Differential PilA pilus assembly by a hospital-acquired and a community-derived *Enterococcus faecium* isolate

Antoni P. A. Hendrickx,^{1†} Claudia M. E. Schapendonk,¹
Miranda van Luit-Asbroek,¹ Marc J. M. Bonten,^{1,2} Willem van Schaik¹
and Rob J. L. Willems¹

Correspondence

Antoni P. A. Hendrickx
ahendric@bsd.uchicago.edu

¹Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

²Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands

Pili are hair-like structures protruding from the cell envelope of bacterial cells. Here, we describe the conditional and differential display of PilA-type pili, and PilE and PilF proteins, encoded from pilin gene cluster 1 at the surface of a hospital-acquired *Enterococcus faecium* bloodstream isolate (E1165) and a community-derived stool isolate (E1039), at two different temperatures. Both strains have virtually identical *pilA* gene clusters, as determined by sequencing. Western blotting and transmission immunoelectron microscopy revealed that PilA and PilF assembled into high-molecular-mass pilus-like structures at 37 °C in the E1165 strain, whereas PilE was not produced at either of the temperatures used; at 21 °C, PilA and PilF were cell-wall-anchored proteins. In contrast, in strain E1039, PilA, PilE and PilF pilin proteins were found to be displayed as cell-wall-anchored proteins at 37 °C only, and they were not associated with pilus-like structures. The discrepancy in pilus assembly between E1039 and E1165 cannot be explained by differences in expression of the genes encoding the predicted sortases in the *pilA* gene cluster, as these had similar expression levels in both strains at 21 and 37 °C. Double-labelling electron microscopy revealed that PilA formed the pilus backbone in E1165, and PilF the minor subunit which was distributed along the PilA pilus shaft and positioned at the tip; however, it was deposited as a cell-wall-anchored protein in a *pilA* isogenic mutant. The differential deposition of surface proteins from pilin gene cluster 1 and differences in pilus assembly in the two strains suggest a complex post-transcriptional regulatory mechanism of pilus biogenesis in *E. faecium*.

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INTRODUCTION

Enterococci are antimicrobial-resistant opportunistic pathogens that are increasingly causing healthcare-associated infections (Murray, 2000; Werner *et al.*, 2008). Both *Enterococcus faecium* and *Enterococcus faecalis* have rapidly emerged in hospitals around the world, and they are responsible for infections in the immunocompromised host (Hidron *et al.*, 2008; Murdoch *et al.*, 2002). Today, enterococci rank third among nosocomial pathogens in the USA, as reported by the National Healthcare Safety Network (Hidron *et al.*, 2008). Hospital-acquired *E. faecium* isolates associated with hospital

outbreaks and invasive infections frequently show high-level resistance to ampicillin and ciprofloxacin, and can be distinguished from community-derived isolates based on their gene content (Leavis *et al.*, 2006, 2007; Willems *et al.*, 2005). Hospital-acquired *E. faecium* isolates are enriched in genes encoding LPXTG-like cell-wall-anchored surface proteins (Hendrickx *et al.*, 2007), including the gene encoding the enterococcal surface protein Esp (Leavis *et al.*, 2004), a collagen- and fibrinogen-binding microbial surface component recognizing adhesive matrix molecule (MSCRAMM) called EcbA, a nidogen and fibrinogen adhesin designated SgrA, and several LPXTG surface protein genes located near one or more predicted sortase genes in pilin gene clusters (PGCs) (Hendrickx *et al.*, 2008, 2009a). Recent *in silico* analysis has revealed the presence of four PGCs in *E. faecium*, and specific sera directed against two putative major pilin subunits, which are designated PilA and PilB and are encoded from PGC-1 and -3, respectively, have been shown to react with two phenotypically distinct pilus-like structures on the surface of a clinical *E. faecium* isolate (Hendrickx *et al.*, 2008).

†Present address: Department of Microbiology, University of Chicago, Chicago, IL 60637, USA.

Abbreviations: BHI, brain heart infusion; CWS, cell wall sorting signal; FACS, fluorescence-activated cell sorting; HSA, human serum albumin; MLST, multilocus sequence typing; MSCRAMM, microbial surface component recognizing adhesive matrix molecule; PBSb, 1 % BSA in PBS; PGC, pilin gene cluster; TEM, transmission immunoelectron microscopy; TSA, trypticase soy agar II.

Pili, also referred to as fimbriae, are hair-like proteinaceous filaments protruding from the bacterial cell wall, and they are implicated in virulence of several Gram-positive pathogens, including *E. faecalis* (Abbot *et al.*, 2007; Dramsi *et al.*, 2006b; Gaspar & Ton-That, 2006; Nallapareddy *et al.*, 2006b; Nelson *et al.*, 2007). In contrast with Gram-negative organisms, pili of Gram-positive organisms are covalently joined polymers that are tightly immobilized to the peptidoglycan wall (Marraffini & Schneewind, 2006; Ton-That & Schneewind, 2003). The prototype heterotrimeric pilus is composed of a major subunit that forms the pilus backbone, a minor pilin subunit distributed along the pilus shaft, and an adhesive tip protein. The putative *E. faecium* major pilin subunits contain an N-terminal signal sequence for initiation of Sec-mediated translocation across the plasma membrane, a HLYPKN pilin motif, an ETxAPEXY E box motif, and a C-terminal cell wall sorting signal (CWS). The CWS comprises an LPXTG-like sortase substrate sequence, and a hydrophobic domain, followed by positively charged amino acids (Schneewind *et al.*, 1992, 1993; Ton-That & Schneewind, 2003). Conservation of these sequence elements in putative major pilin subunits of *E. faecium*, and in pilin subunits from other Gram-positive bacteria, suggests a universal mechanism of pilus assembly that may also be applied to enterococci (Hendrickx *et al.*, 2009b).

