Morphology of *Mycoplasma laidlawii* Type A

II. Effect of Glucose on Growth and Cellular Morphology

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ABSTRACT

ANDERSON, D. L. (University of Minnesota, Minneapolis), M. E. POLLOCK, AND L. F. BROWER. Morphology of *Mycoplasma laidlawii* type A. II. Effect of glucose on growth and cellular morphology. *J. Bacteriol.* **90**:1768-1777. 1965.—Cells of *Mycoplasma laidlawii* A grown in soy peptone-yeast extract (SP-YE) broth reached higher maximal titers than cells grown in SP-YE supplemented with 0.2% glucose, and remained viable longer than glucose-grown cells. Moreover, addition of glucose to SP-YE markedly affected the morphological developmental sequence of the organism. The diameter of glucose-grown cells at 28 to 40 hr was often two to four times that of cells grown without glucose; these large glucose-grown cells deteriorated progressively, and presumably liberated 0.1- to 0.25- μ small bodies. In contrast, pleomorphic 22- to 28-hr cells grown in SP-YE without glucose gave rise to uniform bodies 0.5 to 1.0 μ in diameter; limited numbers of 0.1- to 0.25- μ small bodies were also liberated in nonglucose cultures after 45 to 64 hr. Electron-microscopic counts of 16- to 64-hr cultures agreed well with counts of colony-forming units (CFU) when organisms larger than 0.25 μ were included in the microscopic counts. Small bodies, 0.1 to 0.25 μ in diameter, apparently did not contribute substantially to numbers of CFU. Droplet patterns of cells grown in Arginine Assay Medium and L15 Tissue Culture Medium were cleaner than patterns of cells grown in SP-YE.

Previous light and electron photomicrographs of *Mycoplasma laidlawii* showed spherical bodies which vary in size with culture age (Ruska and Poppe, 1947; Kandler and Kandler, 1954; Kandler, Kandler, and Huber, 1954; Morowitz and Tourtellotte, 1962). Cultures (24-hr) have been reported to contain primarily large cells, whereas elementary bodies about 0.1 μ in diameter are the predominant forms in 6-day cultures; elementary bodies presumably develop into large cells which eventually form inclusions to yield elementary bodies, thus starting a new life cycle (Morowitz and Tourtellotte, 1962).

Anderson, Pollock, and Brower (1965) used an electron-microscopic count technique to elucidate the relationship between specific morphological entities and colony-forming units (CFU) of *M. laidlawii* A in cultures of various ages. Good agreement of electron-microscopic counts with numbers of CFU was obtained when cells larger than 0.25 μ were included in microscopic counts; bodies 0.1 to 0.25 μ apparently did not contribute substantially to colony counts. Colony-forming ability may not be an accurate index of viability,

however, and purified preparations of large and small bodies have not been counted.

An observation that growth curves of *M. laidlawii* A in soy peptone-yeast extract (SP-YE) broth and in SP-YE supplemented with 0.2% glucose differed markedly prompted the present investigation on the effect of glucose on the cellular morphology of *M. laidlawii* in cultures of various ages. A dialyzing flask culture technique (Pollock, 1965) permitted electron-microscopic study of *M. laidlawii* cells without prior concentration by centrifugation. Electron-microscopic counts have been compared with counts of CFU over a range of culture ages in SP-YE and SP-YE with glucose.

MATERIALS AND METHODS

Culture. The organism used was *M. laidlawii* type A (ATCC 14089).

Growth of cells and enumeration of CFU. SP-YE broth and agar media were used for cultivation of *Mycoplasma* and enumeration of CFU (Pollock, 1965).

Other media tested for their effects on total growth, morphology, and microscopic counts of *M. laidlawii* in dialyzing flask cultures were Arginine Assay Medium and L15 Tissue Culture Medium (Difco). These media were placed in the

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FIG. 1. Representative growth curve of *Mycoplasma laidlawii* in SP-YE broth supplemented with 0.2% glucose.

dialysis bag and SP-YE was used in the reservoir of the dialyzing flask in all cases.

Viable and electron microscopic counts. Methods for comparing counts of CFU with electron-microscopic counts were described in detail by Anderson et al. (1965).

Dry weight determinations. Cells from 15-ml samples of cultures were sedimented at $30,000 \times g$ for 1 hr, dried at 110°C for 24 hr, placed in an evacuated desiccator for 24 hr, and weighed; dried cells were kept in the desiccator and weighed periodically until weights were constant.

RESULTS

Effect of glucose on growth. By use of the dialyzing flask technique, cells from an 18-hr culture of *M. laidlawii* were grown in SP-YE broth and in SP-YE broth supplemented with 0.2% glucose to determine the effects of glucose on growth. Growth curves of cells grown with and without glucose were obtained by use of a spot plate technique for enumerating CFU (Pollock, 1965); representative curves are presented in Fig. 1 and 2. The growth curves show that nonglucose-grown cultures reach a higher maximal titer than glucose cultures, and that cells from nonglucose cultures remain viable longer than glucose-grown cells.

