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**ELECTRON MICROSCOPY OF FINE STRUCTURE OF
CORYNEBACTERIUM RENALE
WITH SPECIAL REFERENCE TO PILI*¹**

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INTRODUCTION

Three types of *Corynebacterium renale* were reported in preceding papers from this department. Serological, biochemical properties and nutritional requirements were obviously different among the 3 types of *C. renale* (YANAGAWA et al., 1967; HIRAI & YANAGAWA, 1967).

In this study, morphological investigation was performed using electron microscopy. While conducting this work the authors noticed the organisms of *C. renale* possessed fine fibrils corresponding to fimbriae (DUGUID et al., 1955) or pili (BRINTON, 1959). In addition, the piliation found was not the same among the 3 types of *C. renale*.

The appendages corresponding to pili were first reported by ANDERSON (1949) and HOUWINK & VAN ITERSOM (1950). Since then investigations have accumulated on the pili of gram negative bacteria. Pili of Enterobacteriaceae have been most intensively investigated. However, so far as the authors know, the existence of pili has been known only in gram negative bacteria and not in gram positive bacteria. This paper describes the anatomical features of *C. renale* with particular reference to pili, which is the first description of pili in gram positive bacteria.

MATERIALS AND METHODS

Bacterial strains and cultivation A total of 45 strains of *C. renale*, all the 3 types (YANAGAWA et al., 1967), were used. The types of these strains are shown in table 1. These strains were grown in nutrient broth or on nutrient agar plates for 1~2 days at 37°C. Strain No. 45 was used especially for ultrathin sectioning.

Fixation, embedding, staining and sectioning Shadowed and stained preparations of whole bacterial cells were made as follows: Bacteria grown in broth were fixed by adding osmium tetroxide to the broth culture to give a final concentration of 1%

*¹ According to BRINTON (1965), the authors use the term "pili" rather than "fimbriae".

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fixative. After fixing overnight at 4°C, the bacterial cells were sedimented by centrifugation and washed twice with distilled water. Bacteria grown on agar medium were suspended in distilled water and, after fixing in final 1% osmium tetroxide overnight at 4°C, the cells were sedimented and washed likewise. The washed bacteria were either mounted on the collodion grids and shadowed with palladium or stained with 2% phosphotungstic acid (for positive staining). For negative staining, the washed bacteria were mounted on the carbon coated collodion grids and, after reducing the excess amount of material by absorption with filter paper, stained with 2% phosphotungstic acid which was adjusted with 1N potassium hydroxide to pH 7.2.

Preparation for section was performed in two ways. Strain No. 45, whose culture on agar was viscous, was grown on agar plates for 24 hr. A small mass of the culture (0.5~1 mm in diameter) taken with a loop, was fixed overnight in Millonig phosphate buffer solution (MILLONIG, 1961) mixed with equal volume of 2% osmium tetroxide. Other strains which were not so viscous, were grown in nutrient broth, fixed overnight by adding 1/10 volume of 2% osmium. Fixed bacteria were washed thoroughly and hardened in 2% agar, dehydrated, embedded in Epon and sectioned with JUM 5A type ultramicrotome (Japan Electron Optics Laboratory Co.) using glass knives. Sections were stained with uranyl acetate and lead acetate.

Electron microscopy Preparations were examined in a JEM 7 electron microscope at instrumental magnification up to $\times 100,000$.

RESULTS

Findings obtained by ultrathin sections were mostly those of strain No. 45 (type II). Other strains were also examined but the findings were not significantly different from strain No. 45.

1 Pili

Fine fibrils attached to the surface of the cells were observed in many strains of *C. renale*. The fibrils were considered to be very similar to pili which were already reported in gram negative bacteria. Pili of *C. renale* were detected under the electron microscope in preparations either shadowed or stained with phosphotungstic acid. In figures 1~4, electron micrographs of the shadowed preparation of strains Nos. 5, 35, 46 and 42 are shown. It should be noticed that these strains possess pili but the number of pili varies among them; small in number in strain No. 5 (type I), numerous in strains Nos. 35 (type II) and 42 (type III) and particularly numerous, more than 400, in strain No. 46 (type II).

The table shows the general features of pili, particularly their number and length, in relation to the *C. renale* types. Strains of type II possess pili which are generally 1~10 μ long and numerous. The pili of type II strain are occasionally seen to attained a length of several ten micron. A portion of a long pili is seen in figure 7. On the other hand, pili of the strains belonging to type I, generally 0.1~4 μ long, are characteristic in that they are small in number, or hardly detectable. The pili of the strains of type III vary among the strains; generally, 0.1~2 μ long, small in number, and similar to the strains of type I, however there are some exceptions such as strains Nos. 42 and 43 which have many pili.

