ELECTRON MICROSCOPY OF CYTOPLASMIC STRUCTURES IN FACULTATIVE AND ANAEROBIC *ACTINOMYCES*

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ABSTRACT

OVERMAN, JOHN R. (Duke University Medical Center, Durham, N.C.) AND LEO PINE. Electron microscopy of cytoplasmic structures in facultative and anaerobic *Actinomyces*. J. Bacteriol. **86**:656–665. 1963.—Electron microscopy of cytoplasmic complexes and the cytoplasmic fine structure of *Actinomyces bovis, A. israelii, A. naeslundii*, and *A. propionicus* demonstrated marked differences among these four species. Also included in the present study was *Lactobacillus bifidus*, an organism closely related to the *Actinomyces* species. A relatively small and compact cytoplasmic membrane complex of *A. propionicus* was unique in its morphology. Membrane structures of *A. naeslundii* and *A. israelii* were relatively large and consisted of coils of various sizes of the cytoplasmic membrane. No membrane complexes were found in *L. bifidus* or *A. bovis.* Measurements of cell-wall thickness indicated a significant difference between *A. bovis* and *A. israelii.* On the basis of general morphology, cell-wall thickness, and cytoplasmic membrane complexes, *A. bovis* and *A. israelii* appear to be distinct species. The relation of the fine structure complexity to phylogenetic position of these organisms is considered.

Membranous structures with varying degrees of complexity have been observed in bacteria and fungi of unrelated types (Chapman and Hillier, 1953; Glauert, 1962*a*, *b*; Edwards and Gordon, 1962; Fukushi et al., 1962). These complexes appear to be composed of coils of double-walled membranes, some of which connect with or are a part of the cytoplasmic membrane, whereas in other instances the relationship between the "membrane complex" and the cytoplasmic membrane is not clear (Van Iterson, 1961; Imaeda and Ogura, 1963). The function of these structures is unknown. Some authors have referred to these complexes as "organelles" in recognition of their possible functional significance and mode of origin.

Studies of sections of microorganisms with an electron microscope have illustrated the structural complexity which may exist in many species. The usual determinant in placing microorganisms on a phylogenetic scale is their degree of complexity based on all available criteria, so that fine structure of organisms as studied by electron microscopy should provide some additional measure of complexity. On this basis, *Streptomyces* and *Mycobacterium* species show rudimentary cell-membrane systems as compared with cells of yeasts and algae which are higher in the phylum *Protophyta*. Theoretically, cells lower in the phylogenetic scale than the streptomycetes would show either equally rudimentary cytoplasmic membranes or none at all. Koike and Takeya (1961) found cytoplasmic organelles in cells of *Mycobacterium*, and stated that "the cytoplasmic membrane of bacteria appears to correspond to the cell membrane of cells of higher organisms. Therefore, the extension and concentration of the cytoplasmic membrane into the 'lamellar structure' may suggest the phylogenetic origin of the membrane systems. . . ."

Thus, previous studies of cytoplasmic figures (as they will be named here) raise several important questions. Does the presence of such cytoplasmic figures relate to the phylogenetic position of the organism? Are more complex cytoplasmic structures characteristic of more complex microorganisms? Are cytoplasmic figures of closely related species identical, and, if different, may they be considered a species-specific characteristic?

To attempt to answer such questions, the order Actinomycetales was selected for fine-structure study because of our general interest in this group and because of recent physiological, biochemical, and electron microscopic studies reported on certain of its members (Cummins, 1962; Pine,

Howell, and Watson, 1960; Glauert and Hopwood, 1959). The actinomycetes have long been considered a transitional group in the phylogenetic scale and are thought to be intermediate between true bacteria and higher fungi (Lechevalier, Solotorovsky, and McDurmont, 1961). Recently, a suggested sequence of increased phylogenetic complexity based on biochemical, metabolic, and growth characteristics of members of this order was as follows: *Lactobacillus bifidus*, *Actinomyces israelii*, *A. naeslundii*, *A. propionicus*, and *Nocardia* and *Streptomyces* species (Buchanan and Pine, 1962).

Of these species, cytoplasmic figures have been found in *S. coelicolor* (Glauert and Hopwood, 1959) and *A. israelii* (Edwards and Gordon, 1962), but none of the other members of the proposed progression has been studied with regard to fine structure. The present paper describes the cytoplasmic figures or absence of them in four *Actinomyces* species: *A. israelii*, *A. bovis*, *A. naeslundii*, and *A. propionicus*. Also included in this study was the closely related bacterium *L. bifidus*.

