*Oral Microbiology Immunology 2006: 21: 283–288*
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

# Resistance to acidic and alkaline environments in the endodontic pathogen *Enterococcus faecalis*

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*Nakajo K, Komori R, Ishikawa S, Ueno T, Suzuki Y, Iwami Y, Takahashi N. Resistance to acidic and alkaline environments in the endodontic pathogen* Enterococcus faecalis. *Oral Microbiol Immunol 2006: 21: 283–288.* © Blackwell Munksgaard, 2006.

**Background/aims:** This study aimed to investigate the biochemical mechanisms employed by the endodontic pathogen *Enterococcus faecalis* to confer acid- and alkali-resistance and to compare these with the mechanisms of representative oral streptococci.
**Methods:** *E. faecalis* JCM8728, *Streptococcus mutans* NCTC10449 and *Streptococcus sanguinis* ATCC10556 were used to assess both acid- and alkali-resistance by examining: (i) growth in complex media; (ii) stability of intracellular pH ($pH_{in}$); (iii) cell durability to leakage of preloaded BCECF (2′,7′-bis-(2-carboxyethyl)-5,6-carboxy-fluorescein); and (iv) cell permeability to SYTOX-Green.
**Results:** Growth was initiated by *E. faecalis* at pH 4.0–11.0, by *S. mutans* at pH 4.0–9.0 and by *S. sanguinis* at pH 5.0–9.0. The $pH_{in}$ was similar to the extracellular pH in *S. mutans* and *S. sanguinis* at pH 5–10, while the $pH_{in}$ of *E. faecalis* was maintained at approximately 7.5–8.5 when extracellular pH was 7.5–10 and was maintained at levels equivalent to the extracellular pH when pH < 7.5. Cell membranes of *E. faecalis* were resistant to BCECF leakage when extracellular pH was 2.5–12 and to SYTOX-Green permeability at pH 4–10. The cell membrane durability to extracellular pH in *E. faecalis* was higher than that observed in the *Streptococcus* strains.
**Conclusion:** Compared to *S. mutans*, *E. faecalis* was found to be equally resistant to acid and more resistant to alkalis. The results suggest that pH-resistance in *E. faecalis* is attributed to membrane durability against acid and alkali, in addition to cell membrane-bound proton-transport systems. These characteristics may account for why *E. faecalis* is frequently isolated from acidic caries lesions and from persistently infected root canals where calcium hydroxide medication is ineffective.

Key words: acid-resistance; alkali-resistance; endodontic pathogen; *Enterococcus faecalis*

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Accepted for publication February 23, 2006

*Enterococcus faecalis* is a gram-positive facultative anaerobe found among the commensal microflora of the human intestinal tract. Although the prevalence of *E. faecalis* in root canals was already reported in 1959 (47), this bacterium has recently attracted the attention of dental researchers because it has been isolated frequently from dentin caries and from infected root canals (12, 29, 40, 41). Furthermore, several studies have demonstrated that *E. faecalis* is one of the most commonly isolated bacteria in failed endodontic cases treated with calcium hydroxide [$Ca(OH)_2$] (12, 43). Indeed, this bacterium appears to be highly resistant to $Ca(OH)_2$ (5, 6), which is known as one of the most effective endodontic medicaments because of the bactericidal effect derived from its strong alkaline properties (42). These findings suggest that *E. faecalis* is resistant to both acid environments, such as those of carious dentin including root canals, and alkaline environments such as those of root canals treated with $Ca(OH)_2$.

Several mechanisms have been proposed regarding the methods employed by *E. faecalis* to survive extremes in the pH environment. It has been known that *E. faecalis* can grow at pH 9.6 since the 1930s (39). In recent years, *E. faecalis* has been demonstrated to synthesize a variety of stress proteins when exposed to acids (8) and alkalis (7). However, stress protein synthesis by *E. faecalis* appears to be unrelated to survival at extreme pHs, such as the alkali pH induced by $Ca(OH)_2$ treatment (6, 7). Instead, it is suggested

that a cell membrane-bound proton-transport system is critical to survival under these conditions (6).

