has not been investigated well. Ammonia is known to be toxic to host tissues[6,7] and associated with oral malodor[8]. Ammonia can be produced from urea and arginine through bacterial ureolytic and arginolytic activities, respectively[4,5]. The bacterial degradation of proteins and amino acids, derived from saliva, gingival crevicular fluid and desquamated gingival epithelium, can also be involved in the production of ammonia as well as other cytotoxic end-products, such as butyrate[9-11]. Thus, dental plaque containing a high concentration of ammonia is considered to be pathogenic to periodontal tissues due to the existence of ammonia itself and the coexistence of other cytotoxic by-products from amino acid degradation. In addition, unlike acid production from carbohydrates, ammonia can be constantly produced from urea, arginine, amino acids, and proteins continuously supplied in dental plaque and thus, the ammonia productivity of dental plaque can be estimated by measuring the concentration of ammonia contained within dental plaque.

In this study, therefore, the ammonia concentration of marginal dental plaque was compared between teeth with and without periodontitis. Furthermore, since ammonia is reportedly associated with caries activity, as stated above, the cariogenic potential, pH-lowering activity of dental plaque, was also measured.

Materials and Methods

1. Subjects and measurement of clinical indication

Subjects were 71 patients (20–71 y, 43.2 ± 14.9 y; 37 males and 34 females) who visited Kumagami Dental Clinic in Minamisoma City, Fukushima Prefecture, Japan, from February 2005 to March 2005. No subject had experienced periodontal treatment for over 3 months. All subjects were systemically healthy and were not taking antibiotics. Smokers or expectant mothers were excluded from the present study. All subjects provided written informed consent to participate. This study was conducted under the approval of the ethics committee of Tohoku University, Japan.

An oral examination and medical examination by interview were conducted by one dentist, and only subjects without systemic diseases were selected. As periodontal indicators, the probing depth (PD) and bleeding on probing (BOP) were used. Six sites per tooth were examined, and the deepest PD was adopted for diagnosis. Teeth with PD ≥3 mm were defined as showing periodontitis, according to the Japanese Society of Periodontology, 2008 and the American Dental Association, 1986. A color code probe (Hu-Friedy, USA) was employed for probing depth measurement. Panoramic and dental X-ray photographs were used if necessary to confirm whether bone loss was present. The targeted teeth in the present study had no history of caries.

2. Sampling of dental plaque

The subjects conducted regular tooth brushing the day before sampling. After confirming that the subject had not eaten any food for >2 h and had not used a fluoridated water rinse or fluoride gel, dental plaque was sampled from sound buccal marginal surfaces of the upper first or second molars using a dental spoon excavator (model No. 1; Yamaura Seisakusyo, Tokyo, Japan) by one dentist. The volume of one cup of the spoon excavator was evaluated by weighing water which filled the cup, determined to be approximately 2.4 μL. The dental plaque samples were analyzed immediately by one dental hygienist for the ammonia concentration and pH-lowering activity after the addition of glucose as follows.

3. Measurement of ammonia concentration and pH drop after glucose addition

The spoon-excavator cup of dental plaque was immediately suspended in 100 μL distilled water using a micropipette and a small glass rod, and 20 μL of the suspension was placed on the sensor area of a portable ammonia meter (Amicheck meter; Arcray, Tokyo, Japan), and the ammonia concentration was measured. Then, 30 μL of the suspension was placed on the sensor area of a portable pH meter (model pH BOY-P1; Shindengen Kogyo, Tokyo, Japan), and the pH was confirmed to be higher than 6.5. Then 50 μL of 0.5% glucose was added to the suspension and mixed well. Changes in pH were monitored for 20 min at 37℃. If there was no change after 20 min (data not shown), the pH at 20 min after glucose addition was designated as the minimum pH[1]. The portable ammonia meter adopts a microdiffusion method to separate ammonia from other contaminants in a specimen.

Just before measurement, the portable ammonia meter and portable pH meter were calibrated with NH_4Cl solution and pH standard solution (pH 7 and 4), respec-

Table 1 Comparison of ammonia concentration between subject groups

Subject groups	Ammonia concentration (mM) [a]
Total	4.02 ± 2.90
PD <3 mm (n=22)	2.92 ± 2.23
PD ≥3 mm (n=49)	4.51 ± 3.05 *
BOP (−) (n=56)	3.27 ± 2.32
BOP (+) (n=15)	6.81 ± 3.22 **
PD <3 mm and BOP (−) (n=22)	2.92 ± 2.23
PD ≥3 mm and BOP (−) (n=34)	3.50 ± 2.38
PD ≥3 mm and BOP (+) (n=15)	6.81 ± 3.22 ***

[a] Mean ± SD.
* Significantly different from subjects with PD <3 mm ($p<0.05$) by t-test.
** Significantly different from subjects with BOP (−) ($p<0.001$) by t-test.
*** Significantly different from subjects with PD <3 mm ($p<0.0005$) and subjects with PD ≥3 mm and BOP (−) ($p<0.001$) by Bonferroni test.

Table 2 Comparison of minimum pH between subject groups

Subject groups	Minimum pH [a]
Total	5.55 ± 0.72
PD <3 mm (n=22)	5.75 ± 0.76
PD ≥3 mm (n=49)	5.46 ± 0.69
BOP (−) (n=56)	5.62 ± 0.57
BOP (+) (n=15)	5.28 ± 0.57
PD <3 mm and BOP (−) (n=22)	5.75 ± 0.76
PD ≥3 mm and BOP (−) (n=34)	5.54 ± 0.74
PD ≥3 mm and BOP (+) (n=15)	5.28 ± 0.57

[a] Mean ± SD.

tively. The portable ammonia meter showed linearity within 5–500 μM of NH_4^+ when using NH_4Cl as a standard, and the reading was consistent with the concentration of standard solution (data not shown). The values of the ammonia concentration measured in this study were within the range of linearity and were corrected to mM (mmol per liter of dental plaque).

4. Statistical analysis

The differences in measurement values were compared using the unpaired t-test for 2 groups and Bonferroni test for 3 groups. The associations between the ammonia concentration and pH-lowering activity were analyzed employing Pearson's correlation coefficient.

Results

The ammonia concentration of marginal dental plaque from all subjects was 4.02 ± 2.90 mM (Table 1). The PD distribution of targeted teeth was as follows: 2 mm (n = 22), 3 mm (n = 32), 4 mm (n = 14), and 5 mm (n = 3) with a mean PD of 2.97 mm. First, according to PD, subjects were divided into 2 groups: PD <3 mm and PD ≥3 mm. Subjects with PD ≥3 mm had a significantly higher concentration of ammonia than those with PD <3 mm. When subjects were divided according to the presence or absence of BOP, subjects with BOP (+) showed a significantly higher concentration of ammonia than those with BOP (−). In addition, when subjects were divided into three groups according to PD and BOP, subjects with PD ≥3 mm and BOP (+) had the significantly highest ammonia concentration, followed by subjects with PD ≥3 mm and BOP (−) and those with PD <3 mm. In the present study, there was no subject with PD <3 mm and BOP (+).

The minimum pH was in the order of subjects with PD ≥3 mm and BOP (+), PD ≥3 mm and BOP (−) and PD <3 mm and BOP (−) (Table 2), although there was no significant difference in the minimum pH between the subject groups.

In each group of subjects, the ammonia concentration was weakly correlated with the minimum pH (Fig. 1). There was no correlation between the age and ammonia concentration or the minimum pH and age (data not shown).