Materials and Methods

A. propionicus strain 699 was isolated from a human lacrimal-duct infection; *A. bovis* strain P2S was isolated from a cow; *A. israelii* strain 895 was isolated from a case of human thoracic infection; and *A. naeslundii* strain 279 was obtained from Arden Howell, National Institute of Dental Research, who isolated this strain from a human jaw infection. The details of the biochemical and other characteristics of these organisms have been described previously (Buchanan and Pine, 1962; Pine et al., 1960). *L. bifidus* var. *pennsylvanicus* was obtained from Paul Gyorgy, University of Pennsylvania. All species were grown in Casitone media (Pine and Watson, 1959) for 24 to 72 hr, and samples were removed during the log phase of growth. In the case of the anaerobes in this group, *L. bifidus*, *A. bovis*, and *A. israelii*, cultures were made also on agar plates and incubated under anaerobic conditions. After about 24 hr, the growth was scraped off, fixed, and embedded.

Fixation, staining, and embedding. Organisms from solid or liquid media were centrifuged into a loose pellet and fixed with osmium tetroxide (buffered with acetate and Veronal) at 5 C for 17 hr, following the method described by Moore and Chapman (1959). Samples of the same cultures were separated and fixed by the method of Kellenberger, Ryter, and Séchaud (1958). All of the fixed and dehydrated samples were then embedded in Araldite (Cargille & Sons, New York, N. Y.) and sectioned. Sections were stained with saturated uranyl acetate for 1 hr at room temperature and with 1% potassium dichromate for 2 min (Watson, 1958). Micrographs were made with an RCA-EML or RCA EMU-3F electron microscope.

Results

The results are summarized in Table 1. Considering the facultative and closely related *A. naeslundii* and *A. propionicus*, cytoplasmic figures were present in both, but these contrasted sharply in morphology from each other (Fig. 1 and 2). The cytoplasmic figure shown in Fig. 1a typifies the complete structure found in *A. propionicus*, and all cytoplasmic figures were either this fully developed or appeared to be sections through a portion of it (Fig. 1b and c). The cytoplasmic figure was never found to be in contact or in continuity with the cytoplasmic membrane, but the possibility that such contact may exist cannot be excluded. Although the cultures represented log-phase growth, it was obvious from the individual organisms examined that the sections contained cells showing varying degrees of loss of cytoplasmic components. Although the absence of cytoplasmic components may represent a definite stage of the life cycle (Fitz-James, 1962) of the organism, it is more likely in this case that it represents dead or dying cells. It is of interest that the cytoplasmic

TABLE 1. *Cytoplasmic complexes and cell-wall measurements of Actinomyces species*

Organism*	Oxygen utilization	Cell-wall thickness	Membrane coils or complexes
		A	
Lactobacillus bifidus	Anaerobic	200	None found
(*A. bovis*)	Anaerobic	100	None found
A. israelii	Anaerobic	290	Simple coils
A. naeslundii	Facultative	200	Simple coils
A. propionicus	Facultative	114	Complexcoils

* See Buchanan and Pine (1962), Table 7. The phylogenetic progression is as outlined in this table except that *A. bovis* has been inserted in a possible phylogenetic position.