Kakinuma and Igarashi (17–20) proposed that an ATP-linked potassium/proton antiport system incorporates protons into the cells to maintain the intracellular pH ($pH_{in}$) in alkaline environments, as has been observed in *Enterococcus hirae*, an enterococcal species similar to *E. faecalis*. On the other hand, acid-resistance in *E. faecalis* is the result of the activity of the cell membrane-bound proton-translocating ATPase ($H^+$-ATPase) which maintains $pH_{in}$ by excreting protons from the cells (22). These findings show that cell membrane-bound proton-transport systems are responsible for acid- and alkali-resistance, but for them to function, the bacterium has to produce energy, such as ATP, continuously. However, in treated root canal environments where the pH is maintained at approximately 9.5–11.0 with $Ca(OH)_2$ (2, 33) and nutrients appear to be limited, it should be difficult for the bacterium to obtain sufficient energy through metabolism. To overcome this situation, the bacterium may have additional mechanisms to protect itself from pH impairment.

This study was undertaken to elucidate the biochemical mechanisms associated with acid- and alkali-resistance, excluding those that use ATP-linked proton-transport systems, in non-growing and non-metabolizing *E. faecalis* cells by comparing them with those of representative oral streptococci, *Streptococcus mutans* and *Streptococcus sanguinis*.

## Material and methods

### Bacterial strains and culture media

*E. faecalis* JCM8728, *S. mutans* NCTC10449 and *S. sanguinis* ATCC10556 were used in this study. These bacteria were cultured on a complex medium (10) containing 1.7% tryptone (Difco Laboratories, Detroit, MI), 0.3% yeast extract (Difco Laboratories) and 0.5% NaCl, which was autoclaved before 0.5% glucose and 50 mM potassium phosphate buffer were added using a sterile membrane filter (pore size 0.22 μm; Pall Corporation, East Hills, NY) (TYG culture medium). This medium was also used as a pre-culture medium.

### Growth ability at acidic and alkali pH

Each strain was inoculated in TYG culture medium and pre-cultured at 37°C overnight. The cell culture was transferred (5% inoculum size) to TYG culture media adjusted to pH 3.0–11.0 with HCl or KOH and incubated at 37°C for 48 h. Bacterial growth was conducted in an anaerobic glove box (90% $N_2$ and 10% $H_2$, NH-type; Hirasawa Works, Tokyo, Japan) and was estimated at 48 h after inoculation by measuring optical density at 660 nm ($OD_{660}$) with a spectrophotometer (UV-160, Shimadzu Corporation, Kyoto, Japan). Initial and final pH values of the cell cultures were determined using a pH meter (Model HM-30G, DKK-TOA Corporation, Tokyo, Japan). Bacterial purity was confirmed after each experiment by culturing on blood agar plates.

### The $pH_{in}$ at acidic and alkali pH

The $pH_{in}$ at acidic and alkali pH was estimated using the methods of Futsaether et al. (9) and Iwami et al. (16). Each strain was grown in TYG culture medium at pH 7.0 until the late-log growth phase ($OD_{660}$ 0.9–1.0) in another anaerobic glove box (80% $N_2$, 10% $H_2$ and 10% $CO_2$, NHC-type; Hirasawa Works). The culture was then taken from the glove box, mixed with 2′,7′-bis-(2-carboxyethyl)-5,6-carboxy-fluorescein acetoxymethyl ester (BCECF-AM, Dojindo Laboratories, Kumamoto, Japan) at a final concentration of 0.5 μM and incubated for 15 min at 35°C (16). The BCECF-loaded cells were harvested and washed three times by centrifugation (21,000 ***g*** for 7 min at 4°C) with deionized water. BCECF-AM easily penetrates the cell membrane because of its hydrophobicity, but BCECF, the hydrolyzed product arising from intracellular esterases, is retained within the cells and exhibits fluorescence (9, 32). The cells were suspended in deionized water at an $OD_{660}$ of 5.0 (16).

The BCECF-loaded cell suspensions were diluted in the presence of 150 mM KCl and 0.5 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–KOH buffer (pH 7.0) for experiments at acidic pH, or 0.5 mM *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine)–KOH buffer (pH 7.5) for experiments at alkali pH. The cell suspensions were then incubated for 30 min at 37°C for the depletion of intracellular polysaccharide. The cells were pelleted by centrifugation (21,000 ***g*** for 5 min at room temperature) and stored at 4°C until use.

The reaction mixtures containing BCECF-loaded cells ($OD_{660}$ 1.0), 150 mM KCl and 0.5 mM MOPS–KOH buffer (pH 7.0) or 0.5 mM Bicine–KOH buffer (pH 7.5), were incubated at 37°C with agitation by a magnetic stirrer. Small aliquots of 0.15 M HCl or 0.15–0.8 M KOH were added to the reaction mixture to decrease or increase extracellular pH between 4.0 and 10.0 at intervals of 4 min. The pH of the reaction mixture, and the fluorescence intensity derived from intracellular BCECF were monitored simultaneously using a pH meter and a fluorescence spectrophotometer (Model CAF-110, JASCO Corporation, Tokyo, Japan) at excitation and emission wavelengths of 500 and 540 nm, respectively.