Discussion

From early studies[6,7], ammonia has been considered to be related to the periodontal status, such as gingivitis and periodontitis. Although the measurement of ammonia in dental plaque has been desired, this is difficult due to the small amount of dental plaque obtainable. By employing the method developed in this study, using a portable ammonia meter originally developed for monitoring the ammonia concentration in blood as an indica-

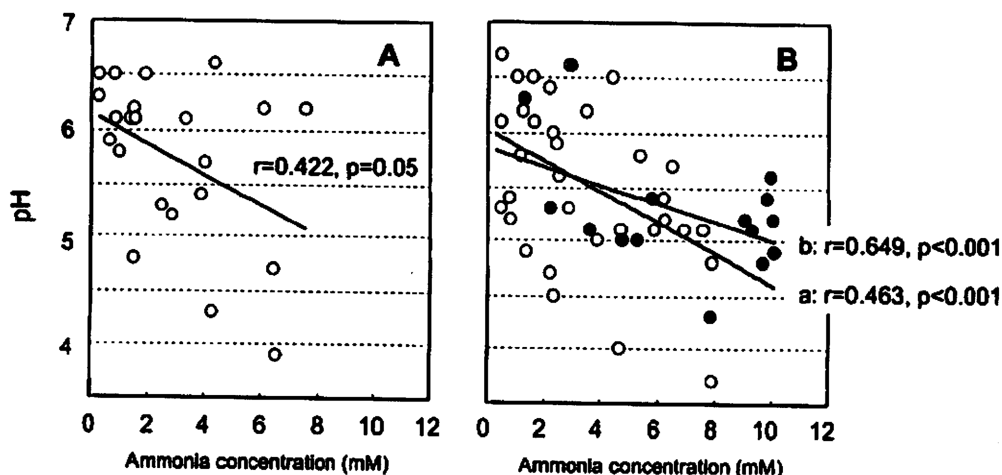

Fig. 1 Correlation between ammonia concentration and minimum pH of subjects with PD <3 mm (A) and PD ≥3 mm (B)
In panel (A), r and p values are indicated. In panel (B), r and p values are indicated for subjects with PD ≥3 mm and BOP (−) (open circle, approximate line a) and subjects with PD ≥3 mm and BOP (+) (closed circle, approximate line b).

tor of renal function impairment, it was possible to measure the ammonia concentration in dental plaque. In addition, it was possible to assess the pH-lowering activity of the same dental plaque sample using a portable pH meter, as described in our previous study[1]. These methods are possibly applicable to chairside measurement.

The ammonia concentration in plaque fluid was reportedly 2 mM[12]–50 mM[13] in previous studies, where plaque fluids were collected by centrifugation from 48-h accumulated whole mouth plaque. Ammonia concentration obtained in the present study was similar to those in previous studies, though the values were relatively low. This might have been due to the method of plaque sampling in the present study, in which the subjects conducted regular tooth brushing the day before sampling and the plaque samples were collected from only marginal areas of teeth. Ammonia concentration obtained in the present study was higher than pure saliva (0.2–0.3 mM)[14,15] and blood (0.011–0.035 mM)[16], supporting that ammonia in dental plaque is mainly produced within dental plaque.

The present study showed that subjects with PD ≥3 mm had a significantly higher concentration of ammonia (Table 1), suggesting that the ammonia level of dental plaque reflects the periodontitis status. This finding supports previous studies in which ammonia was found to be harmful to periodontal tissues[6,7], and indicates the possibility that the ammonia concentration in dental plaque is a risk marker of periodontal diseases, although epidemiological studies investigating the utility of ammonia to predict periodontal diseases are needed.

In vitro studies showed that ammonia exhibits a concentration-dependent growth-inhibiting effect on fibroblasts in the range of 2–20 mM[17] and an inhibitory effect on collagen secretion of fibroblasts even at 2 mM[18], concentrations that are similar to those in most plaque samples in the present study. Although periodontal tissues are probably more resistant to ammonia due to their structural difference from cultured cells, the result of the present study that the ammonia concentration was high in subjects with symptoms of periodontitis suggests that ammonia might affect periodontal tissues persistently and adversely.

The bacteria in marginal plaque produce acids from carbohydrates supplied by food[19,20] while, between meals, the bacteria metabolize salivary components, desquamated epithelium and gingival crevicular fluid[11]. Oral streptococci and *Actinomyces*, the predominant bacteria in marginal plaque[21], contain the enzymes of urease and arginine deiminase that degrade urea and arginine supplied from saliva into ammonia and carbon dioxide, respectively[4,5]. Ammonia production occurs

constantly, and is considered to contribute to maintaining the pH of plaque around neutral[11,22-24]. In addition to ammonia production from urea and arginine, marginal plaque at sites of gingivitis and periodontitis contains proteolytic and/or amino acid-degrading bacteria, such as *Porphyromonas gingivalis, Prevotella intermedia* and *Fusobacterium nucleatum*[21,25], which release ammonia from amino groups of amino acids through the metabolism of proteins and amino acids supplied from saliva, desquamated epithelium, and gingival crevicular fluid. The observation that subjects with BOP (+) exhibited a significantly higher ammonia concentration indicates that bleeding is a factor leading to an increase in the ammonia concentration in marginal dental plaque. Blood components, such as hemoglobin, are bacterial nutrients that stimulate the growth of periodontopathic bacteria, including *P. gingivalis*[26,27], and subsequently enhance bacterial proteolytic and/or amino acid degradation and increase bacterial ammonia production. In the present study, there was no significant difference in the ammonia concentration between subjects with PD $\geq$3 mm/BOP (−) and those with PD <3 mm/BOP (−), although the former showed a higher ammonia concentration than the latter (Table 1). This result suggests that BOP might be more strongly associated with the ammonia concentration than PD itself.

The minimum pH was in the order of subjects with PD $\geq$3 mm and BOP (+), PD $\geq$3 mm and BOP (−), and PD <3 mm, although there was no significant difference between subject groups (Table 2). In addition, the ammonia concentration was weakly correlated with the minimum pH (Fig. 1), suggesting a link between ammonia and acid production in dental plaque. This inverse association may be due to the metabolic characteristics of microbial flora in marginal dental plaque, although a microbiological approach is necessary to clarify this.

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歯肉辺縁部歯垢のアンモニア濃度および pH 低下能：歯周炎部と健全部の比較

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概要：歯垢によるアンモニア産生や pH 低下と齲蝕との関連性はよく知られているが，歯周疾患との関連はあまり知られていない．そこで，歯周炎部と健全部の歯垢中のアンモニア濃度および pH 低下能について比較した．開業歯科医院を訪れた 71 名を対象に上顎第一または第二大臼歯の頬側歯肉辺縁部から歯垢（約 2.4 μL）を採取し，アンモニア濃度およびグルコース添加後の pH 低下（最低 pH）を測定した．また，同部位の歯周ポケット深さ（PD）および bleeding on probing（BOP）を測定し，アンモニア濃度およびグルコース添加後の最低 pH との関連性について比較検討した．歯垢中のアンモニア濃度は，PD≥3 mm，BOP（＋）のグループでは 6.81±3.22 mM であり，PD≥3 mm，BOP（－）のグループ（3.50±2.38 mM）および PD<3 mm，BOP（－）のグループ（2.92±2.23 mM）よりも有意に高かった．最低 pH は，PD≥3 mm，BOP（＋）のグループ（5.28±0.57），PD≥3 mm，BOP（－）のグループ（5.54±0.74），PD<3 mm，BOP（－）のグループ（5.75±0.76）の順に低かったが，各グループ間に統計学的有意差はなかった．しかし，各グループにおいてアンモニア濃度と最低 pH との間に負の相関がみられた．PD≥3 mm，BOP（＋）の歯から採取した歯肉辺縁歯垢中のアンモニア濃度が統計的有意に高かったことから，歯垢中のアンモニアの増加と歯周炎は関連していることが示された．また，アンモニア濃度と最低 pH 値にみられた負の相関は，歯肉辺縁部歯垢細菌叢の代謝特性による可能性が考えられた．

