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Comparative analysis of virulence determinants and mass spectral profiles of Finnish and Lithuanian endodontic *Enterococcus faecalis* isolates

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Introduction: Putative virulence factors of *Enterococcus faecalis* have been proposed by several workers and, by analogy, these have been linked to strains of endodontic origin. However, their distribution within the cell population is unknown. In the present study, isolates were taken from the dental root canals of two defined human populations, Lithuanian and Finnish, and examined for a range of virulence properties. In addition, surface-associated molecules and intracellular proteins were compared using matrix-assisted laser desorption-ionization/mass spectrometry (MALDI-TOF-MS) and ProteinChip™ capture/MS (SELDI-TOF-MS), respectively.
Methods: Twenty-three Lithuanian and 35 Finnish dental root canal isolates were included. The *esp*, *gelE*, *ace* and *efaA* genes were detected by polymerase chain reaction, and cytolysin and gelatinase phenotypes were determined by hydrolysis of horse blood agar and gelatine agar, respectively. Protein extracts and surface-associated molecules of whole cells were analysed by SELDI-TOF-MS and MALDI-TOF-MS, respectively.
Results: Presence of *esp* ($n = 15$), cytolysin ($n = 9$), *ace* ($n = 55$) and *efaA* ($n = 58$) was not statistically different in the two samples, whereas *gelE* and gelatinase production was detected more frequently in the Finnish material (chi-squared, $P < 0.01$). Analysis of protein profiles by SELDI-TOF-MS showed clustering of cytolysin-producing strains, whereas MALDI-TOF-MS generated profiles that clustered according to the samples' origin and, furthermore, to atypical quinupristin–dalfopristin susceptibility.
Conclusion: A high prevalence of virulence factors was demonstrated in both population types. SELDI-TOF-MS and MALDI-TOF-MS proved useful in distinguishing between different *E. faecalis* phenotypes and they may be useful technologies for elucidating the eco-distribution of *E. faecalis* in humans.

Key words: endodontic infections; *Enterococcus faecalis*; virulence determinants; MALDI-TOF; SELDI-TOF

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Enterococci are well recognized opportunistic pathogens in human and animal infections (15). The severity of these infections depends on the host immune response and on the presence of bacterial virulence factors (27). Several studies have reported a high prevalence of enterococcal virulence factors related to specific types of infections such as endocarditis, urinary tract infection and septicaemia (8, 43). A large number of virulence factors have been recognized among enterococci, including gelatinase (45), cytolysin (3), enterococcal surface protein (Esp) (42), *Enterococcus faecalis* collagen adhesin

(Ace) (38), and *E. faecalis* endocarditis-specific antigen (EfaA) (29).

Cytolysin confers cytotoxic and haemolytic activity (18), and the associated genes were first discovered on the plasmid pAD1 (25). It is a two-subunit toxin encoded by the $CylL_S$ and $CylL_L$ genes, the expression of which is regulated through a quorum-sensing mechanism (7). Animal models have suggested that haemolytic *E. faecalis* strains are more virulent than non-haemolytic strains (26, 45), and an association between a haemolytic phenotype and an adverse outcome for patients with bacteraemia caused by *E. faecalis* has been reported (24). A close relationship between the cytolysin and the *esp* genes was reported by Shankar *et al.* (41), who located them to a pathogenicity island on chromosomal DNA. The presence of repetitive integration genes derived from the pheromone-responsive plasmids pAM373 and pAD1 suggested that at least part of this pathogenicity island originates from the insertion of these plasmids into the chromosomal DNA (41). Gelatinase has been associated with an increased mortality in murine peritonitis models (45). However, no association could be demonstrated between the presence of gelatinase and the outcome of bacteraemia in humans (53).

The surface-bound protein Esp was first described in *E. faecalis* by Shankar *et al.* (42), and an Esp variant in *Enterococcus faecium* has been associated with a genetically distinct infective subpopulation of vancomycin-resistant *E. faecium* (VREM) from hospital infections in the USA (54). Esp has been reported more frequently in clinical isolates than in food strains and starter strains (14), and the presence of Esp contributed to colonization and persistence in ascending urinary tract infections (43) in a murine model.

