が良くなったと表5の歯磨き習慣が悪くなったという相反する質問の選択に矛盾がないかを確認するものである。その結果、3つの組合せのいずれにおいても相反する質問の両方を選択しているものは存在しなかった。この点からすれば分析の正当性が担保されたといえる。

子どもに変化があったと認識しているのは有意（$p<0.0001$）に少なかった（表3）。そのなかで、表4から9までの相反する質問に対する選択割合は、歯磨き習慣が悪くなったが0.3％で良くなったが10.7％、歯の色が白くなったが 3.3％で黒くなったが0.3％、口内炎ができやすくなったが 1.1％でできにくくなったが3.0％であった。

これらの質問を設定したのは、フッ化物洗口を実施することによって、フッ化物に頼りすぎて歯磨き習慣などがおろそかになるという心配、歯のフッ素症が生じるという心配、口内炎などの粘膜への副作用の心配があるからである。日弁連では、「保育所、幼稚園、小学校、中学校、特別支援学校等で実施されるフッ素洗口・塗布には安全性、有効性、必要性・相当性、使用薬剤・安全管理、追跡調査、環境汚染に関してさまざまな問題点が認められるにもかかわらず、行政等の組織的な推進施策の下、学校等で集団的に実施されている。これによって、個々人の自由な意思決定が阻害され、安全性・有効性、必要性等に関する否定的見解も情報提供されず、プライバシーも保護されないなど、自己決定権、知る権利及びプライバシー権が侵害されていることから、厚生労働省、文部科学省、各地方自治体及び各学校等の長に対し、学校等で集団的に実施されているフッ素洗口・塗布を中止するよう求める。」という意見書[4]を提出した。

しかし、今回のフォローアップ調査からは、現実には歯磨き習慣などがおろそかになる、歯のフッ素症が生じる、口内炎などの粘膜への副作用が生じるということは認められなかった。

今後は調査規模を拡大し、継続してフォローアップしていくべきである。

E. 結論

フッ化物洗口実施後の安全性確認のフォローアップの一環として、歯科保健習慣や健康への影響について質問紙調査を実施した結果、歯磨き習慣などがおろそかになる、歯のフッ素症が生じる、口内炎などの粘膜への副作用が生じるということは認められなかった。今後は調査規模を拡大し、継続してフォローアップしていくべきである。

F. 文献

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G. 研究発表

なし

表 1　質問紙の内容

質問	回答肢
1. 小学校でフッ素洗口事業を行っていることを知っていますか？	1. 知っている 2. 知らない 3. 知っているが行わせていない
2. 小学校でのフッ素洗口事業によると思われるお子さんの変化について、お気づきの点があればお選び下さい（複数回答可）	1. とくにない 2. 歯みがき習慣が良くなった　3. 歯みがき習慣が悪くなった 4. 歯の色が白くなった　5. 歯の色が黒ずんだ 6. 内炎などができにくくなった　4. 内炎などができやすくなった 8. その他にお気づきの点があれば記入して下さい （　　）

表 2　小学校でのフッ素洗口事業実施の認知度

	度数	相対度数（%）	有意性
1. 知っている	910	93.5	p＜0.00001
2. 知らない	58	6.0	
3. 知っているが行わせていない	5	0.5	

表 3　フッ素洗口事業実施による子ども変化の有無

	度数	相対度数（%）	有意性
1. とくにない	807	82.9	p＜0.0001
2. ある	166	17.1	

表 4　フッ素洗口事業実施によって歯磨き習慣が良くなった

	度数	相対度数（%）	有意性
1. 選択	104	10.7	p＜0.0001
2. 非選択	869	89.3	

表5　フッ素洗口事業実施によって歯磨き習慣が悪くなった

	度数	相対度数（%）	有意性
1. 選択	3	0.3	p＜0.0001
2. 非選択	970	99.7	

表6　フッ素洗口事業実施によって歯の色が白くなった

	度数	相対度数（%）	有意性
1. 選択	33	3.3	p＜0.0001
2. 非選択	944	94.7	

表7　フッ素洗口事業実施によって歯の色が黒くなった

	度数	相対度数（%）	有意性
1. 選択	3	0.3	p＜0.0001
2. 非選択	970	99.7	

表8　フッ素洗口事業実施によって口内炎などができにくくなった

	度数	相対度数（%）	有意性
1. 選択	29	3.0	p＜0.0001
2. 非選択	944	97.2	

表9　フッ素洗口事業実施によって口内炎などができやすくなった

	度数	相対度数（%）	有意性
1. 選択	11	1.1	p＜0.0001
2. 非選択	962	98.9	

表10　フッ素洗口事業実施によって変化に気付いたこと

改善点	人数	悪化点	人数
むし歯になりにくくなった	9	歯が黄ばんできた	2
むし歯ができない	4	洗口液の味がまずい	1
むし歯の進行が停止している	1	歯科医院の定期健診に行かなくなった	1
歯がツルツルになった	1		
自分の歯に興味をもつようになった	1		

研 究 成 果 等

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Effects of the antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) on bacterial viability and metabolism

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The antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) is a strong bactericide when unpolymerized and has the potential to be utilized in various resinous biomaterials. To analyze the antibacterial characteristics of this monomer in detail, the ability of high concentrations of unpolymerized MDPB to kill *Streptococcus mutans* in planktonic or biofilm forms within a short time-period of contact, and the inhibitory effects of low concentrations of MDPB on the metabolic function of *S. mutans*, were examined. High concentrations of MDPB showed effective killing of planktonic and biofilm *S. mutans* cells within 60 s, and complete killing was obtained by contact with 1,000 $\mu g\ ml^{-1}$ of MDPB for 60 s. At a concentration of 4–8 $\mu g\ ml^{-1}$, MDPB demonstrated growth inhibition, inducing elongation of the lag phase and of the doubling time, when the bacterial number was low. Inhibition of the production of acid from *S. mutans* by 8 $\mu g\ ml^{-1}$ of MDPB may have been caused by the inhibition of lactate dehydrogenase activity. At high concentrations, MDPB is lethal to both planktonic and biofilm forms of *S. mutans* in a short time-period, and at low concentrations, MDPB inhibits metabolic enzymatic activity.

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Control of bacterial infection is a key factor to obtain healing where biomaterials are used in a tissue-associated environment. The antibacterial resin monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) is a polymerizable bactericide synthesized by combining a quaternary ammonium with a methacryloyl group (1). When unpolymerized, MDPB acts as a disinfectant because it is an analogue of quaternary ammonium compounds (QACs). In addition, the antibacterial component of the molecule is covalently bound to the backbone of the polymer by copolymerization with other monomers, thus providing an antibacterial component pendantly immobilized in the polymer network (2). The antibacterial component immobilized by the polymerization of MDPB inhibits the growth of contacting bacteria (2, 3). Therefore, incorporation of MDPB is also advantageous to achieve an antibacterial surface, without adverse effects to the physical properties of their carrier materials in a wet environment.

Unpolymerized MDPB has been proven to show strong bactericidal effects against various oral bacteria associated with dental caries (4). Intensive studies have been carried out with the aim of incorporating MDPB into the components of dental adhesives, and the world's first antibacterial dental adhesive system has been successfully commercialized (4). Owing to its unique antibacterial and polymerizable characteristics, MDPB has the potential to be utilized in various resinous biomaterials, in both dental and medical fields.

However, to utilize MDPB under optimum conditions in various resin-based materials in diverse medical fields, further detailed analysis of its antibacterial activity is necessary. Quaternary ammonium compounds (QACs) interact electrostatically with bacterial membranes, which are negatively charged, and exert their effects through membrane damage, resulting in the leakage of intracellular components (5, 6). Therefore, MDPB is expected to be able to show rapid bactericidal effects. In addition, MDPB may interfere with bacterial growth and metabolic function, even at low concentrations, as acid production by various oral streptococci has been reported to be inhibited by low concentrations of the cationic antimicrobial chlorhexidine (7, 8), whose primary mechanism of action is membrane disruption, similarly to that of QACs (9).

The aims of this study were to examine the ability of unpolymerized MDPB to kill *Streptococcus mutans* in planktonic or biofilm forms with a short time-period of contact and to assess the inhibitory effects of low concentrations of MDPB on the activity (such as growth or acid production) of *S. mutans*.

Material and methods

Minimum inhibitory concentration/minimum bactericidal concentration measurements

The minimum inhibitory concentrations/minimum bactericidal concentrations (MICs/MBCs) of unpolymerized MDPB against *S. mutans* NCTC10449 were measured using microdilution methods, as described previously (1, 10), using brain–heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.5% yeast extract (Becton Dickinson) for culture. For comparison, the MIC/MBC values of two resin monomers, 2-hydroxyethyl methacrylate (HEMA; purity 99.8%; MITSUBISHI RAYON, Tokyo, Japan) and 10-methacryloyloxydecyl dihydrogen phosphate (MDP; purity > 90%; Kuraray Medical, Tokyo, Japan), and two antimicrobials, cetylpyridinium chloride (CPC; Wako, Osaka, Japan) and chlorhexidine diacetate (CHX; Sigma, St Louis, MO, USA) were measured. The experiments were performed in triplicate.

Rapid bactericidal effects against planktonic cells with a short time-period of contact

S. mutans NCTC10449 was adjusted to approximately 1×10^4, 1×10^5, or 1×10^6 colony-forming units (CFU) ml^{-1} in 0.01 M PBS, pH 7.4. To 90 μl of bacterial suspension, 10 μl of aqueous solution of unpolymerized MDPB was added to give final concentrations of 10, 50, 250, 500 or 1,000 $\mu g\ ml^{-1}$. After the bacteria had been in contact with MDPB for 20, 40 or 60 s with agitation, the suspension was diluted ×100 by the addition of 9.9 ml of PBS. In the case of 500- and 1,000-$\mu g\ ml^{-1}$ concentrations of MDPB, the solution was further diluted ×10 immediately with PBS. Five portions of 100-μl aliquots of diluted suspension were inoculated onto BHI agar plates, and the number of viable bacteria was counted after 48 h of anaerobic incubation of the plates at 37°C. The tests were repeated three times for each concentration of MDPB and bacterial suspension.

