

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

Janetta Top^{1,*}, Xinglin Zhang², Antoni P.A. Hendrickx³, Sjeef Boeren⁴, Willem van Schaik⁵, Johannes Huebner⁶, Rob J.L. Willems^{1,†}, Helen L. Leavis^{1,†}, Fernanda L. Paganelli^{1,†}

¹Department of Medical Microbiology, University Medical Center Utrecht, PO box 85500, 3584 CX Utrecht, the Netherlands

²College of Agriculture and Forestry, Linyi University, Building 60, Yujingwan, Linyi City, Shandong Province, 276000, China

³Centre for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, the Netherlands

⁴Laboratory of Biochemistry, Wageningen University, PO box 8128, 6700 ET Wageningen, the Netherlands

⁵Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, United Kingdom

⁶Division of Pediatric Infectious Diseases, Hauner Children's Hospital, Ludwig-Maximilian Universität München, Lindwurmstr. 4, 80337 Munich, Germany

*Corresponding author. Department of Medical Microbiology, University Medical Center Utrecht (UMCU), PO box 85500, G04.614, 3584 CX Utrecht, the Netherlands.

E-mail: j.top@umcutrecht.nl

[†]These authors contributed equally to this work.

Editor: [Ana Freitas]

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 $\Delta yajC$ 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-YidC2 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 surface 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

Received 29 January 2024; revised 25 April 2024; accepted 17 May 2024

© The Author(s) 2024. Published by Oxford University Press on behalf of FEMS. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

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* DH5 α 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 μ g/ml (*E. faecium*) and 25 μ g/ml (*E. coli*), spectinomycin 300 μ g/ml (*E. faecium*) and 100 μ g/ml (*E. coli*), and ampicillin 100 μ g/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 μ g/ml) for 24 h. A volume of 20 μ l 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 μ l 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 $\Delta yajC$ 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 OD₆₆₀ of 0.01 in 6 ml TSBg and added to a coverslip coated with poly-L-lysine (0.45 μ m; 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 μ g/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 OD₆₆₀ 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 μ l 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 $\Delta yajC$

To determine the proteome of the supernatant of washed E1162 wild-type and $\Delta yajC$, 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 \times 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)

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, IIBC 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

^aLocus tag of the genes containing the transposon insertion.
^bFold-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 ($\Delta yajC$) and complemented this deletion *in trans* by introducing a complete copy of *yajC* on the vector pEF25 (Top et al. 2013) in $\Delta yajC$ ($\Delta yajC+yajC$). Biofilm formation of the wild-type, $\Delta yajC$ and the complemented strain ($\Delta yajC+yajC$) 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 $\Delta yajC$ when compared to the wild-type strain and this deficiency could be complemented in $\Delta yajC+yajC$, confirming the M-TraM results (Fig. 1A). In the flow cell model, biofilm formation of $\Delta yajC$ was also attenuated (Fig. 1B). Already after 4 h of growth, the biofilm coverage of the slide was less compared to wild-type and $\Delta yajC+yajC$, which was even more pronounced after 17 h. In addition, the live/dead stain revealed reduced numbers of dead (red) cells in $\Delta yajC$, further confirming its biofilm deficient phenotype (Desai et al. 2019).

As a control, we performed growth curves of the wild-type, $\Delta yajC$ and the complemented strain ($\Delta yajC+yajC$) and did not observe clumps or aggregates nor differences in growth rate indicating that the observed deficiency in biofilm formation of $\Delta yajC$ 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 $\Delta yajC$ 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 $\Delta yajC$ (Fig. 2). These results suggested that the proteins involved in initial adherence are loosely attached to the cell surface in the $\Delta yajC$ 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 $\Delta yajC$ in *E. faecium*. We analyzed the proteome of PBS supernatants of washed wild-type, $\Delta yajC$ and complemented strain SDS-PAGE. In the $\Delta yajC$ 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 $\Delta yajC$ and the wild-type strain revealed 272 proteins, of which 152 proteins were only present in the $\Delta yajC$ 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-phosphate 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 $\Delta yajC$ compared to wild-type and complemented strain (Fig. 3B). Next, we verified the presence of these proteins on the surface of washed wild-type, $\Delta yajC$ 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 $\Delta yajC$ (Fig. 3D). This indicates that in these conditions CPs are found attached to the surface of wild-type *E. faecium* cells, but that in $\Delta yajC$ attachment of these proteins is altered resulting in inadequate retaining or capture at the bacterial