TABLE General features of pili in relation to *C. renale* types

TYPE OF <i>C. RENALE</i>	PILI		
	Small in number or hardly detectable, usually short	Not scant but not numerous, usually short	Numerous, usually long
I	1* ¹ , 2, 3, 4, 5, 6, 8, 9, 10, 12, 15, 24, 25, 79 30, 27, 70	11, 106	
II			13, 14, 17, 18, 19, 20, 22, 23, 26, 35, 37, 45, 46, 58, 59, 60, 61, 63, 64
III	40, 47, 48, 103	104	42* ² , 43

*¹ Strain No.

*² Pili of strains Nos. 42 & 43 are rather short.

Diameter and detail of the structure of the pili were observed with the preparations negatively stained. Figure 5 shows pili of strain No. 35 (type II). It is clear that the pili tend to attach side by side and form bundles of pili. The number of pili forming the bundles also varies, 2 to more than 10. The bundle formation was also found in strain No. 42 (type III). The cell surface is often surrounded by the bundles of pili (figs. 6, 7 & 8). The diameter of the finest pili, shown in figure 5, was 25~30 Å. The diameter of individual pilus did not vary significantly among strains of different types. No particular subunit structures could be found in the pili, but this should be studied further.

Two bacteria are connected by bundles of pili (fig. 8). Such findings are particularly recognized in type II strains.

Pili are firmly attached to the isolated cell wall, this was conducted after vigorous shaking with glass beads in Homogenon (Ohtake). Figure 9 shows many pili attached to the isolated cell wall of strain No. 45 (type II). Figure 10 indicates the cell wall of strain No. 9 (type I) which does not possess many pili. This strain does not possess many pili originally.

The origin of the pili was not clear in both shadowed and sectioned preparations. Photographs of the sections may indicate if the bundles of pili originated from the outer-most layer of the cell wall as exemplified in figure 11. This was not the case because the structural appearance differs between the outer-most layer and pili. Pili are attached firmly to the isolated cell wall as described above. We presume that the pili might originate from some place deeper than the cell wall and extend through cell wall. The origin of the pili of *C. renale* needs further investigation.

Pili of *C. renale* are obviously different from flagella. *C. renale* strains used in this

study did not possess flagella morphologically also, no strains showed motility in semi-solid media incubated at 30°C and 37°C for a week.

2 Cell wall

An outer-most layer was found (fig. 12), which was 40~50 Å thick, and connected to an underlying clear zone of 60~80 Å (fig. 13). The outer-most layer, which seems to be the surface layer of the cell wall, does not participate in septum-formation (fig. 15). The outer-most layer is occasionally separated from the underlying zone, especially when 2 bacteria were closely located. This indicates the outer-most layer of the cell wall is not very rigid and may be viscous in nature.

The relationships between the outer-most layer of the cell wall and the pili is not obvious. They are structurally different as mentioned before. However, it frequently occurs that bundles of pili interwind the cell surface (fig. 6) or cells are surrounded by the bundles of pili (figs. 7 & 8).

A dense thick layer, 140~160 Å, is remarkable underneath the outer-most layer and the intermediate clear zone (fig. 13). This layer should be rigid because, beside its thickness, it always exists in variously degenerated cells. In some sections, the cell wall is found to consist of 3 layers, the inner and outer dense layers approximately 30 Å thick and the intermediate broad layer, about 80~100 Å (fig. 15).

3 Cytoplasmic membrane

Cytoplasmic membrane (fig. 11) consists of 2 layers, each 30 Å thick and separated by a lighter inter zone, 20~30 Å thick. The cytoplasmic membrane, 80~90 Å in total thickness, shows the features of the unit membrane, and is generally, clearly seen in degenerating cells.

Figures 14 and 15 show septum formation. As described before, the cytoplasmic membrane and cell wall, not the outer-most layer, participated in septum formation.

4 Cytoplasm and nuclear apparatus

The cytoplasm of *C. renale*, as shown in figure 13, is packed with fine granules which seem to be ribosomes. Very often these granules aggregate to make bigger granules which vary in size (fig. 11). The existence of the larger granules seem to show cell degeneration.

The nuclear region, having a lower density than the cytoplasm, occupies the central area of the cell and is filled with fine fibrils (fig. 16). No limiting membrane was found around the nuclear region.

5 Intracytoplasmic membrane system

The membranous organelle and the intracytoplasmic membrane system, as illustrated in figure 17, are detectable especially in young cells such as the 6 hr culture. The organelles are circular in shape and lamellar in structure. The diameter of these organelles generally ranges from about 60 to 160 m μ . The thickness of the unit membrane of the intracytoplasmic membrane system is the same as that of the cytoplasmic membrane. Both membranes are continuous, particularly where septum formation is initiated (figs. 14 & 18).