Rapid bactericidal effects against biofilm cells with a short time-period of contact

To prepare biofilm samples, collagen discs (13.5 mm in diameter, Sumilon cell tight C-1 cell disk LF; Akita Sumitomo Bake, Akita, Japan) were placed in the wells of a 12-well microplate, and 3 ml of *S. mutans* NCTC10449 suspension at 1×10^6 CFU ml^{-1} was inoculated and incubated anaerobically for 24 h at 37°C. To the biofilm formed on the collagen disc, 45 μl of 500 or 1,000 $\mu g\ ml^{-1}$ MDPB solution was applied. After 20, 40 or 60 s, the collagen discs were washed with distilled water, and bacterial viability was immediately assessed by staining (11). The staining solution was prepared by mixing 3 μl of SYTO9 and 1.5 μl of propidium iodide (LIVE/DEAD *Bac*Light Bacterial Viability kit L7012; Molecular Probes, Eugene, OR, USA), diluted with 1 ml of distilled water. The surfaces of the discs were treated with 90 μl of the staining solution for 15 min, rinsed with distilled water, and observed by fluorescence microscopy. Filters with two wavelengths (FITC, EX 465–495 nm; G-2A, EX 510–560 nm; Nikon, Tokyo, Japan) attached to a fluorescence microscope (Nikon ECLIPSE E600; Nikon) were used. The green/red area was measured on three randomly selected images with ×1,000 magnification, using image-analysis software (Sciam image; Sciam Corporation, Fredrick, ML, USA), and the ratio of dead cells to whole cells was calculated as follows:

$$\%\ \text{Dead cells} = (D/L + D) \times 100,$$

where D is the area stained red and L is the area stained green.

Inhibitory effects on bacterial growth by low concentrations of MDPB

One-hundred microlitres of *S. mutans* NCTC10449 suspension was added to 10 ml of BHI broth, containing 0.5% yeast extract and MDPB, to achieve a bacterial density of 1.0×10^3 CFU ml^{-1}, and incubated at 37°C. One-hundred-microlitre aliquots were taken periodically – every 2 h until 24 h; every 4 h from 26 to 46 h; and after 48, 60, 72, and 96 h of incubation – and bacterial growth was monitored by measuring the optical density (OD) at 550 nm using a spectrophotometer (Model 680 Microplate Reader; BioRad, Hercules, CA, USA). The concentrations of MDPB tested were 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 $\mu g\ ml^{-1}$, and the experiments were performed in triplicate.

Inhibitory effects on bacterial metabolism by low concentrations of MDPB

To assess the effects of low concentrations of MDPB on bacterial metabolism, inhibition of acid production by *S. mutans* NCTC10449 was evaluated (12). The *S. mutans* suspension was adjusted to an OD of 0.1 at 660 nm (1×10^8 CFU ml^{-1}) using 2 mM potassium phosphate buffer, pH 7.0, containing 150 mM KCl and 5 mM $MgCl_2$ (KPB). A reaction mixture containing 2.7 ml of bacterial suspension was preincubated at 37°C, and the pH was adjusted to 7.0. After 4 min of preincubation, 0.2 ml of 30, 60, or 120 $\mu g\ ml^{-1}$ of MDPB solution and 0.1 ml of 10 mM glucose were added, and anaerobic incubation at 37°C was continued for a further 30 min during which acid production was monitored by titration with 6 mM potassium hydroxide (KOH) using a pH-stat system (AUT-211S; Toa Electronics, South San Francisco, CA, USA). The final concentrations of MDPB in the reaction mixture were 2.0, 4.0, and 8.0 $\mu g\ ml^{-1}$. All procedures were conducted under anaerobic conditions in the anaerobic chamber. The experiments were performed in triplicate.

Acid-production rates were determined according to the following formula:

$$\text{Acid-production rate} = (X/C) \times 100,$$

where X is the volume of KOH needed for titration in the presence of MDPB and C is the volume of KOH needed for titration of the control without MDPB.

The end products, including glutamic, lactic, formic, pyruvic, and acetic acids, produced by glucose fermentation, were analyzed as described previously (13). To 1 ml of reaction mixture, 0.1 ml of 0.6 M perchloric acid was added at the end of the acid-production tests, filtered through a polypropylene membrane (pore size 0.20 μm; Toyo Roshi, Tokyo, Japan), and assayed using a carboxylic acid analyzer (Eyela S-3000; Tokyo Rika, Tokyo, Japan).

S. mutans cells, at a concentration of 1×10^8 CFU ml^{-1} in KPB, were preincubated at 37°C for 4 min. Then, MDPB solution at 2.0, 4.0, or 8.0 $\mu g\ ml^{-1}$ and 10 mM

glucose were added, and the pH values were monitored after anaerobic incubation at 37°C for 30 min, 1 h, and 2 h using a pH-measurement device (Sindengen ISFET pH KS723; BAS, Tokyo, Japan). The tests were repeated three times.

Statistical analysis

Statistical significance of the data obtained on rapid bactericidal effects, acid-production rates, the amount of end products, and pH measurement were analyzed using ANOVA with a *post hoc* Fisher's protected least significant difference (PLSD) test at a significance level of 0.05.

Results and Discussion

MIC/MBC measurement

The MIC and MBC values of MDPB and other compounds tested are shown in Table 1. 12-Methacryloyloxydodecylpyridinium bromide was confirmed to have strong antibacterial activity against *S. mutans*, showing much lower MIC/MBC values than the two other resin monomers, HEMA and MDP. 2-Hydroxyethyl methacrylate is a hydrophilic monomer, frequently used as a dentin primer to promote the impregnation of resin components into demineralized dentin (14). 2-Hydroxyethyl methacrylate has been reported to have low toxicity towards various cell types (15), and did not inhibit the growth of *S. mutans*, even at 25 mg ml^{-1}. 10-Methacryloyloxydecyl dihydrogen phosphate is an acidic monomer that is also utilized in dental adhesives (14), and it is considered to exhibit antibacterial activity owing to the low-pH environment it produces. However, its effects against *S. mutans* were much smaller than those of MDPB. The acidity of MDP was reduced by the buffering action of the BHI broth to a level where no significant antibacterial activity was found. Cetylpyridinium chloride and CHX are strongly biocidal against oral bacteria, especially Gram-positive species, including *S. mutans* (16, 17), and are used as oral disinfectants or in various oral hygiene products. While the antibacterial activity of MDPB was lower than that of either CHX or CPC, its effects were comparable with those of other bactericides (such as triclosan) that are included in dentifrices and in mouthrinses (18).

Rapid bactericidal effects against planktonic cells with a short time-period of contact

Figure 1 demonstrates the number of viable cells after contact with MDPB. The initial bacterial numbers before the addition of MDPB were determined to be 1.44×10^5, 2.36×10^4, and 1.07×10^3 CFU. The number of surviving cells decreased with the increase in the concentration of MDPB, regardless of the initial number of bacteria. At a density of 1.44×10^5 CFU of bacteria, contact with 250, 500, and 1,000 $\mu g\ ml^{-1}$ of MDPB significantly reduced, for all contact times, the number of recovered cells compared with the control ($P < 0.05$, Fig. 1A). In the case of 2.36×10^4 CFU of *S. mutans*, the number of

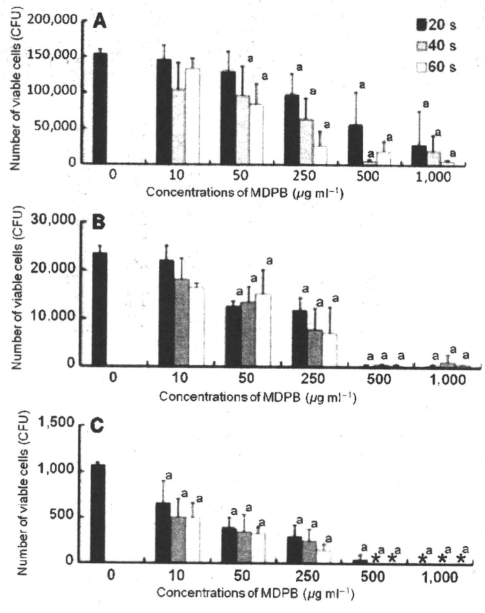

Fig. 1. The number of viable *Streptococcus mutans* NCTC10449 after contact with 12-methacryloyloxydodecylpyridinium bromide (MDPB) solutions for 20 s (black bars), 40 s (grey bars) or 60 s (white bars). Initial cell number: (A) 1.44×10^5 colony-forming units (CFU), (B) 2.36×10^4 CFU, and (C) 1.07×10^3 CFU. For the control, 10 μl of distilled water was used. *No recovery of viable cells. The bars represent the SD of three replicates. a: significantly different from the control (0 $\mu g\ ml^{-1}$ of MDPB) for each bacterial concentration (ANOVA and Fisher's PLSD test, $P < 0.05$).

Table 1

Minimum inhibitory concentration/minimum bactericidal concentration (MIC/MBC) values of resin monomers, including 12-methacryloyloxydodecylpyridinium bromide (MDPB) and antimicrobials against Streptococcus mutans NCTC10449

Resin monomer/antimicrobial	Abbreviation	MIC ($\mu g\ ml^{-1}$)	MBC ($\mu g\ ml^{-1}$)
12-Methacryloyloxydodecylpyridinium bromide	MDPB	7.8	125
2-Hydroxyethyl methacrylate	HEMA	> 25000	> 25000
10-Methacryloyloxydecyl dihydrogen phosphate	MDP	250	> 250
Cetylpyridinium chloride	CPC	0.98	15.6
Chlorhexidine diacetate	CHX	3.91	31.3

The same end-point was obtained for three repetitions of the experiments for all materials.

