APPLIED MICROBIOLOGY, Oct. 1971, p. 546-551

Vol. 22, No. 4
Printed in U.S.A.

Electronic Counting in Growth Studies of *Mycobacterium avium*

CHARLOTTE McCARTHY

National Jewish Hospital and Research Center, Denver, Colorado 80206

Received for publication 30 June 1971

Metabolically uniform cells of *Mycobacterium avium* were obtained by selective filtration. The life cycle of these cells was followed by photomicrographs, electronic enumeration, and sizing and by viability and protein determinations. The cells elongate to form filaments several times their initial length; the increase in mass is reflected by a five- to sixfold increase of total protein in the culture. The filaments then fragment causing the production of viable coccobacilli. The techniques employed to obtain this form of growth are described and comparisons with nocardial growth are noted.

Brieger and Fell (4), 26 years ago, described for *Mycobacterium avium* three forms of slow growth and a "standard" life cycle. Their description of the standard life cycle of *M. avium* growing on chicken embryo extract was as follows: ". . . the bacillus elongates during the first 24 hr. to form a filament several times its original length; this divides repeatedly to form a bunch of 20–30 filaments which on the 2nd or 3rd day break down into short rods, which continue to multiply slowly by ordinary binary fission. If the bacilli are subcultivated in fresh embryo extract the cycle is repeated but not otherwise."

With the introduction of 7H10 agar (11), it was possible to detect stable colony variants of *M. avium* (15) which Brieger and Fell were unable to distinguish on their more complex media. Two colony variants, opaque and transparent, differ in that the transparent variant is more pathogenic for chickens and mice (12, 13), is more drug-resistant (8), and forms less cell mass (10) than the opaque variant.

Because of the existence of two physiologically distinct variants and the possibility of a life cycle in *M. avium*, an attempt was made to define the temporal sequence of events in cell growth and division in this organism. In addition to the measurement of culture density, viability, and protein increases during growth, a Coulter counter was used to determine, electronically, numbers and sizes of the cells at different stages of growth. This method proved useful in delineating events in the "standard" life cycle of *M. avium* that was described by Brieger and Fell (4).

MATERIALS AND METHODS

Organism and medium. An isolate from a patient at National Jewish Hospital and Research Center, Denver, Colo., was used; it was identified as *M. avium*, serotype I, by W. B. Schaefer (*personal communication*). A transparent and an opaque colony variant were purified on 7H10 agar (BBL) and maintained frozen as described previously (10). Minimal B liquid medium (10) was used in all experiments; it consisted of defined salts, 0.2% Tween 80 (Polysorbate 80; Atlas Chemical Industries, Inc.), 0.01% oleic acid, and 0.5% each of dialyzed bovine serum albumin, glycerol, and glucose. The medium was filtered through an 0.22-μm filter (MF; Millipore Corp., Bedford, Mass.) by means of a suction flask and a Pyrex filter holder with a stainless-steel screen support (Millipore Corp.).

Growth studies. Frozen stock concentrates of the cells were thawed, diluted into minimal B medium, and incubated on a rotary shaker at 37 C to a cell density of 2×10^8 to 5×10^8 viable units/ml. The culture was then passed through a 1.2-μm filter (MF; Millipore Corp.); the cells in the filtrate were concentrated by centrifugation, washed, and then resuspended in fresh minimal B medium. These cells, selected for their small size, were incubated on a rotary shaker at 125 rev/min and 37 C. Turbidity measurements were made with a Klett-Summerson colorimeter and a no. 42 filter. Samples were removed at intervals and stored in an ice bath for subsequent protein, viability, and particle determinations.

Assays. Cells for protein and deoxyribonucleic acid (DNA) determinations were harvested by centrifugation at 4 C and then washed twice with cold 0.2% Tween 80. Protein was estimated by the method of Lowry et al. (9) with bovine serum albumin as standard; the assay was modified by heating the cells in 0.5 N NaOH at 90 C for 10 min before addition of

the other reagents. DNA was determined by the method of Burton (6) with deoxyadenosine-5'-monophosphate as standard; the cells (concentrated from 50 ml of culture) were heated at 70 C for 15 min in 0.5 N $HClO_4$ before addition of other reagents.

Viable cell determinations were made by dilution of the culture in S salt solution (10) and plating on 7H10 agar; the plates were incubated at 37 C for 14 days. Smears of the bacteria were fixed with formaldehyde vapor and then stained with the cold acid-fast stain of Aubert (3).

