JOURNAL OF BACTERIOLOGY, Sept. 1970, p. 686-691

Vol. 103, No. 3
Printed in U.S.A.

Induction of Enterococcal L-Forms by the Action of Lysozyme

JAMES R. KING[1] AND HARRY GOODER

Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill 27515

Received for publication 10 June 1970

Suspensions of enterococci were treated with lysozyme in the presence of osmotic stabilizers. The resulting osmotically fragile bodies prepared from *Streptococcus faecium* strain F24 and *S. faecalis* strain E1 gave rise to L-forms under optimal osmotic and nutritional conditions for treatment and subsequent growth. The most critical component of the growth medium, to obtain maximum yields, was the nature and concentration of the added salt. The two most effective salts were sodium chloride and ammonium chloride in the range of 2 to 3% (w/v) added to a suitable agar base. Ammonium chloride was more versatile, because it could be used with either sucrose or polyethylene glycol 4000 as the osmotic stabilizer for preparation and dilution of the osmotically fragile bodies. Sodium chloride would not consistently support growth of *S. faecium* F24 as L-forms when polyethylene glycol 4000 was used as the osmotic stabilizer during lysozyme treatment. Time-course studies of concurrent cell wall removal and L-form induction suggested that maximal induction required only cell wall damage rather than complete wall removal. This method for induction of L-forms from a suspension of enterococci is a significant improvement over other presently known methods.

Gooder and Maxted (9) and Freimer, Krause, and McCarty (6) induced L-phase growth of group A streptococci by use of a phage-associated lysin. Enzymatic induction of group D streptococcal L-forms has not been previously obtained by removal of the streptococcal cell wall, although there are numerous reports of protoplast formation by the action of lysozyme on group D streptococci (1-3, 14). It was of interest to determine whether L-forms could be induced en masse after damage to or removal of the cell wall by lysozyme treatment.

In preliminary studies (8), *Streptococcus faecium* (*faecalis*) strain F24 L-forms were obtained from normal cell suspensions plated onto medium containing 20 units of penicillin per ml. In contrast to penicillin treatment alone, prior lysozyme treatment in the presence of sucrose increased the yield of L-forms 1,000-fold. In this report, a system is described for approximately quantitative conversion of group D streptococci to L-forms in the absence of penicillin in suitable agar media.

[1] Present address: Laboratory of Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014.

MATERIALS AND METHODS

Protoplast formation. Protoplasts were prepared essentially by the method of Bibb and Straughn (2) and Weibull (15). Cultures of *S. faecium* F24 and *S. faecalis* E1 grown at 37 C overnight (stationary phase) in Trypticase Soy broth (BBL, Division of Bioquest Laboratories, Cockeysville, Md.) were harvested and washed three times in distilled water. Sucrose (0.6 M) or polyethylene glycol 4000 (8%, w/v) was used as an osmotic stabilizer in *p*H 7.1 tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (0.01 M, ionic strength 0.01). A mixture was prepared in the buffer-stabilizer to contain 2×10^9 streptococcal colony-forming units (CFU) per ml and 200 μg of lysozyme (Nutritional Biochemicals Corp., Cleveland, Ohio) per ml. The resulting mixture (turbidity equal to 250 Klett units) was incubated at 37 C for time periods up to 2 hr. A similar control tube did not contain lysozyme.

Viable counts of protoplast suspensions. Tenfold serial dilutions of protoplast suspensions were made in a solution of 0.01 M Tris-chloride buffer, *p*H 7.1 (ionic strength 0.01). As an osmotic stabilizer, either 0.6 M sucrose or 8% (w/v) polyethylene glycol 4000 was included in the diluent. These diluents will be abbreviated TSD and PEG, respectively. In most experiments samples from appropriate dilutions were plated out onto the surface of growth medium composed of Tryptone Soy agar (Oxoid, Consolidated

Laboratories, Chicago, Ill.) containing 0.43 M NH_4Cl, 0.5% (w/v) additional glucose, and 2% (w/v) Seitz-filtered horse serum (previously inactivated at 56 C for 30 min). This L-form growth medium will be abbreviated LGM. Colonies were counted after incubation of plates in plastic bags at 37 C for 2 to 5 days.

Chemical methods. Rhamnose (methyl pentose) was determined by the Dische and Shettles (5) quantitative colorimetric method.

Measurement of turbidities. A Klett-Summerson photoelectric colorimeter equipped with a no. 54 filter (wavelength range 500 to 570 nm) was used to measure the turbidity of suspensions.

