heart infusion broth (BHI : Difco Laboratories, Detroit, MI) at 37℃ for 16 hours. Bacterial cells were collected by centrifugation (6,000 rpm, 10 min), and washed once with phosphate-buffered saline, pH 7.2 (PBS). The cell suspensions of bacteria in PBS solutions were adjusted to an optical density of 2.0 at 500 nm (5.0×10^8 colony forming units/m*l*).

2. XF

XF was synthesized by the method of Avigad et al.[13] using levan-sucrase of *Bacillus subtillis* var *saccharolyticus* and provided by Dr. Kitahata (Osaka Municipal Technical Research Institute).

3. Adherence assay in culture system

The effect of XF on the synthesis and adherence of WIG and adherence of mutans streptococci to a glass surface were examined by batch culture. Twenty μ*l* of subcultured *S. mutans* MT8148 was inoculated into 2 m*l* of heart infusion broth (HI : Difco Laboratories) containing 1% sucrose and 0-5% XF, and the bacterium was cultured at 37℃ for 17 hr in a small glass test tube at a 45° angle. After cultivation, bacterial cells and WIG were fractionated into firm-adherent, loose-adherent, and non-adherent fractions. The cultured tube was rotated gently 3 times and non-adherent culture was poured into another test tube. The original test tube was washed with 2 m*l* of PBS. Washing was combined with non-adherent culture and this fraction was referred to as the non-adherent fraction. Two m*l* of PBS was added to the original test tube and shaked by a mixer (Scientific Industries, NY, USA) for 10 sec. The upper fraction by this procedure was the loose-adherent fraction, and the residual fraction on the glass surface was the firm-adherent fraction. In non-adherent and loose-adherent fractions, bacterial cells and WIG were precipitated by centrifugation (25,000 rpm, 20 min) at 4℃. Two m*l* of 0.5 N NaOH was added to each adherent fraction to resolve WIG on the bacterial cell surface, and cells and WIG were separated by centrifugation (25,000 rpm, 20 min) at 4℃. The amount of cells in each fraction was measured by turbidity at 500 nm after suspension in 1 m*l* of PBS. WIG in the supernatant was determined colorimetrically at 492 nm by the phenol-sulfuric acid method[14] using glucose as a standard.

4. Biofilm formation and enamel demineralization in artificial mouth system

1) Preparation of enamel slabs

Enamel slabs with a flat surface, 3.5×3.5×1.5 mm in size, including dentine were prepared from the central part of the labial surface of unerupted bovine lower incisors. The enamel slabs were gradually polished using wet abrasive paper and finally with 1 μm grade polishing film. Nine symmetrical points on the surface of each slab were measured for hardness with a diamond Vicker's indenter on a microhardness tester (Akashi Seisakusho Ltd., MVK-E) loaded with 200 g. Slabs with mean Vicker's hardness values of 280 to 320 were used in the experiments. The area of each enamel slab was measured using a micrometer caliper (Mitsutoyo, CD-15, Kawasaki, Japan).

2) AMS

The AMS consisted of two identical columnar artificial mouths, thermostatic incubator, multiple pH meter, peristaltic pump, and cooling stirrer (Fig. 1). The artificial mouth (60 mm in diameter, 140 mm in height) and surrounding water jacket (140 mm in diameter) were made from transparent vinyl chloride. Warm water was circulated by the thermostatic incubator (Taitech, Saitama, Japan) to maintain the temperature of the artificial mouths at 37℃. A pH electrode with a flat bulb (9 mm in diameter, TOA-DKK, Tokyo, Japan) was set upside down in the center of the artificial mouth with a silicon plug. Four enamel slabs were arranged on the flat surface of a special Teflon holder (24 mm in diameter) around the bulb of the electrode. Another silicon plug with five stainless steel tubes and a thermometer was set on the upper part of the artificial mouth to constantly supply HI medium supplemented with sucrose, PBS, or XF in PBS, and bacterial cell suspension with a peristaltic pump (Furue Science, Tokyo, Japan). The cell suspension ($OD_{500} = 2.0$) was maintained at a low temperature using a cooling stirrer (Iwaki Glass, Tokyo, Japan) during the experiment. Changes in pH underneath the artificial biofilm were continuously monitored with a multiple pH recorder (TOA-DKK, Tokyo, Japan). All procedures were conducted under aseptic conditions.

3) Evaluation of quantity of artificial biofilm and enamel demineralization

After stopping the operation of the AMS, the entire

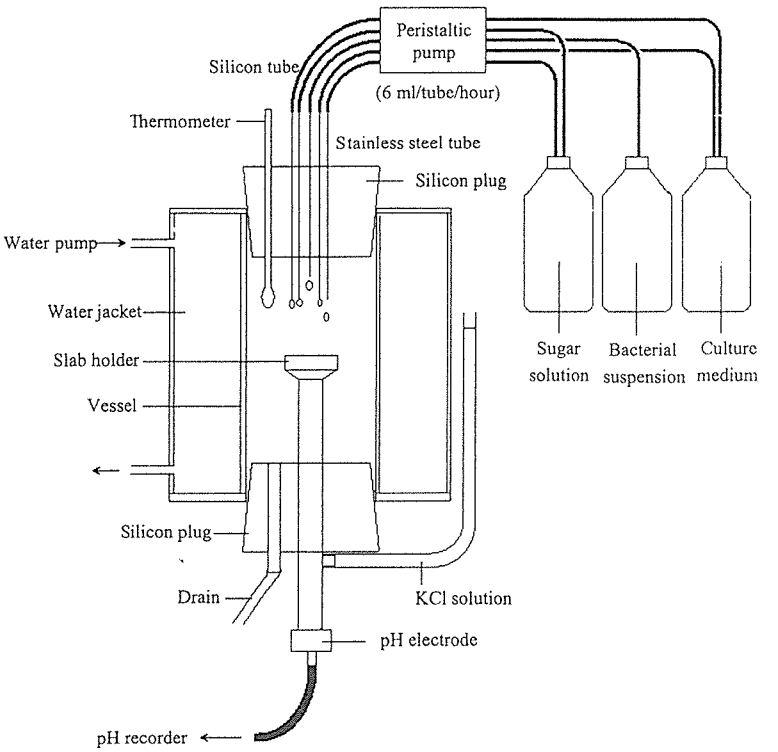

Fig. 1 Cross-sectional appearance of the artificial mouth system

flat electrode and enamel slabs were photographed. The biofilm formed on each enamel slab and flat bulb was carefully removed with a silicon scraper before being immersed in a 0.5 N NaOH solution to resolve the WIG on ice. This was followed by centrifugation (6,000 rpm, 20 min) to separate the bacterial cells from the WIG. Bacterial cells were re-suspended in PBS and the turbidity at 500 nm was measured. In addition, the amount of WIG in the NaOH solution was determined colorimetrically at 492 nm by the phenol-sulfuric acid method. Both cell turbidity and the amount of WIG were used as indicators of the amount of biofilm.