Particle counts and cell volume determinations. A Coulter counter (model B) and Coulter plotter (model H) were used in the electronic determinations of total number of particles and of the particle size distributions. The diluent was Isoton (Coulter Electronics, Inc., Hialeah, Fla.), some lots of which were filtered through an 0.22-μm filter to remove contaminating particles. To kill and fix the bacteria, a 0.2-ml sample of the culture was mixed with 0.05 ml of Isoton which contained 1.6% formaldehyde. This solution was filtered through a 0.22-μm filter before use.

A 30-μm aperture tube was used with machine settings of $\frac{1}{8}$ and 0.354 for the amplification and aperture current reciprocals, respectively.With these settings, the noise level was 500 counts at a lower threshold of 12. The plotter was factory-set at 25 windows, each of which was equivalent to 4 thresholds on the counter. The plotter was standardized with latex beads, 0.8 μm in diameter and 0.27 μm^3 in volume (Coulter Electronics, Inc.); this standard produced a peak at window 8 on the plotter which was calculated to correspond to a threshold of 34 on the counter.

RESULTS

Growth of two colony variants of M. avium. Liquid cultures of the transparent and opaque variants were filtered through 1.2-μm filters. The cells in the filtrates, selected only for their small size, were transferred to fresh media and incubated. Samples were removed at intervals and tested for viability, total protein, and number of particles to quantitate cell growth and division. The cells extensively synthesized protein during the first 22 hr with a five- to sixfold increase in protein in the absence of cell division (Fig. 1). In the next 40 hr, the total protein accumulated to 1.5 to 2 times that of the 22-hr sample, but the viable units increased fourfold in the opaque variant and twofold in the transparent variant culture.

The particle enumeration concurred with the viability determinations for the opaque variant. The discrepancy was very large, however, for the transparent variant, and, after 65 hr of incubation, the number of particles was 20 times that of the viable units. Passage of such a culture through an 0.8-μm filter (MF; Millipore Corp.) removed 45% of the particles and only 0.1% of these were viable (Table 1). The nonviable particles may be globules of compound(s) elaborated by the organism during growth (*unpublished data*).

Synchronization of an opaque variant. The growth cycle of the opaque variant was analyzed in detail since aberrant particle counts were reduced or absent in it (Fig. 1). A 1.2-μm filtrate of a fully grown culture was washed and placed in fresh medium; then samples were removed at intervals for various growth measurements. Plots were made of the distribution of particles having volumes of 0.08 to 0.78 μm^3. The cells, at zero time, were very small as shown in both the photomicrograph and the size distribution plot (Fig. 2). They had a mean volume of 0.15 μm^3 (Table 2) which could be approximated by bacilli 0.40 by 1.18 μm. All of the bacteria elongated in a homologous fashion as indicated by the gradual increase in large particles at each successive sampling time of 2, 4, and 6 hr. After 12 hr of incubation, there was a Poisson distribution of the particles ranging in volume from 0.22 through 0.55 μm^3 (Fig. 2); these had a mean cell volume of 0.31 μm^3 (Table 2) which could coincide with bacilli having dimensions of 0.40 by 2.49 μm. There was no gross change in numbers of viable units or particles during the initial 12-hr period and only a low level of protein synthesis occurred (Table 2). The bacteria thus elongated synchronously during this time which indicates that they were metabolically uniform.

Fragmentation within a synchronized culture. After 26 hr of growth, there was five times as much protein as in the zero-time culture (Table 2); the augmentation of cell mass was verified by the photograph of the enlarged, clumped cells or filaments (Fig. 3). There was a slight decrease in the number of particles due to the clumping (Table 2). Calculation of mean cell volume of such clumped bacteria results in a spurious value and it was therefore not used to assess the size of the filaments. At 45 hr, there was an approximately parallel increase in particles and protein, two- and threefold, respectively, over that of the culture incubated for 26 hr (Table 2).

Fragmentation occurred by the next sampling time, since at 70 hr there was a 5.4-fold increase in yield of particles over that found at 45 hr (Table 2). Indeed more particles may have been produced than the number detected by the electronic counter because there was extensive clumping of the particles (Fig. 3). During this period of rapid expansion in cell numbers, the total quantity of protein synthesized was slight (Table 2).

