REVERSION OF 3A TYPE L FORMS OF *PROTEUS MIRABILIS*

ROBERT A. ALTENBERN

U. S. Army Chemical Corps, Fort Detrick, Frederick, Maryland

Received for publication November 16, 1960

Reversion of L forms to the normal bacterial state has been described in general terms in a variety of bacterial cell-L-form systems (Dienes and Weinberger, 1951). The relatively large 3B type L colony formed on penicillin-containing medium is composed entirely of spherical bodies of varying sizes (2 to 10 μ in diameter) that appear to propagate by a budding process. In contrast, 3A type L forms, also induced by penicillin, produce a tiny, subsurface colony composed of very small, deeply staining granules. Estimated rates of L form reversion vary widely from rapid and efficient reversion of 3B type L bodies to rare and delayed reversion of 3A type L forms and finally to the apparently permanent (stable) 3A L forms encountered in many investigations (Kawatomari, 1958; Medill-Brown, Hutchinson, and Cocklin, 1960; Freimer, Krause, and McCarty, 1959; Sharp, Hijmans, and Dienes, 1957). There is general agreement that 3A type L forms represent a more extensive departure from normal bacterial cells than do 3B L forms (Taubeneck and Schuhmann, 1959) and consequently that reversion to the bacterial state by 3A L bodies is basically a more complex phenomenon than reversion of 3B type L bodies.

The studies reported below present the initial attempts to define quantitatively some parameters of reversion of 3A type L forms of *Proteus mirabilis*. These data show that reversion of such L forms is indeed a rare event but with a definite probability of occurrence which can be altered by modifications of the growth environment. Furthermore, the available evidence indicates that reversion of these L forms is a physiological and not a genetic event. Finally, the quantitative characteristics of reversion of 3A L bodies suggest a general explanation for permanence or impermanence of L forms.

MATERIALS AND METHODS

P. mirabilis strain F was employed in these studies. Stock cultures were maintained on nutrient agar slants stored at 4 C and transferred to fresh medium every 6 weeks. Inocula of cells were prepared by growth in Penassay (Difco) broth on a shaker at 37 C for 24 hr.

The plating medium consisted of Penassay broth compounded from concentrated stock solutions of the individual ingredients plus 0.8% agar, 10^{-2} M $MgSO_4$, and 10% (v/v) sterile defibrinated horse serum (unless otherwise noted). The details of preparation of this medium are described in a recent paper (Altenbern, 1961). The general medium notation defining agar concentration, concentration of penicillin in units per ml, and tonicity (molarity of sodium succinate) as described earlier (Altenbern and Landman, 1960) has been retained. Thus, medium I contains 0.8% agar, 200 units of penicillin per ml, and no succinate, whereas medium II is liquid and contains no agar or penicillin but does contain 0.3 M sodium succinate, sterilized separately and added aseptically. The initial crop of 3A type L colonies was obtained by plating appropriate dilutions of Proteus onto medium I containing glucose-phosphate autoclaved at pH 7.7 to suppress growth of 3B L colonies (Altenbern, 1961). After 6 days of incubation at 30 C, blocks of agar containing a total of 100 to 200 3A L colonies were excised from the plate and suspended in 10 ml of liquid medium II in the 50-ml receptacle of a Servall Omnimixer controlled by a rheostat (Powerstat by Superior Electric Company). The agar blocks were then triturated for 45 to 60 sec in the Omnimixer operating at step 35 of the Powerstat. The resulting suspension was then centrifuged for 30 min in an anglehead centrifuge. The supernatant fluid was carefully withdrawn with a syringe and needle and examined by phase microscopy. Many pilot experiments have shown that any 3B bodies, initially present or purposely admixed, are sedimented by this centrifugation step, leaving only 3A granules, occasional fragments of agar, and a few ghosts of large bodies in the supernatant liquid. Hypertonic medium is essential for retention of viability of 3A L granules

