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Enterococcus faecalis V583 cell membrane protein expression to alkaline stress

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One sentence summary: *Enterococcus faecalis* cell membrane response to extreme alkaline conditions results in a shift of carbohydrate metabolism and supports the formation of a protective capsule.
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Abstract

Enterococcus faecalis is able to adapt to alkaline conditions and is commonly recovered from teeth in which endodontic treatment has failed. The role that *E. faecalis* membrane proteins play in survival strategies to extreme alkaline conditions is unclear. We grew *E. faecalis* V583 in a chemostat at pH 8 and 11 at one-tenth the organism's relative maximum growth rate. Following membrane shaving, isotope-coding protein labels were added at the peptide level to samples and then combined. The relative proportion of membrane proteins were identified using LC-ESI mass spectrometry and MaxQuant analysis. Ratios of membrane proteins were $\log_2$ transformed, with proteins deviating by more than 1 SD of the mean considered to be up- or down-regulated. A total of six proteins were up-regulated in pH 11 including: EF0669 (polysaccharide biosynthesis family); EF1927 (glycerol uptake facilitator), and EF0114 (glycosyl hydrolase). A total of five proteins were down-regulated including: EF0108 (C4-dicarboxylate transporter); EF1838 (PTS system IIC component); EF0456 (PTS system IID component); and EF0022 (PTS mannose-specific IID component). In extreme alkaline conditions, the membrane proteins of *E. faecalis* seem to be involved in a shift of carbohydrate metabolism from the PTS system to glycerol, which supports the formation of a protective capsule protecting the cell.

Keywords: alkaline pH, biofilm, continuous culture, *Enterococcus faecalis*, ICPL, membrane shaving

Introduction

Enterococcus faecalis is commonly recovered from food, the oral cavity, and clinical specimens (Anderson et al. 2016). Endodontic treatment (root canal therapy) of a tooth with a necrotic pulp usually involves the chemo-mechanical debridement of the canal(s) with irrigants such as sodium hypochlorite and often interappointment medicaments such as calcium hydroxide (~ pH 12.5–12.8) placed in the main root canal to help in the elimination of surviving bacteria (Siqueira and Lopes 1999). Calcium hydroxide kills bacteria by direct contact through pH effects and is known to dramatically reduce the bacterial load in root canals with survival of *E. faecalis* reported to fall to 0.001% at pH 11 and 0.00001% at pH 12 (Appelbe and Sedgley 2007). However, *E. faecalis* is one of the most commonly recovered bacteria from persistent root canal infections, suggesting that it has the capacity to survive the chemo-mechanical procedures (Yap et al. 2014) and subsequent nutrient limited environment (Sundqvist et al. 1998).

Specific survival mechanisms maybe used by *E. faecalis* to survive alkaline stress, which include: the activation of ion-transport systems to balance intracellular and external pH levels (Evans et al. 2002); intrinsic resistance; neutralization of medicaments by bacterial cells; and an alteration in gene expression to the changes in the environmental conditions (Siqueira and Lopes 1999). More recently, it has been reported that alkaline stress initiates a reduction in carbohydrate and amino acid metabolism and increased nucleotide synthesis (Ran et al. 2015). A similar reduction in carbohydrate and energy metabolism has been reported in a response to fluoride exposure (Li et al. 2020).

The response of bacteria to specific environments are often mediated by membrane proteins, which are especially difficult to study due to the innate hydrophobicity of the transmembrane domain (Wolff et al. 2008). However, a number of fractionation protocols have been used to enrich for integral membrane proteins, with membrane shaving showing the greatest promise (Cathro et al. 2016).

The aim of this study was to determine the role of cell membrane proteins as an adaptive response by *E. faecalis* to extreme alkaline conditions. We have compared the membrane proteome expression in *E. faecalis* grown in nutrient limited conditions at one-tenth the maximum growth rate (μrel; Zilm and Rogers 2007) to mimic the nutrient limited environment of a root canal; using continuous culture at the physiologically and clinically relevant switch in pH from 8 to 11; with membrane shaving, and postdigest ICPL labeling.

