The Enterococcus faecalis Exoproteome: Identification and Temporal Regulation by Fsr

Jayendra Shankar, Rachel G. Walker, Deborah Ward, Malcolm J. Horsburgh

Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom

Abstract

Analysis of the culture supernatant exoproteins produced by two PFGE clusters of high-level gentamicin and ciprofloxacin-resistant clinical isolates of Enterococcus faecalis from the UK and Ireland revealed two distinct protein profiles. This grouping distinguished OG1RF and GelE metalloprotease-expressing isolates from JH2-2 and other GelE-negative isolates. The integrity of the fsrABDCA operon was found to determine the exoproteome composition, since an fsrB mutant of strain OG1RF appeared very similar to that of strain JH2-2, and complementation of the latter with the fsrABDCA operon produced an OG1RF-like exoproteome. The proteins present in the supernatant fraction of OG1RF were separated using 2D SDS-PAGE and identified by mass spectrometry and comprised many mass and pI variants of the GelE and SprE proteases. In addition cell wall synthesis and cell division proteins were identified. An OG1RF fsrB mutant had a distinct exoprotein fraction with an absence of the Fsr-regulated proteases and was characterised by general stress and glycolytic proteins. The exoproteome of the OG1RF fsrB mutant resembles that of a divIVA mutant of E. faecalis, suggestive of a stress phenotype.

Introduction

Recent years have seen greater study of medically important opportunistic bacterial pathogens due to increased levels of nosocomial infection and antibiotic resistance [1,2]. Of these pathogens, Enterococcus faecalis is prominent due to the frequency of disease and its implication in antibiotic resistance transfer to Staphylococcus aureus and Listeria species [3–6].

The pathogenesis of E. faecalis infections is relatively poorly understood; however, its interaction with the environment via secreted exoproteins and surface-attached proteins facilitate colonisation and pathology [7–9]. Exoprotein virulence factors of E. faecalis identified to date include cytolsin, gelatinase (GelE) and serine protease (SprE). Transcription of the gelE-sprE operon is temporally regulated via FsrA, the response regulator of the density-dependent two-component system. Insertional inactivation of this locus ablates expression of these proteases and the Fsr system is the only known regulatory locus for the genes [10]. In addition to the prominent virulence exoproteins, GelE and SprE, the Fsr system is a global regulator of an array of surface-expressed and metabolic proteins. Microarray analysis of an fsrB mutant identified 119 upregulated and 323 downregulated genes in the later stages of growth [11]. The fsrABDCA locus is similar to the agrBDCA locus of S. aureus, but it lacks the RNAIII riboregulator and transcriptional activation appears to function solely via FsrA [10–13]. Density-dependent activation of the Fsr system occurs via extracellular accumulation of gelatinase biosynthesis activating pheromone (GBAP, the product of fsrD) [14].

The frequency of the gelE-sprE operon among isolates was reported to be 93% of 152 clinical isolates [13]; however, the activity of GelE may not be as high as this would suggest due to variation in the frequency of the fsc locus. Within clinical isolates from urine, 69% lacked GelE activity but 88% carried the gene [14]. Nakayama et al. [14] reported a 23.9 kb deletion, which included the genes fscA, fscB and part of fscC, in 91% of gelE-positive strains lacking GelE activity. More recently, Galloway-Pena et al. [15] analysed genome sequences of 22 E. faecalis strains and identified three major groups based on a highly variable region between fscC (EF_1820) and EF_1841, with the 23.9 kb deletion accounting almost exclusively for an absence of GelE expression [15].

The exoprotein fraction represents a secreted pool of proteins that can interact with the host in commensalism and disease. In S. aureus and Streptococcus (Strep) pyogenes, this protein fraction contains toxins, enzymes and immune modulators [16–17]. This exoprotein fraction was therefore examined by 2D SDS-PAGE in order to identify putative novel virulence factors. Given the demonstrated regulatory role of the fsc locus, its contribution together with gelE to the regulation of exoproteins produced by E. faecalis OG1RF were analysed by using isogenic mutants. Based on exoprotein profiles, 47 clinical strains were analysed and categorised as either OG1RF- or JH2-2-like, indicating GelE activity. However, not all strains that lacked GelE activity contained the 23.9 kb deletion in the fsc locus, indicating that other factors may be involved in the regulation of this virulence factor, such as point mutations in fsc described recently [18].

Citation: Shankar J, Walker RG, Ward D, Horsburgh MJ (2012) The Enterococcus faecalis Exoproteome: Identification and Temporal Regulation by Fsr. PLoS ONE 7(3): e33450. doi:10.1371/journal.pone.0033450

Editor: Roy Martin Roop II, East Carolina University School of Medicine, United States of America

Received November 22, 2011; Accepted February 13, 2012; Published March 12, 2012

Copyright: © 2012 Shankar et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was supported by a BBSRC postgraduate award BB/S/K/2004/11198 to RWG and a Dorothy Hodgkin NERC-BP postgraduate award to JS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: M.J.Horsburgh@Liverpool.ac.uk
† These authors contributed equally to this work.
‡ Current address: Society for General Microbiology, Marlborough House, Spencers Wood, Reading, United Kingdom
Methods

Bacterial growth and DNA amplification

Strains and plasmids used in this study are listed in Table 1. Bacterial strains were cultured in BHI (Merck) or LB (Lab M) broth for *E. faecalis* and *Escherichia coli*, respectively. For standard growth, an overnight broth culture of *E. faecalis* was diluted 100-fold dilution into 50 ml fresh BHI in a 250 ml flask, and incubated with shaking at 250 r.p.m. at 37°C. Antibiotics were included in overnight cultures where appropriate at the following concentrations: 2 mg ml⁻¹ kanamycin, 10 μg ml⁻¹ erythromycin for *E. faecalis* and 100 μg ml⁻¹ ampicillin for *Esch. coli*. To determine protease activity, *E. faecalis* strains were inoculated on BHI agar containing 5% (w/v) dried skimmed milk powder. Following overnight growth at 37°C, plates were examined for zones of clearing, which indicated protease activity by the strains. *E. faecalis* was transformed as described previously [11]. Amplification of the fsr locus by PCR was done using primers for fsrAB (PR21: 5’CGGTAAGCTCACAGAAG and PR22: 5’GGCAGGATTTGAGGTGC) or EF_1841-fsrC described previously [14].