The surface protein Ace mediates adherence to collagen I and IV (33), and the presence of the *ace* gene has been associated with an increased binding to dentine, suggesting that this protein may participate in retention of *E. faecalis* in the root canal of infected teeth (22). It has been reported that serodiagnosis of *E. faecalis* in endocarditis can be based on the presence of a 40-kDa antigen in serum, later identified as EfaA, which is similar to the streptococcal adhesins involved in adhesion to hydroxyapatite coated with saliva (1, 17, 29). The expression of *efaA* seems to confer increased adhesion to heart cells, and the gene encoding EfaA has been suggested for use in identification of *E. faecalis* (44).

The presence and high prevalence of *E. faecalis* in treatment-resistant root canal infections is widely recognized (21, 30, 32, 35, 39). Several features of enterococci, such as their tolerance to high levels of alkalinity (9, 36), low nutritional requirement and adherence to dentine (22), favour their survival in the root canal.

Treatment procedures in endodontic infections show considerable variation from one country to another; however, the high prevalence of *E. faecalis* has been recognized in studies from different regions irrespective of treatment protocol (16, 21, 30, 32, 34, 47). It is therefore likely that the environment provided by the root canal system favours the growth and retention of this organism because of its inherent or acquired virulence factors.

The aim of this study was to assess the prevalence of the above-mentioned virulence determinants in root canal isolates of *E. faecalis* from Finland and Lithuania, to investigate the relative importance of surface-associated molecules using matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS), and to obtain a cross-section of the proteome using surface-enhanced laser desorption/ionization (SELDI)-TOF-MS in providing an overview of the phenotypic traits of isolates in these populations.

Material and methods

Materials

Fifty-eight *E. faecalis* isolates from dental root canal infections were screened. The Lithuanian samples consisted of 23 isolates from 21 individuals and originated from root canals in previously root-filled teeth with asymptomatic apical periodontitis, undergoing endodontic retreatment. In two individuals, two different strains of *E. faecalis* were isolated from the same tooth, these strains were previously typed using pulsed-field gel electrophoresis (37). Both pairs of isolates originating from the same individuals were included in the study because they represented different strains. These patients had been referred for dental treatment to the Institute of Odontology in Vilnius, Lithuania (35). The Finnish material comprised 35 isolates of *E. faecalis* taken from root canals undergoing endodontic therapy. These samples had been sent for microbiological identification to the Oral Microbiological Service Laboratory at the Institute of Dentistry in Helsinki by private practitioners in Finland, for diagnostic purposes. Sampling instructions and transport media were the same for both sets of isolates. Transport and identification were performed as described earlier (34, 48). All clinical isolates were extensively characterized using phenotypic, chemotaxonomic (long-chain cellular fatty acids) and genotypic (16S rRNA) methods (manuscript submitted). Therefore reference strains were excluded from the study.

Detection of virulence genes by polymerase chain reaction

The virulence genes *efaA*, *gelE*, *esp* and *ace* were detected by polymerase chain reaction (PCR) using the primers and cycling conditions listed in Table 1. Sequence analysis (Lark Technologies Inc., Cambridge, UK) was performed on the PCR products detecting *ace* (isolate F17), *efaA* (isolate F25), *gelE* (isolate VP2-70) and *esp* (isolate VP2-77), and the results were compared to GenBank sequences (accession numbers: AF260889, U03756, D85393 and AF034779, respectively); this confirmed the presence of these genes in the respective isolates. These isolates were then used as positive controls for the corresponding PCR.

Table 1. Primer sequences and cycling conditions

Primer	Sequence	Product length (bp)	Cycling conditions	Reference
esp-1	GGTCACAAAGCCCAACTTGT	407	5 min 94°C + 30 (25 s 94°C, 40 s 52°C, 50 s 72°C) + 6 min 72°C	(55)
esp-2	ACGTCGAAAGTTCGATTTCC			
efa A-1	CGTGAGAAAGAAATGGAGGA	499	2 min 94°C + 30 (60 s 94°C, 60 s 56°C, 60 s 72°C) + 10 min 72°C	(13)
efa A-2	CTACTAACACGTCACGAATG			
ace-1	AAAGTAGAATTAGATCACAC	319	2 min 94°C + 30 (60 s 94°C, 60 s 56°C, 60 s 72°C) + 10 min 72°C	(13)
ace-2	TCTATCACATTCGGTTGCG			
gelE-1	AGTTCATGTCTATTTTCTTCAC	402	30 (60 s 94°C, 60 s 56°C, 60 s 72°C)	(13)
gelE-2	CTTCATTATTTACACGTTTG			