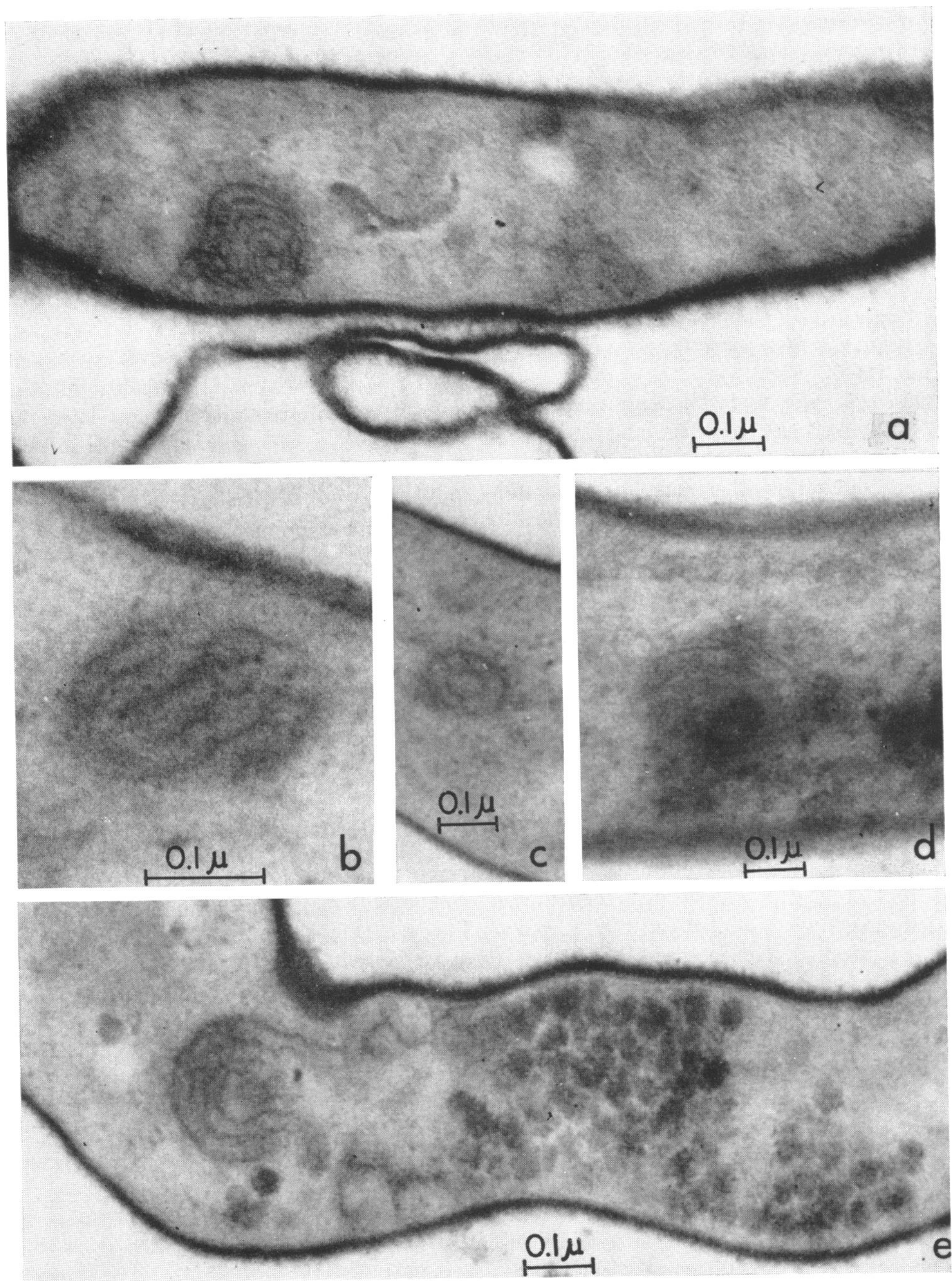

FIG. 1. *Membrane complexes in Actinomyces propionicus; fixation, Chapman; stain, uranyl acetate.* (*a*) *Large complete complex,* × *96,000;* (*b*) *incomplete complex,* × *160,000;* (*c*) *incomplete complex,* × *80,000;* (*d*) *complex partially obscured by granular elements,* × *84,000;* (*e*) *complex forming or dissolving,* × *92,000.*

figure described in *A. propionicus* was most often found in such cells, and it may be that the structure was obscured by the cytoplasmic components. This appears to be the case in the cell shown in Fig. 1d. Figure 1e suggests that some elements of the cytoplasmic figure approach the cytoplasmic membrane but is inconclusive on this point. Figure 1e suggests that the cells of *A. pro-*

pionicus contain a bacteriophage. The dense bodies of Fig. 1e resemble hexagonal heads of the mature virus, as depicted by Kellenberger et al. (1958) in *Escherichia coli*. Similar dark bodies may be observed in the sections of *L. bifidus* (Fig. 3), although in this case the identity of the structure is more questionable.

In contrast to the above, the closely related *A. naeslundii* contained cytoplasmic figures which varied in shape, tended to occupy a relatively

