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NOTE

Detection and identification of non-*Candida albicans* species in human oral lichen planus

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ABSTRACT

***Candida* species were detected and identified in samples from the buccal mucosa, dorsal surface of the tongue and supragingival plaque of subjects with oral lichen planus (OLP). The *Candida* in the samples were cultured on selection agars, and identified by sequence analyses of 18S, 5.8S and 25/28S rRNA. The isolation frequency of *Candida* was higher in subjects with OLP than in those with healthy oral mucosa. Non-*C. albicans* were only isolated from people with OLP. These results support the notion that subjects with OLP are more likely to have oral colonization with *Candida*, and that non-*C. albicans* are specifically present in subjects with this condition.**

Key words Candida, microflora, oral lichen planus, polymerase chain reaction.

Oral lichen planus is a refractory and chronic inflammatory disease which produces white lesions on the oral mucosa and occurs almost exclusively in middle-aged or elderly people. The condition has a long-term, chronic nature, and has been defined by the World Health Organization as a precancerous condition (1, 2). It has been reported that genetic, environmental and lifestyle factors, such as autoimmune diseases, dental materials, medicaments, microbial infection and stress, are associated with the disease (3–5).

In terms of infection with oral microorganisms, *Candida* species has been reported to be associated with OLP (6, 7). Among the *Candida* species, *C. albicans* is the one that has most frequently been identified by culture methods. Recent molecular biological techniques have enabled us to identify *Candida* species other than *C. albicans* (non-*C. albicans*), and have subsequently suggested a relationship between non-*C. albicans* and various infectious diseases (8–12). However, the relationship between non-*C. albicans* and OLP is unknown. Thus, the present study aimed to detect *Candida* species, including non-*C. albicans*, in subjects with OLP by molecular biological methods in order to elucidate any relationships with OLP.

Samples were obtained from patients with OLP (11 women and 4 men; age, 61.2 ± 10.0 years) who were attending the Clinical Department of Oral Diagnosis, Tohoku University Hospital, Sendai, Japan. OLP was diagnosed on the basis of clinical features and histopathological findings. In all cases, the lesions showed the clinical features of the erosive form of OLP. Volunteers with healthy oral mucosa (seven women; age, 51.7 ± 9.8 years) were also examined as controls (Table 1). None of the subjects had received either antibiotics or steroid ointment during the 6 weeks prior to the study, and none were taking immunosuppressants, anticholinergics or cancer chemotherapy agents. Among the 15 patients with OLP, seven (47%) had systemic diseases, namely, diabetes, hypertension and hyperlipidemia (Table 1), but these conditions were well controlled by their regular physicians, and the patients were not chronically immunosuppressed or medically compromised. Furthermore, they had experienced neither periodontitis nor oral candidiasis during

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List of Abbreviations: C. *Candida*; CFU, colony forming units; OLP, oral lichen planus; PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; rRNA, ribosomal RNA.

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Table 1. Clinical features of subjects with oral lichen planus and subjects with healthy oral mucosa in the present study

Subject	Age (years)	Sex	Removable dental appliances	Systemic diseases
Subjects with oral lichen planus				
1	67	Male	–	+‡1
2	75	Female	+†	+‡2
3	77	Female	–	–
4	65	Female	–	–
5	47	Female	–	–
6	52	Female	–	–
7	72	Female	–	–
8	58	Female	–	+‡2
9	47	Female	–	–
10	68	Male	–	+‡1
11	68	Male	–	+‡1
12	59	Female	–	+‡2
13	48	Male	–	–
14	56	Female	–	+‡3
15	59	Female	–	–
Subjects with healthy oral mucosa				
1	59	Female	–	–
2	43	Female	–	–
3	43	Female	–	–
4	52	Female	–	–
5	46	Female	–	–
6	70	Female	–	–
7	49	Female	–	–

†, Removable full (complete) dentures were used by this subject; ‡1, hypertension; ‡2, diabetes; ‡3, hyperlipidemia.

at least the 6 months prior to the study, and had neither salivary gland dysfunction (decrease in saliva secretion) nor smoking habits. Informed consent was obtained from all subjects, and this study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan. Our preliminary calculations using statistical software (Stat Flex Ver. 6, Artic, Osaka, Japan) showed that at least seven subjects were needed for statistical analysis in each group (subjects with OLP and healthy subjects), accordingly 15 patients with OLP and 7 healthy people were selected for the present study.

An area of 1 cm^2 on the surface of the buccal mucosa or rear dorsal surface of the tongue which showed OLP lesions was defined by a window made of sterilized plane paper and firmly scraped 10 times with a sterilized spoon, as described previously (13, 14). In addition, after the sampling sites had been isolated by cotton rolls, samples of supragingival plaque from sites proximal to the upper first molars were collected with sterilized toothpicks.

Each sample (of about 1 mg) was suspended in 1 mL of sterilized 40 mM potassium phosphate buffer (pH 7.0) and dispersed with a Teflon homogenizer. Serial 10-fold dilutions (0.1 mL each) were spread onto the surface of CHROMagar *Candida* (BD, Franklin Lakes, NJ, USA) and Center for Disease Control and Prevention anaerobe 5% sheep blood agar (BD), and incubated at 37°C for 2 days under aerobic conditions, and for 7 days in an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2, respectively.

Sub-cultured colonies of *Candida* isolates on the CHROMagar *Candida* were suspended individually in 1 mL of sterilized water, harvested by centrifugation at 7700 × *g* for 5 min, and the supernatants removed. The genomic DNA of the *Candida* isolates was then extracted from the pellets using the InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions.

The gene sequences of 18 S, 5.8S and 25/28 S rRNA regions were amplified by PCR using fungus-specific primers, primers 1 and 3 (15) and Taq DNA polymerase (HotStarTaq Master Mix, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Primer sequences were: primer 1 (5′- GTC AAA CTT GGT CAT TTA -3′); and primer 3 (5′- TTC TTT TCC TCC GCT TAT TGA -3′) (15). Amplification proceeded using a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed as follows: 15 min at 95°C for initial heat activation and 35 cycles of 30 s at 94°C for denaturation, 30 s at 50°C for annealing, 1 min at 72°C for extension and 10 min at 72°C for final extension. PCR products of 18 S, 5.8S and 25/28 S rRNA regions were digested with *Mwo*I (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Digestion products were separated on 2% agarose gels (High Strength Analytical Grade Agarose, Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM Borate, 1 mM EDTA, pH 8.4), stained with ethidium bromide and photographed under ultraviolet light. The molecular size marker was a 100-bp DNA Ladder (Invitrogen, Carlsbad, CA, USA).

Candida isolates were tentatively identified based on RFLP analysis (15) and representative isolates conclusively identified by sequence analysis as follows. PCR products were sequenced at Hokkaido System Science (Sapporo, Japan) using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (PRISM-3100, Applied Biosystems Japan, Tokyo, Japan). Primer 3, described above, was used to sequence, and the 5.8S rRNA gene sequences were then compared to 5.8S rRNA gene sequence data from the GenBank database using the Blast search program through the web site of the National Center for Biotechnology Information. Phylogenetic analysis of the 5.8S rRNA genes of *Candida* isolates by the neighbor-joining method was performed using DNASIS Pro V2.6 (Hitachi Software Engineering, Yokohama, Japan).

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Fisher's exact probability test, Mann–Whitney's U test, Kruskal–Wallis test, and Tukey's test were used to determine statistical significance. A *P*-value of < 0.05 was considered to be statistically significant.

Among the 15 subjects with OLP, *Candida* species were detected in eight samples from the surface of the buccal mucosa (53%), five from the dorsal surface of the tongue (33%) and eight of supragingival plaque (53%). Meanwhile, among the seven subjects with healthy oral mucosa, *Candida* species were detected in two samples from the surface of the buccal mucosa (29%), one from the dorsal surface of the tongue (14%), and two of supragingival plaque (29%). Overall, the detection frequency of *Candida* species was higher in subjects with OLP ($n = 12$; 80%) than in subjects with healthy oral mucosa ($n = 2$; 29%) ($P < 0.05$).

The mean number of CFU (logarithm CFU/mL) of *Candida* isolates in subjects with OLP was 3.6 ± 4.0, 1.7 ± 2.1 and 2.8 ± 3.2 for the surfaces of the buccal mucosa, dorsal surfaces of the tongue and supragingival plaque, respectively, while, the respective values in subjects with healthy oral mucosa were 1.9 ± 2.2, 1.0 ± 1.4 and 2.1 ± 2.4. Thus, the amounts of *Candida* in subjects with OLP were apparently higher than in subjects with healthy oral mucosa, although the differences were not significant.