The values of $pH_{in}$ were calculated using the calibration curve for fluorescence intensity. To obtain fluorescence intensities at various $pH_{in}$, HCl or KOH were added to the reaction mixture containing the cells ($OD_{660}$ 1.0), 150 mM KCl, 0.5 mM MOPS–KOH buffer (pH 7.0) or 0.5 mM Bicine–KOH buffer (pH 7.5) and 12 μM nigericin which eliminates the pH gradient across the bacterial cell membrane (4, 9, 13, 25, 46). Using this method, $pH_{in}$ became the same as the extracellular pH and could then be measured using a pH meter. Separate calibration curves were prepared for individual experiments (15).

### BCECF leakage at acidic and alkali pH

The BCECF-loaded cells were prepared as described for the investigation of $pH_{in}$ at acidic and alkali pH. BCECF is retained within the cells when their cell membranes remain intact, but the fluorescent dye leaks out easily when the cell membranes are damaged. Consequently, leakage of BCECF from cells could be used as one of the determinants of cell membrane durability.

The cells were resuspended in 150 mM KCl and 0.5 mM MOPS–KOH buffer (pH 7.0) or 0.5 mM Bicine–KOH buffer (pH 7.5) at an $OD_{660}$ of 1.0, and incubated at 35°C with agitation by a magnetic stirrer. Within 2 min of starting incubation, the reaction mixture was adjusted to pH 2.0–12.0 with small aliquots of 0.15 M HCl or 0.15–0.8 M KOH. After an additional incubation of 8 min, the reaction mixture was centrifuged (21,000 ***g*** for 5 min at room temperature). Cell pellets were suspended in 100 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CHES)–KOH buffer (pH 10.0), 1.25 M KCl and 10 μM nigericin and stored at room temperature overnight. The fluorescence intensity of residual BCECF in the cells was determined fluorometrically.

### SYTOX-Green permeability at acidic and alkali pH

Each bacterial strain was grown, harvested, washed and starved as described in the

investigation of $pH_{in}$ at acidic and alkaline pHs, except for the BCECF loading. The cells were resuspended in 150 mM KCl and 0.5 mM MOPS–KOH buffer (pH 7.0) or 0.5 mM Bicine–KOH buffer (pH 7.0) at $OD_{660}$ of 1.0, and incubated at 35°C with agitation by a magnetic stirrer. Within 2 min of starting incubation, the reaction mixture was adjusted to pH 4.0–10.0 with small aliquots of 0.15 M HCl or 0.15–0.8 M KOH. After adding SYTOX-Green (Molecular Probe Inc., Eugene, OR) at a final concentration of 2.5 μM, the reaction mixture was further incubated for 5 min; SYTOX-Green permeates damaged cell membranes and binds to nucleic acids where it fluoresces. Consequently, the penetration of SYTOX-Green can be used to evaluate cell membrane durability. The fluorescence intensity of SYTOX-Green bound to nucleic acids was determined fluorometrically at excitation and emission wavelengths of 504 and 540 nm, respectively.

## Results

### Growth ability at acidic and alkali pH

*E. faecalis*, *S. mutans* and *S. sanguinis* were able to grow at an initial pH of 4.0–11.0 (Fig. 1A-1), pH 4.0–9.0 (Fig. 1B-1) and pH 5.0–9.0 (Fig. 1C-1), respectively. The *E. faecalis* grew well over a wide pH range, whereas *S. mutans* and *S. sanguinis* grew well in the narrow pH range around neutral. The culture pH decreased by 0.0–2.9 pH units during 48 h of growth (Fig. 1A-2, B-2 and C-2). At an initial pH of 11, for example, *E. faecalis* decreased the culture pH to 10.5, while at an initial pH of 9.0, *S. mutans* and *S. sanguinis* decreased the culture pH to 8.1 and 7.8, respectively.