Vicker's hardness values of the enamel slabs were then determined after the experiment and compared with those before the experiment. The differences in hardness (ΔH) were used to infer the degree of demineralization.

4) Statistical analysis

Student's *t*-test was used to compare differences in the mean value of the degree of enamel demineralization and the amount of artificial biofilm in the control and experimental groups.

Results

1. Effect of XF on artificial biofilm formation by *S. mutans* in culture system

S. mutans MT8148 was cultured in HI broth containing 1% sucrose with 0-5% XF in a glass test tube. The amounts of cells in the total adherent (loose plus firm) fraction were significantly inhibited by XF in a dose-dependent manner. The inhibitory rates for 1%, 2.5%, and 5% XF were 68%, 83%, and 86%, respectively (Fig. 2). Figure 3 shows the amounts of WIG and water-soluble glucan (WSG) in the presence of 1% sucrose and each concentration of XF. The amounts of WIG and WSG in each adherent fraction were significantly inhibited by XF in a dose-dependent manner. XF alone contributed minimally to the development of artificial biofilm formation in terms of bacterial cells and WIG.

2. Effect of XF on artificial biofilm formation, biofilm pH environment, and enamel demineralization in AMS

Two identical artificial mouths were operated simultaneously under the same conditions except for sugar supplementation. Changes in pH values beneath the artifi-

cial biofilm are shown in Fig. 4. The pH values of the control (2% sucrose) began to decrease after 8 hr of running the AMS, reaching pH 5.5, which is thought to be a critical pH for enamel demineralization after 13 hr and pH 4.6 after 20 hr. Conversely, the pH values in the experiment (2% XF) remained unchanged for 20 hr. Table 1 shows the changes in Vicker's hardness values (ΔH) of the enamel slabs before and after the experiments with the amount of artificial biofilm indicated by the turbidity of the bacterial cell suspension and the amount of WIG produced. The amount of artificial biofilm and change of microhardness were considerably lower in the case of XF alone.

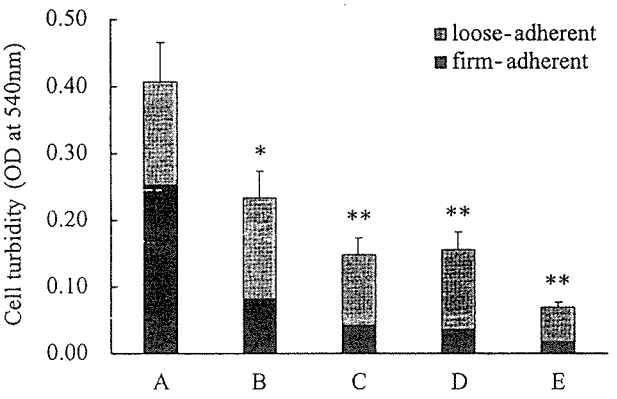

Fig. 2 Amounts of bacterial cells in firm- and loose-adherent fractions in a culture system of *S. mutans* MT8148 in the presence of sugars
A : 1% sucrose, B : 1% sucrose + 1% XF, C : 1% sucrose + 2.5% XF, D : 1% sucrose + 5% XF, E : 1% XF.
* : $p < 0.05$, ** : $p < 0.01$ (Compared with A)

Figure 5 shows the change in pH values of AMS in the presence of 1% sucrose (control) and 1% sucrose plus 1% XF (experiment). The decrease in the pH of the experiment was slightly delayed compared to that observed in the control. The pH values beneath the biofilm on the electrode decreased to 5.5 after 14 hr and 17 hr of AMS operation in the control and experiment, respectively. As shown in Table 2, no significant differences were observed in the amount of biofilm and ΔH from the enamel slabs in the control and experiment.

Figure 6 shows the changes in the pH values underneath the artificial biofilm in the AMS in the presence of 1% sucrose (control) and 1% sucrose plus 2.5% XF (experiment). In the control, pH values began to decrease after 8 hr, reaching 5.5 after 16 hr and 4.7 after 22 hr of operating the AMS. However, the pH values in the experiment did not exhibit any apparent decreases, even after 22 hr. The amount of biofilm produced in terms of bacterial cell turbidity in the experiment was significantly less than that of the control (Table 3). Similarly, no WIG could be detected on the enamel slabs in the ex-

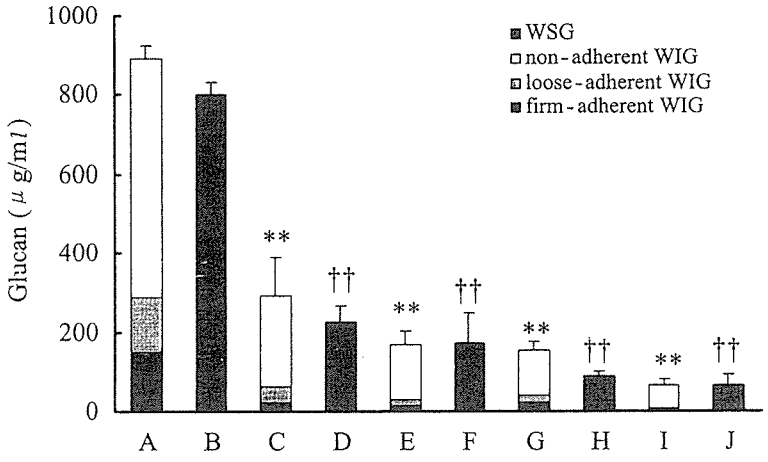

Fig. 3 Amounts of glucans in each adherent fraction in a culture system of *S. mutans* MT8148 in the presence of sugars
A and B : 1% sucrose, C and D : 1% sucrose + 1% XF, E and F : 1% sucrose + 2.5% XF, G and H : 1% sucrose + 5% XF, I and J : 1% XF
** : $p < 0.01$ (Compared with A), †† : $p < 0.01$ (Compared with B)

Table 1 Effect of XF on enamel demineralization and the amount of artificial biofilm formed on bovine enamel slabs by *S. mutans* MT8148 in thc artificial mouth system

	Δ H [a]	OD_{500} [b]	WIG (μg/mm²) [c]
Control (2% sucrose)	252.7 ± 5.1	0.009 ± 0.002	2.503 ± 0.360
Experiment (2% XF)	8.2 ± 4.2 [d]	ND	0.239 ± 0.183 [d]

[a] : Differences in microhardness of 4 enamel slabs before and after experiments (Mean ± SD)
[b] : Bacterial cell turbidity at 500 nm per square mm of 4 enamel slabs (Mean ± SD)
[c] : Amount of total WIG per square mm of 4 enamel slabs (Mean ± SD)
[d] : $p < 0.01$ (Compared with the control group)
ND : Not detected

