Oral Microbiology Immunology 2009: 24: 319–324
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Transient acid-impairment of growth ability of oral *Streptococcus*, *Actinomyces*, and *Lactobacillus*: a possible ecological determinant in dental plaque

M. Horiuchi[1,2], J. Washio[2], H. Mayanagi[1], N. Takahashi[2]
[1]Division of Pediatric Dentistry, [2]Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, Sendai, Japan

Horiuchi M, Washio J, Mayanagi H, Takahashi N. Transient acid-impairment of growth ability of oral Streptococcus*,* Actinomyces*, and* Lactobacillus*: a possible ecological determinant in dental plaque. Oral Microbiol Immunol 2009: 24: 319–324.*

Introduction: Dental plaque pH decreases to about 4 through bacterial fermentation of carbohydrates and this low pH is maintained for from several minutes to about an hour. Repeated acidification causes demineralization of the tooth surface, resulting in caries formation. The acidification also influences plaque bacteria. Severe acidification kills bacteria efficiently, while physiological acidification, the condition occurring in plaque, kills bacteria partially and may impair growth ability. We, therefore, investigated the effects of physiological acidification on representative caries-related bacteria.
Methods: *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, *Streptococcus oralis*, *Lactobacillus paracasei*, and *Actinomyces naeslundii* were used. Effects of physiological acidification at pH 4.0 on cell viability and growth ability, as well as the growth rate of these bacteria at pH 4.0–7.0, were investigated.
Results: Mutans streptococci and *Lactobacillus* grew at pH 4.0 but the growth of *S. sanguinis* and *S. oralis* ceased below pH 4.2 and pH 4.2–4.4, respectively. Acidification at pH 4.0 for 1 h killed 43–89%, 45% and 35–76% of *S. sanguinis*, *S. oralis*, and *Actinomyces*, respectively. Furthermore, assessment of bacterial growth curves revealed that the growth ability of the surviving cells of *S. sanguinis*, *S. oralis* and *Actinomyces* was impaired, but it was recovered within 2–5 h after the environmental pH had returned to 7.0. The acidification neither killed nor impaired the growth of mutans streptococci and *Lactobacillus*.
Conclusions: These results indicate that physiological and transient acidification is not sufficient to kill bacteria, but it causes a temporary acid-impairment of their growth ability, which may function as an ecological determinant for microbial composition in dental plaque.

Key words: acid impairment; acidification; *Actinomyces*; bacterial growth; cell viability; *Lactobacillus*; *Streptococcus*

Nobuhiro Takahashi, Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
Tel.: + 81 22 717 8294;
fax: + 81 22 717 8297;
e-mail: nobu-t@mail.tains.tohoku.ac.jp

Accepted for publication February 4, 2009

Dental plaque on the tooth surface consists of a variety of saccharolytic bacteria. After carbohydrate intake, the pH of dental plaque decreases rapidly to about 4 as the result of bacterial fermentation and this low pH is maintained for from several minutes to about an hour (12, 20). Repeated acidification causes demineralization of the tooth

surface, resulting in dental caries formation.

The acidification also influences bacterial physiology such as growth rate and cell viability. Harper and Loesche (10) demonstrated that when culture pH was lowered from 7.0 to 5.0 the growth rates of *Streptococcus mutans* and *Lactobacillus casei* decreased to 3–30% and 54%, respectively, while *Streptococcus sanguinis* ceased to grow. Experiments with mixed continuous cultures using representative oral bacteria indicated that the reduction of culture pH affected bacterial growth rate and subsequently modified bacterial composition. In the continuous cultures, *S. mutans* and *Lactobacillus rhamnosus* dominated the other bacteria, including non-mutans streptococci and *Actinomyces*, at a pH below 5.5 (3, 4), and the proportions of *S. mutans* and *L. casei* increased gradually after repeated acidification (5) or prolonged acidification (17) while those of the other bacteria decreased. These pH-driven modifications of bacterial composition observed in mixed continuous cultures were similar to the microbial shift from healthy site to carious site *in vivo* (2, 18, 19, 26), where environmental acidification is expected to occur. On the basis of these results, Marsh (15, 16) proposed the 'ecological plaque hypothesis' that environmental acidification causes a microbial shift towards a more acidogenic and aciduric population, resulting in a cariogenic microflora. Recently, Takahashi and Nyvad (24) expanded this hypothesis to 'microbial dynamics' of the caries process, in which the environmental acidification induces microbial acid-adaptation and subsequently increases microbial acidogenicity and acidurance, while the environmental acidification also triggers microbial shift through acid-selection of acidurant bacteria.