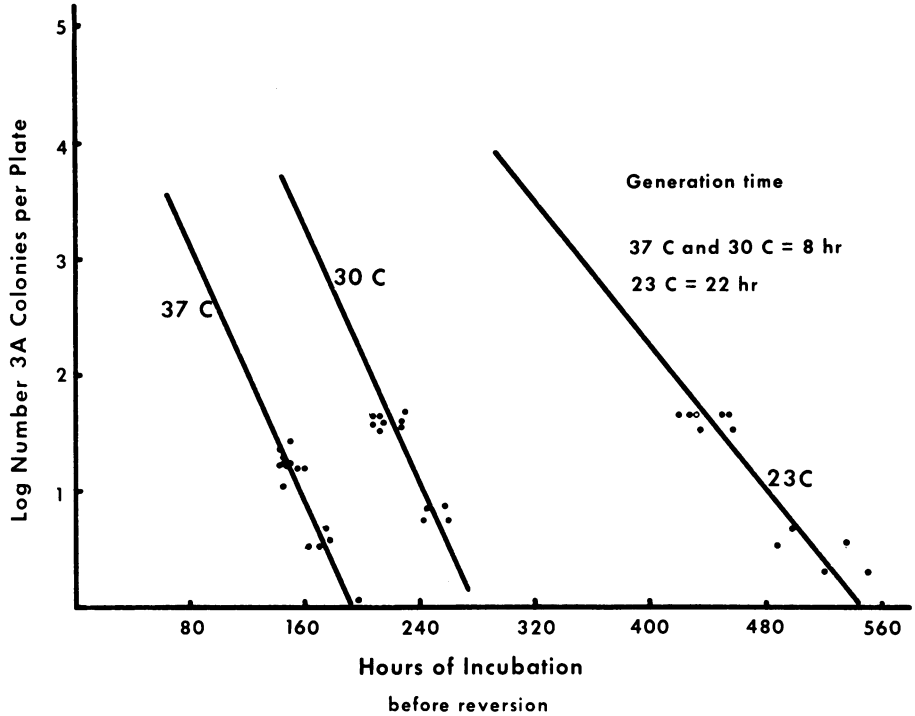

Fig. 1. Reversion of 3A L colonies as a function of number of colonies per plate and the time and temperature of incubation.

in suspension, whereas identical hypertonicity in solid medium completely suppresses growth of 3A type L colonies. Appropriate dilutions (also in medium II) of the final suspension were plated by spreading 0.1-ml amounts onto the surface of agar plates (medium III: 0.8% agar, no penicillin, no succinate). Variations in the composition of the plating medium and the temperature of incubation are specified in the text.

Reversion of 3A L colonies could be easily detected by the formation of a bacterial colony, around a 3 A colony with the characteristic swarming of *P. mirabilis* on soft, isotonic agar. Plates of medium III inoculated with 3A L bodies were examined daily for reversions. For the precise determination of incubation time required for reversion (Fig. 1) the radius of the swarming bacterial colony was measured and divided by the rate of radial swarming. This value (in hours) was then subtracted from the total incubation time to give a value denoting the number of hours of incubation required for a reversion to appear. The rate of radial swarming at a given temperature was steady and remarkably constant from one experiment to the next or on any of several plates in the same experiment. The rates were 8.5 mm per hr at 37 C, 4 mm per hr at 30 C, and 2.4 mm per hr at 23 C. Data presented in other figures were obtained by recording only the days of incubation required for reversion to appear. Occasionally reversions could be detected swarming from two widely separated 3A colonies on the same plate and, in several instances, three independent reversions on the same plate were observed.

RESULTS

Preliminary experiments to determine the period of incubation required for a reversion to appear in a culture of 3A type L forms indicated that reversion occurred relatively early on a plate containing many 3A L colonies, whereas plates containing few 3A colonies required rather long incubation before a reversion could be detected. A precise determination of this relationship between incubation time and colony count by the methods described yielded the data presented in Fig. 1. These lines were calculated from the experimental points by the method of

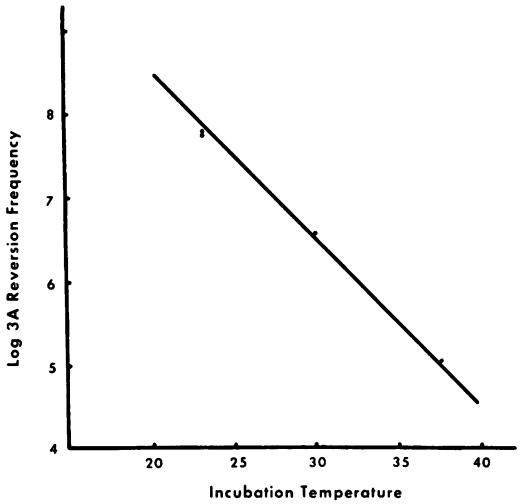

Fig. 2. Relationship of reversion frequency of 3A L forms and temperature of incubation. Each point represents an average of two separate determinations by the methods employed for Fig. 1.