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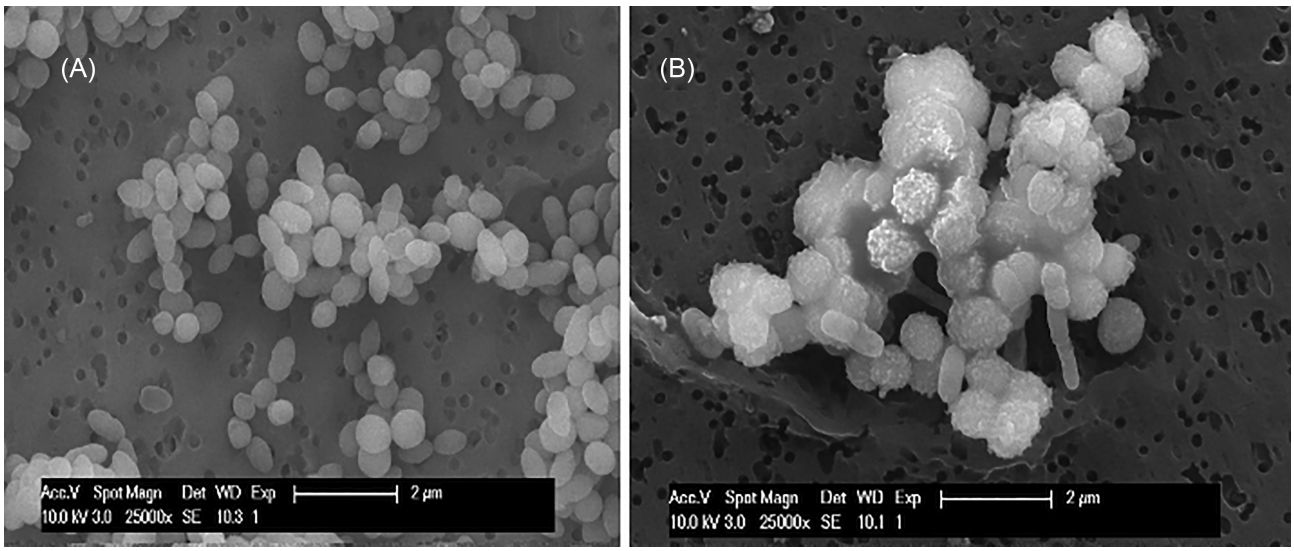

Figure 1. SEM image of *E. faecalis* at steady state at pH 8 (A), and pH 11 (B). Cells at pH 8 and 11 were grown at a MGT = 0.1 μrel.

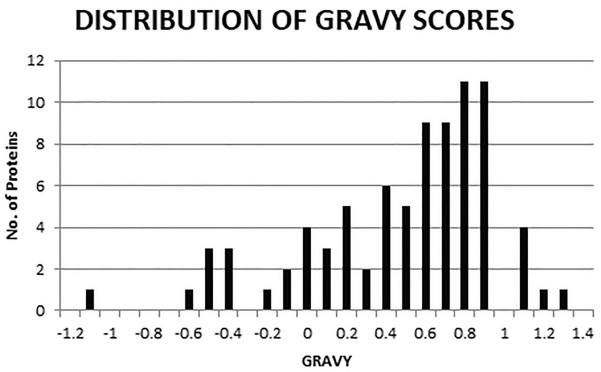

Figure 2. Frequency of GRAVY indices of membrane associated proteins.

Table 1. Predicted localization and number of identified membrane proteins using membrane shaving and searched with UniProtKB (www.uniprot.org).