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Resistance to Acidic Environments of Caries-Associated Bacteria: *Bifidobacterium dentium* and *Bifidobacterium longum*

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Key Words

Acid resistance · Bacterial growth · Bifidobacteriaceae · Cell viability · Intracellular pH · Lactobacilli · Streptococci

Abstract

Oral Bifidobacteriaceae, *Bifidobacterium dentium* and *Bifidobacterium longum*, are known to be isolated together with mutans streptococci and lactobacilli from caries lesions, suggesting that these Bifidobacteriaceae are caries associated and acid resistant. This study aimed to investigate effects of acidification on *B. dentium* and *B. longum*, and to compare them with those on *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus paracasei*. Effects of acidification, growth ability in a complex medium at a pH of 4.0–8.0, cell viability in 2-morpholinoethanesulfonic acid monohydrate (MES)-KOH buffer at pH 4.0, as well as stability of intracellular pH (pH_{in}) at an extracellular pH of 3.5–8.0 estimated using a fluorescent dye, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester in MES-KOH, 3-(*N*-morpholino)propanesulfonic acid-KOH or *N,N*-bis(2-hydroxyethyl)glycine-KOH buffer, were investigated. *B. longum* grew as well as *Streptococcus* strains over a wide pH range, whereas *B. dentium* grew best in the narrow pH range around neutral. The cell viability of *B. dentium* decreased significantly after 2 h of acidification at a pH of 4.0, but this was significantly less than that of the *Streptococcus* and *Lactobacillus* species, whereas *B. longum* maintained almost 100% viability. The pH_{in} was close to the extracellular pH at pH of 5.5–7.5 in the *Bifidobacterium* and *Streptococcus* strains, while at a pH of <5.0, the pH_{in} was higher than the extracellular pH in all the strains, but the pH_{in} maintenance ability of *Bifidobacterium* strains was higher than that of the *Streptococcus* strains. The high survival rate and pH_{in} maintenance ability of bifidobacteria comparable to that of *S. mutans* in the acidic environment may account for why bifidobacteria exist as stable species in acidic caries lesions together with mutans streptococci.

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The family Bifidobacteriaceae consists of 7 genera, *Bifidobacterium*, *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia*, *Scardovia* and *Alloscardovia*, which have been isolated from human and animal sources. These organisms are Gram-positive, nonfilamentous, rod-shaped, anaerobic bacteria, and in humans they are members of the microflora of the gastrointestinal tract [Scardovi, 1986]. The genus *Bifidobacterium* consists of around 36 distinct species though not all of these have been isolated from humans, and molecular studies of the human gut

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bifidobacterial flora indicate that there are a large number of undescribed species yet to be isolated and named [Turroni et al., 2009]. Considerable attention has been paid to *Bifidobacterium* species as probiotic bacteria because they may play a beneficial role in the health of their host via regulatory effects on harmful bacteria and intestinal putrefactive substances, and by enhancement of the immune system [Gibson and Wang, 1994; Gill et al., 2001].

Bifidobacteriaceae also inhabit the human oral cavity. Around 10 taxa of the family Bifidobacteriaceae have been isolated from the oral cavity including dental plaque [Modesto et al., 2006], saliva [Hojo et al., 2007; Beighton et al., 2008] and dental caries lesions [Edwardsson, 1974; Sanyal and Russell, 1978; Maeda, 1980; Moore et al., 1984; Hoshino, 1985; Nyvad and Kilian, 1990; van Houte et al., 1996; Becker et al., 2002; Chavez de Paz et al., 2004; Aas et al., 2005; Chhour et al., 2005; Modesto et al., 2006; Mantzourani et al., 2009a, b]. The predominant species from the dentate mouth is *Bifidobacterium dentium*, while *Scardovia genomosp. C1* [Munson et al., 2004], *Scardovia inopinata* and *Parascardovia denticolens* have been isolated exclusively from the oral cavity. Other taxa including *Alloscardovia omnicolens*, *Bifidobacterium breve*, *Bifidobacterium subtile*, *Bifidobacterium adolescentis*, *Bifidobacterium urinalis* and *Bifidobacterium longum* have also been isolated from the human mouth although they may also be isolated from the human gastrointestinal tract or infections. Many of these taxa, especially *B. dentium* and *B. longum*, have been isolated from root caries lesions and from occlusal caries lesions in children and adults [Mantzourani et al., 2009a, b]. These findings indicate that these Bifidobacteriaceae are caries associated and may be acidogenic and aciduric with the ability to survive and proliferate in the acidic environment of caries lesions. Previously, Bifidobacteriaceae, isolated from advanced root caries lesions, were found to reduce a final pH in glucose-containing medium below 4.2 [van Houte et al., 1996]. These findings indicate that Bifidobacteriaceae are among caries-associated acidogenic and aciduric bacteria in the human oral cavity.

Adaptation and resistance to acidic environments are important factors for the survival of *Bifidobacterium* in the intestinal tract and caries lesions. It is well known that the cell-membrane-bound, proton-translocating ATPase (H^+-ATPase) is an important enzyme for maintaining the intracellular pH (pH_{in}) by excreting protons from the cell of several bacteria under acidic conditions [Kobayashi, 1985; Bender et al., 1986; Hamilton and Buckley, 1991]. Besides, it has been reported that H^+-ATPase activity in *Bifidobacterium animalis* and *Bifidobacterium animalis* subsp. *lactis* increased at acidic pH [Gibson and Wang, 1994; Sánchez et al., 2006]. Conversely, Nakajo et al. [2006] demonstrated that *Enterococcus faecalis*, an alkaline-resistant endodontopathic bacterium, exhibited a high cell membrane acid durability, and concluded that this cell membrane acid durability was responsible for the acid resistance of the bacterium. In caries lesions, energy sources such as carbohydrates are not always available, and bacteria living there have to survive in an acidic environment without carbohydrate supply. Under these conditions, mechanisms for acid resistance or acid durability other than H^+-ATPase may be necessary for the survival of oral *Bifidobacterium*.

The purpose of the present study was to investigate the ability of *B. dentium* and *B. longum* to grow, survive and maintain their pH_{in} when subjected to acidic environments in the absence of an extracellular energy source. For comparative purposes, their abilities have been compared to those of representative caries-associated oral bacteria such as *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus paracasei*.

Material and Methods

Bacterial Strains and Culture Media

B. dentium DSM 20436, *B. longum* DSM 2705, *S. mutans* NCTC 10449, *S. sanguinis* NCTC 7865 and *L. paracasei* DSM 2649 were used in this study. These bacteria were cultured on a complex medium [Gauthier et al., 1984] containing 1.7% tryptone (Difco Laboratories, Detroit, Mich., USA), 0.3% yeast extract (Oxoid, Basingstoke, England) and 0.5% NaCl (tryptone yeast extract glucose, TYG, culture media) which was autoclaved before 0.5% glucose and 50 mM potassium phosphate buffer (PPB) were added, using a sterile membrane filter (pore size: 0.2 μm; Pall Corporation, Newquay, UK). This medium was also used as a preculture medium.

