Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate

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Surface filamentous structures designated pili, and implicated in virulence, have been found on the surfaces of several Gram-positive pathogens. This work describes the conditional expression of two phenotypically distinct pilus-like structures, designated PilA and PilB, on the surface of a hospital-adapted *Enterococcus faecium* bloodstream isolate. *E. faecium* is an emerging Gram-positive opportunistic pathogen that can cause severe disease, particularly in immunocompromised patients. Expression of PilA- and PilB-type pili was analysed during different phases of growth in broth culture. During growth, PilA and PilB pilin subunits were expressed around the cross-wall in early-exponential-phase cells. Polymerization and migration of short PilB-type pili towards the poles occurred in cells from the exponential phase and long polymerized pili were expressed at the poles of cells grown to stationary phase. In contrast, PilA-type pili were not expressed in broth culture, but only when cells were grown on solid media. Furthermore, surface expression of the PilA- and PilB-type pili was regulated in a temperature-dependent manner, as polymerization of two distinct types of pili at the surface only occurred when cells were grown at 37 °C; no pili were observed on cells grown at 21 °C. Hospital-acquired *E. faecium* isolates were specifically enriched in pilin gene clusters, suggesting that conditional expression of pili may contribute to *E. faecium* pathogenesis.

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INTRODUCTION

Enterococci have rapidly emerged as one of the most important nosocomial pathogens causing infections in debilitated patients (Murray, 2000). Although most hospital-acquired infections are still caused by *Enterococcus faecalis*, the ratio of *E. faecalis* to *Enterococcus faecium* infections is rapidly changing in favour of *E. faecium* in hospitals all across the world (Iwen *et al.*, 1997; Murdoch *et al.*, 2002; Top *et al.*, 2007; Treitman *et al.*, 2005). Hospital-acquired *E. faecium* isolates recovered from clinical sites of infection and responsible for nosocomial outbreaks (Leavis *et al.*, 2007; Willems *et al.*, 2005) show high-level resistance to a variety of antibiotics, including ampicillin and ciprofloxacin (Leavis *et al.*, 2006b), and are enriched in a putative pathogenicity island (Leavis *et al.*, 2004), genes encoding LPXTG-like cell-wall-anchored proteins (Hendrickx *et al.*,

2007), insertion sequence elements and genes encoding novel metabolic pathways (Leavis *et al.*, 2007). The cumulative acquisition of resistance determinants and putative virulence genes may have contributed to adaptation and selective advantage of specific *E. faecium* clones in the hospital environment (Leavis *et al.*, 2006a).

Recently, pili (also called fimbriae) have been identified in Gram-positive bacteria (Lauer *et al.*, 2005; Yeung & Ragsdale, 1997; Yeung *et al.*, 1998). Pili are proteinaceous, non-flagellar, covalently joined multimers protruding from the bacterial cell wall, and function as a surface organelle (Marraffini & Schneewind, 2006; Ton-That & Schneewind, 2003). Pili of Gram-positive bacteria have been implicated in biofilm formation and endocarditis (*E. faecalis*) (Nallapareddy *et al.*, 2006), macrophage resistance (Maisey *et al.*, 2008), invasion of and adherence to brain microvascular endothelial cells (*Streptococcus agalactiae*) (Maisey *et al.*, 2007), and adhesion to human tonsil epithelium and skin (*Streptococcus pyogenes*) (Abbot *et al.*, 2007) and to human pharyngeal epithelia (*Corynebacterium diphtheriae*) (Mandlik *et al.*, 2007). Typical heterotrimeric pili are composed of a major subunit which forms the pilus backbone, a minor subunit and a tip, each protein having

Abbreviations: CWS, cell wall sorting signal; PGC, pilin gene cluster; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the DNA sequences of PGC-1 and PGC-3 of *E. faecium* strain E1165 reported in this article are EU909697 and EU909696.

characteristics of Gram-positive cell-wall-anchored proteins. The major pilin subunit contains an N-terminal signal sequence for initiation of Sec-mediated translocation across the plasma membrane and a C-terminal cell wall sorting signal (CWS), which comprises an LPXTG-like sortase substrate motif, a hydrophobic domain and positively charged amino acids (Schneewind *et al.*, 1992, 1993; Ton-That & Schneewind, 2003). A pilus-dedicated sortase catalyses the cleavage of the LPXTG-like motif and mediates pilus polymerization, which involves specific motifs such as the YPK pilin motif with a lysine (K) residue required for pilin polymerization and a conserved E box sequence (YxLxETxAPxGY) with a glutamate (E) residue to incorporate minor pilin subunits (Gaspar & Ton-That, 2006; Swaminathan *et al.*, 2007; Swierczynski & Ton-That, 2006; Ton-That *et al.*, 2004). After polymerization, a housekeeping sortase anchors the pilus via the threonine residue of the last pilin to the amino group of the lipid II precursor within the peptidoglycan (Dramsi *et al.*, 2006; Ton-That & Schneewind, 2003).

The presence of pili has not been documented previously in *E. faecium*. Here, we report the identification of two genetic clusters encoding two distinct types of pili (designated PilA and PilB, previously referred to as Orf1904 and Orf2569, respectively) at the surface of a clinical *E. faecium* isolate, which are both expressed in a growth condition- and temperature-dependent manner.