It is known that environmental acidification kills bacteria. Svensäter et al. (21) demonstrated that acidification at pH 2.3–4.5 for 3 h caused cell death of oral bacteria and that the acid-killing efficiency was dependent on bacterial species in the order of *Actinomyces* > non-mutans streptococci > mutans streptococci > *Lactobacillus*. In addition, Takahashi et al. (23) demonstrated that physiological and transient acidification (pH 4.0 for 1 h) occurring in dental plaque did not kill *S. sanguinis* efficiently but caused a temporary impairment of growth ability. They also showed that the impaired cells were capable of growing again after an 80-min incubation in the culture media at pH 7.0, along with the reactivation of glycolytic enzymes (23). However, no acid-impairment was observed in *S. mutans*. These observations suggest that acid-impairment of growth ability is an important ecological determinant of microbial composition in dental plaque, where repeated acidification occurs daily.

In the present study, we investigated the effects of physiological and transient acidification at pH 4.0 on cell viability and growth ability, as well as growth rate at pH 4.0–7.0, of representative oral bacteria, *Streptococcus*, *Actinomyces*, and *Lactobacillus*.

Materials and methods

Bacterial strains

Actinomyces naeslundii genospecies 1 ATCC 12104, *A. naeslundii* genospecies 2 WVU 627, *Lactobacillus paracasei* subsp. *paracasei* (formerly *L. casei* subsp. *casei*) C12-4, *S. mutans* NCTC 10449, *S. oralis* ATCC 10557, *S. sanguinis* ATCC 10556, *S. sanguinis* NCTC 10904, and *Streptococcus sobrinus* 6715 were used in this study. These bacteria were maintained on blood agar plates. The purity of the culture was confirmed