Predicted localizationUniProtKB	Number of identified proteins
Multipass membrane	54
Integral component of membrane	21
Cell surface/cell wall/cell membrane	8
Cytoplasm	20
No structure information/no localization	32

Materials and methods

Growth conditions

Enterococcus faecalis ATCC V583 strain was purchased from Cryosite (NSW, Australia) and maintained on Columbia blood agar (Oxoid, Victoria, Australia) at 37°C. Culture purity was periodically checked by culturing onto Bile Aesculin agar (Oxoid). *Enterococcus faecalis* was grown by continuous culture using a model C30 BioFlo chemostat (New Brunswick Scientific, Edison, NJ, USA) with a culture volume of 365 ml. The maximum growth rate (μmax) was determined by growing the organism under batch conditions in the chemostat at pH 8 or 11. For growth at pH 8, the medium flow rate was set at 25.19 ml h^{-1} (0.1 μrel) giving a dilution rate of 0.069 h^{-1} and an estimated generation time of 10 h, which is typical of natural ecosystems (Hamilton et al. 1979). Growth pH was maintained at 8.0 by the automatic addition of 2 M KOH or 2 M HCl using a Fermac 260 pH controller (ElectroLab Tewkesbury, UK). After equilibration (10 generations) cell culture samples were harvested daily over a 4-week period and pooled. The pH in the chemostat was then incrementally increased to 11 over 48 h, the maximum growth rate determined and the medium flow rate set at 0.1 μrel (3.3 ml h^{-1}, D = 0.009 h^{-1}) giving an estimated generation time of 77 h. After equilibration of 10 generations (770 h) cell culture samples were harvested daily over a 4-week period and pooled.

Bacteria grown at each pH were harvested by centrifugation (6000 × *g*), at 4°C for 20 min. A volume of 1 ml of each sample was used for SEM analysis. Standard SEM sample processing was undertaken. After the samples had undergone critical point of drying, they were coated with carbon and gold and analyzed under a SEM (Philips XL 30, field emission SEM; Eindhoven, The Netherlands).

Cells were washed twice with saline (0.9% w/v) at 4°C and cells were finally resuspended in 12 ml of ice cold saline. Cells were lysed by two passes (60,000 kPa) through a SLM Aminco French Press (Thermo Fisher, MA, USA). Endogenous proteinase activity was controlled by the addition of 100 μl of bacterial protease inhibitor cocktail (Sigma, St. Louis MO, USA). Nucleic acids were then degraded by the addition of Deoxyribonuclease I (2000 units), Ribonuclease A (1000 units), and $MgCl_2$ (50 mM) and incubated on ice for 60 min. To separate unbroken cells, the suspension was centrifuged twice (8000 × *g* at 4°C for 5 min).

Membrane shaving—the protocol described by Cathro et al. (2016) was utilized for pH 8 and 11 samples and MALDI spectroscopy was used to establish that the relative concentration of peptides in samples was similar.

Peptide ICPL labeling

Both pH 8 and 11 samples were lyophilized and the peptides resuspended in 30 μl TEAB (40 mM, pH 8.5). The samples were then vortexed for 30 s and then sonicated for 5 min.

Table 2. Location, physiological classification, and GRAVY scores of proteins with a $\log_2$ H/L ratio (pH11/pH8) greater or lesser than 1 SD of the mean.

Protein name	Physiological classification	UniProt.org	GRAVY score
EF2096 phage tail protein	Virulence	Integral component of membrane	−0.06
EF1541 membrane protein	Unknown	Riboflavin transporter multipass membrane protein	0.886
EF0669 polysaccharide biosynthesis family protein	Cellular membrane/wall and cell division	Multipass membrane protein	0.596
EF0114 glycosyl hydrolase, family 20	Cellular membrane/wall and cell division	Integral component of membrane	−0.44
EF2938 uncharacterized protein	Unknown	Integral component of membrane	1.297
EF1927 glycerol uptake facilitator protein	Transport and binding	Multipass membrane protein	0.855
EF1838 PTS system IIC component	Transport and binding	Multipass membrane protein	0.8
EF0456 PTS system IID component	Transport and binding	Multipass membrane protein	0.497
EF0177 predicted nucleoside ABC transporter	Transport and binding	Plasma membrane	−0.373
EF0108 C4-dicarboxylate transporter	Transport and binding	Multipass membrane protein	0.812
EF0022 PTS system mannose-specific IID component	Transport and binding	Multipass membrane protein	0.335