On PCR-RFLP analysis targeting the 18 S, 5.8S and 25/28 S rRNA genes, isolates generating restriction DNA fragments of 261, 184 and 141 bp were identified as *C. albicans*, and those generating 414, 174, 171, 86 and 80 bp were identified as *C. glabrata*, as described previously (15). Furthermore, unidentified isolates after PCR-RFLP analysis were subsequently identified as *C. fukuyamaensis* and *C. parapsilosis* by 5.8SrRNA genes sequence analysis (Table 2). Non-*C. albicans*, that is, *C. glabrata*, *C. fukuyamaensis* and *C. parapsilosis*, were detected only in four of the subjects with OLP.

Seven (47%) of 15 subjects with OLP had systemic diseases such as diabetes (20%), hypertension (20%) or hyperlipidemia (7%) (Table 1); in all cases these conditions were well controlled by their regular physicians. Among the *Candida* species, non-*C. albicans* species (78 strains) were more frequently isolated from subjects with OLP and diabetes (51 strains, 65%, $P < 0.05$) or hypertension (14 strains, 18%).

Based on phylogenetic analysis of *C. albicans* and non-*C. albicans* isolates (*C. glabrata*, *C. fukuyamaensis* and *C. parapsilosis*), using the neighbor-joining method, *C. fukuyamaensis* and *C. parapsilosis* were genetically similar to *C. albicans*, while *C. glabrata* was genetically distinct from *C. albicans* (data not shown).

The mean logarithm CFU/mL for bacterial isolates in subjects with OLP was 8.6 ± 9.0, 9.0 ± 9.4 and 8.2 ± 8.3 for the surfaces of the buccal mucosa, dorsal surfaces of the tongue and supragingival plaque, respectively. In subjects with healthy oral mucosa, the respective values were 8.2 ± 8.5, 8.1 ± 8.4 and 8.6 ± 8.8. The amounts of bacteria did not differ ($P > 0.9$) between subjects with OLP and subjects with healthy oral mucosa.

Table 2. Number of isolates of *Candida* species from subjects with oral lichen planus and subjects with healthy oral mucosa

	Subjects with oral lichen planus Number (percentage)	Subjects with healthy oral mucosa Number (percentage)
Buccal mucosa		
C. albicans	412 (93.4)	7 (100.0)
C. glabrata	28 (6.3)	0 (0.0)
C. fukuyamaensis	1 (0.2)	0 (0.0)
C. parapsilosis	0 (0.0)	0 (0.0)
Total	441 (100.0)	7 (100.0)
Dorsal surface of tongue		
C. albicans	5 (21.7)	7 (100)
C. glabrata	12 (52.2)	0 (0.0)
C. fukuyamaensis	4 (17.4)	0 (0.0)
C. parapsilosis	2 (8.7)	0 (0.0)
Total	23 (100.0)	7 (100.0)
Supragingival plaque		
C. albicans	84 (73.0)	76 (100)
C. glabrata	30 (26.0)	0 (0.0)
C. fukuyamaensis	1 (0.9)	0 (0.0)
C. parapsilosis	0 (0.0)	0 (0.0)
Total	115 (100.0)	76 (100.0)

The detection frequency of *Candida* species was significantly higher in subjects with OLP than in those with healthy oral mucosa, thus supporting an association between *Candida* species and OLP (6, 7). The detection frequency and amount of *Candida* species from the dorsal surface of the tongue was apparently lower than from the buccal mucosa and supragingival plaque samples, although this difference was not statistically significant. The environment of the dorsal surface of the tongue is relatively anaerobic because of its anatomical structure, and may therefore support the growth of anaerobes, but not of *Candida* species.

C. albicans was the most predominant of the *Candida* species isolated in the present study (Table 2). The relationship between *C. albicans* and oral mucosal diseases has been reported (7, 16, 17); however, this microorganism is also known to be an opportunistic pathogen and is nonpathogenic to healthy humans in general (9, 11), and was indeed isolated from subjects with healthy oral mucosa in the present study (Table 2). On the other hand, using PCR and sequencing, non-*C. albicans*, that is, *C. glabrata*, *C. fukuyamaensis* and *C. parapsilosis*, were isolated and identified from subjects with OLP, but not from subjects with healthy oral mucosa (Table 2). To our knowledge,

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this is the first study to report the specific isolation of these non-*C. albicans* species from subjects with OLP.

Phylogenetic analysis of *Candida* isolates using the neighbor-joining method revealed that *C. albicans*, *C. fukuyamaensis* and *C. parapsilosis* are genetically similar to one another, while *C. glabrata* isolates, found only in specimens from subjects with OLP, are genetically distinct from these species. The phylogenetic relationship, that is, the great distance between *C. glabrata* and *C. albicans*, is in agreement with previous studies (10, 18–20), suggesting that *C. glabrata* occupies a unique genetic and biological position within *Candida* species.

It has been reported that *C. glabrata*, one of the non-*C. albicans*, is detected more frequently when chemotherapy, such as fluconazole, has been administered to immunocompromised or debilitated patients, such as those with autoimmune diseases (9, 21, 22). However, none of the subjects in the present study suffered from immunosuppressive diseases, and none had received antibiotic or antifungal agents during the 6 weeks prior to the study. Therefore, the occurrence of non-*C. albicans* in the subjects with OLP in the present study supports a possible association between these yeasts and OLP.

C. glabrata has been reported to be less susceptible to killing by human beta-defensins and exhibits various degrees of resistance to the antifungal activity of salivary histatins and mucins (11). In addition, this yeast possesses phospholipase activity, which is capable of promoting destruction of cellular membranes (23, 24). These observations support the possibility that this yeast is pathogenic in OLP, although there is no definite evidence that it can initiate pathogenicity in this condition.

It has been reported that hypoglycemic and antihypertensive agents reduce salivary flow in general (25), and that these medications are associated with a high frequency of isolation of *Candida* species from subjects with OLP (6). However, the subjects in the present study had neither salivary gland dysfunction nor decrease in saliva secretion. In addition, the frequencies and numbers of *C. albicans* were not associated with systemic diseases such as diabetes and hypertension, whereas non-*C. albicans* species were more frequently isolated from subjects with diabetes. These findings suggest that diabetes may be associated with the detection of non-*C. albicans* in subjects with OLP, although further study is required to verify this possibility.

To our knowledge, there have been no reports on the proportions of *Candida* species in oral microflora in subjects with OLP. In the present study, the mean logarithm CFU/mL of bacterial and *Candida* isolates were 8.7 and 3.2, respectively; thus the proportion of *Candida* species in oral microflora of subjects with OLP was approximately 0.0003%. A similarly small percentage of *Candida* species has also been reported in saliva and tongue coating microflora of geriatric edentulous subjects (13, 26). However, because the size of *Candida* cells is estimated to be approximately 1000-times larger than that of bacterial cells, the volume proportion of *Candida* cells among oral microflora of OLP is approximately 0.3%. This proportionate volume of *Candida* cells, irrespective of health- or disease-related properties, may contribute to interactions between bacteria and host tissue through some unique biological characteristics of *Candida* species, including co-aggregation with oral bacteria such as *Streptococcus*, *Actinomyces* and *Fusobacterium* (27–29) and resistance to host antifungal activity (9, 11).

All of the findings of the present study support the notion that subjects with OLP are more likely to be orally colonized with *Candida* than subjects with healthy oral mucosa, and that non-*C. albicans* species are specifically isolated in patients with OLP, particularly those with OLP and diabetes. Further studies, including large-scale studies, are clearly required in order to analyze the association between non-*C. albicans* species and OLP and hence elucidate the potential pathogenic roles of non-*C. albicans* species.

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Metabolomics of Supragingival Plaque and Oral Bacteria

ABSTRACT

Dental caries is initiated by demineralization of the tooth surface through acid production by sugar metabolism of supragingival plaque microflora. To elucidate the sugar metabolic system, we used CE-MS to perform metabolomics of the central carbon metabolism, the EMP pathway, the pentose-phosphate pathway, and the TCA cycle in supragingival plaque and representative oral bacteria, *Streptococcus* and *Actinomyces*. Supragingival plaque contained all the targeted metabolites in the central carbon metabolism, except erythrose 4-phosphate in the pentose-phosphate pathway. After glucose rinse, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and pyruvate in the EMP pathway and 6-phosphogluconate, ribulose 5-phosphate, and sedoheptulose 7-phosphate in the pentose-phosphate pathway, and acetyl CoA were increased. Meanwhile, 3-phosphoglycerate and phosphoenolpyruvate in the EMP pathway and succinate, fumarate, and malate in the TCA cycle were decreased. These pathways and changes in metabolites observed in supragingival plaque were similar to the integration of metabolite profiles in *Streptococcus* and *Actinomyces*.

