JOURNAL OF BACTERIOLOGY, Oct. 1970, p. 566-580

Vol. 104, No. 1
Printed in U.S.A.

Control of Morphogenesis in *Geodermatophilus*: Ultrastructural Studies

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Received for publication 10 April 1970

Geodermatophilus grows in two major forms, a nonmotile irregularly shaped aggregate of coccoid cells (C-form) and a motile budding rod (R-form). Morphogenesis can be controlled by an unidentified factor in Tryptose which is required for maintenance of the organism in the C-form and for differentiation of the R-form to the C-form. Morphogenetic events occur synchronously in the described system. Ultrastructural studies show that the major difference between C- and R-forms is in cells envelope structure. R-form cell walls consist of two layers, an inner transparent membranous layer (10 to 12.5 nm thick) and an outer dense diffuse layer (7.5 to 10 nm). In addition to these layers, the C-form has a thick fibrous layer (30 nm) over the dense layer. This layer appears to be a cementing substance which holds the coccoid cells together.

Geodermatophilus has been proposed recently by Luedemann (8) as a new genus of the *Dermatophilaceae* (*Actinomycetales*). Organisms of the genus *Geodermatophilus* occur in two major forms. One is a motile rod which multiplies exclusively by budding (R-form), and the other is a nonmotile irregularly shaped aggregate of coccoid cells (C-form). Luedemann (8) designated these as the zoospore and thallus stages, respectively. In an earlier report Ishiguro and Fletcher (Bacteriol. Proc., p. 24, 1966) described morphogenesis of an unidentified microorganism which subsequently has been found to be *Geodermatophilus*. They employed slide cultures to keep differentiating cells under continuous surveillance. The present study is an attempt to document differentiation in *Geodermatophilus*.

We devised a method for controlling morphogenesis of this organism, and the growth cycle was studied by electron microscopy. Interpretation of the differentiation process was facilitated by the fact that morphogenetic events occur synchronously in our system.

MATERIALS AND METHODS

Organisms. *Geodermatophilus* strains were isolated from high-altitude Mount Everest soil samples (Ishiguro and Fletcher, Bacteriol. Proc., p. 24, 1966). Strain 22-68 was selected for ultrastructural studies.

Media. Medium TYB contained 2.0% (w/v) Tryptose (Difco, lot #450979), 0.2% Yeast Extract (Difco), 0.2% glucose, and 0.5% NaCl at *p*H 7.0. Agar (1.5%) was added for solid medium designated TYA. Medium CB consisted of 0.2% (w/v) Casamino Acids (Difco, vitamin-free), 0.2% glucose, 1.0% (v/v) vitamin mixture (16), and 1.0% (v/v) trace mineral solution (16) at *p*H 7.0.

General conditions of cultivation. Stock cultures were maintained on TYA slants. They were stored at 10 C and transferred weekly. Broth cultures were incubated at 30 C on a rotary shaker, and growth was followed by optical density readings at 660 nm (OD_{660}) on a spectrophotometer (model DU; Beckman Instruments, Inc., Fullerton, Calif.) against a water blank.

Cultivation of C-form. The C-form inoculum was prepared by transferring one loopful of stock culture to 20 ml of TYB medium. After 2 days of incubation, cells were exclusively in the C-form. For cultivation of the C-form, a sufficient sample of a mid-logarithmic-phase culture (approximately 3 to 4 days old) was added to fresh TYB medium to give an OD_{660} of 0.05 to 0.1.

Morphogenesis of C-form to R-form. The C-form inoculum described above was aseptically washed twice with sterile distilled water. Samples were inoculated into appropriate quantities of CB medium to give an OD_{660} of 0.05 or less.

Morphogenesis of R-form to C-form. The C-form inoculum was transferred to CB medium and allowed to differentiate. Incubation of cultures was terminated after approximately 20 hr. At this time, cells were in the R-form. To allow morphogenesis of the R-form to the C-form, samples of these cultures were inoculated into TYB medium to give an OD_{660} of approximately 0.05.