6 Metachromatic granules

In the cytoplasm dense granules are found, which correspond to metachromatic granules (fig. 19). They contain small dense granules ranging from 2 to 11 m μ in diameter. If

present, they are usually 1 or 2 in number, and located centrally or near the ends of the cell. General features of the granules resemble those reported by KAWATA (1961) in *C. diphtheriae*.

DISCUSSION

The first description of pili in gram positive bacteria is discussed in this paper on *C. renale*. Pili have been known to exist in gram negative bacteria, and have been studied in detail in Enterobacteriaceae, but not in gram positive bacteria.

Pili of *C. renale* exhibited different features among types of this species. Strains of type I possessed pili which were small in number. On the contrary, strains of type II possessed numerous long pili. Pili of the strains of type III were varied, many possessed pili similar to those of type I however some possessed many pili. The authors (YANAGAWA et al., 1967) reported 3 types of *C. renale* on the basis of their serological and biochemical properties. Accordingly, these findings will show additional differences among *C. renale* types.

The pili of *C. renale*, though differing in length and number, exhibit similar thickness, and have a tendency to adhere. In addition, bacteria of type II strains are often connected by thick pili, either long or short, which comprise bundles of pili. Pili of *C. renale*, therefore, might be adhesive, as in the case of gram negative bacteria (DUGUID & GILLIES, 1956).

Pili of type I strain are characteristic in that they are small in number. No regularity was found in the location of pili on the cell surface. The following possibilities are considered to be the reason why pili of type I strain are small in number; 1) they are small in number originally, 2) easily be removed mechanically, and 3) able to grow but are inhibited by unknown factor(s). The second possibility is considered to be excluded because fixing of the bacteria in broth cultures was done carefully. We feel that more work should be done to explain the remaining possibilities. As for the variation of piliation of type III strains, we have no appropriate explanation at the present time.

Pili of type II strains were numerous and long without exception. Often they extended more than several ten micron. This suggests that the synthesis of pili is performed actively in this type of *C. renale*.

Type II strains possess numerous pili. As described in the previous report (YANAGAWA et al., 1967) many strains of type II have been isolated from the urinary tract and urine of apparently healthy cattle. In connection with this fact, it is interesting that DUGUID & GILLIES (1956) noted "possibly the fimbriae (of *Shigella flexineri*) act in promoting harmless commensal growth in some parts of the intestine, so as to procure longer infectivity of patients and carriers".

HASHIMOTO (1965) supposed that piliation in gram negative bacteria might be an expression of a shift from pathogenic to saprophytic life. It is of interest that two kinds of bacterial flora, Enterobacteriaceae in the intestine and *C. renale* (especially type II) in the urinary tract, regardless their difference in gram staining character, have pili.

Since *C. renale* is the first known gram positive bacteria possessing pili, it will be necessary to compare the properties of pili of *C. renale* with those of gram negative bacteria. Studies along this line are in progress.

The pili of some of gram negative bacteria are known to act as organelles of genetic transfer (BRINTON et al., 1964). Whether *C. renale* pili play a role in genetics will be a problem of future study.

General anatomic features of *C. renale*, except pili, are not markedly different from those of other gram positive bacteria. However, the existence of the outer-most layer which was distinct, has not been observed in other bacteria of Corynebacteriaceae such as *C. diphtheriae* (KAWATA, 1961) and *L. monocytogenes* (EDWARDS & STEVENS, 1963). It was not found in *S. aureus* (SUGANUMA, 1965). The outer-most layer is clearly different from the underlying thick layer which is characteristic to gram positive bacteria. The outer-most layer did not participate in septum formation and appeared after the cell was divided. Therefore, we consider that the outer-most layer is assembled on the cell wall soon after cell division is completed.

SUMMARY

The first description of pili of the gram positive bacteria, *Corynebacterium renale* was completed. The number and length of pili were different among types of *C. renale*. Generally, type I strains possessed only a few pili, 0.1~4 μ long, while type II strains possessed numerous long pili, 1~10 μ . Pili of type II strains tended to form thick bundles, attached side by side. Occasionally the bundles attained a length of several ten micron. Piliation of type III strains was various, many strains possessed pili similar to those of type I strains, 0.1~2 μ long, but some possessed many pili. The diameter of the pili was, regardless of types, approximately 25~30 Å. Existence of an outer-most layer of cell wall and a well developed intracytoplasmic membrane system was also noticeable.