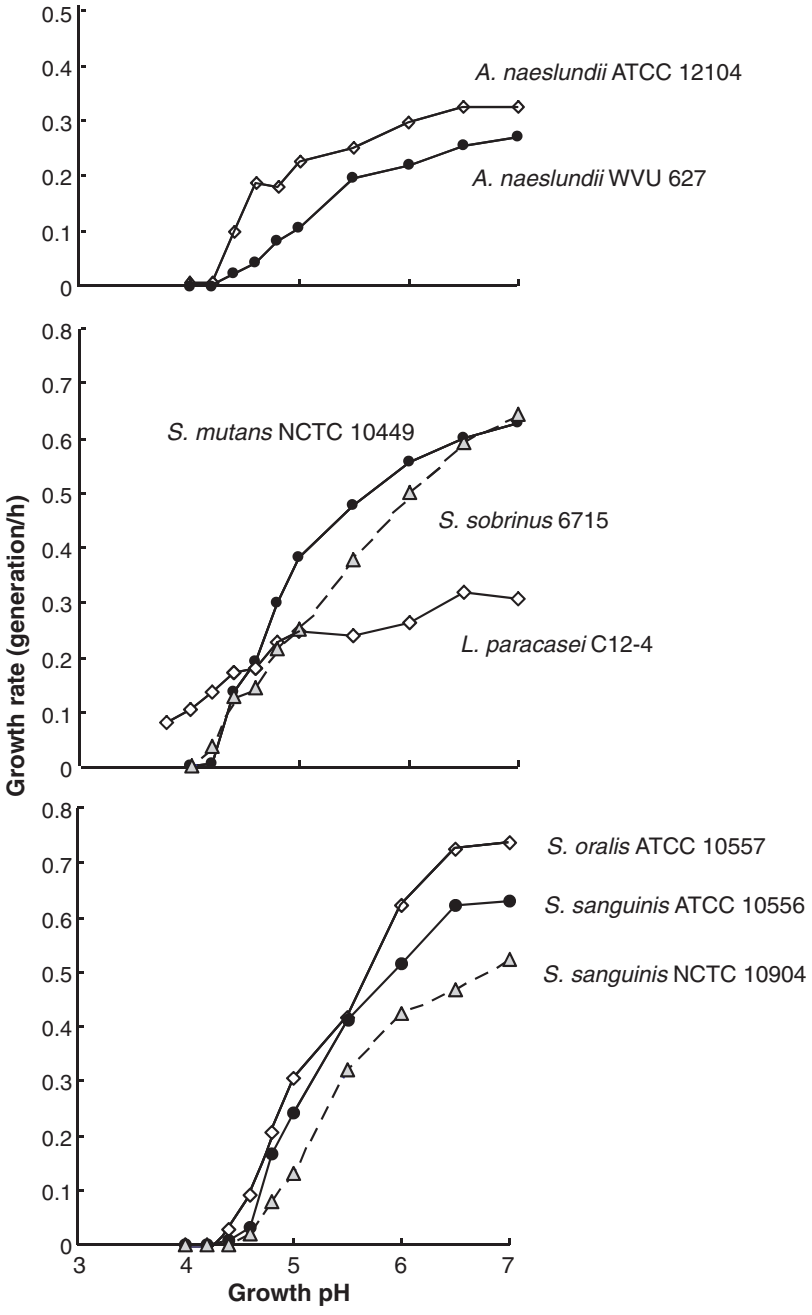

Fig. 1. Bacterial growth rate at various pH values.

by plating the culture on blood agar for all the strains and on mitis-salivarius agar (BBL Microbiology Systems, Cockeysville, MD, USA) for the streptococcal strains. These plates were incubated at 35°C for 2 days under anaerobic conditions.

Growth conditions

The bacteria were precultured in 5 ml trypticase soy broth (BBL Microbiology Systems) supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, MI, USA), 0.5% glucose, 0.1% L-cysteine hydrochloride monohydrate, and 0.04% sodium carbonate anhydrous (TSBYG-1) for 15 h at 35°C in an anaerobic chamber (Hirasawa Works, Tokyo, Japan; gas phase, 10% CO_2, 10% H_2, and 80% N_2). The cultures were transferred into 100 ml trypticase soy broth supplemented with 0.5% yeast extract, 0.5% glucose, 0.1% L-cysteine hydrochloride monohydrate, 0.01% ammonium bicarbonate, and 38 mM potassium phosphate buffer (pH 7.0) (TSBYG-2) and incubated at 35°C in an anaerobic chamber. The pH of the culture was maintained at 6.7–7.0 by regular manual addition of 6 M KOH The culture pH was checked using a handy pH meter (model pH BOY-P1, Sindengen Kogyo, Tokyo, Japan), which can measure the pH of a 30-μl sample. The following experiments were carried out in the same anaerobic chamber. To remove oxygen, all the media, solutions, and buffers were kept in the chamber for at least 3 days before use.

Bacterial growth rate at various pH values

At the exponential phase of growth, 2 ml of the culture was transferred into 5 ml of new TSBYG-2. Just before the experiments, the pH of the medium was adjusted to 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.5, 6.0, 6.5, or 7.0 by addition of 6 M HCl or KOH. The culture was then incubated at 35°C and the growth was monitored photometrically at 660 nm. The pH of the culture was maintained by periodical addition of 6 M KOH as describe above. The growth rate was calculated as generations per hour from initial inclinations of the growth curve.

Acidification of bacterial culture

At the exponential phase of growth [optical density at 660 nm (OD_{660}) 0.6–0.8], the culture (100 ml) was mixed with 100 ml prewarmed sterile TSBYG-2 medium and the pH of the culture was reduced to 4.0 within 3 min using 6 M HCl. The culture was then incubated at 35°C and optical density was monitored at 660 nm.

Survival of bacteria at low pH

After acidification at pH 4.0, the culture was sampled at 0, 0.5, 1, and 2 h. The samples were serially diluted with TSBYG-2 without glucose (pH 7.0) and spread on TSBYG-2 agar plates (pH 7.0). The plates were incubated at 35°C for 2 days and then colony-forming units were counted.

Growth ability of bacteria after acidification

After acidification at pH 4.0, 5 ml of the culture was sampled at 0, 0.5, 1, and 2 h and transferred into 5 ml of new TSBYG-2 (pH 7.0). The culture was then incubated at 35°C and the growth was monitored photometrically at 660 nm. The pH of the culture was maintained by occasional addition of 6 M KOH.

Statistical analyses

Difference in cell viability was analysed by the one-way repeated measures analysis of variance, and significance was examined using the Dunnet's test as *post hoc*. Statistical analysis was performed using StatFlex software version 5.0 (Artech, Osaka, Japan). Differences were considered significant at the level $P < 0.05$.