Exoprotein extraction for 1D PAGE

*E. faecalis* strains were grown overnight in BHI broth, then subcultured for the desired time period before removing aliquots and pelleting cells by centrifugation. TCA was then added to the supernatant at 10% (v/v) final concentration and incubated for 30 minutes on ice. Precipitated proteins were recovered by centrifugation and the protein pellet was washed with buffered wash solution [50 mM Tris-HCl pH 6.8, 95% (v/v) ethanol] and air-dried. The protein pellet was resuspended in 50 mM Tris-HCl (pH 7.5) containing DNase and RNase (both 10 μg ml⁻¹) and incubated at 37°C for 15 min. Exoprotein purification for *E. faecalis* OG1RF fsrB was described in [21]. Proteins were precipitated and washed once more before proteins were resuspended in 150 μl 2D lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 1 mg bromophenol blue] and stored at −80°C until use.

Soluble protein (500 μg) was brought up to 320 μl with rehydration buffer [0 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, 1% (v/v) ASB 14 detergent and 0.5% (v/v) carrier ampholytes (Bio-lyte 3/10, Bio-Rad)]. Samples were kept for 1 h at room temperature with gentle shaking. Samples were in-gel rehydrated and focused on 11 cm, pH 4–7 IPG strips (Bio-Rad) for a total of 40,000 V h⁻¹ [150 V for 1 h, 300 V for 1 h, 600 V for 1 h, 1,200 V for 1 h, 2,000-5,000 V over 1 h (linear gradient), 8,000 V to 40,000 V (steady state)], using a Protean IEF Cell (Bio-Rad). After focusing, strips were equilibrated in 50 mM Tris (pH 6.0), 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol and bromophenol blue, containing 20 mM DTT in the reduction step (15 min) and 25 mM iodoacetamide in the alkylation step (15 min). IPG strips were run in the second dimension on 16x16 cm 12.5% SDS-PAGE gels using a Protean II xi 2D Cell (Bio-Rad). Gels were run in triplicate, silver-stained [22] and scanned (GS-710 Densitometer, Bio-Rad) as grayscale TIF files at 16 bit and 300 dpi and uploaded into the Progenesis ‘SameSpots’ (Non Linear Dynamics) gel image analysis Software. Quantitative analysis was based on average gels created from three gel replicates. Spots in the treated samples with a P value≤0.05 and greater than twofold difference from the control sample were considered statistically significant. For protein identification by mass spectrometry, two gels containing 500 μg each of soluble protein were prepared as above and stained with Colloidal Coomassie Brilliant Blue [23]. Scanned images were uploaded into Progenesis ‘SameSpots’ and matched to the analytical gels.

Exoprotein extraction and 2D SDS-PAGE

Proteins were precipitated from cell culture supernatants using previously described method for *Strep. pyogenes* [20]. Briefly, cells were grown to the required growth phase in dialysed BHI medium and harvested. The cell culture supernatant was filtered (0.22 μm membrane) before precipitating proteins on ice for 60 min by adding TCA:acetone [10% (w/v):5% (v/v)]. Proteins were pelleted by centrifugation and the pellet was washed [50 mM Tris-HCl pH 6.8, 95% (v/v) ethanol] and air-dried. The protein pellet was resuspended in 50 mM Tris-HCl (pH 7.5) containing 50 μM fering enzymes were included in overnight cultures where appropriate at the following concentrations: 2 mg ml⁻¹ kanamycin, 10 μg ml⁻¹ erythromycin for *E. faecalis* and 100 μg ml⁻¹ ampicillin for *Esch. coli*. To determine protease activity, *E. faecalis* strains were inoculated on BHI agar containing 5% (w/v) dried skimmed milk powder. Following overnight growth at 37°C, plates were examined for zones of clearing, which indicated protease activity by the strains. *E. faecalis* was transformed as described previously [11]. Amplification of the fsr locus by PCR was done using primers for fsrAB (PR21: 5’CGGTAAGCTCACAGAAG and PR22: 5’GGCAGGATTTGAGGTGC) or EF_1841-fsrC described previously [14].

**Table 1.** *E. faecalis* strains used in this study.

| *E. faecalis* Strain | Alternative ID | Characteristics | Source |
|----------------------|----------------|----------------|--------|
| OG1RF                |                | Wild type Rif⁻/Fus⁺ | [42]   |
| JH2-2                |                | Wild type Rif⁻/Fus⁺ naturally occurring fsr deficient strain | [45]   |
| OG1RF gefE           | TX2624         | In-frame deletion of gefE | [43]   |
| OG1RF fsrB           | TX2616         | fsrB deletion mutant | [13]   |
| OG1RF fsrB pTEXT5249 | TX2425         | fsrB deletion mutant with fsrABDC complementation on pTEXT5249 | [13]   |
| JH2-2 pTEXT5249      | LV305          | fsr deficient strain with fsrABDC complementation on pTEXT5249 | This study |
| V583                 |                | Van¹ | [44]   |
| EC23, EC117, EC95, EC207, EC238 |                | PGFE cluster 1 BSAC Bacteremia Resistance Surveillance Programme (UK & Ireland) | [24]   |
| EC126, EC137, EC36, EC127, EC216 |                | PGFE cluster 2 BSAC Bacteremia Resistance Surveillance Programme (UK & Ireland) | [24]   |
| 402,463,468,487, 488,489,499 |                | Clinical isolates (Poland) | [30]   |
| IS19-IS48            |                | MLST-typed clinical isolates(Spain) | [26]   |

doi:10.1371/journal.pone.0033450.t001
Protein identification

Spots for identification were excised and digested in-gel with trypsin. Gel plugs were destained with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate, dehydrated in 100% acetonitrile and rehydrated overnight in 10 μl 50 mM ammonium bicarbonate containing 20 ng μl⁻¹ MS grade trypsin (Promega), all at 37°C.