To gain insight into pilus assembly of *E. faecium*, we studied pilin protein display and PilA pilus biogenesis at the surfaces of a clinical wound isolate (E1165), an isogenic *pilA* mutant of E1165 and a community-derived human stool isolate (E1039), at different growth temperatures.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. faecium* strains E1162 and E1165 are clinical isolates harvested from bloodstream and wound infections, respectively, whereas E1360 and E1391 are clinical isolates with an undetermined isolation site. Strains E128, E1039 and E1133 are faecal isolates from healthy human volunteers. All strains have been typed previously by our group using multilocus sequence typing (MLST) (Willems *et al.*, 2005). *E. faecium* strains were grown aerobically at 21 or 37 °C on trypticase soy agar II (TSA) supplemented with 5% sheep blood (Becton Dickinson), or in brain heart infusion (BHI) broth, at 37 °C, unless stated otherwise. When appropriate, chloramphenicol and gentamicin (Sigma-Aldrich) were used at concentrations of 15–34 and 125 µg ml⁻¹, respectively. A temperature-sensitive vector (pTEX5500ts) was used to introduce an insertion–deletion mutation in the *pilA* gene of *E. faecium* E1165. The oligonucleotides used in this study are listed in Table 2.

Identification of PGC-1 in *E. faecium* isolates. Dot-blot analysis was performed on chromosomal DNA isolated from *E. faecium* E128, E1039, E1133, E1162, E1360 and E1391 to determine the presence of the *pilA* and *pilF* genes from PGC-1. Chromosomal DNA isolation was performed 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 and then transferred to a Bio-Rad Bio dot apparatus, vacuum blotted onto an 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 a 100 ng probe. Gene-specific probes were amplified by PCR using primer pairs specific for *pilA* and *pilF* (Table 2). 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).

Antipeptide polyclonal rabbit antisera. Specific polyclonal rabbit antisera were prepared by Eurogentec, according to their 87 day

Table 1. Bacterial strains and plasmids used in this study

NA, Not applicable; ts, temperature sensitive; ST, sequence type.

Strain or plasmid	Relevant characteristics	ST	Reference
Strains			
<i>E. coli</i> DH5α	<i>E. coli</i> host strain for routine cloning	NA	Hanahan (1983)
<i>E. faecium</i>			
E128	Community surveillance isolate from faeces, <i>pilA</i> and <i>pilF</i> negative	82	This study
E1039	Community surveillance isolate from faeces, <i>pilA</i> and <i>pilF</i> positive	42	This study
E1133	Community surveillance isolate from faeces, <i>pilA</i> and <i>pilF</i> positive	117	This study
E1162	Clinical blood isolate, <i>pilA</i> and <i>pilF</i> positive, Cm ^S Gm ^S	17	Heikens <i>et al.</i> (2007)
E1165	Clinical wound isolate, <i>pilA</i> and <i>pilF</i> positive, intermediate Cm ^R Gm ^S	17	Hendrickx <i>et al.</i> (2008)
E1165Δ <i>pilA</i>	E1165 with a disrupted <i>pilA</i> gene, <i>pilF</i> positive Cm ^R Gm ^S	17	This study
E1360	Clinical isolate, undetermined isolation site, <i>pilA</i> and <i>pilF</i> positive	16	This study
E1391	Clinical isolate, undetermined isolation site, <i>pilA</i> and <i>pilF</i> positive	16	This study
E1674	Surveillance isolate from faeces, <i>pilA</i> and <i>pilF</i> positive	110	This study
Plasmids			
pTEX5500ts	Shuttle plasmid, ts in Gram-positive hosts, Cm ^R Gm ^R		Nallapareddy <i>et al.</i> (2006a)
pEF13	pTEX5500ts: <i>pilA</i> -Up, pTEX5500ts with a cloned <i>pilA</i> -Up gene fragment, Cm ^R Gm ^R		This study
pEF15	pTEX5500ts: <i>pilA</i> -Up- <i>pilA</i> -Dn, pTEX5500ts with cloned <i>pilA</i> gene fragments flanking the <i>cat</i> gene, for generating a <i>pilA</i> insertion–deletion mutation, Cm ^R Gm ^R		This study

Table 2. Oligonucleotides used in this study

fw, Forward primer; rv, reverse primer.