A volume of 2 μl of ICPL_0 label (Serva quadruplex kit) was added to the pH 8 sample and 2 μl of ICPL_6 label (Serva quadruplex kit) was added to the pH 11 sample and both were overlayed with Argon. The samples were vortexed for 30 s and then sonicated for 1 min before incubation for 60 min at room temperature. A further 1 μl of ICPL label was added to each sample (0 and 6, respectively) and incubated for a further 60 min at room temperature. A volume of 2 μl of STOP solution was added to each sample and incubated for 20 min at room temperature. Equal volumes of sample pH 8 and sample pH 11 (with ICPL-labels) were loaded into a Protein LoBind tube, mixed gently, and then desalted and concentrated using C18 spin column (Pierec, Rockford, IL, USA). Peptides were eluted using ACN: TFA: H20 (70: 0.5: 29.5, v/v) and freeze-dried. The lyophilized peptides were resuspended using ACN: TFA: H20 (2: 0.1: 97.9, v/v). The volumes of the resulting peptide extracts were reduced by vacuum centrifugation to approximately 2 μl then resuspended with 0.1% TFA in 2% ACN to a total volume of ~10 μl.

Liquid chromatography—electrospray ionization tandem mass spectrometry—the protocol described by Cathro et al. (2016) was utilized.

Protein analysis

Data from the MASCOT search was searched through MAXQUANT (version 1.3.0.5). The ICPL_6-labeled proteins from pH 11 conditions were designated as "H" (heavy)–labeled, whilst ICPL_0-labeled proteins from pH 8 conditions were designated "L" (light).The search allowed for protein quantification on a single H/L count (i.e. just one peptide ratio) and was restricted to ENTFA V583. The proteins were searched using UniProtKB (http://www.uniprot.org) to determine the predicted localization. The total number of membrane-associated proteins and intracellular proteins were determined. The NCBI protein database (http://www.ncbi.nlm.nih.gov) was used to obtain the FASTA format for each membrane-associated protein, which was then used to search with ExPASy ProtParam (http://web.expasy.org/protparam/) for the GRAVY scores (a measure of protein hydrophobicity). The presence of signal peptides and the location of their cleavage sites were performed using SignalP-5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0).

The MAXQUANT H/L ratios of membrane-associated proteins were log2 transformed, and the proteins that deviated by more than 1 SD of the mean were considered to be up- or down-regulated.

Results and discussion

Continuous culture

Continuous culture in a chemostat can be used to study the properties of microorganisms at the slow growth rates that these organisms often achieve in their natural ecosystems (Hamilton et al. 1979). Maximum growth rate at pH 8 was consistent with a mean generation time (MGT) of 1.0 and 7.7 h at pH 11. This reduction is consistent with pH being one of the most important variables to the growth kinetics of *Enterococcus* species (Fisher and Phillips 2009). All chemostat cultures were grown at a growth rate of 0.1 of the maximum growth rate (μrel) to mimic a more nutrient-deprived environment (Zilm and Rogers 2007). The dilution rate of the chemostat was 0.069 h^{-1} (MGT = 10 h) for pH 8 and 0.009 h^{-1} (MGT = 77 h) for pH 11. Sampling of the chemostat occurred after 10 generations in which the bacteria would have reached steady state and adapted to the growth conditions.