Results

Bacterial growth rate at various pH values

All the bacterial strains in exponential growth continued to grow when they were transferred into new culture media. The growth rates of all the bacteria decreased as the pH of the growth media was lowered (Fig. 1). The growth rates of streptococci were higher than those of other bacteria above pH 5.5, although those of *S. sanguinis* and *Streptococcus oralis* declined sharply as the pH was lowered. The growth rate of these streptococci decreased to 4–12% at pH 4.6 and ceased completely at pH 4.2–4.4. In contrast, the growth rates of *S. mutans* and *S. sobrinus* decreased more gradually at acidic pH and remained at 22–31% at pH 4.6. However, no growth was found at pH 4.0. The growth rates of *Actinomyces* were about half the streptococcal growth rates at pH 7.0, but decreased gradually as the pH was lowered. The growth rates were maintained at 14–52% at pH 4.6 and no growth was observed at pH 4.2. The growth rate of *Lactobacillus* was as low as those of *Actinomyces* at pH 7.0, but the *Lactobacillus* strain maintained growth rates of 35 and 26% at pH 4.0 and pH 3.8, respectively.

Bacterial killing by acidification at pH 4.0

The acidification to pH 4.0 killed *A. naeslundii*, *S. sanguinis*, and *S. oralis*. The cell viability of these bacteria decreased significantly as the acidification was prolonged (Fig. 2), and decreased to 24–64%, 10–57% and 55% after 1 h of acidification,

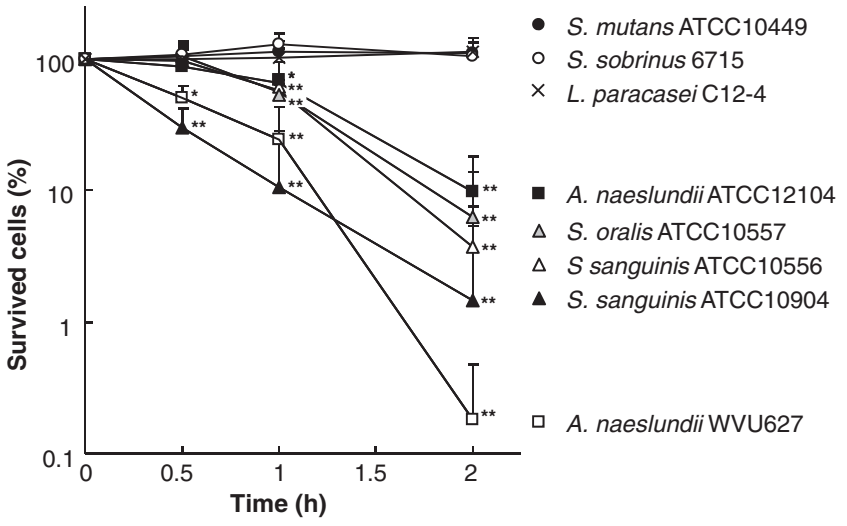

Fig. 2. The effect of acidification at pH 4.0 on cell viability. The mean values with standard deviations of three independent experiments are given. *$P < 0.05$, **$P < 0.01$.

respectively. In contrast, mutans streptococci and *Lactobacillus* maintained almost 100% viability during a 2-h acidification at pH 4.0.

Growth impairment

The bacterial cells acidified at pH 4.0 for 1 h were transferred into new TSBYG-2 media (pH 7.0), and their growth was monitored. At the beginning, the growth rates of *A. naeslundii*, *S. sanguinis*, and *S. oralis* (actual growth curve) were lower than those of control cultures, but they gradually returned to the original level (Fig. 3). There was no effect on mutans streptococci and *Lactobacillus*.

The expected growth curves of *A. naeslundii*, *S. sanguinis*, and *S. oralis* were also calculated from the proportion of viable cells (Fig. 2). For example, assuming that the surviving cells of *S. oralis* (55% of cells were viable after exposure to pH 4.0 for 1 h) started to regrow at the original growth rate at pH 7.0, the growth curve could be estimated by calculation using the following formulae:

1 OD value at n h (OD_n) = OD value contributed by dead cells (ODD) + OD value contributed by growing cells at n h (ODG_n)
2 $ODD = OD_0 \times (100 - VC\%)/100$
3 $ODG_n = (OD_{n-1} - ODD) \times 2^R$

where OD_0, VC% and R are initial OD value, viable cell % at 0 h and growth rate (i.e. generation/hour), respectively (indicated by open squares in Fig. 3).

