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Surface protein EF3314 contributes to virulence properties of *Enterococcus faecalis*

ROBERTA CRETÌ^{1,*}, FRANCESCA FABRETTI^{1,#}, STEFANIE KOCH^{3,§}, JOHANNES HUEBNER^{2,3}, DANIELLE A. GARSIN⁴, LUCILLA BALDASSARRI¹, LUCIO MONTANARO^{5,6}, and CARLA RENATA ARCIOLA^{5,6}

¹Respiratory and Systemic Disease Unit, Department of Infectious, Parasitic, and Immune-mediated Diseases, National Health Institute (ISS), Rome - Italy

²Division of Infectious Diseases, Department of Medicine, University Hospital Freiburg, Freiburg - Germany

³Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA - USA

⁴Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, TX - USA

⁵Research Unit on Implant Infections, Rizzoli Orthopedic Institute, Bologna - Italy

⁶Department of Experimental Pathology of the University of Bologna, Bologna - Italy

Abstract

Purpose—Identification of putative new virulence factors as additional targets for therapeutic approaches alternative to antibiotic treatment of multi-resistant enterococcal infections.

Methods—The EF3314 gene, coding for a putative surface-exposed antigen, was identified by the analysis of the *Enterococcus faecalis* V583 genome for LPXTG-motif cell wall anchor surface protein genes. A non-polar EF3314 gene deletion mutant in the *E. faecalis* 12030 human clinical isolate was obtained. The wild type and the isogenic mutant strain were investigated for biofilm formation, adherence to Hela cells, survival in human macrophages and a *Caenorhabditis elegans* infection model. The aminoterminal portion of the EF3314 protein was overexpressed in *E. coli* to obtain mouse polyclonal antibodies for use in Western blotting and immunolocalization experiments.

Results—The EF3314 gene has an unusually high GC content (46.88% vs. an average of 37.5% in the *E. faecalis* chromosome) and encodes a protein of 1744 amino acids that presents a series of

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Address for correspondence Roberta Creti, PhD, Reparto di Malattie Batteriche Respiratorie e Sistemiche, Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy, roberta.creti@iss.it.

#Present addresses: Renal Division, Department of Medicine, University of Cologne, Germany;

§Children's Hospital, Boston, MA, USA

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None of the authors have a financial interest in any of the products or devices described in this article.

14 imperfect repeats of 90 amino acids covering almost the entire length of the protein. Its global organization is similar to the alpha-like protein family of group B streptococci, enterococcal surface protein Esp and biofilm associated protein Bap from *S. aureus*. The EF3314 gene was always present and specific for *E. faecalis* strains of human, food and animal origin. Differences in size depended on variable numbers of repeats in the repetitive region.

Conclusions—EF3314 is a newly described, surface exposed protein that contributes to the virulence properties of *E. faecalis*.

Keywords

E. faecalis; Surface protein; Adhesion; Biofilm; *C. elegans* infection model

INTRODUCTION

Enterococci are commensals of the human and animal intestinal microflora that have emerged as an important cause of nosocomial infections; the most frequently isolated species is *Enterococcus faecalis*, often in association with implanted medical devices (1–4). The increasing occurrence of enterococci resistant to antibiotics of the last resort against gram-positive infections has fueled research on the molecular mechanism underlying the innate and acquired resistance of enterococci toward antimicrobials agents (5–7). As an opportunistic pathogen, *E. faecalis* does not possess unequivocal virulence factors like toxins and hydrolytic enzymes; some proteins/enzymes are commonly recognized as virulence factors and the knowledge of their specific role in the pathogenesis and contribution to the establishment of infection is increasing (8–11). Rather than the presence of a specific virulence factor, it is the total number of virulence factors possessed by an *E. faecalis* strain that appears to be correlated to its source of isolation (12). This can be partially explained by the concentration of virulence factors in a pathogenicity island identified in *E. faecalis* strains mainly present in nosocomial settings (13, 14). This enterococcal subpopulation appear to colonize new niches of the gastro-intestinal tract of patients within few days of admission to a hospital following antibiotic treatment (15). Much attention has been recently given to the possible involvement of biofilm production by *E. faecalis* in the infectious process (16–22).