Bacterial Growth at Various pH Values

Each strain was inoculated in TYG culture medium and precultured at 37°C over night. The cell culture was transferred (5% inoculum size) to TYG culture media adjusted to a number of pH values in the pH range of 4.0–8.0 with 6 N HCl or 5 N KOH and incubated at 37°C for 48 h. Bacterial growth was conducted in an anaerobic glove box (80% N_2, 10% H_2 and 10% CO_2, NHC type; Hirasawa Works, Tokyo, Japan) and was estimated 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 cell cultures were determined using a pH meter (Model HM-30G; DKK-TOA Corporation, Tokyo, Japan).

Survival of Bacteria at Acidic pH

Each strain was inoculated in TYG culture medium and precultured at 37°C over night. The culture was diluted 1:5 in fresh

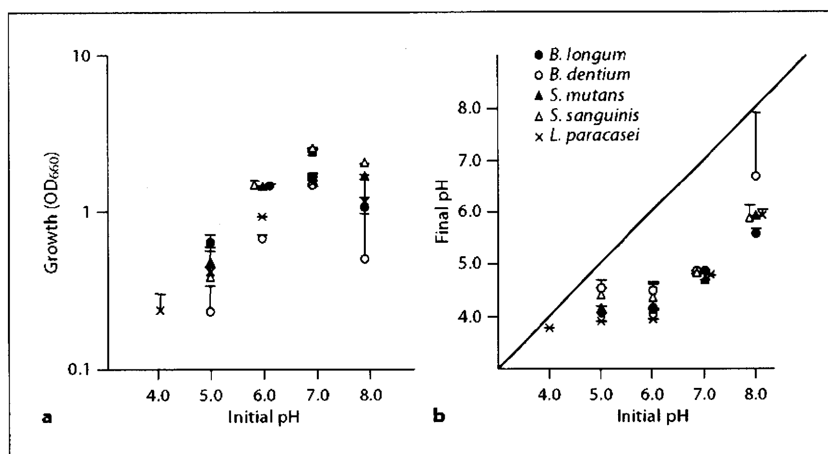

Fig. 1. Bacterial growth (**a**) and final pH of medium (**b**) after 48-hour incubation at initial pH of 4.0, 5.0, 6.0, 7.0 and 8.0 in TYG culture media. Bacterial growth at various initial pH levels and final pH after 48 h in TYG culture media. Bacterial culture in *Bifidobacterium* and *Streptococcus* strains with an OD_{660} of <0.1 at a pH of 4.0 was judged as no growth. Data are presented as means with SD obtained from 3 independent experiments.

TYG culture medium and incubated for an additional 3–6 h in an anaerobic chamber (80% N_2, 10% H_2 and 10% CO_2; MACS-MG-1000 Anaerobic Workstation; Don Whitley Scientific Ltd., Shipley, UK) until an OD of approximately 0.6 at 620 nm, measured using a microplate spectrophotometer (iEMS Reader MF; Labsystems, Helsinki, Finland), was achieved. The culture pH before harvest was >5.5, measured using a portable pH meter (Twin pH; Horiba, Kyoto, Japan). The culture was taken from the glove box, harvested and washed 3 times by centrifugation (21,000 *g* for 3 min at 4°C) in buffer solution (2 mM PPB containing 150 mM KCl and 5 mM $MgCl_2$, pH 7.0). The cell suspensions containing the washed cells (OD_{620} = 0.15), 150 mM KCl and 20 mM 2-morpholinoethanesulfonic acid monohydrate (MES)-KOH buffer (pH 3.8) were adjusted to a pH of 4.0 within 3 min, using 0.1 M HCl or 0.1 M KOH. The suspension was then incubated at 36°C aerobically, and sampled at 0, 1, 2 and 3 h. The samples were serially diluted with PPB (pH 7.0) and spread on TYG agar plates (pH 7.0). The plates were incubated at 37°C for 2–5 days anaerobically and colony-forming units were counted.

pH_{in} of Bacteria at Various pH Values

The pH_{in} was estimated using the method by Breeuwer et al. [1996]. The cell culture of each strain was obtained as described in the subsection Survival of Bacteria at Acidic pH above. The culture was taken from the anaerobic chamber, harvested and washed by centrifugation (21,000 *g* for 3 min at 4°C) in buffer solution, resuspended in the PPB and mixed with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (cFSE; Molecular Probes, Eugene, Oreg., USA) at a final concentration of 1.0 μM and incubated for 15 min at 36°C. The cFSE-loaded cells were collected, washed and resuspended in PPB. Glucose was added at a final concentration of 1 mM and the cell suspension incubated for 30 min at 36°C to eliminate nonconjugated cFSE. The cells were then washed twice, resuspended and incubated for an additional 30 min at 37°C for depletion of intracellular polysaccharide. The cells were pelleted by centrifugation and stored at 4°C until use.

The cFSE-loaded cells were suspended in PPB at an OD_{620} of 5.0. The pH values of the reaction mixtures containing cFSE-loaded cells (OD_{620} = 0.5) were established using 50 mM MES-KOH buffer (pH 3.5–6.5) or 50 mM 3-(*N*-morpholino)propanesulfonic acid-KOH buffer (pH 6.5–7.5) or 50 mM *N,N*-bis(2-hydroxyethyl) glycine-KOH buffer (pH 7.5–8.0) made up in 150 mM KCl. The pH of the reaction mixtures were measured using a portable pH meter (Twin pH).

The reaction mixtures were transferred into microtiter plates and placed on the shaking and thermostatted holder of the microplate reader (Fluoroskan Ascent FL; Thermo Bioanalysis Company, Helsinki, Finland). Fluorescence intensities were measured at excitation wavelengths of 485 and 440 nm, and at an emission wavelength of 525 nm. The 485-to-440-nm ratios were corrected for background signal due to the buffer. The incubation temperature was 36°C. The pH values of the reaction mixtures were redetermined after measuring fluorescence intensity. The values of pH_{in} were calculated using the calibration curve for fluorescence intensity. To obtain the calibration curve, the reaction mixtures were prepared at various extracellular pH values, as described above, and the pH_{in} and extracellular pH were equilibrated by the addition of valinomycin (1 μM) and nigericin (1 μM). The ratios and pH values were determined as described above. Independent calibration curves were prepared for individual experiments.

Statistical Analyses

Difference in cell viability was analyzed by one-way analysis of variance, and significance was examined using the Tukey test. Statistical analysis was performed using Statflex software version 5.0 (Artech, Osaka, Japan). Differences were considered significant at $p < 0.05$.

Results

Bacterial Growth at Various pH Values

The growth of all the bacteria was maximal with an initial medium pH of 7.0, and for each species, the growth decreased as the initial pH of their growth media became more acidic or more alkaline (fig. 1a). The growth of streptococci was higher than those of other bacteria

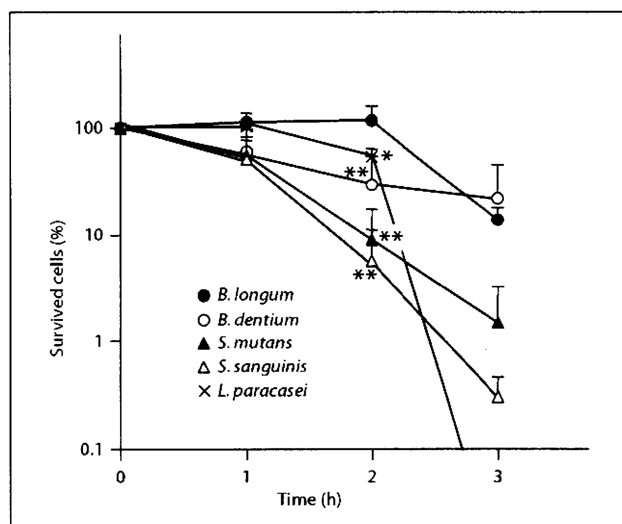

Fig. 2. Effect of acidification at pH 4.0 on cell viability. The mean values with SD of 3 independent experiments are given. * $p < 0.05$, ** $p < 0.01$, relative to the survival rate of *B. longum*.

above a pH of 6.0. However, *B. longum* grew as well as streptococci at a pH of 6.0, and the growth of this strain was relatively high at a pH of 5.0 compared to the other bacteria. The growth of *L. paracasei* was as low as those of both bifidobacteria at a pH of 7.0, but only *L. paracasei* was able to grow at an initial pH of 4.0. Overall, *B. longum* grew well over a wide pH range, whereas *B. dentium* grew best within the narrow pH range around neutral.