Cytolysin and gelatinase activity

Cytolysin confers cytotoxic and haemolytic activity on *E. faecalis*. The presence of cytolysin activity was examined phenotypically on *Brucella* blood agar plates [4.3% weight/volume (w/v) Brucella-agar (BBL Microbiology Systems, Cockeysville, MD), 0.5% (w/v) haemin (Fluka Chemicals, Gillingham, UK), 0.1% (v/v) of a 1% (w/v) ethanol solution of Menadione (Sigma, St Louis, MO), 5% (v/v) defibrinated fresh horse blood]. The cells were inoculated onto the agar plates and incubated at 37°C overnight. Complete haemolysis (beta-haemolysis: translucency around the bacterial colonies) was interpreted as positive for cytolysin production.

Gelatine agar plates (3% w/v gelatine, 1% w/v yeast extract, 1% w/v peptone, 1% w/v agar) were used to determine gelatinase activity. When a translucent halo appeared around the colonies after 24–48 h incubation at 37°C in air, the isolate was considered positive for gelatinase production.

Analysis of proteins by SELDI-TOF-MS

For SELDI-TOF analysis, cell extracts need to be prepared and then adjusted to the equivalent protein concentrations for comparative analysis. For cost- and time-saving reasons, a random selection of just <50% of the isolates (26 strains) was analysed (namely, six Lithuanian and 20 Finnish isolates were analysed by SELDI-TOF-MS (Fig. 1).

Protein extraction

Five plates of each analysed isolate were grown aerobically overnight on Columbia blood agar (Oxoid, Basingstoke, UK), supplemented with 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks, UK) at 37°C. The bacteria were collected and suspended in 2 ml standard lysis solution [48% w/v urea (Sigma, Poole, UK), 2% w/v 3-(3-cholamidopropyl-dimethylammonio)-1-propanesulphonate (CHAPS, Melford Laboratories, Ipswich, UK) and 0.485% w/v Trizma base (Sigma, Poole, UK), in double-distilled water] and 80 µl 25 mM phenylmethylsulphonyl fluoride (Sigma, Poole, UK) and 0.3 g glass beads, grade 13 (< 105 µm) added to the cell suspension. The bacterial cells were broken mechanically using the Mickle Cell Desintegrator (Mickle Laboratory Engineering Co. Ltd, UK). The cell debris and the beads were separated from the resulting crude cell extract by centrifugation for 30 min at 21,000 ***g*** at 4°C. The resulting supernatant was adjusted to 0.4 mg/ml in binding buffer (10 mM ammonium acetate with 25% acetonitrile) and used for the SELDI-TOF-MS analysis on the hydrophobic ProteinChip™ array (Ciphergen Biosystems Ltd, Palo Alto, CA).

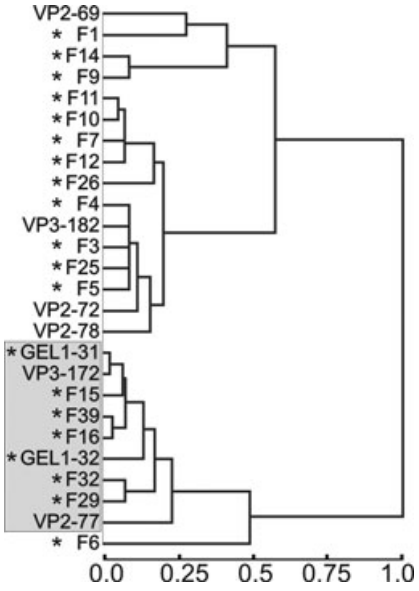

Fig. 1. Cluster analysis of the hydrophobic proteins of 26 strains of *Enterococcus faecalis* using SELDI-TOF-MS. The dendrogram showed two broad phena, one containing all nine cytolysin-positive isolates and a second phenon containing the cytolysin-negative strains. The grey box demarcates the former while the asterisks indicate Finnish isolates.

Hydrophobic ProteinChip™ array (H50)

The active surface of the array contains 16 methylene groups that bind proteins through reverse-phase chemistry. The binding involves proteins that are rich in alanine, valine, leucine, isoleucine, phenylalanine, tryptophan and tyrosine.