In the same way, the expected growth curves of all the strains were obtained, except for mutans streptococci and *Lactobacillus* because of their high viability after acidification (Fig. 2). The actual growth curves of *A. naeslundii*, *S. sanguinis*, and *S. oralis* were always below the expected growth curves. The bacterial cells acidified at pH 4.0 for 0.5 or 2 h showed a similar result, though a 0.5-h acidification resulted in both actual and expected curves closer to control curves while 2-h acidification resulted in curves more separated from the control curves (data not shown).

Furthermore, the duration of the lag phase was calculated as follows (Fig. 4): when line a, the tangent line of the actual growth curve, became approximately parallel to the growth control curve at a logarithmic growth phase, A h was defined as the duration of the lag phase, namely a lag time. Similarly, the expected lag time was calculated from the expected growth curve (B h). The lag time increased as the acidification was prolonged (Table 1). The cells of *A. naeslundii* WVU 627 acidified for 2 h could not resume growth at pH 7.0 at 10 h. The expected lag time was always shorter than the actual lag time. No lag time was observed in mutans streptococci and *Lactobacillus* (Table 1).

Discussion

Around neutral pH, all the oral bacteria tested could grow efficiently, although the growth rates of *Actinomyces* and

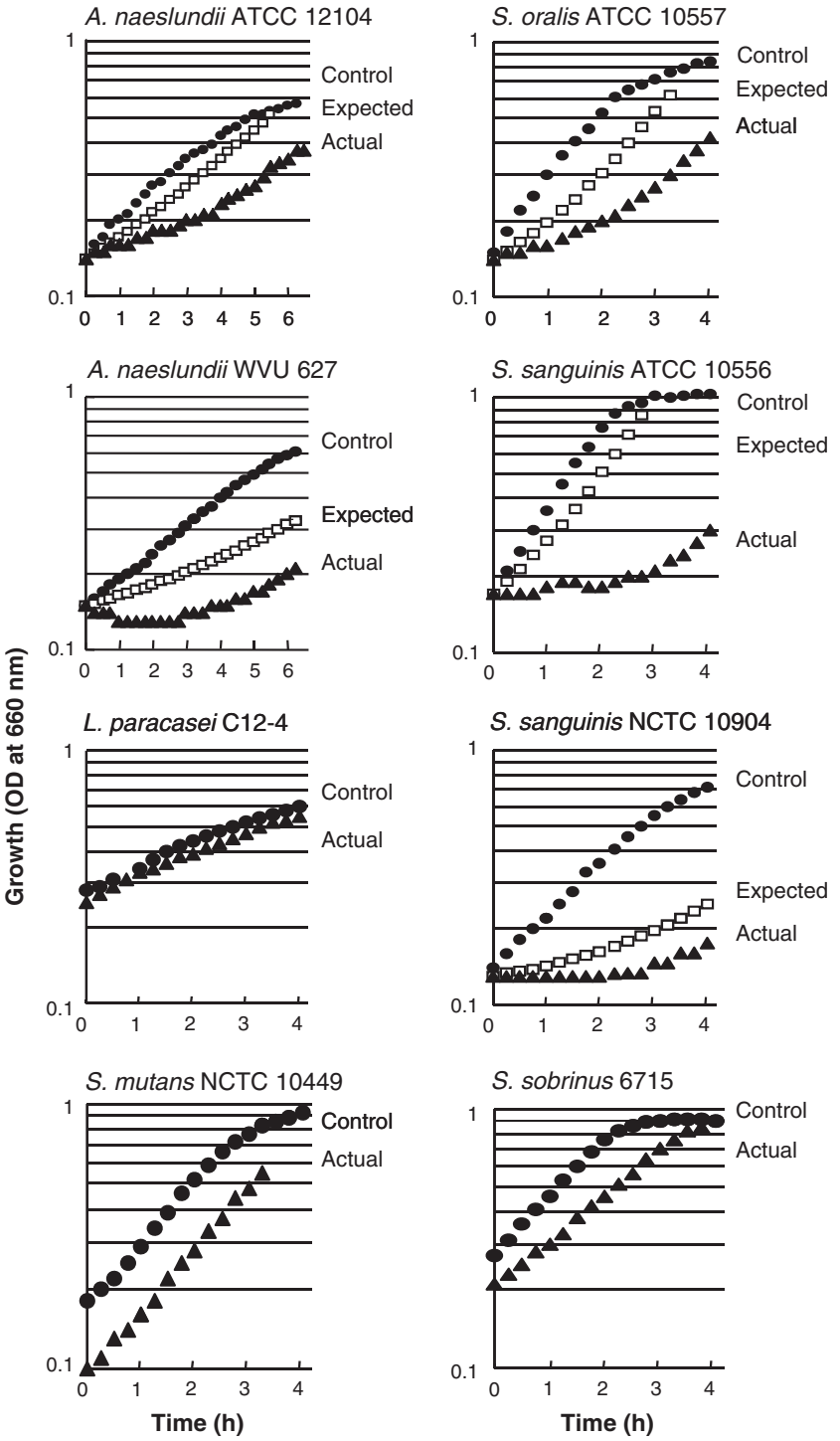

Fig. 3. Comparison between actual (▲) and expected (estimated from number of viable cells) (□) growth curves after acidification at pH 4.0 for 60 min. Control growth curves without acidification (•) are also shown. A representative growth curve from three independent experiments is given for each bacterial strain. Results from three independent experiments were similar.

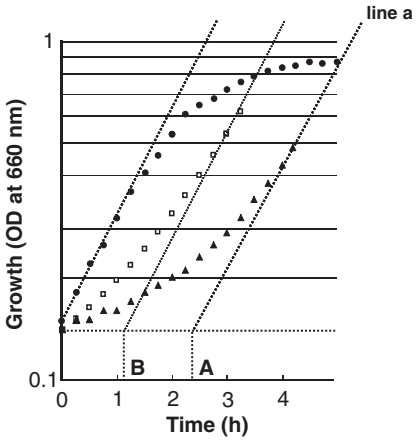

Fig. 4. The method of calculating lag time of growth after acidification. Control growth curve at pH 7.0 without acidification (•), expected growth curve estimated from number of viable cells after acidification at pH 4.0 for 60 min (□) and actual growth curve after acidification at pH 4.0 for 60 min (▲) are shown. Line a is a tangent line of the actual growth curve and is approximately parallel to that of the control growth curve. A, actual lag time; B, expected lag time.