SEM analysis (Fig. 1) after 10 generations at pH 11 showed the appearance of an extracellular matrix compared to growth at pH 8. This observation also coincided with the chemostat chamber becaming coated with a biofilm at pH 11. Collectively these observations are consistent with the formation of floating biofilms (flocs) which are not attached to an interface, but which share the characteristics of biofilms (Zilm and Rogers 2007, Flemming and Wingender 2010). The change in phenotype is consistent with SEM observations made by Distel et al. (2002) of *E. faecalis* exposed to a calcium hydroxide medicaments for 77 days. The production of EPS and its importance in response to alkaline stress has recently been reported by Wu et al. (2019). The authors investigated the role of the WalK/WalR two-component system on the susceptibility of *E. faecalis* to calcium hydroxide in infected root canals. *Enterococcus faecalis* (V583) and the *E. faecalis* A*SwalR* mutant were incubated on root canal blocks for 3 weeks followed by medication with calcium hydroxide for 1 week. The *E. faecalis* parental strain exhibited the highest EPS/bacterial cell ratio, whereas the ASwalR strain exhibited the lowest with a decreased alkaline stress tolerance.

Protein localization and ICPL labeling

A total of 136 proteins were identified from the membrane shaving preparations (Table S1, Supporting Information).

From *in silico* analysis, there are 781 predicted membrane-embedded proteins of *E. faecalis* V583 (Zhou et al. 2008) and approximately 10% were resolved in the present study. This is consistent with the study of Maddalo et al. (2011) who also identified ∼ 10% of the membrane-embedded proteome.

The majority of proteins identified had a positive GRAVY score (the sum of hydropathy values of all amino acids divided by the protein length), and is consistent with the hydrophobic nature of transmembrane protein portions and the efficacy of the membrane shaving protocol. (Fig. 2). It is also consistent with the findings of Ran et al. (2013) who demonstrated that cell surface hydrophobicity increased with increasing pH and time.

Approximately, 90% (123 proteins) were labeled with both ICPL_6 (H) and ICPL_0 (L) indicating a high efficency of labeling (Table S1, Supporting Information).

In conventional ICPL protocols, lysine-containing peptides are labeled with a light or heavy form of the ICPL reagent (Leroy et al. 2010) and quantification is determined by comparing the relative abundance of differentially labeled peptides by mass spectrometry (Paradela et al. 2010). However, as only lysine-containing peptides can be quantified, labeling at the protein level has the disadvantage in that only 60%–70% of proteins may be accurately quantified (Fleron et al. 2010, Leroy et al. 2010). Leroy et al. (2010) described a modified "Post-digest ICPL" protocol to label samples after tryptic digestion, which allowed tagging of the N-terminal primary amine of all peptides in the sample, therefore, making it possible to quantify all identified proteins. To our knowledge, a postdigest ICPL approach has not been used to investigate protein expression in *E. faecalis*.

Only peptides that had either a heavy or light label attached were included and only proteins with a heavy label (pH 11) that were also matched to proteins with a light label (pH 8) were investigated further. The ratio of the ion intensities represents the relative abundance of the protein in the original samples. The predicted localization of the 136 proteins identified were categorized using UniProtKB (www.Uniprot.org; Table 1).

Down-regulated PTS system

Relative abundance of these proteins were calculated to compare expression between growth at pH 11 and pH 8. Comparing the abundance ratios between the two growth conditions, six proteins had a $\log_2$ H/L ratio (pH11/pH8) greater than 1 SD of the mean: phage tail protein EF2096 (3 SD); membrane protein EF1541 (1 SD); polysaccharide biosynthesis family protein EF0669 (2 SD); glycosyl hydrolase, family 20 EF0114 (4 SD); uncharacterized protein EF2938 (1 SD); and glycerol uptake facilitator protein EF1927 (1 SD), whilst five proteins had a $\log_2$ ratio 1 SD less of the mean: PTS system IIC component EF1838 (1D); PTS system IID component EF0456 (2 SD); predicted nucleoside ABC transporter EF0177 (1 SD); C4-dicarboxylate transporter EF0108 (1 SD); and PTS system mannose-specific IID component EF0022 (1 SD; Table 2). The predicted nucleoside ABC transporter EF0177 contained a lipoprotein signal peptide (probability: 0.9984) with a cleavage site between positions 22 and 23 (LAA-CG).