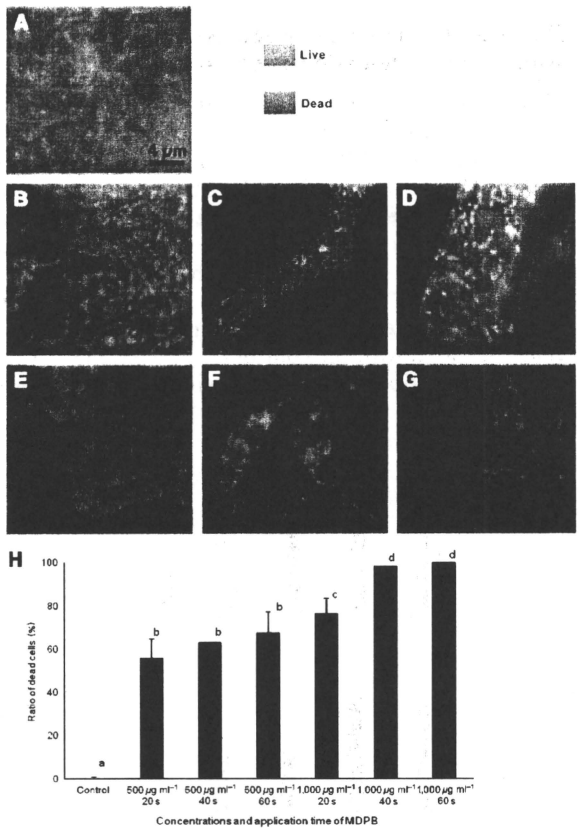

Fig. 2. Fluorescence microscopic images of *Streptococcus mutans* NCTC10449 biofilm stained with viability staining solution, and the ratio of dead cells after contact with 12-methacryloyloxydodecylpyridinium bromide (MDPB). (A) Control (application of distilled water), (B) 500 μg ml^{-1} of MDPB, 20 s, (C) 500 μg ml^{-1} of MDPB, 40 s, (D) 500 μg ml^{-1} of MDPB, 60 s, (E) 1,000 μg ml^{-1} of MDPB, 20 s, (F) 1,000 μg ml^{-1} of MDPB, 40 s, (G) 1,000 μg ml^{-1} of MDPB, 60 s, and (H) ratio of dead cells (%) in biofilm after application of MDPB. Different letters show a significant difference (ANOVA and Fisher's PLSD test, $P < 0.05$). The bar represents the SD of three measurements.

viable cells was significantly decreased by MDPB at concentrations of ≥ 50 μg ml^{-1} at all contact times ($P < 0.05$, Fig. 1B). 10-Methacryloyloxydecyl dihydrogen phosphate exhibited significant killing of 1.07×10^3 CFU of *S. mutans* at all concentrations ($P < 0.05$, Fig. 1C).

IMAZATO *et al.* (4) demonstrated that a dentin primer containing 5% MDPB could kill *S. mutans* in suspension and in demineralized dentin within a short clinical application time of 30 s. These effects are considered to be mainly dependent upon the bactericidal activity of the incorporated MDPB, and unpolymerized MDPB may also exhibit such a rapid killing action. Time–kill studies for planktonic *S. mutans* with MDPB, using longer contact times than in the present study, have previously been performed (10). The results of these studies indicated that 100 μg ml^{-1} of MDPB was not sufficiently potent to completely kill 1×10^6 CFU ml^{-1} of planktonic *S. mutans* in a 100-μl solution (equivalent to 1×10^5 CFU in the present experiment), even after 480 min of contact, but 250 μg ml^{-1} of MDPB could effectively kill all cells in 5 min. Another study, which evaluated the killing effectiveness of MDPB using a viable cell-staining method, reported that 1×10^8 CFU ml^{-1} of *S. mutans* was killed by 10 min of contact with 250 μg ml^{-1} of MDPB (11). The present results indicate that an MDPB concentration of 250 μg ml^{-1} is not capable of eradicating *S. mutans* completely within a short time-period of 60 s, but the higher concentration of 1,000 μg ml^{-1} is sufficient to provide rapid killing effects. As QACs have a positive charge, they can interact rapidly with the bacterial cell surface, which is negatively charged. The test results, using *S. mutans* suspensions, support the possibility of rapid interactions of bacterial cells with MDPB, a derivative of QACs (19).

Rapid bactericidal effects against biofilm cells with a short time-period of contact

We analyzed the biofilm from the bottom to the surface by controlling the focus. Representative images of biofilms stained using a LIVE/DEAD viability staining kit after treatment with MDPB are shown in Fig. 2. After application of 500 μg ml^{-1} of MDPB for 20 s, almost half of the cells were stained green (viable) and the other half were stained red (dead). Exposure to 500 μg ml^{-1} of MDPB for longer periods of time resulted in an increase in the red area (Fig. 2B–D). At a concentration of 1,000 μg ml^{-1}, MDPB killed most of the cells in the biofilm after 20 s of contact (Fig. 2E), and red staining of all areas was observed following 60 s of contact (Fig. 2G).

The ratios of dead cells to live cells after contact with MDPB solutions are shown in Fig. 2H. A significant reduction in the number of viable bacterial cells was obtained for all cases of MDPB application ($P < 0.05$). In the presence of 500 μg ml^{-1} of MDPB, 56.2, 63.2, and 67.8% of the cells (mean values) were dead after 20, 40, and 60 s of contact, respectively. However, there were no significant differences among these three groups ($P > 0.05$). Application of MDPB at 1,000 μg ml^{-1} for 20 s resulted in significantly greater killing effects than 500 μg ml^{-1} of MDPB ($P < 0.05$), with only 23.4% of the cells remaining viable. No green cells were observed in any microscopic fields after treatment with 1,000 μg ml^{-1} of MDPB for 60 s, indicating 100% killing.

Bacterial cells in biofilms are known to be less susceptible to antimicrobials than planktonic microorganisms (20). It has been reported that the application of an antimicrobial component at a concentration 100 times higher than the MIC for planktonic cells could not effectively kill biofilm *S. mutans* cells (21). Several factors are considered to be related to the difference in susceptibility of planktonic and biofilm bacteria to antimicrobials (22). One is the hindrance of penetration of antimicrobials into the biofilm owing to the presence of a copious amount of extracellular matrix, which acts as a molecular sieve. A second factor is that the chemical microenvironment, such as nutrient-depletion or waste

products within the biofilm, may act as antagonists of antimicrobials. A third, and still speculative, hypothesis is that some of the bacteria may differentiate into a protected phenotypic state. In the present study, application of 1,000 μg ml^{-1} of MDPB for 60 s was proven to be able to kill the cells in biofilm at a rate similar to that found for the planktonic bacteria. SANDT *et al.* (23) reported that the hydrophobic nature of QACs was associated with penetration into biofilms. They compared the penetration of CPC and similar molecules with different lengths of alkyl chains into *S. mutans* biofilm and found that the hydrophobic CPC (with longer alkyl chains) demonstrated greater penetration. The hydrophobic nature of MDPB provided by the 12-alkyl chain increased its affinity to exopolysaccarides to enable penetration all the way through the biofilm, of about 20 μm thickness, prepared in this study. The sparse structure of biofilms (i.e. without a dense extracellular matrix) produced under the present experimental conditions may have helped the penetration of MDPB.

Inhibitory effects on bacterial growth by low concentrations of MDPB

Figure 3 shows the growth curves of *S. mutans* incubated in the presence of unpolymerized MDPB. At low concentrations (below the MIC value determined using the standard method) MDPB was found to induce growth inhibition and elongation of the lag phase or the doubling time when small numbers of bacteria (such as 10^3 CFU ml^{-1}) were used. At 1.0–2.0 μg ml^{-1}, MDPB is considered to have caused some damage to the membrane, thereby resulting in the elongation of the lag phase. However, the damage was not severe and bacterial cells showed similar growth ability to the control once they started to divide, reflected by the minimal effect on the doubling time. The elongation of the lag phase in the presence of 4.0–8.0 μg ml^{-1} of MDPB may have been caused by a reduction in bacterial numbers at the start of the experiment. It is clear that bacterial proliferation was inhibited at these concentrations of MDPB because the growth curve showed a decrease. The MIC value for MDPB, determined by the standard method using 1×10^6 CFU ml^{-1} of bacteria, was 7.8 μg ml^{-1}. In the experimental setting using fewer bacteria (10^3 CFU ml^{-1}) and 4.0–8.0 μg ml^{-1} of MDPB, it is possible that MDPB, which initially did not bind to the bacteria, acted further on divided cells.

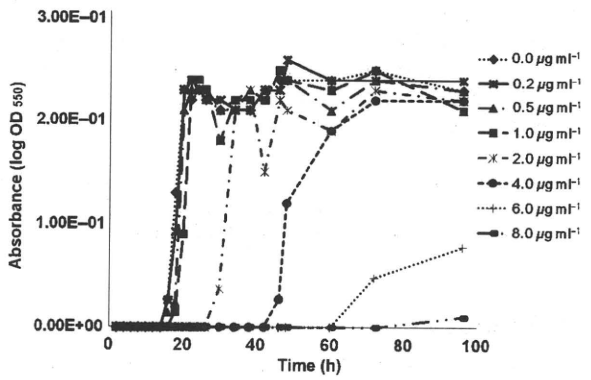

Fig. 3. Growth of *Streptococcus mutans* NCTC10449 in the presence of 12-methacryloyloxydodecylpyridinium bromide (MDPB). The bacterial suspension containing MDPB was incubated at 37°C and the growth was monitored by measuring the optical density (OD) of the aliquot at 550 nm. The *S. mutans* suspension, without the addition of MDPB, served as a positive control, and the experiments were performed in triplicate.