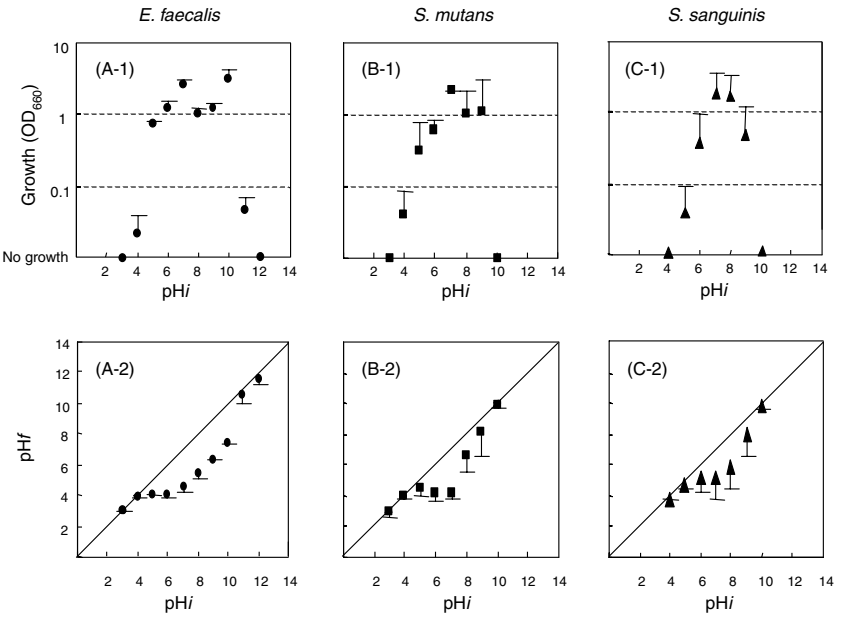


*Fig. 1.* Bacterial growth of *Enterococcus faecalis* JCM 8728 (A-1), *Streptococcus mutans* NCTC 10449 (B-1) and *Streptococcus sanguinis* ATCC 10556 (C-1) at various initial pH levels (pH*i*) in TYG culture media, and final pHs (pH*f*) after 48 h of growth of *E. faecalis* JCM 8728 (A-2), *S. mutans* NCTC 10449 (B-2) and *S. sanguinis* ATCC 10556 (C-2). Data were given in the means with standard deviation obtained from three independent experiments. Bacterial culture with $OD_{660} < 0.03$ was judged as no growth.

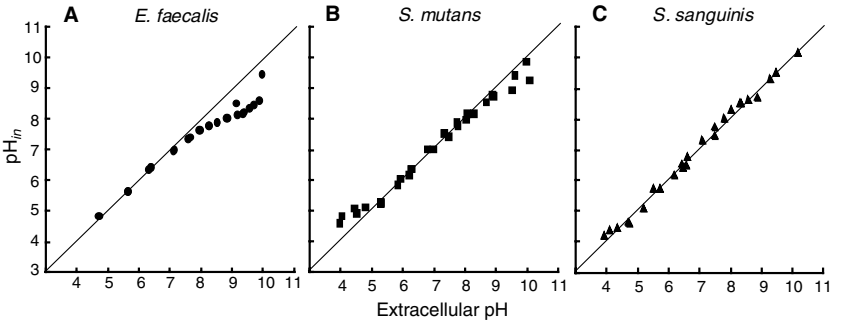


*Fig. 2.* The $pH_{in}$ of *Enterococcus faecalis* JCM 8728 (A), *Streptococcus mutans* NCTC 10449 (B) and *Streptococcus sanguinis* ATCC 10556 (C) at various extracellular pH values. All the data obtained from three independent experiments were plotted.

### The $pH_{in}$ at acidic and alkali pH

When extracellular pH was between 8 and 10, $pH_{in}$ was equal to the extracellular pH in *S. sanguinis*, slightly lower than the extracellular pH in *S. mutans* and stable at approximately 7.5–8.5 in *E. faecalis* (Fig. 2). When extracellular pH was between 5 and 7.5, $pH_{in}$ was similar to the extracellular pH in all the strains. When extracellular pH was between 4 and 5, $pH_{in}$ was maintained at approximately 5 in *S. mutans*, and was equal to the extracellular pH in *S. sanguinis*. However, $pH_{in}$ of *E. faecalis* at an extracellular pH < 5 could not be estimated because the background fluorescence of *E. faecalis* masked the signal fluorescence of $pH_{in}$.

### BCECF leakage at acidic and alkali pHs

*E. faecalis* was resistant to BCECF leakage when extracellular pH was 2.5–12 (Fig. 3A) with residual BCECF > 80%. Both *S. mutans* and *S. sanguinis* were resistant within the pH range of 4–10 (Fig. 3B,C), although *S. mutans* appeared to be more resistant than *S. sanguinis* at alkali pH.