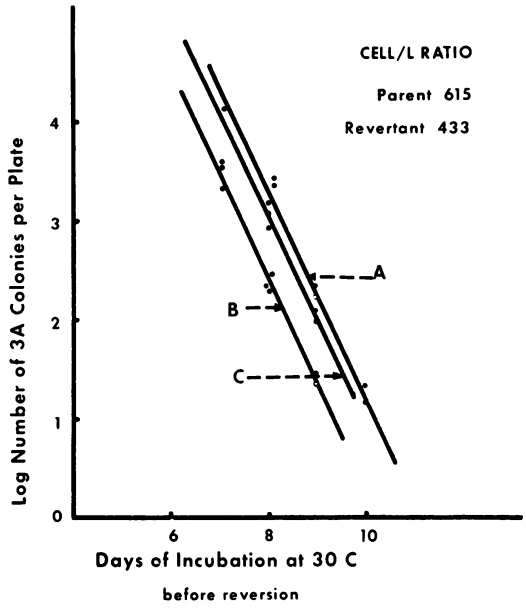

Fig. 3. Comparison of the frequency of reversion of 3A L granules obtained from either parent cells or cells from previous reversions. *A*, 3A L colonies obtained from parent Proteus cells; *B and C*, 3A L colonies obtained from Proteus cells resulting from two separate, previous reversions.

least squares. Two separate determinations gave nearly identical results. By extrapolation of these lines to the ordinate intercept, the frequency of reversion can be determined. For example, the 37 C line in Fig. 1 intersects the ordinate (0 hr of incubation) at a value of 1.3 $\times 10^5$ which means that, at the time of plating, one viable unit in 1.3 $\times 10^5$ viable units is theoretically capable of immediate reversion and requires no further incubation. The relationship between colony count and incubation required for reversion at 30 and 23 C has been similarly extrapolated to zero time. Separate experiments performed with concentrated 3A L body suspensions have shown clearly that the semilogarithmically linear relationship between colony count and reversion time is maintained throughout a 4 log concentration range. The generation times of the 3A L bodies at differing temperatures can be readily derived from the slope of these straight-line relationships and are included in Fig. 1. As with growth of 3B L bodies in liquid media (Altenbern and Landman, 1960), the generation times of 3A type L bodies on agar at 37 and 30 C are nearly identical, whereas the generation time at 23 C is significantly greater.

The strong dependence of reversion frequency upon incubation temperature is illustrated in Fig. 2. The Q_{10} value is nearly 15 indicating the probable participation of many enzymatic reactions leading to manufacture of a normal bacterial revertant. This line extrapolates to a theoretical reversion frequency of 1 (or 10°) at 63 C. All of many attempts to induce mass reversion in 3A L colonies grown at 23 C by exposure to higher temperatures (37 to 45 C) for periods of 1 to 8 hr failed. Such failures indicate that the organizational state of the 3A L body at 37 C leading to frequent reversion cannot be attained by brief exposure of 23 C grown 3A L bodies to higher temperatures, but is probably established only after several generations at the higher temperature.

The nonmutational nature of reversion is demonstrated by the following experiments. Bacterial cells of a revertant from a 3A L colony have been compared to the parent type cells with regard to efficiency of 3A L colony formation when plated on penicillin medium. The results are presented in Fig. 3 as cell/L ratios, as defined in an earlier publication (Landman, Altenbern, and Ginoza, 1958). Several other revertant cultures also exhibited cell/L ratios for the 3A type