The possibility to explore available sequenced genomes looking for potential new virulence determinants is a very important chance to speed up the research on novel therapeutic strategies. We previously identified a new cell wall surface anchor protein gene, *EF3314*, that was always present among *E. faecalis* clinical strains (12). In the present study, we confirmed that the *EF3314* gene is species-specific and present in *E. faecalis* isolates of human, food and animal origin, with differences in size depending on the repetitive region. Additionally, we showed that EF3314 is a surface protein and affects virulence in both human cell and *C. elegans* infection models.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

E. faecalis cells were grown without agitation in Todd-Hewitt broth (THB; Becton Dickinson, Franklin Lakes, NJ, USA) or tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA). For specific purposes they were grown in Biofilm Medium (BFM; 17 g of pancreatic digest of casein, 5 g of NaCl, 3 g of yeast extract, and 2.5 g of dipotassium phosphate per liter) with the addition of 1% glucose when indicated (BFM-G). *Escherichia coli* strains were cultured aerobically in Luria-Bertani (LB) broth or LB agar at 37°C.

Kanamycin at 50 µg/mL and ampicillin at 100 µg/mL (Sigma Chemicals, Milan, Italy) were used where appropriate for *E. coli*; kanamycin 2000 µg/mL was used for *E. faecalis*.

DNA manipulations

Genomic DNA preparations from enterococci were prepared by using the Qiagen DNeasy Tissue Kit (Milan, Italy), according to the manufacturer's instructions. Plasmid DNA was prepared from *E. coli* by the use of QIAprep Spin miniprep (Qiagen). DNA was purified from agarose gels and from polymerase chain reactions (PCRs) by the use of the QIAquick Gel Extraction Kit or the PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Restriction enzymes and modifying enzymes were obtained from Invitrogen (Carlsbad, CA, USA) or New England Bio-labs (Ipswich, MA, USA). Custom primers were ordered from Invitrogen. Electrocompetent enterococci preparation and electroporation (Bio-Rad Gene Pulser II; Bio-Rad, Hercules, CA, USA) were performed according to established protocols (23).

All other methods (DNA ligations, electrophoresis, and transformation of competent *E. coli*) were performed by use of standard techniques (24). For amplification of large DNA fragments (>5 kb) or high fidelity amplification for expression vector cloning, the Elongase mix from Invitrogen was used according to the manufacturer's instructions.

Construction of a nonpolar deletion mutant

A non-polar deletion of the N-terminal portion of the *EF3314* gene was created by use of a established methodology (25). Primers 7 and 8 were used to amplify the region from – 690 to + 54 of the *EF3314* gene and primers 9 and 10 to amplify the region from pos. + 480 to + 1071 (Tab. II). Primers 8 and 9 contained a 21-bp complementary sequence (underlined in Tab. II). Overlap-extension PCR was used to create a PCR product from pos. – 690 to pos. + 1071 lacking a portion of the N-terminal region of the *EF3314* gene (from pos. + 54 to + 480). This fragment was cloned into pCRII vector (Invitrogen) and cut with the restriction enzymes *SpeI* and *XhoI*. The construct was inserted into the mutagenesis vector pTEX4577 (23). The resulting plasmid was transformed into *E. faecalis* 12030 strain (18) by electroporation, and integrants were selected on TSB plates with kanamycin. A single colony was picked, and the insertion of plasmid into the chromosome was confirmed by PCR using the high-fidelity Elongase mix. The integrant was passaged 10 times in liquid culture without antibiotics at the permissive temperature, and colonies were replica-plated to screen for loss of kanamycin resistance. The excision of the plasmid created either a

reconstituted wild-type strain or led to an allelic replacement with the interrupted sequence in the chromosome. The deletion mutant created was designated *E. faecalis* 12030 EF3314 and was confirmed by PCR by use of primers 3 and 4 (Tab. II) and by automated sequencing of the PCR product.