METHODS

Bacterial strains and growth conditions. *E. faecium* strain E1165 is a hospital-acquired wound isolate and strain E1002 is a faecal isolate from a human healthy volunteer. *E. faecium* strains were grown aerobically at 21 °C or 37 °C on Trypticase Soy Agar II supplemented with 5% sheep blood (TSA; Becton Dickinson) or on Brain Heart Infusion (BHI) agar or broth. Growth curves were prepared from cultures in BHI broth inoculated with plate-grown *E. faecium* cells, standardized to OD₆₆₀ ~0.1 and allowed to grow at

21 °C or 37 °C in an environmental air shaker at 225 r.p.m. A total of 131 *E. faecium* isolates collected from 20 countries worldwide were used in this study (Hendrickx *et al.*, 2007). These isolates include 49 hospital-acquired *E. faecium* strains, representing clinical ($n=31$) and hospital outbreak ($n=18$) isolates from hospitalized patients, and 82 non-hospital-acquired isolates including human surveillance isolates ($n=60$, from individuals without known history of *E. faecium* infections) and isolates from various animals ($n=12$), from food products (2) and from the environment (8).

In silico search for genes encoding putative major pilin subunits. The incomplete *E. faecium* TX0016 (TEX16, DO) genome deposited at DDBJ/EMBL/GenBank under the project accession number AAK00000000 was searched for proteins annotated as cell wall surface anchor family protein, von Willebrand factor type A, CnaB-type or surface protein from Gram-positive cocci (anchor region). Furthermore, putative major pilin subunits were identified by searching for the presence of an N-terminal signal peptide sequence, a consensus WxxxVxVYPKN pilin motif, a conserved ETxAPxGY E box motif and an LPXTG-like sortase substrate motif (where X denotes any amino acid), a hydrophobic domain and at least one positively charged amino acid within the last eight residues of the C terminus. The LPXTG-like motif was compared to the consensus [FILMPSVY][AP]X[ATS][GAKNS] and NPX[ST][DGNS] patterns representing distinct sortase substrate motifs (Boekhorst *et al.*, 2005). The presence of an N-terminal signal sequence was analysed using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP>) (SignalP score >0.6) (Bendtsen *et al.*, 2004). The presence of the conserved pilin, E box and LPXTG-like motifs, hydrophobic amino acids and one or more positively charged amino acids within the last 45 residues of the C terminus was examined visually. To identify similarities, BLAST analyses on protein sequences were performed at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST/>). Alignments were made using the Weblogo 2.8.2 site (<http://weblogo.berkeley.edu/>).

Identification of pilin gene clusters (PGCs) in *E. faecium* E1165.

PGC-1 and PGC-3 were amplified from strain E1165 using primers 1900_fw and Int1_rv for PGC-1 and primers 2567_fw and Int2_rv (Table 1) for PGC-3 to yield 6980 bp and 8202 bp fragments, respectively. PCR products were sequenced by using the BigDye Terminator 3.1 reaction kit and an ABI PRISM 3100 capillary DNA sequencer (both from Applied Biosystems) to determine sequence similarity to the corresponding clusters of *E. faecium* TX0016.

Table 1. Oligonucleotides used in this study

Target (gene cluster/ORF)	Primer name*	Oligonucleotide sequence (5'–3')	Start in sequence† (5'–3')
PGC-1	1900_fw	ATGCCATACACTCTCAGAGT	32701
	Int1_rv	CAGTTGAGACGAACTTATGT	39681
PGC-3	2567_fw	GCAGGAGGAATATGATTTTG	92461
	Int2_rv	TAGTTTCATTCCAACACCTGAT	100663
<i>pilA</i>	PilA_fw	TTTTTGTGTGACTAATCCAG	38272
	PilA_rv	ATGGCAATAAATCGGTATGTA	38807
<i>pilB</i>	PilB_fw	GTGTTTGACAGAGGAGACAGC	95541
	PilB_rv	GACAGAATAATTTACTGGGTCG	94418
<i>ddl</i>	ddl_fw	GAGACATTGAATATGCCTTATG	334‡
	ddl_rv	AAAAAGAAATCGCACCG	893‡

*fw, forward; rv, reverse.

†Relative to the start of the contig.

‡Relative to the start codon.

Distribution of PGCs among *E. faecium* isolates. The presence or absence of the putative pilin subunit genes from the PGCs in the 131 *E. faecium* isolates was determined by PCR and Southern hybridizations as described previously (Hendrickx *et al.*, 2007). A chi-squared test was used to assess statistically significant differences.

Anti-peptide polyclonal rabbit antisera. Highly specific polyclonal rabbit antisera were prepared by Eurogentec (Seraing, Belgium) according to their classic 87 day anti-peptide polyclonal antibody protocol, by immunizing two specific-pathogen-free rabbits with unique 15 amino acid residue keyhole limpet haemocyanin-conjugated peptides directed against the N-terminal region of PilA (H₂N-EDVTQKTPPEKVNITC-CONH₂) and PilB (H₂N-CKMTDLPDPLIQNSGK-CONH₂) of the *E. faecium* TX0016 genome sequence. Rabbits were exsanguinated at day 87; the serum was collected and stored at -20 °C.