Identification of growth as L-forms. Subculture was attempted on blood-agar, bile aesculin, and LGM plates. L-forms grew only on LGM, whereas untreated streptococci grew on all three media. Representative colonies were stained by the method of Dienes (4) and typical large bodies were seen.

RESULTS

The initial studies were performed to discover the optimal lysozyme to cell ratio, length of time of lysozyme treatment, suitable growth media, and other features of a system which permitted the growth of lysozyme-treated *S. faecium* F24 cells as L-forms. In these preliminary studies, it was necessary to prevent the growth of any residual streptococci, so penicillin (20 units per ml) was present in the medium LGM. The ability of penicillin to induce L-forms under these circumstances was therefore studied. Suspensions of streptococci were treated as described above, except that lysozyme was omitted. After incubation at 37 C for varying time periods up to 1 hr, dilutions were prepared as if the suspension were protoplasts, and appropriate samples were plated on LGM containing 20 units of penicillin per ml. In numerous experiments, it was possible to grow approximately 10^4 L-forms from suspensions containing 5×10^9 CFU per ml of *S. faecium* F24. This number did not vary when the incubation period ranged from 1 min to 1 hr or if the penicillin concentration was increased to 1,000 units per ml of medium. It was therefore concluded that, in this system, penicillin at 20 units per ml of medium was minimally effective in inducing L-forms and could be utilized in the initial studies to prevent growth of residual streptococci.

Effect of growth medium components. Suspensions of *S. faecium* F24 (5×10^9 to 6×10^9 CFU/ml) were treated with lysozyme for 30 min in the presence of 0.6 M sucrose. The resulting suspension of osmotically fragile cells was diluted in TSD and plated out onto LGM containing 2% (w/v) additional NaCl instead of 0.43 M NH_4Cl. Some components of the growth medium produced differences only in colonial appearance. For comparison, the appearance of a colony of the parent strain *S. faecium* F24 after incubation for 5 days at 37 C is shown in Fig. 1. The diameter of such colonies was usually 3 to 5 mm, and the colonies possessed a smooth, sharp edge and creamy texture. The growth shown in Fig. 2 is an L-form obtained after incubation under similar conditions in a medium devoid of

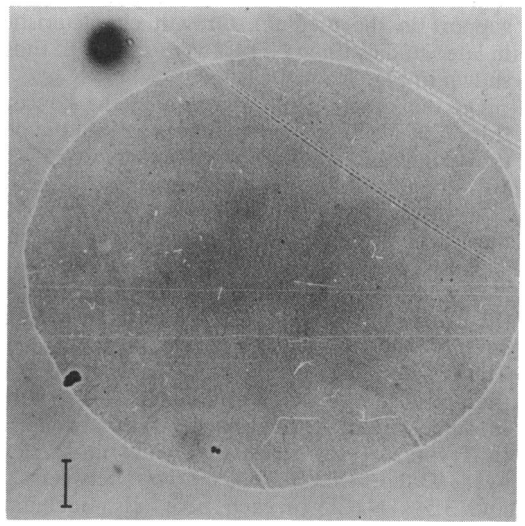

FIG. 1. *Colony of Streptococcus faecium F24. Bar represents 0.4 mm.*

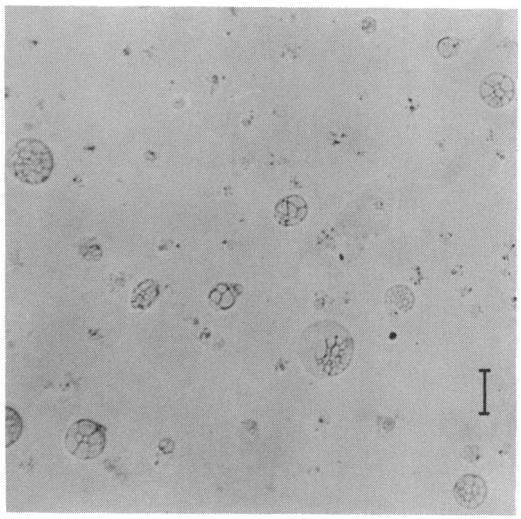

FIG. 2. *Streptococcus faecium F24 L-forms grown in the absence of glucose and horse serum. Bar represents 0.4 mm.*

horse serum and additional glucose. The L-forms exhibited a typical lacy and granular appearance, and they rarely exceeded 0.5-mm diameter. The L-forms appearing on a medium containing 2% horse serum and 0.5% glucose are shown in Fig. 3. The growth, 1 mm in diameter, appeared as a typical "fried egg" (dense granular centers surrounded by much wider halos). In horse serum concentrations up to 10%, the same robust L-form was observed. The photographs shown in Fig. 1, 2, and 3 were made during the preliminary studies when NaCl supplied osmotic support in the medium. Growth characteristics in later studies using NH_4Cl were similar to those shown in Fig. 3.

