Growth and Cell Division of *Mycobacterium avium*

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The rates of cell division and of protein, DNA and RNA synthesis upon transition of *Mycobacterium avium* to and from rich medium were examined. The changes in cell morphology (elongation) were also examined by optical and electron microscopy. Upon transfer from poor to rich medium, the rate of synthesis of RNA increased rapidly, followed by an increase in protein synthesis within 3 h and by an increase in DNA synthesis within 7 h; cell division began after a lag of about 10 h. Upon transfer from rich to poor medium, the preshift rates for protein and DNA synthesis changed to postshift rates after 3 h and 7 h, respectively; RNA synthesis stopped immediately, there was a transient fall in total RNA, and synthesis was resumed at a new rate only after 24 h. After the period of adjustment to new medium, the bacteria entered the postshift growth in which cell size, the increase in cell mass (absorbance at 650 nm) and viable counts, and the rates of synthesis of protein, DNA and RNA were constant. Ultrastructural examination of elongated cells during the adjustment period showed that they had septa at different stages of formation, but no evidence of fragmentation was found. It was concluded that cell division in *M. avium* was by binary fission, and that the notion of a life-cycle was not supported by the present findings.

**INTRODUCTION**

It has been suggested that the ability of *Mycobacterium avium* to survive in the tissues of man and experimental animals might be related to the expression of the genes that specify the smooth transparent (SmT) colony type; since the expression of these genes is not necessary for survival in artificial media, they would be repressed upon transfer of the bacteria in the laboratory, which could lead to colony type variation resulting in the predominance of smooth, opaque and dome-shaped (SmD) colonies (David, 1978). This hypothesis was based on reports concerning the virulence of the SmT and SmD variants (Olitzki et al., 1969; Schaefer et al., 1970), the differential drug-susceptibility of the variants (Wichelhausen & Robinson, 1965; Schaefer et al., 1970) and the genetic basis of the SmT-SmD colony type variation (McCarthy, 1970; Woodley & David, 1976).

However, since the pleomorphism of *M. avium* (Brieger & Fell, 1945; Brieger & Glauert, 1952) has been attributed to a life-cycle (McCarthy, 1971, 1974, 1976, 1978), the diverse physiological states described above could relate to the regulatory processes at the various stages of the life-cycle. The life-cycle of *M. avium* was described by McCarthy (1971) as follows: when *M. avium* cells selected for their small size were placed in a new medium, they elongated to several times their original length and then fragmented into small bacillary units. One can predict that during the elongation process, the rate of increase in cell mass should be higher than the rate of increase in cell numbers, and that during the fragmentation step, there must be an increase in cell numbers for a constant cell mass. Furthermore, the life-cycle implies the operation of regulatory processes that at the cellular level should be expressed by the formation of septa whose inward growth should progress at a constant rate in preparation...
for a fragmentation step. It is the purpose of this paper to show that these predictions were not confirmed under our experimental conditions, which showed that division in *M. avium* was by binary fission.

**METHODS**

*Organism.* Mycobacterium avium ATCC 15769 maintained on Löffenstein–Jensen medium was used in these studies.

**Composition of media.** Experiments on cell elongation were conducted in complete Middlebrook and Cohn 7H9 Tween medium, which contained 4.7 g 7H9 powder (Difco) and 0.5 ml Tween 80 in 900 ml distilled water. After sterilization, 5 × 20 ml ampoules of Middlebrook ADC enrichment (Difco) were added aseptically. The 7H9 powder (Difco) used alone without ADC enrichment or Tween 80 gave the minimal medium. The minimal medium enriched with 0.05 % (v/v) Tween 80 and 0.5 % (w/v) albumin (final concentrations) was used as the poor medium. The minimal medium enriched with 0.05 % (v/v) Tween 80, 0.5 % (w/v) albumin, 0.01 % (w/v) oleic acid, 0.5 % (w/v) glucose and 0.5 % (w/v) glycerol (final concentrations) was used as the rich medium. Preliminary studies had shown that these substrates and concentrations were optimal for the growth of *M. avium* in the conditions required for our shift experiments.

**Bacterial growth.** For elongation studies, an *M. avium* culture grown to about 10⁸ viable counts ml⁻¹ was filtered through a Millipore filter (3 µm pore size) under sterile conditions, and the bacteria in the filtrate were concentrated by centrifugation at 4 °C and resuspended in the same volume of fresh 7H9 Tween medium. Growth was followed during incubation on a shaker at 37 °C: samples of bacteria were taken at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h, stained by the Ziehl–Neelsen process and observed by optical microscopy.

For the shift-down studies, bacteria were first grown to about 10⁸ viable counts ml⁻¹ in 5 ml portions of the rich medium which were then used to inoculate 100 ml portions of rich medium (in 250 ml Erlenmeyer flasks with side-arms), so as to eliminate any possible lag phase during shift experiments. The cultures were grown with agitation at 37 °C and at various times up to 96 h, viable counts were measured by plating 0.1 ml of the appropriate culture dilutions on Middlebrook and Cohn 7H10 agar (Difco) containing OADC enrichment, and counting after 21 d (when the counts were stabilized). Each sampling for viable count determinations was followed by turbidity measurements at 650 nm (taken as an indication of cell mass), as well as RNA, DNA and protein estimations. For these assays, 50 ml of culture was taken out in two portions of 25 ml and immediately frozen at −20 °C until the assays were done.

