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YajC, a predicted membrane protein, promotes *Enterococcus faecium* biofilm formation *in vitro* and in a rat endocarditis model

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Abstract

Biofilm formation is a critical step in the pathogenesis of difficult-to-treat Gram-positive bacterial infections. We identified that YajC, a conserved membrane protein in bacteria, plays a role in biofilm formation of the clinically relevant *Enterococcus faecium* strain E1162. Deletion of *yajC* conferred significantly impaired biofilm formation *in vitro* and was attenuated in a rat endocarditis model. Mass spectrometry analysis of supernatants of washed Δ*yajC* cells revealed increased amounts in cytoplasmic and cell-surface-located proteins, including biofilm-associated proteins, suggesting that proteins on the surface of the *yajC* mutant are only loosely attached. In *Streptococcus mutans* YajC has been identified in complex with proteins of two cotranslational membrane protein-insertion pathways; the signal recognition particle (SRP)-SecYEG-YajC-YidC1 and the SRP-YajC-YidC2 pathway, but its function is unknown. In *S. mutans* mutation of *yidC1* and *yidC2* resulted in impaired protein insertion in the cell membrane and secretion in the supernatant. The *E. faecium* genome contains all homologous genes encoding for the cotranslational membrane protein-insertion pathways. By combining the studies in *S. mutans* and *E. faecium*, we propose that YajC is involved in the stabilization of the SRP-SecYEG-YajC-YidC1 and SRP-YajC-Yid2 pathway or plays a role in retaining proteins for proper docking to the YidC insertases for translocation in and over the membrane.

Keywords: YajC; Biofilm; rat endocarditis model; *Enterococcus faecium*; cotranslational membrane protein insertion pathway; *Streptococcus mutans*

Introduction

Enterococci, gut commensals in a wide variety of hosts, have emerged as one of the major nosocomial multidrug resistant pathogens, ranking among the top three causes of bloodstream, surgical site, and urinary tract infections (Hidron et al. 2008). *Enterococcus faecium*, together with *Enterococcus faecalis*, are responsible for a sizable fraction of difficult-to-treat infections, mostly due to their ability to form biofilms (Donlan and Costerton 2002) and antibiotic resistance, with the latter most apparent in *E. faecium* with increasing rates of ampicillin and vancomycin resistance (Rice 2001, Miller et al. 2020). A biofilm is a complex microbial community that is protected by an extracellular matrix and that can develop from a unicellular planktonic lifestyle (O'Toole et al. 2000, Costerton 2001, Abee et al. 2011). The transition from planktonic to the sessile state is triggered by environmental signals and it can be an important adaptation for survival of microorganisms (Chmielewski and Frank 2003). The clinical relevance of biofilms is related to difficult-to-treat infections, in particular those associated with medical implants and endovascular foreign bodies. Several mechanisms, including release of extracellular DNA, proteins, and polysaccharides contribute to biofilm formation and stability (Abee et al. 2011, Paganelli et al. 2012, 2015, 2016). Although these components are conserved in bacterial species, the molecular pathways leading to release of these factors seem to be mostly species specific. So far, the identified mechanisms involved in biofilm formation in *E. faecium* are related to autolysis and surfaces proteins (Heikens et al. 2007, Hendrickx et al. 2007, 2008, Nallapareddy et al. 2011, Paganelli et al. 2013, Top et al. 2013, 2015). The discovery of new mechanisms of biofilm formation can aid in development of new drugs to treat these infections.

In this study, we adapted an unbiased technique called microarray-based transposon mapping (M-TraM) (Zhang et al. 2012) to perform a genome-wide screening for determinants involved in biofilm formation in *E. faecium*. This screening identified *yajC*, encoding a membrane protein, as a critical determinant of biofilm formation in *E. faecium*. Using a targeted mutagenesis approach in *E. faecium*, we demonstrate that YajC, predicted to be part of two different putative cotranslational membrane protein

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insertion pathways, plays a role in biofilm formation by retaining cytoplasmic and cell surface located proteins at the surface.

Materials and methods

Bacterial strains, plasmids, growth conditions, and determination of growth curves

Enterococcus faecium and *E. coli* strains used in this study are listed in Table S1. The ampicillin-resistant clinical *E. faecium* isolate E1162, for which a genome sequence is available (accession number ABQJ00000000, https://www.ncbi.nlm.nih.gov/assembly/GCF_000172675.1) was used throughout this study (van Schaik et al. 2010). *Enterococcus faecium* were grown in brain–heart infusion medium (BHI) at 37°C. For biofilm assays, tryptic soy broth (TSB) supplemented with 1% glucose (TSBg) was used (Top et al. 2013). *Escherichia coli* DH5α and EC1000 (Leenhouts et al. 1996) were grown in Luria–Bertani medium. Where necessary, antibiotics (Sigma–Aldrich, Saint Louis, MO) were used at the following concentrations: gentamicin 300 µg/ml (*E. faecium*) and 25 µg/ml (*E. coli*), spectinomycin 300 µg/ml (*E. faecium*) and 100 µg/ml (*E. coli*), and ampicillin 100 µg/ml (*E. coli*). Determination of growth curves was performed as described previously (Zhang et al. 2012) using a BioScreen C instrument (Oy Growth Curves AB).