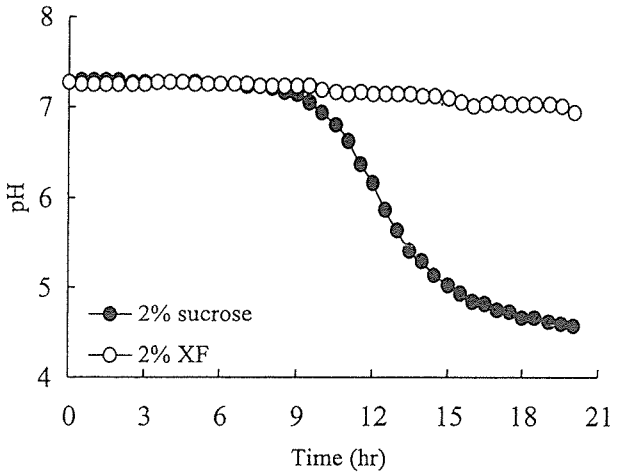

Fig. 4 Changes in pH underneath the artificial biofilm formed by 2% sucrose or 2% XF

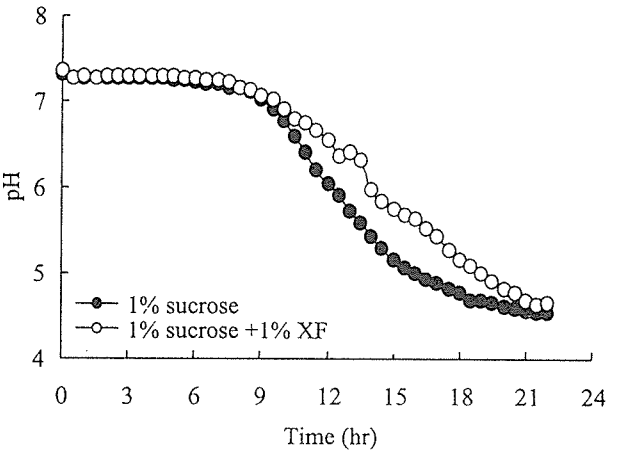

Fig. 5 Changes in pH underneath the artificial biofilm formed by 1% sucrose or 1% sucrose + 1% XF

periment, and the value for ΔH in the experiment was significantly less than that of the control.

Discussion

Adherence of *S. mutans* MT8148 cells and WIG to a glass surface were inhibited by XF in a dose-dependent manner in this study. WSG production was inhibited by XF in a similar manner. Imai et al. [11] reported that 83% of WIG adherence by *Streptococcus sobrinus* 6715 in the presence of 5 mM sucrose was inhibited by 10 mM XF in a culture system. Takeuchi[15] reported that 10 mM XF inhibited both the production and adherence of WIG by crude glucosyltransferase from *S. sobrinus* 6715 in the presence of 5 mM sucrose by 86% and 94%, respectively. Kishi[16] reported that XF effectively inhibited purified GTF from *S. sobrinus* MT3791 and that XF competitively inhibited the sucrose degrading activity of GTF, inhibiting the activity of glucan production. Togashi[17] reported that purified GTF from *S. mutans* MT8148 was competitively inhibited by XF.

Under the culture conditions used in this study, 43% of *S. mutans* MT8148 cells and 33% of WIG were recovered in the total adherent (loose and firm adherent) fraction. Imai[18] reported that the adherence ability of mutans streptococci to a smooth surface was dependent on the ability to produce WIG from sucrose, and that a positive correlation existed between adherent WIG and adherent bacterial cells. Consequently, bacterial cells and WIG were both suitable for use as adherence parameters of *S. mutans*.

In vivo test systems such as animal experiments[19], human plaque pH tests[20], and human intraoral cariogenicity tests (ICT)[21] have been used to assess both the cari-

Table 2 Effect of XF on enamel demineralization and the amount of artificial biofilm formed on bovine enamel slabs by *S. mutans* MT8148 in the artificial mouth system

	Δ H [a)]	OD_{500} [b)]	WIG (μg/mm²) [c)]
Control (1% sucrose)	245.8 ± 8.4	0.014 ± 0.005	4.527 ± 0.991
Experiment (1% sucrose + 1% XF)	244.2 ± 11.3	0.014 ± 0.002	4.183 ± 0.627

a) : Differences in microhardness of 4 enamel slabs before and after experiments (Mean ± SD)
b) : Bacterial cell turbidity at 500 nm per square mm of 4 enamel slabs (Mean ± SD)
c) : Amount of total WIG per square mm of 4 enamel slabs (Mean ± SD)

Table 3 Effect of XF on enamel demineralization and the amount of artificial biofilm formed on bovine enamel slabs by *S. mutans* MT8148 in the artificial mouth system

	Δ H [a)]	OD_{500} [b)]	WIG (μg/mm²) [c)]
Control (1% sucrose)	242.7 ± 5.4	0.009 ± 0.002	1.850 ± 0.692
Experiment (1% sucrose + 2.5% XF)	11.1 ± 3.0 [d)]	0.0005 ± 0.0001 [d)]	ND

a) : Differences in microhardness of 4 enamel slabs before and after experiments (Mean ± SD)
b) : Bacterial cell turbidity at 500 nm per square mm of 4 enamel slabs (Mean ± SD)
c) : Amount of total WIG per square mm of 4 enamel slabs (Mean ± SD)
d) : $p < 0.01$ (Compared with the control group)
ND : Not detected

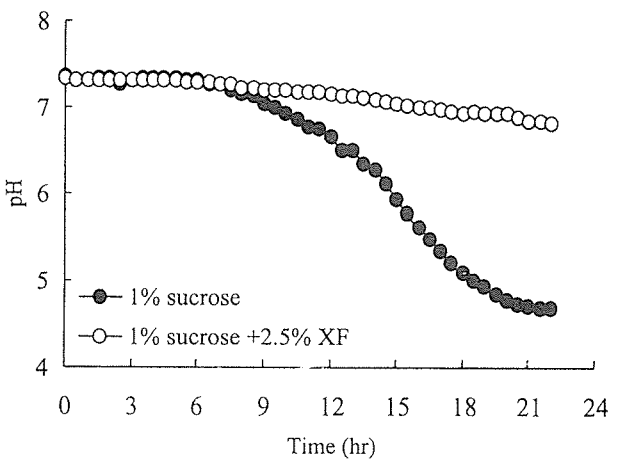

Fig. 6 Changes in pH underneath the artificial biofilm formed by 1% sucrose or 1% sucrose + 2.5% XF

ogenic potential and inhibition of sugar substitutes or enzyme inhibitors. However, *in vivo* test systems have had several associated intrinsic time and economic problems, so that *in vitro* test systems, especially artificial mouth systems, have been developed[22-32]. We improved an AMS originally developed by Hinoide et al.[31]. The characteristics of this system made it possible to assess the important parameters responsible for causing dental caries such as biofilm formation, the pH underneath biofilm, and enamel demineralization.