Electron microscopy. Negative staining was performed with aqueous potassium phosphotungstate (*p*H 7.0) at concentrations ranging from 0.02 to 2% (w/v) as previously described (7). To study the mode of flagella insertion, cells were autolyzed by placing

washed 24-hr R-form cells in sterile distilled water at 4 C for 3 weeks.

Preshadowed carbon replicas were made by the method of Rode and Williams (13) with the following modifications. To avoid collapse of cells, they were fixed by direct addition of glutaraldehyde to the medium to a final concentration of 4%. Specimens were prepared on glass slides and were removed by flotation on 5% hydrofluoric acid (7). Cellular material was digested with 2% sodium hypochlorite (household bleach, The Clorox Co., Oakland, Calif.) for 4 hr.

For thin sectioning, cells were fixed by the method of Kellenberger, Ryter, and Sechaud (5) and embedded in Epon 812 (9). Sections were cut with a diamond knife on a Reichert OMU-2 ultramicrotome. They were stained with aqueous 1% uranyl acetate at 37 C for 20 min and then by one of two lead citrate stains (11, 14) for 5 to 15 min. Specimens were examined with either an RCA EMU-2E or a JEM-T6S electron microscope.

RESULTS

Tryptose (Difco) contains an unidentified factor, M, which controls morphogenesis in *Geodermatophilus*. In broth, factor M is required to maintain the organism in the C-form and for differentiation of the R-form to the C-form. In its absence, growth is exclusively in the R-form. Thus, it is possible to control morphogenesis in two undefined broth media, one with M (TYB) and the other without M (CB). Results for *Geodermatophilus* strain 22–68 are presented below and are summarized diagrammatically in Fig. 1. Identical observations were made on 5 additional strains (1-34, 1-37, 47-45, 47-50, and 47-55).

Typical growth curves for the C-form and the R-form in CB and TYB media are presented in Fig. 2. Morphology of cells in these experiments is shown in Fig. 3–6; a sample of each growing culture was removed and photographed at the time indicated on each photomicrograph. When the C-form was transferred to fresh TYB medium, growth continued exclusively in this form (Fig. 1, types I, II, and III; Fig. 2, curve A; Fig. 3). The C-form differentiated to the R-form in CB medium (Fig. 1, forms I, IV, V, VI, and VII; Fig. 2, curve B; Fig. 4). The C-form did not multiply in this medium and apparently underwent lysis as evidenced by its eventual disappearance from the culture. No differentiation was observed when the R-form was inoculated into CB medium (Fig. 1, types V, VI, and VII; Fig. 2, curve C; Fig. 5). Morphogenesis from R- to C-form occurred upon transfer to TYB medium (Fig. 1, forms VII through XI to I; Fig. 2, curve D; Fig. 6).

Morphogenetic events occurred synchronously, and their timing was highly reproducible under

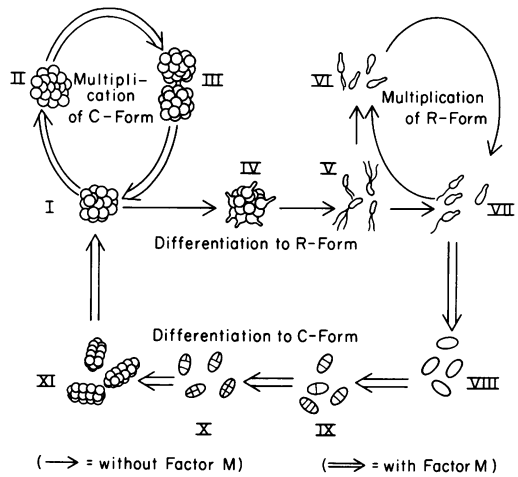

FIG. 1. *Morphogenetic growth cycle of Geodermatophilus strain 22-68. Morphogenetic events designated by single arrows occur in medium CB. Double arrows indicate events which occur in TYB medium.*