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EXPLANATION OF PLATES

PLATE I

- Fig. 1 *C. renale* strain No. 5 (type I), shadowed × 23,000
- Fig. 2 *C. renale* strain No. 35 (type II), shadowed × 35,000
- Fig. 3 *C. renale* strain No. 46 (type II), shadowed × 40,000
- Fig. 4 *C. renale* strain No. 42 (type III), shadowed × 24,400

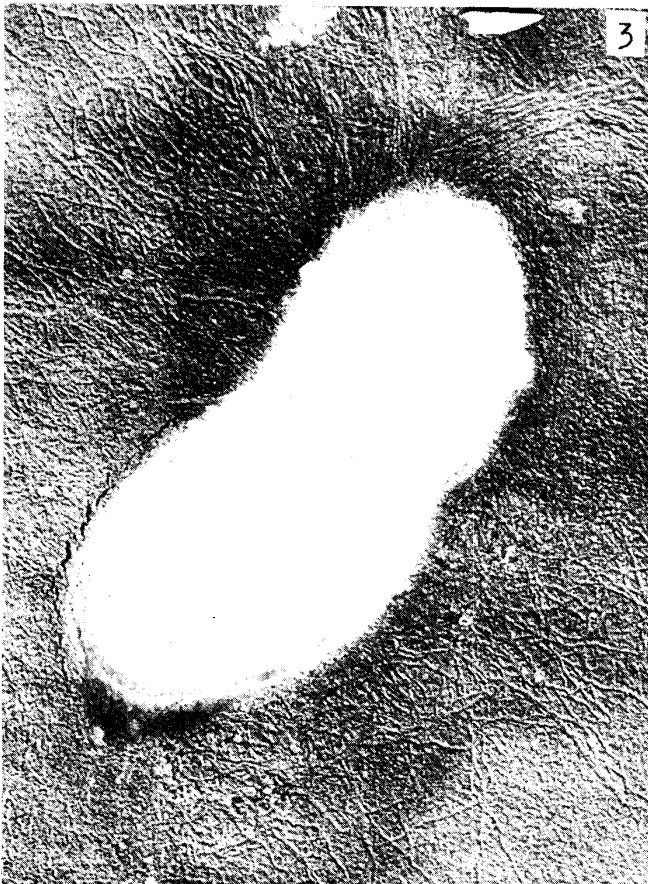
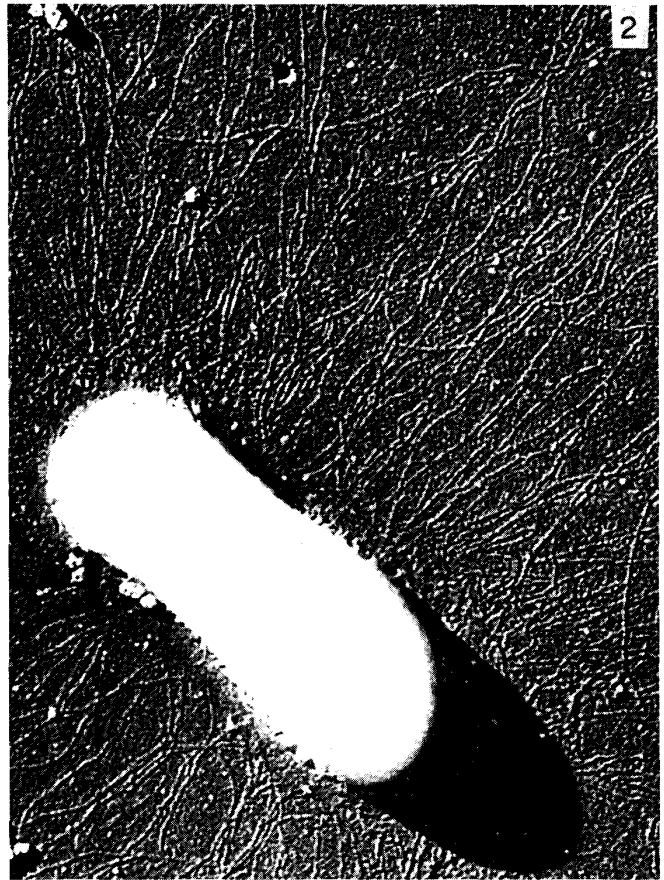


PLATE II

Scale indicates 100 m μ .

Fig. 5 Pili of strain No. 35, negative stain

Fig. 6 Pili of strain No. 42, negative stain

Bacterial cell is surrounded by bundles of pili.

