A METHOD FOR GENTLE LYSIS OF STREPTOCOCCUS SANGUIS AND STREPTOCOCCUS MUTANS

R. J. Eisenberg and K. Lillmars
Department of Microbiology and Center for Oral Health Research
School of Dental Medicine
University of Pennsylvania, Philadelphia, Penna. 19174

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Summary. Methods have been developed to gently lyse strains of Streptococcus sanguis and Streptococcus mutans. For S. sanguis, the procedure involves: 1) acetylation of the cells; 2) exposure of the cells to high concentrations of lysozyme in the presence of a phosphate-sucrose buffer and 3) lysis of the cells with Sarkosyl. For S. mutans glycerol is used as a stabilizer instead of sucrose and dextranase is added along with lysozyme. Data are presented to indicate that 5 strains of each species are lysed by more than 60%. Using the lysing procedure developed for S. mutans, we were able to identify the plasmid of S. mutans LM7 by dye-CsCl and sucrose density centrifugation.

Introduction: The ease with which many bacterial species (especially the gram negative bacteria) are lysed with lysozyme and detergent in the presence of sucrose has enabled many workers to examine cells for the presence of plasmids (1, 2, 3). However, with the exception of Streptococcus faecalis, most streptococci are very resistant to lysis by these procedures. Most strains of S. mutans and S. sanguis are generally refractory to lysis by procedures which are successful for other species. Dunny et al. (2) reported the presence of a plasmid in strain LM7 of S. mutans but also reported that analysis of other strains of S. mutans for plasmids was hampered by the difficulties encountered in lysing the cells. Our own studies of S. sanguis DNA were also frought with difficulties of this kind (Eisenberg and Lillmars, unpublished results). We found great variability in cell lysis among strains of S. sanguis. We also found that lysis of a given strain varied in an unpredictable manner. Moreover, stationary phase cultures were generally refractory to breaking by chemical or physical means.

Because we wanted to examine the DNA of S. sanguis at various phases and conditions of growth, we decided to develop a procedure that would break cells efficiently, reproducibly and without regard to culture age or conditions. This report will describe our procedure developed for strains of S. sanguis. We found that with some modifications, this procedure is also applicable to all strains of S. mutans so far tested.

Results and Discussion: It was reported by Hayashi, et al. (4) that resistance to lysozyme in the genus Bacillus is accounted for in part by the presence of un-acetylated glucosamine in the peptidoglycan component of the cell wall. They reported that acetylation of the cell walls with acetic anhydride increased the sensitivity of the purified cell wall to lysozyme. For this reason, our lysis procedure includes an acetylation step. Although in some cases lysis may occur in young cultures without prior acetylation the lysis of other strains is absolutely dependent on this step. Thus for a given strain grown under a given set of circumstances, this step may be superfluous. Therefore, omission of this step should only be made on a trial and error basis for each new strain. We also found that the commonly used TES buffer system of 0.05M tris, 0.05M NaCl and .005M EDTA, pH 8.0 (1, 2) generally gave poor lysis with S. sanguis and S. mutans. Our lysing procedure involves the use of phosphate buffer containing $MgCl_2$ and NaCl (Table 1). The concentration of sucrose is a critical factor in obtaining cell lysis, especially of stationary phase cultures. In general, high (10-20%) concentrations of sucrose inhibit lysis. We found that optimal lysis of S. sanguis took place at a final concentration of 4% sucrose. However, for S. mutans, it was necessary to replace sucrose with glycerol and to treat the cells with dextranase as well as with lysozyme.