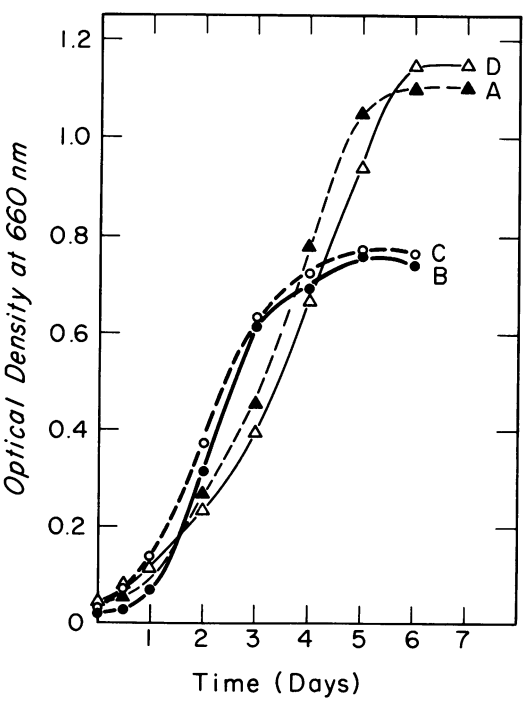

FIG. 2. *Growth of Geodermatophilus strain 22-68 in CB medium and TYB medium. Inocula were prepared as described in text. A, Three-day C-form cells inoculated into TYB medium; B, 3-day C-form cells inoculated into CB medium; C, 2-day R-form cells inoculated into CB medium; D, 20-hr R-form cells inoculated into TYB medium. Morphology of cells in these experiments is shown in Fig. 3–6.*