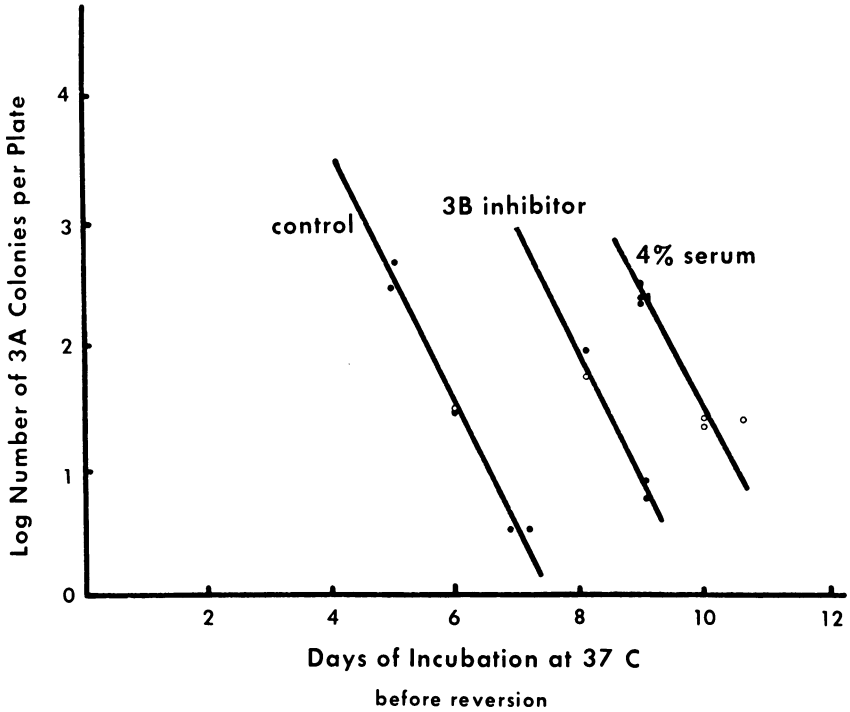

Fig. 4. Modification of reversion frequency of 3A L granules by medium changes. 3B inhibitor generated by autoclaving glucose-phosphate solution at pH 8.8 as described in text.

L colony nearly identical to that obtained from the parent cultures. Additionally, 3A L bodies obtained by plating revertants on penicillin medium possessed a reversion frequency identical to that exhibited by 3A L bodies obtained from the parent cells as indicated by data plotted in Fig. 3. Such evidence strongly suggests that conversion of Proteus cells to 3A type L forms and reversion of these L forms to the normal bacterial state involve no detectable alterations in genotype.

The reversion frequency at 30 or 37 C seems to be remarkably stable and could be altered by only two modifications of the medium. Inclusion in the medium of the inhibitor of 3B type L growth, generated by autoclaving glucose in phosphate buffer at pH 8.8, markedly extended the incubation period required for reversion. Reduction in serum amount to 4% (v/v) also decreased the reversion frequency significantly (Fig. 4). It is to be noted that the generation time was not appreciably altered by these medium changes. The other constituents of the medium, namely, peptone, beef extract, yeast extract, and NaCl, could be varied in amount from 0.1 to 2.0 times that present in Penassay medium without altering either the frequency of reversion or the generation time.

DISCUSSION

Although numerous authors have described the apparently low rate of growth of 3A type L forms, the preceding data estimate the actual generation time of these units. The values obtained are considerably in excess of those derived for 3B type L forms from the same strain of *P. mirabilis* (Altenbern and Landman, 1960). These estimates, however, are made with regard to propagation of 3A bodies capable of reversion and cannot include generation of those particles which are nonviable.

Numerous experiments in this laboratory have indicated that the yield of viable plating centers (ability to form another 3A colony) from a 3A type L colony is always very low. Similar estimates of the percentage of viability of 3B L bodies of *Escherichia coli* have been presented by Lederberg and St. Clair (1958). Such low viability may be due, in part, to the vigorous procedures employed to triturate the 3A colonies

imbedded in the agar as described in the Materials and Methods section. From the reversion data, however, rough calculations suggest that the number of 3A particles per colony which are viable in terms of initiating reversion is, in actuality, quite low, on the order of 0.001 to 0.01% of the total number of granules in the colony. This estimate is based on the number and average size of the colonies (0.1 to 0.2 mm diameter, roughly spherical) on the plate at the time of reversion and the number of 3A particles per colony calculated from the average size of the 3A particles (0.3 to 0.5 μ diameter). The concept that the majority of 3A type granules within a colony are nonviable with regard to either further propagation or reversion is consistent with all observations. Furthermore, these calculations indicate that the percentage for viability of granules is lowest at 37 C and highest at 23 C, suggesting that the relatively high reversion rate at 37 C is not due to increased viability of the 3A granules.