The surface of the hydrophobic ProteinChip™ array was bulk-washed twice with 50% acetonitrile for 5 min and air-dried for 1 h to minimize spot contamination. Five microlitres 0.1% trifluoroacetic acid (Sigma, Poole, UK) was added to the active surface of the array and removed after 5 min.

Then, 2–3 µl of each sample was added to each active spot on the protein array. After a 15-min incubation in the humidity chamber with constant shaking the sample was removed and the array was washed twice with 50% methanol. The spots were left to air dry for 15 min.

Matrix solution preparation

The matrix solution was prepared by dissolving 14 mg sinapinic acid (Fluka Chemicals) in 400 µl of 50% acetonitrile (BDH, Poole, UK) and 0.5% trifluoroacetic acid. The solution was vortexed for 10 min, and sonicated in a water bath for 10 min to ensure the solubilization of sinapinic acid. Then, 0.5 µl of the solution was added twice to each active spot of the ProteinChip™ array and left to air dry for 30 min.

Data acquisition

The captured proteins on the surface of the arrays were analysed using PBSII ProteinChip Array Reader (Ciphergen Biosystems), a laser desorption/ionization time of flight mass spectrometer. The instrument was equipped with a nitrogen laser (wavelength 337 nm) and was operated in positive ion mode; spectral profiles were collected in the mass range of 3000–70,000 Da. The laser energy was set to 190 V, the microchannel plate detector voltage was set at 1900 V, the pulse voltage was set at 3000 V and the source voltage was set at 20,000 V. Five shots per transient (or spot) were acquired and a total of 65 shots were collected from each sample well.

Analysis of whole cell mass spectral profile by MALDI-TOF-MS

Bacterial cultures of all 58 isolates were cultivated as for the SELDI analysis. Micromass target plates [Waters Corporation (Micromass) Ltd, Manchester, UK] were prepared as previously described for gram-positive organisms (28). Twelve replicates were analysed per sample. One microlitre of matrix solution [acetonitrile, water and methanol (1 : 1 : 1), 0.01 M 18-Crown-6 ether and 0.1% formic acid (v/v), saturated with 5-chloro-2-mercaptobenzothiazole at a concentration of 3.0 mg/ml] was then added over the sample wells and allowed to air dry. Lock mass wells were spotted with 1 µl of a 1 : 1 mixture of matrix solution saturated with α-cyano-4-hydroxy-cinnamic acid (14 mg/ml) and peptide mixture [bradykinin, angiotensin I, glu-fibrinopeptide B, rennin, adrenocorticotropic hormone (18–39 clip), all at 1 pmol/µl, bovine insulin 2 pmol/µl and ubiquitin 10 pmol/µl]. All reagents unless otherwise stated were from Sigma (Poole, UK). A MALDI Linear Time of Flight Mass Spectrometer (Micromass) was operated using the MassLynx™ software. Automated calibration of the time of flight tube, followed by automated acquisition of the bacterial spectra, was then performed using the

Real Time Data Selection function in the MassLynx™ software, as previously described (28). Spectral profiles were collected within a mass range of 500–10,000 Da. Ten and 15 spectra were collected from the lock mass wells and the sample wells, respectively. Data were processed using the MicrobeLynx™ software. Individual spectral profiles were lock mass corrected by a factor derived from the alignment of the rennin peak obtained empirically, with the exact mass of 1760 Da. The 15 spectral profiles collected for each sample well were then combined to one average spectrum, which was then background subtracted and smoothed. The average spectra of the 12 replicates per samples were then combined to one average spectrum for that sample. A neighbouring algorithm was used for the cluster analysis (28).

Statistical methods

The prevalence of virulence determinants was compared between the Finnish and the Lithuanian populations by testing the null hypothesis, that the prevalence of virulence factors in both materials was not statistically different, using chi-squared statistics (Fisher's exact test). The minimum level of significance was set to $P \leqslant 0.05$ (Table 2). Associations between the presences of the two virulence determinants were also investigated using the same statistical method.

Distinctive mass ions in each spectrum generated by SELDI-TOF-MS were analysed by CiphergenExpress™ software, which uses UPGMA (unweighted pair group method with arithmetic mean) and the Ward algorithm for comparative analysis.