Particle accumulation continued in the culture resulting in, at 96 hr, three times the 70-hr value (Table 2). It is possible that this particle increase was due to disaggregation of some of the cell

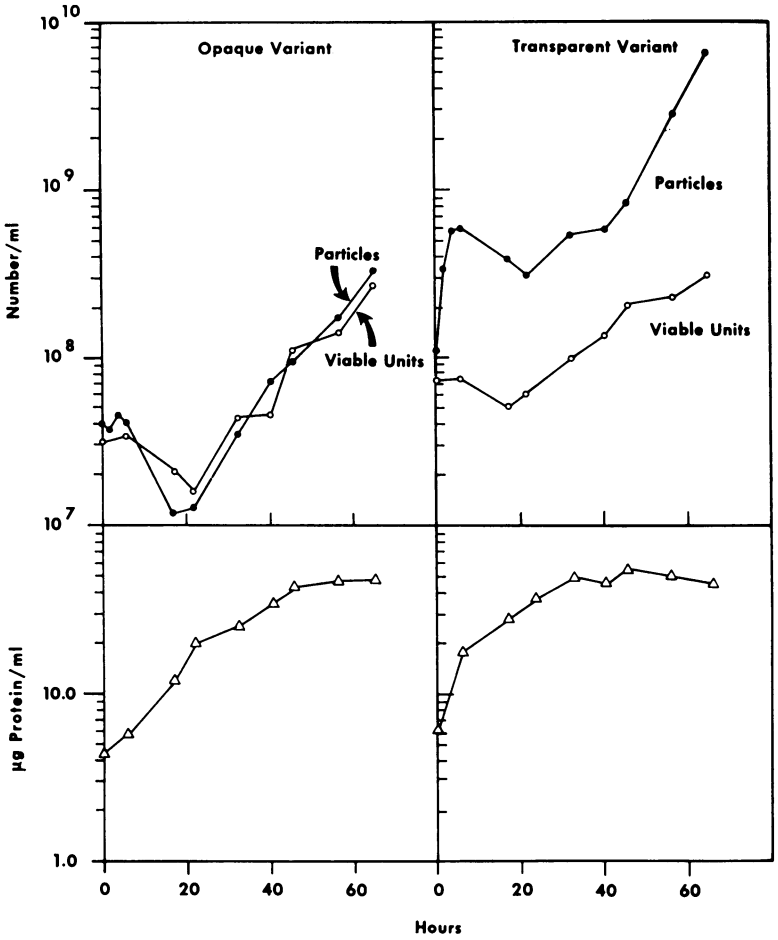

FIG. 1. *Particle, viability, and protein determinations during growth of transparent and opaque colony variants of M. avium. The experimental procedure is described in Materials and Methods.*

TABLE 1. *Removal of nonviable particles in a culture of a transparent variant of M. avium by selective filtration*

Fraction[a]	Particles		Viable units	
	No./ml	Per cent	No./ml	Particles[b] (%)
No treatment	9.2×10^9	100	3.9×10^8	4.2
3.0-µm filtrate	5.3×10^9	58	1.8×10^8	3.4
1.2-µm filtrate	4.8×10^9	52	3.8×10^7	0.8
0.8-µm filtrate	4.1×10^9	45	4.2×10^6	0.1

[a] A culture of a transparent variant was grown under the same conditions as given in Fig. 1 for 110 hr. One portion was not treated and other portions were filtered through 3-, 1.2-, or 0.8-µm filters, respectively, via Millipore Swinney syringes, 25 mm in diameter. Particles were counted on the Coulter counter, and viable units were estimated by plating on 7H10 agar.

[b] Per cent of total particles in the fraction that formed colonies on 7H10 agar.

TABLE 2. *Growth measurements for the synchronized culture of opaque cells illustrated in Fig. 2 and 3*

Time (hr)	Particles/ml	Viable units/ml	Amt (µg) of protein/ml	Klett units	Mean cell volume (µm³)
0	2.6×10^7	1.5×10^7	2.4	1	0.15
2	2.7×10^7	1.2×10^7			0.17
4	3.0×10^7	1.3×10^7			0.19
6	3.1×10^7	1.4×10^7			0.22
12	2.8×10^7	1.1×10^7	3.6	1	0.31
26	2.0×10^7	1.5×10^7	12	16	
45	4.2×10^7	3.5×10^7	34	58	
70	2.3×10^8	1.7×10^8	52	92	
96	7.3×10^8	4.8×10^8	62	127	
117	1.3×10^9	8.4×10^8	80		

clumps (Fig. 3). After 117 hr, the culture was nearly stabilized since there was only a 1.7-fold increase in particles above the measurement taken at 96 hr (Table 2).