Production of the N-terminal portion EF3314 recombinant protein

The N-terminal portion of the *EF3314* gene was amplified by PCR from primers 5 and 6 (Tab.II) with high-fidelity Elongase mix and inserted into *Bam*HI – *Sal*I linearized pQE30 vector (Qiagen) to place the 6X histidine (His)-affinity tag at the amino-terminal of the fusion protein. The recombinant plasmid was sequenced to verify that the correct open reading frame had been maintained and used to transform *E. coli* M15 (pREP4) competent cells for the isopropyl-beta-D-thiogalactoside (IPTG) inducible expression of the His-tagged N-terminal portion of the EF3314 protein. When the culture reached an OD₆₀₀ of 0.7, IPTG was added to a final concentration of 1.5 mM to induce expression. *E. coli* M15 cells transformed with the recombinant pQE16 plasmid encoding the mouse (His)-tagged 16 kDa dihydrofolate reductase protein were used as the positive control. To follow the time course of the protein production, 1 mL samples of *E. coli* M15 cultures were collected at times 0, 2, and 4 hours by centrifugation and boiled for 3 minutes in loading buffer. A 20 µL volume of each sample was subjected to 12% SDS-PAGE and proteins were visualized by staining with Coomassie Blue.

For protein purification, *E. coli* M15 cells expressing the recombinant EF3314 protein portion were collected after incubation for 5 hours with IPTG. The bacteria were disrupted according to the manufacture's instructions. To remove cell debris, the samples were centrifuged and the supernatants were applied to a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen). All purification steps were performed in denaturing conditions according to the manufacturer's instructions.

Peptide sequencing and preparation of the anti-EF3314 polyclonal serum

The identity of the recombinant protein was confirmed by Edman degradation of the first 17 amino acids corresponding to the pEQ30 tag (MRGHHHHHHGS) plus the first six amino acids (SVTSGT) of the purified EF3314 protein portion. BALB/c mice were immunized with the recombinant EF3314 protein for the production of polyclonal antisera by Primm.

SDS-PAGE and immunoblotting

E. faecalis cells were disrupted by sonication at 4°C for 6 minutes (1 min cycles with 1 min cooling intervals). Recombinant EF3314 and *E. faecalis* crude extracts were subjected to SDS-PAGE in separating acrylamide gels (Bio-Rad) 10% W/v and the proteins were visualized by staining with Coomassie Blue as described previously (24). Separated proteins were transferred on to nitrocellulose membrane in a Trans-Blot SD module (Bio-Rad). Nitrocellulose sheets were blocked for 1 hour at 37°C in PBS containing Non-Fat Dry Milk (Bio-Rad) 5% w/v and incubated for 2 hours at room temperature with the mouse anti-EF3314 serum diluted 1:50 in PBS. After washing in TTBS (Tween 20 0.05% v/v in TBS), the sheets were incubated for 1 hour at room temperature with goat anti-mouse IgG alkaline-phosphatase conjugate diluted 1:1000 with PBS and then washed again in TTBS. Binding of

the enzyme-conjugated antibodies was detected with the AP color development reagent and fast red (Bio-Rad).

Biofilm plate assay

Overnight cultures grown either in BFM alone or BFM-G were diluted 1:10 and inoculated into polystyrene microtiter plates (Costar, Milan, Italy) in BFM-G (-/+ G, +/- G conditions) at 37°C for 18 hours. Enterococci were tested for production of biofilm according to the protocol previously described by the authors (26, 27). Biofilm formation was normalized to growth with the biofilm index, calculated as $OD_{\text{biofilm}} \times 0.5/OD_{\text{growth}}$ (28).

Immunoelectron microscopy

Immune mouse serum raised against purified recombinant 3314 protein was used as primary antibody for protein localization. Bacterial pellets from overnight cultures were washed three times with PBS. Drops of bacterial suspensions were placed on carbon-coated grids and air dried. Primary antibody was applied diluted 1:100 in PBS supplemented with 1% BSA and 0.05% Tween 20 for 30 minutes at 37°C. Preimmune mouse serum was used as control. After washing with PBS/BSA, 10 nm-gold-conjugated protein G was used at a dilution of 1:500 and incubated for 30 minutes at room temperature. After washing, samples were examined without further staining, by a Philips 208 electron microscope.