Western immunoblot analysis. To detect PilA and PilB pilin protein expression, E1165 bacteria were resuspended in PBS and harvested by centrifugation (1560 g, 5 min). To extract cell-surface proteins, cell pellets were suspended in 70% formic acid and incubated at 65 °C for 30 min as described previously (Ton-That & Schneewind, 2003; Gaspar & Ton-That, 2006), followed by trichloroacetic acid precipitation and acetone washing. Protein pellets were resuspended in 50 µl PBS + 50 µl sample buffer (100 mM Tris/HCl, 5% dithiothreitol, 2% SDS, 0.004% bromophenol blue and 20% glycerol) and boiled for 5 min. Equal amounts of protein sample of *E. faecium* E1165 were analysed by Western blotting as described previously (Hendrickx *et al.*, 2007).

Dot-blot analysis. Dot-blot analysis was performed on chromosomal DNA isolated from *E. faecium* E1002 to determine the presence or absence of LPXTG-like surface-protein genes from PGC-1 and PGC-3. Chromosomal DNA was isolated as described previously (Hendrickx *et al.*, 2007). The chromosomal DNA was denatured with 0.5 M NaOH in a 96-well microtitre plate for 15 min, transferred to a Bio-Rad Bio dot apparatus, vacuum blotted onto a Hybond-N⁺ nylon membrane and fixed onto the membrane by incubation for 2 min in 0.4 M NaOH followed by neutralization in 10 × SSC for 1 min. Membranes were hybridized overnight at 42 °C with 100 ng probe. Gene-specific probes were amplified by PCR using primer pairs specific for *orf1901*, *pilA*, *pilB*, *orf2570* and *orf2571* as described previously (Hendrickx *et al.*, 2007). Amplified DNA probes were purified with a PCR purification kit (Qiagen) and labelled according to the ECL nucleic acid labelling kit (GE Healthcare). The membranes were exposed to Hyperfilm ECL (GE Healthcare).

mRNA expression analysis by reverse transcription (RT)-PCR. For mRNA expression of two putative major pilin subunit genes (*pilA* and *pilB*) cells were resuspended into PBS to an OD₆₆₀ of 1.0 (~1 × 10⁹ c.f.u. ml⁻¹) and pelleted by centrifugation (6500 g for 1 min). Total RNA was isolated according to Cheung *et al.* (1994). RNA purification and cDNA generation was done as described previously (Hendrickx *et al.*, 2007; Nallapareddy & Murray, 2006). cDNA was used as template for PCR using primer pairs PilA_fw and PilA_rv and PilB_fw and PilB_rv (Table 1). As an internal control, the housekeeping gene *ddl* (encoding D-alanine-D-alanine ligase) was amplified using primers ddl_fw and ddl_rv (Table 1). RNA samples not treated with reverse transcriptase were used as control to detect DNA contamination in the total RNA preparations.

Electron microscopy and immunogold labelling. Transmission immunoelectron microscopy was performed as described previously with some modifications (Hendrickx *et al.*, 2007; Van Wamel *et al.*, 2007). In brief, copper grids (mesh Formvar-carbon coated) were incubated for 30 min with the carbon side on a drop containing *E. faecium* E1165 cells (1 × 10⁹ c.f.u. ml⁻¹) to detect surface-exposed

PilA and PilB expression. Grids were washed three times for 5 min on drops of 0.02 M glycine in PBS and subsequently blocked for 30 min on drops of 1% BSA in PBS (PBSb). The PilA- and PilB-type pili were labelled for 1 h on drops of undiluted specific peptide rabbit immune serum or pre-immune serum in PBSb. Grids were washed four times for 2 min on drops of 0.1% BSA in PBS. Pilus-antibody complexes were labelled by incubation for 20 min on drops with 1:55 diluted protein A-gold label (10 nm) in PBSb. Grids were washed four times for 2 min on drops of PBS, fixed by incubation on drops of 1% glutaraldehyde in PBS and washed again eight times for 2 min on drops of H₂O. For double labelling experiments, the labelling was repeated similarly with a second immune serum, and a 1:70 diluted protein A-gold label (5 nm). Bacteria were stained by incubation of the grids for 5 min on drops containing 1.8% methylcellulose (25 centipoise; Sigma-Aldrich) and 0.4% uranyl acetate (pH 4) and subsequently air-dried for 10 min. Grids were examined using a JEOL 1010 transmission electron microscope (JEOL Europe).

RESULTS

Expression of putative pilus-like structures at the surface of *E. faecium*

To examine *E. faecium* for the presence of putative pilus-like appendages at the surface of the cell, transmission electron microscopy (TEM) was performed on *E. faecium* strain E1165, a clinical hospital-acquired isolate. This strain exhibited good binding to the carbon-coated copper grids, in contrast to many other strains. TEM identified putative pilus-like structures, typically 50 nm to 1 µm long, expressed at the poles of the cell (Fig. 1A).