Inhibitory effects on bacterial metabolism by low concentrations of MDPB

The production of acid by *S. mutans* was inhibited by low concentrations of MDPB (Fig. 4A). After 10 and 15 min, the presence of 8.0 μg ml^{-1} of MDPB reduced the acid-production rates to 81.3 and 74.7% of the control levels, respectively, while 2.0 or 4.0 of μg ml^{-1} MDPB reduced these rates by 1–7%. After 30 min of incubation, the presence of 4.0 μg ml^{-1} of MDPB decreased the acid-production rate by 14%, but no significant difference was observed compared to the control. The presence of 8.0 μg ml^{-1} of MDPB resulted in a

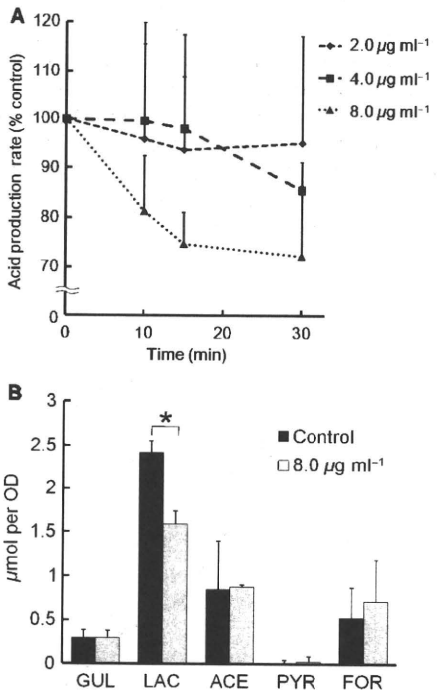

Fig. 4. Acid-production rates and the amount of end products in the presence of 12-methacryloyloxydodecylpyridinium bromide (MDPB). (A) Relative acid-production rate in the presence of low concentrations of MDPB. Acid production of *S. mutans* NCTC10449 was monitored by titration using a pH-stat system. *Significantly different from the control without MDPB (ANOVA and Fisher's PLSD test, $P < 0.05$). (B) The amount of fermentation end products in the presence of MDPB. ACE, acetate; FOR, formate; GUL, glutamate; LAC, lactate; PYR, pyruvate. *Significantly different (ANOVA and Fisher's PLSD test, $P < 0.05$).

significant reduction in acid production after 30 min, whereby the acid-production rates decreased to 72% of the control value.

Figure 4B shows the amounts of fermentation end products in the presence of 8.0 μg ml^{-1} of MDPB. The production of lactate was significantly reduced by MDPB ($P < 0.05$), whereas no significant differences in the production of glutamate, acetate, pyruvate, and formate were observed.

It has been preliminarily confirmed by plating methods that the number of viable bacteria is not affected by incubation with MDPB at 8.0 μg ml^{-1} and 10 mM glucose until 30 min under the experimental conditions used. Accordingly, in this experiment, the effects of MDPB (used at a concentration that has no effect on cell viability) on glucose fermentation were examined. Mutans streptococci metabolize glucose to pyruvate through the Embden–Meyerhof–Parnas pathway and convert pyruvate into lactate and volatile compounds, such as formate, acetate, and ethanol (13). *S. mutans* produces pyruvate formate-lyase (PFL), which catalyzes the first step of the conversion of pyruvate into formate, acetate, and ethanol, and also produces lactate dehydrogenase (LDH), which catalyzes lactate production (24). It is possible for some antimicrobials to target metabolism, for example triclosan, which does not inhibit glycolytic enzymes generally, but acts specifically on pyruvate kinase and lactic dehydrogenase (25). From the analysis of the end products of glucose fermentation, the quantities of formic acid and acetic acid produced by PFL were not significantly affected by MDPB (Fig. 4B). On the contrary, the production of lactic acid was decreased significantly by 8.0 μg ml^{-1} of MDPB. Thus, MDPB appears to inhibit the LDH activity of *S. mutans*, thereby reducing the production of lactic acid (Fig. 5).

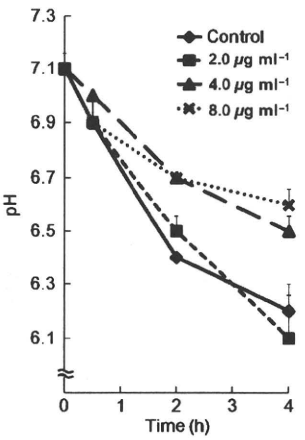

Fig. 5. Decrease in pH of a *Streptococcus mutans* NCTC10449 suspension incubated in the presence of 12-methacryloyloxydodecylpyridinium bromide (MDPB). After 2 and 4 h of incubation, the pH values of reaction mixtures containing 4.0 and 8.0 μg ml^{-1} of MDPB were significantly greater than those of the control and of the suspension containing 2.0 μg ml^{-1} of MDPB (ANOVA and Fisher's PLSD test, $P < 0.05$).

The pH values of the *S. mutans* suspension incubated in the presence of MDPB are shown in Fig. 5. The optimum pH for the activation of PFL in *S. mutans* is 6.8 (26) and that for LDH is 5–6.2 (27). Under glucose-excessive conditions, such as the conditions used in this experiment, the densities of intracellular glycolytic intermediates, such as glucose-6-phosphate (Glc6*P*), fructose-1,6-bisphosphate [Fru(1,6)P_2], 3-phosphoglycerate (Gri*P*), and dihydroxyacetone phosphate (DHAP), increase as a result of active glucose metabolism. These intracellular glycolytic intermediates interact with other metabolic enzymes. Glc6*P* activates pyruvate kinase and Fru(1,6)P_2 activates LDH. On the other hand, Gri*P*3 and DHAP inhibit PFL (28). Therefore, the main end product under conditions of glucose excess is lactate. After 2 h of incubation, the pH of cell suspensions in the presence of 4.0 and 8.0 μg ml^{-1} of MDPB was 6.7, but the pH of the control was 6.4 and the pH of the 2.0 μg ml^{-1} MDPB group was 6.5, closer to the optimum pH value for LDH. It is possible that LDH was activated more strongly in the control and 2.0 μg ml^{-1} MDPB groups to produce the significantly greater decrease in pH than seen in the 4.0 and 8.0 μg ml^{-1} MDPB groups ($P < 0.05$).

Clinically, bacterial infection with formation of biofilm occurs frequently and disturbs healing or regeneration of the tissue. Under the limitations of the present study, short-term exposure to unpolymerized MDPB was found to exhibit strong antibacterial effects against biofilm cells with a relatively thin and sparse structure, as well as planktonic cells, and such an action of MDPB took place quickly. 12-Methacryloyloxydodecylpyridinium bromide is a resin monomer and can be dissolved in organic solvent at much higher concentrations than in water; hence, it is of potential use for various resin-based restoratives, such as resinous tissue or skin adhesive. In addition, the present study indicates that bacteriostatic effects, including inhibition of growth and metabolism, can be exerted even when MDPB is diluted by tissue components or fluids. However, the inhibition of enzymatic activity by cationic agents may be somewhat specific because CHX has also been reported to inhibit lactate production of *S. mutans* to a greater extent than acetate and formate production (7). The effects of MDPB on the metabolic functions of species other than *S. mutans* remain to be determined.

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CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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The Role of Bacteria in the Caries Process: Ecological Perspectives

ABSTRACT

Dental biofilms produce acids from carbohydrates that result in caries. According to the extended caries ecological hypothesis, the caries process consists of 3 reversible stages. The microflora on clinically sound enamel surfaces contains mainly non-mutans streptococci and *Actinomyces*, in which acidification is mild and infrequent. This is compatible with equilibrium of the demineralization/remineralization balance or shifts the mineral balance toward net mineral gain (dynamic stability stage). When sugar is supplied frequently, acidification becomes moderate and frequent. This may enhance the acidogenicity and acidurance of the non-mutans bacteria adaptively. In addition, more aciduric strains, such as 'low-pH' non-mutans streptococci, may increase selectively. These microbial acid-induced adaptation and selection processes may, over time, shift the demineralization/remineralization balance toward net mineral loss, leading to initiation/progression of dental caries (acidogenic stage). Under severe and prolonged acidic conditions, more aciduric bacteria become dominant through acid-induced selection by temporary acid-impairment and acid-inhibition of growth (aciduric stage). At this stage, mutans streptococci and lactobacilli as well as aciduric strains of non-mutans streptococci, *Actinomyces*, bifidobacteria, and yeasts may become dominant. Many acidogenic and aciduric bacteria are involved in caries. Environmental acidification is the main determinant of the phenotypic and genotypic changes that occur in the microflora during caries.

KEY WORDS: acidogenicity, acidurance, acid-induced adaptation, acid-induced selection, *Actinomyces*, *Bifidobacterium*, caries-associated bacteria, caries process, extended caries ecological hypothesis, *Lactobacillus*, mutans streptococci, non-mutans streptococci.

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INTRODUCTION

The supragingival dental biofilm constitutes an ecosystem of bacteria that exhibits a variety of physiological characteristics. In particular, the acid production resulting from carbohydrate metabolism by these bacteria and the subsequent decrease in environmental pH are responsible for the demineralization of tooth surfaces (Marsh and Nyvad, 2008). However, other physiological traits of the biofilm bacteria, such as base formation, may partly dampen the demineralization processes. Therefore, Kleinberg (2002) suggested that it is the proportions and numbers of acid-base-producing bacteria that are the core of dental caries activity.

Much research has identified mutans streptococci (MS) as the major pathogens of dental caries. This is because, first, MS are frequently isolated from cavitated caries lesions; second, MS induce caries formation in animals fed a sucrose-rich diet; third, MS are highly acidogenic and aciduric (Hamada and Slade, 1980; Loesche, 1986); and fourth, MS are able to produce surface antigens I/II and water-insoluble glucan, which promote bacterial adhesion to the tooth surface and to other bacteria (Hamada and Slade, 1980). A systematic literature review by Tanzer *et al.* (2001) confirms a central role for the MS in the initiation of dental caries on enamel and root surfaces.