At 96 h, when the bacteria were still in the exponential phase of growth, they were rapidly centrifuged at 4 °C, washed with poor medium and resuspended in the poor medium at twofold dilution compared with the rich medium. Postshift growth in the poor medium was followed in the same way as for the rich medium.

For shift-up experiments, the method was basically the same, except that the bacteria could be grown only to an absorbance of 0.055 in the poor medium, no washing was necessary during the shift-up and the whole process of shifting could be done in less than 30 min.

**Chemical assays.** Frozen cultures kept at −20 °C were thawed, centrifuged and washed thoroughly with distilled water. The bacterial mass obtained was digested with 1 ml 1 M-perchloric acid at 75 °C for 15 min for DNA extraction and with 1 ml 1 M-NaOH at 90 °C for 10 min for RNA and protein extraction. Protein was assayed by the Lowry method, RNA by the orcinol reagent (Dische, 1953) and DNA by diphenylamine as previously reported for mycobacteria (Brieger et al., 1959). Bovine serum albumin (Sigma), yeast RNA and herring sperm DNA (Calbiochem), subjected to the appropriate extraction conditions, were used as standards.

**Ultrastructural studies.** Normal and elongated cells were prefixed for 6 h in 2% (w/v) glutaraldehyde (Sigma) in cacodylate buffer (0.2 M, pH 7.2), washed repeatedly with the same buffer and postfixed overnight in 1% (w/v) OsO₄ in the same buffer. Bacteria at this stage were longitudinally oriented in agar as suggested by Whitehouse et al. (1977). Specimens in agar were dehydrated in a graded ethanol series and then in propylene oxide and embedded in Epon 812 (Ladd Research Laboratories, Burlington, Vermont, U.S.A.). Sections (40 nm thick) were cut with a LKB Ultratome III using a diamond knife, mounted on copper grids, stained in uranyl acetate and lead citrate, and observed in a Siemens 101 electron microscope.

**RESULTS**

**Shift-up and shift-down experiments**

The generation time of *M. avium* in the poor medium was 91 h (growth rate constant, \( k = 0.011 \) h⁻¹). When bacteria were transferred to the rich medium, growth continued at the preshift rate for about 10 h and then abruptly increased to a rate of 0.065 h⁻¹ (generation
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Fig. 1. Growth of M. avium upon transition from poor to rich medium (shift-up, a) and from rich to poor medium (shift-down, b).

Fig. 2. Growth of M. avium upon transition from poor to rich medium (shift-up, a) and from rich to poor medium (shift-down, b): ○, cell mass (\(A_{650}\)); ●, protein concentration; △, RNA concentration; □, DNA concentration. Insets represent the early postshift events on an expanded time scale.

time of 15.4 h; Fig. 1a). Conversely, when the bacteria were transferred from the rich to the poor medium, the initial growth rate of about 0.065 h\(^{-1}\) changed in about 12 h to a very slow growth rate, and it was several days before growth resumed at the rate of 0.011 h\(^{-1}\), characteristic of the poor medium (Fig. 1b).

The changes in cell mass and the total DNA, RNA and protein contents of the bacteria during these shift experiments are shown in Fig. 2. When the bacteria were transferred from the poor to the rich medium (Fig. 2a), the rate of RNA synthesis increased after a lag of
about 3 h, and DNA synthesis continued at the preshift rate for about 7 h. The rate of increase in cell mass changed upwards after a lag of about 3 h, whereas the rate of increase in viable cell counts changed to a higher value after about 10 h (Fig. 1a). When the bacteria were transferred from the rich to the poor medium (Fig. 2b), the preshift rates for protein and DNA synthesis changed to postshift rates after 3 and 7 h, respectively. RNA synthesis stopped immediately after the transfer; there was a transient fall in total RNA and synthesis was resumed at a new rate only after 24 h. The change in the rate of increase in cell mass occurred about 4 h after the shift, whereas the increase in viable cell counts remained at the preshift rate for approximately 12 h (Fig. 1b).

The above observations showed that under these conditions the cell mass and total RNA, DNA and protein changed proportionally with the growth rate, and that the change from one such state of growth to another resulted in a subsequent change in these growth parameters. Furthermore, the period of adjustment to a richer medium was shorter than the period of adjustment to a poorer medium.

During the shift-up, the higher rate of increase in the cell mass (Fig. 2a) preceded by 7 h the increase in the rate of cell division (Fig. 1a), and during this time the bacteria increased in size before division. During the shift-down, the cell mass increased slightly for a few hours (Fig. 2b), before the cell division reached the new rate (Fig. 1b). The viable units in the rich medium were larger than those in the poor medium as observed by the ratio of cell mass (Fig. 2a, b) to viable cell unit concentration in shift-up and shift-down (Fig. 1a, b).