Peptide mass fingerprints (PMFs) were generated by using a reflectron MALDI-TOF instrument (Micromass). Supernatants (above) were mixed (1:1 ratio) with a saturated solution of α-cyano-4-hydroxycinnamic acid ACN:water:TFA [50:49:1 (v/v/v)]. The acquired spectra were analysed using MassLynx v4.0 (Waters-Micromass) and were all externally calibrated with a mixture of peptides. For each sample, all acquired spectra were combined and processed as follows using MassLynx v 4.0: smoothing, 2× smooth using a Savitzky Golay method set at ±3 channels and background subtraction using a polynomial of order 1 and 40% below the curve in order to reduce background noise. To get accurate mono-isotopic peak data all processed spectra were centred using the top 80% of each peak. Peak lists were generated by using ProteinLynx, part of MassLynx v 4.0. Monoisotopic peptide masses in the mass range of 800–4000 Da were used in the database search. The resulting peptide mass maps were used to interrogate Firmicute sequences to generate statistically significant candidate identifications using the Mascot search engine (http://www.matrixscience.com). Searches were performed allowing for complete carbamidomethylation (alkylation) of cysteine residues, partial oxidation of methionine residues, one missed cleavage and a mass error of 6 u.

Molecular weight search (MOWSE) scores, number of matched ions, percent protein sequence coverage and correlation of gel ions with predicted mass and pI were collectively considered for each protein identification. Inter-genome protein comparisons were performed using Seed-Viewer (http://www.theseed.org).

Results

Distinct exoprotein patterns in E. faecalis strains

Stationary phase exoproteins were purified from culture supernatants of clinical isolates bearing high level gentamicin and ciprofloxacin resistance that were previously divided into two separate PFGE clusters [24]. Culture supernatant exoproteins were separated using SDS-PAGE and compared with those of the commonly used E. faecalis laboratory strains OG1RF and JH2-2. Strikingly, two distinct patterns of proteins were observed that separately grouped each PFGE cluster with the protein profile of either strain OG1RF or JH2-2 (Figure 1 A,B). In addition, a comparison with the exoproteins from several genome-sequenced strains of E. faecalis (V583, HH22 and TXO104) [25] identified OG1RF-like exoprotein expression patterns (Figure 1C). In contrast with OG1RF, strain JH2-2 is negative for gelatinase activity (Zn-metalloprotease) [26] and consequently the clinical isolates were tested for proteolysis on casein agar. The PFGE clusters were discriminated by their ability to hydrolyse casein, with PFGE cluster 2 lacking activity and matching the known phenotype of JH2-2 (data not shown).

Common to exoproteins from strain OG1RF and OG1RF-like strains was the presence of two prominent proteins with molecular masses of approximately 36 and 38 kDa. To discriminate the OG1RF-like and JH2-2-like strains, these two proteins were cut from gels of exoproteins from strain V583, trypsinised and identified from their MALDI-TOF PMFs using the Mascot database. These PMFs ascribed identities to the 36 kDa protein as serine protease (SprE) (5 peptides, 23% coverage) and the 38 kDa protein as gelatinase (GelE) (5 peptides, 21% coverage). These proteases are well-described exoproteins regulated via the Fsr two-component signal transduction system that mediates quorum sensing [10].

Fsr-dependent regulation discriminates OG1RF-like and JH2-2-like exoprotein profiles

The absence of GelE and SprE proteins from the JH2-2-like strains was proposed here to reflect the known lack of conservation within the fsrABDC chromosomal locus [10–15]. To confirm the relationship between the observed OG1RF-like exoprotein profile and the presence of the fsrABDC operon and GelE, the strains TX5264 (OG1RF gelE) and TX5266 (OG1RF fsrB) were compared with strains OG1RF and JH2-2 (Figure 2). The association was also investigated by examining the exoproteins of the complementation strain TX5245 (OG1RF fsrB pTEX5249) (Figure 2). Inactivation of fsrB revealed a marked change to the OG1RF-like exoprotein complement towards that of JH2-2-like strains. A similar, but distinct, exoprotein set was evident with inactivation of gelE in strain OG1RF, although this protein complement contains elevated levels of SaliB (Shankar et al., submitted for publication). In addition, complementation of fsrB with the plasmid-encoded fsrABDC operon produced overexpression of proteins, likely to represent GelE and SprE (Figure 3).

Figure 1. Exoproteins from E. faecalis strains OG1RF and JH2-2 and clinical isolates from separate PFGE clusters. Stationary phase (8 h) culture supernatant from (A) strains OG1RF, JH2-2, PFGE cluster 1 strains EC23, EC117, EC95, EC207, EC238. (B) PFGE cluster 2 strains EC126, EC137, EC36, EC127, EC216. (C) strains V583, HH22, TXO104. GelE (G) and SprE (S) were identified by mass spectrometry. Molecular mass markers are indicated (kDa).

doi:10.1371/journal.pone.0033450.g001
These comparisons revealed that the OG1RF-like exoprotein profile was a signature of a functional fsrABCD locus and downstream gelE-sprE operon. The fsrABCD operon-encoded quorum-sensing locus is a known growth-phase regulator of the proteases GelE and SprE [10–12]. To identify its contribution to temporal exoprotein expression, culture supernatant of strain OG1RF was sampled at early-exponential (3 h), mid-exponential (5 h) and stationary (8 h) growth phases, together with an overnight (14 h) culture sample. Electrophoretic separation identified clear temporal changes to the exoproteome that consisted of nine major protein bands (Figure 4). It was also confirmed that in contrast with OG1RF, strain TX5266 (OG1RF fsrB) revealed an absence of clear temporal changes to the exoproteome (data not shown), supporting the suggestion that Fsr is the sole temporal regulator of the exoproteome in strain OG1RF under the conditions tested.