KEY WORDS: metabolomics, plaque, sugar metabolism, *Streptococcus*, *Actinomyces*.

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INTRODUCTION

Dental caries is initiated by demineralization of the tooth surface through acid production from sugar by supragingival plaque microflora. Biochemical mechanisms of sugar metabolism by plaque bacteria, such as *Streptococcus* and *Actinomyces*, have been investigated for many years, because it is essential to elucidate the metabolic regulation of bacterial acid production, not only to understand caries etiology from a bacterial perspective, but also to develop more effective and safer caries-preventive reagents and protocols.

Metabolomics (metabolome analysis) is the comprehensive identification and quantification of metabolites in biological systems, which is one of the most powerful approaches to metabolism research. In the 1960s, Minakami *et al.* (1965) succeeded in quantifying metabolic intermediates of the Embden-Meyerhof-Parnas (EMP) pathway in human red blood cells by a photometry-coupled enzymatic method using purified glycolytic enzymes. This method was then modified and developed by the authors' laboratory for oral bacteria, including oral *Streptococcus* (Iwami *et al.*, 1975, 1992, 2000; Yamada and Carlsson, 1975a; Iwami and Yamada, 1980, 1985; Abbe *et al.*, 1982; Hata *et al.*, 1990; Takahashi *et al.*, 1991) and *Actinomyces* (Takahashi and Yamada, 1992). Later, Conyers *et al.* (1976) developed thin-layer chromatography using radio-labeled metabolic substrates to quantify glycolytic intermediates, and Thompson and his colleagues (Thompson and Thomas, 1977; Thompson, 1978; Thompson and Chassy, 1983) adopted this method for *Streptococcus lactis*, a species similar to oral streptococci. Furthermore, Ugurbil *et al.* (1978) developed a nuclear magnetic resonance (NMR) method to quantify glycolytic intermediates in intact *Escherichia coli* cells using stable isotopes, and Thompson and Torchia (1984) applied this method to *Streptococcus lactis*. These studies clarified changes in the profile of glycolytic intermediates and enabled us to speculate on the regulatory mechanisms of bacterial glycolysis; however, these methods have long been limited mainly to the EMP pathway, because purified metabolic enzymes for the enzymatic method were not available to determine other metabolites, and both the thin-layer chromatography and NMR methods were not capable of separating and identifying other metabolites.

In the past two decades, metabolomics has developed rapidly, mainly because of the combination of chromatography or electrophoresis for separation with high-resolution and mass spectrometry (MS) for precise identification of biological molecules. Recently, capillary electrophoresis (CE) has been adopted for the separation of metabolites, because most metabolites are polar and ionic small molecules, such as phosphorylated sugars, carboxylic acids, amino acids, and nucleotides. CE-MS is thus suitable for separating and quantifying metabolites in terms of the central carbon metabolism, including the EMP pathway, the pentose-phosphate pathway, and the Krebs tricarboxylic

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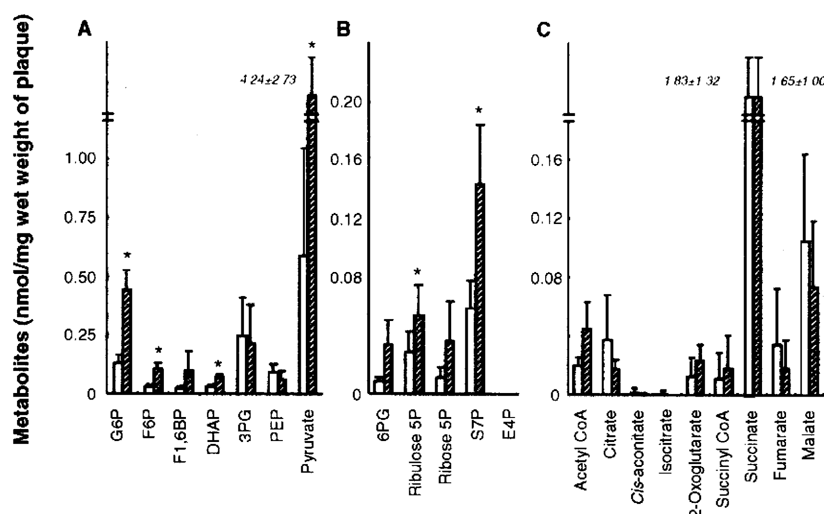

Figure 1. Metabolomics of the EMP pathway **(A)**, the pentose-phosphate pathway **(B)**, and acetyl CoA and the TCA cycle **(C)** of supragingival plaque before and after glucose rinse. Open box, metabolites before glucose rinse; hatched box, metabolites after glucose rinse; vertical bar, standard deviation. Significant difference between before-and-after glucose rinse (*$p < 0.002$). G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6BP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; 6PG, 6-phosphogluconate; Ribulose 5P, ribulose 5-phosphate; Ribose 5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate.

acid cycle (TCA cycle) (Edwards *et al.*, 2006; Monton and Soga, 2007; Timischl *et al.*, 2008; Ramautar *et al.*, 2009). Furthermore, CE-MS requires only a small amount of sample for analysis, leading to the hypothesis that it is applicable for the analysis of human dental plaque and elucidation of the central carbon metabolism in dental plaque.

In the present study, we attempted to perform metabolomics of the central carbon metabolism, the EMP pathway, the pentose-phosphate pathway, and the TCA cycle in supragingival plaque and representative oral bacteria, *Streptococcus mutans*, *Streptococcus sanguinis*, *Actinomyces oris*, and *Actinomyces naeslundii*, using CE-MS, and to elucidate metabolic regulation in dental plaque through the comparison of metabolite profiles between supragingival dental plaque and representative plaque bacteria.

MATERIALS & METHODS

Supragingival Plaque

After informed consent was obtained, three males and two females (age, 25.8 ± 4.6 yrs) were asked to refrain from toothbrushing and to allow dental plaque to accumulate overnight. The volunteers were periodontally healthy, with 0.40 ± 0.45 decayed teeth, and were not taking any antibiotics. After confirming that the volunteers had not consumed any food for at least 2 hrs, we used sterilized toothpicks to collect all the available supragingival plaque from different sides of half of the dentition. Immediately, plaque samples were weighed and mixed with 0.80 mL ice-cold methanol containing internal standards (Internal standard solution-1; Human Metabolome Technologies, Tsuruoka, Japan) and sonicated for 30 sec (55W, US-1R; AS ONE Corporation, Osaka, Japan). The volunteers were asked to rinse with 10% glucose solution (10 mL) for 60 sec, and after 10 min, supragingival plaque was collected from the rest of the dentition, weighed, mixed with 0.80 mL ice-cold methanol, and sonicated for 30 sec.

The samples were mixed well with 0.80 mL chloroform and 0.32 mL Milli-Q water by being vortexed for 30 sec, and centrifuged at 2300 x *g* and 4°C for 5 min. The aqueous layer (500 μL) was distributed into 2 ultrafilter sets (250 μL each) (Ultrafree-MC 5000NMWL UFC3 LCCNB; Millipore, Billerica, MA, USA) and centrifuged at 9100 x *g* and 0°C overnight. The filtrate was dried for 6-9 hrs, suspended in 50 μL Milli-Q water containing internal standards (Internal standard solution-3; Human Metabolome Technologies), and stored at -80°C until analysis.

Internal standard solution-I contains camphor-10-sulfonic acid for calibration of quantification of MS. Internal standard solution-3 contains trimesic acid and 3-hydroxynaphthalene-2,7-disulfonic acid for calibration of retention time for CE.

Bacterial Strains

Streptococcus mutans NCTC 10449, *Streptococcus sanguinis* ATCC 10556, *Actinomyces oris* WVU 627, and *Actinomyces naeslundii* ATCC 12104 were pre-cultured on TYG media containing 1.7% tryptone (Becton Dickinson and Company, Sparks, MD, USA), 0.3% yeast extract, and 0.5% glucose at pH 7 and 37°C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2), and the pre-cultures were transferred to new TYG media (inoculum size, 5%). When the cells reached the logarithmic growth phase (optical density at 660 nm = approximately 1.0), they were harvested by centrifugation, washed twice with 40 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$, and suspended in the same buffer.