On the other hand, variation in the concentration of NaCl in the medium produced quantitative differences in the L-form counts when 7×10^9 streptococcal CFU/ml were treated for 30 min with lysozyme in the presence of sucrose. In these preliminary studies, penicillin (20 units/ml) was used in the medium to retard the growth of *S. faecium* cells unaffected by the lysozyme. In the absence of penicillin, colonies from residual streptococci overgrew the developing L colonies. The effect of varying the NaCl concentration in the presence of a variety of agar bases is shown in Table 1. The optimal concentration for NaCl in Trypticase Soy agar (BBL) and in nutrient agar (Difco, Detroit, Mich.) was between 2.5 and 3.5% NaCl. In each base (also including Tryptone Soy agar, Consolidated Laboratories, Chicago, Ill.), 2.5% NaCl was the minimum final concentration of salt necessary for maximal recovery of developing L-forms.

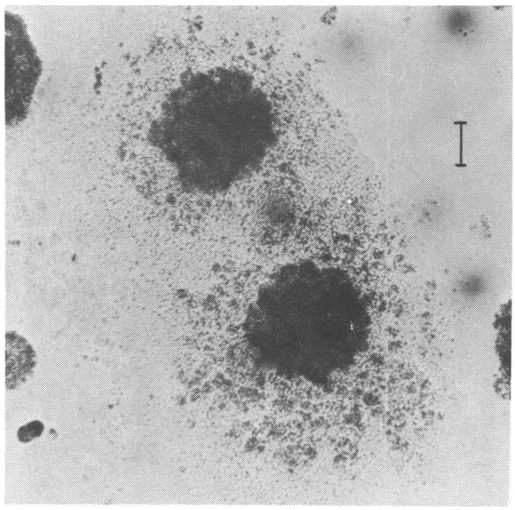

FIG. 3. *Streptococcus faecium F24 L-forms grown in the presence of glucose and horse serum. Bar represents 0.2 mm.*

TABLE 1. *Variation in the L-form count due to the concentration of sodium chloride in the growth medium*

Added NaCl concn	L-form count (CFU/ml)[a]		
	Trypticase Soy	Nutrient agar	Brain Heart Infusion
%			
0	0	0	0
1	0	10^5	10^5
2	4×10^5	10^7	10^7
3	5×10^6	3×10^6	10^6
4	4×10^5	2×10^6	4×10^5
5	10^3	2×10^6	10^4

[a] Growth was in basal medium with 0.5% sodium chloride. Glucose (0.5%, w/v) and horse serum (2%, v/v) were added to each basal medium.

It was also of interest to determine whether salts other than NaCl could be used to fortify the medium. The approximate molarity of 2.5% NaCl is 0.43 M, so all the salts investigated were added individually to the medium at a final concentration of 0.43 M. The following salts, in increasing order of effectiveness in the number of L-forms recovered, were tested: KCl, NaH_2PO_4, $NaNO_3$, Na_2SO_4, $MgCl_2$, Na_2HPO_4, and NH_4Cl. All the salts except NH_4Cl either had objectionable precipitates in the Tryptone Soy agar base or did not have the optimum yields seen in Table 1.

Interrelationship of diluent and salt in the medium. As important as the concentration of the most effective salt in the medium, was the choice of diluent. Protoplasts were prepared in TSD or PEG and plated in the absence of penicillin onto LGM or LGM with 2% additional NaCl instead of NH_4Cl. The results of this experiment are shown in Table 2. The usual yields were obtained with either salt in the medium when TSD was used as diluent. However, low irreproducible yields of L-forms were found on the medium containing 2% NaCl whenever PEG was the stabilizing agent for the protoplasts.