Distinct genetic differences produce a JH2-2-like exoprotein pattern of expression

Previous studies identified that deletion of a 23.9 kb region encompassing the majority of the fsrABCD operon was the major factor (79% of GelE-negative strains) contributing to a gelatinase-negative phenotype [14]. To determine the nature of the genetic differences producing GelE-negative phenotypes, and thus JH2-2-
like exoprotein phenotypes, two separate loci were amplified by PCR; these were: EF1841-\(fsrC\) and \(fsrAB\), and these were amplified from 47 European clinical isolates from the UK [23], Spain [25] and Poland [26] (Table 1), with strains OG1RF and JH2-2 representing \(fsr\)-positive and negative strains, respectively.

JH2-2-like strains were determined by confirming an absence of gelatinase activity on casein agar. Analysis of the clinical isolates indicated four distinct groups: Group 1 strains were GelE-positive and PCR-positive for \(fsrAB\) and EF_1841-EF_1820 \((\text{fsrC})\) (27 isolates); Group 2 strains were GelE-negative and PCR-negative

### Table 2. Identification of exoproteins of \(E. faecalis\) OG1RF.

| Spot | Protein description (Gene) | Calculated mass (kDa)/pI | Peptides matched | Coverage (%) |
|------|---------------------------|--------------------------|-----------------|-------------|
| 1    | GTP-binding protein LepA (EF_2352) | 68.27/4.99 | 10/28 | 20 |
| 2    | D-alanine-D-lactate ligase (EF_2294) | 21.24/4.7 | 6/31 | 52 |
| 3    | GTP-binding protein LepA (EF_2352) | 68.27/4.99 | 5/11 | 16 |
| 4    | DNA primase (EF_1521) | 73.01/5.12 | 6/20 | 19 |
| 5    | DNA polymerase III subunit alpha (EF_1044) | 31.35/4.96 | 5/17 | 35 |
| 6    | Transcriptional regulator, Cro/CI family (EF_2508) | 20.92/8.33 | 4/18 | 34 |
| 7    | VanXYG2; D-alanyl-D-alanine carboxypeptidase (Q30BF0_ENTFA) | 29.43/5.21 | 9/93 | 55 |
| 8    | Acetyltransferase, GNAT family (EF_1296) | 21.98/5.48 | 4/18 | 44 |
| 9    | Alpha-glycerophosphate oxidase (EF_1928) | 67.80/4.94 | 5/10 | 16 |
| 10   | Glycosyl hydrolase, family 20 (EF_0114) | 94.12/4.96 | 11/21 | 18 |
| 11   | Lipoate p-thiolate synthase, LtaS (EF_1264) | 79.96/6.2 | 8/32 | 21 |
| 12   | Hypothetical protein (EF_1995) | 12.32/4.29 | 6/27 | 60 |
| 13   | LtaS (EF_1264) | 79.96/6.2 | 8/27 | 19 |
| 14   | Endo-beta-N-acetylglucosaminidase (EF_2863) | 34.54/5.96 | 7/29 | 26 |
| 15   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 16/105 | 42 |
| 16   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 14/56 | 39 |
| 17   | Septation ring formation regulator EzrA (EF_0370) | 68.11/4.75 | 8/27 | 24 |
| 18   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 9/31 | 38 |
| 19   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 5/19 | 25 |
| 20   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 15/82 | 57 |
| 21   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 10/33 | 32 |
| 22   | GelE (EF_1818) | 55.34/4.99 | 5/13 | 15 |
| 23   | Transcriptional regulator, TetR family (EF_1531) | 20.70/6.63 | 4/21 | 48 |
| 24   | GelE (EF_1818) | 55.34/4.99 | 6/16 | 14 |
| 25   | D-alanyl-D-alanine dipeptidase (EF_2293) | 23.20/5.8 | 4/16 | 26 |
| 26   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 6/13 | 16 |
| 27   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 6/18 | 16 |
| 28   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 7/16 | 19 |
| 29   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 10/34 | 32 |
| 30   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 12/71 | 50 |
| 31   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 7/21 | 19 |
| 32   | Zn-Metalloprotease, GelE (EF_1818) | 55.34/4.99 | 8/16 | 23 |
| 33   | Hypothetical protein (EF_0841) | 39.46/5.61 | 6/58 | 27 |
| 34   | DNA replication protein, putative (EF_1279) | 29.74/6.19 | 5/17 | 31 |
| 35   | Serine protease, SprE (EF_1817) | 31.04/5.54 | 10/57 | 39 |
| 36   | Septation ring formation regulator EzrA (EF_0370) | 68.28/4.75 | 9/20 | 31 |
| 37   | Ribosomal protein L11 RplK (EF_2719) | 14.68/9.52 | 5/23 | 47 |
| 39   | Hypothetical protein (EF_0486) | 8.62/6.23 | 4/26 | 66 |
| 40   | Hypothetical protein (EF_2308) | 3.23/5.91 | 2/15 | 84 |
| 41   | Transcriptional regulator, Cro/CI family (EF_2508) | 20.92/8.33 | 5/27 | 37 |
| 42   | Hypothetical protein (EF_2843) | 8.41/4.6 | 4/21 | 56 |
| 43   | Hypothetical protein (EF_1926) | 20.78/9.32 | 5/25 | 34 |
| 44   | ATP synthase F0, AtpE (EF_2613) | 7.61/6.07 | 4/20 | 63 |

Spot numbers correspond to those in Figure 5.

doi:10.1371/journal.pone.0033450.t002
Proteases GelE and SprE are the dominant exoproteins of *E. faecalis* OG1RF

The exoproteome of *E. faecalis* OG1RF was purified from cell culture supernatants after 8 h growth (representing early stationary phase) and 500 μg protein was separated by using 2D SDS-PAGE (Figure 3). Protein spots of interest were excised and trypsinised and the masses of component peptides were determined by using MALDI-TOF mass spectrometry. PMFs were queried against the MASCOT database and all protein identifications were assigned from the best matches. A list of identified proteins is given in Table 2. Fifty-nine protein spots were excised from the gels, which yielded 44 protein identities matched to *E. faecalis* V583, with 20 unique identities. Nine protein spots were identified as GelE and seven protein spots were identified as SprE, where these existed with several distinct molecular masses and pl values, indicative of post-translational modifications such as charge changes.