However, several well-designed studies have revealed that the level of MS is not necessarily high in caries-associated biofilms, especially the microflora associated with non-cavitated stages of lesion formation (van Houte *et al.*, 1991a; Sansone *et al.*, 1993). Instead, it is proposed that non-mutans acidogenic and aciduric bacteria, including non-mutans streptococci and *Actinomyces* (Sansone *et al.*, 1993; van Houte, 1994; van Houte *et al.*, 1996), are more closely involved with the initiation of caries. In addition, van Ruyven *et al.* (2000) have detected non-mutans aciduric bacteria other than non-mutans streptococci and *Actinomyces* from dental biofilms covering white-spot lesions. They found that these bacteria consisted of various species, including lactobacilli and *Bifidobacterium*.

Given these circumstances, the authors reconsidered the caries process from a microbiological, biochemical, ecological, and clinical perspective, and proposed an extension of the ecological plaque hypothesis (Takahashi and Nyvad, 2008) to explain the relation between dynamic changes in the phenotypic/genotypic properties of plaque bacteria and the demineralization/remineralization balance of the caries process (Fig. 1). In this hypothesis, dental plaque is a dynamic microbial ecosystem in which non-mutans bacteria (mainly non-mutans streptococci and *Actinomyces*) are the key players for maintaining dynamic stability, *i.e.*, a natural pH cycle (dynamic stability stage). Microbial acid-induced adaptation and subsequent acid-induced selection of 'low-pH' non-mutans bacteria play a critical role in destabilizing the homeostasis of the plaque by facilitating a shift of the demineralization/remineralization balance from 'net mineral gain' to 'net mineral loss' (acidogenic stage). Once the acidic environment has been established, MS and other

aciduric bacteria may increase and promote lesion development by sustaining an environment characterized by 'net mineral loss' (aciduric stage).

From the perspective of microbial ecology, dental diseases may be considered a model system of amphibiosis (Ruby and Goldner, 2007), a term invented by the microbial ecologist Theodore Rosebury about 50 years ago (Rosebury, 1962). Amphibiosis is the dynamic adaptation that occurs in response to changing environmental conditions between two dissimilar organisms living together. Under 'normal' conditions, micro-organisms in the oral cavity live in a symbiotic relationship with the host, characterized by mutualism (beneficial to both). However, the nature of a particular symbiosis may shift under changing conditions in a reciprocal manner, with mutualism becoming parasitism (beneficial to one and detrimental to the other) and *vice versa* (Stanier *et al.*, 1970). This dynamic adaptation is the basic principle of endogenous disease processes, including dental caries, and is congruent with the ecological caries hypothesis (Marsh, 1994; Takahashi and Nyvad, 2008). In the present article, the authors will focus on recent microbiological findings about caries-associated bacteria, and re-assess the role of these bacteria in the caries process from an ecological perspective.

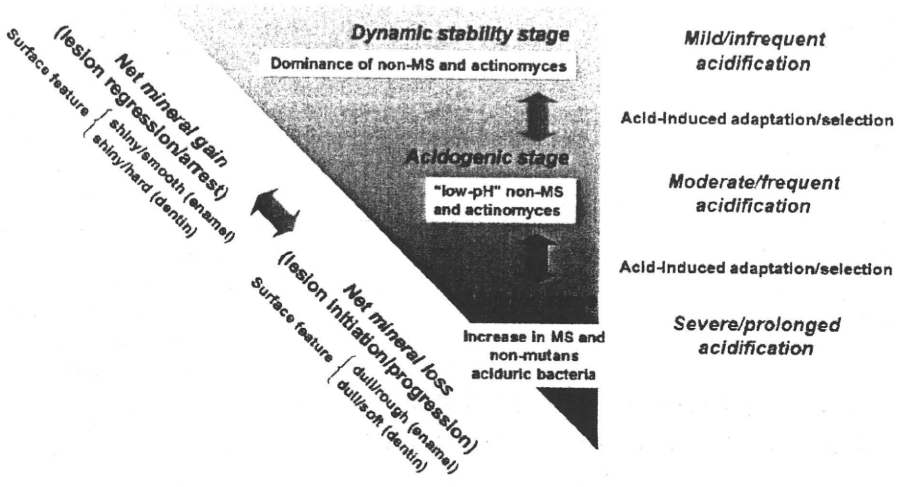

Figure 1. The caries process according to an extended caries ecological hypothesis (modified from Takahashi and Nyvad, 2008).

BACTERIAL MEMBERS IN THE CARIES PROCESS: MICROFLORA OF DENTAL PLAQUE ON CLINICALLY SOUND ENAMEL SURFACES, WHITE-SPOT LESIONS, AND CAVITATED DENTIN LESIONS

Studies have shown that the initial colonizers of newly cleaned tooth surfaces constitute a highly selected part of the oral microflora, mainly *S. sanguinis, S. oralis,* and *S. mitis* 1 (Nyvad and Kilian, 1987), but other genera, such as *Actinomyces,* are also present (Li *et al.*, 2004; Dige *et al.*, 2009). Surprisingly, MS comprise only 2% or less of the initial streptococcal population, regardless of the caries activity of the individual (Nyvad and Kilian, 1990). These observations emphasize that the vast majority of the early colonizers on teeth belong to the 'mitis group'. These bacteria, as well as other viridans group streptococci, are often referred to as the 'non-mutans streptococci', which are genetically distinct from the MS that belong to the 'mutans group' (Kawamura *et al.*, 1995). As the microflora ages, the composition shifts from *Streptococcus*-dominant to *Actinomyces*-dominant (Syed and Loesche, 1978; van Palenstein Helderman, 1981). The predominant genera in mature smooth-surface plaque therefore belong to *Actinomyces* and *Streptococcus,* most of which are non-mutans streptococci (Ximénez-Fyvie *et al.*, 2000). MS are present in very low numbers (Bowden *et al.*, 1975).

The proportion of MS in plaque covering white-spot enamel lesions is often higher than that at clinically healthy sites (van Houte *et al.*, 1991b). Yet, non-mutans streptococci still remain the major bacterial group in white spots (Sansone *et al.*, 1993; van Houte *et al.*, 1996). In fact, it has been shown that, in the absence of MS and lactobacilli, the dissolution of enamel can be produced by members of the early microflora, exclusively (Boyar *et al.*, 1989).

In cavitated lesions in dentin, including rampant caries, MS constitute about 30% of the total flora (Loesche *et al.*, 1984; Milnes and Bowden, 1985; Boue *et al.*, 1987), suggesting that MS are associated with progressive stages of caries. By contrast, MS are encountered less frequently at the advancing front of dentin caries, where lactobacilli, Prevotellae, and *Bifidobacterium* are more prevalent (Edwardsson, 1974; Becker *et al.*, 2002; Martin *et al.*, 2002; Munson *et al.*, 2004; Chhour *et al.*, 2005; Aas *et al.*, 2008; Mantzourani *et al.*, 2009a).

All these findings clearly show that the microflora on the tooth surface changes with caries lesion development, from dominance of non-mutans streptococci and *Actinomyces* to dominance of MS and other non-mutans bacteria, including lactobacilli and *Bifidobacterium.* Recent molecular identification methods have also revealed that the microflora of clinically sound and carious surfaces is much more diverse and comprises hundreds of predominant species, of which 50-60% are not cultivable (Aas *et al.*, 2005, 2008). Nevertheless, these studies suggest, again, that bacterial species other than *S. mutans, e.g., Lactobacillus, Bifidobacterium, Propionibacterium,* non-mutans streptococci, and *Actinomyces,* likely play important roles in the caries process.

Table. Acidogenicity of Representative Caries-associated Bacteria

Bacteria	Final pH	Reference
Non-mutans streptococci	4.2-5.2 [a]	1
Actinomyces	4.3-5.7 [a]	2
Mutans streptococci	4.0-4.4 [a]	1
Lactobacillus	3.6-4.0 [a]	1
Bifidobacterium	3.9-4.0 [b]	3

[a]Final pH when grown in batch culture containing glucose.
[b]Final pH when incubated in glucose solution.
1 Holt, 1984.
2 Johnson *et al.*, 1990.
3 Haukioja *et al.*, 2008.

BACTERIAL METABOLIC PROPERTIES RELEVANT TO CARIES AND SURVIVAL IN THE ORAL CAVITY

Most bacteria in supragingival plaque metabolize various sugars and produce acids through a common glycolytic pathway, the Embden-Meyerhof-Parnas pathway. When sugar is supplied in excess, oral streptococci, including MS and non-mutans streptococci (van Houte *et al.*, 1970; Hamilton, 1976; Takahashi *et al.*, 1991) and *Actinomyces* (Hamilton and Ellwood, 1983; Komiyama *et al.*, 1988), can store the extra sugars as intracellular polysaccharides (IPS), and they can utilize the IPS as an energy source to produce acids when sugar is limited, as occurs between meals. The final pH values of non-mutans streptococci, *Actinomyces*, MS, lactobacilli, and *Bifidobacterium*, when grown or incubated with glucose, are shown in the Table (Holt, 1984; Johnson *et al.*, 1990; Haukioja *et al.*, 2008). In general, on the basis of final pH values, MS, lactobacilli, and *Bifidobacterium* are more acidogenic and aciduric than non-mutans streptococci and *Actinomyces*. It should be realized, however, that the final pH values of non-mutans streptococci and *Actinomyces* can be lower than pH 5.5, the 'critical' pH for the demineralization of enamel.

