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Method for the Lysis of Gram-Positive, Asporogenous Bacteria with Lysozyme

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A method developed for the lysis of oral streptococci that employed the action of lysozyme suspended in dilute tris(hydroxymethyl)aminomethane-hydrochloride buffer containing polyethylene glycol has been adapted for use with lactobacilli, actinomycetes, propionibacteria, and pediococci. Most of the cellular deoxyribonucleic acid was liberated from many strains of bacteria usually thought to be lysozyme resistant. The major observations were as follows: (i) supplementation of the growth medium with L-threonine, L-lysine, or both frequently produced cells that were more susceptible to lysis by lysozyme; (ii) glucose-containing media produced cells that were more easily lysed than those from cultures grown on other substrates; (iii) polyethylene glycol not only served as an osmotic stabilizer, it also enhanced the extent of lysis; and (iv) dilute tris(hydroxymethyl)aminomethane buffer was superior to the buffer systems most commonly employed in published muramidase-based lysis techniques. Stationary-phase cells of *Lactobacillus casei* and *Streptococcus mutans* were more easily lysed than those isolated from log-phase cultures. The method as detailed in this report should be generally applicable for the lysis of gram-positive, asporogenous bacteria.

The need for a gentle method to lyse a particular bacterium is common to many areas of research in microbiology. The preparations of intact membranes, protoplasts, spheroplasts, high-molecular-weight deoxyribonucleic acid (DNA), and plasmids are but a few examples of procedures best initiated with a gentle procedure for removing or weakening the cell wall. Lysozyme (EC 3.2.1.17, mucopeptide *N*-acetylmuramyl-hydrolase) hydrolyzes repetitive *N*-acetyl glucosamine-β-1 $\rightarrow$ 4-*N*-acetylmuramic acid bonds present in the bacterial cell wall. This hydrolysis frequently causes overt cellular lysis of gram-negative bacteria unless an osmotic stabilizer is present to protect the osmo-fragile spheroplasts that result from the action of muramidase. In marked contrast, most strains of the gram-positive genera are resistant to the action of lysozyme (12). The major argument advanced to explain the observed difference in sensitivity to lysozyme is the much greater thickness and density of the gram-positive bacterial cell wall.

Coleman et al. (5) have reported a method for the lysis of most streptococci which employs incubation of cells with lysozyme dissolved in dilute tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer followed by addition of sodium dodecyl sulfate (SDS). A more effective adaptation of this technique has been reported by Chassy (4). In this modified procedure, streptococci are grown in media supplemented with L-threonine, which weakens cell wall cross-links (R. M. McCarron and F. Y. Chang, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K116 p. 166). Cells are harvested and treated with lysozyme dissolved in dilute Tris buffer with polyethylene glycol (PEG) added as an osmotic stabilizer. Stable spheroplast-like structures can be isolated by this technique; lysozyme-treated cells lyse upon addition of SDS. Lactobacilli are thought to be more resistant to muramidase than are streptococci: however, Rogosa has reported a technique for at least partial lysis of a number of species of this genus (12), and Barker and Thorne have succeeded in preparing spheroplasts of *Lactobacillus casei* by the simultaneous action of trypsin and lysozyme (2). Neujahr et al. examined some of the factors contributing to the insensitivity of *Lactobacillus fermenti* to lysozyme (11). Factors which contribute to the resistance of some *Bacillus* strains to lysozyme have also been documented (1, 7). The susceptibility of other gram-positive asporogenous genera to lysozyme remains largely undetermined.

This study was undertaken to determine whether the lysozyme-PEG procedure described previously (4) could be applied to gram-positive genera other than *Streptococcus*. Data are presented to show that the lysozyme-Tris-PEG

technique can be used to effect lysis of many strains of actinomyces, lactobacilli, pediococci, and propionibacterium.

MATERIALS AND METHODS

Cultures. All cultures designated by an ATCC number were obtained from the American Type Culture Collection, Rockville, Md. *Actinomyces viscosus* W1557 and *Actinomyces naeslundii* W1544 and W1527 were provided by S. L. Bragg of the Center for Disease Control, Atlanta, Ga. *A. viscosus* M100 was from the collection of B. Hammond, University of Pennsylvania, Philadelphia, Pa. *A. naeslundii* WVU820 was supplied by M. Gerenscer, University of West Virginia, Morgantown, W. Va. *L. casei* 64H, Cl-17, were kindly provided by J. London, National Institute of Dental Research, Bethesda, Md. *Streptococcus mutans* strains were obtained from L. Thomson, National Institute of Dental Research, Bethesda, Md.

