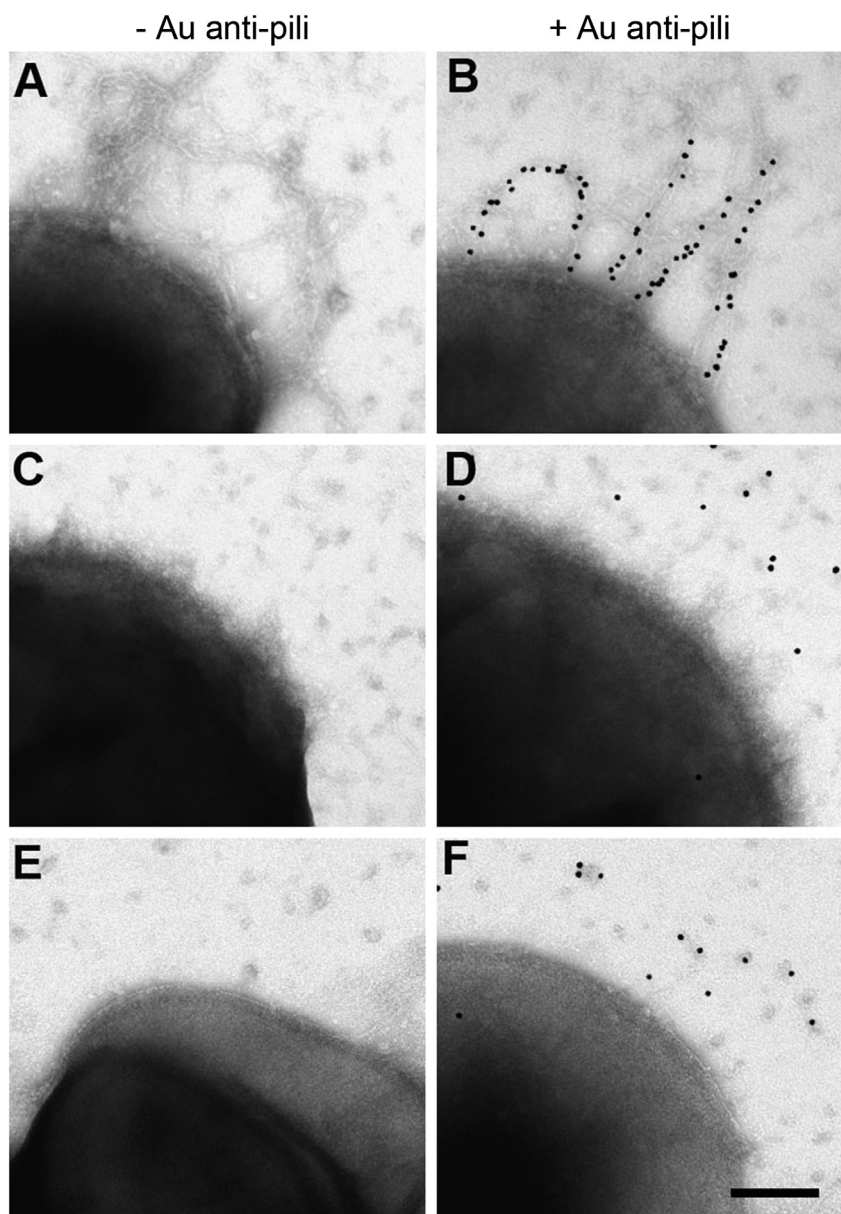
Fig. 5. Transmission electron micrographs visualising sortase-dependent pili on the surface of *F. magna*. *F. magna* strains ALB8 and 505 were grown to stationary phase and then incubated with Fmp1 antiserum labelled with colloidal gold. As a control, *F. magna* ALB8 was also treated with trypsin to illustrate removal of sortase-dependent pili from the cell surface. (A) *F. magna* ALB8 (B) *F. magna* ALB8 with gold labelled Fmp1 antiserum, (C) *F. magna* 505, (D) *F. magna* 505 with gold labelled Fmp1 antiserum, (E) *F. magna* ALB8 trypsinised, (F) *F. magna* ALB8 trypsinised with gold labelled Fmp1 antiserum. The scale bar represents 100 nm.

monomers present. However, it is hard to say, definitively, that *F. magna* strain 505 does not express Fmp1, without genome sequence data. Quantification of total amount of pili structures on the cell surface from 300 bacteria, not considering binding of the gold labelled Fmp1 antibody, identified pili on 78% of ALB8, 41% of 505 and 6% of the trypsinised ALB8 bacteria. These percentages indicate efficient trypsinisation of the cell surface of ALB8 and also suggests that *F. magna* 505 does express pili structures, but these do not contain homologous subunits to the *F. magna* ALB8 pilus.