The most striking observations in this study are that: (1) two proteins associated with nutrient acquisition, glycosyl hydrolase and glycerol uptake facilitator were up-regulated at pH 11, whilst (2) the proteins associated with the PTS system and associated glucose metabolism were down-regulated.

Dale et al. (2015) found that glycosyltranferase genes play a role in *E. faecalis* maintenance of cell envelope integrity, and polysaccharide composition and is consistent with our first observation. However, it seems to be in contrast to the findings of Ran et al. (2015) that in response to alkaline stress, the *GlpF* operon encoding glycerol uptake facilitator protein, *glxK* encoding glycerate kinase and *dhaKL* encoding dihydroxyacetone kinase were significantly down-regulated, suggesting that glycerol metabolism was reduced. This difference maybe explained by the time alkaline stress was imposed on the bacteria. Ran et al. (2015) grew *E. faecalis* in alkaline stress (pH 10) for 24 h, whereas our study in grew bacteria over a 4-week period and samples were pooled after 10 generations. This difference may also reflect the different phenotypic changes observed, Ran et al. (2015) reported poor biofilms on glass surfaces composed of isolated microcolonies with sparse cells. This is in contrast to the evidence of an extracellular matrix and the formation of organized communities in our study which is consistent with the formation of a mature biofilm

Our second observation of a down-regulated PTS system was initially unexpected. The carbohydrate specificity of the PTS system resides in enzyme IIs (EIIs), which consist of an integral membrane domain (Deutscher et al. 2006). In this study, the PTS mannose-specific IID protein (EF0022), PTS system IIC component (EF1838), and PTS system IID component (EF0456) were all down-regulated at pH 11 by more than 1 SD of the mean, clearly indicating a shift away from PTS uptake. The imposed growth rate was equally set for both pH 8 and 11 conditions, and therefore, nutrient availability would be similar. A similar finding of the EII enzyme family in PTS being inhibited or down-regulated in *E. facealis* has been reported by Ran et al. (2015) in response to an alkaline challenge and by Li et al. (2019) to a fluoride challenge.

Glycerol forms one of the repeating units of the extracellular polysaccharide of *E. faecalis* (Hancock and Gilmore 2002) with additional potential sources of glycerol coming from the recycling of lysed cells within the biofilm matrix (Flemming and Wingender 2010). Glycerol can be an important energy source for pathogenic bacteria and enters cells by energy-independent facilitated diffusion. It has recently been reported that compared to glucose, glycerol did not increase either the planktonic growth rate of biofilm development, but significantly increased the metatobolic activity of biofilms, especially at a pH of 11 compared with a pH of 8 (Salem et al. 2022). With the up-regulation of the glycosyl hydrolase protein (EF0114) at pH 11, it is likely that sugar residues would be released from the extracellular polysaccharide and made available for cellular metabolism.

Whilst the down regulation in PTS could be a result of a general stress response similar to that seen for bacteriocin resistance (Opsata et al. 2010), it may reflect an adaption to a specific ecological niche resulting in the choice of an alternative carbohydrate (Bruckner and Titgemeyer 2002).

Conclusions

Comparisons of the membrane protein profiles between *E. faecalis* grown at a host/clinically relevant growth rate at pH 11 compared to pH 8 resulted in a limited number of regulated proteins. Collectively, the membrane proteins seem to be involved in the formation of a protective capsule/EPS that protects the cell from the destructive OH^- ions. The production of the EPS is facilitated by: an increase in polysaccharide biosynthesis; a shift to glycerol metabolism as the favored carbohydrate source with an associated down-regulation of the PTS system: nutrient acquisition via the actions of glycosyl hydrolase and glycerol uptake facilitator.

The roles of membrane proteins in this coordinated response to increased pH may help explain the adaptation of cells to an extreme alkaline pH and therefore survival of the medicaments used in endodontic therapy.

Supplementary data

Supplementary data is available at *FEMSLE* online.

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Conflict of interest. None declared.

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