Table 1 summarizes the procedures chosen to lyse S. sanguis and S. mutans. For each strain 100 ml cultures in brain heart infusion broth (BHI,

Table 1. Procedure for lysing Streptococcus sanguis and Streptococcus mutans

Procedure	S. sanguis	S. mutans
Acetylation	Cells from 100 ml suspended in 6 ml acetic anhydride + 4.2g $NaHCO_3$ in 50 ml H_2O. Incubated 4°, 16 hours.	Same
Lysozyme	Cells suspended in: 1 ml buffered sucrose (20% sucrose in 0.1M phosphate buffer pH 7.0, 10^{-3}M dithiothreitol) 2 ml $MgCl_2$ (0.25 M) 1 ml NaCl (2M) 1 ml lysozyme (7 mg/ml) Incubated at 37° C*	Cells suspended in: 1 ml buffered glycerol (50% glycerol in 0.1M phosphate buffer pH 7.0 + 10^{-3}M dithiothreitol 2 ml $MgCl_2$ (0.25 M) 1 ml NaCl (2M) 1 ml lysozyme + dextranase (7 mg/ml lysozyme, 200 units/ml dextranase) Incubated at 37°C*
Pronase and detergent	1 ml (5 mg/ml) protease (Sigma Type VI) added to above suspension and incubated 30 min at 37°C. Then 0.3 ml of 20% Sarkosyl (Geigy) or sodium dodecyl sulfate (Sigma) was added. The suspension was incubated at 4°C until lysis occurred (30 minutes or less)	Same

* Time of incubation with lysozyme depended on the strain used and the age of the culture. For log phase cultures of S. sanguis strains G9B, Wicky and RC, lysis occurred within 2 hr. S. mutans strain LM7 and FA1 lysed within 1-2 hr. The other strains of S. sanguis and S. mutans required 4 hr of incubation or longer. Lysis of stationary phase cultures of both species required overnight incubation with lysozyme.

Difco) were grown to mid log phase (about 80 Klett units). The growth medium contained 5 μCi/ ml thymidine-methyl ^{3}H (New England Nuclear). The cells were centrifuged at 12,000 xg for 15 min and acetylated overnight at 4°C according to the procedure in Table 1. The pH of the acetylation mixture went from 6.5 to 4.5 during this step. By phase contrast microscopy the cells appeared to be intact and gram positive after acetylation. Moreover, none of the acid precipitable

label present in the cells was lost during acetylation. These observations indicate that the acetylation procedure did not lyse the cells and subject the DNA to possible depurinization.

The cells were then centrifuged at 10,000 xg, washed 3 times with sodium phosphate buffer (0.1 M pH 7.0) and treated with lysozyme (Sigma) and dextranase (Sigma) as shown in Table 1. Generally the cells began to lyse within 2 hours after addition of lysozyme and within 30 minutes after addition of pronase and detergent (Table 1).

We assessed the efficiency of lysis in two ways (Table 2). First, a qualitative assessment was made by microscopic examination of the suspensions after the final step of treatment. By this criterion, most of the strains lysed

Table 2. Degree of lysis of various strains of Streptococcus sanguis and Streptococcus mutans

Species	Strain	Lysis* (Microscopic Examination)	DNA		
			Total Acid precipitable counts**	Acid precipitable counts in supernatant	% of Total counts in supernatant
S. sanguis	G9B	+++	468,125	360,970	77
	M5	++	110,721	78,373	71
	Wicky	+++	322,838	191,056	59
	RC	+++	N.D. ***	N.D.	N.D.
	10558	++	N.D.	N.D.	N.D.
S. mutans	6715	++	377,000	366,920	92
	10449	++	141,330	99,190	88
	FA1	++	564,937	488,750	87
	LM7	+++	467,926	361,061	79
	SL1	++	752,947	440,196	58

* Lysis estimated by microscopic examination
+ = 30-50% + = 50-80% ++ = 100%

** 5% trichloroacetic acid precipitable counts

*** N.D. - not done

completely and none showed less than 60% lysis. We also attempted a more quantitative assessment of lysis by measuring the amount of DNA released into the medium. The suspensions were centrifuged at 3000 xg for 15 min to pellet whole cells. The supernatant was collected and the cell pellets were resuspended in phosphate buffer. We then measured the amount of ^{3}H-thymidine incorporated into acid precipitable material in the original suspension and in the supernatant (or cell-free) fraction. Table 2 shows that at least 58% of the total counts incorporated were present in the cell free supernatant. These data represent a minimum estimate of the amount of DNA released since some of the DNA probably remained associated with large membrane fragments that were pelletted along with the whole cells. When the lysed suspensions were sheared extensively by vortexing, many more acid precipitable counts (up to 100%) were found in the supernatant. However, this treatment would not be desirable for obtaining high molecular weight DNA. We were able to purify the DNA released (with no vortexing) from S. sanguis strain G9B by Marmur's procedure(5). The DNA could be spooled repeatedly indicating that it had a molecular weight of 8-12 megadaltons. This molecular weight would make the DNA satisfactory for transformation studies (5).