All protein matches were also analysed by using SignalP v3.0 (http://www.cbs.dtu.dk/services/SignalP/) [27] to identify potential signal peptide signatures for protein export [28]. PSortb v3.0 (http://www.psort.org/psortb/) [29] was used to determine the most probable localisation of the proteins. Four proteins displayed a signal peptide: GelE [VAA-EE], SprE [AWA-EE], EF_1264 (LtaS) [AYA-VD] and EF_2863 (endo-ß-N-glucosaminidase) [VQA-AS].

The exoproteome of OG1RF fsrB is dominated by metabolism- and stress-related proteins

Analysis of the exoproteome of strain TX5266 (OG1RF fsrB) revealed many minor proteins and this protein pattern appeared to be constant at 5 h (exponential phase, data not shown), 8 h (figure 3) and 14 h (overnight, data not shown). To characterise this fraction in more detail, and determine the contributing proteins, a 500 μg sample was separated by 2D-gel electrophoresis (Figure 6). The exoprotein fraction differed from strain OG1RF due to the greater abundance of high molecular weight, low pl proteins. The major protein identities associated with strain TX5266 (OG1RF fsrB) are listed in Table 3. A key difference between the two exoproteomes is the absence of the GelE and SprE proteases in the fsrB mutant, which dominate the strain OG1RF supernatant fraction. In addition low pl proteins constitute the majority of the exoproteins of strain TX5266 (OG1RF fsrB) including metabolism enzymes (e.g. Eno, Pkg, GpmA, Gap-2) and general stress proteins (DnaK, GroEL, AhpC).

The protein complement in the culture supernatant did not reflect that which would be expected if this fraction was generated solely by cell lysis, due to the absence of the known dominant intracellular proteins, at least within the spots identified here.

Discussion

Analysis of the culture supernatant exoproteins produced by two PFGE clusters of high level gentamicin- and ciprofloxacin-resistant clinical isolates of *E. faecalis* from the UK and Ireland [24] revealed two distinct protein profiles. When compared to the commonly used laboratory strains OG1RF and JH2-2, there was an observed correlation of the exoprotein profiles produced by isolates of these distinct PFGE clusters corresponding to either an OG1RF-like or JH2-2-like exoprotein complement. Since OG1RF and JH2-2 are known to differ in their expression of the virulence-associated Zn-metalloprotease, gelatinase (GelE) [10,13,14], the PFGE cluster strains were tested for casein hydrolysis. OG1RF-like strains were all positive for gelatinase activity and revealed the presence of characteristic prominent protein bands of between ~30 and 40 kDa using 1D SDS-PAGE, which were identified as being GelE and the serine protease SprE.

Previous studies have clearly demonstrated the genetic variance of the *fsrABD-gelE-sprE* locus resulting from deletion of the 23.9 kb *EF_1841-fsrC* region and have demonstrated the correlation between this deletion and the lack of gelatinase activity [10,11,13,15]. More recently, a larger study testing a diverse set of multilocus sequence types (MLSTs) demonstrated that the *EF_1841-fsrC* deletion was highly conserved with common single nucleotide polymorphisms (SNPs) and junction sequences in strains bearing the deletion; its association with the absence of gelatinase expression was shown to be independent of genetic lineage [15].

In this study, using a set of 47 *E. faecalis* isolates from the UK, Spain and Poland, GelE-negative strains were identified by the absence of casein hydrolysis and then tested by PCR amplification to investigate the basis for the JH2-2-like exoprotein phenotype. Three groups of GelE-negative strain genotypes were discriminated by the presence/absence of the 23.9 kb *EF_1841-fsrC* region and *fsrAB*, indicating that in 21 of 22 isolates the absence of regulatory genes explained loss of expression. Thus, for locus variants can produce a JH2-2-like exoprotein supernatant fraction. Recent studies [15] clearly demonstrate that the lack of GelE activity in isolates is almost completely due to variant *fsr* locus deletions from an analysis of 22 *E. faecalis* genome sequences.
Consequently, the underlying genetic cause for the JH2-2-like exoprotein profile was not unambiguously determined here. The finding in this study that the PFGE clusters identified within the high-level gentamicin and ciprofloxacin bacteraemia strains from the UK could also be grouped by their exoprotein profile was tested with ten isolates. Analysis of MLSTs and gelatinase expression [15] suggests that the relationship between genetic lineage and gelatinase expression would not be maintained in a larger set of strains. The precise mechanistic explanation for this deletion and its spread across different MLSTs requires further study.

Temporal regulation of the exoproteome was demonstrated to be dependent on the fsr locus in strain OG1RF, but strain JH2-2, which has the 23.9 kb EF_1841-fsrC deletion [14] maintains an apparently constant exoproteome throughout growth in the conditions studied. This fsr locus deletion was complemented by transformation with pTEX5249, which contains the fsrABDC operon, and temporal expression was restored with a protein complement similar to that of OG1RF. This indicates that fsr function, and thus an OG1RF-like exoproteome, can be regained in this naturally fsr-deficient strain and strains in the environment could have functionality restored via chromosomal transfer or chromosomal transfer.