**Growth and cell division**

McCarty (1971) showed that when *M. avium* cells selected for their small size were placed in a new medium, they elongated to several times their initial length and then
Fig. 4. Fine structure of *M. avium* during the elongation process. (a) Time 0 h: initial dividing short bacillus. (b) Time 48 h: elongated form with complete septa and initial septum formation in the cell in the middle of the series. (c) Time 48 h: elongated form showing an almost complete equatorial septum and an incomplete septum. (d) Time 48 h: protoplasmic bridge formation between two daughter cells. The bar markers represent 100 nm.
fragmented into a number of small viable units. The elongation of *M. avium* is illustrated in Fig. 3. When the initial short bacilli were compared by electron microscopy with the 24 h and 48 h elongated bacilli, some marked differences were noted. At 48 h nearly all cells were elongated to three to seven times their initial length (Fig. 3d), and due to clumping, presented a cord-like aspect. Elongated forms of *M. avium* seen in the electron microscope (Fig. 4) had several division septa in certain cases. Figure 4(b) shows an elongated form of *M. avium* with many septa, clearly showing different daughter cells. This could have indicated an elongation and fragmentation process if it had not been for the presence of various intermediate forms of elongated bacteria with several division septa at various stages of completion. For example, Fig. 4(c) shows an elongated form, where the septum in the middle is complete and another septum is half-formed. In addition, we sometimes observed protoplasmic bridges between dividing daughter cells indicating binary fission (Fig. 4d). We think that the bacteria divide by binary fission, but that the daughter cells may remain attached to each other. This explains why, during the adjustment to new growth conditions, the rate of increase in cell mass is higher than the rate of cell division (see Figs 1 and 2). New septa are formed before the separation of the two daughter cells is completed. With optical microscopy, this gives appearance of an elongation process.

**DISCUSSION**

As long ago as 1952, cell elongation with mycelium production, followed by fragmentation was proposed as a mode of growth of *M. avium* (Brieger & Glauert, 1952) and later supported by the findings of McCarthy (1971). Our observations utilizing optical microscopy agreed well with the previous reports (Fig. 3), but when 24 h and 48 h preparations were observed in the electron microscope, the division septa in the elongated forms were at different stages of formation and only seldom did we observe cells with several complete septa. In some cases after a cell division, the two daughter cells were still attached but one of the two daughter cells had started to divide again by binary fission. Repetition of the same event would lead to forms with several septa, and any physical shock could separate these forms, thus greatly increasing the particle count compared with viable counts measured before such a physical shock.

According to our data, the rate of synthesis of RNA in *M. avium* increased rapidly upon enrichment of the medium. It was followed by an increase in protein synthesis within about 3 h and by an increase in DNA synthesis within about 7 h. These increases in the cellular concentration of the macromolecules caused an increase in cell mass, but division of the bacteria at the postshift rate was delayed. Consequently, during the initial adjustment period of unbalanced growth, the bacteria were larger than they were later during their exponential growth. Therefore, it appeared that during unbalanced growth, the control of the process of division of the cell wall took longer to adjust to a new environment. Thus, the long, filamentous, nocardia-like forms of *M. avium* observed under certain experimental conditions may be caused by the conditions that interfere with the division of the cell wall (septum formation). Shift-up experiments with *Bacillus megaterium* KM (Sud & Schaechter, 1964) showed that preshift rates of wall and DNA synthesis were maintained for similar periods, which resulted in cells with a higher growth rate but with less wall material per unit mass. Thus, contrary to earlier interpretations (McCarthy, 1971), we think that the elongation process may not be representative of a life-cycle with its implication that a differentiation mechanism is operative in *M. avium*. The following considerations are presented to support the above conclusions.

In *M. avium* the sequence of events, but not the timing, upon transition to and from rich medium was identical to observations reported for other bacteria, e.g. *Salmonella typhimurium* (Kjeldgaard et al., 1958), *Escherichia coli* (Cooper, 1969) and *Bacillus megaterium* (Sud & Schaechter, 1964). The possible mechanism for the maintenance of a
preshift rate of DNA synthesis following a shift-up has been explained previously (Helmstetter & Cooper, 1968; Cooper & Helmstetter, 1968). The increased postshift rate of cell division after a shift-up would be due to an increased rate of initiation of chromosome replication (Maaløe & Kjeldgaard, 1966).

The earlier observations in S. typhimurium showed an elongation before division into two daughter cells (Schaechet et al., 1958), while in M. avium the elongation appeared to proceed without division. However, electron microscopic study of these elongated forms showed that the size of the individual daughter cells, estimated by measuring the distances between the division septa (irrespective of their completion), was about constant. Therefore, cell division after a shift-up would be due to an increased rate of initiation of chromosome replication (Maaløe & Kjeldgaard, 1966). BALANCED GROWTH was established as demonstrated by the proportional increase in viable cell counts, cell mass and RNA, DNA and protein concentrations. Our results are thus similar to observations made by Winder & Rooney (1970) with other mycobacterial species.

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