To ensure that this lysis method is applicable to plasmid studies, a sample of supernatant obtained from S. mutans LM7 (obtained from D.B. Clewell) was centrifuged to equilibrium in a CsCl gradient containing ethidium bromide. Figure 1 indicates the presence of a satellite peak with a density greater than that of the bulk of the chromosomal DNA. This satellite was not present when ethidium bromide was absent. The satellite peak fractions (# 11-18) were pooled, treated with isopropanol, dialyzed and centrifuged in a neutral sucrose gradient (5-20%) according to the method of Barth and Grinter (6). Two peaks of radioactivity were obtained with S values of 18S and 23S. These results compare favorably with the reported values of 17.5 S and 21.5 S for the LM7 plasmid (2).

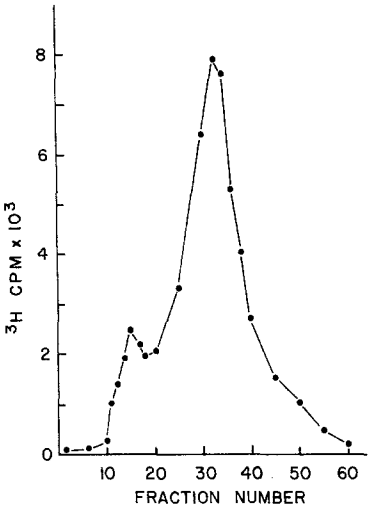

Figure 1. Dye CsCl buoyant density centrifugation of supernatant lysate of S. mutans LM-7 prepared according to the procedure in Table 1. A 1 ml amount of lysate was centrifuged to equilibrium in a Beckman 40 fixed angle rotor at 33,000 rpm for 60 hours at 20°C. The gradient contained 100 μg/ml ethidium bromide, CsCl at a starting density of 1.55 and a final volume of 4 ml. The gradient was fractionated and the amount of radioactivity in each fraction (0.05 ml) was determined by liquid scintillation spectrometry. (Intertechnique) using Aquasol (New England Nuclear).

In that study, lysis was achieved with TES-lysozyme. We conclude that our method allowed us to isolate a plasmid of known molecular size from S. mutans. In other experiments we found a third small peak of 63S DNA (212 megadaltons). This material probably represents chromosomal DNA and indicates that the lysis method may allow for isolation of high molecular weight chromosomal DNA.

It should be noted that in our hands, LM7 was the only strain of S. mutans that could be lysed in the presence of sucrose and in the absence of dextranase. Thus it is not surprising that Clewell, Dunny and co-workers were able to lyse this strain by the standard procedure using TES buffer and lysozyme (2). Whether this strain behaves so differently because of the presence of a plasmid or for another reason will have to await further investigation.

Finally, it should be noted that the method of lysis reported here might be appropriate with modifications for obtaining protoplasts of S. sanguis or S. mutans. We did not attempt to establish optimal conditions for protoplast formation, but we did note that protoplasts were commonly present in preparations of S. sanguis and S. mutans prior to addition of detergent. Calandra, Nugent and Cole (7) recently reported a method to obtain protoplasts of S. sanguis utilizing raffinose instead of sucrose as a stabilizer and a phage associated lysin obtained from Group C streptococci. It might be possible to employ raffinose as a stabilizer instead of sucrose or glycerol in our acetylation-lysozyme procedure or in the procedure involving TES buffer-lysozyme (1, 2). Although the procedure reported here has been quite successful in lysing streptococci, it has not worked well with members of the genus Actinomyces. Studies employing raffinose as a stabilizer for lysis of organisms in that genus are underway.

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