Identification of genes involved in biofilm formation in *E. faecium* by mapping of transposon insertion sites

We used the high throughput technique M-TraM for a genome-wide screening (Zhang et al. 2012) to identify genes involved in biofilm formation in *E. faecium* and followed a similar approach as performed previously by Amini et al. (2009). We grew a transposon library constructed in the E1162 strain (Zhang et al. 2012) in 20 ml BHI with gentamicin (100 µg/ml) for 24 h. A volume of 20 µl of overnight culture was transferred to a glass slide coated with poly-L-lysine that was placed in a glass Petri dish (14 cm) with 40 ml TSBg. Biofilm were allowed to grow on the glass slide for 24 h at 37°C and at 120 rpm. After 24 h growth, 20 µl of the planktonic phase was transferred to a new poly-L-lysine coated glass slide and again biofilms were allowed to grow for 24 h. By repeating this three times, mutants present in the library that were deficient for biofilm formation were enriched. After four passages, 1 ml from the planktonic phase was collected and total genomic DNA from the enriched library and that of the original library was isolated and hybridized to an *E. faecium* E1162 microarray to assess the relative abundance of clones enriched in comparison to the entire library (Fig. S1) (Zhang et al. 2012). Microarray data generated in this study have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4524.

Generation of a *yajC* markerless deletion mutant and *in trans* complementation

We generated a markerless mutant in the *yajC* gene in *E. faecium* using a previously described approach (Zhang et al. 2012). For the amplification of the flanking regions of *yajC*, we used primers yajCup-F-XhoI, yajCup-R-EcoRI, yajCdn-F-EcoRI, and yajCdn-R-XhoI (Table S2). Generation of the deletion mutant was confirmed by PCR using the yajC check-up and check-down primers (Table S2).

A plasmid for the *in trans* complementation of the Δ*yajC* was constructed by amplification of the *yajC* gene using the yajCpEF25-F-BamHI and yajCpEF25-R-HindIII primers listed in Table S2 as described by previously (Top et al. 2013).

Biofilm semistatic model and confocal laser scanning microscopy

A biofilm semistatic model was performed as previously described (Paganelli et al. 2013). In brief, overnight bacterial cultures were diluted to an OD_{660} of 0.01 in 6 ml TSBg and added to a coverslip coated with poly-L-lysine (0.45 µm; diameter, 12 mm; Becton Dickinson) inside a well from a six-well polystyrene plate (Corning Inc.). Biofilms were grown at 37°C for 24 h at 120 rpm. After 24 h, the coverslips were washed once with 0.85% NaCl, and the biofilms were chemically fixed in 8% glutaraldehyde (Merck) for 20 min and washed again with 0.85% NaCl. The biofilms were stained with 15 µg/ml propidium iodide (PI) in 0.85% NaCl for 15 min. Pictures were analyzed with LAS AF software (Leica) and biofilm was quantified using Comstat (Heydorn et al. 2000). Matlab R2010b software (The MathWorks). The average thickness and biomass of the biofilms was measured at five randomly chosen positions. Statistical analysis of the data was performed using a two-tailed Student's *t*-test.

Flow cell biofilm model

Dynamic biofilms were studied in a Stovall flow cell system (Life Science, Inc., Greensboro, N.C.) as described previously (Paganelli et al. 2013). In brief, 24 h TSB cultures were diluted until an OD_{660} of 0.01 and inoculated in the chambers. Biofilms were grown in TSB diluted in PBS [1:10 (v/v)] with 1% glucose under a flow of 0.13 ml/min during 17 h. Biofilm development was scanned at regular intervals of 7 min (40x objective) with a DFC360 FX digital camera kit SP5 (Leica). After 17 h, the flow was increased to 0.5 ml/min to wash away loose cells. Final biofilms were stained with live/dead stain (BAC light kit; Invitrogen). Images were acquired using a confocal microscope (Leica SP5).

Initial polystyrene adherence assay

The initial adherence assay was performed in triplicate, as described previously with minor modifications (Heikens et al. 2007, Paganelli et al. 2013). Overnight cultures in TSBg of *E. faecium* strains were either washed two times with PBS, or not, and washed cells were resuspended again in the same volume. Next, 100 µl of bacterial suspension was added in triplicate to the polystyrene plate and incubated at 37°C for 2 h. Bacteria were stained with 0.2% crystal violet and eluted with 96% ethanol. Statistical analysis of the data was performed using a two-tailed Student's *t*-test.

Analysis of the supernatant proteome of E1162 wild-type and the Δ*yajC*

To determine the proteome of the supernatant of washed E1162 wild-type and Δ*yajC*, 4 ml of TSBg overnight grown cells were washed 1x with 1 ml phosphate-buffered saline (PBS), resuspended in 1 ml PBS and incubated for 1 h at 4°C while gently shaking. After incubation, cells were centrifuged for 5 min at 13 000 × *g*. Supernatant was collected for total protein analysis and concentrated with a 10 k filter (Amicon Ultra—Merck Millipore). Total protein was loaded on a 12.5% SDS-PAGE gel, which was run for 5 min. After Colloidal Blue staining, gel slices were cut to include all proteins and in gel digestion procedure was started as described before (Ince et al. 2010). Proteins were identified by nano liquid chromatography MS/MS (Lu et al. 2011). MS/MS data were analyzed with MaxQuant (Cox et al. 2009) using a specific *E. faecium* E1162 database downloaded from the Uniprot website)

together with a contaminant database and filtered with Perseus as described before (Smaczniak et al. 2012).

