Acidogenicity of *Streptococcus mutans* at Different pH Conditions in a Chemostat.

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INTRODUCTION

Dental plaque on the tooth surfaces is implicated in the etiology of two prevalent diseases affecting industrialized societies i.e. dental caries and periodontal diseases[1]. The microflora on enamel is however influenced by a variety of environmental factors, the chemostat studies have been used *in vitro* to establish constant and stable long-term environments for bacterial growth which can provide a homogeneous population of cells, and allows accurate and precise manipulation of culture variables[2-4].

It is well known that *Streptococcus mutans* is one of the most dominant bacteria in the initiation of dental caries caused by rapid acid formation from sugar substrate[5]. The activity of the phosphoenol-pyruvate-phosphotransferase, and hence acid production of *S. mutans* was found to vary according to the conditions of growth in a chemostat studies[6-10].

On the other hand, the caries process, that is the decalcification of dental enamel, takes place at a relatively low pH[11]. The ability of plaque microorganisms to sustain acid production at a low pH (aciduricity), thus during down the pH of plaque is considered to lie an important caries-associated microbial evidence trait[12]. Hence, we attempt to determine the relationship between the acidogenicity of *S. mutans* Ingbritt 1600 grown under different pH (6.8, 5.8, 4.8) conditions using a chemostat system under glucose-limiting conditions and the rate of acid production at of different pH stat levels (7.0 and 5.0).

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MATERIALS AND METHODS

1. Bacterial strain and growth

S. mutans IB-1600 was grown in a New Brunswick chemostat (model 30, New Brunswick Scientific Co. Inc., Edison, N. J.) with a working volume of 350 ml using either Todd Hewitt Broth (THB; Balt. Biol. Labs. Cockeysville, Maryland, USA), TTY[13] or modified TTY medium (decreased concentration of buffering salt 1/8). Dilution rate (D) was 0.1 hr^{-1} (doubling time 6.9 hr), and culture pH was continuously controlled at three different values (6.8, 5.8 and 4.8) in each growth medium by the automatic addition of 1 mol/L KOH. Before the end of each chemostat experiment the culture pH was returned to the initial pH 6.8.

2. Collection of samples

Total of 220 ml of culture broth was collected (approximately 7, 10, 13 generation times of cultivated cells respectively) without changing of dilution rate after reaching steady state under each three different culture pH conditions. Cells were washed twice by potassium buffer (100 mmol/L), and harvested by centrifugation (10,000×g) for 10 min at 4°C, followed by spatulation on filter paper (grade 6B, VWR Scientific, Inc., San Francisco, CA) to remove excess moisture. After calibration by wet weight, the cell sediments were resuspended in a 150 mmol/L KCl, 1 mmol/L $MgSO_4$ solution (20 mg wet weight of cells/ml), and maintained on ice until use in pH stat experiments.

3. Analysis of protein

The bicinchoninic acid (BCA) protein assay method (Pierce Chemical C., IL) was used to estimate the content of porteins in cell suspensions with known concentrations

(100–600 μg/ml) of bovine serum (Sigma Chemical Co.) as a standard.

4. Glycolytic activity

As an index of acidogenicity, glycolytic activity was evaluated from two criteria i.e. glycolytic rate and pH free fall.

(1) Glycolytic rate

The rate of acid production from glucose challenge was determined at constant pH levels of 7.0 and 5.0 in a pH stat (Metrohm Co., Herisau, switzerland) at 37°C. Two ml of cell suspensions (wet weight of 20 mg/ml) was placed in the pH stat vessel, adjusted to the desired pH level with either 0.1 N KOH or 0.1 N HCl. The cells were held at the respective pH levels for 2 min and then 1% glucose (40 μl of a 500 mg/ml solution) was added to the vessel. The pH maintained at a constant pH level by the addition of 0.1 N KOH over a 10 min period. The endogenous glycolytic rate (wet weight of 20 mg/ml) in the absence of a glucose challenge was also measured at pH 7.0 level in a pH stat over a 10 min period. Glycolytic rate values were obtained from μmole of metabolic acid neutralized per mg protein per min.

(2) pH free fall experiment after addition of glucose

Terminal pH achieved in 15 min after addition of 1% glucose was determined under free fall conditions. The cell suspension (wet weight of 20 mg/ml) was placed in the vessel of the pH stat at 37°C, the pH 7.0 was adjusted with 0.1 N KOH. After 2 min, 1% flucose was added to the vessel. The change in pH free fall was recorded for 15 min.

The mean values and standard deviation of each tested were obtained from 3 numbers (exhaust samples at 7, 10, 13 generation times of cells grown under same culture pH level), and analyzed a Student's *t*-test.

RESULTS

1. The effect of culture medium on glycolytic rate

The glycolytic rate of cells grown at pH 6.8 varies according to the medium used are given in Fig. 1.

For both pH conditions of 7.0 and 5.0 which were challenged a 1% glucose in a pH stat, the values for the THB growth conditions revealed the least and modified TTY was the highest. There are statistical differences between the values of THB and that of TTY or modified TTY medium, however no such difference was observed between the values of TTY and that of modified TTY ($p<0.05$).