Adherence to HeLa cell monolayer and THP-1 survival test

A standard adhesion assay was performed as previously described (9). Briefly, HeLa cells were seeded at 5×10^4 per well, on sterile glass coverslips placed into 24-well plates, 24 hours before infection. Semiconfluent monolayers (2×10^5 cells/well) were inoculated with stationary phase bacterial suspensions normalized at different bacteria-to-cell ratios. After incubation for 1 or 2 hours at 37°C and/or 4°C in a modified atmosphere of 5% CO₂, monolayers were washed five times with PBS and lysed for 5 minutes with 1% Triton X-100. Viable bacteria were quantified by plating aliquots of serial dilutions on trypticase soy agar (TSA).

THP-1, a myelomonocytic human cell line expressing Fc and C3b receptors, was maintained in RPMI 1640 medium supplemented with 10% FCS and 2 mM glutamine in an atmosphere of 95% air and 5% CO₂. Cells were differentiated by incubation with phorbol miristate acetate (0.16 µM; Sigma) for 24 hours at 37°C in 24- or 96-well plates, as described by Scoreaux et al (29). Macrophages were infected for 1 hour at 37°C, thoroughly washed with PBS, and further incubated for 3 and 24 hours in medium supplemented with 10% FCS and gentamycin 250 µg/mL. At the different time points, duplicate wells were washed and lysed with 0.1% Triton X-100 in PBS for 5 minutes. Lysates were diluted in PBS and plated on TSB agar plates to quantify viable bacteria. Throughout the experiment, viability of cells was confirmed by trypan blue dye exclusion and counting with an hemocytometer.

Results are reported as the percentage of the inoculum recovered after infection. All assays were conducted in duplicate and repeated independently at least three times.

Assay of *C. elegans* killing

Assay conditions as well as statistical evaluation were performed as already described (30, 31). In brief, a synchronized population of N2 wildtype worms were grown on NG plates containing a lawn of OP50 (standard conditions for laboratory growth of *C. elegans*). At the L4 stage the worms were moved onto BHI (Brain Heart Infusion) plates containing a lawn of either *E. faecalis* 12030 or its isogenic mutant strain. Nalidixic acid (10 µg/mL) was added to the medium to selectively prevent growth of *E. coli*. The number of dead and alive worms was counted once a day, and the dead ones were removed. Sixty to ninety worms were used in each replicate of the experiment. The data was analyzed using GraphPadPrism 3.0 (GraphPad Software, San Diego, CA, USA). Survival was plotted by the Kaplan-Meier method and the curves compared using the log-rank test, which generates a p-value testing the null hypothesis that the survival curves are identical. P-values less than 0.05 were considered statistically significant.

RESULTS

Structural features of the deduced EF3314 protein

The *E. faecalis* V583 EF3314 gene consists of 5495 base pairs and possesses an unusually high GC content (46.88% vs. an average of 37.5% in the *E. faecalis* chromosome). The deduced protein sequence is 1744 amino acids long and presents features characteristic of gram-positive surface adhesins: a signal sequence directing export of the mature protein outside the cytoplasm corresponding to the first 60 amino acids; an N-terminal region of 300 amino acids; a series of 14 imperfect repeats of 90 amino acids covering almost the entire length of the protein; and a LPX-TG-motif cell wall anchor domain at the C-terminal portion (Fig. 1A). The first three repeats are identical while the others present some variations in the amino acid composition (Fig. 1B). The global organization of the EF3314 protein is similar to other gram-positive repeat-containing proteins like the alpha-like protein family of group B streptococci (32), the enterococcal surface protein Esp and the biofilm associated protein Bap from *S. aureus* (33).

The search for similar protein sequences in the data bank using either FASTA3 and Blastp programs retrieved as first hits a putative surface protein of *Lactococcus lactis* subsp. *cremoris* (38.9% identity, 73% similarity) and the *Staphylococcus aureus* biofilm associated protein Bap (31.8% identity, 66.7% similarity) restricted, in this case, to the repeat and carboxy-terminal region of the proteins, while the N-terminal portion was distinctive.

Distribution and expression of EF3314 protein among *E. faecalis* isolates

The N-terminal portion of the protein EF3314 corresponding to the amino acid positions 61–357 was over-expressed in *E. coli*. The signal sequence was avoided as well as the repeat region for maximizing the expression of the heterologous protein. The recombinant protein was successfully expressed by introducing the His-tag at the N-terminal portion while poor levels were obtained when the His-tag was placed at the C-terminus (data not shown).