### SYTOX-Green permeability at acidic and alkali pH

The *E. faecalis* was resistant to permeation by SYTOX-Green at an extracellular pH between 4 and 10 and showed low and constant fluorescence intensities (Fig. 4A). Both *S. mutans* and *S. sanguinis* were resistant at pH 5–9 (Fig. 4B,C), although their fluorescence intensities were higher than those of *E. faecalis*. At pH > 9 and <5, both *S. mutans* and *S. sanguinis* showed high permeability to SYTOX-Green (Fig. 4B,C).

## Discussion

In this study, *E. faecalis* was observed to be more alkali-resistant in growth than either *S. mutans* or *S. sanguinis*, and in terms of the acid-resistance of its growth this bacterium showed similar acid-resistance to *S. mutans* and more acid-resist-

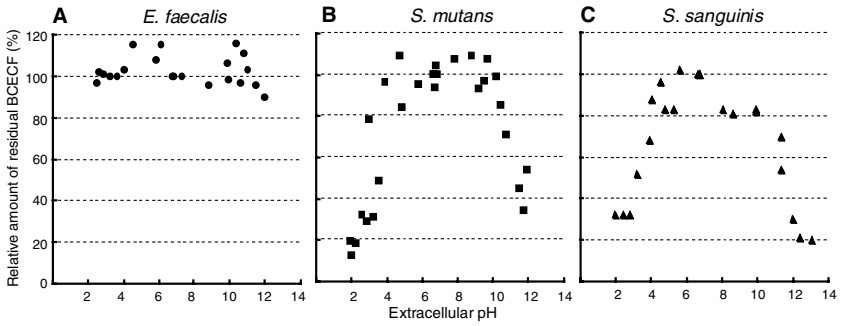


*Fig. 3.* The percentage of residual intracellular BCECF in *Enterococcus faecalis* JCM 8728 (A), *Streptococcus mutans* NCTC 10449 (B) and *Streptococcus sanguinis* ATCC 10556 (C) at various extracellular pH levels. The value at pH 7.0 was regarded as 100. All data obtained from three independent experiments were plotted.

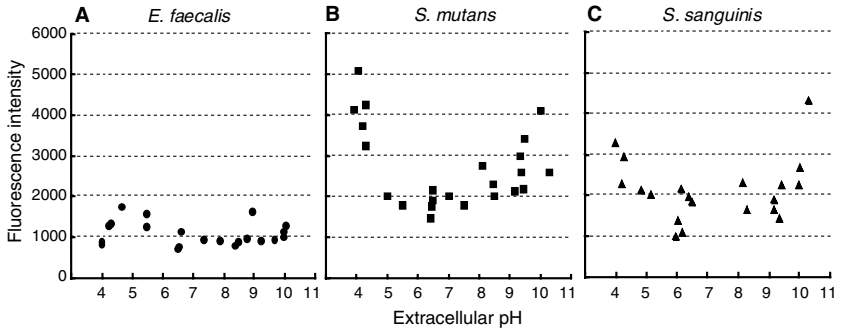


*Fig. 4.* Fluorescence intensity of SYTOX-Green into the cells for 5 min of *Enterococcus faecalis* JCM 8728 (A), *Streptococcus mutans* NCTC 10449 (B) and *Streptococcus sanguinis* ATCC 10556 (C) at various extracellular pH levels. All the data obtained from three independent experiments were plotted.

ance than *S. sanguinis* (Fig. 1). Although the culture pH decreased during growth, initial culture pH is thought to be critical for these bacteria to initiate growth. It was reported that *E. faecalis*, *S. mutans* and *S. sanguinis* initiated growth at pH 5.0, 5.0 and 5.5, respectively (14). The most alkali pH for *E. faecalis* to initiate growth was reported to be approximately 10 (28, 29, 39), but these values for *S. mutans* and *S. sanguinis* were determined for the first time in the present study. In general, the results of the present study were consistent with those previous reports, but the bacterial species in the present study appeared to be more pH-resistant than those in the previous studies. This discrepancy could be the result of differences among bacterial strains and among growth conditions, including the culture media.

The bacterial growth yields were different among the initial pH values (Fig. 1). The growth yield may be influenced by a change in metabolic pathway, an accumulation of end products and a limitation of energy source although these possibilities need to be elucidated. Growth medium composition such as the concentration and type of buffer may also influence bacterial growth and change in culture pH during growth.