As mentioned previously, XF was not used as a substrate for GTF and it was found to inhibit GTF activity and the production of WIG. In ICT in human subjects, 2% XF did not inhibit enamel demineralization in the presence of 2% sucrose[33]. However, enamel demineralization by 2% XF alone was significantly lower than that observed when exposed to 2% sucrose, indicating that XF appears to be a low-cariogenic sugar. It is not known whether or not XF produces the same results in the AMS as those in ICT. As shown in Fig. 4 and Table 1, 2% sucrose caused marked decreases in pH, the production of large amounts of biofilm, and considerable enamel demineralization. These results indicate that the use of the AMS facilitated the assessment of the cariogenicity of sucrose. Conversely, 2% XF alone did not decrease the pH in the AMS, and the amount of biofilm and enamel demineralization were considerably low, suggesting that XF might be a low- or non-cariogenic sugar. These results corroborated previous findings obtained by ICT. Using *S. sobrinus* 6715, Hinoide et al.[31] re-

ported that xylitol, a non-cariogenic sugar alcohol, did not decrease the pH of their AMS, the prototype of the system used in our study, over a period of 24 hr.

The capacity of 1% and 2.5% XF to differentially inhibit the cariogenicity of 1% sucrose was interesting. While pH values under conditions of the simultaneous addition of 1% XF and 1% sucrose were slightly delayed compared with 1% sucrose alone in AMS, 1% XF did not suppress the formation of biofilm and enamel demineralization (Fig. 5 and Table 2). There was also a discrepancy between the results obtained in the culture system and those from the AMS. In the culture system, 1% XF significantly inhibited the adherence of *S. mutans* cells and WIG to glass surfaces in the presence of 1% sucrose (Figs. 2 and 3). This discrepancy might be ascribed to the differences in experimental conditions between the static conditions of the culture system and the flowing system of the AMS. However, 2.5% XF significantly inhibited the formation of biofilm on enamel slabs, pH decrease underneath the biofilm, and enamel demineralization (Fig. 5 and Table 3). These results suggested that an appropriate concentration of XF could inhibit, in part, the cariogenicity of sucrose.

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人工口腔装置におけるスクロースのう蝕原性に及ぼす二糖類キシロシルフルクトシドの影響について

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概要：甘味二糖類キシロシルフルクトシド（XF）が歯面へのミュータンスレンサ球菌の付着や，非水溶性グルカンの合成を効果的に阻害することが報告されている．本研究は *in vitro* の実験系と，う蝕誘発過程に重要なバイオフィルム形成，バイオフィルム直下の pH，エナメル質脱灰度を同時に検討できる人工口腔装置による評価系を用いて，XF のう蝕誘発性およびスクロースのう蝕誘発性に及ぼす XF の抑制効果を検討した．ガラス試験管に 1% スクロースと 1～5% XF をそれぞれ含む培地に，*S. mutans* MT8148 を植菌し，培養を行った．その結果，XF はガラス管壁付着性の非水溶性グルカン量と菌体量を濃度依存的に抑制した．また人工口腔装置において，1% XF は 1% スクロース存在下における人工バイオフィルム形成やエナメル質脱灰度は抑制しなかったが，2.5% XF は人工バイオフィルム直下の pH をほとんど低下させず，人工バイオフィルム形成量，エナメル質脱灰度を有意に低下させた．以上のことから，XF は濃度によってスクロースのう蝕誘発性を部分的に抑制することが示唆された．

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索引用語：キシロシルフルクトシド，人工口腔装置，う蝕誘発性，バイオフィルム形成，ミュータンスレンサ球菌

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原　著

口腔ケアの評価指標とreal-time PCRによる舌苔中細菌数との関連

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概要：口腔ケアの評価に用いられることの多い臨床的指標と，舌苔湿重量ならびに舌苔中細菌数との関連を検討し，比較的簡便に得られる臨床的指標が口腔内の細菌量を反映しているかを検討した．調査対象は体系的な口腔ケアが行われているリハビリテーション病院に入院した者27名とし，入院時と入院2か月後の両時点で同一の口腔内指標を採得した．臨床的指標には口中VSC測定値，舌苔スコア，CPIを用い，舌苔中細菌数の測定にはreal-time PCRを用いた．その結果，入院時と入院2か月後の比較において，舌苔スコア，CPI，CH_3SH濃度および舌苔湿重量に有意な減少が認められ，H_2S濃度と舌苔中総細菌数には減少傾向が認められた．一方，舌苔1mg当たりの細菌数はほぼ同等の値となった．臨床的指標と舌苔中細菌数の関連において，入院時と入院2か月後のいずれにおいても，口中H_2S濃度と舌苔1mg中細菌数および舌苔中総細菌数の間に有意な関連を認めた．舌苔スコアとVSC測定値を独立変数，舌苔中総細菌数を目的変数とした重回帰分析では舌苔スコアとH_2S濃度が有意な変数として選択された．これらのことから，口腔ケアの臨床的指標として舌苔量を評価することに加えて口中気体のH_2S濃度を測定することで，舌苔中細菌数の変動をより詳細にとらえていると考えられ，それらを口腔ケアの指標とすることの妥当性が確認された．

索引用語：口腔ケア，舌苔，VSC，Real-time PCR

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緒　言

専門的な口腔ケアが要介護高齢者の呼吸器疾患に対する予防効果があることが報告されて以来[1-5]，入所者に対する口腔ケアに取り組む医療施設や養護施設が増加し，その効果について数多く報告されている[6-12]．それら報告において，口腔ケアの効果を評価するアウトカム指標として，舌苔や口臭を用いる例が多くみられる．高齢者や病弱者の誤嚥性肺炎予防のためには口腔内の肺炎起炎菌を減少させることが重要であるが，口腔ケアは肺炎起炎菌を特異的に減少させるのではなく，口腔内の総細菌数を減少させる方法である[4,13]．舌苔付着量の変化は口腔内の総細菌数の変動を反映しているものと考えられる．なぜなら舌苔は舌背表面に堆積する細菌性バイオフィルムの一つであり，唾液や歯垢への細菌の受容・供給相互関係にあると考えられている[14-16]こと，さらに付着面積が大きいために舌苔の口腔内総細菌量への影響が少なくないものと考えられるからである．一方，口臭の揮発性硫黄化合物（VSC）濃度は舌苔付着量との関連が強いことから[17-22]，口臭強度が間接的に口腔内細菌量を反映する可能性がある．舌苔や口臭の評価は非侵襲的であり，無歯顎者や唾液分泌が減少した高齢者に対しても適用が可能である．このような理由から，舌苔や口臭の評価が口腔ケアのアウトカム指標としてこれまで多く用いられてきたものと考えられる．しかし，これまでの報告における評価法は簡便な視診や官能試験によるものがほとんどであり，舌苔や口臭と口腔内細菌量との関連は明瞭とはいえない．

そこで本研究では，入院中に口腔ケアによる口腔内状態の改善が期待できるリハビリテーション病院におい

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表1　対象者の入院原因となった傷病と入院時食事状況

		食事形態			
		常　食	刻み食*	経　管**	計
傷病名	脳血管疾患	11	7	2	20
	脊髄損傷	3	1	0	4
	脳　炎	0	1	0	1
	脳挫傷	1	0	0	1
	神経疾患	0	1	0	1
計		15	10	2	27