Effect of glucose on cellular morphology and microscopic counts. The effect of glucose on morphology and microscopic counts was determined by inoculating SP-YE broth and SP-YE broth containing 0.2% glucose with approximately 3×10^6 cells per milliliter from an 18-hr culture of *M. laidlawii*. These large inocula were used to observe cellular morphology prior to 20 hr without concentrating cells by physical means; under these conditions, the organisms were in early stationary phase at 16 hr. Cell samples were

FIG. 2. Representative growth curve of *Mycoplasma laidlawii* in SP-YE broth without glucose.

removed from culture flasks at intervals of 16, 19, 22, 25, 28, 42, and 64 hr, fixed with 3.3% glutaraldehyde, and dialyzed successively against Veronal-acetate buffer with 3.3% glutaraldehyde, Veronal-acetate buffer, and 0.2 M ammonium acetate, by methods described previously (Anderson et al., 1965). Fixed cells were mixed with $0.264\text{-}\mu$ latex reference particles prior to dialysis to eliminate effects of volume change on counts. The dialyzed mixture was sprayed on specimen grids for electron microscopy. At 16 and 19 hr, cells of the two cultures were quite similar in gross morphology (Fig. 3 to 10); ring forms were observed in both cultures at 19 hr (Fig. 6 and 8). At 22 and 25 hr, differences in morphology of cells from glucose and nonglucose cultures were apparent (Fig. 11 to 18); ring forms were present in both cultures at 22 hr, but the average cell size was much greater in the glucose cultures. Some cells were filamentous (Fig. 11), and in many instances cytoplasm was concentrated at the edges of cells to give characteristic ring forms (Fig. 13). Several attached, electron-dense units often constituted a ring structure (Fig. 16). The flattened areas in the center of these rings appeared to consist solely of the limiting membrane of the cells. Most of the 25-hr glucose-grown cells were spherical and very large at 1 to $3\text{ }\mu$, whereas nonglucose cells were small at 0.5 to $1\text{ }\mu$ and highly pleomorphic with extensions, branches, and buds (Fig. 17 and 18). These trends continued in 28-hr cultures (Fig. 19 to 22); glucose-grown cells were two to three times the diameter of the nonglucose cells and had pockets or areas of low electron density. Large numbers of individual cells 0.5 to $1\text{ }\mu$ in diameter were visible in nonglucose cultures. At 42 hr, many glucose-grown cells were still two to four times the size of nonglucose cells and were highly pleomorphic

FIG. 3 to 18. Electron micrographs of 16- to 25-hr cells of *Mycoplasma laidlawii* grown in SP-YE broth or glucose supplemented SP-YE and fixed with glutaraldehyde. (3) 16-hr cells, SP-YE with glucose; (4 and 5) 16-hr cells, SP-YE; (6 and 7) 19-hr cells, SP-YE with glucose; (8-10) 19-hr cells, SP-YE; (11-13) 22-hr cells, SP-YE with glucose; (14-16) 22-hr cells, SP-YE; (17) 25-hr cells, SP-YE with glucose; (18) 25-hr cells, SP-YE. Scale markers denote 1 μ .

FIG. 19 to 30. Electron micrographs of 28-hr to 14-day cells of *Mycoplasma laidlawii* grown in SP-YE broth or glucose-supplemented SP-YE and fixed with glutaraldehyde. (19 and 20) 28-hr cells, SP-YE with glucose; (21 and 22) 28-hr cells, SP-YE; (23 and 24) 42-hr cells, SP-YE with glucose; (25) 42-hr cells, SP-YE; (26 and 27) 64-hr cells, SP-YE with glucose; (28) 64-hr cells, SP-YE; (29) 14-day cells, SP-YE with glucose; (30) 13-day cells, SP-YE. Scale markers denote 1 μ .

(Fig. 23 to 25). Some large cells appeared to fragment to yield clusters of 3 to 20 dense units (Fig. 23), and flattened structures with beaded borders, composed of units 0.1 to 0.25 μ in diameter, were common (Fig. 24). Most of the nonglucose-grown cells were present as individual, uniform units of rather homogeneous density at this time (Fig. 25). At 64 hr, numerous 0.1 to 0.25- μ small bodies were especially evident in the glucose-grown preparation (Fig. 26), but occurred also in the nonglucose culture. An increased number of rings and pleomorphic forms were evident at this stage in the glucose-grown culture, whereas the nonglucose-grown cells remained uniform and homogeneous in density (Fig. 26 to 28). At 13 days (306 hr), the very large cells found in earlier glucose-grown preparations were rare; considerable cellular debris and large numbers of small bodies were evident (Fig. 29). Small bodies and cellular debris were also present in nonglucose-grown preparations at 14 days, but the cells were slightly smaller and the 10 to 15 $m\mu$ particles so evident in glucose-grown cultures were far less numerous (Fig. 30). Finally, 13- to 14-day cells from both glucose- and nonglucose-grown cultures were not flattened on the film, as were cells from younger cultures; both large and small cells produced long shadows, indicating preservation of three-dimensional morphology.