Mouse polyclonal anti-EF3314 serum raised against the purified N-terminal portion of the EF3314 protein specifically reacted with the recombinant portion of the EF3314 protein

(Fig. 2A). We previously reported that the *EF3314* gene is *E. faecalis*-specific and present in all isolates tested so far (12). To verify whether this gene was expressed, total protein extracts from twenty randomly chosen strains were examined by Western blot with the mouse polyclonal anti-EF3314 serum. All strains examined showed the appropriate band (Fig. 2B). Mouse serum was also used to verify the presence of the protein on the bacterial surface by immunoelectron microscopy (Fig. 3).

Biofilm assay of parental and mutant strain

No differences in biofilm formation were observed between *E. faecalis* 12030 and its isogenic mutant when grown in conditions that were not favorable to biofilm formation, i.e. when 1% glucose was present since the first overnight growth (+/+G) (34). When the additional carbon source was added only in the second overnight subculture growth (-/+ G), significantly less biofilm was formed by the 12030 3314 compared to the wild type (Fig. 4A).

Adherence to HeLa cells and survival in macrophages of parental and mutant strain

The ability of *E. faecalis* 12030 strain and its isogenic deletion mutant to adhere to HeLa cells was tested by allowing the bacteria to adhere for either one or two hours. While no significant differences were observed at 1 hour post-infection, after two hours the rate of attachment of the deletion mutant strain was significantly lower than the 12030 strain (Fig. 4B). To evaluate whether the differential attachment to epithelial cells would translate into a differential survival ability of the 12030 3314 mutant strain compared to the wild type, the interaction of both strains was tested with professional phagocytes, i.e. THP-1 cells. As predicted, significantly higher adherence and survival rates of 12030 were observed compared to the EF3314 deletion mutant derivative (Fig. 4C).

C. elegans infection model

To see if the 12030:3314 significantly affected pathogenesis in an animal model we infected *C. elegans* with strain 12030 as well as the isogenic mutant 12030 EF3314. It has previously been shown that a number of *E. faecalis* virulence determinants that affect pathogenesis in mammalian models also affect pathogenesis in the worm model by causing death to occur significantly more slowly than a wildtype isogenic strain (30, 31, 35). A synchronized population of wildtype *C. elegans* was grown to the L4 stage and then placed on lawns of 12030 EF3314 and 12030 where they become infected by consuming the pathogens. Survival over time was followed by counting the number of worms alive and dead at approximately 24-hour intervals. *C. elegans* feeding on strain 12030 EF3314 died significantly more slowly compared to those infected with the wild type 12030 strain (Fig. 5) with a p-value of 0.0010. These data suggest that EF3314 contributes positively to the pathogenic process in *C. elegans*.

DISCUSSION

The ability of *E. faecalis* to cause infection in humans has become of particular relevance primarily in the hospital environment, where enterococci resistant to multiple antibiotics are

leading nosocomial pathogens of urinary tract, bloodstream and surgical-wound infections (16).

Enterococcal genomic studies are unraveling the factors involved in transforming a commensal into a pathogen. The analysis of the genome sequence of *E. faecalis* V583 strain, the first vancomycin-resistant clinical isolate reported in the United States, demonstrated that, in addition to antibiotic resistance, the strain had also acquired a number of genes conferring infectivity and virulence encoded within a pathogenicity island (13–15, 36). A recent comparative genomics study revealed that there is variation in the penetration of antibiotic resistance determinants and virulence traits within the major *E. faecalis* lineages (37).

By the analysis of the V583 genome sequence for the identification of new surface-associated proteins as potentially exposed antigens and/or as factors that may influence the ability of enterococci to colonize host tissues or implanted medical devices, we have focused our studies on a chromosomal gene, *EF3314* encoding a LPTXG motif anchor membrane protein.

The *EF3314* gene possesses an unusually high GC content (46.88% vs. 37.5% average). Generally a higher GC content in genomic regions is due to constraints on nucleotide bias at replication origins or at the ribosomal RNAs operons (38), but, when present in protein-coding genes, is often a sign of a lateral gene transfer event.