Further electron microscopy studies were carried out to examine the interaction of pili on the surface of *F. magna* ALB8 with components of human skin, see Fig. 6. Following a 72 h incubation with a human skin biopsy, *F. magna* ALB8 bacteria, as indicated by black arrows, can be seen to have penetrated into the deeper layers of the epidermis, see Fig. 6(A). Under higher magnification and immunolabelling using anti Fmp1 antiserum, pili on the surface of

F. magna ALB8 can be seen interacting and binding to the surface of a keratinocyte, see Fig. 6(B). As was seen in Fig. 5(B), the anti Fmp1 antiserum appears to bind to individual Fmp1 backbone subunits along the pilus. In Fig. 6(C), pili on the bacterial surface can be seen binding to keratin in the epidermis.

These electron micrographs visually confirm the presence of pilus structures on the cell surface of *F. magna* ALB8. They also suggest a biological function for this structure as they can be seen in the direct binding of *F. magna* ALB8 bacteria to keratinocyte cells in the epidermis.

4. Discussion

In the past decade, sortase-dependent pili have been increasingly identified in Gram-positive bacteria, both in the major enterococcal and streptococcal pathogens and in commensals, such as lactobacilli
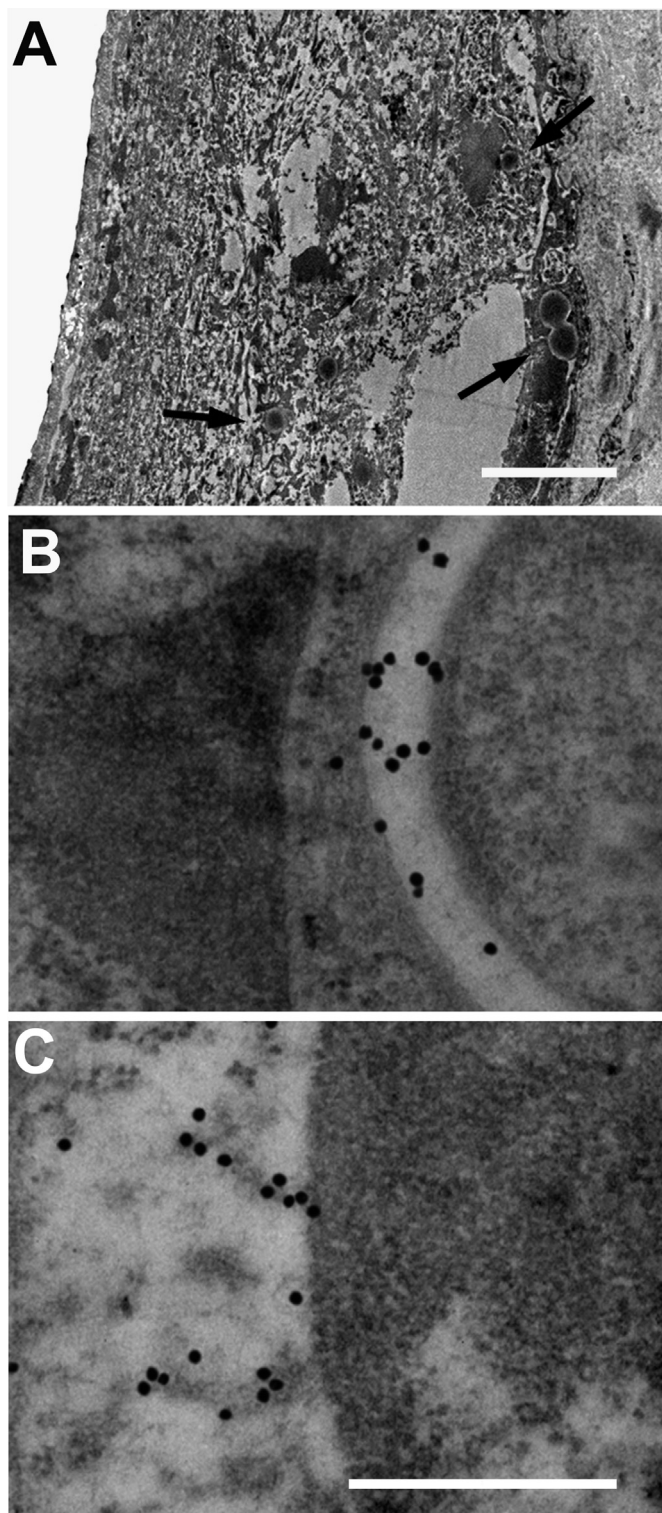
Fig. 6. Transmission electron micrographs visualising *F. magna* sortase-dependent pili interacting with keratinocytes in human skin. *F. magna* strain ALB8 was incubated with a human skin biopsy followed by immunolabelling with anti Fmp1 antiserum. (A) Trans section of human skin epidermis with black arrows pointing to *F. magna* ALB8 bacteria. The scale bar represents 5 μm. (B) Gold labelled Fmp1 pili on the surface of *F. magna* ALB8 interacting with a human keratinocyte (C) Gold labelled Fmp1 pili on the surface of *F. magna* ALB8 interacting with keratin in the epidermal region of human skin. The scale bar represents 100 nm.

and bifidobacteria. Here we describe for the first time, the presence of sortase-dependent pili in the commensal and opportunistic pathogen, *F. magna*. Sortase-dependent pili have been reported to have many functions, with the main role being an adhesive organelle, enabling bacterial colonization of host tissues [45]. However, they have also been associated with biofilm formation, adhesion to proteins of the extracellular matrix and interaction with the innate immune system [45]. *F. magna* is found as a commensal in the skin, oral cavity and in the gastrointestinal and urogenital tracts, so production of sortase-dependent pili could help in adhesion and effective colonisation of host tissues at these sites [2,52].