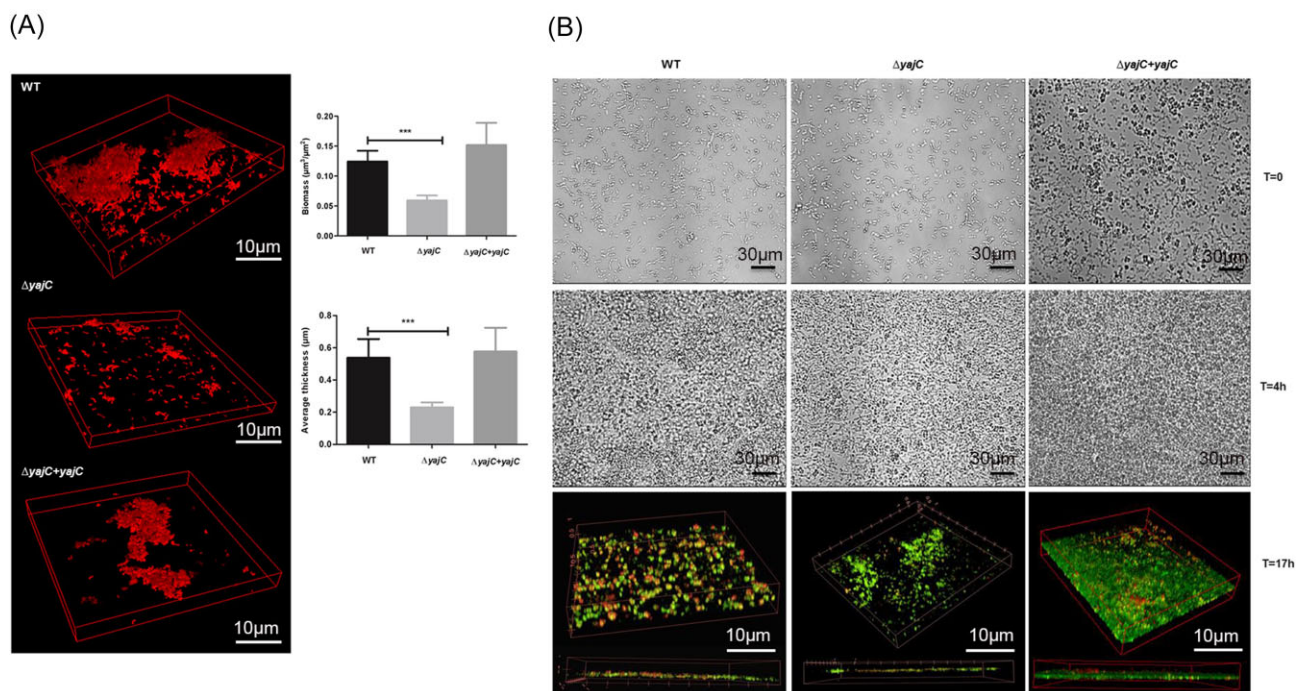


Figure 1. The effect of targeted *yajC* mutation on semistatic and flow cell biofilm model. (A) Confocal imaging of biofilm formation of wild-type, $\Delta yajC$ and $\Delta yajC+yajC$ in a semistatic biofilm model. Cells were grown for 24 h on poly-L-lysine-coated glass slides, in TSBg, at 120 rpm, 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, $\Delta yajC$ and $\Delta yajC+yajC$ 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).

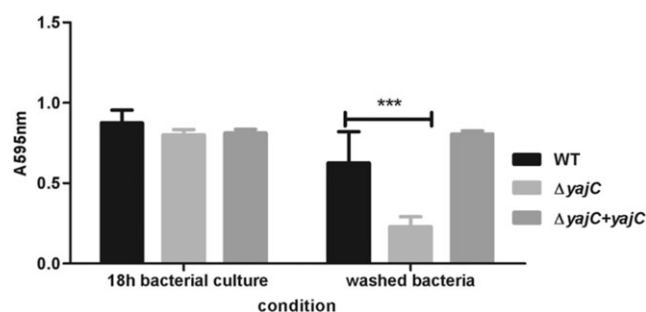


Figure 2. The effect of targeted *yajC* mutation on initial adhesion. E1162 wild-type, $\Delta yajC$ and $\Delta yajC+yajC$ were cultured overnight, washed two times with PBS, or not, 100 μl of bacterial suspension was added in triplicate to a polystyrene plate and incubated for 2 h at 37°C without shaking. 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 $\Delta yajC$ relative to wild-type and complemented strain.