Bacteria need to maintain their $pH_{in}$ against extracellular pH extremes for survival. Many mechanisms by which $pH_{in}$ is maintained relative to extracellular alkaline pH values have been reported. Kakinuma and Igarashi (17–20) proposed that in *E. faecalis* an ATP-linked potassium/proton antiport system functions to incorporate protons into cells against intracellular alkalization. In addition, Evans et al. (6) have demonstrated that a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), diminished alkali-resistance of *E. faecalis*, supporting the involvement of a cell membrane-bound proton-transport system in the alkali-resistance. Since the potassium/proton antiport system requires ATP to function, *E. faecalis* needs to generate ATP by metabolizing energy sources at alkali pH. Not only can *E. faecalis* ferment carbohydrates but it can also degrade proteins such as gelatin (21). Thus, it could be possible that *E. faecalis* obtains energy from nitrogenous compounds available in the root canals and so drives an ATP-linked proton-transport system. However, under conditions such as those found in root canals after endodontic therapy using $Ca(OH)_2$, it is unlikely that metabolic substrate is supplied sufficiently. Consequently, the potassium/proton antiport system is unlikely to function efficiently and additional mechanisms are thought to be involved.

In the present study, *E. faecalis* was able to maintain its $pH_{in}$ at approximately 8–8.5 when extracellular pH was 7.5–10 in the absence of energy sources, namely without an ATP supply (Fig. 2A). In addition, this bacterium exhibited low leakage of cell-loaded BCECF (Fig. 3A) and low permeation of SYTOX-Green (Fig. 4A), indicating that the cell membrane was durable at alkali pH and capable of retaining small intracellular molecules without leakage. This ability to maintain $pH_{in}$ and protect cell membranes from alkali-impairment may enable *E. faecalis* to survive extreme alkaline environments, such as those of a root canal medicated with $Ca(OH)_2$, without energy substrates. Perez et al. (33) demonstrated that dentinal pH decreased to around 9.5 within 2–3 weeks after placement of $Ca(OH)_2$ in root canals. At this pH, *E. faecalis* would not only survive but it would grow again when metabolic substrates were supplied (Fig. 1A).

In *S. mutans* and *S. sanguinis*, however, $pH_{in}$ was not maintained (Fig. 2B,C), and cell-loaded BCECF leaked out from cells (Fig. 3B,C) and SYTOX-Green permeated into cells (Fig. 4B,C) at alkali pH. These observations indicate that the cell membranes of these streptococci are more vulnerable to alkaline environments than the membrane of *E. faecalis*, resulting in the alkali-labile growth in *S. mutans* and *S. sanguinis* (Fig. 1B,C).

The cell membrane of *E. faecalis* was highly acid-durable (Figs 3A and 4A), suggesting that the capacity for acid-resistant growth (Fig. 1A) is attributed to the acid-durability of the cell membrane, although the $pH_{in}$ maintenance at an extracellular pH < 5 could not be determined. While *S. mutans* had lower acid-durability of cell membrane than *E. faecalis* (Figs 3B and 4B), this bacterium maintained its $pH_{in}$ at approximately 5 when extracellular pH was <5 without an energy source (Fig. 2B). This $pH_{in}$ stability may compensate for the acid-durability of the cell membrane, which is weakened at an extracellular pH < 5 (Figs 3B and 4B), and contributes to acid-resistant growth in *S. mutans* (Fig. 1B). In the present study, however, the mechanism of the $pH_{in}$ stability was not elucidated.

In addition, *E. faecalis*, *S. mutans* and *S. sanguinis* are known to have $H^+$-ATPase which functions to maintain $pH_{in}$ at acidic extracellular pH by expelling protons from cells when ATP is supplied; the pH minima for $H^+$-ATPase activity in these bacteria are 4.0, 4.0 and 4.5, respectively (3). The most acidic pH values for these bacteria to initiate growth (Fig. 1) appeared to reflect these pH minima, suggesting that $H^+$-ATPase can confer acid resistance on these bacteria in the presence of energy sources (22).

Although both *E. faecalis* and *S. mutans* were found to be acid-resistant in this study, they are isolated from different oral acidic sites; *S. mutans* has frequently been isolated from dental plaque and dental caries, including enamel caries, dentin caries and infected root canals (24, 30, 38), while *E. faecalis* has not usually been isolated from dental plaque (11) or enamel caries (31), but is mainly isolated from dentin caries including infected root canals (27, 29, 35, 41, 43). The discrepancy in the distribution of these two bacterial species could be the result of the different extent to which they adhere to tooth surfaces; *S. mutans* is known to colonize the enamel of the tooth surface and promote plaque formation (1, 26, 34, 45), whereas the ability of *E. faecalis* is still unclear. While both bacteria are capable of adhering to type I collagen consisting of dentin (36, 37, 44), *E. faecalis* is more adhesive to dentin and invasive of dentinal tubules than *S. mutans* (23, 36), possibly accounting for the relatively increased frequency of isolation of *E. faecalis* from dentin caries and infected root canals.