Media and growth of bacteria. *Actinomyces* and *Streptococcus* strains were maintained through biweekly passage in fluid thioglycolate broth (Difco Laboratories). Cultures for experiments were grown at 37°C in brain heart infusion (Difco). *Lactobacillus* and *Pediococcus* strains were maintained by monthly transfer in litmus milk (Difco). Cells for experiments were prepared by growth at 37°C in lactobacillus carrying medium (6). *Propionibacterium* strains were transferred weekly in a medium composed of 1% yeast extract (BBL) and 1% glucose (Difco) in water. Two days of incubation at 30°C were required for growth of *Propionibacterium* strains. Cultures were grown overnight in the appropriate medium, and a 0.1 ml inoculum was added to the corresponding medium (10 ml) containing 0.5 μCi of [^{3}H]thymine per ml (New England Nuclear), 0.5 μCi of [^{3}H]thymine per ml (New England Nuclear), and 250 μg of deoxyadenosine per ml (Sigma). The radioactivity-containing cultures were incubated at 37°C for 16 h, except in the case of *Propionibacterium* spp. which were allowed 40 h at 30°C for complete growth. These conditions produced early stationary-phase cells used in all experiments except those designed to study the effect of cell age on lysis.

Lysis of cells. The turbidity (Gilford 300-N microspectrophotometer) of each culture at 600 nm was determined and used to estimate dry weight as well as the amount of lysozyme to be added. Cultures (10 ml) of cells were harvested by centrifugation, washed once with 0.01 M Tris-hydrochloride, pH 8.2, or other buffers being studied, and resuspended in 1 ml of the same buffer. The suspension was diluted with 2 ml of 24% (wt/vol) 20 M PEG (Carbowax 20,000, Fisher) in water. A 1-ml amount of a solution of lysozyme (Sigma grade I) in 0.02 M Tris-hydrochloride, pH 8.2, (or other buffers as indicated) was added, and the suspension was mixed by inverting the tubes 5 to 10 times. Incubations with lysozyme were carried out at 37°C for various times (indicated in legends), but usually for 1 h. The amount of lysozyme added was calculated to be 1.2 mg/1.0 mg (dry weight) of cells. In the absence of a standard curve relating dry weight to turbidity, it has been found that use of 1 mg of lysozyme per 1.0 optical density unit at 600 nm per ml of culture gives essentially the same results with all bacteria examined so far. Duplicate tubes containing no lysozyme were prepared as controls for each experiment. The cells were sedimented by centrifugation (10,000 × *g*, 10 min), and 50 μg of the lysozyme-PEG supernatant was checked for release of DNA during the lysozyme incubation by estimation of acid-precipitable radioactivity (5% trichloroacetic acid). The supernatant was discarded, and the pellet of lysozyme-treated bacteria was resuspended completely in 1.8 ml 0.1 M Tris-hydrochloride–0.01 M ethylenediaminetetraacetic acid, pH 8.5 (TE buffer). The bacteria frequently were difficult to resuspend because of clumping. Microscopic examination for morphological changes was made at this point; lysis was induced by the addition of 0.2 ml of 10% SDS (Sigma) followed by incubation at 37°C for 15 min. When the technique is used for isolation of high-molecular-weight DNA, care should be taken to avoid mechanical shearing of the DNA in all operations subsequent to the addition of SDS; however, it was found that passage of the lysate 10 times through a 26-gauge needle improved analytical precision because shearing of the viscous, DNA-containing solutions made more accurate sampling possible. Lysates were frequently completely clear and gelatinous; however, the lysates of many completely lysed strains remained turbid at this point. A 50-μl sample was taken for the estimation of total cellular DNA; after centrifugation the DNA released was determined by measurement of the acid-precipitable DNA remaining in the supernatant.

Calculation of percent lysis. The counts per minute released into the SDS supernatant of controls without lysozyme (usually 1 to 3% of the total) were subtracted from the counts per minute found in the SDS supernatant of lysozyme-treated cells to give net [^{3}H]DNA released. The net counts per minute of DNA released was divided by the counts per minute of total DNA and multiplied by 100 to give percent DNA released. These "DNA released" values are reported as percent lysis.