On the other hand, the higher GC content of the *EF3314* gene compared to the *E. faecalis* genome could be attributed to intrinsic characteristics of the *EF3314* gene. In fact, while the GC content of the first and third codon positions of the *EF3314* gene (53.7% and 31.2%) is not different from the GC content of the first and third codon positions of all the *E. faecalis* coding regions (48.7% and 30.4%), there is a significant difference between the *EF3314* second codon positions (55.7 %GC content) and second codon positions of the rest of the genome coding regions (34.7 % GC content). The second codon position is distinctive for each amino acid (39) and indeed the EF3314 protein is rich in amino acids (Ser, Ala, Thr, Gly) whose second codon position is G or C, constituting up to 49.4% of the amino acid content.

The highest homology of the deduced EF3314 protein was with a hypothetical surface protein gene identified both in *Lactobacillus lactis* subsp. *cremoris* MG1363 and SK11 genomes (40). Annotation describes the deduced protein (1349 amino acids) as a subtilisin-like serine protease, yet no experimental evidence of its function has been reported. The EF3314 protein also presents a lower but significant sequence homology with the *S. aureus* biofilm-associated protein (Bap) as well as gram-negative surface proteins: a 6310 aa hypothetical surface adhesion protein of *Pseudomonas putida* (60.3% GC genomic content, 30.2% amino acid identity) and 1578 aa putative outer membrane ligand of *Bordetella bronchiseptica* (63.0 %GC content, 28.9 % identity). The gene organization of the chromosomal region comprising the *E. faecalis* *EF3314* gene and *Lactococcus*, *Bordetella* and *Pseudomonas* genomes are not comparable and, for this reason, the hypothesis of a

lateral gene transfer between high %GC gram-negative and low %GC gram-positive bacteria cannot be demonstrated.

As pointed out above, the structural organization of the EF3314 protein is similar to other well characterized gram-positive cell surface proteins (*S. aureus* Bap protein, *E. faecalis* surface protein Esp), whose involvement in biofilm formation and adhesion has been hypothesized (33, 41, 42). Additionally, there is similarity to the alpha-like proteins of group B streptococci that promote *S. agalactiae* invasion of epithelial cells by interaction with host cell glycosaminoglycans and are involved in the first step of infection (43, 44). These proteins exhibit size variability, also within the same strain, probably as a means to evade the human immune system (32, 33, 45). We have previously demonstrated that the EF3314 gene is always present in all the *E. faecalis* isolates tested and is *E. faecalis*-specific (12). Analysis of different *E. faecalis* strains demonstrated that the EF3314 gene exhibits size variability depending on a variable number of the internal repeat units (data not shown).

Furthermore, the nucleotide portion of the 12030 EF3314 gene, utilized for the expression experiments, was sequenced and compared to the corresponding region of the V583 EF3314 gene (pos. 183 – 1071). Fifteen nucleotide differences, of which three leading to nonsynonymous amino acid substitutions were observed, demonstrating a polymorphism of the EF3314 protein among *E. faecalis* strains. It will be useful to investigate whether these amino acid differences may contribute to a difference in the properties of EF3314 proteins.

Based on the immunolocalization results and the functional properties of the homologous proteins, we investigated the role of the EF3314 protein as a possible surface-exposed virulence factor. *E. faecalis* is often isolated from biofilms on the surfaces of indwelling medical devices (4). The development of biofilm is a multi-step and multi-factorial process; the dissection and elucidation of the factors involved in each stage are difficult and sometimes controversial (46). The identification of a molecular mechanism contributing to *E. faecalis* biofilm production have been recently reported (22, 27). According to our experimental results, EF3314 may be reasonably considered a new *E. faecalis* cell-anchor surface protein implicated in biofilm formation. The specific step in which the EF3314 protein enters the biofilm formation process is still unknown; however, because the lack of the EF3314 protein renders the isogenic deletion mutant strain significantly less capable of forming biofilm in favorable growth conditions, it is possible that the EF3314 protein plays a role in the biofilm tridimensional architecture.