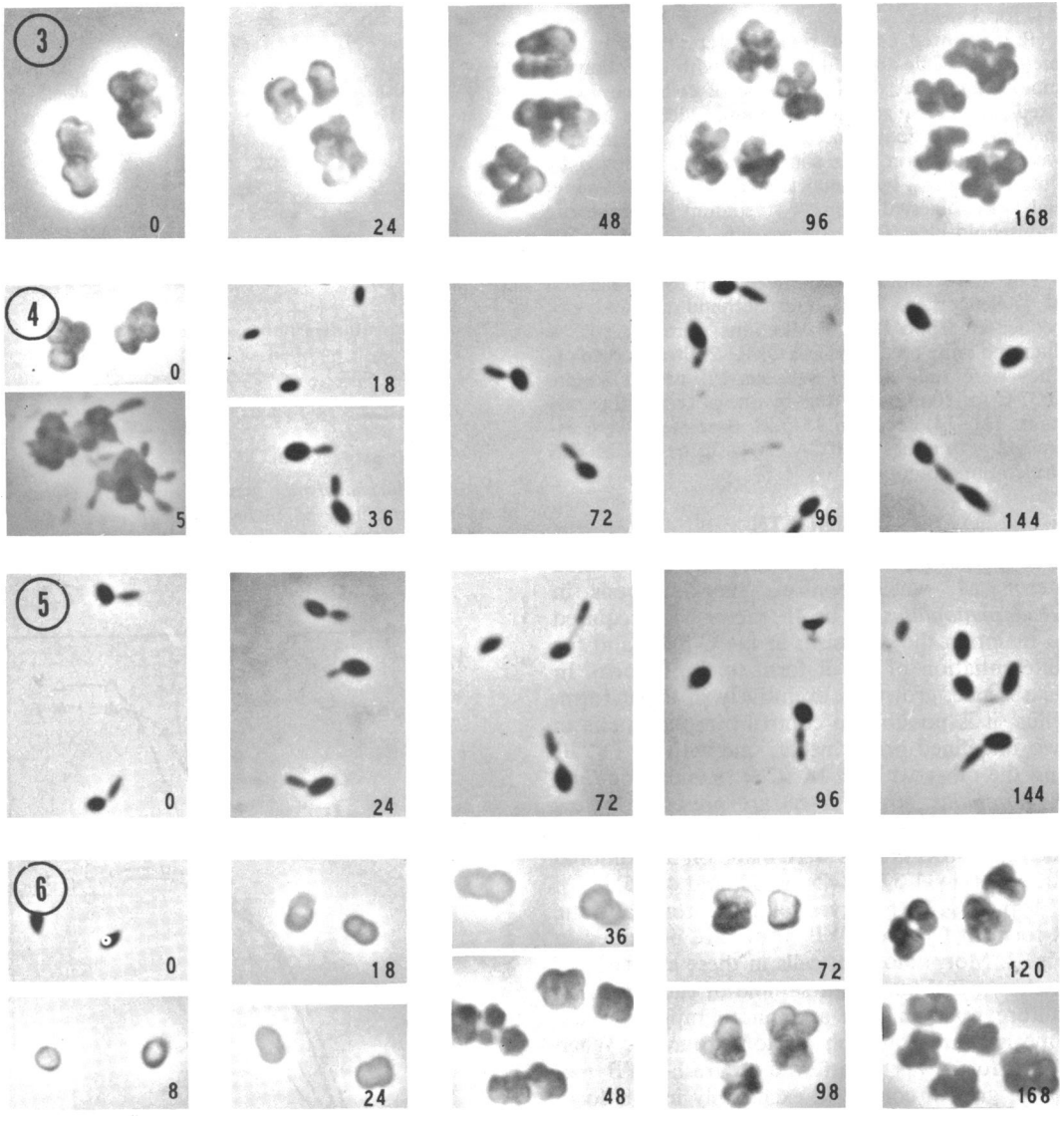

FIG. 3. *Morphology of C-form inoculated into TYB medium. Numerals in lower corners refer to time in hours* $\times 2{,}200$.

FIG. 4. *Morphogenesis of C-form to R-form in CB medium.* $\times 2{,}200$.

FIG. 5. *Morphology of R-form in CB medium.* $\times 2{,}200$.

FIG. 6. *Morphogenesis of R-form to C-form in TYB medium.* $\times 2{,}200$.

the described conditions. Inoculum age did not affect the ability of the organism to differentiate when placed in the appropriate medium, but did cause changes in timing and synchrony of morphogenetic events.

Ultrastructure of the C-form. The C-form was demonstrated to be an aggregate of coccoid cells by carbon replication. Although aggregates were irregular in shape, areas of regularly arranged cells were consistently observed, suggesting that a regular pattern of division existed in certain regions (Fig. 7). Obvious surface ultrastructure was not evident.

In sections, each coccoid cell was delimited by a membrane and cell wall (Fig. 8). Composition of the granular inclusions, labeled i, is unknown, but the inclusions appear similar to polysaccharide granules described in *Stigmatella aurantiaca*

FIG. 7. *Carbon replica of C-form showing an area of regularly arranged coccoid cells.* ×57,700.

(10, 15). They were characteristically spherical and of uniform size. Mesosomes of the coiled tubule type were common (Fig. 9). Surface structure was difficult to resolve. The cell wall consisted of at least two layers (Fig. 8). A transparent membranous inner layer (9–11 nm thick), t, was usually obscured by a thick fibrous outer layer (29–32 nm), f, and seen in only a few sections (*also see* Fig. 17 and 18). The outer layer stained poorly with heavy metals. Thick sections had to be overstained to clearly demonstrate its presence, and the density of the cytoplasm (Fig. 8) may be attributed to this. This layer was relatively uniform in thickness and appeared to be a rigid structure. The presence of a transparent region (TZ) between adjacent cells suggests that this region is an integral part of the cell envelope and may represent a "cementing factor" which holds the cells together.