The sortase-dependent pili locus in *F. magna* ALB8 was found to contain two structural sortase-dependent pili subunits, Fmp1 and Fmp2. Based on amino acid sequence analysis, Fmp1 was predicted to be the major pilus backbone protein as it contained an IPXTG sorting motif, a pilus motif and an E-box − all features characteristic of major pilus proteins [15]. The locus also contained three sortases, which have been predicted to be class C sortases based on analysis with sortases in *S. pneumoniae*. Class C sortases have been shown to be involved in pilus formation and are characterised by the presence of a C-terminal hydrophobic domain and a catalytic site TLXTC, followed by a conserved proline residue.

Furthermore, sortases Srt959 and Srt960 of *F. magna* contained the characteristic catalytic site TLXTC followed by a conserved proline residue, strengthening their identity as class C sortases. The third sortase, Srt961 was not found to contain any catalytic motif so it could have possibly been inactivated as it may be no longer required for sortase-dependent pili polymerisation. The locus contains only two pilus subunits, unlike *S. pneumoniae*, which contains three, so it may only require two sortases for polymerisation. The fourth sortase in the genome of *F. magna* ALB8, Srt562, was classified as a class A sortase. Class A sortases have a broad substrate range and are responsible for anchoring proteins with an LPXTG cell wall sorting signal (CWSS) and are usually not clustered with their substrates [49].

Further characterisation of the pilus was carried out by recombinant production of the major pilus subunit, Fmp1. Trypsinised cell wall extracts separated on a gradient gel and immunoblotted with Fmp1 antiserum displayed the characteristic high molecular weight ladder of protein bands, representing covalently bound sortase-dependent pili of different lengths released from the *F. magna* cell surface. Visual confirmation of the presence of sortase-dependent pili on the cell surface was seen using transmission electron microscopy. In this study, gold labelled Fmp1 antibodies bound to individual monomers of the Fmp1 backbone protein all along the pilus. In addition, trypsinisation of the *F. magna* ALB8 cell surface was shown to be effective in releasing sortase-dependent pili from the cell surface and as a result, only low background binding to trypsin released Fmp1 monomers was seen.

Further transmission electron microscopy studies using ultra thin sections of human skin demonstrated the likely ability of pili on the surface of *F. magna* to adhere to keratinocytes in the epithelial layer of human skin. This could be an important function for sortase-dependent pili in *F. magna* as adherence to host tissue via keratinocytes could assist in initial colonisation by *F. magna* of human skin. It could also assist *F. magna* in moving through the epithelial layers and to set up colonisation at deeper tissue sites. As can be seen in Fig. 6(A), *F. magna* ALB8 that were added to the surface of a skin biopsy, were able to bind and penetrate to the basement membrane layer of human skin and this could have been aided through binding of its pili to keratinocytes.

Fmp2, the minor pilus subunit, could function either as an anchoring pilus linking the whole polymer to the cell wall, or an accessory pilus, in which it could be located either at the tip or decorated along the sides, possibly functioning as an adhesin and in

binding host proteins [15]. An analysis carried out using the Conserved domain database to search for conserved domains in Fmp2 found two Cna protein B-type domains [53]. This domain was originally found in the collagen-binding surface protein of *S. aureus* and it is thought that this domain forms a stalk to present the collagen binding domain away from the cell surface [54]. The presence of this domain in Fmp2 suggests that this minor pilus protein could also have an adhesive function and bind to extra-cellular matrix proteins. Future studies will examine the role that Fmp2 plays on the pilus.

The Fmp1 antiserum was used to probe trypsinised cell wall extracts of other *F. magna* isolates by Western blot, but no pattern of high molecular weight bands could be found in any of the strains (data not shown). However, the genome of *F. magna* ATCC 29328, which was also probed with Fmp1 antiserum, has been sequenced [41], and a putative sortase-dependent pili locus was identified on the chromosome. Furthermore, the percentage identity between the backbone pilus proteins in this strain and the ALB8 strain is only 31.97%, explaining why no high molecular weight protein ladder was found with the Fmp1 antiserum. This suggests that pilus backbone proteins may differ in sequence between different strains, depending on their isolation/colonisation site. Or, sortase-dependent pili may only be found in some strains of *F. magna*. Future genome sequencing projects will help to shed light on this.

5. Conclusions

This study has identified the presence of pili on *F. magna* for the first time. The presence of sortase-dependent pili was discovered through bioinformatic analysis, transmission electron microscopy and recombinant protein production of the major pilus subunit. Transmission electron micrographs suggested that sortase-dependent pili on *F. magna* could aid in adhesion to host tissues via keratinocytes in human skin and extracellular matrix proteins, shedding further light into how it succeeds as a commensal and opportunistic pathogen. The role of sortase-dependent pili in host-*F. magna* interactions will be further investigated in future studies.

Acknowledgements

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