* 全粥，とろみ食を含む
**経鼻管栄養摂取

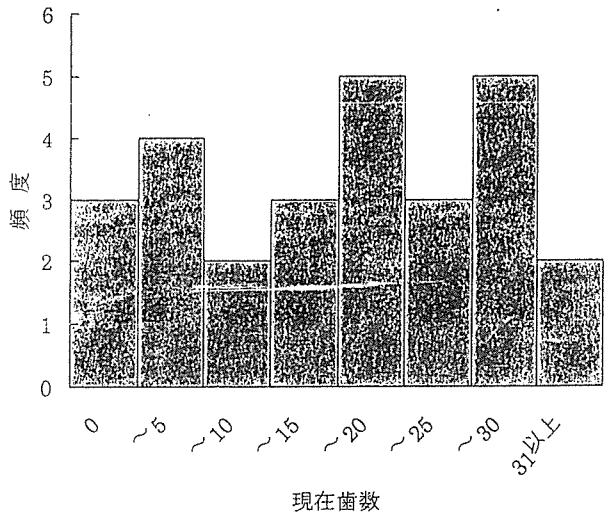

図1　対象者の現在歯数の分布

て，入院時と入院2か月後に，口腔ケアの評価に用いられることの多い臨床的指標と，細菌学的指標を採得した．臨床的指標にはポータブルガスクロマトグラフィによる口臭測定，視診による舌苔スコア，CPIを用い，細菌学的指標として舌苔湿重量の測定ならびにreal-time PCRを用いた舌苔中細菌量の定量を行った．それらの関連を分析することにより，比較的簡便に得られる臨床的指標が口腔内の細菌量を反映するかを検討した．

対象および方法

1．対象

岩手県内某リハビリテーション病院において，2004年8月の1か月間に入院した者27名（男性21名，女性6名，平均年齢±標準偏差：58.7±16.5歳）を対象とした．当該リハビリテーション病院は，傷病のための急性期医療をほかの病院で行った後の回復期医療を担当する医療機関であり，口腔ケアについても歯科衛生士を中心とした体系的な口腔ケアを行っている．入院時と入院2か月後の同時刻に，口臭測定，口腔内検査（硬組織検査，舌苔スコア評価，歯周組織検査）および舌苔採取を行った．口腔内検査は，十分にキャリブレーションを行った2名の検査者が両検査時点で異なる対象者を調査した．

対象者の入院原因となった傷病と入院時食事形態の分布を表1に示す．両調査時点での食事形態に関する差違は，経管栄養からとろみ食に移行した者が1名，その他刻み食の刻み程度が大きくなった者が2名であった．対象集団の1人平均現在歯数は16.0±10.8であり，無歯顎者は3名であった．対象集団の現在歯数の分布を図1に示す．

2．口臭測定

ポータブルガスクロマトグラフィ（Oral Chroma®，ABILIT，大阪）を用いて口中気体の硫化水素（H_2S）とメチルメルカプタン（CH_3SH）の測定を通法[23]で行った．すなわちディスポーザブルシリンジを被験者の口腔内に挿入し，30秒間鼻呼吸のもと口唇閉鎖させ，口中気体1.0 ml を採取し，0.5 ml を廃棄，残りの0.5 ml をガスクロマトグラフィに注入し測定した．

3．舌苔スコア

舌苔付着状況をMiyazakiら[21]の視診による基準に従い，4段階で評価した．すなわち，0：舌苔がほとんど認められない，1：舌苔が舌背の1/3未満を覆っている，2：舌背の1/3以上2/3未満を覆っている，3：舌背の2/3以上を覆っている．

4．歯周組織検査

有歯顎者に対しては歯周組織の状態をすべての残存歯についてCPIの基準[24]で評価し，記録された最も重度なコードを個人のCPIコードとした．

表2　入院時と入院2か月後における評価指標の比較

アウトカム指標	入院時		入院2か月後
H_2S（ppb）	231±76.5		88.5±24.7
CH_3SH（ppb）	77.1±30.0	—*—	27.7±10.4
CPI	2.57±0.24	—**—	1.48±0.31
舌苔スコア	1.96±0.21	—**—	1.04±0.17
舌苔付着量（mg）	24.4±5.60	—*—	12.8±1.92
舌苔1mg中細菌数（対数平均値）	1.55×10^{10} (9.71 ± 0.21)		1.77×10^{10} (9.71 ± 0.17)
舌苔中総細菌数（対数平均値）	5.48×10^{11} (10.9 ± 0.29)		3.21×10^{11} (10.7 ± 0.21)

数値は平均値 ± 標準誤差

* $p<0.05$, ** $p<0.01$：Wilcoxon 順位検定

5. 舌苔湿重量

前報[16,22]同様に舌苔を採取・定量した．すなわち舌背の有郭乳頭から舌尖部の付着物を滅菌歯ブラシにて可及的に採取し，5 ml PBS に懸濁した．懸濁液を超音波処理（20 W，5秒，5回）により均一化した後，懸濁液中の舌苔湿重量を測定し，舌苔付着量とした．

6. 舌苔中細菌数の定量

一定量（4 mg）に調整した舌苔試料から Wizard Genomic DNA Purification Kit (Promega, MI, USA) を用いて，ゲノム DNA を精製後，20 μl の Tris-EDTA 緩衝液に溶解し，TaqMan プローブを用いた real-time PCR (ABI PRIZM 7700 system, Applied Biosystems, USA) に供した．PCR には，精製したゲノム溶液 0.1 μl，1× TaqMan Universal PCR Master Mix (Applied Biosystems)，200 nM の 16 S rRNA ユニバーサルプライマー，250 nM TaqMan プローブからなる 20 μl 溶液を用いた．プライマーとプローブの設計は既報[25]に準じた．スタンダードには 16 S rRNA 領域を挿入したプラスミドを使用した．その総塩基長に平均分子量を乗じて重量を求め，重量当たりのゲノムコピー数を算出し，これを細菌数とした．測定は2回行い，平均 Ct 値をスタンダード直線に当てはめて舌苔 1 mg 当たりの細菌数を求めた．また，舌苔 1 mg 中の細菌数に採取した舌苔付着量を乗じ，舌苔中総細菌数とした．

7. 口腔ケア

看護職員による口腔ケア介入は調査期間中，対象者の自立度に応じて通常と同様に以下のように行われた．1. 声かけ・実地指導：処置を要する歯科疾患がなく，適切なオーラルセルフケアが行える者に対して実施．2. 看護職員による口腔清掃：適切なセルフケアが不可能な者に対して歯科衛生士，看護師による定期的口腔清掃を実施．3. 歯科治療：明らかな歯疾，義歯不調がある場合に実施．