The cell suspension was incubated in air at 37°C for 10 min, and glucose was added to the cell suspension at a final concentration of 10 mM. Before and 10 min after glucose addition, 2.0 mL cell suspension (containing approximately 50 mg wet weight of cells) was taken and filtered through a membrane filter (pore size, 0.40 μm; Millipore). The residual cells on the filter were immediately washed twice with 20 mL ice-cold Milli-Q water, and the filter was immersed in 2.0 mL methanol containing internal standards (Internal standard solution-1; Human Metabolome Technologies) in a plastic dish (5-067-2; AS ONE Corporation). The dish was sonicated

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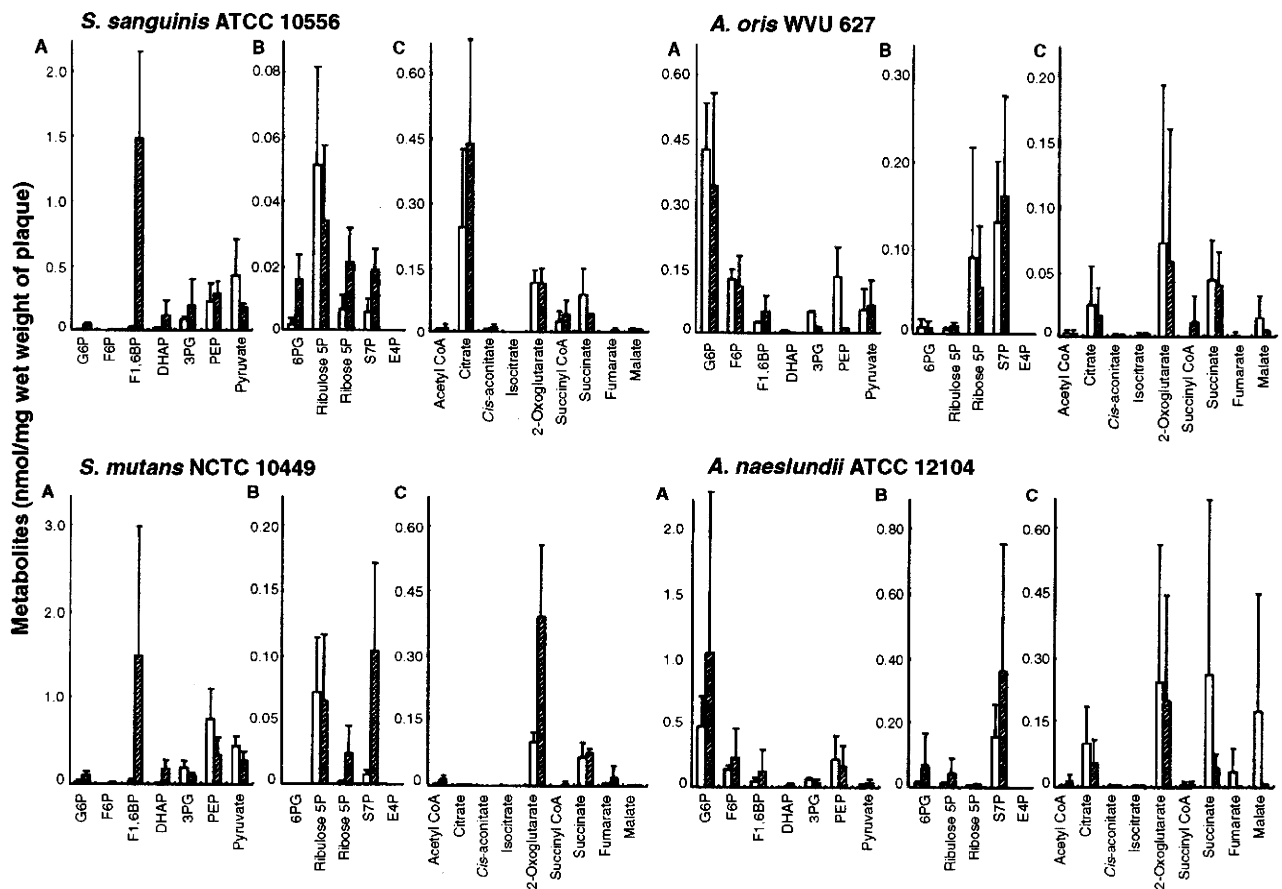

Figure 2. Metabolomics of the EMP pathway **(A)**, the pentose-phosphate pathway **(B)**, and acetyl CoA and the TCA cycle **(C)** of representative plaque bacteria before and after glucose addition. The experiments were performed in triplicate. See the legend to Fig. 1 for abbreviations of metabolites.

for 30 sec (55W, US-1R; AS ONE Corporation) for efficient extraction of metabolites. A 1.6-mL portion of the methanol extracts was mixed well with 1.6 mL chloroform and 0.64 mL Milli-Q water, vortexed for 30 sec, and centrifuged at 2300 x *g* and 4°C for 5 min. The aqueous layer (250 µL) was treated as described above.

CE-MS Conditions

CE-MS was carried out by CE (G1600AX; Agilent Technologies, Waldbronn, Germany) equipped with a time-of-flight mass spectrometer (TOFMS) (G1969A; Agilent Technologies). Separations and detections of metabolites were performed as described previously (Soga *et al.*, 2002, 2003). A fused silica capillary (H3305-2002; Human Metabolome Technologies), sheath liquid (H3301-1020; Human Metabolome Technologies), and electrolytes (H3302-1021; Human Metabolome Technologies) were used for analysis. The applied voltage was set at +30 kV, the electrospray ionization was operated in the negative ion mode, and the capillary voltage was set at 3.5 kV. The flow rate of heated dry nitrogen gas (300°C) was maintained at 7 L/min. All standard metabolites and chemicals used were of analytical or reagent grade. The quantitative error of the CE-MS was less than 10%.

We analyzed the data with calculating software (MassHunter Workstation Software Qualitative Analysis; Agilent Technologies), using data obtained from standard metabolite solutions. All metabolites included in the EMP pathway, the pentose-phosphate pathway, and the TCA cycle were identified and quantified, except glyceraldehyde 3-phosphate, 2-phosphoglycerate (the EMP pathway), and xylulose 5-phosphate (the pentose-phosphate pathway), whose standards were not available. Lactate was also quantified for supragingival plaque samples, while acetate and formate were not, because their mass-to-charge ratio (m/z) values are too small for the present CE-MS.

Statistical Analysis

Differences in the amounts of metabolites before and after glucose addition were analyzed by the paired *t* test. The p value was adjusted from 0.05 to 0.002 based on the Bonferroni correction for multiple comparisons.

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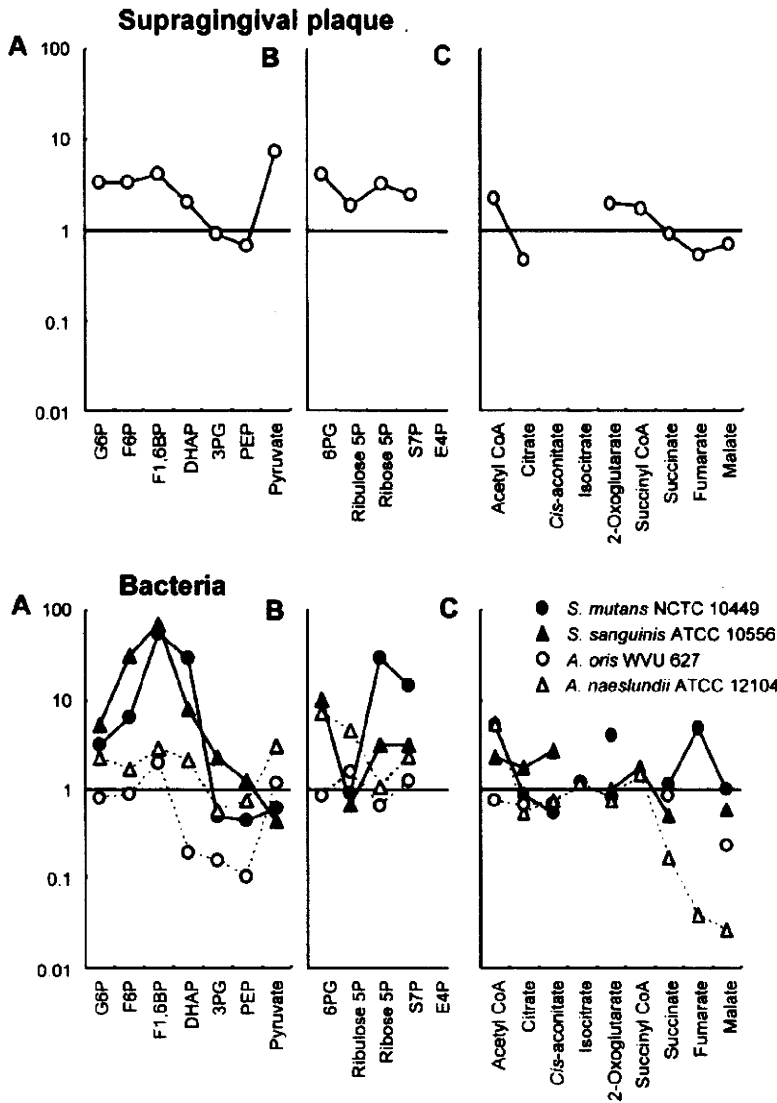

