Induction of L-type colonies from *Streptococcus mutans*

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It is considered that *Streptococcus mutans* (*Str. mutans*) plays an important role in the initiation and development of dental caries. But the genetics of this species is yet to be clarified. The reason is that because its cell walls are hard, the genetic transformation does not readily progress. It occurs at an extremely low frequency^{1,2)}.

L-forms are cell wall-defective organisms, and differ largely from *Mycoplasmas* in that they do not require cholesterol for multiplication and the GC contents of gene DNA are higher³⁾. It has been shown that L-type colonies can not grow in the same medium as the parent strain increases, but a change sets in on the stable L-forms as a subculture using a suitable agar medium advances, and the maintenance of the gene of the parent strain can be held⁴⁾. Several researchers⁵⁻¹²⁾ have demonstrated that the genetic transformation of cell wall-defective microbial forms, such as L-forms, protoplasts and spheroplasts, are readily caused by cell fusion, transfection and so on.

The purpose of this study is to induce L-type colonies with an aim of obtaining stable L-forms from the strains of cariogenic *Str. mutans*.

Materials and Methods

The strains were anaerobically incubated for 8 h at 37°C in Trypticase soy broth (TSB, BBL) containing 0.5% yeast extract (Difco). Then the cells were harvested by centrifugation, washed twice with phosphate buffered saline pH 7.0 and suspended in a deionized water to an optical density at 600_{nm} 2.0

(Baush & Lomb, spectronic 88 with 1/2" test tube). An aliquot of this cell suspension was then mixed with an equal volume of 0.05 M Tris-Maleate NaOH buffer pH 6.5 containing 200 µg N-Acetylmuramidase SG¹³⁾ (Seikagaku Kogyo Co., Ltd.) per ml, 0.01 M magnesium chloride and 2% sodium chloride. As a control, a mixture of the buffer containing only salts and the cell suspension was used. After incubation for 60 min at 37°C, 0.1 ml of the reaction mixture was spread on the agar plates prepared for induction of L-type colonies and incubated anaerobically at 37°C for two weeks. During the period, attention was paid to the growth of the colonies presenting a so-called fried egg appearance. Viable cell counts, expressed in the number of colony-forming units (CFU) per ml, in the above control reaction mixture, were determined by the pour-plate technique using TSB agar plate containing 0.5% yeast extract. The composition of the medium used to induce L-type colonies was as follows: TSB as a basal medium, 0.5% yeast extract, 1.0% agar purified (Difco), 5% horse serum (M.A. Bioproducts, screened for *Mycoplasmas*) heated for 30 min at 56°C, potassium penicillin G (Meiji Seika Co., Ltd.) to 100 µg per ml, and several kinds and concentrations of chemical compounds are listed in Table 1.

The electron microscopic photograph of L-type colony was prepared according to the method described by Siegel, et al.¹⁴⁾

Results and Discussion

Table 2 shows the strains used in this

Table 1 The kinds and concentrations of chemical compounds added to the medium for induction of L-type colonies of *Str. mutans* strains

Chemical compound	NaCl	MgCl ₂	NH ₄ Cl	Glycine
Concentration	0(%)	0(mM)	0(mM)	0(%)
1	10	10		0.5
2	20	20		1.0
3	50	50		1.5
4	100	100		
5				

study, the presence and number of L-type colonies induced, and the kinds and concentrations of the additions in a suitable medium for each strain of cariogenic *Str. mutans* to induce L-type colonies. From eleven strains including all serotypes of (a) to (g) of *Str. mutans*, E-49(a), HS-6(a), BHT(b), Fa-1(b), OMZ-70(c), 67-1(c),

6725(c), OMZ-176(d), P-4(e), OMZ-175(f) and 6715(g), L-type colonies were induced. However, from five strains, GS-5(c), Ingbritt(c), LM-7(e), KIR(g) and AHT(g), L-type colonies failed to appear. Five- to seven-day incubation was required for the appearance of L-type colonies in these strains. The number of the colonies induced differed among the strains and were divided into three grades of (+) to (###). The kinds and optimal concentrations of the chemical compounds in a suitable medium also differed among the strains. In other words, in a concentration of sodium chloride participating in the osmotic pressure of the cells, an addition of 2% was suitable for OMZ-70 strain. However, 3% was suitable for HS-6, BHT, 67-1, 6725 and P-4 strain, 4% for E-49 and Fa-1, and both 2 and 3% were adequate for OMZ-175, while both 3 and 4% were suitable for OMZ-176 and 6715 strain, respectively.

Dienes and Sharp¹⁵⁾ described that, in the

Table 2 Induction of L-type colonies from sixteen strains of *Str. mutans*

<i>Str. mutans</i> strain	Sero type	L-type colonies induced	Optimum conc. of the additions			
			NaCl (%)	MgCl ₂ (mM)	NH ₄ Cl (mM)	Glycine (%)
E-49	a	††*	4	0	0	0
HS-6		+	3	10-20	0	0
BHT	b	###	3	10-20	0	0
Fa-1		+	4	0	10-100	0
OMZ-70	c	††	2	10-20	0	0.5-1.0
67-1		+	3	10-20	0	0
6725		+	3	10-20	0	0
GS-5		—				
Ingbritt	d	—				
OMZ-176		###	3, 4	10-20	0	0
P-4		+	3	10-20	0	0
LM-7		—				
OMZ-175	f	††	2, 3	10-20	0	1.0-1.5
6715	g	+	3, 4	10-20	0	0
KIR		—				
AHT		—				

*; —: 0, +: 1-100, ††: 101-1000, ###: over 1000 of L-type colonies per 10⁸ viable cells (CFU)

induction of L-forms from several species of bacteria, the effect of an addition of inorganic substances, such as magnesium chloride, ammonium chloride and others varied, largely depending on the type of the inorganic substances added. In this study, an addition of magnesium chloride was proved effective at concentrations of 10 to 20 mM on the nine strains except for two strains of E-49 and Fa-1 in which no L-type colony was obtained by the addition. Only on Fa-1 strain, ammonium chloride was successful at concentrations of 10 to 100 mM. Michel and Hijmans¹⁶⁾ reported that glycine had the effect on the induction of group A streptococcal L-forms. In this paper, in the two strains, i.e. OMZ-70 and OMZ-175, an addition of glycine was effective at concen-

trations of 0.5 to 1.0% and 1.0 to 1.5%, respectively.

In the microscopic photograph of a colony (Fig. 1, from *Str. mutans* BHT strain) obtained by the above-mentioned method, the so-called fried egg appearance was seen, and in the electron microscopic photograph of this colony (Fig. 2), the cell wall-defective forms and various sized cells were observed.

From the above observations and the facts that horse serum screened for *Mycoplasmas* was used and no L-type colonies appeared when only the medium prepared for induction was incubated, it was concluded that L-type colonies obtained in this study were true cell wall-defective colonies from cariogenic *Str. mutans*.

Fig. 1 L-type colony of *Str. mutans* BHT strain ($\times 40$). So-called fried egg appearance is seen.

Fig. 2 Electron micrograph of L-type colony of *Str. mutans* BHT strain ($\times 24,000$). All of the cells lack a cell wall and are surrounded by unit membrane.

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