8. 統計解析

入院時と入院2か月後における評価指標の比較には，Wilcoxon 順位検定を用いた．両時点での指標相互の関連分析には Spearman の順位相関分析を行った．臨床的指標と舌苔細菌数の関連の検討には重回帰分析を行った．対象者の入院原因となった傷病については，そのほとんどが脳血管疾患であり，その他も中枢神経を傷害して機能障害をきたす傷病であることから，傷病別の分析は行わなかった．すべての統計解析には SPSS 11.5 J (SPSS Japan，東京）を使用した．

9. 研究倫理

研究に先立ち，岩手医科大学歯学部倫理委員会（受付番号 01025）および調査対象施設研究倫理委員会の承諾を得た．調査現場においては，対象者とその主たる近親者から調査に関する説明を行った後，文書による同意を得て行った．

結　果

1. 口腔ケア前後における口腔内状況の比較

入院時と入院2か月後の口腔内状況の比較を，表2に示す．臨床的指標では舌苔スコア，CPI，CH_3SH 濃度に有意な減少が認められ，H_2S 濃度は統計学的に有意な差ではなかった（$p=0.156$：Wilcoxon 順位検定）が減少傾向にあった．また，採取した舌苔の分析で得られた細菌学的指標では，舌苔付着量の平均値が有意に減少していた．舌苔中総細菌数は統計学的に有意な差ではなかった（$p=0.144$：Wilcoxon 順位検定）が減少傾向にあった．また，舌苔 1 mg 当たりの細菌数の対数平均値は口腔ケア前後でほぼ同等の値であった（$p=0.990$：対応のある t 検定）．また，入院時の食事形態別を，常食とそれ以外

表 3　口腔ケアの指標および現在歯数相互の関連

	H_2S	CH_3SH	CPI	現在歯数	舌苔スコア	舌苔湿重量（mg）	舌苔 1mg 中細菌数	舌苔中総細菌数
H_2S	1							
	1							
CH_3SH	0.725 **	1						
	0.756 **	1						
CPI	− 0.307	− 0.118	1					
	− 0.138	− 0.134	1					
現在歯数	0.101	− 0.148	− 0.541 **	1				
	− 0.115	− 0.091	0.179	1				
舌苔スコア	0.365	0.225	0.080	− 0.128	1			
	0.297	0.046	0.310	− 0.357	1			
舌苔湿重量（mg）	0.133	0.270	− 0.079	0.029	0.671 **	1		
	0.400	0.306	0.080	− 0.120	0.703 **	1		
舌苔 1mg 中細菌数	0.444 *	0.278	− 0.265	0.112	0.416 *	0.186	1	
	0.456 *	0.110	0.000	0.226	0.228	0.308	1	
舌苔中総細菌数	0.458 *	0.290	− 0.190	0.151	0.684 **	0.620 **	0.783 **	1
	0.557 **	0.212	− 0.070	0.131	0.403 *	0.619 **	0.892 **	1

表中の数値は Spearman の順位相関係数，セル上段に入院時，下段に入院 2 か月後を示す（* $p < 0.05$, ** $p < 0.01$）．
CPI については有歯顎者（24 名）についての分析結果を示す．

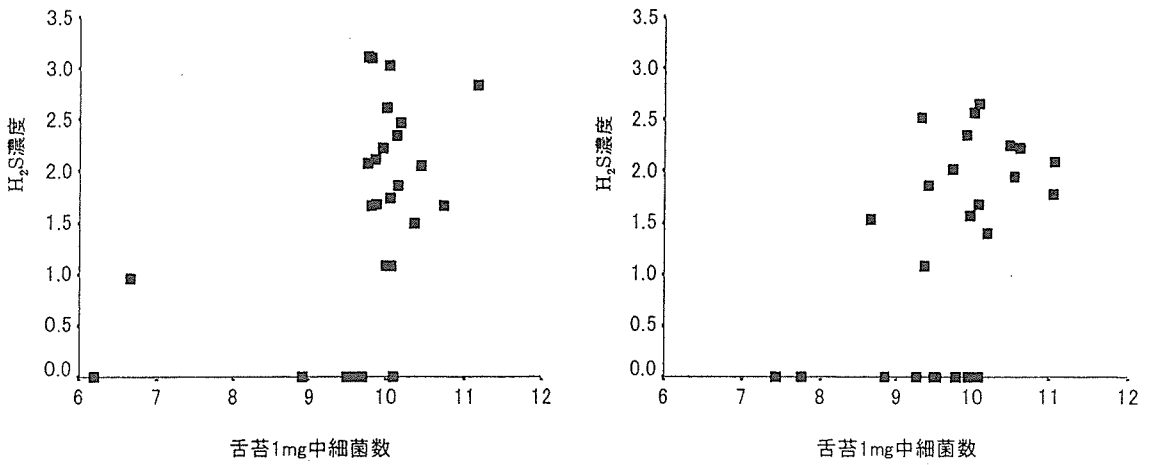

図 2　入院時と入院 2 か月後における口中 H_2S 濃度と舌苔 1 mg 中細菌数の関連*
*口中気体の H_2S 濃度ならびに舌苔 1 mg 中細菌数はいずれも
対数変換値 = $\log_{10}$（実測値 + 1）で表した．

の摂取形態に分類して比較したところ，2 調査時点のいずれの評価項目についても差は認められなかった．

2．臨床的指標と細菌学的指標の関連

入院時と入院 2 か月後の両時点における臨床的指標と細菌学的指標の関連を，表 3 に示す．口中気体の H_2S 濃度と舌苔 1 mg 中細菌数および舌苔中総細菌数の間に有意な相関を認めた．両時点におけるそれらの関連を図 2，3 に示す．CH_3SH 濃度は H_2S 濃度と高い相関を呈したが，ほかの評価項目との関連は有意ではなかった（表 3）．さらに，口中気体の VSC 濃度と舌苔スコアを説明変数とし，舌苔中総細菌数を目的変数とした重回帰分析を行ったところ，入院時点では舌苔スコアのみが有意な説明変数であったが，入院 2 か月後の時点では舌苔スコアと H_2S が有意となり，偏回帰係数は H_2S のほうが大