The physiological processes leading to reversion remain unknown. It is known that, after prolonged incubation on penicillin-containing medium, 3A type colonies change to 3B colonial morphology and that these "derived" 3B bodies revert to normal bacterial forms on penicillin-free medium with the same efficiency as do 3B bodies which have grown on hypertonic media and have not passed through the 3A type morphology. The medium manipulations which alter reversion frequency of 3A L forms, namely, addition of the 3B inhibitor or reduction in amount of serum, suggest that the crucial step in reversion is the formation of a lipid-rich membrane around a 3A granule.

Finally, these data provide an interesting insight into the phenomenon of "permanent" L forms. With Proteus 3A type L forms, permanence or impermanence is a relative matter depending upon the temperature of incubation, the medium constitution, and the number of colonies per plate. In nearly all respects, detection of reversion in plates of 3A type L forms of Proteus simulates the Luria-Delbrück fluctuation test for mutation rate determination. Incubation of a small number of colonies at an unfavorable temperature, such as 23 C, for an insufficient length of time would readily give the impression that these L forms are permanent. As in the fluctuation test, transfer of these units to a fresh plate before reversion has occurred returns the system to the same reversion probability as on the initial plates. By suspending and transferring successively 3A bodies of Proteus to fresh penicillin-free medium at the proper intervals, the culture can be maintained as a "permanent" L form indefinitely. At any time, however, prolonged incubation leads to reversion even if only a single colony is present on the plate. The selection, by unknown factors, of a mutant blocked in one of the reactions involved in cell wall formation would, of course, yield a truly permanent L form (Lederberg and St. Clair, 1958). However, it is reasonable to assume that 3A type L forms from various bacterial species possess differing reversion rates, perhaps some so low that the growth on a single plate cannot attain the size necessary to insure appearance of the revertant and thus be judged "permanent" by default.

SUMMARY

Reversion of 3A type L particles of *Proteus mirabilis* to the bacillary state is a function of the number of colonies on the plate and incubation time and temperature. The frequency of reversion on soft serum agar varied from 7.7×10^{-6} at 37 C to 1.5×10^{-8} at 23 C. The generation time of 3A type L units was approximately 8 hr at 37 and 30 C and approximately 22 hr at 23 C. Bacterial cells obtained from reversion of 3A type L particles are no more efficient than original, parent cells in forming 3A type L colonies, and the 3A L units so obtained revert to bacteria with the same frequency as 3A L units obtained from parent bacilli. Reversion frequency may be altered by medium modifications which do not change the generation time of the 3A L bodies. In light of these observations, an interpretation of the phenomenon of permanence of L forms is offered.

REFERENCES

ALTENBERN, R. A. 1961 Critical factors influencing growth of L forms of *Proteus mirabilis*. J. Bacteriol., **81**, 586–594.

ALTENBERN, R. A., AND O. E. LANDMAN 1960 Growth of L-forms of *Proteus mirabilis* in liquid media. J. Bacteriol., **79**, 510–518.

DIENES, L., AND WEINBERGER, H. J. 1951 The L-forms of bacteria. Bacteriol. Rev., **15**, 245–288.

FREIMER, E. H., M. KRAUSE, AND M. MCCARTY

1959 Studies of L forms and protoplasts of group A streptococci. I. Isolation, growth, and bacteriologic characteristics. J. Exptl. Med., **110,** 853–874.

KAWATOMARI, T. 1958 Studies of the L-forms of *Clostridium perfringens*. I. Relationship of colony morphology and reversibility. J. Bacteriol., **76,** 227–232.

LANDMAN, O. E., R. A. ALTENBERN, AND H. S. GINOZA 1958 Quantitative conversion of cells and protoplasts of *Proteus mirabilis* and *Escherichia coli* to the L-form. J. Bacteriol., **75,** 567–576.

LEDERBERG, J., AND J. ST. CLAIR 1958 Protoplasts and L-type growth of *Escherichia coli*. J. Bacteriol., **75,** 143–160.

MEDILL-BROWN, M., W. G. HUTCHINSON, AND E. COCKLIN 1960 The L forms of *Proteus mirabilis*. Ann. N. Y. Acad. Sci., **79,** 374–379.

SHARP, J. T., W. HIJMANS, AND L. DIENES 1957 Examination of the L forms of group A streptococci for the group-specific polysaccharide and M protein. J. Exptl. Med., **105,** 153–159.

TAUBENECK, U., AND E. SCHUHMANN 1959 Beobachtungen über die Entstehung der L-Kolonien bei *Proteus mirabilis*. Zentr. Bakteriol., Parasitenk., Abt. II, **113,** 60–62.