GAPDH, ATP synthase, and Tu localization

GAPDH, ATP synthase, and Tu localization in *E. faecium* was performed on TSBg overnight cultures washed with PBS. Washed bacteria (10 µl) were placed on a cover slip coated with poly-L-lysine and dried at 55°C for 20 min. Cells were washed with 3 ml Hanks' balanced salt solution (HBSS) (Sigma–Aldrich), fixed with 3% paraformaldehyde for 15 min and washed again with HBSS. Cells were incubated with antirecombinant GAPDH monoclonal antibody (Thermo Fisher Scientific), anti-Complex V alpha subunit (or ATP synthase) monoclonal antibody (Thermo Fisher Scientific) and antirecombinant Tu (or elongation factor Tu) polyclonal antibody (Thermo Fisher Scientific) (1:200 in HBSS with 1% BSA) for 1 h on ice and subsequently washed with PBS. A secondary IgG antibody Alexa Fluor 488 goat antirabbit or antimouse (Life Technology) (1:500 in HBSS with 1%BSA) was added and incubated for an additional 1 h on ice to detect binding to first antibodies for *E. faecium*. Cells were washed once more and incubated with 5 µg/ml FM 5–95 dye (Invitrogen) for 2 min on ice. Fluorescence was analyzed in the confocal microscope (Leica SP5). Alexa 488 and FM 5–95 were excited at 488 nm. As a control, bacteria were treated as described above, but omitting the first antibodies. The amount of proteins present in the cell surface was calculated in ImageJ (Schneider et al. 2012) by the ratio between green (protein of interested) and red signal (number of bacteria).

SDS-PAGE and western blot

Protein samples were equally mixed with sample buffer (100 mM Tris-HCl, 5% dithiothreitol, 2% SDS, 0.004% bromophenol blue, and 20% glycerol) and boiled for 5 min. Western blotting was carried out as described previously (Hendrickx et al. 2007). Proteins were visualized using the ECL Plus western blotting detection system (GE Healthcare, Diegem, Belgium) and ImageQuant LAS 4000 biomolecular imager GE Healthcare.

Rat endocarditis model

To assess the role of YajC in infective endocarditis, two groups of five female Wistar rats (Charles River Laboratories Germany GmbH) were infected with *E. faecium* E1162 wild-type strain and with the Δ*yajC*, respectively, as described previously (Haller et al. 2014). Valve vegetations of one rat per group (one inoculated with wild-type and one inoculated with the Δ*yajC*) were fixed in 4% formalin and further analyzed by microscopy. Fixed samples were processed for scanning transmission electron microscopy as described previously (Wang et al. 2013). Samples were mounted onto specimen mounts and coated with 80% Pt–20% Pd to 10 nm using a Cressington 208HR sputter coater at 20 mA prior to examination with a Phenom World tabletop scanning electron microscope (SEM). The SEM was operated with an acceleration voltage of 5 kV. After weighting of the valve vegetations of the four remaining rats from each group, 500 µl TSB was added per sample and the vegetations were homogenized on ice using a tissue homogenizer. Serial dilutions of the samples were made in TSB and plated. Quantitative assessment was performed by weighing of the vegetations as well as culturing serial dilutions on agar plates incubated over night at 37°C (Haller et al. 2014).

Mouse colonization model

Intestinal colonization by wild-type E1162 and Δ*yajC* was tested as previously described (Zhang et al. 2012, Top et al. 2013). In brief, specific pathogen free 10-week-old male Balb/c mice (16 mice in total) were purchased from Charles River Laboratories Inc. (Maastricht, the Netherlands) and housed as described previously. A total of 2 days before inoculation of bacteria, mice were administered subcutaneous injections of ceftriaxone (Roche, Woerden, The Netherlands; 100 µl per injection, 12 mg/ml) two times daily and one time at the day of inoculation. For the remaining duration of the experiment, cefoxitin (0.125 g/l) was added to sterile drinking water. The inoculum of 2×10^9 CFU/300 µl Todd Hewitt Broth E1162 or Δ*yajC* was prepared as described previously (Zhang et al. 2012, Top et al. 2013). Collection of samples and determination of bacterial outgrowth was performed as previously described (Zhang et al. 2012, Top et al. 2013).

Ethics statement

The rat endocarditis experiment was performed with permission of the regional administrative authority Freiburg (animal welfare committee of the University of Freiburg; Regierungspräsidium Freiburg Az 35/9185.81/G-07/72) and in accordance with the German animal protection law (TierSchG). The rats were handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS.

The mouse colonization study was approved by the Animal Ethics Committee Utrecht and the Animal Welfare Body Utrecht as part of a project, which was licensed by the Central Authority for Scientific Procedures on Animals (CCD) (license number: AVD115002016568).

Results

Identification of genes involved in biofilm formation in *E. faecium* by M-TraM

We applied the M-TraM (Zhang et al. 2012) approach to identify new genes potentially involved in *E. faecium* biofilm formation. Therefore, we enriched a transposon library constructed in the clinical *E. faecium* strain E1162 (van Schaik et al. 2010) for mutants deficient in biofilm formation in a semistatic biofilm model. After each round of enrichment, the planktonic fraction of the library was passed onto a new glass slide coated with poly-L-lysine (Fig. S1). After four passages the amount of biofilm formation was only a fraction of the initial culture, indicating a considerable enrichment of biofilm defective mutants in the planktonic phase of the fourth passage. The genetic content of the library in the planktonic phase after the fourth passage was compared to the total transposon library grown in BHI broth using DNA microarrays. This revealed that the relative abundance of transposon insertions in 16 genes was significantly increased after enrichment for biofilm deficiency and as a result, these genes were considered to be putatively involved in biofilm formation in *E. faecium* (Table 1). The transposon insertion mutant that was the most (>70-fold) enriched, contained a transposon insertion in the gene with locus tag EfmE1162_0936, designated *yajC*. Therefore, the focus of this study was the characterization of *yajC* and its role in biofilm formation.

YajC plays a role in biofilm formation *in vitro*

The *yajC* gene is part of the core genome of Gram-positive and Gram-negative bacteria and is predicted to encode a protein containing an N-terminal transmembrane domain and cytosolic C-terminus (Fig. S2) (Hallgren et al. 2022). In *Streptococcus mutans*, YajC has been described to be part of two putative cotranslational membrane protein insertion pathways, the signal

Table 1. Top genes putatively involved in biofilm formation in *E. faecium* according to M-TraM analysis.

