

The prevalence of virulent clonal strains of mutans streptococci *in vivo* and co-culture succession of the strains *in vitro*

—Virulence potential of mutans streptococci

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ABSTRACT

Purpose: To examine selected putative virulent properties of mutans streptococci in two groups with different caries activity and to examine co-culture hybridization of the strains *in vitro*. **Methods:** A set of strains from caries-free subjects (115) and another set from caries-active subjects (165) were isolated. Each strain was characterized for three virulence determinants. The clinical bacteria were then cocultured by three strains exhibiting the highest levels of virulence. Isolate colonies of last filial generation bacteria were enrichment-incubated and estimated for virulence again. RAPD-PCR and MLEE analyses were processed for parental bacteria and last filial generation one. **Results:** No difference associated with caries activity of the subjects from whom the isolates originated. Virulent properties of a filial generation strains was not different in the same generation, but was very different from their parent strains. **Conclusion:** The coexist properties of virulent polyclonal strain of MS may hold in a very general conditional sense in a dental plaque ecosystem *in vivo*, however, one of the co-culture strains may become dominant and displaced the others as the result of continuous ecological succession *in vitro*.

Keywords: Ecological Phenomenon; Mutans Streptococci; Co-Culture; Virulent; *in Vitro*

1. INTRODUCTION

Dental caries is a transmissible and poly-microbial ecological disease, in which mutans streptococci (MS), mainly *Streptococcus mutans* and *Streptococcus sobrinus* play the major role. The main virulent factors of MS

include adhesion, acidogenicity and aciduricity [1-3]. Primary mechanism for adherence of *S. mutans* is the production of glucan from sucrose via glucosyltransferases (GTFs) [4,5]. Acid production and aciduricity are other important caries-inducing virulent factors [6-8]. MS is generally considered to be the principal aetiological agent of dental caries [9,10]; however, they are widely distributed not only in populations with moderate or high caries prevalence [11,12] but also in populations having no or low caries experience [13,14]. Caries can also develop in the absence of MS species [15].

So Takahashi and Nyvad present a new view of caries, the Extended Caries Etiological Hypothesis, that it is no longer just a consideration of which specific bacteria are present, but rather what those bacteria are doing. They are behaving as acidogenic/aciduric bacteria contributing to the dental caries disease process [16]. The virulence properties of MS have the coexist properties of a polyclonal epidemic [17]. The three highest virulent characters (GTFs activity, acid production and aciduricity) were not assembled together on a same strain [18]. Kuramitsu and Wang suggested that *S. gordonii*, and perhaps additional nonmutans streptococci, can attenuate some of the virulence properties of *S. mutans* [19].

Despite its importance, we have only a limited understanding of the biological nature of MS virulent strains colonization. How the prevalence of virulent factors of MS from strain to strain in a dental plaque ecosystem *in vivo*. How do polyclonal strains replace one another in ecological succession *in vitro*? To attempt to determine what happens to ecological sites where more than one *S. mutans* strain is present, co-culture of this study was performed by the 3 clinical strains which exhibited the highest level of virulence properties in glycosyltransferase activity, acid production, and acid tolerance, re-

spectively. The last filial generation bacteria on an agar plate were picked up and estimated for virulence properties again. Genetic studies were processed to understand how parent bacteria and last filial one variations.

2. MATERIALS AND METHODS

2.1. Subjects

The experimental procedures were approved by the Ethics Committee of Ninth People's Hospital, Medical College, Shanghai Jiao Tong University.

The study consisted of 100 subjects aged between 5 and 25 years (13.5 ± 4.7). Groups of caries-free and caries-active groups were 50 and 50, with the DMF (decayed, missing, filled) in caries-free ($dmft = 0$) and caries-active ($dmft > 4$), respectively. A total of 280 MS (both *sobrinus* and *mutans* species) was obtained from dental biofilm of the subjects. MS form Groups of caries-free and caries-active groups were 115 and 165.