Fig. 7 Thick pili of strain No. 45, positive stain $\times 17,500$

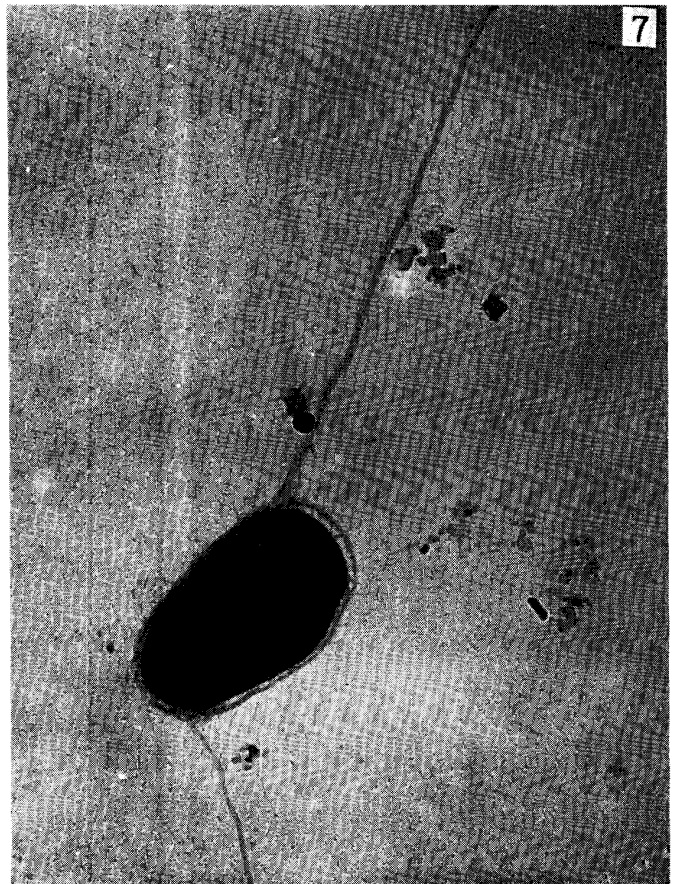
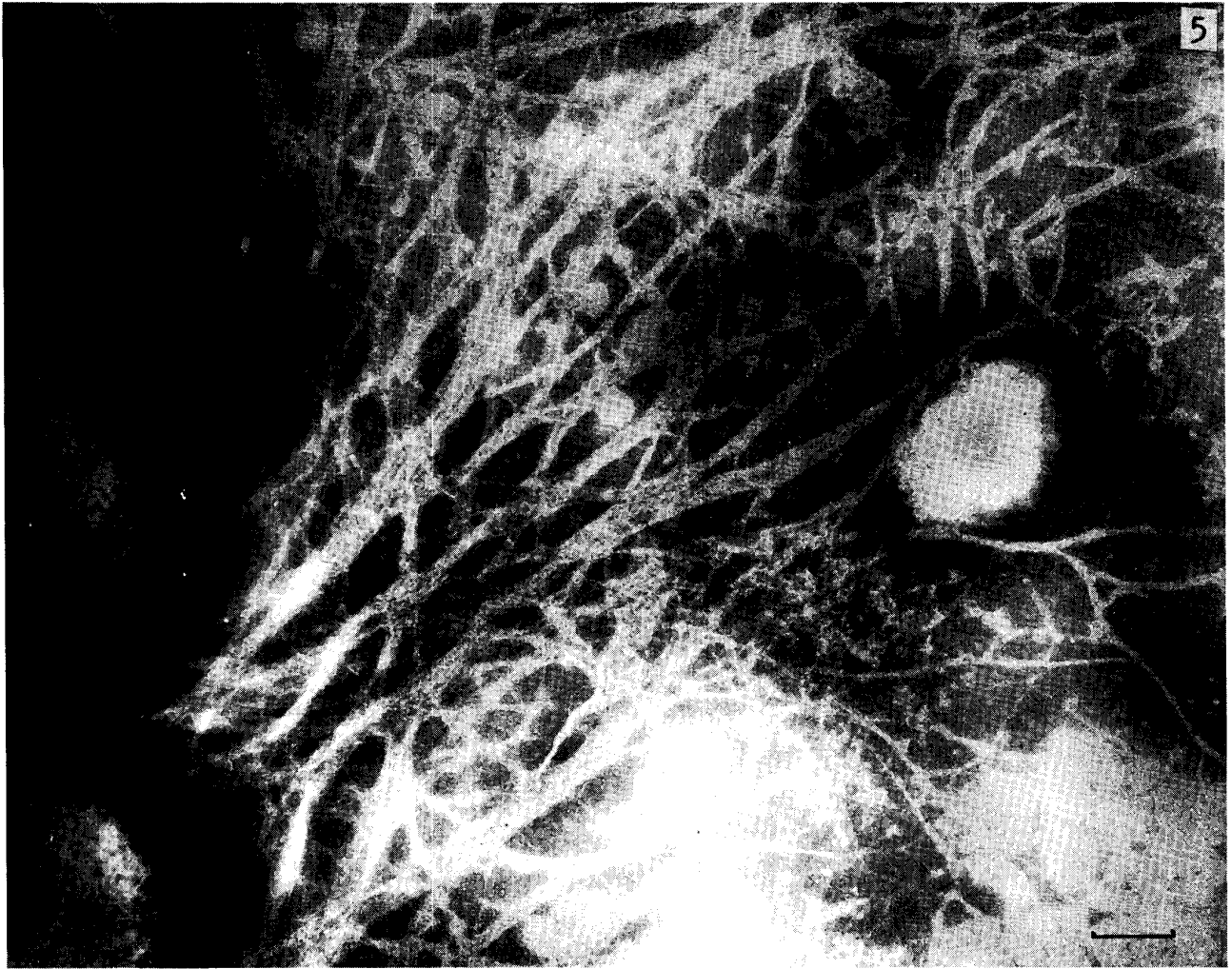