Figure 3. Changes in amounts of metabolites of the EMP pathway (**A**), the pentose-phosphate pathway (**B**), and acetyl CoA and the TCA cycle (**C**) after glucose addition in supragingival plaque and representative plaque bacteria. The rate of change was calculated as (amount of metabolite after glucose addition) / (amount of metabolite before glucose addition). See the legend to Fig. 1 for abbreviations of metabolites.

RESULTS

Metabolomics of Supragingival Plaque before and after Glucose Intake

The CE-MS method applied in the present study was capable of the comprehensive identification and quantification of metabolites in the central carbon metabolism, the EMP pathway, the pentose-phosphate pathway, and the TCA cycle, contained in a small amount of supragingival plaque (mean, 12.3 mg wet weight; range, 5.5-22.8 mg wet weight). The results showed that supragingival plaque contained all the targeted metabolites in the central carbon metabolism, with no detection of erythrose 4-phosphate (E4P) in the pentose-phosphate pathway and low detection of *cis*-aconitate and isocitrate in the TCA cycle (Fig. 1). After the glucose rinse, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (F1,6BP), dihydroxyacetone phosphate (DHAP), and pyruvate in the EMP pathway, and 6-phosphogluconate (6PG), ribulose 5-phosphate, and sedoheptulose 7-phosphate (S7P) in the pentose-phosphate pathway and acetyl CoA increased. Meanwhile, 3-phosphoglycerate (3PG) and phosphoenolpyruvate (PEP) in the EMP pathway and succinate, fumarate, and malate in the TCA cycle decreased. Lactate was also increased from 1.74 ± 0.82 nmol to 13.1 ± 12.7 nmol *per* mg wet weight of plaque by glucose rinse (not shown).

Metabolomics of Representative Oral Bacteria during Glucose Fermentation

S. sanguinis had all the targeted metabolites in the central carbon metabolism, except E4P and isocitrate (Fig. 2). After glucose addition, G6P, F6P and F1,6BP in the EMP pathway, S7P in the pentose-phosphate pathway, and citrate in the TCA cycle increased. *S. mutans* showed a similar profile of metabolites with some specific aspects: a decrease in 3PG and PEP, an increase in 2-oxoglutarate, and no detection of 6PG. *A. oris* and *A. naeslundii* had a similar profile of metabolites, in which G6P and F6P were high regardless of glucose addition, and 3PG and PEP were decreased by glucose addition.

Comparison of Metabolomics between Supragingival Plaque and Bacteria

Changes in metabolite amounts after glucose addition are shown in Fig. 3. Increases in G6P, F6P, and F1,6BP and decreases in PEP in supragingival plaque were similar to those of oral bacteria. Changes in metabolites in the pentose-phosphate pathway and the TCA cycle were also similar between supragingival plaque and plaque bacteria, with some aspects specific to bacterial species.

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DISCUSSION

The present study revealed that the EMP pathway, the pentose-phosphate pathway, and the TCA cycle were functioning in supragingival plaque microbiota *in vivo*. No detection of E4P in the pentose-phosphate pathway and low detection of isocitrate and *cis*-aconitate in the TCA cycle suggest no or low function of metabolic pathways for corresponding metabolites *in vivo*. The metabolomics of plaque bacteria showed a similar metabolite profile, with no or low detection of E4P, isocitrate, and *cis*-aconitate. No detection of 6PG in *S. mutans* was consistent with the previous study, indicating the defect of glucose 6-phosphate dehydrogenase responsible for 6PG formation in *S. mutans* (Brown and Wittenberger, 1971). *S. mutans* was reported to have a part of the TCA cycle, in which 2-oxoglutarate can be formed *via* citrate, isocitrate, and *cis*-aconitate during growth in the absence of glutamate (Cvitkovitch *et al.*, 1997). Meanwhile, *Actinomyces* was suggested to utilize the TCA cycle for complete oxidation of carbohydrates to carbon dioxide and reducing power under highly aerated conditions (de Jong *et al.*, 1988), although enzymatic activity is still unclear for the formation of succinyl CoA from 2-oxoglutarate (Buchanan and Pine, 1965). These previous results were obtained under specific experimental conditions different from those in the oral cavity, and thus further study is needed to elucidate the function of enzymes responsible for the metabolic reactions. Roughly, the metabolite profile in supragingival plaque seemed to share the characteristics of both *Streptococcus* and *Actinomyces*, although there were some exceptions, suggesting cohabitation of these bacteria in supragingival plaque (Kolenbrander, 2000; Ximénez-Fyvie *et al.*, 2000), *e.g.*, high levels of G6P and F6P as found in *Actinomyces*, and high levels of metabolites in the pentose-phosphate pathway as found in both *Streptococcus* and *Actinomyces*.

After glucose rinse, the metabolite profile of supragingival plaque changed markedly, and the changes were similar to those of both *Streptococcus* and *Actinomyces*. The increase in G6P, F6P, and F1,6BP after glucose addition in *S. mutans* and *S. sanguinis* was consistent with that reported in previous studies of streptococcal cells (Thompson and Thomas, 1977; Abbe *et al.*, 1982). The decrease in 3PG and PEP in *S. mutans* indicates that the PEP-dependent sugar phosphotransferase system functioned for glucose uptake (Thompson and Thomas, 1977). The increase in G6P and F6P and the decrease in 3PG and PEP in *A. oris* and *A. naeslundii* were similar to those reported in previous studies (Takahashi and Yamada, 1992). The increase in G6P and F1,6BP observed in *Streptococcus* indicates the activation of glycolytic key enzymes, pyruvate kinase and lactate dehydrogenase, resulting in the acceleration of glycolysis, because G6P and F1,6BP are activators for streptococcal pyruvate kinase (Yamada and Carlsson, 1975b; Abbe *et al.*, 1983), and F1,6BP is an absolute activator for streptococcal lactate dehydrogenase (Brown and Wittenberger, 1972). Upon glycolysis activation, it was revealed that metabolites in the pentose-phosphate pathway were increased, while succinate, fumarate, and malate in the TCA cycle were decreased, indicating mutual regulation between these metabolic systems. These metabolic regulations may also function in supragingival plaque *in vivo*.

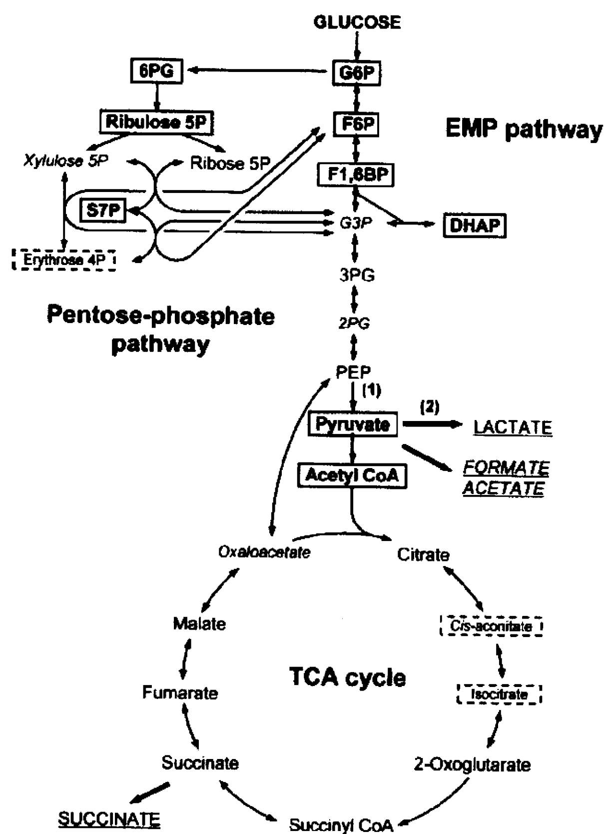

Figure 4. Expected metabolic pathways in supragingival plaque. Metabolites framed by broken lines, no or low detection; metabolites framed by solid lines, increased by glucose rinse; activation of pyruvate kinase (1) and lactate dehydrogenase (2) by G6P, F6P, and F1,6BP; metabolites in italics, not detectable by the CE-MS system in the present study. See the legend to Fig. 1 for abbreviations of metabolites.