2.2. Bacterial Isolates and Species Confirmation

Plaque biofilm samples were plated on Mitis Salivarius Bacitracin (MSB) agar plates (Difco Laboratories, Detroit, MI). They were cultured in an Anaerobic Work Station (BUG BOX DUAL, Ruskinn, England) with 85% of N_2 , 15% of CO_2 at $37^\circ C$ for 2 days. In order to detect MS, preliminary identification was done by Gram's staining method. Further classification was done to examine their sensitivity to antibiotics and fermentation of carbohydrates etc, by using a biochemical appraisal and verification system, API 20 Strep test kits according to the manufacturer's instructions (bioMerieux Vitek Inc.). *S. mutans* UA159 and *S. sobrinus* 6715 were used as standard laboratory strains.

2.3. GTFs Activity, Acid Production and Aciduricity Assay

Identified MS were incubated anaerobically in Trypticase soy broth (TSB) in an incubator at $37^\circ C$ for 18 h. The samples of bacterial supernatants were obtained by means of centrifugation. Extracellular GTFs activity was determined by means of direct enzyme assay [20]. Each supernatant was exposed to ^{14}C glucosyl-sucrose (Shanghai Institute of Nuclear Research of Chinese Academy of Science, Shanghai, China, final concentration = 100 mmol/L sucrose, 40 μ mol/L dextran 9,000 in buffered-KCl, pH 6.5) for 4 h at $37^\circ C$, to form glucans. GTFs activity by direct enzyme assay was expressed as micromol glucans formed per milliliter supernatant.

The acid sensitivity (tolerance) was assayed by using broth micro-dilution method on 96-microwell plates. Bacteria were incubated anaerobically in 5 ml of WILKINS-CHALGREN anaerobe broth (Oxoid, England) in the incubator at $37^\circ C$ for 24 hours to a density equal to

that of a no. 1 McFarland standard. For the evaluation of acid sensitivity, strains were sub-cultured into fresh anaerobe medium at pH of 5.5, 5.0, 4.5, 4.0, 3.5, 3.0 and 2.5 (medium pH was adjusted with HCl). The control was treated in the same way except the medium at pH 7.5. After 24 hours incubation, bacterial growth was observed by a stereoscope and recorded. At the same time, from every microwell, 50 μ l of suspension was removed and smeared on an agar plate. Bacterial colony was counted correspondingly after 48 hours incubation. The pH value at which there was no bacterial growth was considered as acid sensitivity.

For the evaluation of acid production, the strains were cultured in the anaerobe broth with glucose in microwell plate at pH of 7.5. After 24 hours incubation in an anaerobic chamber, acid production of bacterial cultures was measured by an Orion pH meter (Model 501, Orion Research, Inc., Cambridge, MA). Un-inoculated broth (control) was also cultured under the same conditions. The experiments of virulent assay were performed in quadruplicate and the result was presented by means.

2.4. Co-Culture *in Vitro*

Hybridization co-culture was performed by 3 strains with ratio of 1:1:1 (v/v/v) anaerobically in Trypticase soy broth (TSB) in an incubator at $37^\circ C$, which exhibited the highest levels of aciduricity, acidogenicity and GTFs activity, respectively. Each respective strain was cultured alone under the same condition as control. After one week of co-culture, during which the growth medium was replenished daily, the cultures was plated out, individual colonies were enrichment incubated and assayed for aciduricity, acidogenicity and GTFs activity again.

2.5. RAPD-PCR Analysis

A total of 26 of 96 strain colonies from last filial generation were next subjected to RAPD-PCR analysis. The protocol for DNA amplification was based on Pereira [21], with a little modification. For DNA extraction, cells were washed in 0.1 M Tris buffer, lysed with lysozyme + 10% SDS and extracted with phenol-chloroform. DNA was precipitated with sodium acetate and ethanol, and its concentration was calculated by measuring A_{260} . Quality was estimated from A_{260}/A_{280} ratio. RAPD working primers [21] were OPA-3 (5'-AGTCA-GCCAC-3'), OPA-5 (5'-AGGGGTCTTG-3'), and OPA-18 (5'-AGGTGACCGT-3') from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The RAPD reaction was performed in a total volume of 50 μ l consisting of 5 μ l template DNA, 10 \times PCR buffer (750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 3.5 mM $MgCl_2$, 200 μ M deoxynucleoside triphosphates, 1.25 U of Taq DNA po-