Primer name	Oligonucleotide sequence (5'–3')
PilA-Up_fw	TTCCGCGGCCGCTATGGCCGACGTCGTC-GACGCGTGGTTATA-AGTCATTTGAAGAT
PilA-Up_rv	CGCGGATCCGCGATAAACAGTAACA-AATGGA
PilA-Dn_fw	AACTGCAGAACCAATGCATTGGAAATT-TCGTTAATTCAAAAAC
PilA-Dn_rv	GCCTTAAGGCCGTTAGGAAATATTACGGAT
PilA_fw1	AACGAAATCCACGCTATTTG
PilA_fw2	TTTACGTATGAAGCTTTTAA
PilA_rv1	ATCAAGGAACGCAATTGAAT
PilA_rv2	GCACAGCTCAAAGCAAACAA
Srt1903_fw	CATCAAAAAGATTATCTTTA
Srt1903_rv	TAAGAAAAAGCGAATCATGG
Srt1905_fw	TAAGTAAAGTCCATCACCTCC
Srt1905_rv	GGTGTTTAAAGGAAAATATAG
Tuf_fw	GACACGCCACTACGCTCACG
Tuf_rv	AGCTCCGTCCATTTGAGCAG
Cam_F	TTTAATAAAATTGATTTAGA
Cam_R	CCTGAATAGAGTTCATAAAC

antipeptide polyclonal antibody protocol, by immunizing two specific-pathogen-free rabbits with unique 15 aa keyhole limpet haemocyanin-conjugated peptides equivalent to the N-terminal regions of PilA (GenBank accession no. ZP_00603529; H₂N-EDVTQKTPPEKVNITC-CONH₂), PilF (GenBank accession no. ZP_00603526; H₂N-CEKV-IVDPSNPIQNVK-CONH₂) and PilE (GenBank accession no. ZP_00603527; H₂N-SSDETVDSSVDSSEE-CONH₂) of *E. faecium* strain TX0016 (also termed TX16 or DO) genome sequence. Rabbits were killed on day 87; serum was collected and stored at –25 °C.

Western immunoblot analysis of surface protein extracts. To detect PilA, PilE and PilF pilin protein production, E1165 and E1039 bacteria were grown at 21 and 37 °C on TSA agar, then resuspended in PBS and harvested by centrifugation (1560 g, 5 min). To extract the surface proteome including pili, cell pellets were suspended in 70 % formic acid and incubated at 65 °C for 30 min, followed by trichloroacetic acid precipitation and acetone washing (Hendrickx *et al.*, 2008; Ton-That & Schneewind, 2003). Protein pellets were resuspended in a solution containing 50 µl PBS and 50 µl sample buffer (100 mM Tris/HCl, 5 % DTT, 2 % SDS, 0.004 % Bromophenol Blue and 20 % glycerol, v/v), and boiled for 5 min. Equal amounts of protein samples of *E. faecium* E1039 and E1165 were analysed by Western blotting, as described previously (Hendrickx *et al.*, 2008).

Fluorescence-activated cell sorting (FACS) analysis. Bacteria were collected from TSA agar plates and resuspended in RPMI 1640 (Cambrex Bioscience) containing 0.05 % human serum albumin (HSA), to an OD₆₆₀ of 1.0 (approximately 1 × 10⁹ bacteria ml^{–1}). For each sample, 100 µl of the cell suspension was pelleted by centrifugation (6500 g, 1 min), and 50 µl RPMI/HSA containing 1:100 rabbit anti-PilA immune serum was added. Bacteria were resuspended and incubated on ice. After 30 min, 1 ml cold RPMI/HSA was added, and the bacteria were isolated by centrifugation (6500 g, 1 min). Bacteria were resuspended in 50 µl RPMI/HSA containing 1:50 goat anti-rabbit FITC (Sigma-Aldrich), and incubated for 30 min on ice. Bacteria were washed with 1 ml cold RPMI/HSA, and after centrifugation (6500 g, 1 min), bacteria were resuspended in 50 µl RPMI/HSA. Before analyses in the FACSCalibur

(BD Biosciences), bacteria were resuspended in 300 µl RPMI/HSA. All measurements were performed in one machine, using the same parameters each time. The data were normalized for bacterial size, and experiments were performed twice. The mean fluorescence was used as a measure of cell-surface-associated PilA. Rabbit pre-immune serum and bacteria incubated without anti-PilA rabbit immune serum were used as negative controls.

Electron microscopy and immunogold labelling. Transmission immunoelectron microscopy (TEM) was performed as described previously, with some modifications (Hendrickx *et al.*, 2007, 2008). In brief, copper grids (mesh Formvar-carbon coated) were incubated for 30 min with the carbon side on a drop of 1 × 10⁹ c.f.u. ml^{–1} *E. faecium* E1039, E1162 or E1165 cells, to detect surface-exposed PilA, PilE or PilF. 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, PilE and PilF proteins were labelled for 1 h on drops of undiluted or 1:100 diluted specific peptide rabbit immune sera or pre-immune sera in PBSb. Grids were washed four times for 2 min on drops of 0.1 % BSA in PBS. Surface protein–antibody complexes were labelled by incubation for 20 min on drops of 1:70 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 eight times for 2 min on drops of H₂O. For double-labelling experiments, the labelling was repeated similarly with a 1:100 dilution of a second immune serum, and 1:70 diluted protein-A–gold-label (5 nm). After fixation with 1 % glutaraldehyde in PBS, bacteria were stained by incubation of the grids for 5 min on drops containing 1.8 % methylcellulose (25 mPa s; Sigma-Aldrich) and 0.4 % uranyl acetate (pH4), and they were subsequently air-dried for 10 min. Grids were examined using a JEOL 1010 transmission electron microscope (JEOL Europe).