In silico characterization of putative pilin loci of *E. faecium*

The unsequenced *E. faecium* strain E1165 expressed putative pilus-like structures at its surface. Therefore, we assessed whether the partially sequenced and annotated genome of *E. faecium* TX0016 contains ORFs encoding putative major pilin subunit genes, located in the vicinity of putative sortase genes. Putative major pilin subunits were identified by the presence of an N-terminal signal sequence, a conserved pilin motif, an E box motif and a CWS. The genome search identified four putative major pilin subunit genes, located in four distinct gene clusters containing LPXTG-like surface protein genes and putative pilus-dedicated sortases. Putative *E. faecium* PGC-1 contains six ORFs: *orf1901*, *orf1916*, *orf1902*, *orf1903*, *orf1904* and *orf1905* (Fig. 1B). *orf1901* is predicted to encode a putative cell-wall-associated protein with a VPKTG sortase substrate motif. The predicted *orf1904* gene product, designated PilA, is a putative major pilin subunit with an N-terminal signal sequence, a pilin motif, a motif resembling the conserved E box motif, and a VPMTG sortase substrate motif (Fig. 1C). The putative PilA major pilin protein displays similarities to uncharacterized cell-wall-anchored surface proteins. The *pilA* gene is located between two putative sortase-encoding genes, of which *orf1903* resembles a pilus-dedicated sortase enzyme, and *orf1905* a housekeeping sortase. *orf1903* has low (34%)

Fig. 1. Putative pili, pilin gene clusters and major pilin subunit proteins in *E. faecium*. (A) TEM micrograph of a negatively stained *E. faecium* E1165 cell. Bar, 100 nm. (B) Schematic representation of the genomic organization of four putative pilin gene clusters identified from the *E. faecium* TX0016 genome sequence as published at DDBJ/EMBL/GenBank. The position and presumed direction of transcription of ORFs is indicated by arrows and the ORF number is depicted above the arrows. The black arrows indicate putative sortase genes; grey arrows indicate putative major pilin genes; white arrows indicate putative minor or tip pilin genes or genes with unknown function. Thin lines connect genes encoding putative major pilin subunits. The numbers at the beginning and the end of the pilin gene clusters are positions relative to the start of the contig. (C) Structural organization of the putative major pilin proteins. Signal sequences (SS) are depicted in grey, conserved pilin motifs in dotted grey, E box motifs in black, and the CWSs are striped. (D) Alignment of regions of the conserved pilin motifs and E box motifs found in the four putative major pilin proteins. Amino acid sequences in large letters are highly conserved.

similarity to the EF1094 (also designated *srtC* or Bps) pilus-associated sortase protein of *E. faecalis* V583 (Nallapareddy *et al.*, 2006) and other putative pilus-dedicated sortases of *Corynebacterium diphtheriae* and *Clostridium perfringens* (data not shown). In contrast, the *orf1905* sortase did not display similarities to pilus-dedicated sortase enzymes, but it showed 31 % similarity to the SrtA (EF3056) housekeeping sortase of *E. faecalis* V583.

PGC-2 contains four ORFs, *orf2007*, *orf2008*, *orf2009* and *orf2010* (Fig. 1B). The *orf2007* gene product resembles a putative pilus-dedicated sortase, showing similarity (44 %) to the EF1094 pilus-associated sortase of *E. faecalis* V583. The *orf2008* gene is predicted to encode a major pilin subunit protein with an N-terminal signal sequence, a putative pilin motif, an E box motif, and a canonical LPATG sortase substrate recognition sequence (Fig. 1C). The *orf2008* gene product has 32 % similarity to the Bee protein of *E. faecalis*, which has been implicated in biofilm formation (Tendolkar *et al.*, 2006). Interestingly, *orf2009* also contains, in addition to all features of a Gram-positive LPXTG-like surface protein, a putative pilin motif, and may represent a minor pilin subunit. *orf2010* is predicted to encode the largest LPXTG-like protein in this PGC.

PGC-3 contains four ORFs, *orf2568* to *orf2571* (Fig. 1B). This cluster closely resembles the organization of and has

high similarity to the *ebpABC-srtC* (previously designated EF1091–EF1094) PGC of *E. faecalis* V583 (Nallapareddy *et al.*, 2006). A putative sortase (*orf2568*) is located upstream of a putative major pilin subunit, *pilB*, previously designated *orf2569*. *PilB* contains a pilin and E box motif and exhibits 74 % similarity to the putative major EbpC (EF1093) pilin protein of *E. faecalis* V583 (Fig. 1C). *orf2570* and *orf2571* (the latter contains an E box motif) share similarities of 48 % with *ebpB* and 57 % with *ebpA* of *E. faecalis* and putatively encode a minor subunit and a pilus tip, respectively.

We recently identified a cluster of three LPXTG-like surface protein genes and a sortase gene (*orf903* to *orf908*) located on a putative pathogenicity island, which was specifically enriched in hospital-acquired *E. faecium* (Hendrickx *et al.*, 2007). This cluster possibly constitutes a fourth *E. faecium* PGC (Fig. 1B). In the majority of the hospital-acquired *E. faecium* isolates, the presence of multiple premature stop codons suggests that this is a non-functional locus. However, in 9.2 % of the isolates we analysed, this cluster comprises ORFs encoding three surface-exposed proteins, *orf903*, *orf904.5* and *orf906.7*, of which the *orf904.5* product contains a pilin motif and is a putative major pilin subunit (Fig. 1C). *orf908* tentatively encodes a pilus-dedicated sortase, since it shares 52 % similarity to the EF1094 pilus-associated sortase protein of *E. faecalis* V583.