The *E. faecium* $\Delta yajC$ 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 $\Delta yajC$ were on average half of the weight of those formed by the wild-type (5 mg in $\Delta yajC$ 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 $\Delta yajC$ 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.

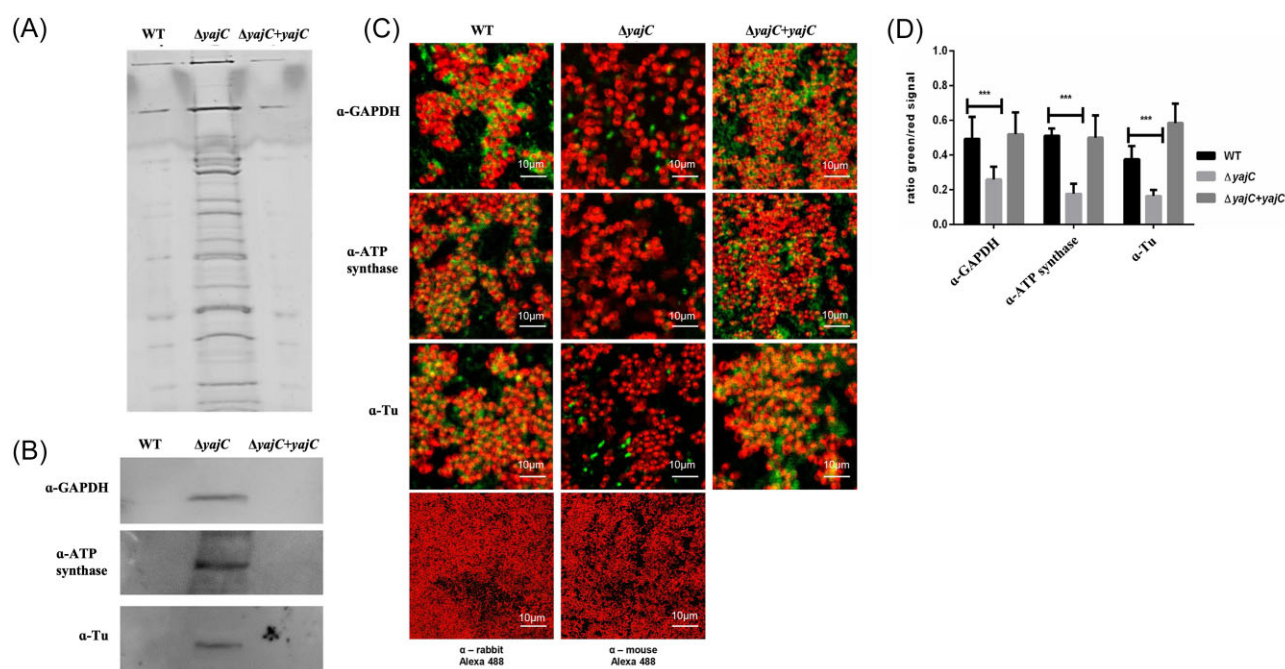


Figure 3. The effect of targeted *yajC* mutation on protein attachment. (A) Proteins present in the supernatant of wild-type, $\Delta yajC$ and $\Delta yajC+yajC$ after wash with PBS pH 7 were separated through a 12.5% SDS page gel with Coomassie blue stain. (B) Presence of GAPDH, ATP synthase, and Tu in the supernatant of washed *E. faecium* E1162 wild-type, $\Delta yajC$ and $\Delta yajC+yajC$ 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, $\Delta yajC$ and $\Delta yajC+yajC$ 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 μ m). (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 holotranslcon or Sec system (Duong and Wickner 1997a,b). This holotranslcon 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, fucosyltransferases, 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 $\Delta yajC$ 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 $\Delta yajC$ 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 $\Delta yajC$ 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