Mutagenesis of *pilA* by insertional inactivation. The *pilA* gene of *E. faecium* E1165 was disrupted by construction of an insertion–deletion mutation, as described previously (Heikens *et al.*, 2007; Nallapareddy *et al.*, 2006a). In brief, a 397 bp *pilA* fragment, designated PilA-Up, was amplified from genomic *E. faecium* E1165 DNA by using primers PilA-Up_fw and PilA-Up_rv, which contain *SalI* and *BamHI* restriction sites, respectively (Table 2). The PCR product was digested with *SalI* and *BamHI*, and ligated into similarly digested pTEX5500ts, resulting in pEF13. Similarly, a 392 bp fragment, designated PilA-Dn, was amplified by using primers PilA-Dn_fw and PilA-Dn_rv, including restriction sites *NsiI* and *EcoRI* incorporated at the 5' ends of the primers to facilitate directional cloning into pEF13, resulting in pEF15 (Table 1). The pEF15 plasmid was introduced into competent *E. faecium* E1165 by electroporation. After transformation, gentamicin-resistant colonies were picked and grown overnight at 42 °C in BHI broth supplemented with gentamicin; they were subsequently plated on BHI agar plates containing chloramphenicol (15 µg ml^{–1}) and grown at 42 °C. Single-crossover integration into PilA-Up complementary sequences of *E. faecium* E1165 was confirmed by PCR. Single-crossover mutants were grown overnight for eight serial passages in BHI culture supplemented with consecutive increasing concentrations of chloramphenicol (from 15 to 34 µg ml^{–1}), at 42 °C, to cure pEF15. Selection for double-crossover recombination was performed by replica plating on BHI agar plates containing either chloramphenicol or gentamicin. The insertional inactivation of the *pilA* gene in chloramphenicol-resistant gentamicin-susceptible double-crossover mutants was analysed by PCR with primers PilA_fw1 and PilA_rv1, sequencing and Southern hybridization.

Southern blot analysis. Southern blot analysis was performed, as described previously (Hendrickx *et al.*, 2007), on *EcoRI*-digested chromosomal DNA isolated from *E. faecium* E1165 and E1165Δ*pilA*

to confirm disruption of the chromosomal *pilA* gene. Probes were generated by PCR (in 50 µl volumes) by using primer pairs Cam_F and Cam_R, and PilA_fw2 and PilA_rv2, on the templates pTEX5500ts and chromosomal DNA of E1165, respectively, as shown in Table 2.

Generation of cDNA from total mRNA and PCR. *E. faecium* isolates E1039 and E1165 were assayed for mRNA expression of the putative *srt1903* and *srt1905* genes. Total *E. faecium* RNA isolation, RNA purification (including enzymic digestion of DNA) and cDNA generation were performed as described previously (Hendrickx *et al.*, 2007). cDNA was used as a template for PCR using primer pairs Srt1903_fw and Srt1903_rv, and Srt1905_fw and Srt1905_rv, as listed in Table 2. As an internal control, the housekeeping gene *tuf* (encoding the elongation factor Tu) was amplified by using primers Tuf_fw and Tuf_rv (Table 2). RNA samples not treated with reverse transcriptase were used as controls to detect DNA contamination in the total RNA preparations.

RESULTS

Characteristics of the proteins putatively encoded from PGC-1 of *E. faecium* E1165

The previously identified PGC-1 of *E. faecium* E1165 (GenBank accession no. EU909697) contains six putative ORFs: *orf1901* (*pilF*), *orf1916*, *orf1902* (*pilE*), *orf1903* (class C sortase), *orf1904* (*pilA*) and *orf1905* (class A sortase) (Fig. 1). As previously reported, the *orf1901*, *orf1902* and *orf1904* gene products, designated PilF, PilE and PilA, respectively, are predicted to encode cell-wall-associated proteins with LPXTG-like sortase substrate motifs (Hendrickx *et al.*, 2008). The PilE pilin protein is predicted to be a secreted protein (Sillanpää *et al.*, 2008). Indeed, the SignalP 3.0 server predicted that this protein contains an N-terminal signal sequence. However, visual examination of the amino acid sequence identified six SS repeats and four SD repeats distributed along the protein, and a deviant IVSTG cell-wall-anchor motif, followed by a hydrophobic transmembrane region and a positively charged tail, suggesting that PilE is a putative LPXTG-like surface protein. The IVSTG motif of PilE has been compared with the consensus (FILMPVY)(AP)X(ATLS)(GAKNS) and NPX(ST)(DGNS) patterns representing distinct sortase substrates (Boekhorst *et al.*, 2005; Janulczyk & Rasmussen, 2001). However, to date, these putative sortase substrate sequences do not allow the valine (V) on the second position of the IVSTG motif. Furthermore, PilE contains a putative YPKSE pilin motif. Visual examination has revealed that PilA, PilE and PilF are predicted to contain five, two and four *N*-glycosylation sites (N[ST](P)), respectively, suggesting that these pilin subunits may be glycosylated (Power & Jennings, 2003).

orf1916 putatively encodes a small 6.8 kDa protein containing an N-terminal signal peptide, without a cell wall anchor, and it is possibly a secreted hydrophobic polypeptide.