The culture pH decreased by 0.0–2.5 pH units during 48 h of growth (fig. 1b). At an initial pH of 7.0, all the bacteria decreased the culture pH to a similar level, i.e. around a pH of 4.65–4.8. *B. longum* decreased the culture pH to the same extent as *S. mutans*. *B. dentium* was less able to grow in the medium so that the decrease in culture pH was less than that observed with the other bacteria.

Survival of Bacteria at pH 4.0

The cell viability of *L. paracasei, B. dentium* and streptococci decreased significantly after a 2-hour acidification at a pH of 4.0, whereas *B. longum* maintained almost 100% viability during the acidification period (fig. 2). However, with incubation extended to 3 h, *B. dentium* and *B. longum* exhibited the greatest ability to survive at a pH of 4.0, with cell viability maintained at 21.9 and 13.7%, respectively. The survival of the other 3 species was poor, with survival of <1.5% after 3 h.

pH_{in} at Various pH Values without Energy Source

When the extracellular pH was above 7.5, the pH_{in} was equal to the extracellular pH in *B. longum*, and slightly lower than the extracellular pH in *B. dentium* and streptococci (fig. 3). When the extracellular pH was between 5.5 and 7.5, the pH_{in} was similar to the extracellular pH in all the strains. When the extracellular pH was between 4.0 and 5.5, the pH_{in} was maintained in *B. longum, B. dentium* and streptococci at 5.5–6.5, 5.25–6.0 and 4.75–5.5, respectively. The pH_{in} of *L. paracasei* could not be estimated because the cFSE was not loaded to bacterial cells of *L. paracasei*.

Discussion

In the present study, *B. longum* was more acid resistant in growth than all the other bacteria tested at a pH of 5.0, while *L. paracasei* was able to grow at a pH of 4.0 (fig. 1a). In contrast, *B. dentium* was less acid resistant in growth than other bacteria. It has been reported that *S. mutans, S. sanguinis* and *L. paracasei* are able to initiate growth at pH of 4.0–5.0, 4.5–5.5 and 3.75, respectively [Harper and Loesche, 1984; Nakajo et al., 2006; Horiuchi et al., 2009]. The present study is consistent with these previous reports, and the first to report the critical pH values for initial growth of *B. dentium* and *B. longum*. All the bacteria were able to decrease the culture pH below 5.0 at an initial pH of 5.0–7.0, indicating that the bacteria, including bifidobacteria, are capable of creating an acidic environment in dental plaque and caries lesions.

Although *B. dentium* was not as acid resistant in the growth studies as *B. longum*, both bifidobacteria were found to be similarly acid durable for 3 h in the cell viability test at a pH of 4.0 (fig. 2), and more acid durable than the streptococci and *L. paracasei*. To survive in acidic environments, bacteria maintain their pH_{in} more alkaline than the extracellular pH. It has been reported that, in oral streptococci, mainly H^+-ATPase functions to maintain pH_{in} against extracellular acidic pH values [Bender et al., 1986; Hamilton and Buckley, 1991]. Matsumoto et al. [2004] also demonstrated that the ability of bifidobacteria to survive an acidic environment was related to an increased H^+-ATPase activity responsible for pumping excess protons from the cytoplasm to the external environment. In the present study, however, there was no energy source available for the H^+-ATPase function during the cell viability test, probably explaining the drastic reduction in viable numbers of streptococci during the 3-hour acidification at a pH of 4.0.

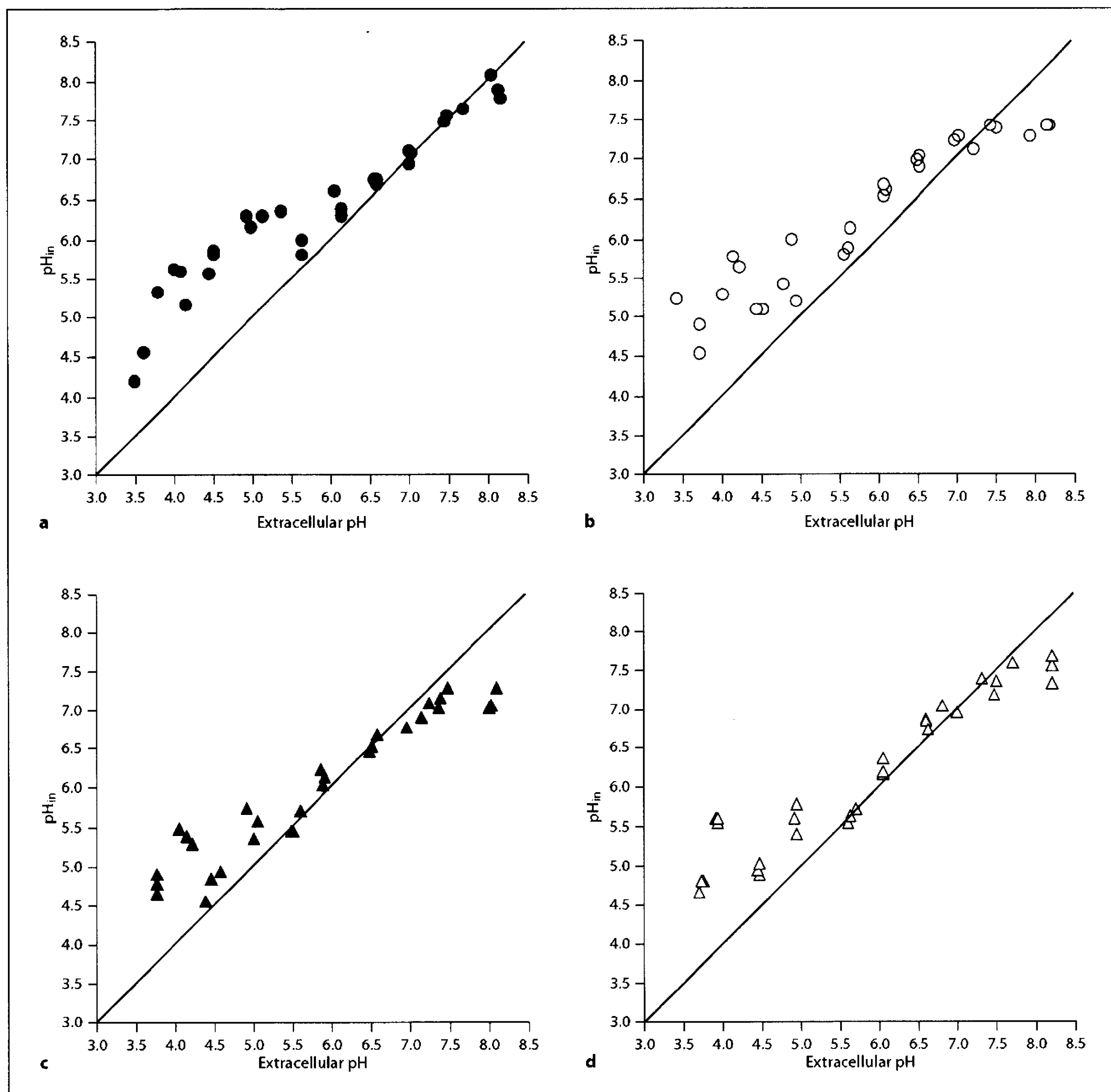

Fig. 3. pH_{in} of *B. longum* (**a**), *B. dentium* (**b**), *S. mutans* (**c**) and *S. sanguinis* (**d**) at different extracellular pH values.