Locus Tag[a]	Annotation	Fold-change[b]
EfmE1162_0936	Preprotein translocase subunit YajC	71.51
EfmE1162_0935	Queuine tRNA-ribosyltransferase	46.89
EfmE1162_1879	ABC transporter, ATP-binding protein	13.65
EfmE1162_1566	ABC transporter, permease protein	10.67
EfmE1162_1563	Cytidine deaminase	8.63
EfmE1162_1028	Phosphate ABC transporter, phosphate-binding protein	7.99
EfmE1162_1562	Deoxyribose-phosphate aldolase	6.79
EfmE1162_2142	Nitrate transport ATP-binding protein NrtD	6.69
EfmE1162_0665	PTS system, fructose-specific family, IIABC components	6.01
EfmE1162_2009	Cation diffusion facilitator family transporter	5.73
EfmE1162_1550	Putative phosphoglucomutase	5.62
EfmE1162_1564	Basic membrane protein family	5.55
EfmE1162_1567	ABC transporter, permease protein	5.38
EfmE1162_1365	ComG operon protein 1	5.26
EfmE1162_1565	Ribose import ATP-binding protein RbsA	4.85
EfmE1162_0666	1-phosphofructokinase	4.49

[a]Locus tag of the genes containing the transposon insertion.
[b]Fold-change derived from the ratio of the unselected control library to the biofilm deficient enriched library.

recognition particle (SRP)-SecYEG-YajC-YidC1 and the SRP-YajC-YidC2 pathway (Lara Vasquez et al. 2021). Genome analysis of *E. faecium* E1162 (van Schaik et al. 2010) revealed the presence of all genes encoding the proteins involved in the proposed pathways in *S. mutans*, suggesting that similar pathways exist in *E. faecium* (Table S3). However, although transposon insertions were identified in these genes, none appeared differential abundant in the M-TRAM experiment (data not shown). To confirm that YajC contributes to biofilm formation in *E. faecium*, we constructed a markerless deletion mutant (Δ*yajC*) and complemented this deletion *in trans* by introducing a complete copy of *yajC* on the vector pEF25 (Top et al. 2013) in Δ*yajC* (Δ*yajC*+*yajC*). Biofilm formation of the wild-type, Δ*yajC* and the complemented strain (Δ*yajC*+*yajC*) was tested in two biofilm models, a semistatic model and a flow cell model. In the semistatic model, a significant decrease in biofilm biomass and thickness was observed in Δ*yajC* when compared to the wild-type strain and this deficiency could be complemented in Δ*yajC*+*yajC*, confirming the M-TraM results (Fig. 1A). In the flow cell model, biofilm formation of Δ*yajC* was also attenuated (Fig. 1B). Already after 4 h of growth, the biofilm coverage of the slide was less compared to wild-type and Δ*yajC*+*yajC*, which was even more pronounced after 17 h. In addition, the live/dead stain revealed reduced numbers of dead (red) cells in Δ*yajC*, further confirming its biofilm deficient phenotype (Desai et al. 2019).

As a control, we performed growth curves of the wild-type, Δ*yajC* and the complemented strain (Δ*yajC*+*yajC*) and did not observe clumps or aggregates nor differences in growth rate indicating that the observed deficiency in biofilm formation of Δ*yajC* is not due to growth defects (Fig. S3).

YajC plays a role in initial cell adherence

Biofilm formation is a complex process, ranging from attachment of single cells to dispersion from a mature structure, and different genes specifically contribute to each phase of biofilm formation (O'Toole et al. 2000, Chmielewski and Frank 2003). In order to identify whether YajC is involved in single cell adhesion, we performed an initial adherence assay in a 96-well polystyrene plate. We observed a small reduction in attachment by the Δ*yajC* mutant compared to wild-type when incubated directly from overnight culture (Fig. 2). However, when cells were washed with PBS and then incubated in a 96-well plate, the difference in cell adhesion was significantly larger between wild-type and Δ*yajC* (Fig. 2). These results suggested that the proteins involved in initial adherence are loosely attached to the cell surface in the Δ*yajC* mutant.

Deletion of *yajC* results in increased amounts of proteins in the supernatant of washed cells

In *S. mutans*, deletion of the genes encoding for YidC1 or YidC2, belonging to the family of membrane insertases and part of the cotranslational membrane protein insertion pathways, resulted in increased protein secretion (Palmer et al. 2012). As YajC is in complex with both proteins in *S. mutans*, we investigated whether a similar phenotype could be observed for Δ*yajC* in *E. faecium*. We analyzed the proteome of PBS supernatants of washed wild-type, Δ*yajC* and complemented strain SDS-PAGE. In the Δ*yajC* mutant, more proteins were detected in the supernatant when compared to wild-type and complemented strain after washing the cells with PBS, suggesting loose protein attachment to the cell membrane (Fig. 3A). Nano liquid chromatography MS/MS of all proteins present in the supernatant of washed Δ*yajC* and the wild-type strain revealed 272 proteins, of which 152 proteins were only present in the Δ*yajC* supernatant (Table S4). Of these proteins, 94% were predicted as intracellular or cytoplasmic proteins (CPs) and 6% as transmembrane or extracellular proteins including proteins implicated in biofilm formation as PilA (Fms21) and the PilB (Fms9/EbpA$_{fm}$) tip protein (Table S4) (Hendrickx et al. 2007, Sillanpää et al. 2008).