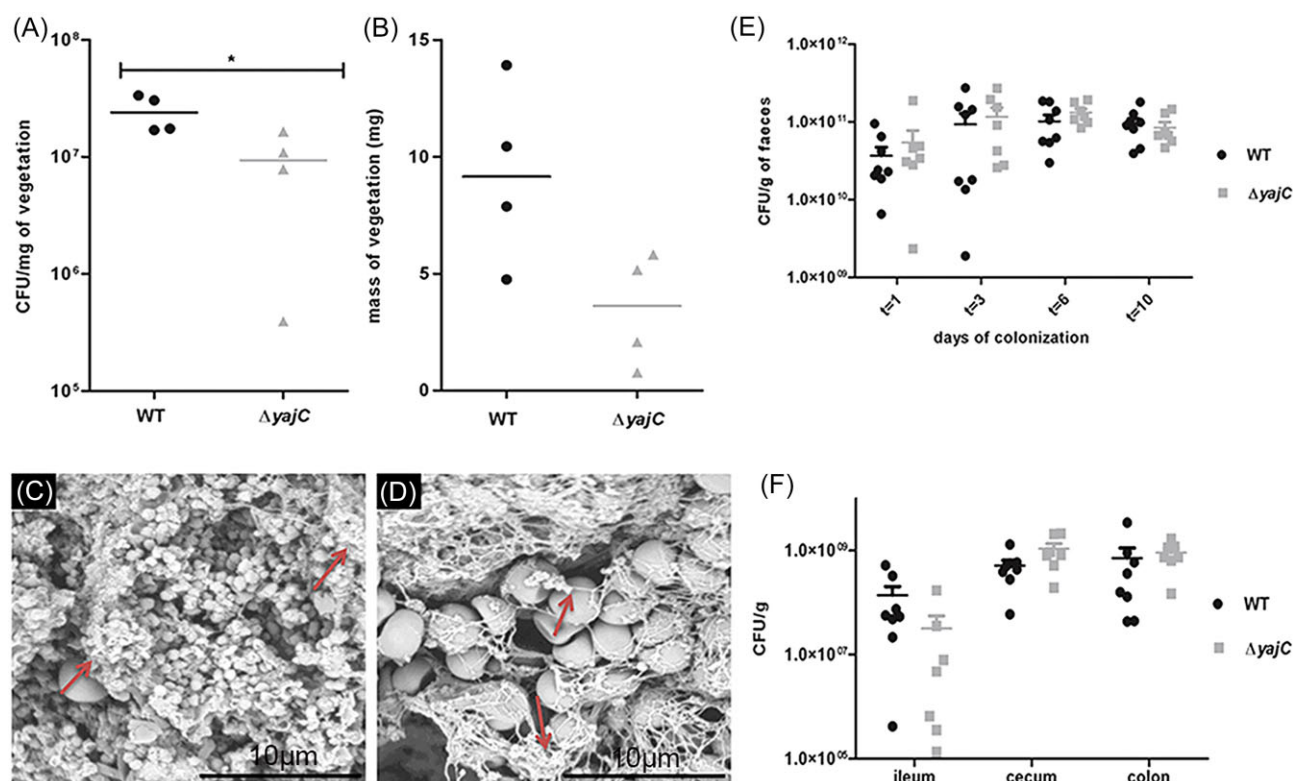


Figure 4. The effect of *yajC* mutation in the rat endocarditis model. Endocarditis of *E. faecium* wild-type and $\Delta yajC$ was measured by determining CFU/ml heart valve vegetations (A) and by determining the mass of vegetations in milligrams (B) in a rat endocarditis model. Vegetations on the heart valve formed by wild-type (C) and $\Delta yajC$ (D) were visualized by Phenom World tabletop SEM with 10 000x magnification (scale bars in C and D, 10 μ m). 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 $\Delta yajC$. During 10 days, colonization of *E. faecium* was determined in stool pellets by CFU enumeration of the mice at different time points (E). After 10 days of CFU counts of E1162 and $\Delta yajC$ were also determined in the ileum, cecum, and colon (F). Data are expressed as CFU per gram of stool/faecal 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 retention.

We also determined a difference in the presence of ATP synthase at the cell surface between *E. faecium* E1162 wild-type and the $\Delta yajC$ 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 $\Delta yajC$ 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* $\Delta yajC$ 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* $\Delta yajC$ 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 $\Delta yajC$ 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).

Acknowledgment

The authors wish to thank René Scriwanek for help with the TEM micrographs.

Author contributions

Janetta Top (Conceptualization, Formal analysis, Methodology, Writing – original draft), Xinglin Zhang (Methodology, Writing – review & editing), Antoni P.A. Hendrickx (Methodology, Writing – review & editing), Sjeef Boeren (Methodology, Writing – review & editing), Willem van Schaik (Supervision, Writing – review & editing), Johannes Huebner (Supervision, Writing – review & editing), Rob J.L. Willems (Conceptualization, Supervision, Writing – review & editing), Helen L. Leavis (Conceptualization, Funding acquisition).