PLATE III

- Fig. 8 Strain No. 45, positive stain
Two cells are connected by pili. $\times 26,400$
- Fig. 9 Cell wall of strain No. 45 with numerous pili attached $\times 25,000$
- Fig. 10 Cell wall of strain No. 9 (type I) attached by only a small number
of pili $\times 25,000$
- Fig. 11 Thin section of strain No. 45
Scale indicates 100 m μ .
P (bundle of pili)
OL (outer-most layer of cell wall)
CW (cell wall)
CM (cytoplasmic membrane)
IMS (intracytoplasmic membrane system)
These abbreviations are also used in the following figures.

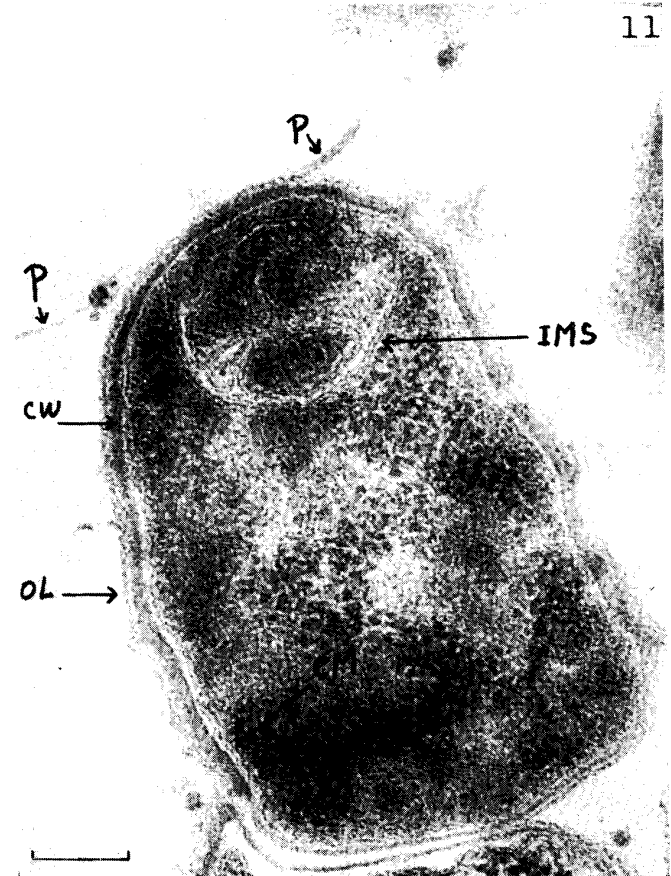
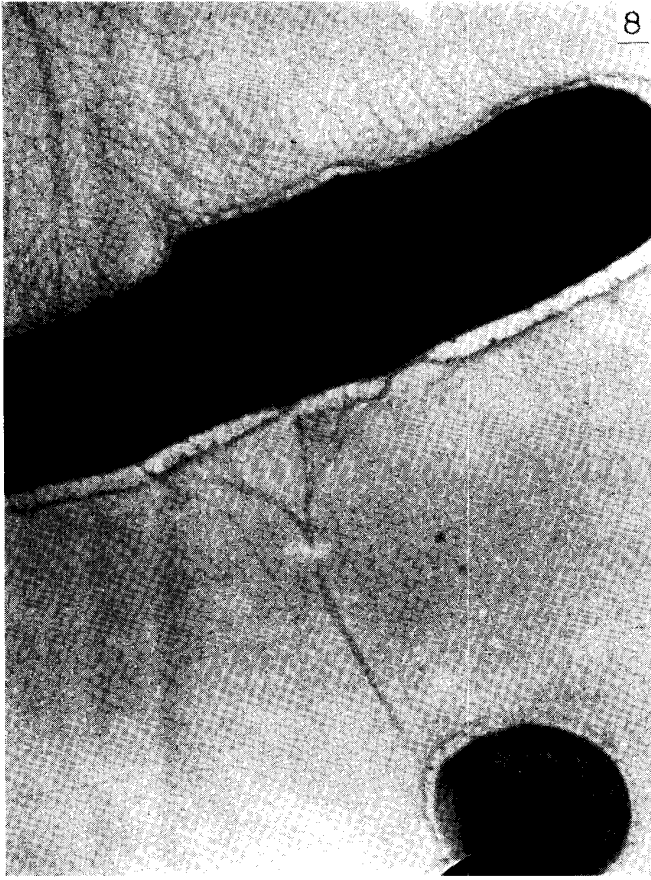