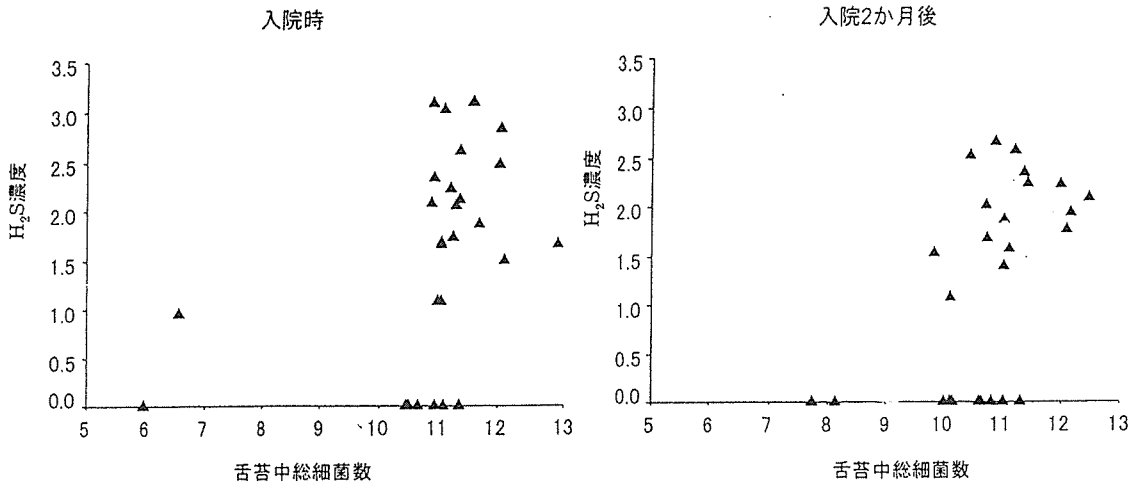

図3　入院時と入院2か月後における口中 H_2S 濃度と舌苔中総細菌数の関連*
* 口中気体の H_2S 濃度ならびに舌苔総細菌数はいずれも
対数変換値 = $\log_{10}$（実測値 + 1）で表した.

表4　口中 VSC 濃度および舌苔スコアと舌苔総細菌数の関連*

入院時			
選択された変数**	非標準化係数（標準誤差）	標準化係数	有意確率
舌苔スコア	0.884（0.204）	0.638	0.0004
			R^2=0.407
入院2か月後			
選択された変数**	非標準化係数（標準誤差）	標準化係数	有意確率
H_2S 濃度	0.493（0.178）	0.458	0.011
舌苔スコア	0.487（0.215）	0.374	0.034
			R^2=0.434

* 分析には H_2S 濃度，CH_3SH 濃度の対数変換値（$\log_{10}$（実測値 +1））を用いた.
** H_2S 濃度，CH_3SH 濃度，舌苔スコアを初期の投入変数としたステップワイズ重回帰分析（変数減少法）で，有効な説明変数として選択された変数.

きかった（表4）.

考　察

本研究で対象としたリハビリテーション病院では，全身だけでなく口腔を対象としたリハビリテーションが行われており，口腔ケアについても一般の急性期医療施設に比べて高い水準のケアがなされているものと考えられる．しかし高水準の体系的な口腔ケアが供給されている医療施設であっても，原疾患の重症度や生活自立度，口腔内状態などによってケアの内容は異なる．また全身のリハビリテーションによって，オーラルセルフケアのレベル，食事形態，口腔機能などが変化することから，純粋に口腔ケアのみのアウトカム評価は困難である．それゆえ，本研究では，同一集団の，異なる口腔内状態にある2時点（表2）での舌苔中細菌数と臨床的指標の関連を分析することを目的とした.

舌苔と口臭強度の関連については，これまでに多くの報告がある[17-23]．われわれも，歯周病健全者の舌苔付着量と口中総VSC濃度[22]や，無歯顎高齢者の舌苔スコアと口中H_2S濃度に関連のあること[23]を報告してきた．本研究結果でも統計学的に有意ではなかったが，舌苔スコア，舌苔付着量という舌苔の量的評価項目と口中気体のH_2S濃度，CH_3SH濃度にある程度の関連が認められた．この結果に加え，本研究では口腔ケア前後の両時点で，口中気体のH_2S濃度が舌苔1mg当たりの細菌数および舌苔中総細菌数と強い関連を呈した(表3，図2，3)．本研究で得られた舌苔中細菌数は1mg当たりのゲノムコピー数として1.59×10^{6}～1.43×10^{11}に分布しており（平均値$=1.55\times10^{10}$)，これは舌苔細菌の凝集程度が一様ではなく，ばらつきがあることを示している．すなわち，舌苔を視診や重量で量的に把握するだけでは舌苔の蓄える細菌量を把握するのに十分ではないと考えられる．一方，口中H_2S濃度と舌苔中細菌数が正の相関を呈したことは，口腔内の細菌量を評価するうえで，VSC測定が舌苔の量的評価を補う指標になりうることを示唆するものである．重回帰モデルにおいて，入院時点の舌苔中総細菌数の有意な説明変数は舌苔スコアだけであったが，入院2か月後ではH_2Sが細菌数を推定するうえで舌苔スコアよりも有効な変数となった．両時点での重回帰モデルにおける有効な説明変数の差違は，入院時点では舌苔が多量に付着している者が多く，舌苔付着量が総細菌数に大きく反映されるが，入院2か月後には口腔ケアにより舌苔付着量が低下し，舌苔の総量よりも舌苔の単位重量当たりの細菌数が総細菌数に反映されるためと考えられる．それゆえ，ある程度口腔清掃状態が良好な場合には，舌苔スコアだけでなく，H_2S濃度を測定することによって，舌苔中細菌量をよりよく反映することが示された．

本研究では，舌苔中細菌数を16S rRNAを標的としたreal-time PCRによって定量した．これまでに舌苔を検体としてreal-time PCRを行った研究がいくつかなされている[26-28]が，湿重量当たりの細菌数は示されておらず，本研究と細菌数の定量結果を比較することはできない．Lyons ら[29]は歯垢を試料としたreal-time PCRで，歯肉溝内にペーパーポイントを挿入して得た細菌数を2.10×10^{7}～2.15×10^{15}と報告している．これも歯垢重量当たりの細菌数の結果ではないが，培養可能総菌数についての従来の知見である1g当たり10^{11}個[30]に比して多く評価されているものと考えられる．Suzuki ら[31]は16S rRNAを標的としたreal-time PCRによる総菌数の定量では実際の細菌数よりも多く評価される可能性を指摘している．その理由として16S rRNAの領域を複数もつ細菌が多く存在すること，培養法では全細菌に対して同時に至適条件で培養することが不可能であるのに対してPCRでは細菌の培養条件にかかわらず検出可能であることなどが挙げられている．本研究で得られた舌苔中細菌数の絶対値としての精度については，培養法との比較検討など，今後さらに研究を進めなければならない．しかし舌苔スコアなどの舌苔の量的評価に加えて口中H_2S濃度を用いることにより，少なくとも舌苔中の細菌数の相対的変化を推定できるものと考えられた．以上のことから，舌苔の量的評価と口臭測定を臨床的な評価項目に用いることは，口腔ケアの細菌学的効果の判定にも有効であると考えられた．

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Relationship between Clinical Indicators and the Total Amount of Bacteria in the Tongue Coat Assessed by Real-time PCR in Oral Care Evaluation

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Abstract：We assessed the relationship between clinical and bacteriological indicators on the efficacy of oral care. The subjects comprised 27 patients of a rehabilitation hospital in Iwate prefecture. We evaluated the oral status of the subjects clinically via inspection of the tongue coat volume (tongue coat score), CPI, and measurement of oral malodor (VSC：H_2S and CH_3SH levels). Subsequently, tongue coat samples were collected from the subjects for biological assessment. These samples were weighed and applied to real-time PCR to quantify the total bacterial cells. Examinations were performed both on admission and two months after admission.