Therefore, *B. dentium* and *B. longum* must possess mechanisms to protect themselves from acidification in the absence of an energy source. In fact, the pH_{in} analyses revealed that *B. dentium* and *B. longum* are more capable of maintaining their pH_{in} than streptococci at acidic extracellular pH without energy source (fig. 3), suggesting the involvement of other mechanisms in pH_{in} maintenance.

In the present study, the pH_{in} of *L. paracasei* could not be measured using cFSE. This might be due to the efflux of loaded cFSE during the pretreatment with glucose because loaded cFSE can be excreted in the presence of

energy sources in a bacterial-strain-dependent manner [Breeuwer et al., 1996]. Meanwhile, *L. paracasei* is known to maintain its pH_{in} in acidic growth medium by the results of radioisotope assays with [^{14}C]benzoic acid [Kashket, 1987; Nannen and Hutkins, 1991], suggesting that this bacterium is capable of maintaining its pH_{in} probably using H^+-ATPase in the presence of energy sources contained in the growth medium. Although the mechanism of the pH_{in} maintenance ability in the absence of energy sources in *L. paracasei* is not clear at this time, the observation that viable cells of *L. paracasei* decreased rapidly at a pH of 4.0 (fig. 2) indicates that *L. paracasei* does not have a high pH_{in} maintenance ability in the absence of an energy source.

Overall, the findings in the present study demonstrate that bifidobacteria possess a high ability to maintain their pH_{in} against extracellular acidification without energy sources, i.e. without an ATP supply, suggesting other mechanisms such as cell membrane durability and intracellular alkaline production. In *S. mutans* [Fozo and Quivey Jr., 2004], *Streptococcus gordonii, Streptococcus salivarius* and *Lactobacillus casei* [Fozo et al., 2004], growth at acidic pH induced alterations in their membrane composition with increased levels of long-chained, monounsaturated fatty acids, suggesting that the acid durability of these bacterial cell membranes could be attributed to the composition of the membrane fatty acids. Besides, it has been reported that the cell membrane of *B. animalis* contained a high level of long-chained, monounsaturated fatty acids [Ruiz et al., 2007]. However, since in this study the culture pH before bacterial harvest was as low as 4.7, the fatty acid composition of bifidobacteria grown at neutral pH was unknown. Nevertheless, one should consider the possibility that the membrane components of bifidobacterial cells are rendered impermeable to protons in acidic conditions. In addition, Sánchez et al. [2007] observed an intracellular accumulation of NH_4^+ in *B. longum*, suggesting that bifidobacteria have an ability to produce NH_4^+ from intracellular substrates such as amino acids. In an attempt to identify the relationship between acid resistance and other phenotypic properties, Collado and Sanz [2007] compared acid-resistant and acid-sensitive *B. longum* and *Bifidobacterium catenulatum* strains. The acid-resistant derivatives showed better ability to grow in the presence of bile salt (1–3%) and NaCl (6–10%), higher resistance at elevated temperatures, higher fermentative ability, and higher enzymatic activities, but were more sensitive to antibiotics than the parental strains. Acid resistance may therefore also be associated with resistance to other stresses. In the bifidobacteria tested in the present study, compositions of cell membrane fatty acids and generation of intracellular NH_4^+, as well as other mechanisms, are possibly responsible for the survival of these bifidobacteria in acidic conditions although, clearly, further studies are needed to clarify the mechanisms underlying bifidobacterial acid resistance.

B. dentium has been isolated more frequently than *B. longum* from dentine caries together with streptococci and lactobacilli [van Houte et al., 1996; Mantzourani et al., 2009a, b] though *B. dentium* was less acid resistant for growth than *B. longum* (fig. 1) and had a similar cell viability and pH_{in} maintenance ability under acidic conditions to *B. longum* (fig. 2, 3) in the present study. Crociani et al. [1994] and Modesto et al. [2006] also demonstrated that *B. dentium* was able to utilize polysaccharides such as amylopectin and amylose for their fermentation, while *B. longum* was not able to utilize these polysaccharides. In addition, the *B. dentium* derived from human caries lesions is able to utilize raffinose, a trisaccharide for growth [Xiao et al., 2010]. These findings suggest that *B. dentium* is at an advantage in the oral cavity where polysaccharides and oligosaccharides are available as additional energy sources. In addition, Mukai et al. [1997] reported that *B. longum* was not capable of adhering to type I collagen consisting of dentine, possibly explaining the low detection frequency of *B. longum* in caries lesions.

In conclusion, the present study demonstrates that *B. dentium* and *B. longum* were more acid resistant with respect to their ability to survive prolonged exposure to low pH. However, *B. dentium* was less able to grow in acidic conditions than was *B. longum* or *S. mutans*, while the ability to control their pH_{in} appeared to be more pronounced in the bifidobacterial species than in the other strains tested. The high survival rate and pH_{in} maintenance ability of both bifidobacteria in the acidic environment may account for the ability to proliferate caries lesions in the presence of streptococci and lactobacilli.

Acknowledgments

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REVIEW (New Strategy of Study for Oral Microbiology!)

Challenge to Metabolomics of Oral Biofilm —From "What Are They?" to "What Are They Doing?" —

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Key words : metabolomics biofilm carbohydrate metabolism

Abstract : There has been extensive bacteriological research into oral biofilm. Most of these studies examined the bacterial composition of oral biofilm and the bacterial species specific to oral diseases. It is thought that the various metabolites, intracellular metabolic intermediates and end-products produced by metabolic activities of oral biofilm directly relate to oral diseases, such as dental caries and periodontitis. However, the metabolic properties, including functional metabolic pathways and their metabolic regulations, have not been clarified sufficiently because the comprehensive identification and quantification of metabolites, that is, metabolomics (or metabolome analysis) are technically difficult. Recently, a new device consisting of capillary electrophoresis (CE) and a time-of-flight mass spectrometer (TOFMS) has been developed, facilitating the metabolomic investigation of sugar metabolism in oral biofilm. The results clearly showed that oral biofilm metabolomics is possible even with a very small sample, and that almost all metabolites in the central carbon metabolic pathways, the EMP pathway, the pentose-phosphate pathway, and the TCA cycle are present in the oral biofilm *in vivo*. Metabolomics using CE-TOFMS may offer a new frame of knowledge to answer the question "What are bacteria doing in oral biofilm?" from a metabolic perspective, and possibly provide insight into the relationship between the metabolic properties of oral biofilm and the etiology of oral disease, as well as the influences of medicines, such as fluoride, or a sugar substitute, such as xylitol, on metabolism of the oral biofilm.

Introduction

There has been extensive bacteriological research into oral biofilm, most of which have asked "What are they?", that is, what kinds of bacteria compose oral biofilm. In particular, bacteria specific to oral diseases have been targeted using culture methods and molecular biological methods[1—5)].

However, "What are they doing?", that is, the metabolic properties including functional metabolic pathways and their metabolic regulations, of bacteria or the entire microflora has not been sufficiently investigated. Bacteria produce a variety of metabolites, including intracellular metabolic intermediates and metabolic end-products. The enzymatic activities related to these metabolites and the resultant various metabolic products may contribute directly to oral diseases. It is known that organic acids produced by

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bacterial sugar metabolism cause dental caries[6], while short-chain fatty acids, ammonia and sulfur compounds produced through bacterial metabolism of proteins and amino acids cause periodontitis[7–9].