Effect of time of lysozyme treatment on cell wall damage and the maximum number of L-forms induced. The buffer-sucrose solution would seem to be the more useful diluent in this system of L-form induction from protoplasts of group D streptococci. However, we wished to know the degree of cell wall damage during the treatment with lysozyme to assess if protoplasts were formed and also to relate the extent of cell wall removal and the maximal induction of L-forms from a population of *S. faecium* F24 cells. Rham-

TABLE 2. *Effect of the presence of sodium chloride or ammonium chloride in the growth medium on the yield of L-forms obtained with sucrose or polyethylene glycol as the osmotic stabilizer*

Growth medium contained	Diluent	
	Sucrose (0.6 M)	Polyethylene glycol 4000 (8%)
0.43 M NaCl	5.4×10^{8}[a]	Few colonies seen
0.43 M NH_4Cl	2.0×10^{8}	8.6×10^{8}

[a] L-form count (CFU per ml) when 2×10^{9} CFU of strain F24 were treated with lysozyme.

nose release was chosen as an index of cell wall damage, but the sucrose in TSD interfered with the methylpentose determinations. Therefore, in subsequent experiments, PEG was used as the standard diluent for protoplasts and LGM was the assay medium for L-form growth.

It was also shown that, when fewer streptococcal cells (approximately 2×10^{9} CFU/ml) were treated with 200 μg of lysozyme per ml, L-forms far out-numbered residual *S. faecium* colonies in the absence of penicillin in the medium. In many such experiments, no *S. faecium* colonies were found on agar plates devoid of penicillin when dilutions of the lysozyme-treated streptococci were plated so as to allow 300 to 500 L-forms to develop per petri plate. A rare *S. faecium* colony surrounded with L-form colonies is shown in Fig. 4. The L-form viable counts and degree of rhamnose removal for a typical experiment are shown in Fig. 5. Samples were withdrawn from the lysozyme-cell mixtures at the indicated times. Rhamnose determinations were performed on the material remaining in solution after centrifugation at 5,000 × *g* for 15 min. It was found that 9×10^{8} L-form CFU/ml were induced after treatment with lysozyme for 120 min. At that time, less than 10% of the rhamnose remained in a sedimentable form. Furthermore, after washing the sedimented damaged cells with PEG, no group D antigen could be detected by serological techniques, and more than 90% of the cell wall receptor sites for bacteriophage P13 had been lost as shown by phage adsorption studies. It would appear that the osmotically sensitive bodies formed after 2 hr of treatment with lysozyme were protoplasts. However, it could not be proved that the formation of protoplasts in suspension was necessary for L-form induction, as the maximum number of L-forms occurred after 30 min of treatment with lysozyme. At this time, 20% of the total rhamnose remained attached to the sedimented cells. During the succeeding 90 min of exposure to lysozyme to reduce the rhamnose to its minimum value, some lysis of the treated cells occurs, and this is reflected in the lower number of L-forms recovered at 120 min.

Similarly, a time-course study of the L-form induction and rhamnose release was performed by using *S. faecalis* E1 (Fig. 6). After 120 min of treatment with lysozyme, 1.6×10^{8} L-form CFU/ml were induced, whereas the maximum yield of L-forms (2×10^{9} CFU/ml) was found after 75 to 90 min of treatment. Of the rhamnose in the original suspension, 40% was found still attached to the cells after 120 min of treatment with lysozyme, indicating that a considerable amount of cell wall remained attached to the cells. It appeared that it was not necessary to produce protoplasts of strain E1 in order to induce the

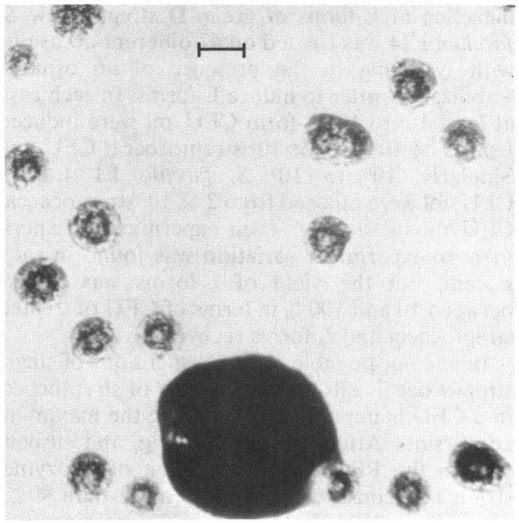

FIG. 4. *Normal Streptococcus faecium F24 colony surrounded by many L-forms. Bar represents 1.0 mm.*

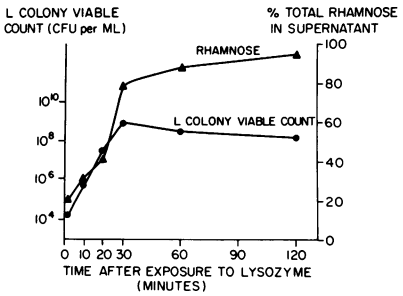

FIG. 5. *A suspension of S. faecium F24 (2×10^{9} CFU/ml) was treated with lysozyme (200 μg/ml) for the indicated time periods in PEG.*