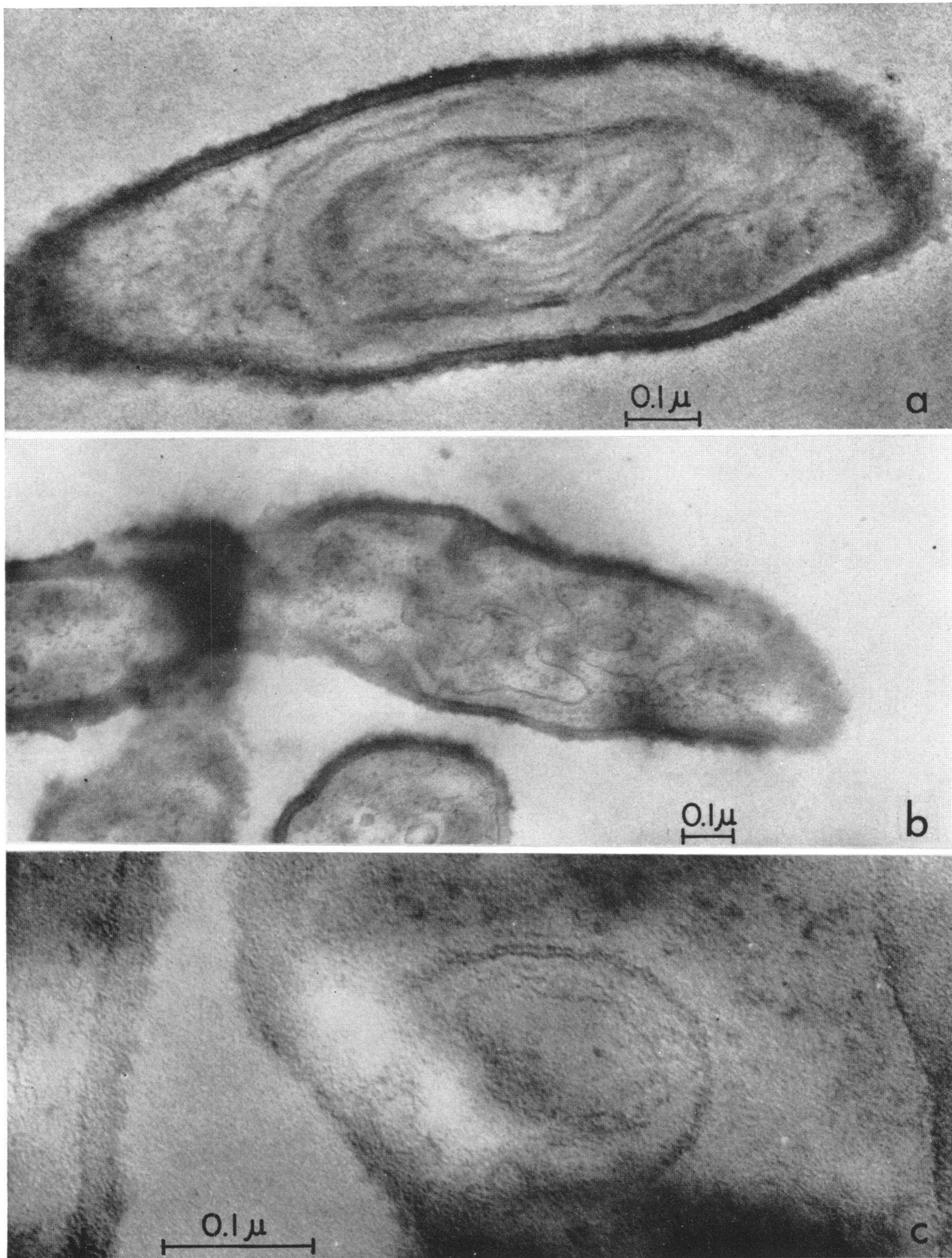

FIG. 2. *Loose unit membrane complexes in Actinomyces naeslundii; fixation, Chapman; stain, uranyl acetate. Width of membranes in a and c = 75 A; (a) × 98,000; (b) × 64,000; (c) × 204,000.*

large portion of the cell, and appeared to make contact with or be a part of the cytoplasmic membrane (Fig. 2). The width of the membrane in the cytoplasmic figure measured about 75 A (Fig. 2a and c) and thus would conform to the unit membrane criterion (Robertson, 1959). No figures found in *A. naeslundii* showed the compact and apparently highly organized structure of those described in *A. propionicus*.

The strict anaerobes provided an interesting contrast to the facultative organisms. Despite very extensive examination, no cytoplasmic fig-

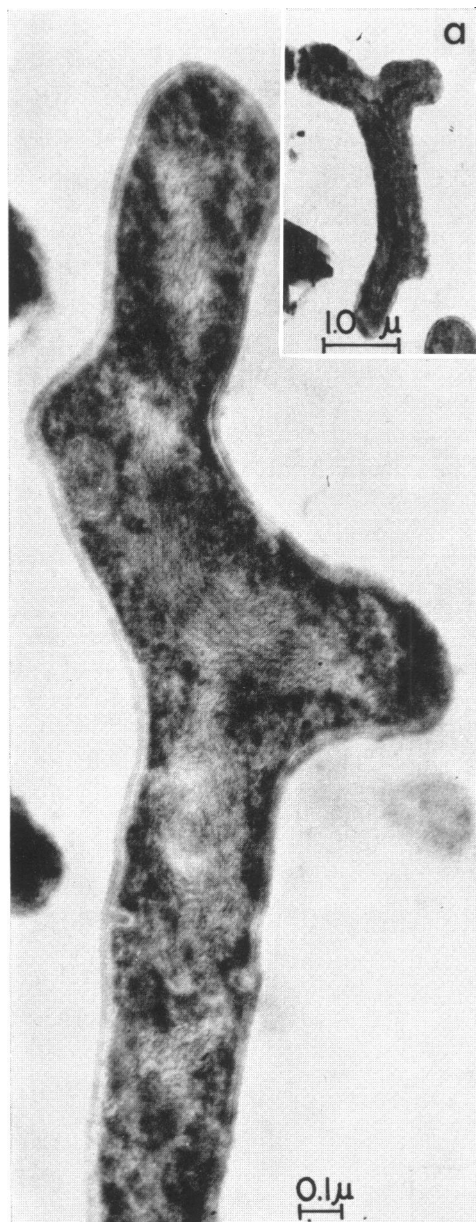

FIG. 3. *Micrograph of Lactobacillus bifidus representative of many micrographs, none of which contained cytoplasmic complexes; fixation, Kellenberger;* × *56,000. Inset (a) shows typical "bifid" appearance of L. bifidus; fixation, Chapman; stain, uranyl acetate;* × *10,000.*