Western blot analysis of the supernatants using specific antibodies against two predicted CPs, i.e. glyceraldehyde-3-phophate dehydrogenase (GAPDH), elongation factor Tu and a predicted membrane bound protein ATP synthase, revealed that these proteins were present in higher quantities in the washed supernatant of Δ*yajC* compared to wild-type and complemented strain (Fig. 3B). Next, we verified the presence of these proteins on the surface of washed wild-type, Δ*yajC* and complemented cells by confocal laser scanning microscopy (Fig. 3C). This revealed that these intracellular proteins were detected at the surface of the wild-type and complemented strains but were less abundant on the surface of Δ*yajC* (Fig. 3D). This indicates that in these conditions CPs are found attached to the surface of wild-type *E. faecium* cells, but that in Δ*yajC* attachment of these proteins is altered resulting in inadequate retaining or capture at the bacterial

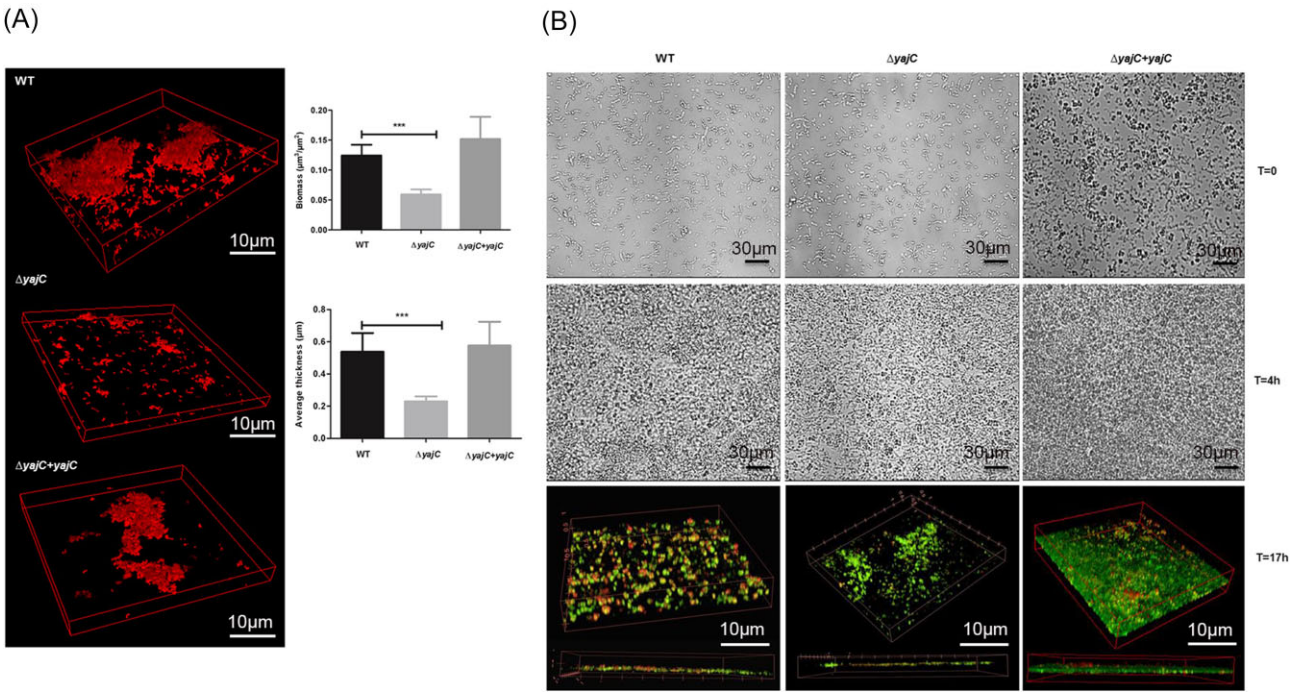

Figure 1. The effect of targeted *yajC* mutation on semistatic and flow cell biofilm model. (A) Confocal imaging of biofilm formation of wild-type, Δ*yajC* and Δ*yajC*+*yajC* in a semistatic biofilm model. Cells were grown for 24 h on poly-L-lysine-coated glass slides, in TSBg, at 120 rpm, at 37°C, chemically fixed in 8% glutaraldehyde and stained with PI (red) (scale bars in A, 10 μm). The biomass and average thickness of biofilms was measured at five random positions of three biological replicates and analyzed with Comstat/Matlab software. Pictures were taken at 63x magnification with 2.5 optical zoom. Asterisks represent significant differences (***$P < .001$) with the wild-type strain as determined by an unpaired two-tailed Student's *t*-test. (B) Biofilms of wild-type, Δ*yajC* and Δ*yajC*+*yajC* in a flow cell biofilm model. Biofilms were grown for 17 h in a Stovall flow cell system, in 1:10 diluted TSB with 1% glucose (0.13 ml/min), at 37°C. Pictures were taken at 40x magnification with 2.5 optical zoom. Cells were stained with syto 9 and PI after 17 h of growth (scale bars in bright filter picture and confocal images, 30 μm and 10 μm, respectively).

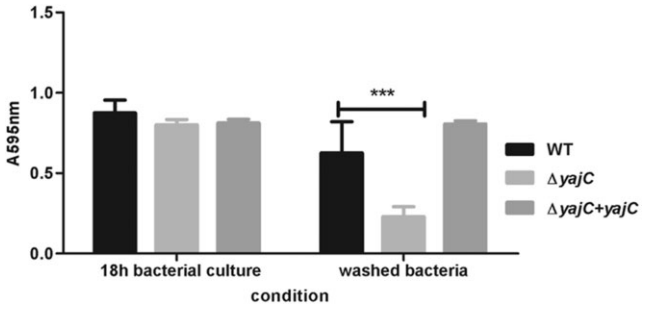

Figure 2. The effect of targeted *yajC* mutation on initial adhesion. E1162 wild-type, Δ*yajC* and Δ*yajC*+*yajC* were cultured overnight, washed two times with PBS, or not, 100 μl of bacterial suspension was added in triplicate to a polystyrene plate and incubated for 2 h at 37°C without shaking. Cell attachment was measured by absorbance of crystal violet at 595 nm and repeated three times. Asterisks represent significant difference (***$P < .001$) with the wild-type strain as determined by an unpaired two-tailed Student's *t*-test.

surface, which is in line with the results showing higher abundance of GAPDH, Tu and ATP synthase in the supernatant of Δ*yajC* relative to wild-type and complemented strain.