Results

Prevalence of virulence determinants

Cytolysin was present in nine of the 58 isolates; the genes *esp*, *ace* and *gelE* were found in 15, 55 and 42 isolates, respectively, and *efaA* was found in all isolates (Table 2). Gelatinase production was positive in 27 of the 58 isolates (Table 2). The presence of *esp* was higher in the Finnish samples than in the Lithuanian samples, although this difference was not significant. A strong association was also found between the presence of *esp* and cytolysin (chi-squared, $P < 0.0005$). Further, 15 *gelE*-positive strains did not produce gelatinase. An association between the origin of the isolates, *gelE* and gelatinase was found and was highly significant (chi-squared, $P = 0.001$ and $P = 0.003$ respectively, Table 2).

SELDI-TOF-MS analysis

The data obtained by SELDI-TOF-MS is complex, especially where studies involve a large number of isolates. Here, distinctive mass ions in each spectrum were analysed by CiphergenExpress™ software, which uses UPGMA and the Ward algorithm for comparative analysis. Thus, comparison of the intracellular hydrophobic proteins using SELDI-TOF-MS revealed that all nine isolates that possessed cytolysin activity had similar profiles and therefore clustered into one phenon of the dendrogram (Fig. 1). By representing their mass intensities on a 'gel view' image, the distinctive protein patterns of these strains can be seen against two representative strains from the second phenon (i.e. cytolysin-negative strains) (Figs 1 and 2). As predicted, there were a large number of overlapping mass ions in the spectra of all isolates; however, several biomarker proteins (15,000–16,300 Da) were evident in the spectra of the cytolysin-positive isolates.

MALDI-TOF-MS analysis

The Waters MicrobeLynx™ cluster package enabled direct cluster analysis of the spectral properties of each isolate. Interestingly, this dendrogram, based upon the surface-associated molecules of the cell, showed a tendency to delineate the Finnish and Lithuanian isolates (Fig. 3). Thus, at a similarity level of approximately 65%, the dendrogram could be arbitrarily divided into three phena. Isolates from the Finnish population were recovered in phenon 1 ($n = 17$ isolates) and 3 ($n = 14$ isolates) (Fig. 3). Phenon 2 encompassed all the Lithuanian isolates ($n = 23$) and four Finnish isolates. Nine of the 15 isolates that possessed an atypical susceptibility to quinupristin–dalfopristin, as previously described (37), were found in the same phenon and comprised 12 isolates (Fig. 3).

Discussion

E. faecalis is the most frequently isolated species in treatment-resistant root canal infections. Whether it represents a potential threat to general health is not yet known because endodontic infecting microorganisms are localized in a restricted area within the root canal system and little is known on the extraradicular presence of *E. faecalis* (49).

Expression of cytolysin in the present study (16%) was similar to the prevalence of *cylA* reported in bacteraemia and endocarditis specimens that were not clonally related (15% and 11%, respectively) (23). When clonally related strains were considered, *cylA*, which is necessary for cytolysin activation, was more frequently found in bacteraemia isolates in the abovementioned study. This is in contrast to the incidence of the reported haemolysin activity by Sedgley *et al.* (40) in an endodontic material originating from Sweden, where none of the 31 *E. faecalis* isolates expressed haemolysin, although bacteriocin production was reported in 14 isolates. The authors reported the presence of the *cylA* gene, which is involved in cytolysin expression, in six isolates. The terms bacteriocin and haemolysin refer to the cytotoxic and beta-haemolytic activities of the same toxin, now usually designated cytolysin (18, 25, 27). The discrepancy between the results in the present study and those reported by Sedgley *et al.* (40) may be the result of different

Table 2. Prevalence of virulence determinant among Finnish and Lithuanian isolates

	Origin				All isolates		
Virulence determinants	Lithuania ($n = 23$)	%	Finland ($n = 35$)	%	Total ($n = 58$)	%	*P*-value
esp	3	13	12	33	15	26	0.124
gel E	11	48	31	89	42	72	0.001
Gelatinase	5	22	22	63	27	47	0.003
Cytolysin	2	9	7	20	9	15	0.295
ace	23	100	32	91	55	95	0.270

efaA was present in all 58 strains.
P-value calculated using chi-squared test.