**Table 3. Identification of exoproteins of E. faecalis strain TX5266 (OG1RF fsrB).**

| Spot | Protein description (Gene) | Calculated mass (kDa)/pI | Peptides matched | Coverage (%) |
|------|---------------------------|--------------------------|-----------------|-------------|
| 1a   | RNA polymerase omega subunit, RpoZ (EF_3126) | 11.53/5.36 | 6/35 | 51 |
| 2a   | 30S ribosomal protein S6, RpsF (EF_0007) | 11.59/5.01 | 7/11 | 48 |
| 3a   | Dps Family protein (EF_3233) | 17.93/4.56 | 6/11 | 33 |
| 4a   | Fumarate reductase subunit (EF_2556) | 53.83/5.26 | 12/35 | 25 |
| 5a   | Alkyl hydroperoxide reductase, AhpC (EF_2739) | 21.3/4.5 | 16/22 | 43 |
| 6a   | N-acetylmuramoyl-L-alanine amidase (EF_1823) | 30.32/4.89 | 3/4 | 16 |
| 7a   | Hypothetical protein (EF_1470) | 14.62/5.61 | 5/26 | 38 |
| 8a   | ABC transporter, ATP-binding protein (EF_2394) | 28.40/4.73 | 9/13 | 37 |
| 9a   | Glycine betaine/carnitine/choline ABC transporter (EF_0863) | 34.72/5.14 | 16/21 | 41 |
| 10a  | Fructose-bisphosphate aldolase class II, Fba (EF_1167) | 31.02/4.86 | 7/14 | 26 |
| 11a  | Adenylate kinase (EF_0228) | 24.25/5.05 | 9/15 | 36 |
| 12a  | Phosphoglycerate mutase L, GpmA (EF_0195) | 26.05/5.09 | 11/21 | 34 |
| 13a  | Endo-beta-N-acetylglucosaminidase (EF_2863) | 35.54/5.96 | 6/10 | 25 |
| 14a  | Tail protein, putative (EF_1829) | 37.97/5.27 | 8/21 | 27 |
| 15a  | Hypothetical protein (EF_0375) | 35.43/7.07 | 9/19 | 28 |
| 16a  | Endolysin, Ply-1 (EF_1293) | 40.14/5.70 | 11/1 | 30 |
| 17a  | Catabolite control protein A, CcpA (EF_1741) | 36.19/5.27 | 13/14 | 30 |
| 18a  | Chitinase, family 2 (EF_0361) | 38.31/5.31 | 6/10 | 13 |
| 19a  | Phosphotransacetylase Pta (EF_0949) | 35.59/4.97 | 7/19 | 30 |
| 20a  | Translation elongation factor, Ts, Tsf (EF_2397) | 32.11/4.87 | 18/43 | 51 |
| 21a  | L-lactate dehydrogenase Ldh (EF_0255) | 35.52/4.77 | 14/38 | 47 |
| 22a  | Pyruvate dehydrogenase E1 component, PdhB (EF_1354) | 35.37/4.67 | 12/46 | 37 |
| 23a  | Basic membrane protein family (EF_0177) | 37.78/4.88 | 8/15 | 21 |
| 24a  | Basic membrane protein family (EF_0177) | 37.78/4.88 | 4/15 | 14 |
| 25a  | Arginine deiminase, ArcB (EF_0105) | 38.13/5.02 | 19/28 | 46 |
| 26a  | Pyruvate dehydrogenase complex E1 component, PdhA (EF_1353) | 41.35/5.25 | 8/12 | 24 |
| 27a  | Glyceraldehyde 3-phosphate dehydrogenase, Gap-2 (EF_1353) | 35.92/5.03 | 7/17 | 24 |
| 28a  | Acetate kinase, AckA (EF_1983) | 43.49/4.96 | 12/15 | 33 |
| 29a  | Phosphoglycerate kinase, Pgk (EF_1963) | 42.42/4.90 | 16/36 | 49 |
| 30a  | Arginine deiminase, ArcB (EF_0105) | 38.13/5.02 | 20/26 | 41 |
| 31a  | Enolase, Enol (EF_1961) | 46.48/4.56 | 12/34 | 36 |
| 32a  | Lipoteichoic acid synthase, LtaS (EF_1264) | 79.96/6.2 | 12/24 | 15 |
| 33a  | Lipoteichoic acid synthase, LtaS (EF_1264) | 79.96/6.2 | 19/36 | 30 |
| 34a  | Efr/YidS/Ycf5/YnhG family protein, putative (EF_2860) | 52.77/6.74 | 20/38 | 35 |
| 35a  | DnaK (EF_1308) | 65.54/4.59 | 12/20 | 23 |
| 36a  | GroEL (EF_2633) | 55.41/4.66 | 18/24 | 28 |
| 37a  | Putative ATPase, CbiA (Q9AL20) | 34.43/4.17 | 7/25 | 25 |
| 38a  | Glutamate dehydrogenase, GdhA (EF1415) | 49.62/5.42 | 11/33 | 29 |

Spot numbers correspond to those in Figure 6.
doi:10.1371/journal.pone.0033450.t003
temperate bacteriophage-mediated generalised transduction [30,31].

GelE was observed on 2D gels within the 31–44 kDa region. Significant processing of GelE via C-terminal proteolytic cleavage is required for its activation [32] and the presence of GelE spots in this region with minor variations in the pI, indicates post-translational processing of GelE such as charge changes. SprE was identified from seven spots on 2D SDS-PAGE gels, which could be due to GelE-dependent proteolysis or SprE-catalysed self-cleavage [33]. Different isoforms of SprE were previously demonstrated to have different activities [33] and like GelE, pI variants of SprE were observed in this study.