PilA is a cell-wall-anchored protein in various *E. faecium* isolates

Recently, we showed that PilA-type pili were assembled on the surface of a clinical *E. faecium* isolate E1165 (Hendrickx

Fig. 1. Schematic representation of the genomic organization of PGC-1 of *E. faecium* E1165, and E1039 and E1165 gene products (GenBank accession nos NZ_ACO501000003 and EU909697). The positions and presumed directions of transcription of ORFs are indicated by arrows. The ORF numbers are given below the arrows, while the gene names are given in bold above the arrows. The blue arrows indicate minor pilin proteins, and the white arrow indicates a gene with unknown function. The red arrow indicates the putative major pilin subunit. The black and green arrows indicate class C and class A sortase genes, respectively. The putative LPXTG-like surface proteins are shown below PGC-1. SS, signal sequence. The pilin and pilin-like motifs are shown in red and yellow, respectively. The repeats in PilE are shown by vertical black lines.

et al., 2008). To analyse the display of PilA on the surfaces of other *E. faecium* isolates, three clinical (E1162, E1360 and E1391) and four non-clinical (E128, E1039, E1133 and E1674) isolates were selected, and PCR and DNA–DNA hybridizations confirmed the presence of *pilA* in these strains, except in E128, which was used as a negative control. Based on MLST, the E1162, E1165, E1360 and E1391 isolates belong to the polyclonal hospital-acquired genetic lineage, whereas E128, E1039, E1133 and E1674 are of non-hospital-acquired origin (Table 1).

Surface exposure of PilA was demonstrated by FACS analysis in five *pilA*-positive strains grown on TSA agar at 37 °C. PilA protein levels varied among the isolates analysed, with the highest level found at the surface of E1039 (Fig. 2). Strain E1360 displayed a low mean fluorescence, which was comparable to that of the *pilA*-deficient strain (E128), suggesting that PilA was not produced in this isolate. The flow cytometry analyses were confirmed by TEM on negatively stained and anti-PilA immunogold-labelled bacteria. Gold particles were associated with the bacterial cell wall at different levels, but were not associated with pilus-like structures, suggesting differential PilA pilus assembly among *E. faecium* isolates (data not shown). Surface exposure of PilA at the cell wall of E1039 was the highest compared with the other isolates, and was therefore subjected to further analyses in this study.

Fig. 2. Flow cytometry analysis of surface display of PilA on *E. faecium* strains. Three clinical (E1162, E1360, and E1391) and four non-clinical (E128, E1039, E1133 and E1674) isolates were grown on TSA at 37 °C in aerobic conditions to analyse PilA protein levels by flow cytometry. A representative of two independent experiments is shown. Bars, SD.

Comparison of the PGC-1 sequence of E1039 and E1165 reveals heterogeneity in *orf1916* and *pilE*

The sequence of PGC-1 of *E. faecium* strain E1039 (GenBank accession no. NZ_ACOS01000003) (van Schaik *et al.*, 2010), which did not assemble PilA pili, was compared with the recently sequenced PGC-1 of *E. faecium* E1165, which is a strain that produces PilA pili. In general, the proteins putatively encoded from the ORFs from PGC-1 of E1039 and E1165 exhibited a very high overall similarity of >97% (Table 3). The organization and transcriptional direction of the ORFs in this PGC were similar to each other, demonstrating that PGC-1 is conserved among *E. faecium* E1039 and E1165. The sortase genes *srt1903* and *srt1905* of E1039 and E1165 did not contain mutations leading to amino acid changes in the active site amino acid residues or the TLxTC catalytic pocket (Ilangovan *et al.*, 2001; Mazmanian *et al.*, 1999). Furthermore, conserved elements required for pilus assembly, such as the CWS of the putative minor pilin protein PilF, the HLYPKN pilin

Table 3. Similarities between the proteins of PGC-1 of *E. faecium* E1039 and E1165

PGC-1 protein	Putative function	Amino acid identity (%)
Srt1905	Class A housekeeping sortase	98.7
PilA	Major pilin subunit	98.9
Srt1903	Class C pilus-dedicated sortase	98.8
PilE	PilF linker protein	97.8
Orf1916	Secreted peptide	97.7
PilF	Minor pilin subunit	99.3

motif, the E box sequence and the CWS of the major PilA pilin subunit, were identical among the E1039 and E1165 strains. The largest sequence heterogeneity in PGC-1 was found in the putative PilE and Orf1916 proteins. PilE of E1039 did not contain a CWS, but it did contain a C-terminal IYPKS sequence, which resembles a pilin motif (Fig. 1). Orf1916 contained two amino acid substitutions in the hydrophobic polypeptide.