In this article, we will introduce a new research concept, "metabolomics, a comprehensive analysis of metabolites" to investigate the metabolic properties of each bacterial species and the entire microflora, and present some preliminary results of the metabolomics of oral biofilm before and after an *in vivo* glucose rinse.

What Is Metabolomics?

In living cells, there are many metabolites, such as phosphorylated sugars, carboxylic acids and amino acids, derived from various metabolic activities. The comprehensive identification and quantification of these metabolites is called "metabolomics" or "metabolome analysis", in which "metabolome" means the comprehensive profile of metabolites. The metabolome is the final product of cellular processes, including the expressions of genes, mRNA and proteins and metabolic enzymatic reactions.

Metabolomics is an omics-research for the next generation, such as genomics, transcriptomics and proteomics, which allows the direct investigation of cellular function. Genomics is the comprehensive analysis of DNA sequences and demonstrates DNA information ; transcriptomics is the comprehensive analysis of mRNA and demonstrates which information is transferred from DNA to mRNA. Proteomics is the comprehensive analysis of proteins translated from mRNA and demonstrates which information is passed from DNA to proteins. However, these three omics-research approaches are insufficient to understand the dynamic reactions in living cells, because the final phenotypic properties are determined by the function of proteins, which are detected as various metabolites, signaling molecules and other factors in the living cells. Metabolomics is essential for covering the information. Recently, metabolomics research has been growing rapidly in various research fields[10–23], from bacterial cells[12–17], such as *Escherichia coli*[12,13] and *Bacillus subtilis*[14,15], to mammalian cells, such as cancer cells of the stomach and large intestine[18,19], and body fluids such as blood[20–22].

Past Metabolomic Approach to Bacterial Metabolism

In the 1960s, Minakami *et al.* succeeded in quantifying the metabolic intermediates of the Embden-Meyerhof-Parnas (EMP) pathway in human red blood cells using a photometry-coupled enzymatic method with purified glycolytic enzyme[24]. This method was then improved and used to quantify metabolites of the EMP pathway in *Streptococcus mutans* in our laboratory[25]. Since then, the metabolite profiles of the EMP pathway in oral saccharolytic bacteria, such as oral *Streptococcus* and *Actinomyces*, have been investigated, and bacterial metabolism and its regulation have been elucidated[25–37]. Furthermore, the inhibitory effects of fluoride and xylitol on bacterial sugar metabolism were investigated, leading to elucidation of the inhibitory mechanisms[38–41].

However, the targeted metabolites were usually limited, because a significant amount of sample (~ 100 μL) was required to analyze only one metabolite ; thus, it was difficult to measure various metabolites in the same sample. For the same reason, it has been impossible to use clinical specimens, such as oral biofilm. This problem has resulted in a missing link between *in vitro* data from bacterial experiments and *in vivo* data on oral biofilm.

In addition, only metabolites in the EMP pathway were targeted, because enzymes to analyze metabolites other than the EMP pathway were not commercially available. Bacterial sugar metabolism could be related with other central carbon metabolism, such as the pentose phosphate pathway and the Krebs tricarboxylic acid cycle (TCA cycle), while bacterial amino acid metabolism, mainly functioning in periodontitis-associated bacteria, could be totally different from sugar metabolism, according to the study of *Porphyromoans gingivalis*, in which metabolic pathways were expected from the detection of metabolic enzymes and the stoichiometry of end-products[42].

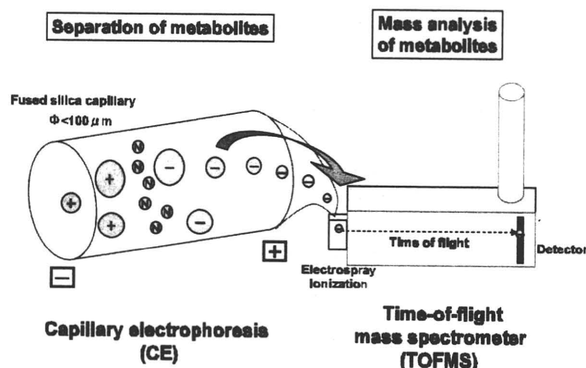

Fig. 1 Conceptual image of CE-TOFMS

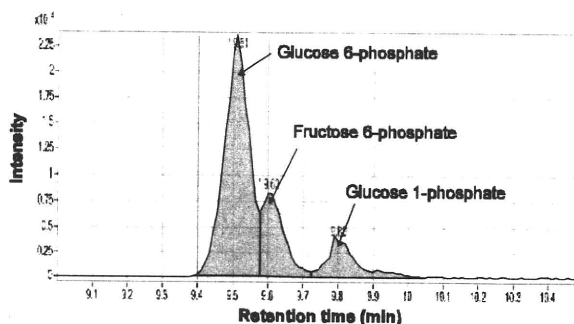

Fig. 2 CE analysis of glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate
These metabolites have the same m/z value, but were differentiated by CE. Representative data from 3 independent experiments are shown, and all data were similar between experiments.

Advancement of Metabolomic Technology

It has long been assumed that it would be technically difficult to analyze metabolites comprehensively and simultaneously within a short time frame due to limits on the separation and identification of metabolites.

In recent years, new devices consisting of capillary electrophoresis (CE) and a time-of-flight mass spectrometer (TOFMS) have been developed[14,43] (Fig. 1). CE is capable of separating small ionic molecules precisely. The majority of metabolites can be separated by CE, since 80—90% are small ionic molecules, such as phosphorylated sugars, carbonic acid and amino acids. It is necessary to identify and quantify these separated metabolites precisely with high sensitivity, and TOFMS has the advantage of accurate mass analysis. CE-TOFMS has resolved the technical difficulties of metabolomics and opened a new era of metabolomics research.

CE-TOFMS

1. CE

Capillary electrophoresis (CE) consists of a microtube with a diameter of 100 μm or less (Fig. 1). Samples are injected into the capillary and a high voltage is applied to both ends of the capillary tube, causing cations to move to the cathode and anions to the anode. In the capillary, the movement velocities of ionic molecules differ according to the ionic radius and the electric charge ; therefore, ionic molecules in the sample can be separated with high resolution.

For instance, using HPLC, it is difficult to separate three phosphorylated glucoses that are structural isomers with the same mass ; glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate ; however, CE can separate these isomers because there is a slight difference in their ionic radii (Fig. 2). In addition, the sample volume required for analysis is only a few microliters due to the very small diameter of the capillary. This is suitable for clinical specimens such as tissues, blood, saliva and oral biofilm. Moreover, by replacing the anode with the cathode at the ends of the capillary, both cationic and anionic metabolites can be separated.