In conclusion, the present study demonstrated that *E. faecalis* was similar in acid-resistance to *S. mutans*, but more alkali-resistant than *S. mutans* and *S. sanguinis*. The high acid- and alkali-resistance observed in *E. faecalis* could be the result of cell membrane durability against acid and alkaline substances, in addition to ATP-linked proton-transport system functioning. The pH-resistance of *E. faecalis* may account for why this bacterium is frequently isolated from both acidic caries lesions and persistently infected root canals treated with $Ca(OH)_2$ medicaments.

## Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (no. 16390601, no. 17659659, no. 17791350) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

## References

1. Aoki H, Shiroza T, Hayakawa M, Sato S, Kuramitsu HK. Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. Infect Immun 1986: **53**: 587–594.
2. Ardeshna S, Qualtrough A, Worthington H. An *in vitro* comparison of pH changes in root dentine following canal dressing with calcium hydroxide points and a conventional calcium hydroxide paste. Int Endod J 2002: **35**: 239–244.
3. Bender GR, Sutton SVW, Marquis RE. Acid tolerance, proton permeability, and membrane ATPases of oral streptococci. Infect Immun 1986: **53**: 331–338.
4. Distler W, Kagermeier A, Hickel R, Krönke A. Lactate influx and efflux in the '*Streptococcus mutans* group' and *Streptococcus sanguis*. Caries Res 1989: **23**: 252–255.
5. Engström B. The significance of enterococci in root canal treatment. Odontologisk Revy 1964: **15**: 87–106.
6. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 2002: **35**: 221–228.
7. Flahaut S, Hartke A, Giard JC, Auffray Y. Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. Appl Environ Microbiol 1997: **63**: 812–814.
8. Flahaut S, Hartke A, Giard JC, Benachour A, Boutibonnes P, Auffray Y. Relationship between stress response towards bile salts, acid and heat treatment in *Enterococcus faecalis*. FEMS Microbiol Lett 1996: **138**: 49–54.
9. Futsaether CM, Kjeldstad B, Johnsson A. Measurement of the intracellular pH of *Propionibacterium acnes*: comparison between the fluorescent probe BCECF and 31P-NMR spectroscopy. Can J Microbiol 1993: **39**: 180–186.
10. Gauthier L, Vadeboncoeur C, Mayrand D. Loss of sensitivity to xylitol by *Streptococcus mutans* LG-1. Caries Res 1984: **18**: 289–295.
11. Guggenheim B. Streptococci of dental plaques. Caries Res 1968: **2**: 147–163.
12. Hancock H, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **91**: 579–586.
13. Harold FM, Pavlasova E, Baarda JR. A transmembrane pH gradient in *Streptococcus faecalis*. Origin and dissipation by proton conductors and *N*,*N*-dicyclo-hexylcarbodiimide. Biochem Biophys Acta 1970: **196**: 235–244.
14. Harper DS, Loesche WJ. Growth and acid tolerance of human dental plaque bacteria. Arch Oral Biol 1984: **29**: 843–848.
15. Hegyi P, Rakonczay Z, Gray MA, Argent BE. Measurement of intracellular pH in pancreatic duct cells: a new method for calibrating the fluorescence data. Pancreas 2004: **28**: 427–434.
16. Iwami Y, Kawarada K, Kojima I et al. Intracellular and extracellular pHs of *Streptococcus mutans* after addition of acids: loading and efflux of a fluorescent pH indicator in streptococcal cells. Oral Microbiol Immunol 2002: **17**: 239–244.
17. Kakinuma Y. Lowering of cytoplasmic pH is essential for growth of *Streptococcus faecalis* at high pH. J Bacteriol 1987: **169**: 4403–4405.
18. Kakinuma Y, Igarashi K. Mutants of *Streptococcus faecalis* sensitive to alkaline pH lack $Na^+$-ATPase. J Bacteriol 1990: **172**: 1732–1735.
19. Kakinuma Y, Igarashi K. Potassium/proton antiport system of growing *Enterococcus hirae* at high pH. J Bacteriol 1995: **177**: 2227–2229.
20. Kakinuma Y, Igarashi K. Isolation and properties of *Enterococcus hirae* mutants defective in the potassium/proton antiport system. J Bacteriol 1999: **181**: 4103–4105.
21. Kayaoglu G, Orstavik D. Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. Crit Rev Oral Biol Med 2004: **15**: 308–320.
22. Kobayashi H. A proton-translocating ATPase regulates pH of the bacterial cytoplasm. J Biol Chem 1985: **260**: 72–76.
23. Love RM. *Enterococcus faecalis* – a mechanism for its role in endodontic failure. Int Endod J 2001: **34**: 399–405.
24. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 2002: **13**: 171–183.
25. Loveren CV, Fielmich AM, Brink BT. Comparison of the effects of fluoride and the ionophore nigericine on acid production by *Streptococcus mutans* and the resultant *in vitro* enamel demineralization. J Dent Res 1987: **66**: 1658–1662.
26. Matsumura M, Izumi T, Matsumoto M, Tsuji M, Fujiwara T, Ooshima T. The role of glucan-binding proteins in the cariogenicity of *Streptococcus mutans*. Microbiol Immunol 2003: **47**: 213–215.
27. Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J 1998: **31**: 1–7.
28. Mundt JO. Enterococci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds. Bergey's manual of systematic bacteriology, Vol. 2. Baltimore, MD: Williams & Wilkins, 1986: 1063–1065.
29. Nakajo K, Nakazawa F, Iwaku M, Hoshino E. Alkali-resistant bacteria in root canal systems. Oral Microbiol Immunol 2004: **19**: 390–394.
30. Nascimento MM, Hofling JF, Goncalves RB. *Streptococcus mutans* genotypes isolated from root and coronal caries. Caries Res 2004: **38**: 454–463.
31. Nyvad B, Kilian M. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. Caries Res 1990: **24**: 267–272.
32. Paradiso AM, Tsien RY, Machen TE. $Na^+$-$H^+$ exchange in gastric glands as measured with a cytoplasmic-trapped, fluorescent pH indicator. Proc Natl Acad Sci U S A 1984: **81**: 7436–7440.