lymerase (Fermentas, Lithuania), and 1 μ M of primer. The amplification was done in a Perkin Elmer 9600 Thermal Cycler. PCR cycling procedures were as follows; 50 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min. A final step of extension was applied at 72°C for 10 min. Fifteen microliters of PCR products were analysed by electrophoresis on 4% denaturing PAGE (polyacrylamide gel electrophoresis) and displayed by silver staining.

2.6. Multilocus Enzyme Electrophoresis Analysis (MLEE)

Soluble proteins were extracted by grinding 100 mg freeze-dried bacteria with pestle and mortar in liquid nitrogen and 5 ml buffer solution (0.1M Tris-HCl, pH 6.8). The mixture was centrifuged for 20 min at 17,000 rpm and the supernatant collected. The protein content in supernatant was estimated. Protein content was adjusted to 1 mg/ml per sample.

PAGE was performed by the method described by Laemmli [22]. Proteins were analyzed on a dual vertical slab mini-gel system (Bio-Rad Mini-Protean, Hercules, CA, USA). From each sample, 20 μ l of extract was loaded on a polyacrylamide gel. The separation gel (10%) and stacking gel (3.5%) were prepared from an acrylamide monomer solution (Roth, Karlsruhe, Germany). Electrophoresis was carried out at a constant current of 35 mA through the stacking gel, and at 90 mA through the

separation gel at 4°C. After the running time, gels were revealed for enzyme active band detection of glucose dehydrogenase (GDH), glucose-6-phosphate dehydrogenase, glucosyltransferase (GTF), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), mannitol-1-phosphate dehydrogenase (M1P), mannose-phosphate isomerase (MPI), nucleoside phosphorylase (NSP), phenylalanyl-leucine peptidase (PLP), and glutamic-oxalacetic transaminase (GOT). After appearing, the bands were scored according to their respective relative mobilities [23].

2.7. Statistical Analysis

The GTFs activity and acidogenicity of strains between groups from caries-free and caries-active subjects were compared by means of student-t tests.

3. RESULTS

The distribution of MS in dental plaque of different carious experience persons was different. The detection rate of MS isolated from caries-active (61%) were higher than that isolated from caries-free patients (35%) ($P < 0.005$), however, the distribution of virulence potential strains of MS was almost the same in caries-free subjects and caries-active subjects (Figure 1). The three principal virulence factors were not put together in one strain (data not shown). There was no significant difference between the two groups ($P > 0.05$) of caries-free and