2. TOFMS in combination with CE

TOFMS is a method of mass spectrometry, in which the mass of ions can be measuried very precisely. Metabolites separated by CE are ionized by electrospray, accelerated by an electric field under a high vacuum, and the time that it takes for the metabolite to reach the detector is measured. The velocity of the ion depends on the mass-to-charge ratio (m/z) ; thus, by measuring the flight time, the m/z of the ion is determined precisely (Fig. 1). By comparison with standard molecules, CE-TOFMS can identify and quantify most small ionic metabolites

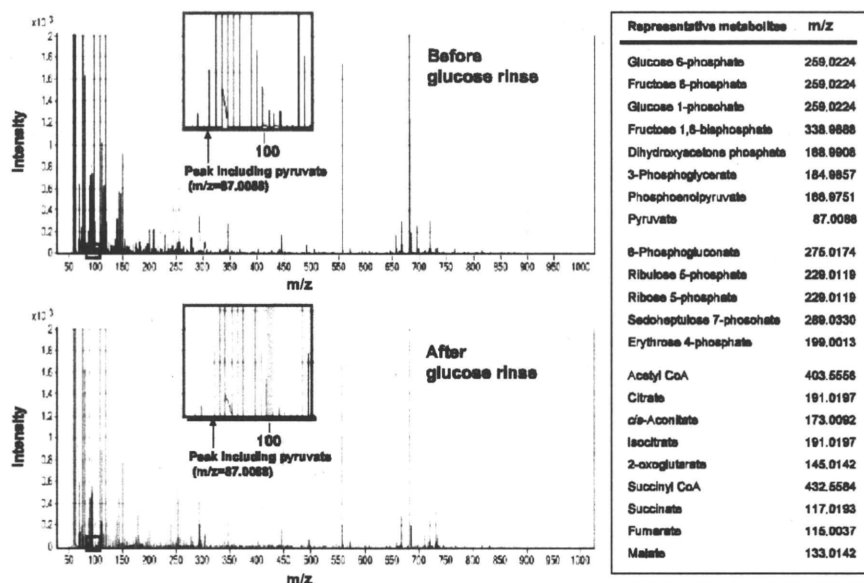

Fig. 3 Metabolome analyzed by TOFMS before and after glucose rinse
Each peak was identified by m/z value using TOFMS. The peaks framed in the small gray box were enlarged, in which the peak with an m/z of 87.0077 included pyruvate. The m/z values of representative metabolites in the central carbon metabolic pathways are also listed.

of living cells.

Metabolomics of Oral Biofilm

1. Preparation of oral biofilm sample

Supragingival plaque was used as the oral biofilm sample. After informed consent was obtained, 3 healthy volunteers were asked to accumulate oral biofilm overnight. Oral biofilm was collected using sterilized toothpicks from half of the dentition of the volunteers before and after rinsing with 10% glucose solution (10 mL) and placed in a pre-weighed plastic tube. It was confirmed that the volunteers had not consumed any food for at least 2 h before sampling. Immediately, samples were weighed with an electronic scale (CP-64, Sartorius, Tokyo), mixed with ice-cold methanol (0.80 mL) containing internal standards (Internal standard solution-1 ; Human Metabolome Technologies) and sonicated (30 s) to extract metabolites from the sample. Furthermore, the samples were mixed with chloroform (0.80 mL) and Milli-Q (0.32 mL) by vortexing (30 s) and centrifugation (2,300 ×*g*, 4℃, 5 min) to remove materials such as phospholipids that may interfere with the analysis. The aqueous layer (500 μL) was filtrated through an ultra-filtration membrane to remove proteins, and the filtrate was dried completely and suspended in Milli-Q water (50 μL) containing internal standards (Internal standard solution-3 ; Human Metabolome Technologies).

2. CE-TOFMS conditions

The sample (5 μL) was analyzed using CE (G1600AX ; Agilent Technologies, Waldbronn, Germany) equipped with TOFMS (G1969A ; Agilent Technologies) in the negative ion mode. Acquired data were analyzed by software (MassHunter WorkStation Software Qualitative Analysis ; Agilent Technologies). In this study, almost all metabolites in the EMP pathway, the pentose-phosphate pathway and the TCA cycle were identified and quantified using data obtained from standard metabolite solutions (Human Metabolome Technologies).

Table 1. Metabolites detected in oral biofilm before and after glucose rinse.

	Metabolite (nmol/mg of oral biofilm)	
	Before (n=3)	After (n=3)
EMP pathway		
Glucose 6-phosphate	0.122±0.041	0.437±0.122
Fructose 6-phosphate	0.030±0.006	0.108±0.036
Fructose 1,6-bisphosophate	0.026±0.013	0.112±0.114
Dihydroxyacetone phosphate	0.037±0.006	0.076±0.014
3-Phosphoglycerate	0.175±0.173	0.116±0.102
Phosphoenolpyruvate	0.090±0.047	0.041±0.014
Pyruvate	0.776±0.446	5.218±3.359
Pentose phosphate pathway		
6-Phosphogluconate	0.008±0.003	0.024±0.008
Ribulose 5-phosphate	0.024±0.017	0.047±0.026
Ribose 5-phosphate	0.009±0.008	0.045±0.035
Sedoheptulose 7-phosohate	0.049±0.020	0.124±0.043
Erythrose 4-phosphate	nd	nd
TCA cycle		
Acetyl-CoA	0.022±0.007	0.043±0.025
Citrate	0.035±0.030	0.016±0.008
cis-Aconitate	0.003±0.004	0.001±0.001
Isocitrate	0.002±0.003	nd
2-Oxoglutarate	0.014±0.013	0.027±0.015
Succinyl-CoA	0.005±0.009	0.007±0.012
Succinate	1.705±0.879	1.674±0.982
Fumarate	0.029±0.050	0.006±0.010
Malate	0.098±0.067	0.054±0.027

Data are presented as the mean±SD.
nd : not detected.

3. Metabolomic Data

The metabolites in the EMP pathway, the pentose phosphate pathway and the TCA pathway were successfully detected and identified comprehensively (Fig. 3), even with a very small amount of oral biofilm (less than 10 mg). Differences in the metabolite profile after the glucose rinse were also detected. In addition, by comparing with data of standard metabolites, the amounts of metabolites were calculated (Table 1).

These data are still preliminary but clearly show that almost all the metabolites in the central carbon metabolic pathways, the EMP pathway, the pentose phosphate pathway and the TCA cycle, are functioning in oral biofilm *in vivo*. These results are similar to previous studies using planktonic cells of *Streptococcus*[25] and *Actinomyces*[36] *in vitro*, though the previous studies focused mainly on the EMP pathway. In addition, there were many unidentified signals that differed after the glucose rinse compared to findings before rinsing (Fig. 3), suggesting that metabolic pathways other than the central carbon metabolism are also functioning in oral biofilm.

Conclusion and the Future Research

Metabolomics using CE-TOFMS not only facilitate the elucidation of the metabolic dynamics of individual bacteria[12–15], but also allow the expansion of our research into how oral bacteria function within the overall oral microflora, that is, the ecosystem of oral

biofilm, including the interaction between individual bacteria and the interaction between bacteria and environmental factors.

There are several types of oral biofilm, supragingival plaque, subgingival plaque, tongue coating and so on in the oral cavity, which are directly related to oral diseases, such as dental caries, periodontitis and oral malodor. Microbiological studies have shown differences in microflora between the types of oral biofilm[44,45], while there is no information about metabolomes except for end-product formation, such as organic acids in oral biofilm. In the future, the metabolomics of oral biofilm may clarify the whole range of metabolic activities in oral biofilm and the difference in metabolic function between oral biofilms. This approach may further clarify the relationship between metabolomes of oral biofilm and the etiology of oral disease, as well as the influences of medicines, such as fluoride, or sugar substitutes, such as xylitol, on the metabolism of oral biofilm. Comparative studies on metabolomes might lead to finding new biomarkers relevant to oral diseases and the establishment of a more efficient and safer protocol for the prevention and treatment of oral diseases.

Metabolomics is useful not only for bacteria or microflora, but is also for analysis of body fluids, including saliva and blood, mammalian cells and tissue from either healthy or unhealthy subjects. Metabolomics using CE-TOFMS offers new possibilities for research and a new frame of knowledge to answer the question "What are they doing?".

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