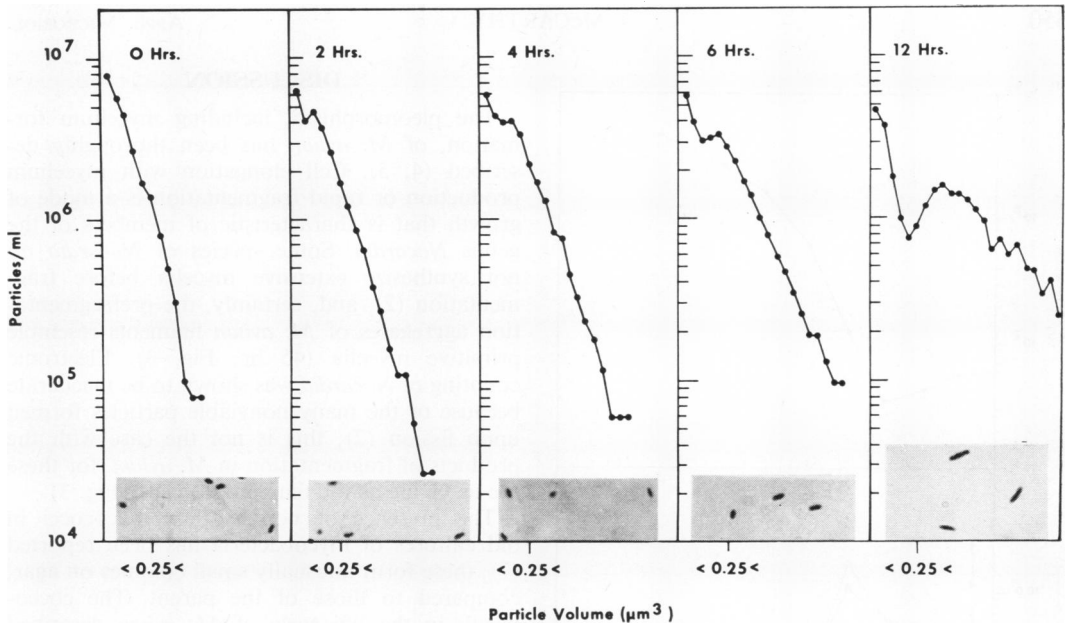

FIG. 2. *Elongation of a synchronized culture of an opaque variant of M. avium during the first 12 hr of incubation of cells from a 1.2-μm filtrate. The size distribution range in each graph is 0.08 through 0.78 μm³. The 0.25-μm³ mark approximates the size of standard latex beads, 0.8 μm in diameter. The magnification, 1,125 ×, is the same in all photographs.*