The *E. faecium* Δ*yajC* mutant is attenuated in a rat endocarditis model, but not in a mouse colonization model

Enterococci can cause endocarditis in humans and biofilm formation is thought to be an important step in the pathogenesis of this type of infection (Paganelli et al. 2016). Therefore, we tested if YajC contributes to infective endocarditis in a rat endocarditis model by comparing number of bacteria in the vegetations on the aortic valve formed by wild-type *E. faecium* and the *yajC* mutant. As observed in Fig. 4(A) and (B), *E. faecium* vegetations on heart valves formed by Δ*yajC* were on average half of the weight of those formed by the wild-type (5 mg in Δ*yajC* compared to 10 mg in the wild-type) and contained a significant decreased number of bacteria compared to *E. faecium* wild-type. SEM of the heart revealed altered vegetations in rat infected with Δ*yajC* relative to rat infected with the wild-type strain, demonstrating the *in vivo* relevance of YajC in *E. faecium* pathogenesis (Fig. 4C and D). In contrast, we did not observe a significant difference in number of colony-forming units (CFUs) from the ileum, cecum, colon, and faeces in a mouse colonization model (Fig. 4E and F). These latter results suggest that YajC is not essential for gastrointestinal colonization and that the *yajC* mutation did not have a significant impact on cell viability.

Discussion

Despite *E. faecium* ranks as one of most prevalent nosocomial pathogens, our knowledge of factors that play a role in *E. faecium* pathogenesis is relatively limited. Biofilm formation is considered an important step in *E. faecium* pathogenesis of catheter or other foreign body-related infections (Paganelli et al. 2012). In this study, we identified YajC as a new important player in biofilm formation of *E. faecium*. We demonstrated that disruption of the *yajC* gene resulted in impaired biofilm formation *in vitro* and *in vivo* probably due to altered protein membrane retainment of effector proteins like pilin proteins, at the cell surface.

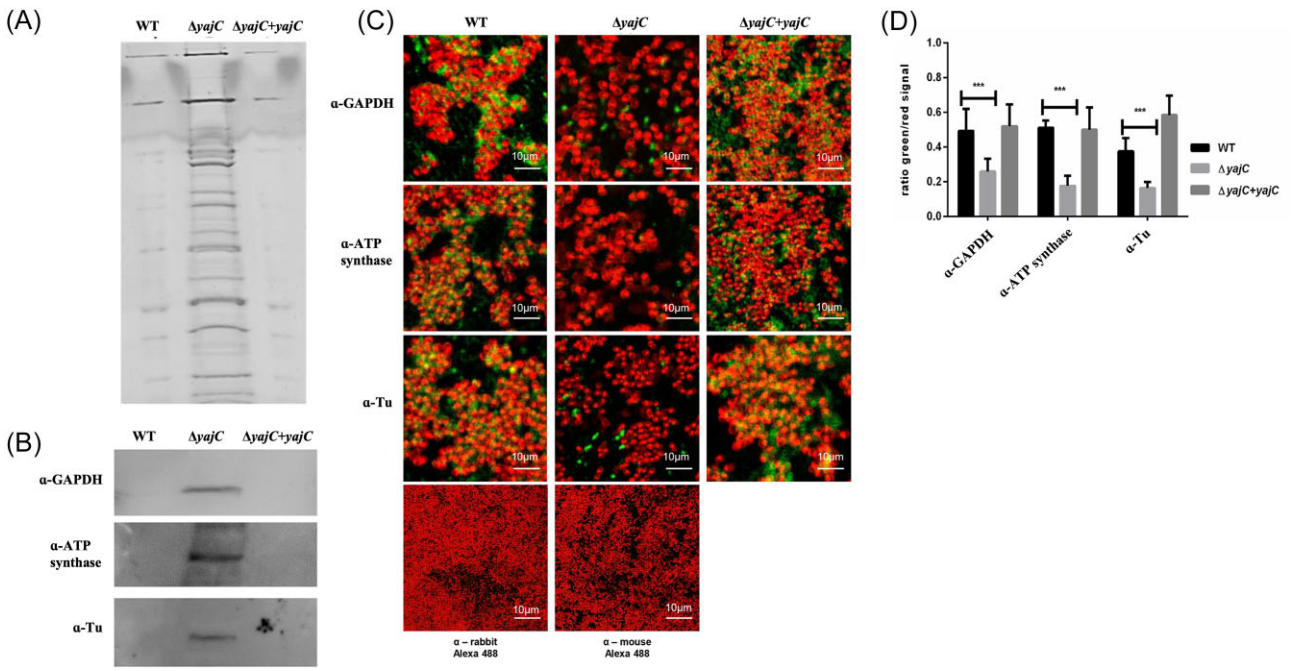

Figure 3. The effect of targeted *yajC* mutation on protein attachment. (A) Proteins present in the supernatant of wild-type, Δ*yajC* and Δ*yajC*+*yajC* after wash with PBS pH 7 were separated through a 12.5% SDS page gel with Coomassie blue stain. (B) Presence of GAPDH, ATP synthase, and Tu in the supernatant of washed *E. faecium* E1162 wild-type, Δ*yajC* and Δ*yajC*+*yajC* was analyzed by western blot using α-GAPDH, α-ATP synthase, or α-Tu antibodies. (C) Cell surface exposure of GAPDH, ATP synthase and Tu on washed *E. faecium* wild-type, Δ*yajC* and Δ*yajC*+*yajC* was analyzed by confocal microscopy. Washed cells were incubated with α-GAPDH, α-ATP synthase, or α-Tu antibodies and goat α-rabbit or goat α-mouse Alexa 488 (green). Bacterial membranes were stained with FM 95–5 (red) (scale bars in A, 10 μm). (D) Ratio between green (protein of interested) and red (bacteria) was calculated in ImageJ software. Asterisks represent significant difference (***$P < .001$) with the wild-type strain as determined by an unpaired two-tailed Student's *t*-test.