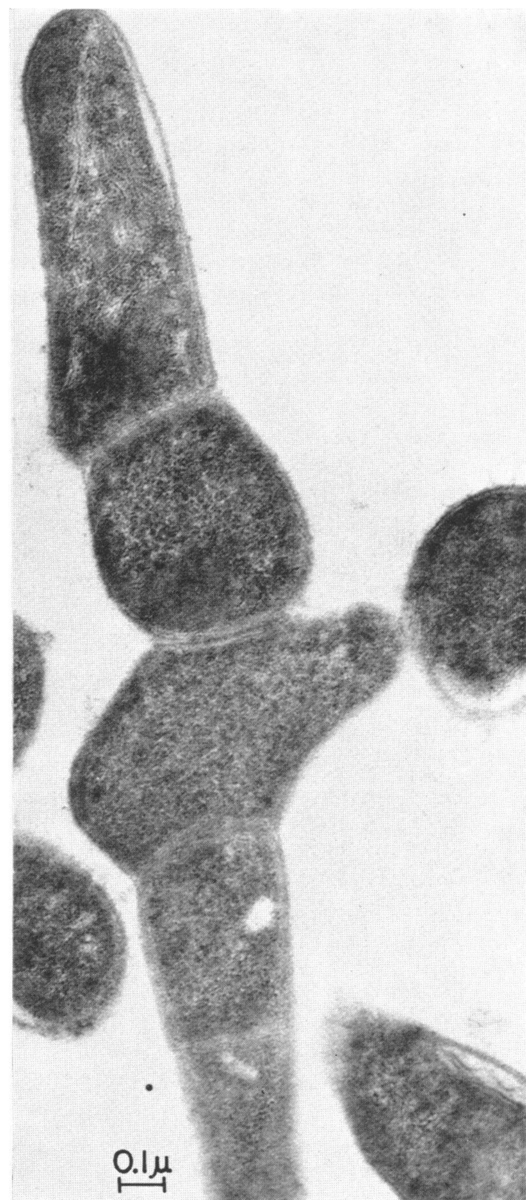

FIG. 4. *Fine granular cytoplasm without complexes in Actinomyces bovis; fixation, Chapman; stain,* $KMnO_4$ *;* × *60,000.*

ures were found in either *L. bifidus* or *A. bovis* (Fig. 3, 4, and 5). In some sections of *A. bovis*, short, straight membranelike structures were observed, but these did not appear to show any organization or coiling of any sort, even the loose type described for *A. naeslundii* (Fig. 5). They most probably represented the fibrous material of the nuclear bodies at a stage of growth when they appear to run parallel (Fitz-James, 1962). The membrane systems described by Edwards and Gordon (1962) were not present in our cultures of *A. bovis* by any of our methods of fixation or staining. Membranes apparently identical to those described by Edwards and Gordon (1962) were present in *A. israelii* (Fig. 6 and 7).

In addition to the cytoplasmic figures described above, it was obvious that differences in cell-wall thickness were present among these five organisms. Table 1 contains averages of cell-wall measurements of these organisms demonstrating their diversity in this regard. The most striking and clearly the most significant difference was that between *A. bovis* and *A. israelii*. The present studies suggest that, in regard to thickness, *A. propionicus* was intermediate between the thick *A. israelii* and *A. naeslundii* and the much thinner *A. bovis*. It is recognized that measurements of the above type are influenced by many factors, and thus are less precise than would be desired. Despite this qualification, the difference in cell-wall thickness between *A. bovis* and *A. israelii* was considered significant.