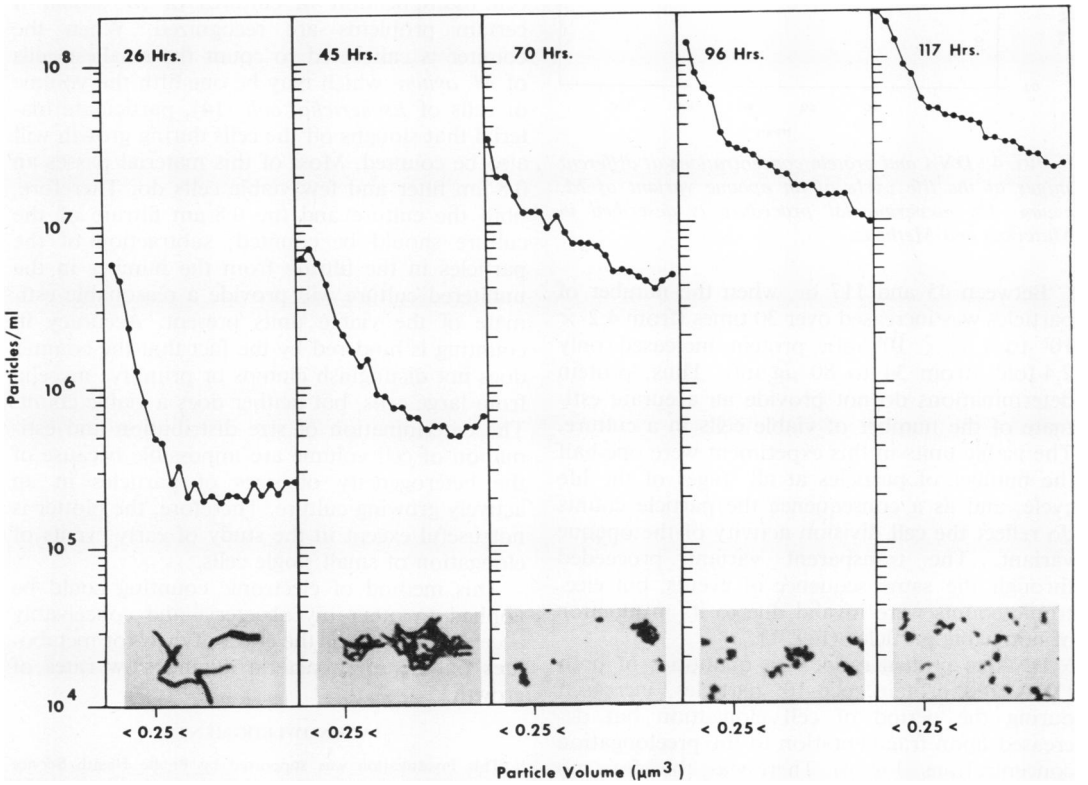

FIG. 3. *Fragmentation within a synchronized culture of an opaque variant of M. avium. The relative volume marker and the magnification of the photographs are the same as for Fig. 2.*

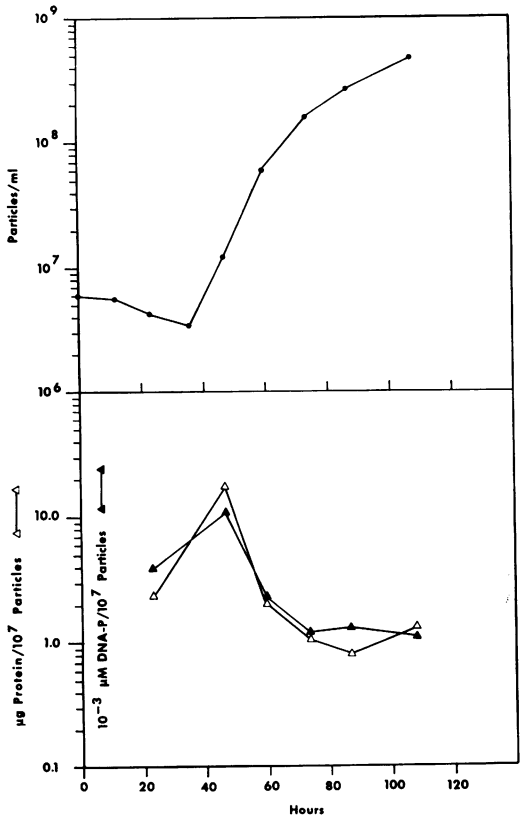

FIG. 4. *DNA and protein concentrations at different stages of the life cycle of an opaque variant of M. avium. The experimental procedure is described in Materials and Methods.*

Between 45 and 117 hr, when the number of particles was increased over 30 times (from 4.2×10^7 to 1.3×10^9/ml), protein increased only 2.4-fold (from 34 to 80 μg/ml). Thus, protein determinations do not provide an accurate estimate of the number of viable cells in a culture. The viable units in this experiment were one-half the number of particles at all stages of the life cycle, and as a consequence the particle counts do reflect the cell division activity of the opaque variant. The transparent variant proceeded through the same sequence of events, but electronic counts were invalid due to its production of nonviable particles (Fig. 1).

DNA to protein ratio. The quantities of both DNA and protein, per 10^7 particles, increased during the period of cell elongation but decreased upon fragmentation to the preelongation concentrations (Fig. 4). There was, therefore, no obvious imbalance in the proportions of DNA and protein at different stages of the life cycle of *M. avium*.