Electron-microscopic counts were compared with numbers of CFU in 16-, 19-, 22-, 25-, 28-, 42-, and 64-hr cultures and in 13- to 14-day cultures to determine the relationship between morphological entities and viable units (Fig. 31 and 32). *Mycoplasma* and reference polystyrene latex of known concentration were enumerated in most cases in five droplet patterns using counting techniques described by Anderson et al. (1965). Microscopic and colony counts of cells in both media correlated quite well at all culture ages with the exception of the 13- to 14-day cultures. The colony and microscopic counts of nonglucose-grown cells at 64 hr and 13 days were greater than for glucose-grown cells at comparable ages; no colony-forming ability was demonstrated in the glucose-grown cultures at 14 days. Large numbers of 0.1- to 0.25- μ small bodies were present at 64 hr and 13 to 14 days, especially in glucose-grown cultures, but these units were not included in the microscopic counts.

Dry weight determinations provided an additional means for comparing glucose- and nonglucose-grown cells. A comparison of cultures at 45 hr demonstrated that the dry weight per CFU of glucose-grown cells was about five to seven times that of nonglucose-grown cells.

Morphology of glucose- and nonglucose-grown

FIG. 31. Comparison of electron microscopic counts with counts of CFU of *Mycoplasma laidlawii* grown for various periods in SP-YE broth supplemented with 0.2% glucose. No CFU were detected at 336 hr.

FIG. 32. Comparison of electron-microscopic counts with counts of CFU units of *Mycoplasma laidlawii* grown for various periods in SP-YE broth without glucose. CFU at 306 hr titered 4.2×10^7 per milliliter.

cells in time-sequence study was essentially the same, regardless of whether the inoculum was grown in SP-YE or glucose-supplemented SP-YE. Also, morphological differences between glucose- and nonglucose-grown cells were just as marked when formaldehyde, rather than glutaraldehyde, was used as the fixative (Fig. 33 to 36).

Use of chemically defined media. Tissue Culture Medium L15 and Arginine Assay Medium were used within the bag in the dialyzing flask culture technique to compare total growth, cellular morphology, and electron-microscopic counts with results obtained using SP-YE; SP-YE was used in the reservoir in all cases. Electron-microscopic preparations of cells grown in Tissue Culture Medium L15 and Arginine Assay Medium were considerably cleaner than SP-YE preparations; this was most apparent at the edges of droplet patterns (Fig. 37 to 39). These were 4-day cultures, however, which accounts for the observed debris. Note particularly the cell envelopes or "ghosts" present in the Arginine Assay preparation. This was the only medium used in which large numbers of presumably intact membranes could be observed in old cultures.

FIG. 33 to 36. Electron micrographs of cells of *Mycoplasma laidlawii* grown in SP-YE broth or glucose supplemented SP-YE and fixed with formaldehyde. (33) 24-hr cells, SP-YE; (34) 24-hr cells, SP-YE with glucose; (35) 28.5-hr cells, SP-YE; (36) 28.5-hr cells, SP-YE with glucose. Scale markers denote 1 μ .

FIG. 37 to 39. Electron micrographs of edges of droplet patterns containing 0.264- μ latex particles and 4-day cells of *Mycoplasma laidlawii* grown in various media in dialysis bags with SP-YE broth in the reservoirs. (37) SP-YE broth. Note debris especially evident at the edge of the droplet pattern. (38) L15 Tissue Culture Medium. (39) Arginine Assay Medium. Note empty cell membranes and overall cleanness of the preparation. Scales denote 1 μ .

FIG. 40. Electron micrograph of a droplet pattern containing 0.264- μ latex and 140-hr cells of *Mycoplasma laidlawii* grown in L15 Tissue Culture Medium in a dialyzing flask culture with SP-YE broth in the reservoir. Note that three-dimensional morphology of many of the *Mycoplasma* is preserved as evidenced by long shadows. Scale denotes 1 μ .

Although 2- to 4- μ cells were numerous in glucose-supplemented SP-YE cultures at 25, 28, and 42 hr, cell membranes were not found in older preparations of these cultures.

Three-dimensional morphology of 140-hr cells of *M. laidlawii* grown in L15 Tissue Culture Medium, and of old cells in general, was more effectively preserved with aldehyde fixation than

young cells as evidenced by long shadows in electron micrographs (Fig. 40).