Display of PilA, PilE and PilF pilin proteins on the surface of *E. faecium* E1039 under different environmental conditions

To show the presence of the PilA, PilE and PilF proteins at the surface of *E. faecium* E1039, TEM was performed on negatively stained and immunogold-labelled E1039 bacteria grown on TSA agar at 21 and 37 °C. Using specific peptide antisera directed against PilA, PilE and PilF, gold particles were associated with the cell wall of strain E1039 at 21 °C (Fig. 3a–c) and at lower levels at 37 °C (Fig. 3b, d, f). The PilA, PilE and PilF proteins were equally distributed in high densities at the surface of the cells. Although the PilE protein in E1039 lacks the deviant IVSTG putative sortase substrate motif, the protein was present at the cell wall of E1039. No gold particles were associated with the cell wall when cells were incubated with the individual rabbit pre-immune sera (Fig. 3d) or when immune sera were omitted (conjugate control; protein-A–gold) (Fig. 3e).

Since the DNA sequences of PGC-1 of E1039 and E1165 were highly similar, we hypothesized that particular environmental conditions relevant for the *in vivo* lifestyle of *E. faecium* may induce PilA pilus assembly in the non-hospital-acquired E1039 isolate. Therefore, E1039 and E1165 strains were grown under anaerobic conditions on TSA and BHI agar at 37 °C, in aerobic conditions on BHI at 37 °C, and on TSA and BHI agar at the elevated temperature of 42 °C, and in filter-sterilized human male urine at 37 °C. The E1039 and E1165 cells grown under these different conditions were harvested, and subjected to TEM analysis using anti-PilA immune serum. Strain E1165 assembled PilA-type pili at the surface during all the different growth conditions; however, in strain E1039, anti-PilA immune serum labelled PilA at the surface, but PilA pilus polymerization was not induced (data not shown).

PilF, but not PilE, is associated with pili in *E. faecium* E1165

To corroborate whether the PilE and PilF pilin proteins were associated with the cell wall and pili, respectively, single-labelling TEM experiments were performed on *E. faecium* E1165 cells grown on TSA agar at 21 and 37 °C. In the TEM experiments, cells were labelled with either rabbit anti-PilE or anti-PilF immune serum, followed by protein-A–gold (10 nm). In contrast with E1039, no gold particles bound to the cell wall or to the pili of E1165 when cells were grown at either temperature and incubated with

Fig. 3. TEM micrographs of *E. faecium* E1039. Strain E1039 was grown at 21 or 37 °C and labelled individually with rabbit anti-PilA, -PilE or -PilF immune serum, followed by protein-A-gold (10 nm). Samples were negatively stained. Temperatures and serum labels were as follows: (a) 21 °C, anti-PilA serum; (b) 37 °C, anti-PilA serum; (c) 21 °C, anti-PilE serum; (d) 37 °C, anti-PilE serum; (e) 21 °C, anti-PilF serum; (f) 37 °C, anti-PilF serum. (g) Representative micrograph of E1039 incubated with rabbit pre-immune serum followed by protein-A-gold (10 nm). (h) E1039 incubated with protein-A-gold only (conjugate control). Bars, 200 nm.

anti-PilE serum (data not shown). Using anti-PilF serum, PilF was associated with pili present at the poles in 40 % of the cells (Fig. 4a), and cells without pili contained cell-wall-associated PilF or did not express PilF at all. Gold particles were equally distributed along pilus shafts at 37 °C (Fig. 4b), and were associated with pilus tips (Fig. 4c) and the base of the pilus (Fig. 4d). PilF was associated with the cell wall at 21 °C (Fig. 4e) and did not assemble into pili. Rabbit pre-immune serum did not react with pilus-like structures or with the cell wall (Fig. 4f).

PilF is assembled into PilA-type pili of *E. faecium*

To investigate whether the PilF pilin subunit was associated with PilA-type pili, a double-labelling TEM experiment was performed. *E. faecium* E1165 cells were grown at 37 °C, and initially labelled with rabbit anti-PilA immune serum, followed by protein-A-gold (5 nm). They were then fixed, and subsequently incubated with anti-PilF serum, followed by protein-A-gold (10 nm). TEM analysis confirmed that both PilA and PilF immune sera bound to the same type of

pili (Fig. 5a). We showed that in PilA-type pili, PilA forms the major pilin subunit and PilF forms a minor subunit, which is also positioned at the pilus tip. Multiple (typically 3–5 gold particles) PilF pilin subunits were also positioned at the base of some PilA pili (Fig. 5b). PilF was also found to be distributed along the PilA pilus shaft.

To confirm that PilF is assembled into PilA-type pili, the *pilA* gene was inactivated by an insertion-deletion mutation in the multiresistant clinical *E. faecium* E1165 isolate. Correct insertion-deletion mutation in the *pilA* gene was confirmed by PCR, sequencing and Southern hybridization, and it was demonstrated that in the double-crossover E1165 Δ *pilA* mutant, a part of the *pilA* gene was replaced by the chloramphenicol (*cat*) cassette (data not shown). Subsequent TEM analysis demonstrated that in the E1165 Δ *pilA* strain, PilF bound to the cell surface only, and was not associated with pili (Fig. 5c). In addition, Western blotting performed on surface protein extracts of wild-type and E1165 Δ *pilA* cells revealed the absence of high-molecular-mass PilF pilin species (data not shown).