RESULTS

Application of the PEG-lysozyme method to genera other than *Streptococcus*. Twenty strains of three nonsporeforming, gram-negative genera were subjected to the PEG-lysozyme technique to assess their susceptibility to lysis. The percent lysis varied from 100% observed for *A. viscosus* M100 to 11% for *P. rubrum* (Table 1). Considerable variation occurred within a species (*A. viscosus*, 22 to 100%) as well as within a single genus (*Propionibacterium* spp., 11 to 97%). The results indicate that the method of lysis originally developed for streptococci has broad applicability among gram-positive genera. In experiments not shown, strains of Lancefield group A, B, D, F, and H streptococci, as well as clinical isolates resembling *Corynibacterium* spp. but unidentified as to species, were all easily lysed.

Stimulation of lysis by addition of threo-

nine and lysine to the growth medium. Previous studies (4) have shown that incorporation of 10 mM L-threonine into the growth medium gives rise to cells of *S. mutans* that are more susceptible to lysozyme than those cultured in brain heart infusion alone. Seven strains of *S. mutans* were grown in media containing L-threonine, and the resulting cells were subjected to the PEG-lysozyme method. To determine whether L-lysine could enhance lysozyme resistance, cultures were prepared on media containing 10 mM L-lysine. A combination of both amino acids was tested to look for possible antagonism or synergism. As can be seen in Table 2, four of the seven strains of *S. mutans* studied were lysed to a greater extent when grown in media supplemented with L-threonine. The enhancement varied from 80 to 94%. An almost identical enhancement of lysis was observed for the same four strains grown in L-lysine-supplemented media. Although no synergism was observed for combinations of these two amino acids, in certain cases (for example, *S. mutans* AHT), an antagonism appeared to negate the effect of addition of the individual amino acids. In one instance, *S. mutans* NCTC 10449, any amino acid supplementation of the growth medium was detrimental to lysis. To date, 28 strains have been tested in this manner; the lysis of 19 strains was stimulated at least 50% by amino acid supplementation of the growth medium.

TABLE 1. *Lysis of representatives of three genera of gram-positive bacteria by the lysozyme-PEG technique*

Bacteria	% Lysis
A. viscosus M 100	100
A. viscosus W 1528	73
A. viscosus W 1557	22
A. naeslundii WVU 820	32
A. naeslundii W 1544	54
Pediococcus pentosaceus ATCC 25744	97
P. cervisiae ATCC 8081	87
P. acidilacti ATCC 25740	58
Propionibacterium pentasascens ATCC 4875	94
P. rubrum ATCC 4871	11
P. zeae ATCC 4964	97

An identical analysis was conducted on 13 strains of the genus *Lactobacillus* (12 species). Most strains of the genus *Lactobacillus* were efficiently lysed (Table 2). More than 50% of the cellular DNA was isolated from 11 of the 13 strains assayed. With about half of the strains studied, incorporation of L-lysine, L-threonine, or both amino acids in the growth medium pro-

TABLE 2. *Effect of the addition of L-threonine and L-lysine to the growth medium on the lysis of strains of S. mutans and Lactobacillus spp.*

Bacteria	% Lysis with addition to growth medium:[a]			
	None	Threonine	Lysine	Lysine + threonine
S. mutans[b] AHT	31	60	62	27
S. mutans BHT	41	79	72	54
S. mutans OMZ 70	98	86	83	101
S. mutans NCTC 10449	99	72	62	59
S. mutans SL-1	47	87	90	95
S. mutans LM 7	31	56	51	59
L. acidophilus[c] ATCC 19992	87	96	97	101
L. arabinosus ATCC 8014	34	27	21	28
L. brevis ATCC 14869	87	97	94	97
L. bulgaricus ATCC 11842	26	85	100	98
L. casei ATCC 7469	48	100	88	97
L. casei ATCC 64H	49	66	67	64
L. coryniformis ATCC 25600	80	101	104	103
L. fermentum ATCC 14931	27	51	47	58
L. lactis ATCC 12315	90	94	93	96
L. leichmanii ATCC 14797	63	73	75	78
L. plantarum ATCC 14917	89	94	90	86
L. salivarius ATCC 11742	41	100	101	104
L. zeae ATCC 15820	6	17	18	22

[a] Medium supplemented with 10 mM L-threonine, 10 mM L-lysine, or a combination of both as indicated in the column headings.