In the foregoing studies, a direct comparison was possible between the methods of Chapman and Hillier (1953) and Kellenberger et al. (1958). In general, the nuclear material of the cell appeared better preserved by the latter method on the basis that electron-dense material in the nuclear region was found in strands (Fig. 3) and no large masses of this dense material were seen. With the method of Chapman and Hillier (1953), however, an electron-dense material, homogenous and of varying shapes, appeared in the nuclear region of many but not all cells (Fig. 1a, 3a, and 6), and its structure was suggestive of coagulated chromatin. However, in terms of membrane or cytoplasmic-figure preservation, the two methods seemed equally good. In terms of cell-wall measurements, the method of Chapman and Hillier (1953) appeared desirable, since the wall structure was better visualized, particularly in the outermost portion. With the method of Kellenberger et al. (1958), the outer part of the cell wall was associated with debrislike material of varying amounts so that precise measurement of thickness was difficult. The cell in Fig. 3 was a rare exception; Fig. 7c was more typical. In general, the outer portion of the cell wall fixed by Chapman and Hillier's method appeared sharper in outline and more uniform in thickness than samples of the same culture fixed by the alternate technique. Fig. 6 was typical of cell-wall visualization by this method. Generally, however, cell-wall thickness by both methods appeared to be without signifi-

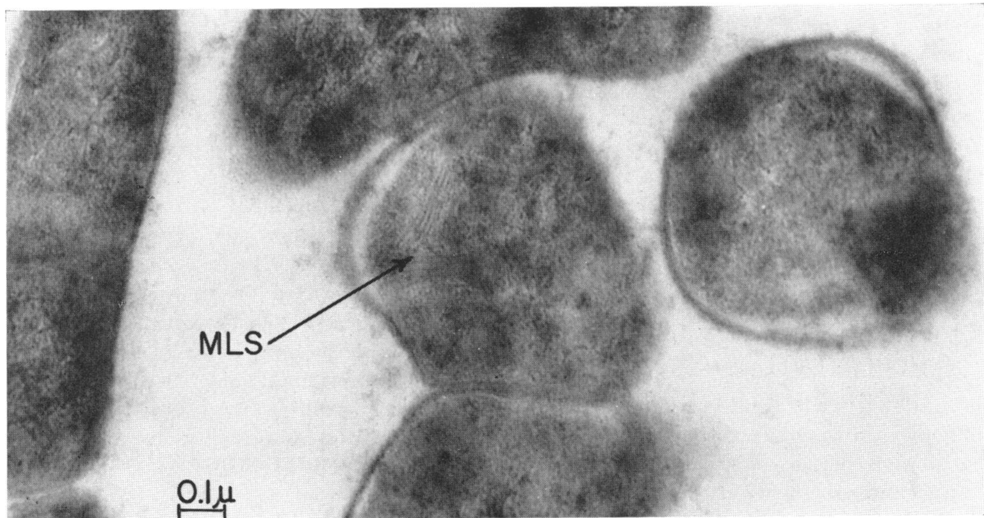

FIG. 5. *Membranelike structures (MLS) rarely seen and never coiled in Actinomyces bovis; fixation, Kellenberger; × 56,000.*

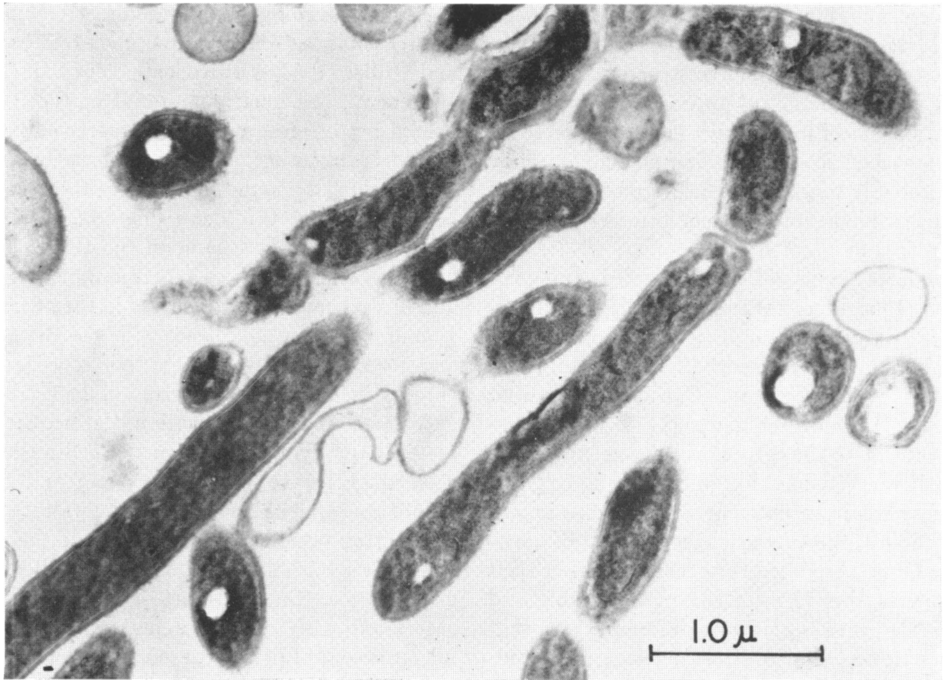

FIG. 6. *Rodlike morphology of Actinomyces israelii in contrast to short buds of A. bovis; fixation, Chapman; stain, uranyl acetate;* × *23,000.*