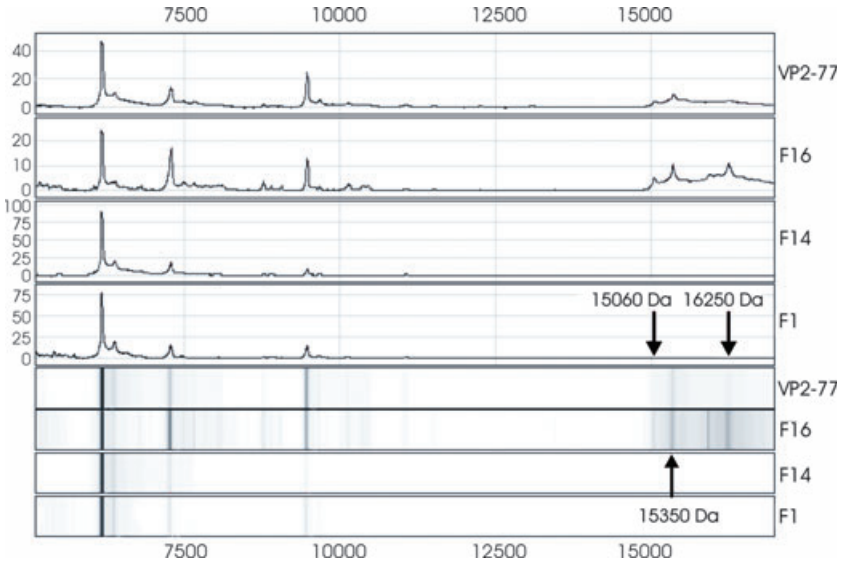

Fig. 2. Representative mass spectral profiles of isolates belonging to each phenon are shown as 'gel view' images. Protein biomarkers in the mass range 15,060, 15,350 and 16,250 Da may be used to identify strains that possess cytolysin activity.

methodological approaches. The haemolytic phenotype has been shown to contribute to *E. faecalis* proliferation in the bloodstream (24), which may increase the risk of endocarditis in predisposed individuals.

In a study by Duprè *et al.* in 2003 (13), *E. faecalis* samples were screened for the presence of *efaA* and two of the 15 isolates were negative, whereas all isolates were found to harbour *efaA*, using the same primer; this is in concordance with Singh *et al.* (44), who suggested that this gene is omnipresent in *E. faecalis*. However, others have reported a lower prevalence of this gene (2, 14).

Ace has been recognized as a collagen-binding MSCRAMM protein (38), and Duh *et al.* (12) found *ace* to be omnipresent in their *E. faecalis* isolates but absent from all other enterococci tested. We used the *ace* primers designed by Duprè *et al.* (13), and found that all Lithuanian isolates were positive, whereas 9% of the Finnish isolates gave a negative PCR result. Duprè *et al.* (13) found only 60% of their *E. faecalis* isolates to be *ace*-positive. This is in contrast with the results reported by Duh *et al.* (12) who suggested *ace* as a possible identification target for *E. faecalis*. The *ace1* primer used in the present study has a GC content of 30%, which is less than ideal and may have caused some false-negative results in both the Duprè *et al.* study (13) and the present study.

Both *esp* and cytolysin are enriched in medical isolates, according to several reports (14, 24, 42). The frequency of *esp* in the present study (26%) was similar to the isolation rate in bloodstream samples (29 of 100) reported by Shankar *et al.* (42) but was higher than that reported in stool isolates (one of 34) and lower than the prevalence in endocarditis isolates (14 of 33) (42), which assigns these isolates to the same category as virulent strains. It was, however, found to be less frequent than in the endodontic material studied by Sedgley *et al.* (40), where 20 of 33 isolates were *esp*-positive. This is noteworthy because no apparent factors other than geographic origin and possibly treatment procedures differed between their sample and those used in the present study.

Both *esp* and the cytolysin genes may be located on the same pathogenicity island (41), although the cytolysin genes can also be encoded within a pheromone-responsive plasmid (25), and it has been hypothesized that these factors may act synergistically (19). Consequently, the strong association found between *esp* and cytolysin in the present study may represent the presence of particularly virulent strains.