Multiplication of individual cells was by binary fission. Sections of cells in the process of cross-wall formation are presented in Fig. 10. Since single cells were never observed in actively growing C-form cultures, it was unlikely that each aggregate developed from a single coccoid cell which had detached from a preexisting aggregate. Closer examination by phase contrast microscopy revealed that cell aggregates varied considerably in size and that larger C-forms had well-defined division clefts which separated them into units of approximately equal sizes (Fig. 3, 6, 11). These units were also equivalent to the smallest clumps observed. In addition, bridges were noted between separating clumps. Bridges could not be conclusively demonstrated in sections and carbon replicas; however, they were clearly shown in negatively stained preparations although their structure remains obscure (Fig. 12, 13). We propose the following mechanism for multiplication of the C-form. (i) Coccoid cells multiply by binary fission but remain integrated as part of the C-form, causing growth of the aggregate; (ii) when a maximal size is attained, a division cleft forms which divides the aggregate into approximately equal fractions; (iii) upon separation, each resulting aggregate repeats this multiplication cycle. The proposed process is presented diagrammatically in Fig. 1 (types I, II, and III). What determines "maximal size" and what mechanisms are involved in division-cleft formation and aggregate separation are intriguing questions which remain unanswered. It seems conceivable that the latter processes may involve localized digestion of the proposed cementing factor.

Ultrastructure of C-form to R-form morphogenesis. Small buds appeared on coccoid cells of the C-form approximately 4 hr after transfer to CB medium (Fig. 4). Figure 14 is a tangential section through one of these buds. The outer fibrous cell wall layer completely covered these young buds. However, in well-developed buds, this structure did not extend beyond a short stalk-like region (labeled f in Fig. 15) which formed between the coccoid cell and the bud. A septum developed on the stalk, and upon its completion, the mature bud detached as the R-form leaving the stalk as part of the parent cell (Fig. 16). Not more than a single bud was observed on each coccoid cell. Our results clearly show that the R-form arises by budding of the C-form as reported by Ishiguro and Fletcher (Bacteriol. Proc., p. 24, 1966). No evidence was found for the germ tubes or pores suggested by Luedemann (8).

It was reported previously that the C-form disaggregated into its component coccoid cells (8). Although we have made similar observations, we are not certain that this is an essential part of the growth cycle, since it does not always occur. Disaggregation was evident in one preparation examined. The apparent cause was degradation of the outer cell wall layer which then appeared as fibrous material around individual coccoid cells (Fig. 17), supporting the proposal that this layer represents a cementing factor. The cementing layer is only partially digested in the C-form shown in Fig. 18, and the coccoid cells are in the process of disaggregation. Cell walls of disaggregated coccoid cells consisted of an outer diffuse dense layer (7 to 10 nm thick) and a transparent inner layer (9 to 13 nm). Their ultrastructure was identical to walls of developing buds (Fig. 15, 16, 19). It is clear that C-form to R-form differentiation results in changes in cell wall structure.

It was consistently observed that the parent cell remained intact by the formation of a complete septum before release of the R-form. However, degenerate cells were common in all preparations examined, and their numbers increased upon further incubation. No trace of intact coccoid cells was found in samples collected at 72 hr. Although disaggregated C-form cells appeared normal (Fig. 17), lysed cells were common in this preparation. A section of a clump which contained two lysed cells, labeled E, is shown in Fig. 18. However, lysis cannot be attributed solely to loss of the outer cell wall layer since it also occurred in aggregated C-forms. The cementing layer of lysed cells retained the original shape of the cell, suggesting that it is a rigid structure. But this apparent rigidity may actually be due to the presence of the inner cell wall layers which were not resolved in our micrographs. No multiplica-