Alignment of the identified putative major pilin subunits of *E. faecium* did not reveal a significant degree of amino acid identity, except for the pilin and E box motifs, which were highly conserved among the *E. faecium* pilin proteins analysed (Fig. 1D). The consensus pilin motif of putative major pilin proteins of *E. faecium* is LxxIHLYPKNxx, with a conserved HLYPK motif, which is different from the consensus Gram-positive pilin motif, WxxxVxVYPKN (Mandlik *et al.*, 2008). Similarly, the consensus E box motif of *E. faecium*, YxxxETxAPExY, was also different from the consensus E box motif (YxxxETxAPxGY) of other Gram-positive major pilin subunits.

PGCs-1 and -3 of *E. faecium* TX0016 and E1165 are conserved

In this paper, the focus is on the *pilA* and *pilB* genes located in PGC-1 and PGC-3, respectively. To identify homologues of *pilA* and *pilB* of *E. faecium* TX0016 in strain E1165, PGC-1 and PGC-3 were amplified by PCR from genomic *E. faecium* E1165 DNA and sequenced. PGC-1 and PGC-3 of *E. faecium* E1165 exhibit very high overall similarity (99 and 100 %, respectively) to PGC-1 and PGC-3 of *E. faecium* TX0016. The organization and transcriptional direction of the ORFs in PGC-1 and PGC-3 are similar, demonstrating that both PGCs are conserved in *E. faecium* E1165.

Putative pilin subunit genes are enriched among hospital-acquired *E. faecium* isolates

The presence of the putative pilin subunit genes from the four PGCs was analysed among 131 *E. faecium* isolates, of which 49 were hospital-acquired and 82 non-hospital-acquired. Based on PCR and Southern hybridization, both *orf1901* and *pilA* (PGC-1) were present in the genomes of 71 % (35/49) of the hospital-acquired isolates and in 48 % (39/82) of the non-hospital *E. faecium* isolates ($P=0.004$). The three putative pilin subunit genes from PGC-2 (*orf2008*–*orf2010*) were detected in 80 % (39/49) of the hospital-acquired isolates and in only 43 % (35/82) of the non-hospital strains ($P<0.001$). The ORFs *pilB* to *orf2571* were present in 94 % (46/49) of the hospital-acquired strains and in 74 % (61/82) of the non-hospital strains ($P=0.002$). These data, in combination with the previously reported distribution of PGC-4 (Hendrickx *et al.*, 2007), show that hospital-acquired *E. faecium* isolates are significantly enriched for putative pilin genes.

Expression of PilA and PilB multimers at 37 °C

Expression of the PilA and PilB pilin proteins (of PGC-1 and -3) in *E. faecium* strain E1165 grown at 37 °C on TSA plates was studied at both the mRNA and protein level. mRNA transcripts of *pilA* and *pilB* were detected in strain E1165 (data not shown). Using specific rabbit anti-peptide immune sera directed against the two putative major pilin subunits, monomeric forms of PilA and PilB pilin proteins were detected at the predicted molecular masses of 64 and 61 kDa

respectively (Fig. 2A). In addition, as observed for other Gram-positive pili, bands of high molecular mass were detected corresponding to PilA and PilB multimers, and PilA and PilB monomers coupled to sortase enzymes or associated with peptidoglycan. Rabbit pre-immune sera did not react with these protein monomers or multimeric pilin species (data not shown). The patterns of PilA and PilB multimeric pilin species were different, suggesting that *pilA* and *pilB* encode two different types of pili. To exclude potential cross-reactivity of the rabbit anti-PilA peptide immune serum, the E1002 PilB pili overexpression strain was included in this study. *E. faecium* strain E1002 is deficient for *orf1901* and *pilA* from PGC-1, and harbours the *pilB*-*orf2570*–*orf2571* genes from PGC-3 (Fig. 2B) as determined by PCR and DNA–DNA hybridizations using gene specific probes. The anti-PilA peptide immune serum did not react with PilB monomers or high-molecular-mass pilin species (Fig. 2A, lane 3), whereas the anti-PilB peptide immune serum did react with the PilB monomer and pilin species (Fig. 2A, lane 4).

Expression of PilA and PilB pili at the surface of *E. faecium* E1165

To detect expression of PilA and PilB pili at the surface of *E. faecium* E1165, single-labelling TEM experiments were

Fig. 2. (A) Western blots of surface protein extracts of *E. faecium* E1165 and E1002 grown on TSA plates at 37 °C. Blots were probed with rabbit anti-PilA (lanes 1 and 3) and rabbit anti-PilB (lanes 2 and 4) immune sera followed by goat anti-rabbit IgG-HRP conjugate. Predicted monomeric PilA (64 kDa) and PilB (61 kDa) pilin proteins are indicated by arrows, high-molecular-mass pilin species are indicated by the bracket. (B) Presence and absence of PGC-1 and PGC-3 in strains E1165 and E1002 as determined by PCR and dot-blot hybridizations. + indicates presence and – indicates absence of the LPXTG-like surface protein genes in the PGC.