The analysis of the proteins generated by SELDI-TOF-MS using the hydrophobic surface chip, HP50 (Ciphergen Biosystems) showed that isolates producing cytolysin possessed similar mass spectral profiles (see Fig. 2). According to a model published by Booth *et al.* (4), the expression of cytolysin relies on the presence of an operon with five open reading frames, three of which are involved in the activation of the final cytolysin protein which is a lantibiotic formed by a combination of two peptides $CylL_L$ and $CylL_S$, (4, 6). The same authors analysed the amino acid composition of the intracellular forms of the $CylL_L$ and $CylL_S$ peptides and found alanine, valine, phenylalanine and leucine in large amounts (55% in both), which should confer strong binding to the hydrophobic ProteinChip™ (HP50). This may partly explain the clustering of the protein profiles of the cytolytic strains. A quorum-sensing mechanism regulates the expression of $CylL_L$ and $CylL_S$ (7). According to this model, the presence of target cells, such as horse erythrocytes in the Columbia blood agar plates, promotes binding of $CylL_L$, leaving the $CylL_S$ unit for uptake, which in turn will up-regulate $CylL_L$ expression. Accordingly, the cell lysate of cytolysin-positive strains analysed by SELDI-TOF-MS should be enriched in intracellular $CylL_S$, which would differentiate them from the non-cytolytic strains in the cluster analysis of the protein profiles (see Fig. 2). Mass ions within the cluster of peaks 15,060, 15,350 and 16,250 Da in the spectrum of strains may serve as useful biomarkers for future studies.

The prevalence of cytolysin in this study was comparable to that in endocarditis isolates (23) and the presence cytolysin has been shown to increase the probability of a mortal outcome in animal models of experimental endocarditis and in patients with bacteraemia (5, 24). This implies the necessity for further investigations assessing the potential risk that this may represent in patients prone to endocarditis, in particular in view of prophylactic guidelines during endodontic retreatment cases.

A higher prevalence of both the *gelE* gene and the gelatinase phenotype was found in the Finnish than in the Lithuanian material. The higher incidence of *gelE* and gelatinase, as well as the higher prevalence of *esp* and cytolysin (although the latter was not significant) may be caused by a bias towards more virulent isolates in the Finnish material: the Lithuanian isolates originate from root canal samples taken from patients with asymptomatic apical periodontitis, whereas the Finnish strains come from samples taken from root canals by private practitioners and sent in for identification at the Finnish Oral Microbiology Service Unit. It is likely that the Finnish samples were collected from patients presenting subjective or clinical symptoms, which would have motivated the extra cost and time for this procedure, although no data are available to support this. Both *esp* and gelatinase have been