cant difference. The organisms measured for cell-wall thickness were selected as those representative of most of the cells in any given section, and all contained their full complement of visible cytoplasmic structures. Specifically, no apparently dead or dying cells were included, since cell-wall thickness may vary with age of the individual cell. It should also be noted that the micrographs used to illustrate the cytoplasmic structures were not particularly desirable for cell-wall measurements, and the micrographs which were used for cell-wall measurements were selected for the clarity of cell-wall structure and the flatness of the section in this measured area. Thus, the micrographs accompanying the present paper are not representative of cells used for cell-wall measurements.

Discussion

The results described fall into four categories: (i) differences in the morphology of the cytoplasmic membrane complexes (or cytoplasmic figures, as we prefer to call them) in closely related species, including a previously undescribed type of cytoplasmic figure; (ii) cell-wall thickness measurements to aid in differentiating *A. bovis* and *A. israelii* into separate species; (iii) the comparison of relative general morphology between the species; and (iv) the possible relation of the presence or absence of cytoplasmic figures to the phylogenetic position of the organisms.

There is no question that the morphology of cytoplasmic figures varies in closely related species. In addition, the present work suggests that, at least in *A. propionicus*, the compact highly organized structure is not an extension of the cytoplasmic membrane. On the basis of the regular, helical arrangement of the coils and the uniformity of size and shape, the cytoplasmic structure described for *A. propionicus* appears distinct from those reported for other microorganisms. In contrast, the cytoplasmic figures of *A. naeslundii* and *A. israelii* appear to be coils of the cytoplasmic membrane. Sometimes the coils are restricted to a small localized area (Fig. 7a), and in other cases the complex or figure appears to occupy most of the cell (Fig. 7b).

The relation of *A. bovis* to *A. israelii* has not been clear in the past, and some laboratories have considered *A. israelii* to be either identical to *A.*