Lactobacillus were about half those of oral streptococci under experimental conditions. As the environmental pH was lowered, however, the growth rates of mutans streptococci rose above those of *S. sanguinis* and *S. oralis* at pH 4.8 and above those of *Actinomyces* at pH 4.4. *Lactobacillus* showed a similar tendency but the growth rates became higher than those of mutans streptococci at pH 4.4. These results were consistent with those of previous studies using a continuous culture of mixed bacteria (3–5), although the pH values causing growth limitation seemed to be relatively lower than those from continuous culture studies. This discrepancy could be because of methodological differences between batch culture and continuous culture. The lower acid tolerance of bacteria in continuous culture can be ascribed to their growth under nutrient limitation with a concomitant lower capacity for adapting to the acid conditions. Continuous culture is generally accepted as the method of choice when evaluating bacterial growth at acidic pH. Overall, the present study, using a batch culture method, supports the previous finding that acid-tolerance of bacterial growth rate is an ecological determinant of microbial composition in supragingival plaque, as assumed in the 'ecological plaque hypothesis' (15, 16).

The acid-tolerance of bacterial growth rate indicates the bacterial ability to grow at different pH as described above, but this does not provide information regarding the acid-impairment of bacteria under acidic conditions. Acid-killing is known as one of the determinants of bacterial acid-impairment (1, 6, 9, 14). In the present study, the acidification to pH 4.0 gradually killed *A. naeslundii*, *S. sanguinis*, and *S. oralis*, while mutans streptococci and *Lactobacillus* survived the acidification. These results were similar to previous reports (21, 23), suggesting that *A. naeslundii*, *S. sanguinis*, and *S. oralis* are killed gradually at pH 4.0.