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](https://femsmcjournal.com) online.

Conflict of interest: The authors declare noncompeting interests.

Funding

Part of this work was supported by ZonMW VENI grant 91610058 to H.L.L. from the Netherlands Organization for Health Research and Development. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

References

- Abee T, Kovács ÁT, Kuipers OP et al. Biofilm formation and dispersal in Gram-positive bacteria. *Curr Opin Biotechnol* 2011;**22**:172–9.
- Amini S, Goodarzi H, Tavazoie S. Genetic dissection of an exogenously induced biofilm in laboratory and clinical isolates of *E. coli*. *PLoS Pathog* 2009;**5**:e1000432. <https://doi.org/10.1371/JOURNAL.PP.AT.1000432>.
- Beck K, Eisner G, Trescher D et al. YidC, an assembly site for polytopic *Escherichia coli* membrane proteins located in immediate proximity to the SecYE translocon and lipids. *EMBO Rep* 2001;**2**:709–14.
- Chmielewski RAN, Frank JF. Biofilm formation and control in food processing facilities. *Compr Rev Food Sci Food Saf* 2003;**2**:22–32.
- Costerton JW. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol* 2001;**9**:50–2.
- Cox J, Matic I, Hilger M et al. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat Protoc* 2009;**4**:698–705.
- Desai S, Sanghrajka K, Gajjar D. High adhesion and increased cell death contribute to strong biofilm formation in *Klebsiella pneumoniae*. *Pathogens* 2019;**8**:277.
- Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002;**15**:167–93.
- Duong F, Wickner W. The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. *EMBO J* 1997a;**16**:4871–9.
- Duong F, Wickner W. Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme pathway. *EMBO J* 1997b;**16**:2756–68.
- Ebner P, Götz F. Bacterial excretion of cytoplasmic proteins (ECP): occurrence, mechanism, and function. *Trends Microbiol* 2019;**27**:176–87.
- Haller C, Berthold M, Wobser D et al. Cell-wall glycolipid mutations and their effects on virulence of *E. faecalis* in a rat model of infective endocarditis. *PLoS One* 2014;**9**:e91863. <https://doi.org/10.1371/JOURNAL.PONE.0091863>.
- Hallgren J, Tsigos KD, Damgaard Pedersen M et al. DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks. *Biorxiv* 2022. <https://doi.org/10.1101/2022.04.08.487609>.
- Hasona A, Crowley PJ, Levesque CM et al. Streptococcal viability and diminished stress tolerance in mutants lacking the signal recognition particle pathway or YidC2. *Proc Natl Acad Sci USA* 2005;**102**:17466–71.
- Heikens E, Bonten MJM, Willems RJL. Enterococcal surface protein esp is important for biofilm formation of *Enterococcus faecium* E1162. *J Bacteriol* 2007;**189**:8233–40.
- Hendrickx APA, Bonten MJM, van Luit-Asbroek M et al. Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate. *Microbiology* 2008;**154**:3212–23.
- Hendrickx APA, Van Schaik W, Willems RJL. The cell wall architecture of *Enterococcus faecium*: from resistance to pathogenesis. *Fut Microbiol* 2013;**8**:993–1010.
- Hendrickx APA, van Wamel WJB, Posthuma G et al. Five genes encoding surface-exposed LPXTG proteins are enriched in hospital-adapted *Enterococcus faecium* clonal complex 17 isolates. *J Bacteriol* 2007;**189**:8321–32.
- Heydorn A, Nielsen AT, Hentzer M et al. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 2000;**146**(Pt 10):2395–407.
- Hidron AI, Edwards JR, Patel J et al. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect Control Hosp Epidemiol* 2008;**29**:996–1011.
- Hofbauer B, Vomacka J, Stahl M et al. Dual inhibitor of *Staphylococcus aureus* virulence and biofilm attenuates expression of major toxins and adhesins. *Biochemistry* 2018;**57**:1814.
- Ince IA, Boeren SA, van Oers MM et al. Proteomic analysis of Chilo iridescent virus. *Virology* 2010;**405**:253–8.
- Lara Vasquez P, Mishra S, Kuppuswamy SK et al. Protein interactomes of *Streptococcus mutans* YidC1 and YidC2 membrane protein insertases suggest SRP pathway-independent- and -dependent functions, respectively. *mSphere* 2021;**6**. <https://doi.org/10.1128/MSPH.ERE.01308-20>.
- Leenhouts K, Buist G, Bolhuis A et al. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* 1996;**253**:217–24.
- Lu J, Boeren S, de Vries SC et al. Filter-aided sample preparation with dimethyl labeling to identify and quantify milk fat globule membrane proteins. *J Proteomics* 2011;**75**:34–43.
- Miller WR, Murray BE, Rice LB et al. Resistance in vancomycin-resistant enterococci. *Infect Dis Clin North Am* 2020;**34**:751–71.
- Mishra S, Crowley PJ, Wright KR et al. Membrane proteomic analysis reveals overlapping and independent functions of *Streptococcus mutans* ffh, YidC1, and YidC2. *Mol Oral Microbiol* 2019;**34**:131.
- Nallapareddy SR, Sillanpää J, Mitchel J et al. Conservation of Ebp-type pilus genes among enterococci and demonstration of their role in adherence of *Enterococcus faecalis* to human platelets. *Infect Immun* 2011;**79**:2911–20.
- O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol* 2000;**54**:49–79.
- Paganelli FL, de Been M, Braat JC et al. Distinct SagA from hospital-associated clade A1 *Enterococcus faecium* strains contributes to biofilm formation. *Appl Environ Microb* 2015;**81**:6873–82.
- Paganelli FL, Huebner J, Singh KV et al. Genome-wide screening identifies phosphotransferase system permease BepA to be involved in *Enterococcus faecium* endocarditis and biofilm formation. *J Infect Dis* 2016;**214**:189–95.
- Paganelli FL, Willems RJ, Leavis HL. Optimizing future treatment of enterococcal infections: attacking the biofilm?. *Trends Microbiol* 2012;**20**:40–9.
- Paganelli FL, Willems RJLW, Jansen P et al. *Enterococcus faecium* biofilm formation: identification of major autolysin AtlAefm, associated Acm surface localization, and AtlAefm-independent extracellu-