DISCUSSION

Growth curves of *M. laidlawii* in SP-YE and in SP-YE supplemented with 0.2% glucose indicated that nonglucose-grown cells reached titers two- to fivefold higher than cells grown in broth containing glucose. Furthermore, cells grown in SP-YE without glucose retained their ability to form colonies much longer than glucose-grown cells, as indicated by the prolonged stationary phases in nonglucose cultures. The accelerated death rate of glucose-grown cells can perhaps be explained largely as a pH effect. The pH of glucose-grown cultures often dropped to values of 6.1 in 40 hr, as compared with a pH of 7.6 to 7.8 for nonglucose-grown cultures. Glucose was not detected in SP-YE medium in preliminary analyses.

The morphology of glucose-grown cells differed markedly from that of nonglucose-grown organisms after 24 hr of growth, regardless of whether or not the culture used as inoculum was grown in SP-YE with glucose. Cells in glucose-supplemented SP-YE gradually increased in size with time, and presumably released large numbers of 0.1- to 0.25- μ small bodies after 28 to 40 hr of growth. On the contrary, cells grown in SP-YE without glucose were pleomorphic at about 24 hr, occurring as rings, discs, asters, and branched filaments; these forms closely resembled the organisms of contagious bovine pleuropneumonia (Turner, 1935) and the organism of caprine pleuropneumonia (Klieneberger-Nobel and Cuckow, 1955). The pleomorphic forms in nonglucose cultures gradually gave rise to uniform spheres about 0.5 to 0.75 μ in diameter as observed in shadowed preparations; these organisms flattened on the specimen films and hence were undoubtedly smaller than 0.5 to 0.75 μ . Both glucose and nonglucose cultures produced small bodies about 0.1 to 0.25 μ in diameter, but these forms were generally far more numerous in glucose cultures. These results are interpreted to indicate that the nutritional environment, in this case the presence or absence of glucose, affects the developmental cycle of *M. laidlawii* and dictates the size, overall morphology, and relative numbers of morphological types. Furthermore, cellular morphology has been shown to depend upon culture age. Small bodies, 0.1 to 0.25 μ in diameter, which may be equivalent to "elementary bodies," are rare in cultures grown in SP-YE without glucose and do not appear in glucose-grown cultures until about 28 to 40 hr. The fact that these bodies are numerous in glucose-grown cultures in which

the pH is dropping rapidly supports the supposition that they might be formed in response to a toxic or nutritionally deficient environment. It is doubtful that 0.1- to 0.25- μ small bodies are formed to any great extent during logarithmic growth; their formation is rather a part of the stationary and death growth phases. Inasmuch as these bodies have been considered "minimal reproductive units," it is worth noting that they apparently do not contribute substantially to the number of CFU in comparing colony counts with electron-microscopic entities (Anderson et al., 1965). In the present study, inclusion of small bodies increased electron-microscopic counts as many as 20 times, and accordingly indicated, in many instances, at least a 10-fold excess of morphological units over colony-forming cells. Colony-forming ability, however, may not be an accurate index of viability.

Dry weight determinations substantiated electron-microscopic observations on size differences between glucose- and nonglucose-grown cells. Calculations of dry weight per CFU in 45-hr cultures indicated that glucose-grown cells were up to seven times heavier than cells grown in SP-YE without glucose.

Comparison of electron-microscopic counts with numbers of CFU indicated good agreement for both glucose- and nonglucose-grown cultures up to 64 hr. In older glucose-grown cultures, microscopic units greatly exceeded CFU.

By use of the dialyzing flask technique, *M. laidlawii* was grown in Arginine Assay Medium and L15 Tissue Culture Medium to titers comparable with those obtained in SP-YE. These media had a particular advantage over SP-YE for electron-microscopic counts and studies of cellular morphology in that droplet patterns of cells grown in these media were much cleaner (Fig. 37 to 39). The fact that appreciable numbers of intact membranes or "ghosts" in old cultures were observed only in Arginine Assay Medium suggests that some type of lytic system existed in SP-YE and L15 cultures which dissolved cytoplasmic membranes.

Only cultures containing large numbers of cells can be directly counted by use of the electron-microscopic spray technique unless cells are concentrated by some means; growth of *M. laidlawii* to concentrations of 2×10^{10} to 5×10^{10} per milliliter by use of the dialyzing flask culture method permitted electron-microscopic counts without prior concentration by physical means. The youngest cultures observed in the present study were in late logarithmic-early stationary growth phases. Studies of logarithmic-phase cultures are of equal importance in understanding

the life cycle and means of reproduction of *M. laidlawii*, but the present techniques are applicable to these studies only if cells are concentrated by some means or if very large inocula and short interval sampling procedures are used with the dialyzing flask technique to provide larger numbers of organisms in logarithmic growth.

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