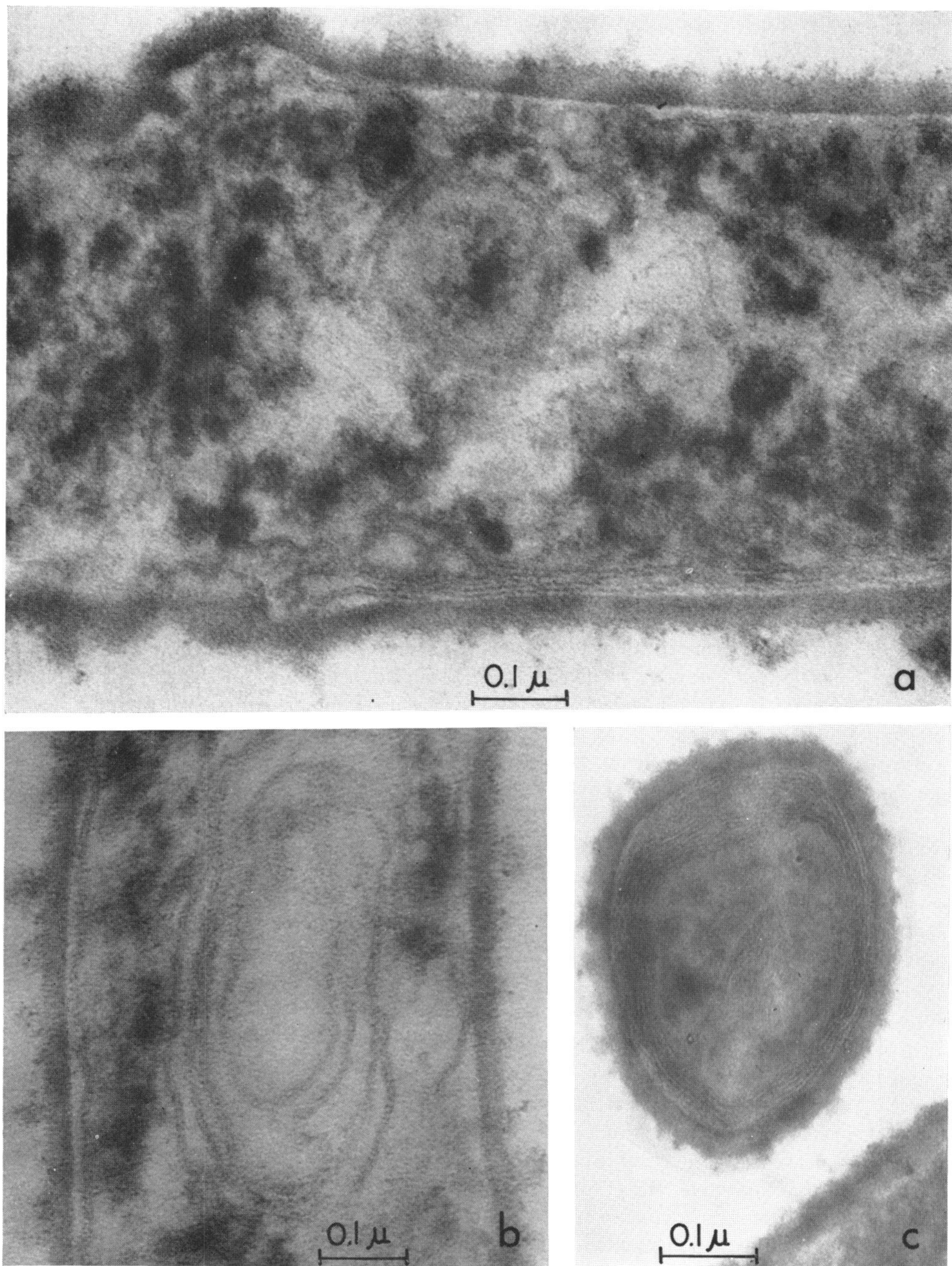

FIG. 7. *Membrane complexes of Actinomyces israelii. Continuity present between coils and the cytoplasmic membrane. Membrane width = 75 A; fixation, Kellenberger; (a) × 130,000; (b) × 120,000; (c) × 130,000.*

bovis or a variant of it. Of the methods for separating these two species (Pine et al., 1960; King and Meyer, 1962; Cummins, 1962), only one has shown itself to be completely reliable and absolute, this being the analysis of cell-wall components as described by Cummins and Harris (1958, 1959). These workers found that *A.* ***bovis*** contained rhamnose and fucose in the cell walls,

whereas *A. israelii* had only galactose as its major sugar component. More recently, MacLennan (1961) showed the presence of relatively large amounts of 6-deoxy-L-talose in the cell walls of *A. bovis*. A comparison of the morphology of these two organisms, as revealed by electron microscopy, showed differences in cell-wall thickness which may reflect the differences in chemical composition. In addition, the general morphology of *A. bovis* reported here has been strikingly different from that of *A. israelii*. Its conically shaped cells with budding tips and total absence of the cytoplasmic figure readily separated it from the rod-like branching form of *A. israelii* which contained cytoplasmic figures.

The problem of the possible relation of cytoplasmic figures to phylogenetic position is most interesting. Considering the phylogenetic order published prior to the onset of the present studies, the progression read *L. bifidus*, *A. israelii*, and *A. naeslundii* and *A. propionicus*, for the reasons stated (Buchanan and Pine, 1962). *A. bovis* was not included, for the data were insufficient to give it a definite position. *A. bovis* may be considered less complex because it appears to lack the fermentation potential of *A. israelii*, being restricted in its growth to relatively few sugars. However, the fine-structure studies definitely show a great difference in cytoplasmic organization, with many membrane systems in *A. israelii* and essentially none in *A. bovis*. Thus this morphological evidence may correlate with the aforementioned biochemical and physiological data.

The status of *L. bifidus* in these studies is in doubt, since a related organism, *L. acidophilus*, has cytoplasmic figures which are similar to those found in *A. israelii* (Glauert, 1962*a*). Physiologically and biochemically, *L. bifidus* resembles the members of the genus *Actinomyces*, and many place it in this genus. From this standpoint, it is of interest to note that, of the eight strains of *L. bifidus* studied by Cummins and Glendenning (1957), all had rhamnose and glucose in the cell wall, and two had amino acid components not unlike those of *A. bovis*. Consequently, the best evaluation that can be given now is that, on a physiological basis, *L. acidophilus* and *L. bifidus* are closely related but their relative phylogenetic position has not been established. No cytoplasmic membrane coils were seen in *L. bifidus* in the present study despite careful searching. This suggests that *L. bifidus* is more primitive (less complex) than *L. acidophilus*. The cell-wall thickness of *L. acidophilus* has been reported by Glauert (1962) to be as great as 800 A. No such measurements were found for *L. bifidus* in the present study, the maximum being about 350 A. Therefore, further studies are needed to distinguish more clearly *L. acidophilus* and *L. bifidus* on a morphological basis, but the results reported here would suggest a closer relationship between *A. israelii* and *L. acidophilus* at a more complex level, with *L. bifidus* and *A. bovis* occupying equal but somewhat lower steps.

Phylogenetic classification is fraught with inconsistencies and pitfalls, and the present work may only coincidentally fit the progression for this small group of organisms. However, correlation of the present morphological findings with the previously published biochemical and physiological data appears good and cannot be dismissed when phylogenetic status is being considered.

Acknowledgments

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