A GENTLE METHOD FOR THE LYSIS OF ORAL STREPTOCOCCI

by

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SUMMARY

A number of strains of oral streptococci, previously thought to be virtually lysozyme resistant, have been successfully lysed by a new procedure. The bacteria are grown in Brain-Heart Infusion supplemented with D,L-threonine which inhibits cell-wall cross linking. Spheroplasts are formed by treatment with lysozyme in 0.01 M Tris-HCl pH 8.2-12% polyethylene glycol. The spheroplasts are harvested by centrifugation and lysed by the addition of 1% sodium dodecyl sulfate. High MW DNA and plasmids have been isolated from cells treated in this manner.

INTRODUCTION

Research in genetics, metabolism, membrane structure and antigen isolation with streptococci has been frequently frustrated by the lack of gentle yet efficient means of generating easily lysed spheroplasts. Many strains have been thought to be lysozyme (EC 3.2.1.17, mucopeptide N-acetylmuramylhydrolase) resistant, especially if the cells are older (i.e. late log and stationary phase). A number of authors have cited methods for the lysis of specific strains; the most applicable method is that reported by Coleman, van de Rijn and Bleiweis in which numerous species are demonstrated to be sensitive to the action of lysozyme in dilute buffer followed by the addition of SDS* (1).

Recently Eisenberg and Lillmars concluded that two viridans species from the oral cavity, Streptococcus sanguis and Streptococcus mutans, could be lysed effectively by lysozyme after acetylation of cell wall muramic acid residues with acetic anhydride. For lysis of S. mutans it

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* SDS: sodium dodecyl sulfate

was necessary to incubate in the presence of dextranase which presumably removes cell-associated polysaccharides (2). This procedure, though more tedious, does not appear to provide any advantage over that reported by Coleman et al. (1).

The need still exists for a method to prepare stable spheroplasts from which may be obtained high MW DNA and other subcellular components. The procedure described here utilizes PEG*, reported by Kruse and Hurst to stabilize spheroplasts of Streptococcus lactis (3). Optimal growth media, and lysozyme-buffer concentrations for the preparation of spheroplasts from four species of oral streptococci are presented.

MATERIALS AND METHODS

Growth and Lysis: Bacteria were grown in the indicated medium supplemented with 20mM glucose, 250 µg/ml deoxyadenosine, and 5 µCi/ml [^{3}H] thymidine (New England Nuclear) at 37° for 16 or 24 hr. Cells were harvested by centrifugation at 27,000 xg for 15 min and washed twice with a volume of 0.01 M Tris-HCl pH 8.2 equal to the volume of the original culture. The cells were then suspended in a 1/4 volume of 2x buffer, followed by a 1/2 volume of 24% (W/V) PEG and a 1/4 volume of 2x buffer containing 4 mg/ml lysozyme (Worthington Biochemicals, 9000 U./ml). The concentrations and volumes were chosen such that the apparent absorbancy (turbidity) at 600nm would be 1.0; this is based on the turbidity of the starting culture at the time of harvest. The lysozyme-PEG solutions were incubated at 37° for the time specified, usually 1 hr, and the resulting spheroplasts collected by centrifugation at 27,000 xg for 15 min. The sedimented spheroplasts were gently resuspended in 1% SDS in 0.01 M Tris-HCl-0.01 M EDTA pH 8.0. The volume is 1/10 that of the lysozyme-PEG buffer. In most cases a viscous solution results. Unless high MW DNA is desired it is necessary to shear by vigorous vortexing to allow accurate sampling. The SDS suspensions were allowed to lyse for 15 min at 22-25° prior to centrifugation at 27,000 xg to remove unlysed cells and cell debris. Percent lysis was calculated from the acid precipitable DNA remaining in this supernatant fraction.

RESULTS AND DISCUSSION

It is generally thought that the growth media and conditions of growth influence susceptibility to lysozyme. McCarron and Chang have reported that threonine interferes with cell wall cross-linking in S. mutans BHT by a penicillin-like inhibition of lysine incorporation (4).

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* PEG: polyethylene glycol (20 M)

Table I

Growth Media	% Cellular DNA Released		
	no addition	ultrafiltered	+ threonine
Brain-Heart Infusion	56	48	87
Todd-Hewitt Broth	51	34	73
Jordan's Medium	53	31	67
Trypticase-Soy Broth	34	23	51

Table I. Lysis of S. mutans SL-1 cultured on various media. The media used were: Brain-Heart Infusion (DIFCO), Todd-Hewitt Broth (DIFCO), Typticase-Soy Broth (BBL) and Jordan's media (ref. 5). All were with or without the addition of 20 mM D,L-threonine, or prior ultrafiltration through an Amicon CH-3 microfiber concentrator. Cultures were inoculated and allowed to stand 16 hr at 37°. After harvest and washing, the cells were suspended in 0.01 M Tris-HCl pH 8.2-12% PEG-1 mg/ml lysozyme and incubated 1 hr at 37°. Spheroplasts were harvested by centrifugation and lysed with 1% SDS in 0.01 M Tris-HCl-0.01 M EDTA pH 8.0.

Cells grown in threonine should be more easily lysed. S. mutans SL-1 was cultured in various media with and without the addition of 20mM D,L-threonine. The data presented in Table I demonstrate that Brain-Heart Infusion-threonine produces late stationary phase cells that are readily lysed. After 60 min the lysozyme-PEG solution contained many clumped spheroplasts; all of the cells had lost the coccobacillary form. In each case media containing threonine produced cells more susceptable to lysozyme treatment. Another factor that might make cells more difficult to lyse is the surface absorption of macromolecules from the complex growth media: such cells might have "masked" or hidden lysozyme-sensitive sites. However, as can be seen in Table I media freed of macromolecules produced cells consistantly more difficult to lyse.