Were the cells that survived the acidification affected? Our previous study indicated that acidification at pH 4.0 for 1 h impaired the growth ability of *S. sanguinis* cells temporarily through a reversible inhibition of glycolytic enzymes, and that the impaired cells started to grow again at pH 7.0 with a longer lag phase (23). In the present study, a similar phenomenon was observed in *A. naeslundii*, *S. sanguinis*, and *S. oralis*. The cells that had survived during the acidification at pH 4.0 started to grow again, but the growth was apparently slower than the expected growth calculated from the number of surviving cells. These findings clearly indicate that physiological and transient acidification impairs the bacterial growth ability temporarily and that the growth ability recovers after the time required for repair.

Temporary bacterial impairment has not been considered as an ecological determinant in dental plaque because no clear influence, such as killing of bacteria, has been observed. However, it should be recognized that a usual method for counting live bacterial cells is to spread bacterial cells on agar plates and incubate them for 1–2 days for colony formation. Using this method it is difficult to detect a delay in bacterial growth. The combination of counting live cells on agar plates and monitoring bacterial growth in liquid media, as adopted in the present study, could enable us to observe temporary and reversible acid-impairment of bacterial growth if the bacteria are not acid-killed. The transient acidification occurs in dental plaque at least three times a day after each meal, probably having a significant influence on bacterial growth ability after pH recovery to neutral, and subsequently functioning as a driving force of ecological modification of dental plaque microflora.

In immature dental plaque, the pH falls to around 5, while the plaque pH becomes more acidic, to around 4, as the plaque matures (7). Transient acidification to around pH 5.5, as occurs in immature plaque, may increase bacterial acidogenicity and acidurance through a series of bacterial acid-adaptation mechanisms. These include the induction of H^+-ATPase, stress proteins, and metabolic enzymes for alkaline production and the reinforcement of the bacterial membrane against H^+-penetration (1, 22, 25), probably along with a shift of energy usage from growth to maintenance metabolism (8, 13) and a resultant decrease in bacterial growth rate. However, a frequent and greater acidification to pH 4.0, as occurs in mature plaque, may weaken the competitiveness of *A. naeslundii*, *S. sanguinis*, and *S. oralis* through a reversible but repetitive impairment of growth ability. Furthermore, prolonged acidification around pH 4, as occurs in established caries lesions (11),

Table 1. Actual and expected lag times

Bacterial strains		Acidification time (h) 0.5	1	2
A. naeslundii ATCC 12104	Actual	1.87 (0.58)[1]	2.97 (1.04)	nc
	Expected	0.36 (0.09)	0.53 (0.07)	4.44 (0.95)
A. naeslundii WVU 627	Actual	2.26 (0.57)	3.52 (0.28)	nc
	Expected	1.14 (0.12)	1.98 (0.47)	20.2 (4.03)
S. oralis ATCC 10557	Actual	0.98 (0.57)	2.22 (0.69)	6.82 (4.11)
	Expected	0.39 (0.27)	0.63 (0.57)	4.41 (2.49)
S. sanguinis ATCC 10904	Actual	1.46 (0.30)	4.85 (1.51)	nc
	Expected	1.62 (0.59)	3.05 (1.13)	7.24 (4.06)
S. sanguinis ATCC 10556	Actual	0.82 (0.30)	2.65 (1.23)	7.19 (2.74)
	Expected	0.16 (0.10)	0.21 (0.19)	2.06 (1.94)
S. mutans NCTC 10449	Actual	0.13 (0.15)	0.34 (0.22)	0.33 (0.30)
	Expected	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
S. sobrinus 6715	Actual	0.14 (0.12)	0.00 (1.19)	0.00 (1.02)
	Expected	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
L. paracasei C12-4	Actual	0.33 (0.32)	0.28 (0.27)	0.00 (0.20)
	Expected	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

[1]The mean values with standard deviations (h) in parenthesis of three independent experiments are given.
nc, not calculated because of poor growth at 12 h after acidification.

may exclude these bacteria through acid-killing and leave more acidogenic and aciduric bacteria, such as mutans streptococci and *Lactobacillus*, as demonstrated by continuous culture studies (3–5, 17).

Acknowledgments

This study was partly supported by Grants-in-Aid for Encouragement of Young Scientists (Nos. 06771620 and 07771650 to N.T.) and Grants-in-Aid for Scientific Research B (Nos. 16390601 and 19390539 to N.T.) from the JSPS, Japan.