In addition, non-mutans streptococci and *Actinomyces* have a variety of extracellular glycosidases (Schaal, 1984; Beighton and Whiley, 1990; Whiley and Beighton, 1998; Paddick *et al.*, 2005) that can liberate sugars and amino-sugars from glycoproteins such as the mucin contained in saliva. Studies have identified sialidases in many species, including *Streptococcus oralis*, *Streptococcus mitis*, and *Actinomyces naeslundii* (Beighton and Whiley, 1990; Bradshaw *et al.*, 1994). *S. oralis* also expresses *N*-acetyl-β-D-glucosaminidase and β-D-galactosidase, in addition to α-1-fucosidase and mannosidase activity (Byers *et al.*, 1999). Furthermore, mannosidase production has been identified within the viridans group streptococci (Homer *et al.*, 2001), and all non-mutans streptococci grow on amino-sugars (Byers *et al.*, 1996; Whiley and Beighton, 1998). This is an advantage for non-mutans streptococci and *Actinomyces* to survive in the oral cavity, where salivary glycoproteins are always available. However, most MS and lactobacilli do not have these metabolic features, except that fucosidase activity has been shown in *Lactobacillus rhamnosus* (Bradshaw *et al.*, 1994). Furthermore, most non-mutans streptococci can utilize arginine/arginine-containing peptides available in saliva through the arginine deiminase system, which degrades the arginine molecule to ammonia and carbon dioxide with production of ATP. Overall, this metabolic pathway produces alkali and neutralizes the intracellular and the environmental pH (Burne and Marquis, 2000). The arginine deiminase system is helpful for non-mutans streptococci not only to utilize arginine as an energy source, but also to survive under the acidic conditions in the oral cavity.

The *Actinomyces* have a unique glycolytic system (Takahashi *et al.*, 1995), in which they utilize high-energy polyphosphate and pyrophosphate compounds for synthesis of hexokinase and phosphofructokinase, respectively, acting as phosphoryl donors instead of ATP. This means that the *Actinomyces* are able to exploit a surplus ATP to synthesize polyphosphate as an energy reservoir, and salvage energy from pyrophosphate, a high-energy phosphoryl-bond-containing by-product from the metabolism of polymers such as nucleic acids and glycogens. In addition, the *Actinomyces* are often ureolytic (Kleinberg, 2002; Liu *et al.*, 2006) and can utilize lactic acid as a carbon source for growth (Takahashi and Yamada, 1996). These diverse physiological characteristics of *Actinomyces* seem to be advantageous to survival and domination in supragingival plaque (Takahashi and Yamada, 1999b).

THE ROLE OF CARIES-RELATED BACTERIA IN THE CARIES PROCESS ACCORDING TO THE EXTENDED CARIES ECOLOGICAL HYPOTHESIS

Dynamic Stability Stage

Many micro-organisms in dental plaque formed on clinically sound surfaces can produce acids from sugary foods, and the acids can demineralize the dental hard tissues. However, if the acidification episodes are mild and infrequent, homeostatic mechanisms in the plaque (Marsh and Martin, 1999) may easily restore the mineral balance toward net mineral gain in favor of 'remineralization' (Manji *et al.*, 1991). This dynamic environment brings the microflora to a stable stage, with dominance of non-mutans streptococci and *Actinomyces* (dynamic stability stage, Fig. 1). A chemostat study with 9 representative oral bacterial strains (Bradshaw and Marsh, 1998) revealed that 10 times the daily glucose supply (glucose pulse) at pH 7.0 established a stable microbial composition characterized by dominance of non-mutans streptococci and *Actinomyces*, which resembles that of oral biofilms on clinically sound enamel surfaces (Fig. 2A). In the chemostat, the growth medium contained a relatively high level of hog gastric mucin and a limited level of glucose, as in the oral cavity between meals, and a glucose pulse gave a temporary increase of glucose in the environment, which mimics mealtimes. In a person with healthy eating habits, sugar is always limited in the oral cavity (Carlsson, 1997), except for regular mealtimes, resulting in mild and low frequencies of acidification (dynamic stability stage, Fig. 1). Both non-mutans streptococci and *Actinomyces* have an ability to utilize glycoproteins and amino acids supplied continuously in saliva, along with dietary sugars provided at infrequent meals, supporting their co-existence with other bacteria in a nutritionally fluctuating environment.

Acidogenic Stage

Acid-induced Adaptation: Phenotypic Changes of Microflora

When sugar is supplied frequently or salivary secretion is too scarce to neutralize the acids produced, the pH decreases in the plaque become more severe and frequent. This change in the environment may enhance the acidogenicity and acidurance of the non-mutans bacteria adaptively. Takahashi and Yamada (1999a) have shown that when non-mutans streptococci, including *S. sanguinis, S. oralis, S. gordonii,* and *S. mitis,* were exposed to an acidic environment, they increased their acidogenicity. These bacteria were grown first at pH 7.0, and afterward at pH 5.5 for 0.5, 1, and 1.5 hrs, respectively (Fig. 3). The bacteria were then harvested, washed, and incubated with glucose, and the final pH values were measured as a marker of acidogenicity. Their acidogenicity expressed as final pH values varied (pH 4.04-4.33) without incubation at pH 5.5, but after incubation at pH 5.5 for 0.5, 1, and 1.5 hrs, all the bacteria increased their acidogenicity (pH 3.96-4.24 after 0.5 hr, pH 3.93-4.12 and pH 3.90-4.19 after 1.5 hrs, respectively). These bacteria were also able to increase their acidurance adaptively (Fig. 3). Bacteria initially grown at pH 7.0 were killed by acid stress in a strain-dependent manner following exposure to pH 4.0 for 1 hr (survival rate: 0.0009-71%), but after pre-acidification at pH 5.5 for 1 hr, all the bacteria increased their acidurance (survival rate: 0.4-81%). The biochemical mechanisms underlying the acid-induced adaptation are thought to involve the following mechanisms (Quivey *et al.*, 2000): (1) an increase in proton impermeability of the cell membrane; (2) induction of proton-translocating ATPase (H^+-ATPase) activity that expels proton from cells; (3) induction of the arginine deiminase system that produces alkali from arginine or arginine-containing peptides; and (4) induction of stress proteins that protect enzymes and nucleic acids from acid denaturation. In non-mutans streptococci, the increase in activities of H^+-ATPase and arginine deiminase and expression of stress proteins (homologues of heat-shock protein, Hsp60 and Hsp70) were observed following incubation at pH 5.5 (Takahashi and Yamada, 1999a).

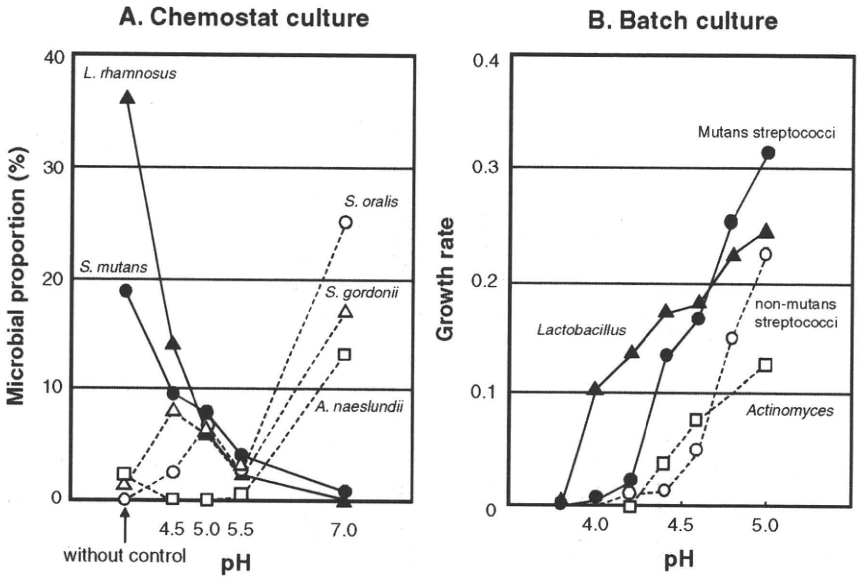

Figure 2. Growth ability at different pH values of representative oral bacteria. **(Panel A)** Results of chemostat study, modified from the data by Bradshaw and Marsh (1998). The culture pH was allowed to fall to either a preset value of 7.0-4.5 or without pH control. Five bacterial strains are shown in the panel, while the other 4 strains (*Neisserria subflava, Veillonella dispar, Prevotella nigrescens,* and *Fusobacterium nucleatum*) are omitted. **(Panel B)** Results of batch culture study, modified from the data by Horiuchi *et al.* (2009). Bacteria were grown at various pH values with pH control by the periodic addition of alkaline, and bacterial growth rates were calculated from the logarithmic growth phase. Data are given by means of 2 strains of MS, 2 strains of non-mutans streptococci, and 2 strains of *Actinomyces*.

Acid-induced Selection of 'Low-pH' Non-mutans Bacteria: Genotypic Change of Microflora

Acidification of dental plaque microflora due to frequent sugar intake or poor salivary secretion can be a driving force to enhance the acidogenicity and acidurance of the non-mutans bacteria, resulting in establishment of a more acidic environment. Even if acid-induced adaptation occurs, non-mutans bacteria such as non-mutans streptococci and *Actinomyces* are still so heterogeneous with respect to acidurance (van Houte *et al.*, 1991b, 1996) that the population of more aciduric strains, *i.e.*, 'low-pH' non-mutans bacteria, will increase selectively in this environment. Microbial acid-induced adaptation (phenotypic change of the microflora) as well as acid-induced selection (genotypic change of the microflora) will cause a shift in the acidogenic potential of the microflora, which, provided the demineralization/remineralization balance is disturbed over an extended period of time, may lead to initiation/progression of dental caries (acidogenic stage, Fig. 1).