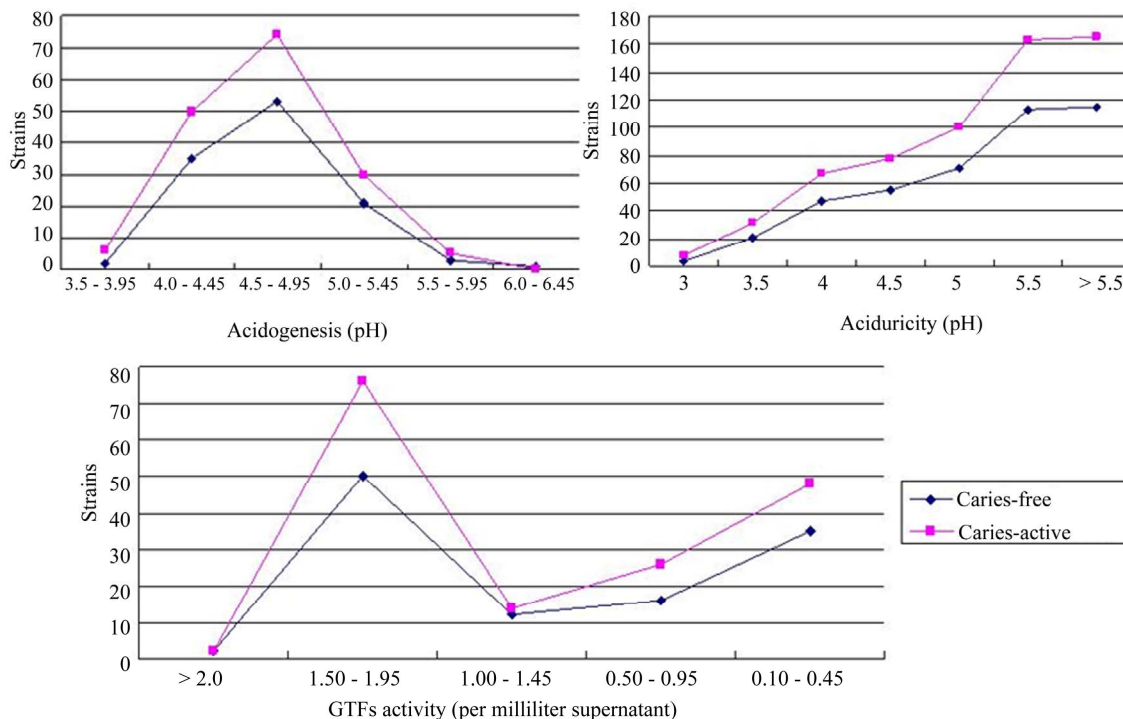


Figure 1. Acidogenicity, aciduricity and GTFs activity distribution of mutans streptococcus strains of clinical isolates from caries-active and caries-free subjects.

caries-active in GTFs activity and acidogenicity (**Table 1**).

In the test of co-culture, a total of 96 individual colonies were isolated and characterized biochemically. Virulent properties of a filial generation strains was not different in the same generation, but was very different from their parent strains (**Table 2**). The virulent potential of the control strains did not change after one week culture alone (data not shown). A total of 26 of 96 strain colonies from last filial generation were next subjected to RAPD-PCR analysis. RAPD-PCR data showed that the band profiles of filial generation bacteria were not significantly different from each other but different from any ones of the parent strains (**Figure 2**). It may suggest that one strain (strain A) of last filial generation may become dominant and displaced the others (**Table 2**, **Figures 2** and **3**). In the results of MLEE, only GOT, NSP, PLP, MPI, LAP as well as MIP showed activity for *S. mutans* strain (**Figure 3**). The results of MLEE were similar to that of RAPD-PCR. RAPD was proved to be more discriminatory than MLEE.

4. DISCUSSION

This work described an analysis of acidogenicity, aciduricity and GTFs activity amongst 280 clinical strains of MS. It was found that the distribution of virulence potential of MS was nearly the same in strains from caries-free subjects and caries-active subjects. Three highest virulent characters (GTFs activity, aciduricity, and acidogenicity) were not fixed together on a same strain. The result is consistent with the study by Guo [18]. So we may proposed a hypothesis that MS strains with varying degree of virulence potential may play different functions *in vivo* (for example one strain plays a function of

GTFs activity, other strain plays a function of acid production and another strain plays a function of aciduricity), which makes them a good collective group to obtain the best potential of cariogenic.

Co-culture of MS strains was performed by the 3 clinical strains which exhibited the highest level of different virulent potential. The results of filial generation obtained neither a combination group of MS strains nor one strain with the highest virulence potential. Virulent properties of a filial generation strains was not different in the same generation, but was very different from their parent strains. RAPD-PCR and MLEE analysis revealed that the band profiles of filial generation bacteria were not significantly different from each other but different from any ones of the parent strains. In the micro-ecological environmental condition *in vitro*, one strain may become dominant and displace the others. This cannot exclude simple adaptation; however, the data indicated the different ecological succession outcomes of polyclonal virulent strains between *in vitro* and *in vivo*.