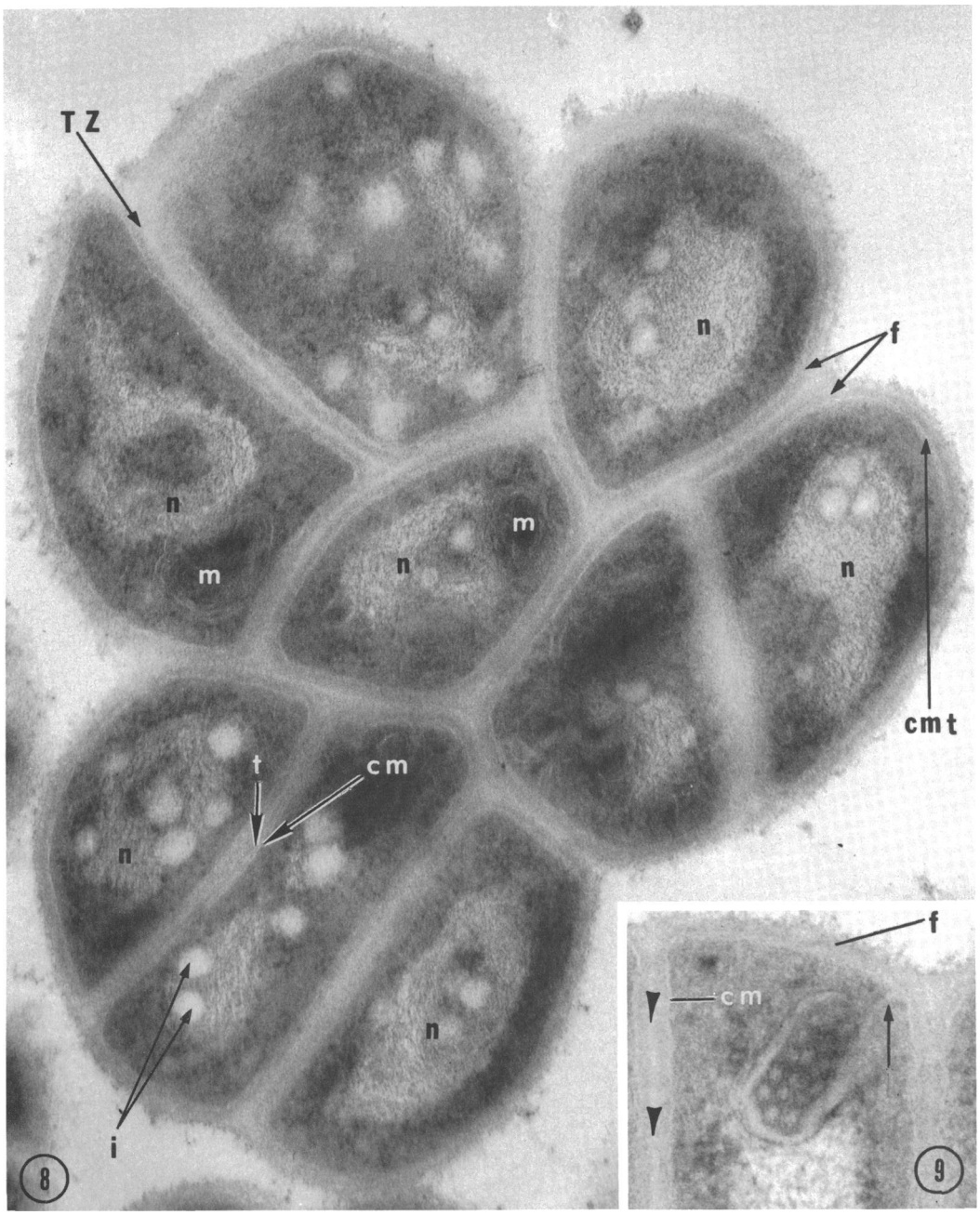

FIG. 8. *General features of C-form. Irregularly arranged coccoid cells individually bound by cytoplasmic membrane (cm) and cell wall with at least two layers, a fibrous outer layer (f) and a transparent inner layer (t). The latter layer is not resolved from cm in some areas (cmt). Note transparent zone between cells (TZ). Dense cytoplasm contains fibrillar nucleoplasm (n), densely stained tubular mesosomes (m), and granular inclusions (i). Section has been over-stained with lead to resolve surface layers. ×40,800.*

FIG. 9. *Cross section of mesosome in C-form. Note attachment (arrow) to cytoplasmic membrane (cm). Fibrous cell wall layer (f) and transparent region between cells (arrowheads) are resolved. ×84,300.*