Fig. 4. TEM micrographs of PilF associated with pili of *E. faecium* E1165. E1165 cells were grown at 21 or 37 °C, and labelled individually with rabbit anti-PilF immune serum, followed by protein-A-gold (10 nm); samples were negatively stained. (a) PilF is associated with pili at the pole of the cell (bar, 200 nm). (b, c) Detail of E1165 grown at 37 °C, showing that PilF is associated along a thin pilus of 1 µm (b) or that PilF is positioned at the tip of pilus-like structures (c) (bars, 100 nm). (d) Detail of a pilus, showing that PilF is positioned at the tip, along the pilus shaft, and present in an elevated concentration at the base of the pilus (arrow) (bar, 40 nm). (e) E1165 grown at 21 °C, showing that PilF is associated at the cell wall (bar, 200 nm). (f) E1165 incubated with rabbit pre-immune serum, followed by protein-A-gold (10 nm) (bar, 200 nm).

Differential surface display of PilA, PilE and PilF in *E. faecium* E1039 and E1165

To analyse the differences in deposition of the PilA, PilE and PilF LPXTG-like proteins at the surfaces of *E. faecium* strains E1039 and E1165 in more detail, Western blotting was performed on surface protein extracts of cells grown on TSA plates at 21 and 37 °C. This confirmed our previous results showing that PilA assembled into high-molecular-mass pilus-like structures in E1165 at 37 °C, whereas the monomeric subunit was produced at the predicted molecular mass of 64.8 kDa at the surface when cells were grown at 21 °C (Fig. 6a). In contrast, PilA did not assemble into high-molecular-mass pili in *E. faecium* E1039, but displayed at the surface at 37 °C; protein levels were higher at 21 °C.

Anti-PilE serum reacted with a ~90 kDa protein band in surface protein extracts of E1039 at 21 °C, and at lower levels at 37 °C, while the PilE protein was not detected in cell wall extracts of E1165 at either temperature (Fig. 6b). The observed molecular mass of PilE did not correlate with the predicted molecular mass of 22.8 kDa (after post-translational modification), suggesting association of PilE with another protein. In strain E1039, the anti-PilF sera

reacted with three bands of 75, ~90 and ~100 kDa at 37 °C, and with one predominant protein band of ~75 kDa at 21 °C. Anti-PilF serum reacted with a smear of protein-reactive material in E1165, which ranged into the stacking gel, suggesting that the PilF pilin protein is associated with pili (Fig. 6c). The monomeric PilF subunit was produced at predicted molecular mass of 70.4 kDa (after post-translational modification) at 21 °C. Pre-immune sera were used as negative controls, and did not react with any of the protein products.

mRNA expression of the housekeeping sortase and pilus-dedicated sortase of PGC-1

Total RNA was isolated from the clinical E1165 and community-derived E1039 *E. faecium* strains after growth on TSA plates at 21 and 37 °C. mRNA expression of *srt1903* (pilus-dedicated sortase), *srt1905* (housekeeping sortase) and *tuf* (internal housekeeping control) genes was analysed by PCR on cDNA generated from total mRNA. mRNA transcripts of *srt1903* and *srt1905* were detected in comparable levels in the E1039 and E1165 strains at both temperatures (Fig. 7), indicating that strain- or

temperature-dependent differences in expression of the putative-dedicated sortases do not cause the observed differences in pilus assembly between E1039 and E1165. The control *tuf* housekeeping gene was expressed in all isolates analysed. No DNA contamination was detected in control *tuf* reactions of the DNase-treated total mRNA preparations in which the reverse transcriptase reaction was omitted.

DISCUSSION

E. faecium isolates of clinical and non-clinical origin express PilA-type pili differently, showing the complexity of pilus assembly and pilin protein production among *E. faecium* isolates from different sources, and of distinct genetic background. More specifically, we demonstrated the differential assembly of PilA-type pili at the surface of a human *E. faecium* clinical hospital-acquired bloodstream isolate (E1165) and a community-derived stool isolate (E1039) grown at 21 and 37 °C. While PilA and PilF proteins were associated with the cell wall of E1165 grown at 21 °C, and assembled into high-molecular-mass pilin species at 37 °C, PilA, PilE and PilF pilin proteins appeared to be cell-wall-associated and not pilus-associated in E1039, when grown at 37 or 21 °C. At 21 °C, all three proteins were displayed at the surface in higher levels. Hence, in the community-derived stool isolate, not only was pilus assembly abrogated, but also the level of surface protein production was differentially regulated when compared with strain E1165. mRNA expression analysis of the pilus-dedicated sortase and housekeeping sortase genes located in PGC-1 revealed comparable levels of transcripts, suggesting that the differential PilA pilus assembly is controlled post-transcriptionally. Which environmental conditions that are relevant for the lifestyle of *E. faecium* induce PilA pilus polymerization in the E1039 strain or in the other tested *E. faecium* isolates, such as growth in the presence of microbial communities in the gastrointestinal tract, in bile, serum or blood, or growth under fluctuating pH or carbon dioxide concentrations, remain to be elucidated.