The niche concept was defined by G. E. Hutchinson. A species can only live within a certain environment, or within a certain environment a species can live. According to the competitive exclusion principle, no two species can occupy the same niche in the same environment for a long time [24]. Different strains of the same species are more likely to occupy the same ecological niche, and hence undergo more intense competition than different species [25]. In the study of Rukayadi, if two different bacterial strains occupy the same niche on the leaf surface, then the antagonism between them can occur only when they use the same nutrient source and occupy the same niche [26]. The nontoxic strains applied to soil occupy the same niches as the natural occurring toxic

Table 1. GTFs activity and acidogenicity of clinical isolates of mutans streptococci.

| | Caries-free (115 Strains) | Caries-active (165 Strains) | <i>P</i> -value (Student-t test) |
|--|---------------------------|-----------------------------|----------------------------------|
| GTFs activity (per milliliter supernatant) | 0.9611 ± 0.6630 | 1.2218 ± 0.6799 | 0.0790 |
| Acidogenicity (pH) | 4.8624 ± 0.5857 | 4.5657 ± 0.4543 | 0.0893 |

The result of aciduricity is not an absolute term and state at **Figure 1**.

Table 2. The actual virulence properties of each strain of last filial generation and parental bacteria of 3 strains which exhibited the highest levels of aciduricity, acidogenicity and GTF activity.

| | GTFs activity (per milliliter supernatant) | Aciduricity pH | Acidogenicity pH |
|--|--|----------------|------------------|
| Strain of the most potential acidogenic capacity | 0.27 | 5.0 | 3.9 |
| Parental bacteria | | | |
| Strain of the most potential aciduricity capacity | 1.67 | 3.0 | 5.59 |
| Strain of the most potential GTFs activity capacity | 2.2 | 4.5 | 4.28 |
| Strain colonies of last filial generation (mean ± SD, <i>n</i> = 96) | 1.36 ± 0.08 | 4.0 ± 0.04 | 4.2 ± 0.3 |

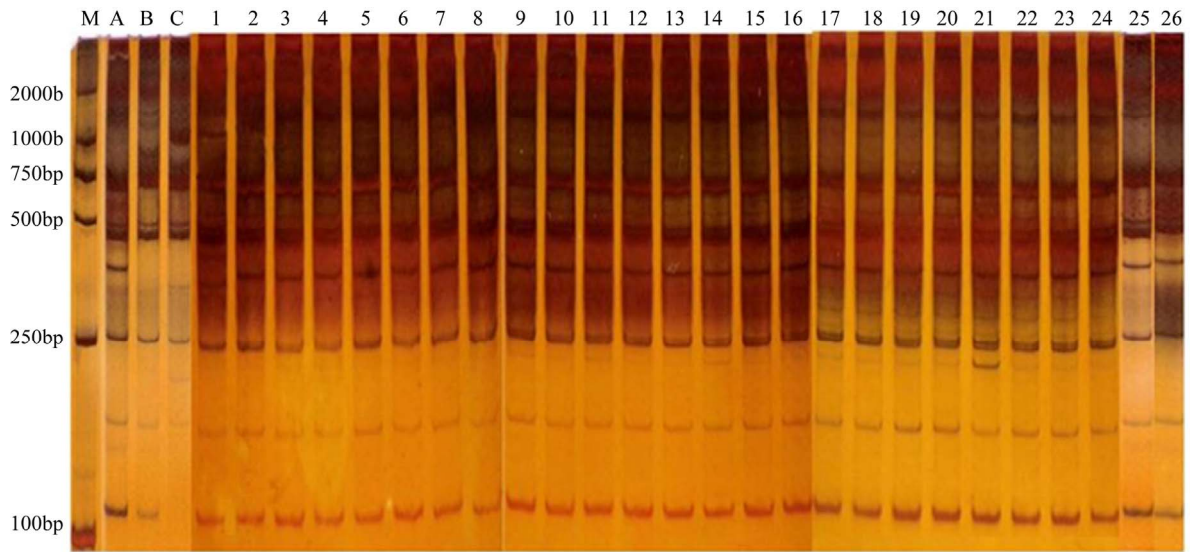


Figure 2. RAPD-PCR analysis of the gene transformation of three parental generation and last filial generation mutans streptococci strains. M: marker, A, B and C: parental generation strains, A: acidogenicity strain, B: aciduricity strain, C: GTFs activity strain. 1 - 26: mutans streptococci strains of last filial generation. Bands spectrum from filial generation mutans streptococci were almost same but different from their three parental generation strains.