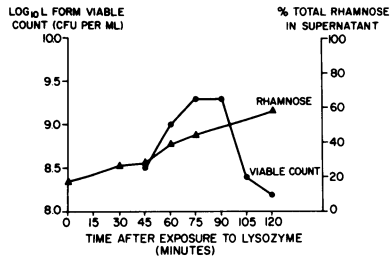

FIG. 6. *A suspension of S. faecalis E1 (5 × 10⁹ to 7 × 10⁹ CFU/ml) was treated with lysozyme (200 μg/ml) for the indicated time periods in PEG.*

maximum number of L-forms from a suspension of these streptococci. Electron microscopic examination of strain E1 cells treated for 120 min with lysozyme revealed visible quantities of wall material remaining on every cell.

Reproducibility of the methods for enzymatic induction of L-forms of group D streptococci. *S. faecium* F24 was treated on 47 different occasions with lysozyme in the presence of an osmotic stabilizer in order to induce L-forms. In each case at least 10^8 to 10^9 L-form CFU/ml were induced from 2×10^9 to 7×10^9 streptococcal CFU/ml. Similarly, 10^8 to 10^9 *S. faecalis* E1 L-form CFU/ml were induced from 2×10^9 streptococcal CFU/ml in each of seven experiments. Experiment-to-experiment variation was found in each system, but the yield of L-forms was always between 10 and 100% in terms of CFU of treated streptococci and L-forms recovered.

It was not possible to use suspensions of single streptococcal cells, so the number of streptococci in a CFU is important in assessing the maximum conversion. After harvest, washing, and suspension in the PEG for the addition of lysozyme, strain F24 consisted primarily (more than 90%) of single cocci or diplococci. The remaining 10% of the CFU had no chain greater than six cocci in length, so that the input total number of cocci was no greater than twice the reported streptococcal CFU. After lysozyme treatment, the enzyme-damaged cells consisted primarily of single cocci or diplococci, but some clumping was observed. Relative counts under phase microscopy showed that the total number of protoplasts was no greater than twice the L-form CFU reported.

DISCUSSION

Some of the added medium components affected only the growth characteristics rather than the quantitative yield of L-forms obtained from the suspensions of enterococci treated by lysozyme. However, it was considered necessary to recognize the variations under the control of medium constituents so as to score the number of L-forms obtained after lysozyme treatment. No experiments were undertaken to determine the physiological basis for these effects. Representative subcultures of both colonial types continued to propagate as L-forms under appropriate tonicity and nutrient conditions.

The relationship of strain F24 L-forms to the parent strain was impossible to ascertain because of the stability of the F24 L-forms upon subculture. The strain E1 L-forms reverted after several subcultures. A system for mass reversion of strain F24 protoplasts to their parent strain (King and Gooder, Bacterial. Proc., p. 68, 1965) suggested that the colonies were indeed derived from *S. faecium*. Furthermore, King, Theodore, and Cole (11) showed that progeny of such L-forms possess identical distributions of membrane proteins, and Hoyer and King (10) showed the close relationship of the homologous parent–L-form pair by deoxyribonucleic acid-deoxyribonucleic acid hybridizations.

The group D streptococcal system for conversion of bacteria to their L-forms differs significantly from other similar systems based upon the action of muralytic enzymes. The most apparent difference is the lack of complete quantitative conversion. In the *Bacillus subtilis* system (13), essentially 100% of the parent strain viable units subjected to the lysozyme treatment were committed to growth in the L-phase under optimal conditions. With group A streptococci (6, 9), results similar to the presently described system for *S. faecium* strain F24 were reported. With strain E1, many experiments showed quantitative conversion under optimal conditions, but this was not found in every experiment.

The induction of high yields of L-forms was not dependent upon protoplast formation. Protoplasts were formed from strain F24 after 120 min of treatment with lysozyme and could be grown as L-forms; however, the maximum yield of induced L-forms was obtained at 30 min of treatment. The maximum number of E1 L-forms was induced with 75 to 90 min of treatment with lysozyme, but protoplasts were not formed after 2 hr. In this respect, the system may be similar to that reported by Landman (12) for *B. subtilis* and lysozyme, in which a stage of wall removal is reached when the spheroplast is able to multiply as an L-form, although complete wall removal and the formation of a protoplast has not occurred in the initial treatment.