[b] *Streptococcus* strains incubated for 30 min at 37°C with 1.2 mg of lysozyme per mg (dry weight) of cells.

[c] *Lactobacillus* strains incubated for 60 min at 37°C with 1.2 mg of lysozyme per mg (dry weight) of cells.

duced cells that were more susceptible to the action of lysozyme, although for a few strains supplementation was without effect. Supplementation of the growth medium with amino acids did not adversely affect lysis of any strain studied. These data, presented in Table 2, are representative of values obtained with 34 strains of lactobacilli.

Effect of growth substrate on lysis. An attempt was made to determine whether a change in the growth substrate could produce cells that were more readily lysed. Before growth of cells in DNA labeling media, the cultures to be studied were adapted to media containing one of six different substrates (i.e., D-glucosamine, D-gluconate, L-malate, pyruvate, ribitol, citrate) by two successive transfers. The adapted cells were inoculated into labeling medium containing the appropriate substrate in lieu of glucose and incubated for 24 h. Turbidimetric estimation of growth rate indicated that growth was slower, but was completed by this time for all cultures except those growing on malate, a poor substrate. The cell yield per milliliter of culture indicated that none of the alternate substrates was equivalent to glucose. In each case where glucose was replaced by another substrate in the growth medium, the resulting cells were more difficult to lyse. With *L. casei* 64H the lysis measured after growth on alternate substrates varied between 17 and 53% of that observed with glucose. The effects of changes of growth substrate were even more striking with *L. casei* Cl-17; the cells produced on other substrates were lysed only 7 to 29% as effectively as those harvested from glucose-containing medium.

Examination of the effect of incubation buffer. A number of different reaction conditions have been employed in studies concerned with the effect of lysozyme on *Streptococcus* and *Lactobacillus* species (5, 12). The use of 0.01 M Tris-hydrochloride, pH 8.2, as previously reported (4, 5), gave the best overall results (Table 3) with the four strains of bacteria tested. The addition of 5 mM ethylenediaminetetraacetic acid produced as much as a 25% inhibition of lysis. Raising either the buffer concentration or the pH reduced the efficiency of lysis. An almost complete loss of lysis was observed when 0.02 M phosphate buffer, pH 7.0, was substituted for Tris buffer. As much as a 12-fold reduction in observed lysis resulted from the inclusion of 5 mM $MgCl_2$–10 mM NaCl in the incubations. These results confirm previous observations that dilute Tris-hydrochloride buffer at pH 8.2 is superior to other reaction conditions studied.

Influence of age of cells on lysis efficiency. To determine the optimal physiological state for lysis, a series of cultures was inoculated at staggered intervals, harvested, and subjected to the PEG-lysozyme technique. The 8-h cultures were in the mid-exponential phase of growth (50 to 75% final turbidity), 12-h cultures were late in the exponential phase (75 to 90% final turbidity), and all subsequent times yielded resting or stationary-phase cells. The data presented in Fig. 1 show that stationary-phase cells were the most easily lysed by this technique. Cells remained susceptible to lysis for at least 24 h of incubation after the cessation of growth. Actively growing cultures were more difficult to lyse (Fig. 1).

TABLE 3. *Effect of various incubation conditions on the extent of lysis*[a]

Lysozyme incubation buffer	% Lysis			
	S. mutans SL-1	*S. mutans* OMZ 70	*L. casei* Cl-17	*L. casei* 64H
0.01 M Tris-hydrochloride, pH 8.2	85	96	82	55
No lysozyme	2	3	2	6
+5 mM EDTA	71	81	60	42
0.01 M Tris-hydrochloride, pH 9.0	76	65	73	42
+5 mM EDTA	62	51	61	37
0.1 M Tris-hydrochloride, pH 8.2	61	84	72	46
+5 mM $MgCl_2$ and 10 mM NaCl	5	17	12	19
0.02 M sodium phosphate, pH 7.0	18	22	11	9
+0.05 M $MgCl_2$ and 0.4 M NaCl	4	5	6	6

[a] The extent of lysis observed with the various buffers was recorded after incubation for 1 h at 37°C as described in the text. EDTA, ethylenediaminetetraacetic acid.