33. Perez F, Franchi M, Peli J. Effect of calcium hydroxide form and placement on root dentine pH. Int Endod J 2001: **34**: 417–423.
34. Pieringer RA. The metabolism of glyceride glycolipids. I. Biosynthesis of monoglucosyl diglyceride and diglucosyl diglyceride by glucosyltransferase pathways in *Streptococcus faecalis*. J Biol Chem 1968: **243**: 4894–4903.
35. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J 2003: **36**: 1–11.
36. Rich RL, Kreikemeyer B, Owens RT et al. Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. J Biol Chem 1999: **274**: 26939–26945.
37. Sato Y, Okamoto K, Kagami A, Yamamoto Y, Igarashi T, Kizaki H. *Streptococcus mutans* strains harboring collagen-binding adhesin. J Dent Res 2004: **83**: 534–539.
38. Sansone C, Van Houte J, Joshipura K, Kent R, Margolis HC. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res 1993: **72**: 508–516.
39. Sherman JM. The streptococci. Bacteriol Rev 1937: **1**: 3–97.
40. Siqueira JF, Uzeda M. Disinfection by calcium hydroxide paste of dentinal tubules infected with two obligate and one facultative anaerobic bacteria. J Endod 1996: **22**: 674–676.
41. Siren E, Haapasalo M, Ranta K, Salmi P, Kerosuo E. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. Int Endod J 1997: **30**: 91–95.
42. Sjögren U, Figdor D, Spångberg L, Sundqvist G, The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991: **24**: 119–125.
43. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998: **85**: 86–93.
44. Switalski LM, Patti JM, Butcher W, Gristina AG, Speziale P, Hook M. A collagen receptor on *Staphylococcus aureus* strains isolated from patients with septic arthritis mediates adhesion to cartilage. Mol Microbiol 1993: **7**: 99–107.
45. Tamesada M, Kawabata S, Fujiwara T, Hamada S. Synergistic effects of streptococcal glucosyltransferases on adhesive biofilm formation. J Dent Res 2004: **83**: 874–879.
46. Tsujimoto K, Semadani M, Huflejt M, Packer L. Intracellular pH of halobacteria can be determined by the fluorescent dye 2′,7′-bis (carboxyethyl)-5(6)-carboxyfluorecein. Biochem Biophys Res Commun 1988: **155**: 123–129.
47. Winkler KC, Amerongen JV. Bacteriologic results from 4,000 root canal cultures. Oral Surg Oral Med Oral Pathol 1959: **12**: 857–875.