The metabolomics approach in the present study revealed that the central carbon metabolism is basically functioning in microbiota of supragingival plaque *in vivo* (Fig. 4), and these pathways are likely similar to the integration of metabolic pathways determined in representative plaque bacteria, *Streptococcus* and *Actinomyces in vitro*. These results suggest that bacterial metabolic studies *in vitro* could be valid for the estimation of *in vivo* bacterial metabolic phenomena if the experimental conditions were well-considered, and also that the metabolomics approach to microbiota, such as dental plaque, may give insight into the whole metabolic system and its mutual regulation within the microbiota. Metabolomics concerning the effects of polyols and fluorides on the acidogenicity of dental plaque is now in progress in our laboratory, in which the metabolic regulatory mechanisms expected from previous *in vitro* studies can be validated *in vivo*.

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ORIGINAL

Autoactivation of Proteolytic Activity in Human Whole Saliva

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Abstract : Proteolytic activity is reportedly present in saliva and seems to play a role in oral diseases such as periodontitis and dental caries. The present study aimed to investigate the autoactivation of proteolytic activity in whole saliva and its influence on salivary proteins. Whole saliva obtained from 10 healthy volunteers (mean age, 27.3±1.5 yr) by chewing paraffin displayed both gelatinolytic (1.21±0.52 unit/mL) and collagenolytic (0.05±0.02 unit/mL) activities using fluorescent-labeled substrates. These activities were partly inhibited by EDTA. Gelatinolytic and collagenolytic activities were significantly increased ($p<0.01$) in whole saliva by incubation at 37℃, and reached 5.6- and 8.8-times the original activities at 12 h, respectively. However, gelatinolytic activities in the supernatant or sediment of whole saliva showed no or low autoactivation. Gelatin zymography suggested that proteases in whole saliva mainly consisted of high-molecular-weight complex forms (>300 and 120 kDa) and a latent form (92 kDa) of matrix metalloproteinase-9 (MMP-9), and that these species were autoactivated at 37℃ and truncated to a 42-kDa protein through 100-, 67-, and 50-kDa proteins. Moreover, SDS-PAGE analysis indicated that salivary proteins in whole saliva were gradually degraded and completely disappeared over 12 h. The present study revealed that whole saliva exhibits mainly gelatinolytic activity, which can be autoactivated in whole saliva and degrade salivary proteins.

Introduction

Host-derived proteases are known to contribute to the growth and turnover of human tissues by degrading extracellular matrix, but are also involved in inflammatory and tumorous diseases[1,2]. Previous studies have indicated that proteases present in the oral cavity are related to the etiology of oral diseases such as dentin caries[3,4] and periodontitis[1,5–8]. The demineralization of dentin is caused by acids produced by oral bacteria[9], while the bacteria isolated from dentinal lesions are incapable of degrading the dentin organic matrix prepared by the acid-demineralization of dentin specimens[10], suggesting the involvement of non-bacterial proteases in the degradation of dentinal proteins. In pH-cycling experiments examining the formation of dentin caries[11], the presence of protease at a neutral pH stage enhanced the formation of artificial dentinal lesions. The observation that matrix metalloproteinase (MMP) inhibitors suppressed caries progression in rats[12] supports the involvement of proteases in dental caries etiology.

Human saliva reportedly displays proteolytic

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activities[13]. In these activities, MMPs, including MMP-8 (collagenase-2), MMP-2 (gelatinase A), and MMP-9 (gelatinase B) have been identified by zymography[3] and immunoassays[14], while non-MMP-type gelatinases have also been found as smear images and weak bands in zymography[3]. In addition, these proteolytic activities were found in dentin caries lesions and dental plaque covering such lesions[15]. These activities are considered to originate mainly from the gingival crevices surrounding the teeth[1,5,13,15–17] and partly from parotid saliva[5], and the responsible enzymes subsequently exist as a mixture in whole saliva. Moreover, similarly to digestive enzymes, salivary MMPs are secreted as latent forms and subsequently activated to active forms by environmental acidification[3].

In most previous studies, supernatants of whole saliva were used to assess salivary proteolytic activity[18], and MMPs included in supernatants were subjected to detailed investigation[3]. Therefore, the present study aimed to measure both gelatinolytic and collagenolytic activities in whole saliva, and to focus attention on their physiological properties such as autoactivation and their influence on salivary components in whole saliva.

Materials and Methods

1. Saliva samples

After informed consent was obtained, whole saliva was collected from 10 healthy male volunteers (mean age, 27.3 ± 1.5 yr) by chewing a sheet of paraffin for 5 min. Immediately after collection, a portion of whole saliva was centrifuged (12,000 rpm, 10 min), and separated into supernatant and sediment. The same volume of deionized water as the separated supernatant was added to the sediment and suspended. A portion of the supernatant was subsequently sterilized using a sterile membrane filter (pore size, 0.22 μm ; PES 33 mm Millex-GP, Nihon Millipore, Tokyo, Japan).

2. Proteolytic activities in saliva samples

Samples of whole saliva and separated fractions were incubated for 0, 1, 2, 4, 6, and 12 h at 37℃, and assayed for collagenolytic and gelatinolytic activities. These activities were measured employing an Enz Check Gelatinase/Collagenase Assay Kit (D-12060/D-12054 ; Molecular Probes, OR, USA) using fluorescent-labeled substrates (DQ-collagen from bovine skin, DQ-gelatin from pig skin)[19]. Saliva samples were pre-incubated for 2 min at 37℃ while stirring in 50 mmol/L Tris-HCl buffer (pH 7.6) containing 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.2 mmol/L sodium azide (final concentration of saliva, 33%). The substrate (40 μL of 500 μg/mL DQ-gelatin or DQ-collagen) was added to the reaction mixture, and the increase in fluorescence resulting from substrate degradation was monitored for 6 min using a fluorescence spectrophotometer (model 650 ; Hitachi, Tokyo, Japan ; excitation wavelength, 485 nm ; emission wavelength, 530 nm). Enzymatic activity was calculated using standard gelatinase (MMP-9, 0.5 units/mL, Life, Yamagata, Japan) as a control. For the inhibition experiment, EDTA was added to the assay mixture at a final concentration of 30 mmol/L, as 30 mmol/L EDTA was sufficient to completely abolish MMP activity[19].

3. Bacterial count in whole saliva

Whole saliva samples incubated at 37℃ for 0 and 12 h were dispersed by a glass homogenizer for 5 min and serially diluted with sterilized potassium phosphate buffer solution (40 mmol/L, pH 7.0). Diluted samples were plated on blood agar (BD, Tokyo, Japan) and skim milk agar plates. Skim milk agar plates contained 0.75% skim milk in FAA (Lab M, Bury, UK) and were used for the identification of proteolytic bacteria in whole saliva[20]. Agar plates were incubated for 7 days at 37℃ under anaerobic (atmosphere : N_2, 80% : H_2, 10% : CO_2, 10%) and aerobic conditions in air, and then colony forming units (CFU) on agar plates were counted.

4. Gelatin zymography

The composition of gelatinolytic activity in whole saliva was determined by zymography[21,22]. Each sample of whole saliva (4 μL) was mixed with 6 μL of sample buffer (4% SDS, 20% glycerol, 0.01% bromophenol blue, and 10 mmol/L EDTA in 0.2 mol/L Tris-HCl at pH 6.8) and electrophoresed, together with a

pre-stained molecular standard (Full-Range ; GE Healthcare Ltd., Tokyo, Japan), on a 6.7% polyacrylamide gel containing 1 mg/mL gelatin. After electrophoresis, gels were washed for 30 min in 50 mmol/L Tris-HCl buffer (pH 7.6) containing 2.5% Triton X-100 and 5 mmol/L $CaCl_2$, and incubated at 37℃ for 36 h in incubation buffer containing 1% Triton X-100, 5 mmol/L $CaCl_2$, and 0.02% NaN_3 in 50 mmol/L Tris-HCl buffer (pH 7.6). Gels were stained with Coomassie Blue R-250 (Bio-Rad) and destained by 10% acetic acid in 40% methanol. For the inhibition of the experiment, 20 mmol/L EDTA was added to the incubation buffer to inhibit the degradation of gelatin by MMPs.