Comparison of the exoproteomes of OG1RF and an isogenic fosB mutant separated by 2D electrophoresis revealed the absence of the cell density-regulated proteases, GelE and SprE. The proteases are predominant in the OG1RF proteome with the remaining proteins identified comprising: cell wall synthesis/modification enzymes lipoteichoic acid synthase (LtaS), endo-b-N-acetylglucosaminidase; cell division proteins (EzrA); and several proteins not clearly assigned to cellular pathways. LtaS (EF_1264) is a transmembrane protein required for the production of the PGP backbone chain of LTA. In S. aureus, proteolytic cleavage by the signal peptidase Spb irreversibly inactivates LtaS during growth [34] and its presence in the exoprotein fraction in the E. faecalis OG1RF exoproteome could reflect similar turnover. Whether the released, cleaved protein which contains a predicted sulphatase superfamily domain contributes an alternative function that benefits the cell, such as mucin desulphation, is not known. The OG1RF exoproteome is apparently limited in potential virulence-enhancing determinants. The strain is atypical among genome-sequenced isolates since it has a CRISPR CAS-bearing strain with minimal evidence of horizontal gene acquisition [35]. Comparison of the exoproteome with other clinical isolates is likely to reveal a more extensive variable complement of proteins in E. faecalis. Together with the expected absence of the GelE and SprE proteases, the 2D exoproteome of the fosB mutant comprised many glycolytic and general stress proteins. Comprehensive studies of the S. aureus exoproteome [21,36] have identified many of the proteins revealed in the E. faecalis exoproteome, suggesting that many of these proteins with defined intracellular functions contribute to the exoproteome. Moreover, some of these have been identified as having extracellular functions e.g. enolase in staphylococci, streptococci and lactobacilli [37]. The E. faecalis TX5266 (OG1RF fosB) exoproteome contains many proteins with identifiable secretion signals supporting their extracellular location. This exoproteome was more extensive as judged by the number of spots and their diversity of functions. The wild-type and fosB mutant exoproteomes share few proteins in common, at least with respect to those identified here, and consequently they were not quantitatively compared. The OG1RF fosB mutant was previously described as having an autolysis defect [30] and we have confirmed this finding and shown that the strain has increased dead cells during culture (Shankar et al, submitted for publication). Despite the fact that protein spot identification was not exhaustive in this study it is apparent that many of the proteins match those of a ΔfleH mutant [39] and ΔsalB mutant (Shankar et al, submitted for publication). This overlap requires further investigation. The mechanisms underpinning the Fsr-dependent changes in the exoproteome are unclear, but could result from differences in protease expression, cell surface and autolysis changes in the mutant, differences in viability, or a combination of these factors. The OG1RF fosB mutant exoproteome differs from that of the gelE mutant due to the presence of SalB in the gelE mutant fraction (Fig. 2A). The reason for this visibly pronounced difference is unclear but it likely reflects FsrA-dependent transcriptional differences between the strains, either directly or indirectly.

Recent protein fractionation studies have begun to examine the secreted proteins of E. faecalis. Several studies have reported efforts to catalogue and characterise the surface-associated and membrane proteins. Analysis of the surface-associated protein fraction described recently [40] reveals overlap with several of the proteins identified here (e.g. glycolytic, general stress proteins). Exoproteins potentially interact with the environment in several ways via release from the cell and/or via binding back to the cell surface.

The recent and ongoing explosion of genome sequencing in the enterococci will enable comparative in silico analyses to determine the pool of potential secreted proteins and several studies have begun to address this [40,41]. The variability of exoproteins among strains will be dramatic mainly due to the high frequency of those bearing a deletion of the fos locus; however, the variance of exoproteins among fosR-replete strains will be important and this study will facilitate this comparative approach.

Acknowledgments

We are grateful to Mark Prescott and Brian Wareing for technical assistance with mass spectrometry.

Author Contributions

Conceived and designed the experiments: JS RGW MJH. Performed the experiments: JS RGW. Analyzed the data: JS RGW DW MJH. Wrote the paper: JS RGW MJH.

References

1. Bonten MJ, Willems R, Weinstein RA (2001) Vancomycin-resistant enterococci: why are they here and where do they come from? Lancet Infect Dis 1: 314–325.

2. Willems RJ, van Schaik W (2009) Transition of Enterococcus faecium from commensal organism to nosocomial pathogen. Future Microbiol 4: 1125–1135.

3. Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, et al. (2003) Infection with vancomycin-resistant Staphylococcus aureus containing the vanA resistance gene. N Engl J Med 348: 1342–1347.

4. Biavasco F, Giovanetti E, Miele A, Vignaroli C, Facinelli B, et al. (1996) In vitro conjugative transfer of VanA vancomycin resistance between enterococci and a commensal organism to nosocomial pathogen. Science 299: 1999–2002.

5. Miller D, Urdaneta V, Weltman A (2002) Public health dispatch: Vancomycin-resistant enterococci: a new public health problem. Morbidity and Mortality Weekly Report 51: 902.

6. Savet D, Boulton M, Stolman G, Johnson D, Stobierski M, et al. (2002) Staphylococcus aureus resistant to vancomycin – United States. Morbidity and Mortality Weekly Report 51: 565–567.

7. Gilmore MS, Ferretti JI (2003) The thin line between gut commensal and pathogen. Science 299: 1999–2002.

8. Hancock LE, Gilmore MS (2006) Pathogenicity of Enterococci. In: Fischetti V, Novick R, Ferretti J, Portnoy D, Rood J, eds. Gram-Positive Organisms: ASM Publications.

9. Tendolkar PM, Baghdayan AS, Shankar N (2003) Pathogenic Enterococci: new developments in the 21st century. Cell Mol Life Sci 60: 2622–2636.

10. Qin X, Singh KV, Weinstock GM, Murray BE (2001) Characterization of the Fsr system of Enterococcus faecalis: the Fsr-regulating operon. J Bacteriol 183: 3372–3382.

11. Bourgogne A, Hilsenbeck SG, Dunny GM, Murray BE (2006) Comparison of the exoproteomes of OG1RF and an isogenic fsrB deletion mutant by transcriptional analysis: the Fsr system of Enterococcus faecalis is more than the activator of gelatinase and serine protease. J Bacteriol 188: 2875–2884.