Influence of lysozyme concentration and PEG on lysis of *L. casei* 64H and *S. mutans* SL-1. To determine the optimal ratio of lysozyme to cell mass, equal amounts of *L. casei* 64H and *S. mutans* SL-1 were incubated with varying concentrations of lysozyme. The results indicated that for both bacterial strains ratios of 80 μg of lysozyme per mg (dry weight) of cells, or higher, gave essentially complete lysis. Higher ratios of lysozyme to cells were of no additional value, and with some strains excess lysozyme actually decreased the efficiency of lysis (data not shown).

The effect of PEG in the lysis procedure was evaluated by treating equal amounts of *L. casei* 64H cells or *S. mutans* SL-1 cells with varying

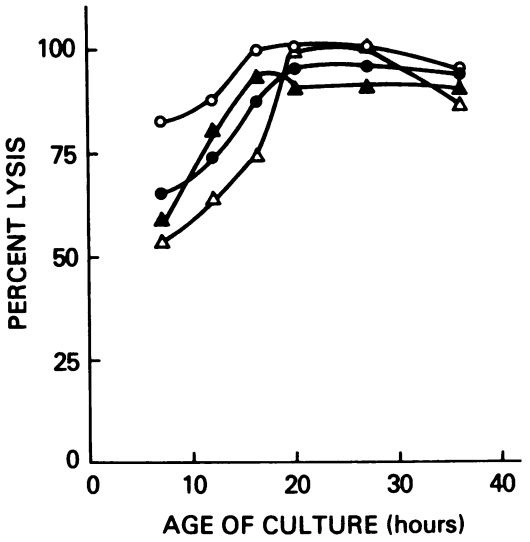

FIG. 1. *Degree of lysis observed upon exposure of cells of differing age to the lysozyme-PEG technique. Cultures were inoculated at staggered intervals, harvested, and subjected to the PEG-lysozyme procedure. Incubations were for 90 min at 37°C. The percent lysis data obtained for S. mutans strain SL-1 are represented by* (○) *and those for S. mutans 3720 are represented by* (●). *The data observed with L. casei 64H are plotted as* (△), *and those for L. casei Cl-17 are plotted* (▲).

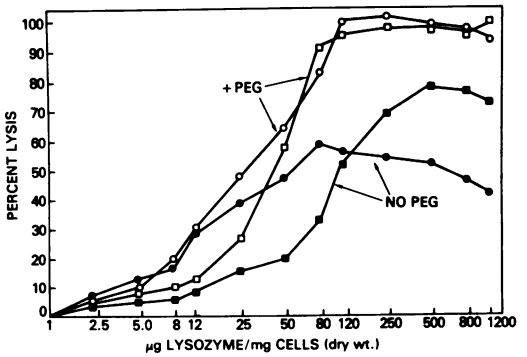

FIG. 2. *Examination of the optimal lysozyme concentration and the role of osmotic stabilizers wth Lactobacillus casei 64H and Streptococcus mutans SL-1. Varying amounts of lysozyme per milligram (dry weight) of L. casei 64H* (○) *or S. mutans SL-1* (□) *were added to 0.01 M Tris-hydrochloride, pH 8.2, in the presence of 12% (wt/vol) PEG. In a duplicate experiment, PEG was omitted from both L. casei 64H* (●) *and S. mutans SL-1* (■). *L. casei 64H was incubated for 90 min at 37°C, whereas S. mutans SL-1 was incubated for 60 min at 37°C.*

amounts of lysozyme in the absence of PEG. The results indicated that PEG was functional as both an osmotic stabilizer and as a stimulant of lysis (Fig. 2). In the absence of PEG, the amount of lysozyme required for maximal lysis of *L. casei* 64H remained unchanged, but the extent of lysis was reduced approximately 50%. With *S. mutans* SL-1 a fourfold increase in the ratio of lysozyme to cells was required to attain maximal lysis when PEG was omitted from incubations. The extent of lysis was only 75% as complete as was observed with PEG.