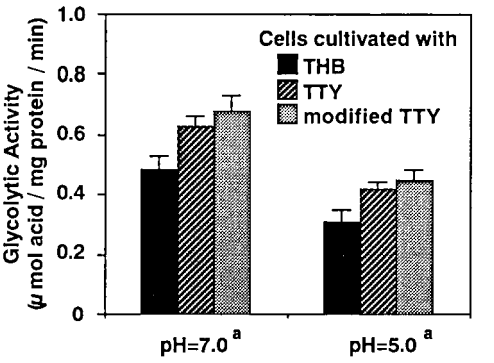

Fig. 1. The glycolytic rate of cells grown at pH 6.8 with three different medium. [a]: 1% glucose challenge.

2. The ffect of culture pH conditions

Glycolytic rate for culture pH conditions of 6.8, 5.8, 4.8 and returned again 6.8 were measured. The pH stat condition of 7.0 and 5.0 experiments were initiated with a 1% glucose challenged, and for endogenous metabolism at pH 7.0 there was no glucose challenge.

Fig. 2 shows the glycolytic rate with modified TTY medium according to three different culture pH conditions. In the case of glucose challenged, glycolytic activity was highest for cells cultivated at pH 5.8 and that at pH 6.8 was the lowest for both pH 7.0 and 5.0 of pH stat conditions. No significant difference was observed between glycolytic rate of cells cultivated at pH 5.8 and that at pH 4.8 ($p<0.05$).

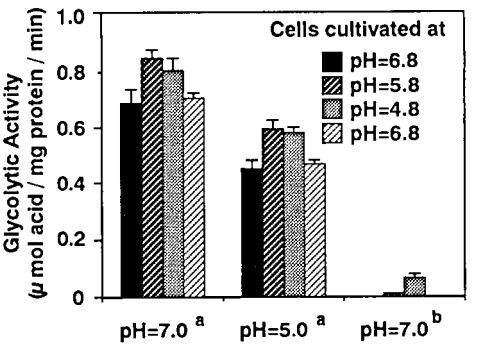

Fig. 2. The glycolytic rate of cells cultivated with modified TTY medium according to three different culture pH conditions (pH 6.8, 5.8, 4.8 and again 6.8). [a]: 1% glucose challenge, [b]: absence of glucose challenge.

Fig. 3 shows glycolytic rate with THB medium according to three different culture pH conditions. There was a similar tendency with modified TTY medium. Glycolytic rate with cells cultivated both at pH 4.8 and 5.8 were higher than that at pH 6.8 for both pH levels of 7.0 and 5.0 in the pH stat conditions ($p<0.05$).

Endogenous metabolism at pH 7.0 in the pH stat, due to endogenous carbolystic was highest for cells grown at pH 4.8 follwed by cells grown at pH 5.8 and 6.8 ($p<0.05$).

For both glycolytic values with modified TTY and THB medium for cells grown again at pH 6.8 returned to their initial values under all conditions tested (pH 7.0, 5.0 and endogenous in the pH stat).

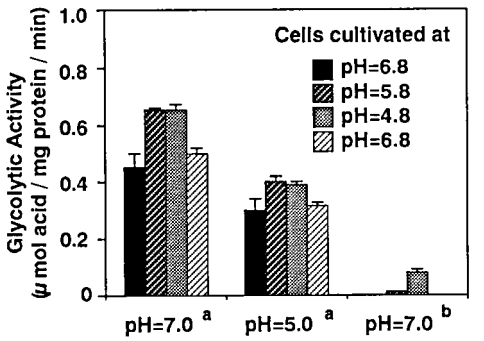

Fig. 3. The glycolytic rate of cells cultivated with THB medium according to three different culture pH conditions (pH 6.8, 5.8, 4.8 and again 6.4). [a]: 1% glucose challenge, [b]: absence of glucose challenge.

3. pH free fall experiment after addition of glucose

Fig. 4 and 5 shows pH fall after the addition of 1% glucose for cells grown under three different pH in modified TTY and THB medium.

Terminal pH of cells for both medium when grown at pH 6.8 was the highest, while cells grown at pH 4.8 was the lowest ($p<0.05$).

4. Relationship between glycolytic activity or temrinal pH and culture pH

For both medium, a negative correlation was observed between glycolytic rate except pH stat level 7.0 with modified TTY medium and culture pH (Table 1). While, terminal pH showed a positive relationships with culture pH for both medium (Table 1).

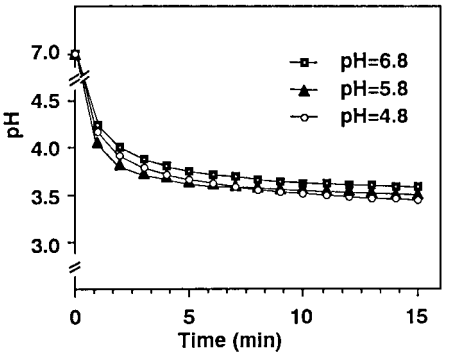

Fig. 4. The pH free fall after the addition of 1% glucose for cells grown under three different culture pH conditions (pH 6.8, 5.8, 4.8 and again 6.8) in modified TTY medium.