At two months after admission, clinical indicators were improved, especially the tongue coat score, CPI, and CH_3SH level. For the bacteriological indicators, the tongue coat volume was reduced significantly and the mean number of bacterial cells in the whole tongue coat sample tended to decrease. However, the mean number of bacterial cells per 1 mg of tongue coat was almost the same. A correlation was observed between the H_2S level and the number of bacterial cells per 1 mg of tongue coat. In step-wise multiple regression models in which the dependent variable was the number of bacterial cells in the whole tongue coat, the tongue coat score and H_2S level were both significant variables. In conclusion, assessment of the tongue coat volume and oral malodor via the H_2S level were efficient clinical indicators to deduce bacteriological changes after oral care.

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Key words：**Oral care, Tongue coat, Oral malodor, Real-time PCR**

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ORAL MICROBIOLOGY
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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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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.

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}.

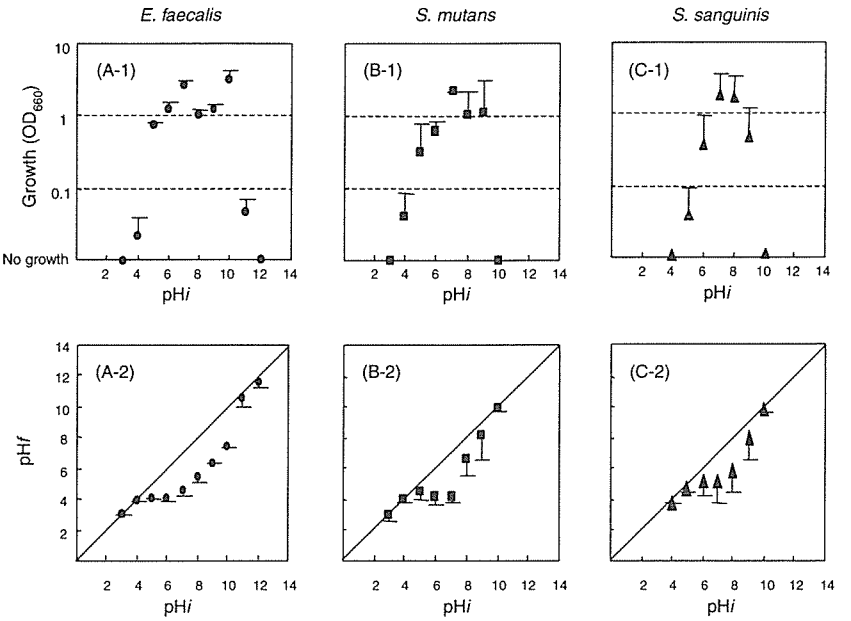

Fig. 1. Bacterial growth of *Enterococcus faecalis* JCM 8728 (A-1), *Streptococcus mutans* NCTC 10449 (B-1) and *Streptococcus sanguinis* ATCC 10556 (C-1) at various initial pH levels (pH*i*) in TYG culture media, and final pHs (pH*f*) after 48 h of growth of *E. faecalis* JCM 8728 (A-2), *S. mutans* 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.

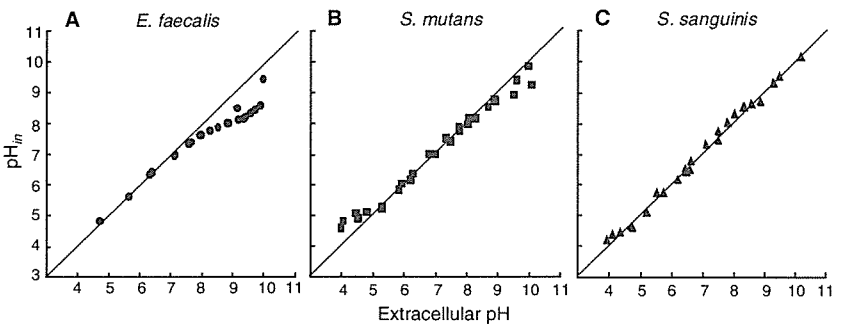

Fig. 2. The pH_{in} of *Enterococcus faecalis* JCM 8728 (A), *Streptococcus mutans* NCTC 10449 (B) and *Streptococcus sanguinis* ATCC 10556 (C) at various extracellular pH values. All the data obtained from three independent experiments were plotted.

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-

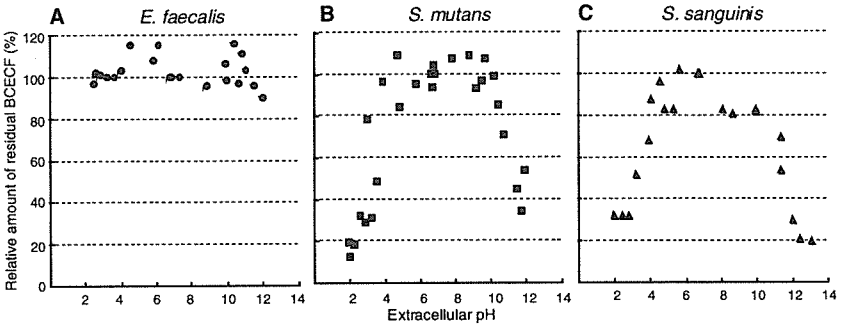

Fig. 3. The percentage of residual intracellular BCECF in *Enterococcus faecalis* JCM 8728 (A), *Streptococcus mutans* NCTC 10449 (B) and *Streptococcus sanguinis* ATCC 10556 (C) at various extracellular pH levels. The value at pH 7.0 was regarded as 100. All data obtained from three independent experiments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

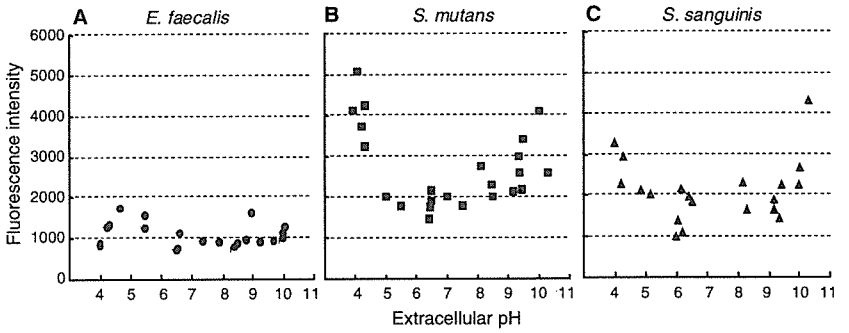

Fig. 4. Fluorescence intensity of SYTOX-Green into the cells for 5 min of *Enterococcus faecalis* JCM 8728 (A), *Streptococcus mutans* NCTC 10449 (B) and *Streptococcus sanguinis* ATCC 10556 (C) at various extracellular pH levels. All the data obtained from three independent experiments were plotted.

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

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