The function of PEG as an osmotic stabilizer was evaluated by measuring the lysis that occurred during incubation with lysozyme in both the presence and absence of PEG. In the presence of PEG, no DNA was released during incubation with lysozyme (data not shown). In the absence of PEG, at lower ratios of lysozyme to cells, significant quantities of DNA were released during incubation with lysozyme. For example, treatment of *L. casei* 64H with 12 μg of lysozyme per mg (dry weight) of cells resulted in 10% overall lysis, but 60% of this lysis occurred during lysozyme incubation when PEG was omitted. With both *L. casei* 64H and *S. mutans* SL-1 it was observed that no cell lysis occurred during lysozyme treatment in the absence of PEG when the ratio of lysozyme to cell mass was greater than 120 μg of lysozyme per mg (dry weight) cells. In fact, cells treated in this fashion could be centrifuged to remove lysozyme and resuspended in TE buffer without any significant lysis occurring in an additional 2 h of incubation at 37°C. SDS induced rapid lysis of such lysozyme-treated suspensions. It appeared that higher quantities of lysozyme were capable of "autostabilization" of lysozyme-treated cell walls, thus eliminating the need for a stabilizer to be present during lysozyme incubation.

DISCUSSION

The primary objective of this study was to determine whether the PEG-lysozyme technique developed for the lysis of oral streptococci (4) could be employed to lyse other asporogenous, gram-positive bacteria. The results show that many strains of *Actinomyces*, *Pediococcus*, *Propionibacterium*, and *Lactobacillus* can be lysed by this procedure. No attempt was made to achieve 100% lysis for each strain studied. In light of the known effects on lysis efficiency of variation in growth medium, addition of amino acids, and increases in incubation time, it should be possible to attain complete lysis of the majority of the strains reported here.

A number of factors appear to influence lysis efficiency. Increases in the amount of lysozyme used were not particularly effective in increasing extent of lysis; however, lengthening the period

of incubation improved lysis (see the legend to Fig. 2). Caution should be exercised in exposure of cells to prolonged incubation at 37°C because intracellular degradative changes are possible. For example, it has been reported that the 32-megadalton lactose plasmid of *S. lactis* is specifically degraded during long incubations with lysozyme; the yield of other plasmids was not affected (9). Another factor likely to produce a large change in lysis efficiency is the growth medium. Previous work has shown that some basal media are superior to others in producing cells that are susceptible to lysozyme (4). It was found that supplementation of the growth medium with L-threonine, L-lysine, or both usually produced streptococci or lactobacilli cells that were more easily lysed. The action of threonine can be explained by its known interference with the establishment of cell wall cross-links (4; McCarron and Chang, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K116, p. 166). The mode of action of L-lysine is more difficult to explain. Cells produced on growth substrates other than glucose also proved difficult to lyse. Although no simple mechanistic interpretation can be advanced to explain these observations, it is obvious that the composition of the growth medium can affect the susceptibility of bacteria to lysozyme and should be evaluated with strains that are difficult to lyse.

The choice of buffers has probably been a major factor in determining the success of many attempted lysis experiments. Based on experience with gram-negative organisms, many investigators have included ethylenediaminetetraacetic acid in lysozyme incubation buffers with gram-positive organisms. The data reported here indicate that this practice diminishes the overall extent of lysis. Other studies have relied on higher pH values, higher buffer concentrations, or phosphate buffers. In the procedure examined here, all of these substitutions interfered with lysis. This observation could have been anticipated from the finding of Metcalf and Diebel (10) that *S. faecalis* and *S. faecium* strains can be lysed by the action of lysozyme dissolved in distilled water. Thus, the use of NaCl, $MgCl_2$, other salts, or chelating anions should be avoided because these additives appear to interfere with the lysis of gram-positive, asporogenous bacteria.

The final factor which contributes to the effectiveness of the technique described here is the choice of the stabilizer. PEG appears to have more value as a stimulant of lysis than as an osmotic stabilizer. At the higher lysozyme concentrations studied, addition of a stabilizer was not even necessary. But, the fact that more complete lysis was obtained when PEG was used indicates a specific facilitation of lysis by PEG. Sucrose or raffinose was not able to stimulate lysis as effectively as PEG (data not shown). In other studies of lysozyme susceptibility, it has been observed that stationary-phase cells are more resistant to lysozyme than those isolated from growing cultures (3, 8). This resistance has been attributed to an increase in cell wall *O*-acetyl groups and decrease in *N*-acetyl groups (3, 8). The results reported here indicate that use of PEG appears to overcome the increased resistance of "older" cells.

In conclusion, it is suggested that the procedures described here can be successfully applied to a great many bacteria currently thought to be lysozyme resistant. This should allow isolation of cellular components difficult or impossible to obtain by other methods.

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