performed on immunogold-labelled (10 nm) and negatively stained cells grown on TSA plates at 37 °C. Using the rabbit PilA and PilB anti-peptide immune sera, 10 nm gold particles were clearly associated with distinct pilus-like structures protruding from the *E. faecium* cell wall (Fig. 3A, E). Interestingly, expression of PilA pili was observed at the surfaces in approximately ~40 % of the cells and expression of PilB pili in ~15 % of the cells examined by TEM. Cells without pili expressed cell-wall-associated major pilin subunits or no pilin subunits at all (data not shown) (Dramsi *et al.*, 2006; Nelson *et al.*, 2007). The PilA pili were highly expressed at the poles of dividing enterococci and appeared as rigid, thin (~2 nm and not electron-dense) appendages, which were relatively short (to ~300 nm) (Fig. 3B), but could extend up to 1 µm long (Fig. 3C). No peritrichous PilA pili expression was observed in the case of single cocci. PilB pili were detected at the poles of the cell (Fig. 3F), and in the case of single cocci, peritrichous pili expression was observed (Fig. 3G). The PilB pili appeared more flexible, extended over 1 µm and were thicker (~4 nm, electron-dense) compared to PilA pili. No gold particles were detected along pilus shafts when cells were incubated with rabbit pre-immune sera (Fig. 3D, H) or with protein A-gold (conjugate control; data not shown).

Pili encoded by *pilA* and *pilB* are two distinct types

To corroborate that *pilA* and *pilB* encode two distinct types of pili, a double-labelling TEM experiment was performed on cells grown on TSA plates at 37 °C. Pili of *E. faecium* E1165 cells were initially labelled with rabbit anti-PilB immune serum followed by protein A-gold (5 nm), fixed with glutaraldehyde, and subsequently incubated with anti-PilA serum and protein A-gold (10 nm). TEM confirmed that the two immune sera bound to distinct pili (Fig. 4) and that cells displayed expression of the two distinct types of pili simultaneously.

Temperature-dependent downregulation of expression of PilA- and PilB-type pili at 21 °C

To determine the influence of temperature on expression levels of PilA- and PilB-type pili, *E. faecium* E1165 cells were grown at 21 °C on TSA plates, and protein and pili expression levels were analysed by Western blotting and single-labelling TEM experiments using anti-PilA and anti-PilB rabbit antisera. Interestingly, only monomeric forms of PilA and PilB pilin protein were detected at 21 °C, at a lower level compared to cells grown at 37 °C, and no polymerization into PilA and PilB multimers was observed (compare Figs 2A and 5A). In addition, cells analysed by TEM did not display expression of either PilA- or PilB-type pili. Instead, major pilin subunits were expressed at the surface or cells did not show expression of pilin subunits at all (Fig. 5B). The observation that expression of both types of pili was decreased at 21 °C suggests common regulation. The fact that putative major pilin proteins were still

expressed at the surface of the cell indicates that polymerization of monomers into pili may readily occur upon a temperature switch.

PilA- and PilB-type pili expression in different stages of growth

To analyse *pilA* and *pilB* expression in different stages of growth, *E. faecium* E1165 cells were grown in BHI broth and a growth curve was made (Fig. 6A). At three consecutive time points, early-exponential, exponential and stationary phase of growth, cells were harvested and transcription of the two major pilin genes, *pilA* and *pilB*, and the *ddl* internal control was analysed by PCR on cDNA generated from total mRNA. Transcripts of *pilB* were detected in all phases of growth, but decreased from early-exponential to stationary phase in strain E1165, indicating downregulation of PilB-type pili expression in later stages of growth (Fig. 6B). Control PCRs on DNase-treated total mRNA samples in which the reverse transcriptase reaction was omitted were negative and *ddl* was constitutively expressed in all samples (Fig. 6B). Although high-level mRNA transcripts were detected, low levels of pilin proteins were extracted from cells grown to early-exponential and exponential phase (Fig. 6C) and no long PilB-type pili were expressed at the surface. Instead, major pilin subunits were observed in early-exponential-phase cells close to the septum or cross-wall in the centre of a dividing cell (Fig. 7A), in which already a low level of polymerization of PilB-type pili occurred (Fig. 7B). In some early-exponential-phase cells, expression of PilB pilin subunits was directed more towards the poles, possibly the result of newly synthesized peptidoglycan pushing old peptidoglycan from the cross-wall to the poles. In exponential-phase cells, *pilB* mRNA expression levels decreased (Fig. 6B), whereas expression of PilB multimeric pilin species and polymerized pili, mainly at the 'old poles' increased (Figs 6C and 7C). In these cells, pili were ~400 nm in length, and less pilin protein was observed around the cross-wall. In stationary phase, no pilin subunits were observed around the cross-wall. Very long (1 µm) PilB-type pili were expressed and PilB subunits were detected at the poles of the cells (Fig. 7D), suggesting pilin subunit translocation and polymerization, which is in line with detection of mRNA transcripts and high levels of PilB multimers (Fig. 6B, C).