- lar DNA release. *mBio* 2013;**4**. <https://doi.org/10.1128/MBIO.00154-13>.
- Palmer SR, Crowley PJ, Oli MW et al. YidC1 and YidC2 are functionally distinct proteins involved in protein secretion, biofilm formation and cariogenicity of *Streptococcus mutans*. *Microbiology* 2012;**158**:1702.
- Palmer SR, Ren Z, Hwang G et al. *Streptococcus mutans* yidC1 and yidC2 impact cell envelope biogenesis, the Biofilm Matrix, and biofilm biophysical properties. *J Bacteriol* 2018;**201**. <https://doi.org/10.1128/JB.00396-18>.
- Rice LB. Emergence of vancomycin-resistant enterococci. *Emerg Infect Dis* 2001;**7**:183–7.
- Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;**9**:671–5.
- Sillanpää J, Nallapareddy SR, Prakash VP et al. Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*. *Microbiology* 2008;**154**:3199–211.
- Sillanpää J, Nallapareddy SR, Singh KV et al. Characterization of the *ebp fm* pilus-encoding operon of *Enterococcus faecium* and its role in biofilm formation and virulence in a murine model of urinary tract infection. *Virulence* 2010;**1**:236–46.
- Smaczniak C, Li N, Boeren S et al. Proteomics-based identification of low-abundance signaling and regulatory protein complexes in native plant tissues. *Nat Protoc* 2012;**7**:2144–58.
- Top J, Paganelli FL, Zhang X et al. The *Enterococcus faecium* enterococcal biofilm regulator, EbrB, regulates the *esp* operon and is implicated in biofilm formation and intestinal colonization. *PLoS One* 2013;**8**:e65224. <https://doi.org/10.1371/JOURNAL.PONE.0065224>.
- van Schaik W, Top J, Riley DR et al. Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. *BMC Genomics* 2010;**11**:239.
- Wang YT, Oh SY, Hendrickx APA et al. *Bacillus cereus* G9241 S-layer assembly contributes to the pathogenesis of anthrax-like disease in mice. *J Bacteriol* 2013;**195**:596–605.
- Zhang X, Paganelli FL, Bierschenk D et al. Genome-wide identification of ampicillin resistance determinants in *Enterococcus faecium*. *PLoS Genet* 2012;**8**:e1002804. <https://doi.org/10.1371/journal.pgen.1002804>.