5. Degradation of salivary proteins

Whole saliva samples were incubated at 37℃ for 0, 1, 2, 4, 6, and 12 h, and stored at −20℃ prior to use. After thawing, the samples were centrifuged (12,000 rpm, 10 min), and 5 μL of each supernatant was mixed with 5 μL of deionized water and 10 μL of sodium dodecyl sulfate (SDS)-sample buffer (2% SDS, 6% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue in 50 mmol/L Tris-HCl at pH 6.8). The mixture was boiled for 5 min and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Ready gel-J with resolving gel of 15% T and concentrating gel of 4%C ; Nippon Bio-Rad, Tokyo, Japan ; Mini-Protean 3 Electrophoresis System ; Bio-Rad, Tokyo, Japan). An SDS-PAGE molecular standard (Broad ; Bio-Rad, CA, USA) was used. Following electrophoresis, gels were stained with Coomassie Blue R-250 (Bio-Rad) and destained by acetic acid in methanol. Salivary proteins were identified according to the database of Ghafouri *et al.*[23].

6. Statistical analyses

Data analysis was performed using Stat Flex version 5.0 for Windows (Artec, Osaka, Japan). A paired t-test was applied to the data for bacterial CFUs. The Tukey test was applied to the data on variations in activity over time. The Wilcoxon signed-rank test was applied to the data for gelatinase activity during incubation in whole saliva and sediment.

Results

1. Proteolytic activities in whole saliva and their autoactivation

All whole saliva samples collected from healthy volunteers displayed gelatinolytic and collagenolytic activities, with gelatinolytic activity being over 20 times higher than collagenolytic activity (Fig. 1). Gelatinolytic and collagenolytic activities in the supernatant were 29 and 53% of those in whole saliva, respectively, those activities in filter-sterilized supernatant were 27 and 53%, respectively, while those activities in sediment were 205 and 164%, respectively. Following incubation at 37℃, both collagenolytic and gelatinolytic activities in whole saliva were increased with time and reached 5.6- and 8.8-fold compared to the control at 12 h, respectively (Fig. 1). However, gelatinolytic activities in sediment were increased only 1.5-fold compared to the control at 12 h, while gelatinolytic activities in the supernatant decreased and ceased during incubation (Fig. 1). The presence of 30 mmol/L EDTA inhibited gelatinolytic activity to 42%, and the incubation of whole saliva in the presence of 30 mmol/L EDTA did not increase gelatinolytic activity. During incubation, pH values of all samples were stable at around 7.

2. Bacterial count in whole saliva

Numbers of bacteria recovered from whole saliva under anaerobic conditions before and after a 12-h incubation were $(2.5 \pm 1.1) \times 10^8$ and $(1.7 \pm 0.9) \times 10^8$ CFU/mL, respectively. Under aerobic conditions, bacterial CFUs were $(2.9 \pm 1.0) \times 10^8$ and $(3.7 \pm 0.5) \times 10^8$ CFU/mL. No significant differences in bacterial numbers were seen between before and after incubation under both aerobic ($p = 0.41$) and anaerobic ($p = 0.13$) conditions. Moreover, there was no significant difference in CFUs of proteolytic bacteria in whole saliva determined using skim milk agar plates under anaerobic conditions between before ($(1.1 \pm 0.4) \times 10^7$ CFU/mL) and after $(1.3 \pm 0.7) \times 10^7$ CFU/mL) incubation ($p = 0.75$).

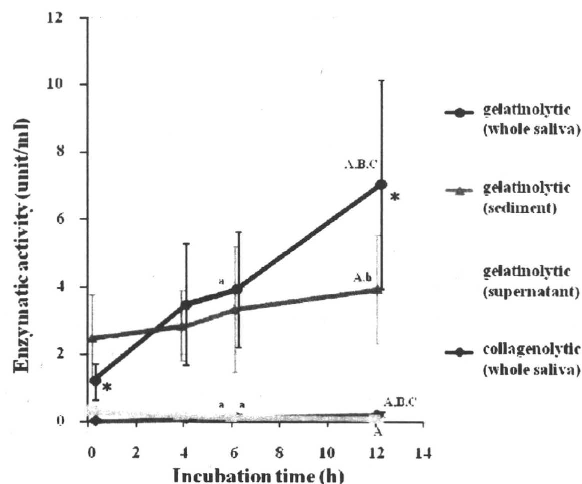

Fig. 1 Time-dependent change of salivary collagenolytic and gelatinolytic activities in whole saliva, supernatant, and sediment

Data are given as the mean ± standard deviation from 5 independent experiments. a and A, significant difference from 0 h ; b and B, significant difference from 4 h ; C, significant difference from 6 h ; a and b, significant difference at $p<0.05$; A, B and C, significant difference at $p<0.01$; *, significant difference from whole saliva and sediment at $p<0.05$.

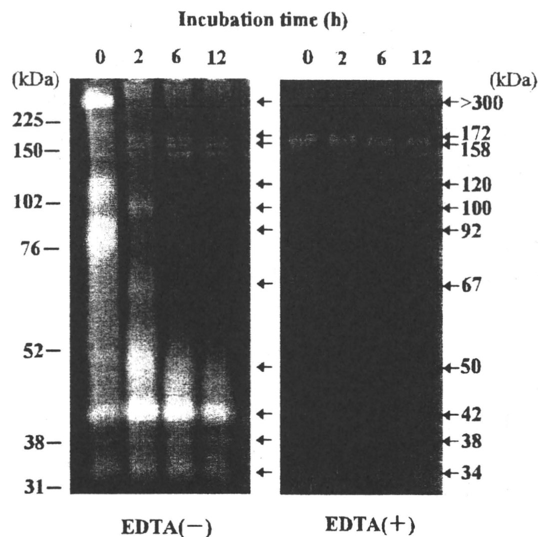

Fig. 2 Time-dependent change of compositon of gelatinolytic enzymes in whole saliva

After electrophoresis, gels were washed with Tris-HCl buffer, and incubated at 37℃ for 36 h in incubation buffer with and without EDTA.

3. Gelatinolytic enzymes detected by zymography

In whole saliva, various gelatinolytic bands were observed (Fig. 2). Major bands appeared at >300, 120, and 92 kDa and a weak band appeared at approximately 42 kDa. The major bands disappeared within 2-h incubation, along with the appearance of smaller bands (100, 67, and 50 kDa) and an increase of the 42-kDa band. These small bands disappeared with the incubation time, while the 42-kDa band remained throughout incubation. In the presence of 20 mmol/L EDTA, no clear band was observed except for weak 172- and 158-kDa bands, which were stable during incubation for 12 h (Fig. 2).

4. Degradation of salivary proteins during incubation

In whole saliva at 0 min, several salivary proteins were shown as clear bands in SDS-PAGE (Fig. 3). These bands gradually faded out with the incubation time and had completely disappeared by 12 h. However, incubation in the presence of 30 mmol/L EDTA repressed the degradation of salivary proteins.

Discussion

Human whole saliva was confirmed to possess gelatinolytic and collagenolytic activities, with the former as the major proteolytic activity (Fig. 1). The salivary supernatant showed a lower proteolytic activity than whole saliva, while the salivary sediment showed higher proteolytic activity than whole saliva, suggesting that the enzymatic properties of saliva were changed by separation.

The presence of 30 mmol/L EDTA inhibited gelatinolytic activity to about half, and the incubation of whole saliva in the presence of 30 mmol/L EDTA did not increase gelatinolytic activity, suggesting that at least half of the activity is derived from MMP-like enzymes, since it is known that 30 mmol/L EDTA is sufficient to completely inhibit MMPs[19]. In addition, EDTA may inhibit the activation system in whole saliva, although further study is needed to clarify this

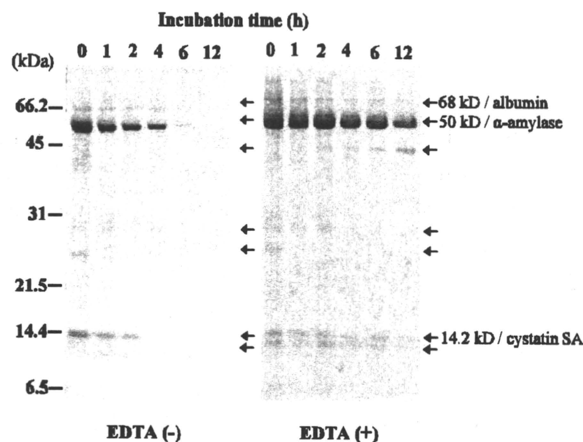

Fig. 3 Time-dependent degradation of salivary proteins
Whole saliva samples were incubated with or without EDTA for 0—12 h. Equal amounts of saliva samples were separated by SDS-PAGE. Arrows indicate salivary proteins.

mechanism.