The EF3314 protein may also be involved in the early interaction steps with epithelia. Within the first hour of contact between human epithelial cells and the 12030 strain or its isogenic deletion mutant, there are no significant differences in the number of bacteria attached to the cell surface but when, after two hours, specialized structures are presumably expressed and exposed on the bacterial surfaces (47, 48), the 12030 EF3314 strain is less effective in the attachment than the wild type strain. These data led to the hypothesis that the EF3314 protein actively contributes to the specific recognition of, and adherence to, the human host epithelium. The possible role of the EF3314 surface protein as a new *E. faecalis* adhesin is also suggested by the lower adherence rate and survival in macrophages by the isogenic deletion mutant compared to the wild type 12030 strain.

The infection animal model supported the hypothesis of a contribution of the EF3314 protein to the *E. faecalis* virulence. An attenuated killing of *C. elegans* was observed when fed with the *E. faecalis* deletion mutant, indicating the EF3314 protein is a potential virulence determinant affecting pathogenesis in the worm.

In conclusion, this study investigated the characteristics and function of a new large repetitive surface protein of *E. faecalis*, EF3314, and presented evidence that this protein may contribute to the virulence properties of *E. faecalis* by influencing the interaction between the bacterium and its host.

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Fig. 1. Structure of the EF3314 deduced protein (A). Alignment of the 90 amino acid repeat units present in the V583 EF3314 protein. Identical or similar amino acids between the units are highlighted in dark and light grey, respectively (B).

Fig. 2.

Western immunoblotting of recombinant EF3314 protein (**A**) and of selected *E. faecalis* sonicates (**B**), after separation by SDS-PAGE, using hyperimmune mouse polyclonal EF3314 serum. In column 1 of panel A pre-immune serum was used. Columns M indicate the molecular markers in kDa: Precision Plus Protein Standards (Bio-Rad, panel **A**) and SeeBlue Plus2 pre-stained standard (Invitrogen, panel **B**).

Fig. 3. Immunolocalization of the EF3314 protein. Polyclonal mouse antiserum raised against the recombinant EF3314 protein recognized a specific target on the bacterial surface of *E. faecalis* 12030 strain, evidenced by arrows (**B**) but not on its isogenic deletion mutant (**A**).

Fig. 4. Comparison between *E. faecalis* 12030 strain and its isogenic deletion mutant in biofilm formation (A), adherence on HeLa epithelial cells (B), and survival in human THP-1 macrophages (C). Conditions -/+ G and +/+ G are described in the text.

Fig. 5.

C. elegans killing by *E. faecalis* 12030 and 12030 EF3314 strains. A significant attenuation in the killing was observed when nematodes were fed on lawns of *E. faecalis* 12030 (solid line) compared to the isogenic deletion mutant strain (dotted line). Survival was plotted by the Kaplan-Meier method and the curves compared using the log-rank test, generating a p-value equal to 0.0010. This experiment was repeated three times with similar results.

TABLE I

PRIMER SEQUENCES USED IN THIS STUDY

	Primer Name	Sequence (5' → 3') ^a	Position with respect to the first nucleotide of V583 <i>EF3314</i> gene
1	EF3314-F	AGAGGGACGATCAGATGAAAAA	+ 36
2	EF3314-R	ATTCCAATTGACGATTCCTTC	+ 602
3	EF3314 Ro2 left	CTTCCTCATTTTCCTCTCATTGG	– 206
4	EF3314 Ro5 right	GCACTTTCAGTCTTGTTCTTCG	+ 58 after the stop codon
5	EF3314-BAMHI	GGGAAAGGATCCGTTACCTCTGGCACAATCAGT	+ 184
6	EF3314-SALI	GGGAAAGTCGACGGATACACCGTCTGCTGTTGC	+ 1072
7	EF3314-1	GCCTATGGAAAGGTCTCAAGAG	– 690
8	EF3314-2	<u>ACTAGCGCGGCCGCTTGCTCCTTT</u> CATCTGATCGTCCCTCTTT	+ 54
9	EF3314-3	<u>GGAGCAAGCGGCCGCGCTAGT</u> ACTGCCGTTTTGAATGCAGCC	+ 480
10	EF3314-4	GGATACACCGTCTGCTGTTGC	+ 1071

^aNucleotides in bold represent the restriction cloning sites; the underlined nucleotides represent the 21-bp overlap used in the overlap-extension PCR.