DISCUSSION

The pleomorphism, including mycelium formation, of *M. avium* has been thoroughly described (4, 5). Cell elongation with mycelium production or rapid fragmentation is a mode of growth that is characteristic of members of the genus *Nocardia*. Some species of *Nocardia* do not synthesize extensive mycelia before fragmentation (2), and, certainly, the prefragmentation aggregates of *M. avium* filaments resemble primitive mycelia (45 hr; Fig. 3). Electronic counting of *Nocardia* was shown to be inaccurate because of the many nonviable particles formed upon fission (2); this is not the case with the products of fragmentation in *M. avium*, for these are as viable as the filamentous cells (Fig. 3).

The presence of non-acid-fast mycococci in old cultures of mycobacteria has been reported (7); these form unusually small colonies on agar, compared to those of the parent. The coccobacilli in the life cycle of *M. avium* described here are acid-fast and they produce typical colonies; these are therefore genuine mycobacteria and not mycococci.

The Coulter counter may be used to estimate cell multiplication in cultures of *M. avium* if certain problems are recognized. When the counter is calibrated to count the smallest cells of *M. avium*, which may be one-fifth the volume of cells of *Escherichia coli* (14), particulate material that sloughs off the cells during growth will also be counted. Most of this material passes an 0.8-μm filter and few viable cells do. Therefore, both the culture and the 0.8-μm filtrate of the culture should be counted; subtraction of the particles in the filtrate from the number in the unfiltered culture will provide a reasonable estimate of the viable units present. Accuracy in counting is hindered by the fact that the counter does not distinguish clumps or primitive mycelia from large cells, but neither does a viable count. The determination of size distribution and estimation of cell volume are impossible because of the heterogeneity of sizes of particles in an actively growing culture. Therefore, the plotter is not useful except in the study of early events of elongation of small single cells.

This method of electronic counting could be applied to other mycobacteria and conceivably accelerate studies on the effect of drugs or metabolites on the mycobacteria having slow rates of growth.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-09541 from the National Institute of Allergy and Infectious Diseases.

The technical assistance of David Kenney and Caroline Otsuka is gratefully acknowledged.

LITERATURE CITED

1. Adams, J. N. 1966. Studies on the fragmentation of *Nocardia erythropolis* in liquid medium. Can. J. Microbiol. 12:433–441.
2. Adams, J. N., and N. M. McClung. 1962. Comparison of the developmental cycles of some members of the genus *Nocardia*. J. Bacteriol. 84:206–216.
3. Aubert, E. 1950. "Cold" stain for acid-fast bacteria. Can. J. Public Health 41:31–32.
4. Brieger, E. M., and H. B. Fell. 1945. Warm-stage observations on the initial development of the avian tubercle bacillus cultivated in embryo extract. J. Hyg. 44:158–169.
5. Brieger, E. M., and A. M. Glauert. 1952. A phase-contrast study of reproduction in mycelial strains of avian tubercle bacilli. J. Gen. Microbiol. 7:287–294.
6. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315–322.
7. Csillag, A. 1970. A simple method to obtain the mycococcus form of *Mycobacterium phlei*. J. Gen. Microbiol. 62:251–259.
8. Engbaek, H. C., B. Vergmann, and I. Baess. 1970. Nonphotochromogenic mycobacteria serotype Davis. Acta Pathol. Microbiol. Scand. 78:619–631.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
10. McCarthy, C. 1970. Spontaneous and induced mutation in *Mycobacterium avium*. Infec. Immun. 2:223–228.
11. Middlebrook, G., and M. L. Cohn. 1958. Bacteriology of tuberculosis: laboratory methods. Amer. J. Public Health 48:844–853.
12. Olitzki, A. L., C. L. Davis, W. B. Schaefer, and M. L. Cohn. 1969. Colony variants of avian-battey group mycobacteria intracerebrally injected into mice. Pathol. Microbiol. 34: 316–323.
13. Schaefer, W. B., C. L. Davis, and M. L. Cohn. 1970. Pathogenicity of transparent, opaque, and rough variants of *Mycobacterium avium* in chickens and mice. Amer. Rev. Resp. Dis. 102:499–506.
14. Shehata, T. E., and A. G. Marr. 1970. Synchronous growth of enteric bacteria. J. Bacteriol. 103:789–792.
15. Vestal, A. L. and G. P. Kubica. 1966. Differential colonial characteristics of mycobacteria on Middlebrook and Cohn 7H10 agar-base medium. Amer. Rev. Resp. Dis. 94:247–252.