Transcripts of *pilA* were detected in all phases of growth, suggesting constitutive expression of PilA-type pili (Fig. 6B). Monomeric forms of PilA pilin protein were detected by Western blotting, demonstrating constitutive PilA expression during the different phases of growth (Fig. 6C). Interestingly, no polymerization of PilA into PilA multimers was detected at any stage of growth in broth culture; this was subsequently confirmed by TEM (data not shown). Pilin subunits were observed close to the cross-wall in the centre of the cell when cells were grown to early-exponential phase (data not shown). This was also the case

Fig. 3. TEM micrographs of PilA-type and PilB-type pili of *E. faecium* E1165. E1165 was grown on TSA plates at 37 °C and subsequently labelled with anti-PilA or anti-PilB peptide immune serum followed by protein A-gold (10 nm particles) and negative staining. (A) *E. faecium* cells incubated with anti-PilA rabbit immune serum, showing details of a PilA pilus, decorated with 10 nm gold particles. (B) Highly expressed PilA-type pili at the poles appearing as rigid and thin (2 nm) appendages, relatively short (to ~300 nm) in length. (C) Similar to (B), but occasionally pili extended up to 1 µm long. (D) *E. faecium* cells incubated with PilA rabbit pre-immune serum not displaying gold labelling. (E) *E. faecium* cells incubated with anti-PilB rabbit immune serum, showing details of a PilB pilus, decorated with 10 nm gold particles. (F) PilB-type pili appear flexible, thick (4 nm) and less abundantly expressed at the poles of the cell compared to PilA-type pili. (G) Peritrichous PilB-type pili expression occurs in single cells. (H) *E. faecium* cells incubated with PilB rabbit pre-immune serum not displaying gold labelling. Bars, 200 nm.

Fig. 4. TEM micrograph of PilA- and PilB-type pili expressed on a single cell. *E. faecium* E1165 was labelled with rabbit anti-PilB immune serum followed by protein A-gold (5 nm particles), fixed, and consecutively labelled with anti-PilA immune serum followed by protein A-gold (10 nm particles). The PilA-type pili are indicated by black arrows, PilB-type pili by white arrows. Bar, 100 nm.

for cells grown to exponential or stationary phase. However, polymerized PilA pili were observed when cells were grown on solid TSA and BHI media, instead of liquid BHI medium, suggesting that PilA pilus polymerization is induced in a contact-dependent manner.

DISCUSSION

We describe conditional expression of pili on the surface of hospital-acquired *E. faecium*. Pili are 'hair-like' surface-exposed structures involved in adhesion to multiple types of human cells and biofilm formation, two processes critical in the pathogenesis of bacterial disease. In contrast to pili of Gram-negative bacteria, little is known concerning conditional pilus expression in Gram-positives. We demonstrate that *E. faecium* can express two distinct types of pili at the surface of a single cell, and that these pili are encoded by two different and physically separated PGCs. Both *pilA* and *pilB* encode two different major pilin subunits that polymerize into distinct multimeric pilus structures. Five lines of evidence support this interpretation. First, *in silico* analysis revealed that the PilA and PilB proteins contain an N-terminal signal sequence to facilitate Sec-mediated translocation, a pilin motif required for pilus polymerization, an E box motif possibly involved in incorporation of minor pilin subunits and a CWS

Fig. 5. Temperature-dependent downregulation of PilA- and PilB-type pili expression at 21 °C. *E. faecium* E1165 was grown on TSA plates at 21 °C. (A) Surface protein extracts analysed by Western blots probed with rabbit anti-PilA (left) and rabbit anti-PilB (right) immune sera. Only PilA and PilB monomeric pilin subunits are expressed, as indicated by the arrow, and no multimers were observed (compare Fig. 2A). (B) TEM micrographs of cells grown at 21 °C and labelled with anti-PilA (upper part) or anti-PilB (lower part) immune serum followed by protein A-gold (10 nm) and negative staining. Major pilin subunits are associated with the cell wall and no surface-exposed pili are expressed. Bars, 200 nm.

Fig. 6. Expression of PilA- and PilB-type pili in different phases of growth. (A) Growth curve of *E. faecium* E1165 in BHI at 37 °C. Cells were harvested in the early-exponential (EE), exponential (E) and stationary phase (S) as indicated by the arrows. (B) mRNA expression of *pilA*, *pilB* and *ddl* in cells isolated in EE, E and S phases of growth. In the upper part, mRNA of *pilB* is depicted, which decreased from EE- to S-phase cells. The *pilA* gene is constitutively expressed in all growth phases. Control *ddl* PCRs on total mRNA preparations in which the RT reaction was omitted were all negative, demonstrating absence of DNA contamination. Control *ddl* RT-PCRs (internal housekeeping control) with *E. faecium* specific *ddl* primers were all positive. The results are presented as amplified PCR products electrophoresed on the same ethidium-bromide-stained 1.5% agarose gel. (C) Surface protein extracts of EE-, E- and S-phase cells analysed by Western blots incubated with rabbit anti-PilB (left) and rabbit anti-PilA (right) immune sera. The PilB pilin protein levels increase from EE- to S-phase cells. The monomeric PilA pilin protein is constitutively expressed (arrow) while pilin multimers are absent.