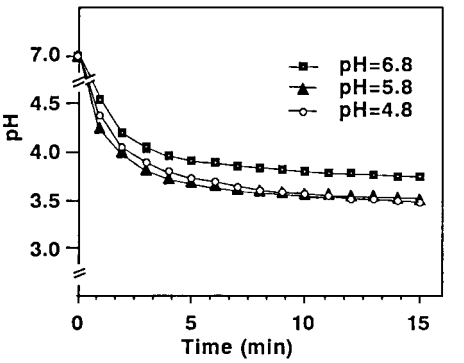

Fig. 5. The pH free fall after the addition of 1% glucose for cells grown under three different culture pH conditions (pH 6.8, 5.8, 4.8 and again 6.8) in THB medium.

Table 1 Correlation coefficient between glycolytic rate or terminal pH and culture pH.

	Correlation coefficient with culture pH	
	TBH medium	modified TTY medium
Glycolytic rate with pH stat conditions at		
pH 7.0[a]	−0.848*	−0.502
pH 5.0[a]	−0.797*	−0.781*
pH 7.0[b]	−0.920*	−0.922*
Terminal pH	0.941*	0.956*

n = 12

[a] 1% glucose challenge

[b] absnece of glucose challenge

* $p<0.01$

DISCUSSION

The acidic end products of the metabolism of glycolytic sugars by streptococci are directly involved in the initiation and progression of dental caries, and the plaque ecosystem in the final stages of the caries process is dominated by a more aciduric microflora in which *S. mutans* play a significant role[14].

It has been reported previously that the glycolytic activity (pH 7.0) condition in pH stat of *S. mutans* Ingbritt is higher when grown at pH 5.5 than when grown at pH 6.5 in chemostat system[8,9], and many *S. mutans* strains are moderately aciduric since they can metabolize and grown at pH values under 5.5[15].

In this experiments, no differences were observed between the glycolytic rate of *S. mutans* Ingbritt 1600 with cells cultivated at pH 4.8 and that at pH 5.8. However, endogenous metabolism at pH 7 in the pH stat, due to endogenous carbolystic for cells grown at pH 4.8 was significantly higher than that at pH 5.8 and 6.8., which indicate that glycolysis of *S. mutans* grown at low pH would become a highly potential. In fact, terminal pH of cells achieved after addition of glucose grown at pH 4.8 was the lowest and there are relationship between the culture pH and the terminal pH or glycolytic rate except at pH 7.0 in the pH stat with modified TTY medium.

Increase in glycolytic activity by the cell at low pH can be attributed to an increased need for ATP to drive proton extrusion via H^+/ATPase, in order to maintain a more alkaline internal pH[16,17]. Glycolytic activity of *S. mutans* Ingritt 1600 with cells cultivated at low pH might increase, when pH level lower than 5.0 in the pH stat experiments is used. It is considered that when glucose is rinsed in the oral conditions, the pH of superficial plaque is neutralized with salivary buffering action eventhough it becomes near the critical pH 5.5. However, the pH of substrate cells fall down with a simulatenous acid production of cells even in a glucose limiting environments for a surrouding with glucan. This pH free fall experiments showed that the terminal pH of *S. mutans* Ingbritt 1600 was achieved below pH 3.5 when cells were grown at pH 4.8.

On the other hand, glycolytic activity different from medium to medium. THB medium involved total of 1% of sodium chloride, sodium carbonate and disodium phosphate. As sodium ions are inhibitory to acid production by plaque organisms[9], glycolytic rate of cells cultivated with THB medium in all conditions in pH stat level would be lower than that with TTY or modified TTY medium. More over, glycolytic activity of cells may be influenced with buffering action of the meidum. When cells are cultivated with a medium in which few salts are included, buffering action on cell wall is decreased. At the pH of cell wall fall down due to acid production, hydrogen ion will be released instead of proton. In the case of low buffering action on the cell wall, amount of hydrogen ions were decreased, which indicated that a high amount of KOH is needed for neutralization.

The values of glycolytic rate and terminal pH of cells grown at final culture pH level returned to that of grown at initial same culture pH level, indicating acidogenicity is acquired by phenotipic adaptation.

SUMMARY

Streptococcus mutas IB-1600 was grown in a chemostat using three kind of medium, with a dilution rate of 0.1 hr^{-1}. Culture pH was controlled continuously at three different values (6.8, 5.8, 4.8, and again 6.8). Glycolytic activity and terminal pH achieved after addition of glusoce were evaluated using a pH stat experiments. Glycolytic acitivty differed from medium to medium. A relationship was observed between the culture pH and glycolytic acitivity or terminal pH. The values of glycolytic rate and terminal pH of cells grown at final culture pH level returned to that of grown at initial same culture pH level.

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