PLATE IV

Scale indicates 100 m μ .

- Fig. 12 Thin section of strain No. 45
- Fig. 13 A part of figure 12
- Fig. 14 Thin section of strain No. 45
N (Nuclear region)
- Fig. 15 Thin section of strain No. 45
S (septum)

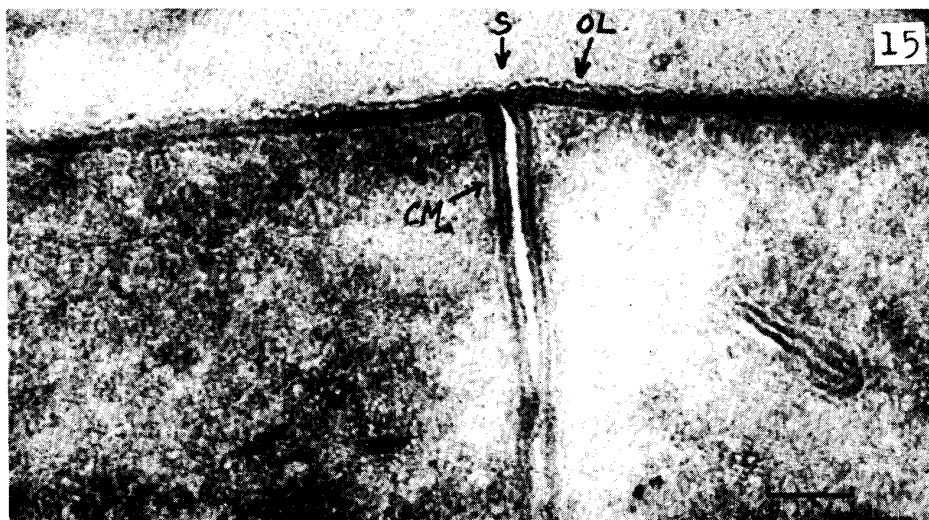
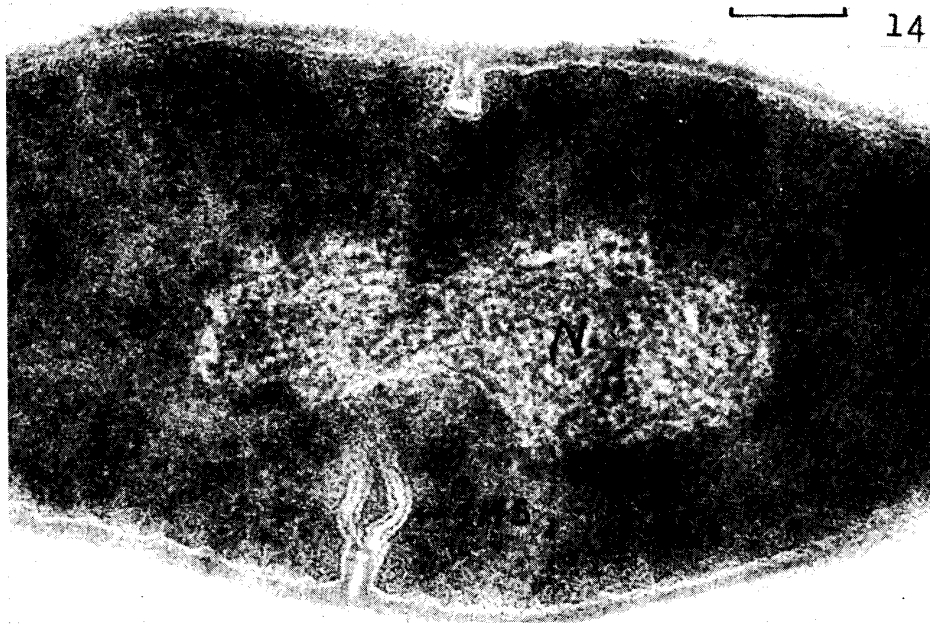
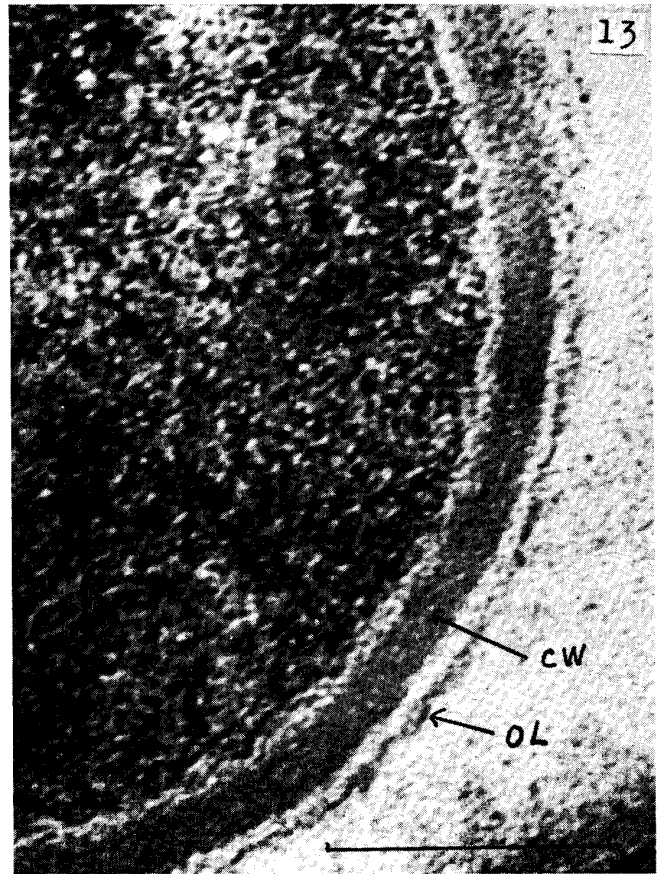
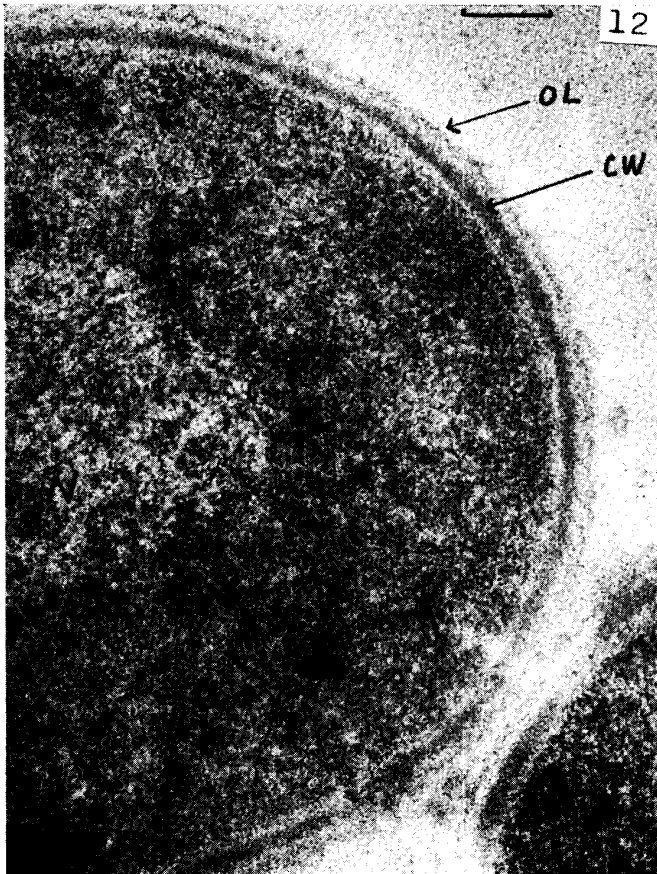


PLATE V

Scale indicates 100 m μ .

- Fig. 16 Thin section of strain No. 45, showing nuclear apparatus
- Fig. 17 Thin section of strain No. 45, showing intracytoplasmic membrane system
- Fig. 18 Thin section of strain No. 45, showing intracytoplasmic membrane system connected by cytoplasmic membrane at the position of septum formation
- Fig. 19 Thin section of strain No. 45, showing metachromatic body (MB)