Double-labelling TEM analysis of the wild-type E1165 and the isogenic *pilA* mutant demonstrated that the PilA protein is the major component of PilA-type pili, and that it forms the pilus backbone. TEM analyses also showed that the PilF protein was found at the pilus base, was equally distributed along the pilus shaft and was associated with the PilA pilus tip in the clinical *E. faecium* E1165 strain at

Fig. 5. TEM micrographs of PilF associated with PilA-type pili. *E. faecium* E1165 was grown at 37 °C and labelled with rabbit anti-PilA immune serum, followed by protein-A-gold (5 nm particles). Cells were fixed and consecutively labelled using anti-PilF immune serum, followed by protein-A-gold (10 nm particles). (a) Distribution of PilF along the PilA pilus shaft. (b) PilF (arrowheads) is positioned at the PilA pilus tip and at the pilus base. (c) An isogenic *pilA* mutant, E1165Δ*pilA* did not display PilF/PilA pili. Bars, 200 nm.

Fig. 6. Western blots of surface protein extracts of *E. faecium* E1039 and E1165 grown on TSA agar at 21 and 37 °C. Blots were probed with rabbit anti-PilA (a), rabbit anti-PilE (b) and rabbit anti-PilF immune sera (c) (left lanes), and with the respective pre-immune sera (right lanes), followed by goat anti-rabbit IgG-horseradish-peroxidase conjugate. Molecular mass values (kDa) are shown on the left. Predicted monomeric PilA (64.8 kDa) is indicated by an arrow.

37 °C. Therefore, PilF was designated a minor pilin subunit, which may represent an adhesin that potentially binds to biotic or abiotic surfaces. It has been demonstrated that the sorting signal IPNTG of the BcpB minor pilin subunit (which lacks a pilin motif) of *Bacillus cereus* is distinct from the LPXTG sorting signal of the major pilin subunit BcpA, and is specifically cleaved by the pilus-dedicated sortase, and not by the housekeeping sortase, thereby initiating polymerization (Budzik *et al.*, 2009). This sortase-specific cleavage of the minor pilin protein indicates that BcpB is positioned at the BcpA pilus tip of *B. cereus*. Initiation of PilA pilus polymerization may be more complex in *E. faecium*. The fact that both PilA and PilF proteins harbour VPXTG sorting motifs suggests that another signal, in addition to the sorting substrate motif that determines sortase specificity, is required to initiate pilus polymerization in *E. faecium*.

Recently, it has been shown that the minor pilin subunit (SpaB) is required to stop SpaA-type pilus polymerization of *Corynebacterium diphtheriae*, and to anchor pilus polymers to the cell wall through incorporation of the minor pilin subunit in the base of the growing pilus shaft (Mandlik *et al.*, 2008). Localization of PilF at the base of PilA pili, as observed in our study, suggests that PilF may also be required for termination of pilus polymerization. However, construction of a *pilF* mutant will be needed to provide evidence for this hypothesis.

The role of PilE in pilus biogenesis remains unresolved and requires further characterization. In E1165, PilE protein was not associated with PilA-type pili or expressed at the cell wall at 21 or 37 °C. PilE, which lacked the deviant IVSTG motif in E1039, is an SE-rich protein, and the SD repeats or S-rich repeat domains of multiple MSCRAMMs are predicted to adopt a long coiled structure to expose the ligand-binding domain to the extracellular environment (Deivanayagam *et al.*, 2000; Hartford *et al.*, 1997). The presence of SD repeats and S-rich repeats in PilE suggests that this protein may adopt a comparable structure. Since cell-surface exposure of PilE correlated with absence of assembled pili in E1039, we suggest that surface deposition of PilE abrogates pilus biogenesis. To prove this, conclusive

Fig. 7. mRNA expression of sortase genes *srt1903* and *srt1905* of PGC-1. The mRNAs of the community-derived isolate E1039 and the clinical isolate E1165, grown at 21 and 37 °C, are shown. Control *tuf* RT-PCRs (internal housekeeping control) with *E. faecium*-specific *tuf* primers were all positive. mRNA expression levels were similar for the *srt1903* pilus-dedicated sortase gene (*srt1903*) and the *srt1905* housekeeping sortase gene of the two strains at both temperatures. The results are presented as PCR products separated on an ethidium-bromide-stained 1.0% agarose gel. M, Molecular mass markers.

experimental evidence has to be obtained using additional knockout mutations in genes of this PGC. However, to date, attempts to construct isogenic *pilE* and *pilF* mutants using the pTEX5500ts vector have failed.

Only in 2005, the first Gram-positive PGCs were identified and the roles of the different proteins encoded by these PGCs were elucidated. Our study has disclosed novel findings with respect to temperature- and strain-dependent regulation, as well as differential assembly of pili at the surface of a clinically relevant Gram-positive species. Increasing the knowledge of pilus assembly will increase our understanding of the different steps implicated in Gram-positive pathogenesis in general and in *E. faecium* in particular. Understanding colonization and virulence factors is important since the incidence of *E. faecium* infections with acquired resistance to the three commonly used classes of antimicrobials, i.e. aminoglycosides, β -lactams and glycopeptides, continues to increase (Werner *et al.*, 2008). Treatment of these infections will become even more challenging when resistance against antibiotics from other, recently introduced, classes of antimicrobials, such as daptomycin and linezolid, emerges (Aksoy & Unal, 2008; Kainer *et al.*, 2007; Montero *et al.*, 2008). Therefore, novel alternative treatment and prevention strategies such as passive or active immunization are urgently required. Gram-positive pilin proteins may be promising novel targets for immune therapy (Gianfaldoni *et al.*, 2007; Mora *et al.*, 2005).

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