Aciduric Stage

Acid-induced Selection of Aciduric Bacteria by Temporary Acid-impairment

Although 'low-pH' non-mutans bacteria can increase their acidurance and acidogenicity, and take over the dominant position in supragingival plaque, MS and lactobacilli are more competitive under severely acidic conditions. Following a rapid exposure to pH 4.0, as often observed in mature dental plaque after a sugar exposure, non-mutans streptococci and *Actinomyces* partially lost their viability, while MS and lactobacilli were able to survive (Fig. 4A) (Horiuchi *et al.*, 2009). Furthermore, when non-mutans streptococci and *Actinomyces* initially treated at pH 4.0 in growth media were returned to pH 7.0, they started to

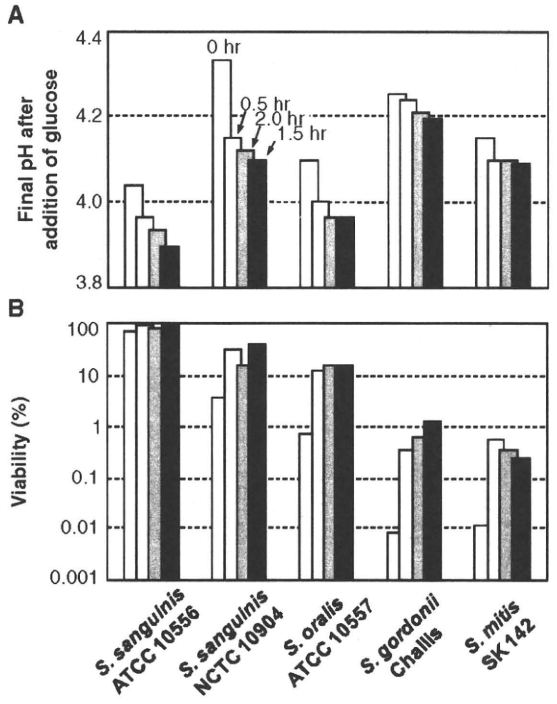

Figure 3. Acid-induced adaptation of non-mutans streptococci. The data were modified from Takahashi and Yamada (1999). **(Panel A)** Acidogenicity (final pH values) after acidification at pH 5.5. **(Panel B)** Acidurance (survival after 1 hr at pH 4.0) after acidification at pH 5.5.

grow again. However, the bacterial growth (actual growth curve) was much slower than that expected from the number of surviving bacterial cells (expected growth curve) (Fig. 4B) (Horiuchi *et al.*, 2009). Delay of the growth after acidification was common among non-mutans streptococci and *Actinomyces*, and ranged from 0.00 to 1.51 hrs after 0.5-hour acidification, from 1.54 to 2.44 hrs after one-hour acidification, and 2.41< hr after two-hour acidification. It should be noted that some non-mutans streptococci and *Actinomyces* strains did not start to grow by 10 hrs after a two-hour acidification, indicating that they required a considerable time for growth to start again, although the cultures contained a significant number of viable bacteria (Fig. 4A). No viability loss and delay were observed in MS and lactobacilli, clearly indicative of their high acidurance. These observations suggest that acidification impairs bacterial growth ability temporarily in a strain-dependent manner, and that the acid-impaired bacteria need a considerable time to recover their growth ability. Correspondingly, Takahashi *et al.* (1997) have shown that a transient acidification temporarily inactivated the glycolytic enzymes, which returned to original levels after the pH had returned to neutral, although the mechanisms have not yet been clarified.

Under these conditions, non-mutans streptococci and *Actinomyces*, probably except for some aciduric strains of non-mutans streptococci and *Actinomyces* (Nyvad and Kilian, 1990; Aas *et al.*, 2008), will be eliminated and replaced by more aciduric bacteria, such as MS and lactobacilli (aciduric stage, Fig. 1), leading to a pronounced net mineral loss and rapid lesion progression. Since *Bifidobacterium* is also acidogenic and aciduric, similar to lactobacilli and more so than MS (van Houte *et al.*, 1996; Haukioja *et al.*, 2008), as shown in the Table, they may also overcome the competition and increase their proportion of the microflora.

Acid-induced Selection of Aciduric Bacteria by Prolonged Acidification

A chemostat experiment with 9 representative oral bacteria (Bradshaw and Marsh, 1998) showed that when the pH was allowed to fall to a preset value of 5.0, MS and lactobacilli became dominant, while non-MS and *Actinomyces* started to be excluded from the consortium (Fig. 2A). When pH was further allowed to fall to 4.5 and without control (final pH = 3.83), MS and lactobacilli increased dramatically. Similar results were obtained from a batch-culture experiment (Horiuchi *et al.*, 2009); at pH ≤ 5.0, MS and lactobacilli were able to grow faster than non-mutans streptococci and *Actinomyces* (Fig. 2B). At pH ≤ 4.6, lactobacilli grew faster than MS, consistent with the chemostat results that the proportion of lactobacilli became higher than that of MS at pH ≤ 4.5. Given these observations, it is suggested that prolonged acidic conditions around pH 5 may cause the emergence of MS and lactobacilli in the microbial flora, and that more severe acidic conditions around pH 4 may exclude the non-mutans streptococci and *Actinomyces*. In the oral cavity, prolonged acidic conditions (pH ≤ 5) can occur in carious cavities (Dirksen *et al.*, 1962; Hojo *et al.*, 1994), where clearance of acids is disturbed. This may be the reason MS and lactobacilli are frequently isolated from established carious cavities. It is noticeable that all caries lesions with pH < 5 were designated as 'active' lesions and contained lactic acid exclusively (Hojo *et al.*, 1994).

At the aciduric stage, acid-induced selection by acid-impairment and growth competition are the major reasons for the shift in the composition of the microflora. However, acid-induced adaptation may still occur in aciduric bacteria, such as MS and lactobacilli (Belli and Marquis, 1991; Ma *et al.*, 1997; Svensäter *et al.*, 1997), in which both the acidogenicity and acidurance are enhanced under severe and prolonged acidic conditions. The basic biochemical reactions in response to acid stress are therefore similar to those described above in the acidogenic stage.

CLINICAL MICROBIOLOGICAL OBSERVATIONS IN SUPPORT OF THE EXTENDED ECOLOGICAL CARIES HYPOTHESIS

Early Childhood Caries (ECC) and MS: Which Comes First, the MS or Poor Eating Habits/Low Socio-economic Status?

ECC refers to any dental caries in the primary dentition. ECC can destroy the primary dentition of toddlers and small children, and, if left untreated, it can lead to pain, acute infection, nutritional insufficiencies, and learning and speech problems (AAPD, 2008). In its less severe stage, ECC is characterized by smooth-surface lesions of the primary teeth and is called 'rampant caries' (Milnes, 1996).

In ECC lesions, the MS have been frequently isolated, and their proportion of the microflora was high (van Houte *et al.*, 1982; Milnes and Bowden, 1985). In addition, it has been reported that both the detection frequency of MS in saliva and the proportion of MS in plaque were associated with the severity

of ECC, suggesting that the MS are a major pathogen of ECC. A systematic review has confirmed that the presence of MS, in both plaque and saliva of young caries-free children, appears to be associated with a considerable increase in ECC risk (Thenisch *et al.*, 2006). These findings remind us of 'the mutans story', in which the MS were claimed to be the major pathogen in caries because of insoluble glucan formation and excessive acid production in response to a sucrose-containing diet.

However, recent studies have reported that eating habits and socio-economic status of children and their caregivers are good predictors of ECC (Nunn *et al.*, 2009). In addition, oral health promotion programs based on repeated preventive guidance initiated during the mother's pregnancy were successful in reducing the incidence of severe ECC in young children (Plutzer and Spencer, 2008). In this context, ECC also follows the steps of the extended ecological plaque hypothesis. Frequent acidification of plaque by poor eating habits, such as frequent intake of sugared beverages and snacks, increases the acidogenic/aciduric bacteria and subsequently leads to dominance of the MS, with progression of caries lesions. Likewise, the detection of lactobacilli and *Bifidobacterium* in ECC lesions (Becker *et al.*, 2002; Aas *et al.*, 2008) is in accord with the extended ecological hypothesis, since both bacterial genera are aciduric enough to colonize and proliferate in acidic caries lesions (Nakajo *et al.*, in press).

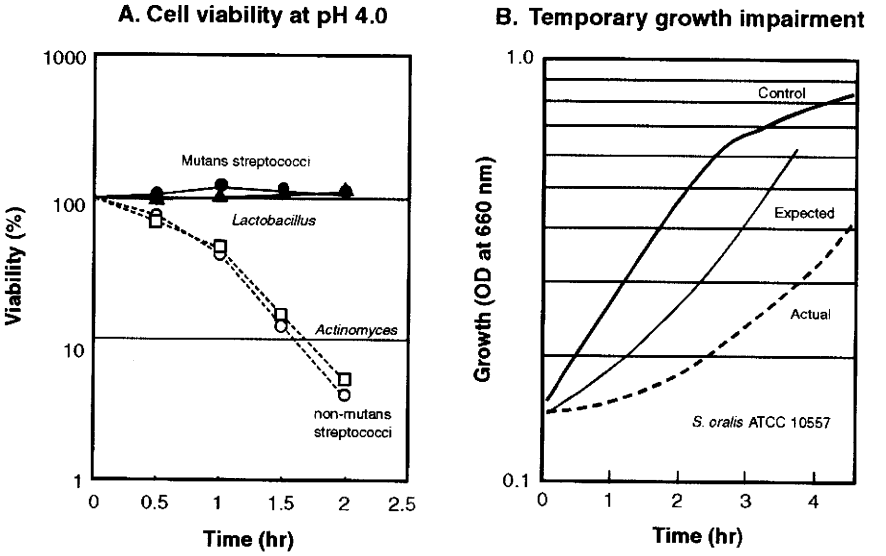

Figure 4. Effect of severe acidification on representative oral bacteria. The data were modified from Horiuchi *et al.* (2009). **(Panel A)** Cell viability at pH 4.0. The bacterial cells grown at pH 7.0 were exposed to pH 4.0 for 0, 0.5, 1, 1.5, and 2 hrs in buffer solution. The treated bacterial cells were plated on blood agar and counted for colony-forming units after anaerobic incubation. **(Panel B)** Temporary growth impairment at pH 4.0. Bacteria (*Streptococcus oralis* ATCC 10557) grown at pH 7.0 were exposed to pH 4.0 for 1 hr in growth medium, and then incubated in growth medium at pH 7.0. Actual = Actual growth curve determined by measurement of optical density of culture medium. Expected = Expected growth curve calculated from surviving cell numbers after the exposure at pH 4.0 for 1 hr. Control = growth without acid-exposure.