containing an LPXTG-like sortase recognition sequence. Second, expression analysis by RT-PCR detected two specific mRNA transcripts and Western blotting revealed two distinct PilA and PilB multimeric pilin species. Third, the rabbit anti-PilA immune serum did not cross-react with the PilB monomeric subunit or the PilB high-molecular-mass pilin species. Fourth, in TEM experiments, specific anti-PilA and anti-PilB peptide sera reacted with two phenotypically distinct pilus structures at the surface

of *E. faecium* cells grown at 37 °C. The PilA-type pili were thin, rigid, relatively short and occasionally expressed as long polymers at the poles of dividing cells. The PilB-type pili were thicker than PilA-type pili, more flexible and expressed as long polymers at the poles of dividing cells. The observation that PilB-type pili are thick suggests incorporation of additional pilin proteins along the pilus shaft or a bundled multi-fibre structure (Hilleringmann *et al.*, 2008). Finally, double-labelling TEM demonstrated expression of two distinct types of pili simultaneously on a single *E. faecium* cell. The fact that PilA- and PilB-type pili were differentially expressed to form a heterogeneous population of *E. faecium* cells expressing PilA- or PilB-type, both types or no pili at all indicates that cells grown in colonies on solid media are in different phases of growth and may favour phase variation, which plays a role in immune evasion by limiting exposure of pilin antigens to the immune system, or adaptation and survival in changing environments (Holden & Gally, 2004; Kearns *et al.*, 2004; van der Woude, 2006).

Expression of PilA- and PilB-type pili at the surface of *E. faecium* was regulated in a temperature-dependent manner. In contrast to high-level expression of both types of pili on surfaces of *E. faecium* cells grown at 37 °C, no pili were observed when the bacteria were grown at 21 °C. Instead, cell-wall-associated major pilin subunits were detected at the surface. This suggests that during prolonged survival of *E. faecium* in the hospital environment, both types of pili are not expressed. Contamination of skin and indwelling medical devices by *E. faecium*, accompanied by a temperature shift to 37 °C, results in upregulation of pili biogenesis and may initiate intimate attachment, followed by colonization and finally infection.

The PilA and PilB major pilin subunits are expressed around the cross-wall in cells grown to early-exponential phase. The cross-wall is a thick layer of newly synthesized peptidoglycan at which the cell separates into two newly formed cells (Giesbrecht *et al.*, 1998). A similar finding was recently reported for the LPXTG-like cell-wall-anchored protein A of *Staphylococcus aureus* (DeDent *et al.*, 2007). This suggests that PilB pilin proteins are possibly translocated around the cross-wall and deposited into newly synthesized murein sacculi during separation of the cross-wall at its midline at cell division. Murein sacculi are rigid exoskeletal organelles in which surface proteins are immobilized (Salton, 1952; Cole & Hahn, 1962; Ghuyssen, 1968; Navarre & Schneewind, 1999). Possibly, the pilus-dedicated sortase is also located at these cell division sites, as a low level of polymerization of PilB-type pili was observed, but this remains to be investigated. During further growth, novel murein sacculi arise and migration of old murein sacculi towards the poles occurs. Indeed, labelled PilB pilin proteins migrated towards the poles and the pili were longer than those of early-exponential-phase cells. These pili were typically ~300 nm long and not abundantly expressed at the surface. In stationary phase, cells display high-level pili expression and pilin subunits

Fig. 7. TEM micrographs of PilB-type pili expression in different phases of growth. *E. faecium* cells were harvested in early-exponential (EE), exponential (E) and stationary (S) phase, and labelled with anti-PilB immune serum followed by protein A-gold (10 nm) and negative staining. (A) Cells harvested from an EE culture display PilB pilin protein expression around the cross-wall (black arrows). (B) Similar to (A); detailed micrograph of low-level of PilB-type pili polymerization at the cross-wall (black arrows). (C) Cells in E phase display PilB pilin protein expression around the cross-wall, to a lesser extent compared to EE cells, and pili are observed at the poles. (D) S-phase cells do not show pilin protein expression around the cross-wall; instead PilB pilin subunits and long (1 μ m) PilB-type pili are observed. Bars, 200 nm.

were detected predominantly at the old poles. The finding of differential PilB expression and biogenesis during growth suggests that pili of this type are expressed in a growth-phase-dependent manner. However, environmentally relevant cues, such as fluctuations in pH, oxygen and carbon dioxide concentrations, increasing cell density, and cell-to-cell signalling in different phases of growth in liquid media may also influence pili expression. Which of these factors affect PilB expression remains to be elucidated.

The PilA-type pili were expressed when the bacteria were grown on solid media, but not in liquid media, and

expression was not growth phase-dependent. These pili are possibly expressed upon cell-cell or cell-substratum contact. Alternatively, shearing, enzymic cleavage or degradation of PilA pili may occur in broth cultures, and could account for the lack of polymerized PilA structures at the surface of cells grown in liquid cultures. The localization of *pilA* between a putative housekeeping and a putative pilus-dedicated sortase gene in PGC-1 suggests that polymerization by the pilus-dedicated sortase may be followed instantly by cell wall anchoring of the PilA pilus by the housekeeping sortase. Possibly, the PilA-type pili may be involved in initial adherence of *E. faecium*.

The finding that PGCs were enriched in hospital-acquired *E. faecium* isolates implies that conditional pili expression may confer a selective advantage in the hospital environment and may promote *E. faecium* pathogenesis. Recently, active and passive immunization with recombinant pilus subunits of *Streptococcus pneumoniae* protected mice against a lethal challenge, suggesting that Gram-positive pili are promising novel targets for immune therapy to treat infections (Gianfaldoni *et al.*, 2007). Hospital-acquired *E. faecium* are multi-resistant, which limits the choice of antibiotics and complicates effective therapy to treat infected patients. A novel strategy to combat these multi-antibiotic-resistant bacteria could be the development of vaccines that target pili.

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