12. Hancock LE, Perego M (2002) Two-component signal transduction in Enterococcus faecalis. J Bacteriol 184: 5819–5825.

13. Qin X, Singh KV, Weinstock GM, Murray BE (2000) Effects of the plasmid-borne lucrative factor (fle) locus on production of gelatinase and a serine protease. Infect Immun 68: 2579–2586.

14. Nakayama J, Kariyama R, Kunno H (2002) Description of a 23.9-kilobase chromosomal deletion containing a region encoding fsp genes which mainly
determines the gelatinase-negative phenotype of clinical isolates of Enterococcus faecalis in urine. Appl Environ Microbiol 60: 3152–3155.

15. Galloway-Pena JR, Bourgogne A, Qin X, Murray BE (2011) Diversity of the fsrE region of the Enterococcus faecalis genome but conservation in strains with partial deletions of the fsr operon. Appl Environ Microbiol 77: 442–451.

16. Lowy FD (1998) Staphylococcus aureus infections. N Engl J Med 339: 520–532.

17. Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background. Electrophoresis 9: 255–262.

18. Neuza Teixeira N, Santos S, Marujo P, Yokohata R, Iyer V, et al. (2012) Incongruent gelatine genotype and phenotype in Enterococcus faecalis is due to shutting off the ability to respond to the GBAP quorum-sensing signal. Microbiology 158: 519–529.

19. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

20. Chaussée MA, McDowell EJ, Chauasse MS (2008) Proteomic analysis of proteins secreted by Staphylococcus pyogenes. Methods Mol Biol 431: 15–24.

21. Burlak C, Hammer CH, Robinson MA, Whitney AR, McGavin MJ, et al. (2007) Global analysis of community-associated meticillin-resistant Staphylococcus aureus exoproteins reveals molecules produced in vitro and during infection. Cell Microbiol 9: 1172–1190.

22. Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, et al. (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis 21: 3666–3672.

23. Neuhoff V, Arlot D, Taube D, Ehrhardt W (1998) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255–262.

24. Woodford N, Reynolds R, Turton J, Scott F, Sinclair A, et al. (2003) Two widely divergent gelatinase genotypes and phenotypes in Enterococcus faecalis: an essential gene involved in cell division, cell growth and chromosome segregation. Microbiology 151: 1381–93.

25. Paulsen IT, Banerjee L, Myers GS, Nelson KE, Shedder R, et al. (2003) Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. Science 299: 2071–2074.

26. Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, et al. (2006) Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol 44: 2220–2228.

27. Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2: 953–971.

28. von Heijne G (1996) The signal peptide. J Membr Biol 153: 195–201.

29. Nakai K, Horton P (1999) PSORT—a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 24: 34–36.

30. Yasmin A, Kenny JG, Shankar J, Darby AC, Hall N, et al. (2010) Comparative genomics and transduction potential of Enterococcus faecalis temperate bacteriophages. J Bacteriol 192: 1122–1130.

31. Manson JM, Hancock RE, Gilmore MS (2010) Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits. Proc Natl Acad Sci U S A 107: 12269–12274.

32. Del Papa MF, Hancock RE, Thomas VC, Perrego M (2007) Full activation of Enterococcus faecalis gelatinase by a C-terminal proteolytic cleavage. J Bacteriol 189: 8835–8843.

33. Kawalec M, Potempa J, Moon JL, Travis J, Murray BE (2005) Molecular diversity of a putative virulence factor: purification and characterization of isoforms of an extracellular serine glutamyl endopeptidase of Enterococcus faecalis with different enzymatic activities. J Bacteriol 187: 266–275.

34. Wormann ME, Reichmann NT, Maline CJ, Horowll AR, Grundling A (2011) Proteolytic cleavage inactivates the Staphylococcus aureus lipoteichoic acid synthase. J Bacteriol 193: 5279–5291.

35. Bourgogne A, Garsin DA, Qin X, Singh KV, Sillanpaa J, et al. (2008) Large scale variation in Enterococcus faecalis illustrated by the genome analysis of strain OGI/RF. Genome Biol 9: R110.

36. Ziebarth AK, Kusch H, Dogter M, Jaglia S, Sibbald MJ, et al. (2010) Proteomics uncovers extreme heterogeneity in the Staphylococcus aureus exoproteome due to genomic plasticity and variant gene regulation. Proteomics 10: 1634–1644.

37. Antikainen J, Kuparin V, Lahrezami K, Korbonen TK (2007) Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. FEMS Immunol Med Microbiol 51: 526–534.

38. Waters CM, Antioporta MH, Murray BE, Dunny GM (2003) Role of the Enterococcus faecalis GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. J Bacteriol 185: 3613–23.

39. Ramirez-Arcos S, Liao M, Marshall S, Rigden J, Dillon JA (2005) Enterococcus faecalis divIIM: an essential gene involved in cell division, cell growth and chromosome segregation. Microbiology 151: 1381–93.

40. Boehle LA, Riaz T, Egge-Jacobsen W, Skaugen M, Busk OJ, et al. (2011) Identification of surface proteins in Enterococcus faecalis V583. BMC Genomics 12: 135.

41. Mardaldo G, Chovanec P, Stenberg-Bruezell F, Nielsen HV, Jensen-Seaman MI, et al. (2013) A reference map of the membrane proteome of Enterococcus faecalis. Proteomics 11: 3935–3941.

42. Dunny GM, Brown BL, Clewell DB (1978) Induced cell aggregation and mating in Streptococcus faecalis: evidence for a bacterial sex pheromone. Proc Natl Acad Sci U S A 75: 3479–3483.

43. Singh KV, Qin X, Weinstock GM, Murray BE (1998) Generation and testing of mutants of Enterococcus faecalis in a mouse peritonitis model. J Infect Dis 178: 1416–1420.

44. Sahm DF, Kissinger J, Gilmore MS, Murray PR, Mulder R, et al. (1989) In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. Antimicrob Agents Chemother 33: 1508–1510.

45. Jacob AE, Hobbs SJ (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. J Bacteriol 117: 360–372.