Ecology of the Microflora in Patients with a Dry Mouth

Acidification of the biofilm could also happen because of hyposalivation, which reduces clearance of sugars and acids after carbohydrate consumption. Therefore, patients with a dry mouth run a higher risk of caries, particularly if their oral hygiene practices are poor. Hyposalivation can be caused by head and neck irradiation for the treatment of cancer, autoimmune diseases such as Sjögren's syndrome, hormonal disorders, neurological disorders, or psychogenic illness, but the most common reason for decreased salivation is medication. Radiation treatment for head and neck cancer produces a particularly aggressive form of dry mouth. In patients with permanent hyposalivation due to radiation treatment, acidification of plaque after a sugar challenge was significantly higher and more prolonged than in control patients (Eliasson *et al.*, 2006). These patients were also colonized by higher numbers of lactobacilli, MS, and *Candida* species in approximal plaque, suggesting that the acidic environment created by severe hyposalivation can be attributed to the propagation of aciduric bacteria. *Candida* species are known to be acidogenic and aciduric (Samaranayake *et al.*, 1986; Klinke *et al.*, 2009), but it could not be excluded that acquired suppression of immune defense mechanisms as a result of cancer therapy may partly explain the emergence of these species (Budtz-Jörgensen, 1990).

Longitudinal analyses of the microflora in patients receiving radiation treatment (Brown *et al.*, 1976) have demonstrated a rapid increase in the proportion of MS parallel with the onset of rampant caries. Increases of *Lactobacillus* species were observed to lag behind those of the MS, suggesting that the acidic environment created by hyposalivation severely destabilized the homeostasis of the microflora. In this case, the MS may be associated with the onset of caries, while lactobacilli are opportunists favored by the environmental change created by lesion initiation, because lactobacilli are more aciduric than MS (Fig. 2).

Interestingly, Brown and co-workers (Brown *et al.*, 1976) showed that deletion of dietary sucrose in the irradiated patients suppressed the emergence of MS and lactobacilli, and the levels of these bacteria remained considerably lower than in irradiated patients on an unrestricted diet. These longitudinal observations are congruent with the reciprocal adaptive microbial changes described in the extended caries ecological hypothesis.

Root-surface Caries

Root surface caries was, for a long time, thought to be induced specifically by *Actinomyces* (Jordan and Hammond, 1972; Sumney and Jordan, 1974). This idea was probably ascribed to the sampling technique combined with the selective culturing techniques applied in these studies, Thus, it is to be expected that samples of softened carious dentin have a higher content of Gram-positive pleomorphic rods compared with samples containing superficial layers of plaque, because of the selective invasion of *Actinomyces*-like bacteria into demineralized root tissue (Nyvad and Fejerskov, 1990). Recent molecular studies have confirmed the abundance of *Actinomyces* in carious root dentin (Preza *et al.*, 2009).

S. mutans was detected in only half of the root caries lesions (Preza *et al.*, 2009). Furthermore, as with enamel caries, MS may comprise only a small proportion of the microflora of root-surface caries lesions. van Houte *et al.* (1996) reported that non-MS and *Actinomyces* spp. were dominant in dental plaque covering root-surface caries and that the isolated *Actinomyces* strains were heterogenous with respect to acidogenicity: Strains isolated from root-surface caries were more acidogenic than those from clinically sound root surfaces. Brailsford *et al.* (2001) observed a similar phenomenon in individuals with root-surface caries. These authors found that aciduric bacteria able to grow at pH 4.8 comprised 21.6% of the total microflora in root-surface caries lesions (lactobacilli and *Actinomyces* were dominant), whereas aciduric bacteria comprised 10.7% in clinically sound root surfaces (*Actinomyces*-dominant). However, in individuals without root-surface caries, aciduric bacteria comprised only 1.4% of total microflora in clinically sound root surfaces. These findings point to an association between acidogenic/aciduric *Actinomyces*, *i.e.*, 'low-pH' *Actinomyces*, and root-surface caries.

Recently, Mantzourani *et al.* (2009b) demonstrated that the family *Bifidobacteriaceae*, including *Bifidobacterium*, *Scardovia*, and *Parascardovia*, was associated with cavitated root caries lesions, together with MS, lactobacilli, and yeasts (*Actinomyces* were not examined), indicating that the acidic environment of the lesions provided a suitable habitat for the proliferation of these aciduric micro-organisms. Collectively, the information obtained so far supports the contention (Bowden, 1990; Nyvad, 1993) that the ecological succession of the microflora in root-surface caries follows the same pattern as that observed for cavitated dentin caries.

CONCLUSIONS AND FUTURE DIRECTIONS FOR RESEARCH

Our review of the literature supports the concept that dental caries is an endogenous disease, which is caused by a change from mutualistic symbiosis to parasitic symbiosis in the microbial ecosystem, *i.e.*, a microbial shift from dynamic stability *via* acid-induced adaptation and selection to an aciduric stage, according to the extended ecological plaque hypothesis. In this hypothesis, the entire consortium of acidogenic/aciduric bacteria, not only the MS, contributes to the caries process—a view that is compatible with the mixed-bacteria ecological approach proposed by Kleinberg (2002).

Acid production is the direct causative factor in the demineralization of tooth surfaces, but acid production is also an environmental determinant that influences both the phenotypic and genotypic properties of the oral microflora through acid-induced adaptation and selection. It is important to appreciate, however, that the enrichment of acidogenic/aciduric bacteria is a result of microbial acid formation during the caries processes—not the causative factor *per se*—and thus the removal of specific aciduric bacterial species such as the MS, through vaccination, gene therapy, or antimicrobial treatment, may not be an effective approach for long-term caries control. Rather, environmental control of the microflora should be achieved by avoiding acidification of the dental biofilm. Practical solutions to this strategy may include mechanical plaque control, reduction/substitution of the intake of sugary foods, and/or application of pH-neutralizing techniques such as saliva stimulation.

The caries process usually progresses rather slowly because of alternating de- and remineralization episodes in the biofilm. However, if the local environment changes—for example, in response to frequent sugar intake or low salivary secretion combined with insufficient oral hygiene—the equilibrium between the de- and remineralization episodes may favor a net mineral loss. These processes may lead to rampant caries, of which ECC and radiation caries are classic examples. Even so, it is salutary to know that the caries processes can be reversed, depending on the local environmental conditions. Therefore, it is important to learn how we can stimulate a mutualistic microflora to sustain clinically healthy conditions. Future microbiological studies of caries should therefore focus on a better understanding of the physiological mechanisms that serve to maintain the dynamic stability in dental biofilms. In this context, because of their association with mildly acidogenic environments, the non-mutans streptococci and *Actinomyces* may be interesting candidates for further analysis of their acid-base metabolic processes (Burne and Marquis, 2000; Kleinberg, 2002).

In recent years, investigators have advanced molecular identification methods in the attempt to resolve the microbiological foundation of caries. Hence, several elegant molecular studies have tried to elucidate the microbiological differences between clinically healthy and carious conditions (Becker *et al.*, 2002; Corby *et al.*, 2005; Aas *et al.*, 2008). While these studies have concluded that the microflora involved in caries is much more complex than hitherto anticipated, the analyses have not always shown a clear-cut pattern between health and disease. In addition to inter-individual differences (Aas *et al.*, 2005; Preza *et al.*, 2009), this may partly be because of methodological shortcomings. In some studies, the bacteria were pooled from several surfaces/lesions in the same person. Because of intra-oral environmental variability (Kleinberg and Jenkins, 1964; Fejerskov *et al.*, 1994; Haffajee *et al.*, 2009), pooled plaque samples cannot be expected to give a clear picture of the microbiome at a site. Another shortcoming may relate to the fact that refined gene technology does not match the crude style of lesion classification commonly used. Most studies of caries microbiology do not take into account novel knowledge about the dynamic metabolic processes in caries, and as a result, lesion activity is seldom defined. There is evidence that site-specific sampling of

well-defined surfaces and lesions may reveal microbial patterns of particular ecological interest. It has thus been reported that the overall composition of the microflora may change from a state of diversity to a state dominated by smaller numbers of aciduric species with increasing lesion activity of root caries (Nyvad and Kilian, 1990; Preza *et al.*, 2009). Therefore, for verification of the extended ecological plaque hypothesis, including the role of the non-mutans bacteria in the caries process, future molecular studies need to apply a refined caries lesion classification (Nyvad *et al.*, 1999) that has been validated for lesion activity (Nyvad *et al.*, 2003).

In view of our current philosophy, stressing the importance of environmental acidification, we propose that, to fully understand the ecological processes in caries, prospective molecular studies should involve not only bacterial identification and quantification, but also metabolic characterization of, *e.g.*, lesion pH *in vivo* (Fejerskov *et al.*, 1992), and/or the use of more sophisticated methodologies, such as metabolome analysis (metabolomics) and metagenome analysis (metagenomics), for reconstruction of metabolic networks in the microflora. Metabolomics can monitor a remarkable spectrum of metabolites in the microflora with the combination of chromatographic/electrophoretic separation and mass spectrometry-aided identification of metabolites (Takahashi *et al.*, in press), while metagenomics can identify metabolically functional genes, such as genes coding metabolic enzymes, comprehensively (Tringe *et al.*, 2005; Gianoulis *et al.*, 2009). We believe that such comprehensive approaches to analyzing the composition and function of well-defined microbial communities may offer new insight into the microbial ecosystem in caries, although these methodologies cannot give information about the spatial organization of the predominant genera and species (Dige *et al.*, 2009; Zijnge *et al.*, 2010).

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