Both gelatinolytic and collagenolytic activities in whole saliva increased with the incubation time at 37℃ (Fig. 1), indicating that whole saliva contains inactive forms of protease and a system to activate inactive enzymes. Uitto *et al.*[5] demonstrated that preincubation at 22℃ for 3 h increased collagenolytic activity in whole saliva, but longer preincubation decreased such activity. They measured collagenolytic activity by the incubation of whole saliva with ^{14}C-collagen for 18 h and, therefore, the long incubation for 18 h might have influenced the stability of collagenolytic activity. In the present study, proteolytic activity was monitored in real time using fluorescence-labeled substrates, facilitating a precise assessment of the autoactivation of both gelatinolytic and collagenolytic acitivities. Furthermore, gelatinolytic activity was not increased in the supernatant (Fig. 1), suggesting that the activation requires both supernatant and sediment. During incubation, pH values of all samples were stable around 7, excluding the possibility of acid-activation of MMPs, as previously reported[3].

Some oral bacteria are known to possess proteolytic activity[20], and may thus contribute to proteolytic activity in saliva. The numbers of total and proteolytic bacteria in whole saliva were unchanged during incubation in the present study, suggesting that the increase of proteolytic activity during incubation was due to the activation of salivary proteases. However, bacteria in whole saliva might increase and/or release protease during incubation, and, thus, the bacterial contribution needs to be clarified.

Gelatin zymography revealed that whole saliva contains various gelatinolytic enzymes (Fig. 2). High-molecular-weight bands (>300 and 120 kDa) may represent gelatinolytic MMPs in complex with other proteins, such as MMP-9/neutrophil gelatinase-associated lipocalin[24,25], whose molecular weight is 120 kDa, although non-MMP-type gelatinases can also be involved. The appearance of smaller proteins (100- and 67-kDa proteins) during 2-h incubation and the subsequent disappearance of these proteins and maintenance of 42-kDa protein (Fig. 2) suggest the molecular size shift of gelatinolytic enzymes from >300-, 120-, and 92-kDa proteins to 42-kDa protein *via* 100-, 67-, and 50-kDa proteins by the autoactivation of gelatinolytic MMPs. It has been reported that 67- and 92-kDa proteins are an active form[26] and a latent form of MMP-9[27], respectively, while 50- and 42-kDa proteins are truncated forms of the enzyme[3,28]. These observations indicate that salivary gelatinolytic activity is mainly attributed to MMP-9 and not to bacterial proteases. Mäkelä *et al.* demonstrated that, in the presence of a reducing agent, mercaptoethanol, high-molecular-weight complexes (> 200 kDa) were converted to 68—72-kDa proteins, which resembled an active form of MMP-2[7], suggesting that the weak 67-kDa band detected by zymography after 2-h incubation (Fig. 2) might represent an active form of MMP-2 converted from high-molecular-weight complexes.

Acid-activation of salivary gelatinase with a molecular size shift from 135- and 92-kDa proteins to 82-kDa protein has been reported in salivary supernatants[3]; however, in the present study, gelatinolytic activity was activated without acidification and the molecular sizes of enzyme species were different, suggesting the presence of an independent autoactivation system of proteolytic activities in whole saliva, which may not function in supernatants separated from whole saliva. Further study is needed to clarify

the activating system of salivary proteases, including bacteria-saliva interaction.

On the other hand, 172- and 158-kDa proteins seem to belong to non-MMP-type enzymes because of their resistance to EDTA (Fig. 2), and these results were consistent with the observation of weak but stable gelatinolytic activity in the presence of EDTA.

All salivary proteins contained in whole saliva gradually disappeared during incubation (Fig. 3), suggesting that proteolytic activity in saliva can degrade salivary proteins. The repression of protein degradation by EDTA was consistent with the inhibition of proteolytic activity detected using a fluorescent substrate. Salivary proteins are generally accepted to have several beneficial effects on the oral cavity, such as anti-microbial effects (peroxidase, secretary IgA, lactoferrin, histatin, *etc.*), mucosal protection (mucin, cystatin, *etc.*) and the remineralization of tooth surfaces (statherin, proline-rich protein, *etc.*)[29]. However, salivary proteases might degrade these beneficial proteins if saliva is retained in the oral cavity for a long time.

In conclusion, the present study revealed that whole saliva contains mainly gelatinolytic enzymes, which can be autoactivated in whole saliva by incubation at 37℃ around a neutral pH, with the molecular shift of proteolytic enzymes. This autoactivation of salivary proteases means that when saliva remains in the oral cavity for a long time, particularly in specific areas such as marginal and interproximal sites of teeth, the interface between dentures and mucosa, and cavitated carious lesions, proteolytic enzymes in saliva can be autoactivated. Furthermore, the activated enzymes might degrade host-defense proteins contained in saliva, and further contribute to the degradation of host proteins, such as collagenous proteins contained in dentin and gingival tissue, once these host tissues are impaired and compromised. These mechanisms might induce and/or promote dentin caries, including root surface caries and periodontal disease, such as gingivitis and periodontitis, as previous studies have suggested[16,17].

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Original

Ammonia Concentration and pH-lowering Activity of Marginal Dental Plaque from Teeth with and without Periodontitis

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Abstract: While the pH-lowering activity and ammonia productivity of dental plaque are known to be associated with caries, the association with periodontal diseases is not clear. Thus, the pH-lowering activity and ammonia concentration of dental plaque were compared between teeth with and without periodontitis. Plaque samples (approximately 2.4 μL) were obtained from marginal areas of sound buccal surfaces of upper first or second permanent molars of 71 subjects, and the probing depth (PD) and bleeding on probing (BOP) of the same tooth were recorded. Samples were measured for minimum pH after glucose addition and ammonia concentration. The ammonia concentration in subjects with PD ≥3 mm and BOP (+) (6.81 ± 3.22 mM) was significantly higher than in subjects with PD ≥3 mm and BOP (−) (3.50 ± 2.38 mM) and subjects with PD <3 mm and BOP (−) (2.92 ± 2.23 mM). The minimum pH was in the order of subjects with PD ≥3 mm and BOP (+) (5.28 ± 0.57), PD ≥3 mm and BOP (−) (5.54 ± 0.74), and PD <3 mm and BOP (−) (5.75 ± 0.76), although there was no significant difference between subject groups. In addition, the ammonia concentration was weakly correlated with the minimum pH in each subject group. Marginal plaque from teeth with PD ≥3 mm and/or BOP (+) contained a significantly higher concentration of ammonia, indicating that periodontitis increases the ammonia level in marginal plaque. The inverse association between the ammonia concentration and minimum pH may be due to the metabolic characteristics of microbial flora in marginal dental plaque.

Key words: Ammonia concentration, pH-lowering activity, Dental plaque, Pocket depth, Bleeding on probing

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Introduction

Dental plaque, microflora consisting of marked numbers and species of bacteria, is a metabolic system that produces many kinds of metabolites, such as lactic acid and ammonia, and subsequently these metabolites influence host tissues and the host defense system. Acid production from carbohydrates by dental plaque has a long history of study as a risk marker of dental caries. Acid production can be evaluated as a pH drop after the addition of carbohydrates to dental plaque, and the association between caries activity and the pH-lowering activity of dental plaque from sound enamel surface was shown in preschool children[1]. In addition, the activity of urease and the arginine deiminase system in dental plaque, the main systems for ammonia production, are reportedly associated with caries resistance[2,3]. This seems to be reasonable because ammonia production is able to counter acid production in dental plaque[4,5]. These previous studies clearly indicate that the caries status is associated with the metabolic activity of dental plaque in the oral cavity, and suggest that the pH-lowering activity and ammonia productivity are candidates for caries risk markers.

On the other hand, the association between the metabolic activity of dental plaque and the periodontal status

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