YajC is predicted to be a membrane protein. It was first described in the Gram-negative bacterium *E. coli* as part of an integral membrane heterotrimeric complex with SecD and SecF all encoded by the *secDF* operon, which is in association with a membrane-embedded trimeric complex of SecY, SecE, and SecG (SecYEG) forming SecYEGDF-YajC also referred to as holotranslocon or Sec system (Duong and Wickner 1997a,b). This holotranslocon interacts with YidC, an integral membrane protein, which is involved in insertion of membrane proteins into the cytoplasmic membrane (Beck et al. 2001).

Different to *E. coli*, Gram-positive bacteria like *S. mutans* but also *E. faecium* lack the SecDF complex but produce two YidC paralogs, suggesting a different function for YajC (Lara Vasquez et al. 2021). Over the last 20 years the group of Brady studied the membrane biogenesis in *S. mutans*. Based on protein-binding assays, three putative models for cotranslational membrane insertion pathways in *S. mutans* were proposed: (1) the SRP-YajC-Yid2 pathway, (2) SecYEG-YajC-YidC1 pathway, and (3) YidC1 and/or YidC2 autonomous pathway, independent of SRP and SecYEG-YajC, suggesting a role for YajC in pathways 1 and 2 (Lara Vasquez et al. 2021). The generation of mutants in *yidC1* and *yidC2* in *S. mutans* resulted in altered protein secretion, reduced biofilm formation, and reduction and alteration of the exopolysaccharide structure and composition (Hasona et al. 2005, Palmer et al. 2012, 2018, Mishra et al. 2019). Biofilm-forming ability of *S. mutans* is dependent on secretion of glucosyltransferases, fuctosyltransferases, and the cell surface-localized adhesin P1 (Palmer et al. 2012). Deletion of *yidC1* in *S. mutans* resulted in increased secretion of two glucosyltransferases while a decrease was observed in the *yidC2* mutant.

To our knowledge, no *yajC* mutants were constructed in *S. mutans* and therefore the role of YajC is still unknown. However, the results from the current study in *E. faecium* suggest that YajC is involved in the retainment of proteins at the membrane as we observed increased amounts of proteins in the culture supernatant after washing of the Δ*yajC* strain. Proteome analysis of these supernatants revealed several proteins, which have been shown to be important in adherence to host cells and biofilm formation, including the tip protein of PilB, also designated endocarditis and biofilm associated protein ($EbpA_{fm}$) or Fms9 (EfmE1162_1256) and the major subunit of PilA, also designated Fms21 (EfmE1162_0571) (Sillanpää et al. 2008, 2010, Hendrickx et al. 2013). The fact that these proteins were increased in the supernatant of the washed wild-type *E. faecium* strain, suggests that they were no longer retained at the cell surface of the Δ*yajC* mutant, which likely explains the significant reduction in initial adherence and biofilm formation *in vitro* and the significant reduction of vegetations on the aortic valve in the rat endocarditis model.

Furthermore, two CPs, GAPDH and elongation factor Tu, which were detected at the surface of the wild-type and complemented strain, were absent at the cell surface of the Δ*yajC* mutant but present in the washed supernatant. For long, the presence of CPs, which lack signal sequences, on the cell surface of both Gram-positive and Gram-negative bacteria has been subject for research. There is growing evidence that these proteins have different functions on the cell surface compared to their intracellular function and can contribute to e.g. biofilm formation, while they are often found in the extracellular biofilm matrix (see review Ebner and Götz 2019). In *S. mutans*, protein interactome analysis revealed an interaction between YidC2 and GAPDH, but not