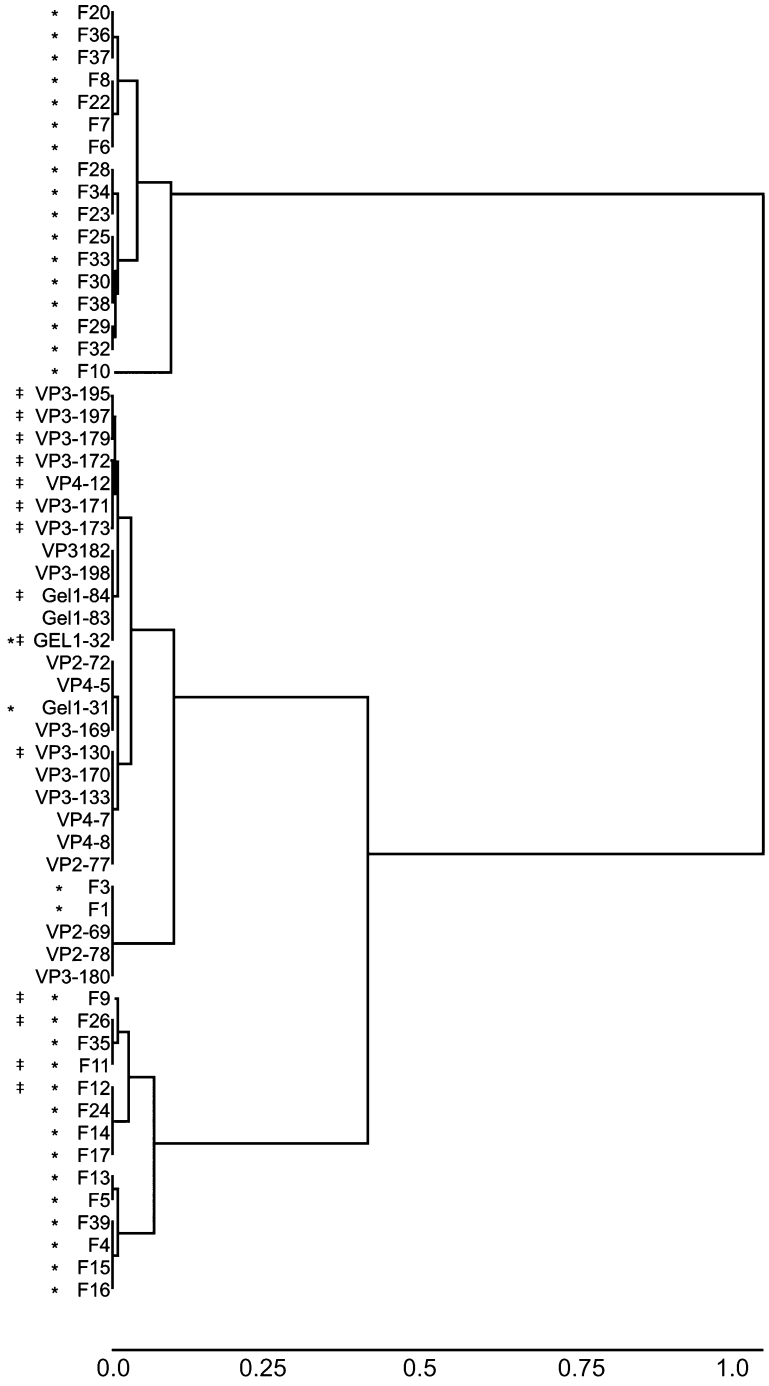

Fig. 3. Cluster analysis of the surface-associated molecules of 58 isolates of *Enterococcus faecalis* using MALDI-TOF-MS. At an arbitrarily set level of approximately 65% the dendrogram comprised three phena. Isolates from the Finnish population were recovered in phenon 1 ($n = 17$ strains) and 3 ($n = 14$ strains) while phenon 2 encompassed all the Lithuanian isolates ($n = 23$) and four Finnish isolates. Nine of the 15 strains that possessed an atypical susceptibility to quinupristin–dalfopristin were found in the same cluster, comprising 12 isolates. The asterisks denote the Finnish origin of the strains while quinupristin–dalfopristin-susceptible strains are marked by a ‡.

associated with biofilm formation (20, 31, 52), and *E. faecalis* has been shown to be capable of forming biofilm on gutta-percha points and on the root canal surface of medicated teeth (11, 50). Further research is required to investigate a possible role for these factors in the colonization and virulence of endodontic infections.

Analysis of surface protein profiles using MALDI-TOF-MS revealed clustering of strains of different geographic origin. A previous study on a smaller selection of the present material using fluorescence-amplified fragment length polymorphism techniques (manuscript submitted) revealed genetic differences between endodontic and non-endodontic isolates, whereas the origin of the isolate was not associated with a particular genotype. One factor common to the present and previously mentioned studies was that the technologies used identified proteomic and genetic differences respectively between quinupristin–dalfopristin-susceptible and non-susceptible strains. A possible explanation for this clustering is that quinupristin–dalfopristin resistance, normally mediated by a membrane-bound protein (Lsa) (46), is probably absent in the susceptible strains because of mutations in the encoding *lsa* gene as previously described (10, 37). The similarities detected by MALDI-TOF-MS among the isolates of similar geographic origin are likely to be related to post-transcriptional differences rather than genetic ones because these were not revealed by fluorescence-amplified fragment length polymorphism analysis (manuscript submitted). Studies on full genome analysis of microorganisms are today showing beyond doubt that the major virulence factors of bacterial pathogens are surface-associated (51). To date there is no high throughput method to screen for such properties. Results demonstrate unequivocally that MALDI-TOF-MS of intact cells is a useful biological tool for screening such factors, particularly in discerning subtle differences in surface-associated post-translational modifications that are not apparent from genome analysis.

Use of prophylactic antibiotics in patients at risk for endocarditis should be considered in root canal revision treatment because *E. faecalis* is highly associated with these infections (39). Considering the high prevalence of the virulence determinants *esp*, *GelE*, gelatinase and cytolysin, in particular in the Finnish material, we hypothesize that these factors all play a role in the positive selection of virulent *E. faecalis* strains in the root canal and may represent a risk in the event of an enterococcal bacteraemia of endodontic origin.

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