References

1. Belli WA, Marquis RE. Adaptation of *Streptoccocus mutans* and *Enterococcus hirae* to acid stress in continuous culture. Appl Environ Microbiol 1991: **57**: 1134–1138.
2. Boue D, Armau E, Tiraby G. A bacteriological study of rampant caries in children. J Dent Res 1987: **66**: 23–28.
3. Bowden GH, Hamilton IR. Environmental pH as a factor in the competition between strains of the oral *Streptococcus mutans*, *S. sanguis*, and "*S. mitior*" growing in continuous culture. Can J Microbiol 1987: **33**: 824–827.
4. Bradshaw DJ, Marsh PD. Analysis of pH-driven disruption of oral microbial communities *in vitro*. Caries Res 1998: **32**: 456–462.
5. Bradshaw DJ, Mckee AS, Marsh PD. Effects of carbohydrate pulses and pH on population shifts within oral microbial communities *in vitro*. J Dent Res 1989: **68**: 1298–1302.
6. Casiano-Colón A, Marquis RE. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl Environ Microbiol 1988: **54**: 1318–1320.
7. Chida R, Igarashi K, Kamiyama K, Hoshino E, Esashi M. Characterization of human dental plaque formed on hydrogen-ion-sensitive field-effect transistor electrodes. J Dent Res 1986: **65**: 448–451.
8. Even S, Lindley ND, Cocaign-Bousquet M. Transcriptional, translational and metabolic regulation of glycolysis in *Lactococcus lactis* subsp. *cremoris* MG 1363 grown in continuous acidic cultures. Microbiology 2003: **149**: 1935–1944.
9. Gorden J, Small PLC. Acid resistance in enteric bacteria. Infect Immun 1993: **6**: 364–367.
10. Harper DS, Loesche WJ. Growth and acid tolerance of human dental plaque bacteria. Arch Oral Biol 1984: **29**: 843–848.
11. Hojo S, Komatsu M, Okuda R, Takahashi N, Yamada T. Acid profiles and pH of carious dentin in active and arrested lesions. J Dent Res 1994: **73**: 1853–1857.
12. Igarashi K, Kamiyama K, Yamada T. Measurement of pH in human dental plaque *invivo* with an ion-sensitive transistor electrode. Arch Oral Biol 1981: **26**: 203–207.
13. Kashket ER. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol Rev 1987: **46**: 233–244.
14. Lin J, Lee IS, Frey J, Slonczewski J, Foster JW. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. J Bacteriol 1995: **177**: 4097–4104.
15. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994: **8**: 263–271.
16. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology 2003: **149**: 279–294.
17. McDermid AS, McKee AS, Ellwood DC, Marsh PD. The effect of lowering the pH on the composition and metabolism of a community of nine oral bacteria grown in a chemostat. J Gen Microbiol 1986: **132**: 1205–1214.
18. Milnes AR, Bowden GH. The microflora associated with developing lesions of nursing caries. Caries Res 1985: **19**: 289–297.
19. Sansone C, van Houte J, Joshipura K, Kent R, Margolis HC. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surfaces. J Dent Res 1993: **72**: 508–516.
20. Schachtele CF, Jensen ME. Comparison of methods for monitoring changes in the pH of human dental plaque. J Dent Res 1982: **61**: 1117–1125.
21. Svensäter G, Larsson U-B, Greif ECG, Cvitkovitch DG, Hamilton IR. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immunol 1997: **12**: 266–273.
22. Takahashi N. Microbial ecosystem in the oral cavity: metabolic diversity in an ecological niche and its relationship with oral diseases. In: Watanabe M, Takahashi N, Takada H ed. Interface Oral Health Science International Congress Series 1284. Oxford: Elsevier, 2005: 103–112.
23. Takahashi N, Horiuchi M, Yamada T. Effects of acidification on growth and glycolysis of *Streptococcus sanguis* and *Streptococcus mutans*. Oral Microbiol Immunol 1997: **12**: 72–76.
24. Takahashi N, Nyvad B. Caries ecology revisited: microbial dynamics and the caries process. Caries Res 2008: **42**: 409–418.
25. Takahashi N, Yamada T. Acid-induced acidogenicity and acid tolerance of non-mutans streptococci. Oral Microbiol Immunol 1999: **14**: 43–48.
26. van Houte J, Lopman J, Kent R. The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. J Dent Res 1996: **75**: 1008–1014.