A puzzling feature of the system that is not explained is the large number of lysozyme-damaged cells that fail to survive as streptococci or L-forms on the growth medium employed here. This is most apparent in the samples removed during the first 0.5 hr of lysozyme treatment (Fig.

5). Although 2×10^9 CFU streptococci were present initially, less than 10^4 streptococci and between 10^4 and 10^8 CFU L-forms were recovered during this time. It would appear that the damaged streptococci cannot recover as normal cells on the growth medium nor are they yet fully potentiated to grow as the L-form. It is realized that the release of rhamnose as an index of cell wall damage to the cell population is not necessarily an indication of what is occurring with an individual streptococcus in the population.

Although lysozyme treatment does not always convert 100% of the viable group D streptococci to L-forms, the yield with this method far exceeds that reported for alternative methods such as penicillin induction on a gradient plate. Young and Armstrong (16) incorporated penicillin as the inducing agent in the medium, but these workers could obtain less than 1% of the original inoculum as enterococcal L-forms. Our method of L-form induction by treatment with lysozyme increases the lowest yields obtainable to 10% of the original inoculum. The high conversion rate, dependence of the phenomenon upon the length of lysozyme treatment, and reversion under the influence of medium constituents (7) rules out a possible conversion system due to simple mutation.

ACKNOWLEDGMENTS

We thank W. R. Straughn for the strain *S. faecium* F24 and W. R. Maxted for the strain *S. faecalis* E1.

This investigation was supported by Public Health Service grants AI-04577 and GM-01138 from the National Institutes of Health and by a contract (no. DR-49-193-MD 2532) from the U.S. Army Medical Research and Development Command under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiology Board.

LITERATURE CITED

1. Abrams, A. 1958. *O*-acetyl groups in the cell wall of *Streptococcus faecalis*. J. Biol. Chem. **230**:949–959.
2. Bibb, W. W., and W. R. Straughn. 1962. Formation of protoplasts from *Streptococcus faecalis* by lysozyme. J. Bacteriol. **84**:1094–1098.
3. Chesbro, W. R. 1961. Lysozyme and the production of osmotic fragility in enterococci. Can. J. Microbiol. **7**:952–955.
4. Dienes, L. 1939. L organism of Klieneberger and *Streptobacillus moniliformis*. J. Infec. Dis. **65**:24–42.
5. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methylpentoses, and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
6. Freimer, E. H., R. M. Krause, and M. McCarty. 1959. Studies of L forms and protoplasts of group A streptococci. I. Isolation, growth and bacteriologic characteristics. J. Exp. Med. **110**:853–874.
7. Gooder, H. 1968. Streptococcal protoplasts and L form growth induced by muralytic enzymes, p. 40–51. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts and L forms. The Williams & Wilkins Co., Baltimore.
8. Gooder, H., and J. R. King. 1963. Induction of L-type growth in a group D streptococcus, *Streptococcus faecalis* F24. J. Elisha Mitchell Sci. Soc. **79**:72.
9. Gooder, H., and W. R. Maxted. 1961. External factors influencing structure and activities of *Streptococcus pyogenes*, p. 151–173. *In* G. G. Meynell and H. Gooder, (ed.), Microbial reaction to the environment. Cambridge University Press, Cambridge, England.
10. Hoyer, B. H., and J. R. King. 1969. Deoxyribonucleic acid sequence losses in a stable streptococcal L form. J. Bacteriol. **97**:1516–1517.
11. King, J. R., T. S. Theodore, and R. M. Cole. 1969. Generic identification of L-forms by polyacrylamide gel electrophoretic comparison of extracts from parent strains and their derived L-forms. J. Bacteriol. **100**:71–77.
12. Landman, O. E. 1968. Protoplasts, spheroplasts and L forms viewed as a genetic system, p. 319–332. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts, and L forms. The Williams & Wilkins Co., Baltimore.
13. Landman, O. E., and S. Halle. 1963. Enzymically and physically induced inheritance changes in *Bacillus subtilis*. J. Mol. Biol. **7**:721–738.
14. Shockman, G. D., J. J. Kolb, B. Bakay, M. J. Conover, and G. Toennies. 1963. Protoplast membrane of *Streptococcus faecalis*. J. Bacteriol. **85**:168–176.
15. Weibull, C. 1953. The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. J. Bacteriol. **66**:688–695.
16. Young, L. S., and D. Armstrong. 1969. Induction, colonial morphology and growth characteristics of the L-form of *Streptococcus liquefaciens*. J. Infec. Dis. **120**:281–291.