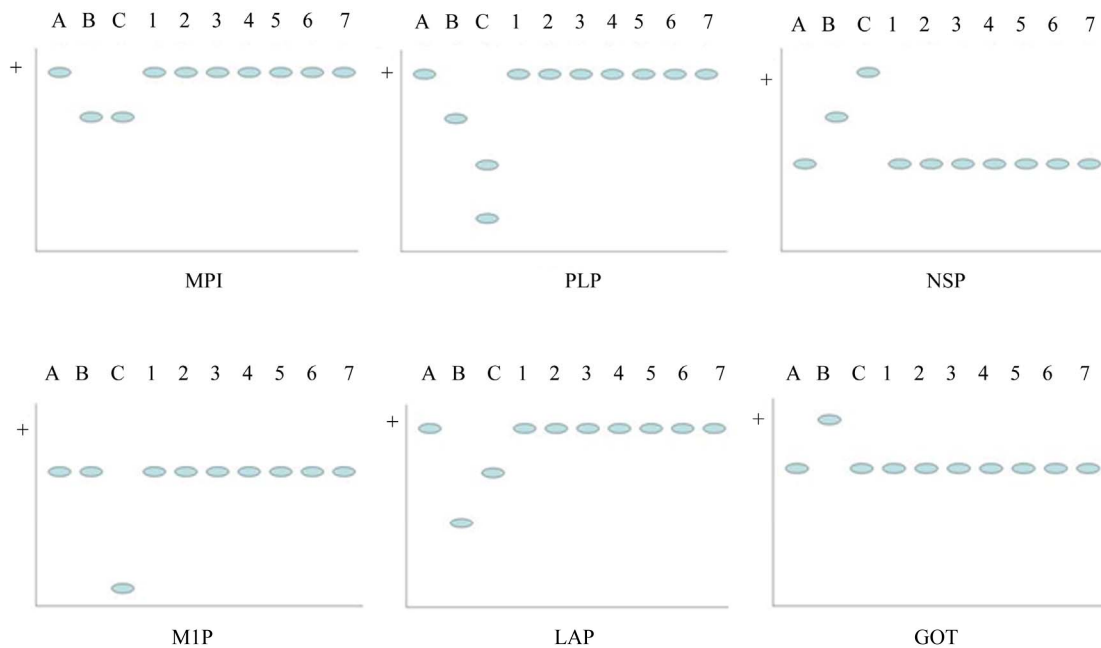


Figure 3. Multilocus enzyme electrophoresis analysis of three parental generation and filial generation mutans streptococcus strains. A, B and C: parental generation strains, A: acidogenicity strain, B: aciduricity strain, C: GTFs activity strain. 1 - 7: mutans streptococci strains of last filial generation. GOT: glutamic-oxalacetic transaminase, NSP: nucleoside phosphorylase, PLP: phenylalanyl-leucine peptidase, MPI: mannose-phosphate isomerase, LAP: leucine aminopeptidase, MIP: mannitol-1-phosphate dehydrogenase. Enzyme loci from filial generation mutans streptococci were almost same.

genic strains. They are capable of competing and displacing toxigenic strains [27]. These data are more in agreement with our results which indicate that different strains of the same species undergo more intense competition if they need protection from the surrounding harsh

ecological niche. A group of different strains of the same specie can not occupy the same ecological niche *in vitro*.

In conclusion, the coexist properties of virulent polyclonal strain of MS may hold in a very general conditional sense in a dental plaque ecosystem *in vivo*, how-

ever, one of the co-culture strains may become dominant and displaced the others as the result of continuous ecological succession *in vitro*.

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ABBREVIATIONS

GTF: glucosyltransferases; RAPD-PCR: Random amplified polymorphic DNA - Polymerase Chain Reaction.