Using S. mutans SL-1 grown on Brain-Heart Infusion-threonine, the most commonly used lysozyme-buffer systems were evaluated to determine which is optimal for the preparation of spheroplasts and their subsequent lysis. The data presented in Fig. 1 indicate that 0.01 M Tris-HCl pH 8.2 (1) is superior to buffers used in other references cited (2,3). It is clear from these data that the choice of buffer systems is critical;

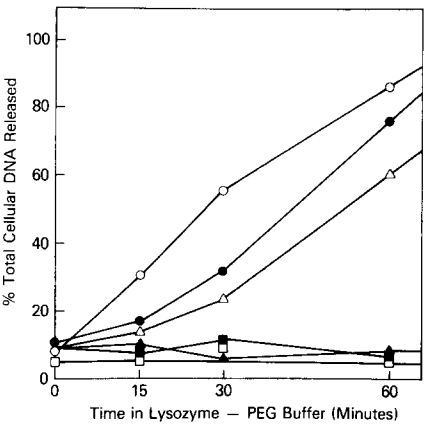

Figure 1. Time course of lysozyme action on 24 hr cultures of S. mutans SL-1 in various buffers. The conditions of growth and assay are described in Materials and Methods. The curves represent 1 mg/ml lysozyme in 12% PEG in the following buffers: 0.01 Tris-HCl pH 8.2 (O, ref. 1), 0.01 M Tris-HCl pH 8.2-5 mM EDTA (●), 0.05 M Tris-HCl pH 9.0-5 mM EDTA (△), 0.01 M Tris-HCl pH 8.2-no lysozyme (▲), 0.1 M Tris-HCl pH 8.0-5 mM $MgCl_2$-10 mM NaCl (□ , ref.3), 0.02 M phosphate pH 7.0-0.05 M $MgCl_2$-0.4 M NaCl (■ , ref. 2).

in general, dilute buffers without chelating agents, salts or metal ions give superior results. Since no DNA could be detected in lysozyme-buffer-PEG supernatants it is condluded that 12% PEG is adequate to stablize spheroplasts; in its absence a variable amount of direct lysis occurs (data not shown).

In order to determine the general applicability of this method, various oral streptococci were cultured in Brain-Heart Infusion-threonine and lysed. The results presented in Table II show that 10 S. mutans strains can be lysed completely; frequently in 30 min. Prior acetylation and dextranase treatment appears unnecessary. S. mitis strains were equally easy to lyse. The S. sanguis strains studied were slightly more difficult to lyse, but, after 1 hr, 80% or greater lysis was achieved in each case. Amongst the oral streptococci, S. salivarius was the most difficult to spheroplast (by microscopic evaluation) and, as can been seen in Table II, the most difficult to lyse. Values ranged from 18 to

Table II

Strain	% Cellular DNA Released	
S. mutans	30 min	60 min
AHT	60	97
E49	90	92
BHT	79	107
Fa-1	88	94
NTCC 10449	72	94
OMZ70	86	95
OIHI	111	102
SL-1	87	94
LM-7	46	77
B14	104	101
S. mitis		
ATCC 9811	88	105
ATCC 15909	67	101
S-3	81	87
S. sanguis		
ATCC 10558	94	87
T-175	74	81
JC-43	70	79
S. salivarius		
ATCC 25975	52	77
ATCC 27945	13	40
ATCC 9222	55	61
ATCC 9759	11	18

Table II. Extent of lysis for various strains of oral streptococci after 30 or 60 min of lysozyme treatment. Cultures were prepared and lysed as described in Table I and in Materials and Methods. Brain-Heart Infusion-threonine was the growth media.

77% lysis for the four strains studied. It is possible that these lower values are due to binding of DNA to the particulate fraction rather than incomplete lysis; the assay technique gives a minimal estimate of lysis. Alternatively, S. salivarius is known to produce more polysaccharide and "slime" than the other oral streptococci studied here; perhaps this provides a barrier against lysozyme attack. Even in the most difficult case, S. salivarius, the extent of lysis is acceptable for most purposes. In some cases a strain will not appear as classical spheroplasts after incubation, but will lyse completely upon addition of SDS. No attempt

was made to examine other commercially available lysozyme preparations or the effect of lower concentrations of lysozyme.

This lysis procedure has been used to prepare DNA from various strains of S. mutans. After sucrose density centrifugation and fractionation, electron microscopy has revealed both supercoiled and relaxed circular plasmid DNA (unpublished observations). In addition, the method has been applied to Lactobacillus coryniformis and Lactobacillus casei. Dye-CsCl bouyant density gradients have shown a heavy satellite peak indicative of plasmid or CCC-DNA in the latter strain. The general applicability of this technique to a variety of gram-positive bacteria is being examined.

From these results it appears that there are sufficient N-acetyl muramic acid residues in the cell wall structure of these streptococci to render them lysozyme sensitive without prior acetylation. Furthermore, while dextranase might facilitate the lysis of some strains, it is generally not necessary. In this study threonine has been used in an attempt to inhibit lysine incorporation into cell wall cross-links. It is probable from the results reported here that the observation made by McCarrow and Chang with S. mutans BHT is a general phenomena amongst oral streptococci (4).

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