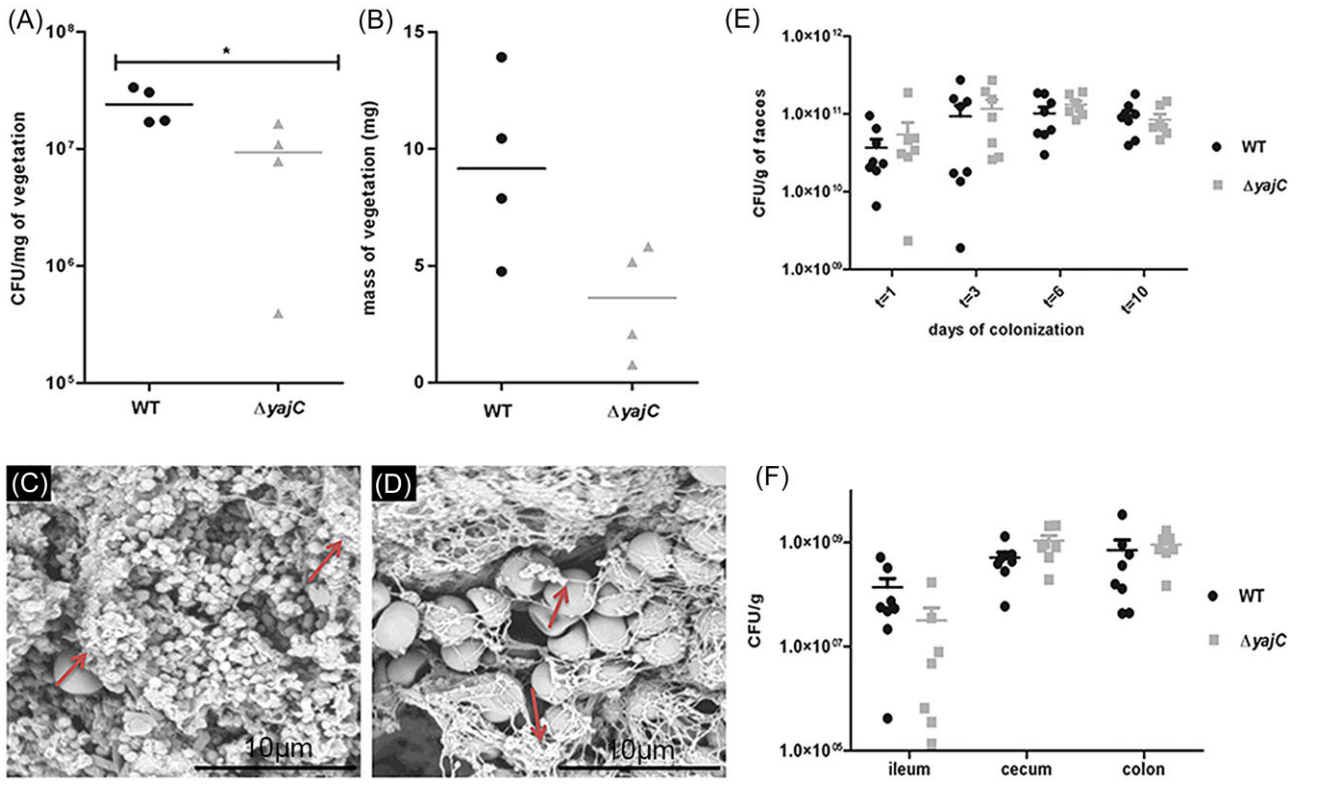

Figure 4. The effect of *yajC* mutation in the rat endocarditis model. Endocarditis of *E. faecium* wild-type and Δ*yajC* was measured by determining CFU/ml heart valve vegetations (A) and by determining the mass of vegetations in milligrams (B) in a rat endocarditis model. Vegetations on the heart valve formed by wild-type (C) and Δ*yajC* (D) were visualized by Phenom World tabletop SEM with 10 000x magnification (scale bars in C and D, 10 µm). Arrows indicate *E. faecium* in a biofilm structure. For the mouse colonization model (E and F), mice were orally inoculated with wild-type *E. faecium* or Δ*yajC*. During 10 days, colonization of *E. faecium* was determined in stool pallets by CFU enumeration of the mice at different time points (E). After 10 days of CFU counts of E1162 and Δ*yajC* were also determined in the ileum, cecum, and colon (F). Data are expressed as CFU per gram of stool/fecal contents and means are shown for eight mice per group. Asterisk represents significant differences (*$P < .05$) as determined by an unpaired two-tailed Student's *t*-test between the indicated samples.

YidC1, while the translation elongation factor Tu interacted with both YidC1 and YidC2. This suggests that the YidC1 and YidC2 insertases play a role in translocation of CPs to the cell surface (Lara Vasquez et al. 2021), but that YajC is necessary for membrane retainment.

We also determined a difference in the presence of ATP synthase at the cell surface between *E. faecium* E1162 wild-type and the Δ*yajC* mutant. While the ATP synthase F_1 alpha and beta subunits were identified at the cell surface in E1162 wild-type, they were detected in the supernatant in the Δ*yajC* mutant. Also this resembles the findings in *S. mutans*, where F_1F_0 ATPase activity was decreased in both *yidC1* and *yidC2* mutant strains relative to the wild-type strain (Hasona et al. 2005, Palmer et al. 2012).

Since the phenotypes that we observed for the *E. faecium* Δ*yajC* mutant in the current study resembles that of the *yidC1* and *yidC2* mutants in *S. mutans*, we hypothesize that also in *E. faecium* YajC is in complex with both YidC1 and YidC2, and is thus part of the co-translational membrane protein insertion pathways implicated in transport of CPs as well as extracellular proteins over the membrane. Comparison of proteins identified in the *E. faecium* Δ*yajC* secretome with the protein interactome of *S. mutans* YidC1 and YidC2 revealed nine proteins specifically bound to YidC1, nine proteins bound to both YidC1 and YidC2, and eleven bound to YidC2 (Table S4) (Lara Vasquez et al. 2021).

In conclusion, our findings indicate that YajC is involved in membrane biogenesis as part of the SRP-SecYEG-YajC-YidC1 pathway and SRP-YajC-Yid2 pathway. As part of these pathways, YajC may play a role in retaining proteins for proper docking to the YidC insertases for translocation in and over the membrane or is involved in the stabilization of the SRP-SecYEG-YajC-YidC1 and SRP-YajC-Yid2 protein complexes. This would corroborate with our findings of increased release of proteins in the supernatant of the Δ*yajC* after washing the cells. As YajC is part of two different pathways, it could be an interesting candidate as target to either prevent biofilm formation and/or destabilize and kill *E. faecium*. In *Staphylococcus aureus* a small molecule screen identified a potent compound which was able to reduce biofilm formation and toxin production and appeared to target YidC (Hofbauer et al. 2018).

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Author contributions

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tion, Supervision, Writing – review & editing), and Fernanda L. Paganelli (Conceptualization, Formal analysis, Methodology, Writing – original draft)

Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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