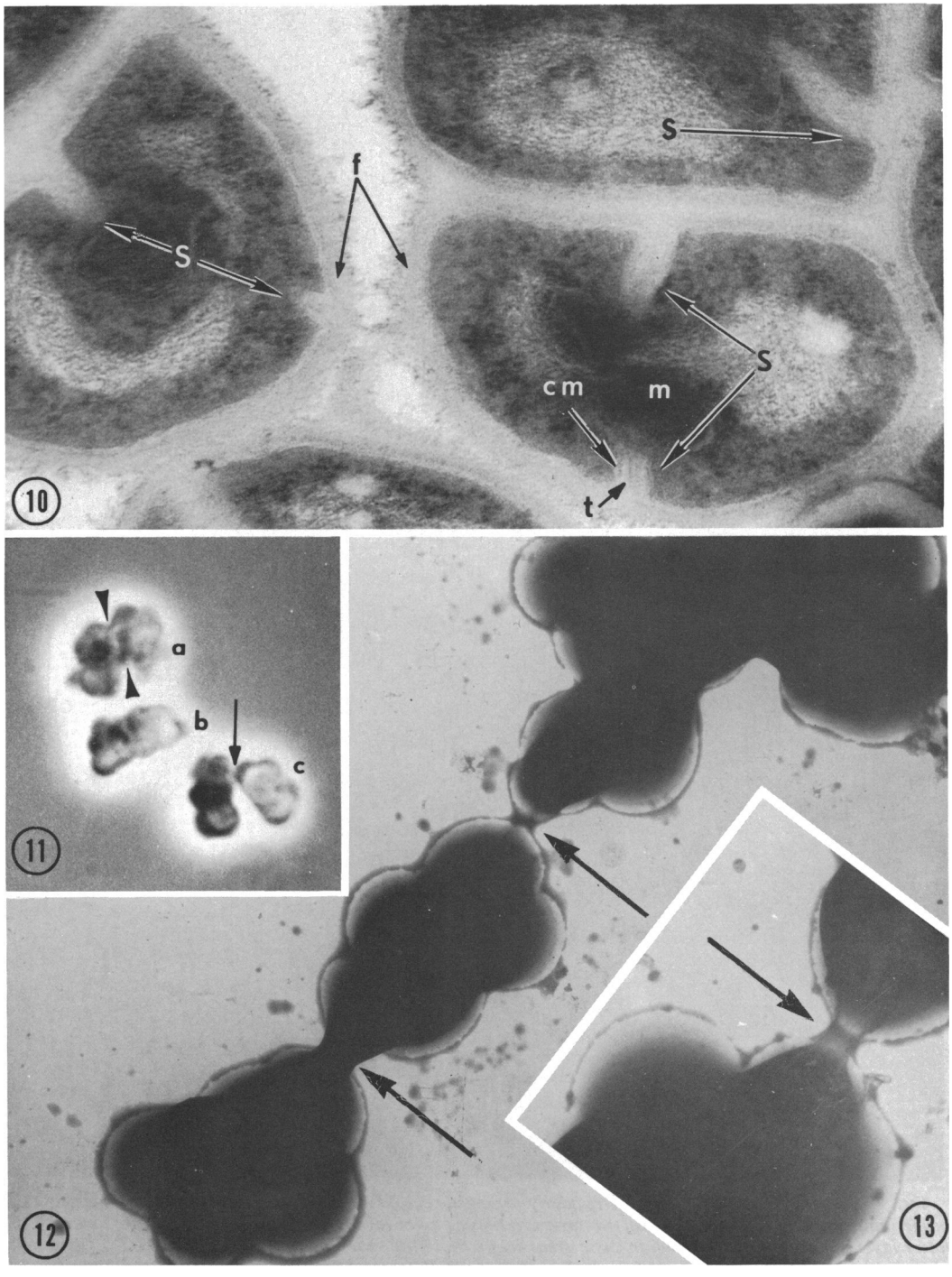

FIG. 10. *Septum formation (S) in coccoid cells of C-form multiplying by binary fission.* ×*62,700.*

FIG. 11. *Separation of C-form. (a) Division cleft (arrowheads) in C-form; (b) growing C-form; (c) bridge (arrow) between separating C-form. Phase contrast,* ×*3,300.*

FIG. 12 and 13. *Bridges (arrows) between separating C-forms. Negatively stained with 2% potassium phosphotungstate. Fig. 12,* ×*15,000; Fig. 13,* ×*27,000.*

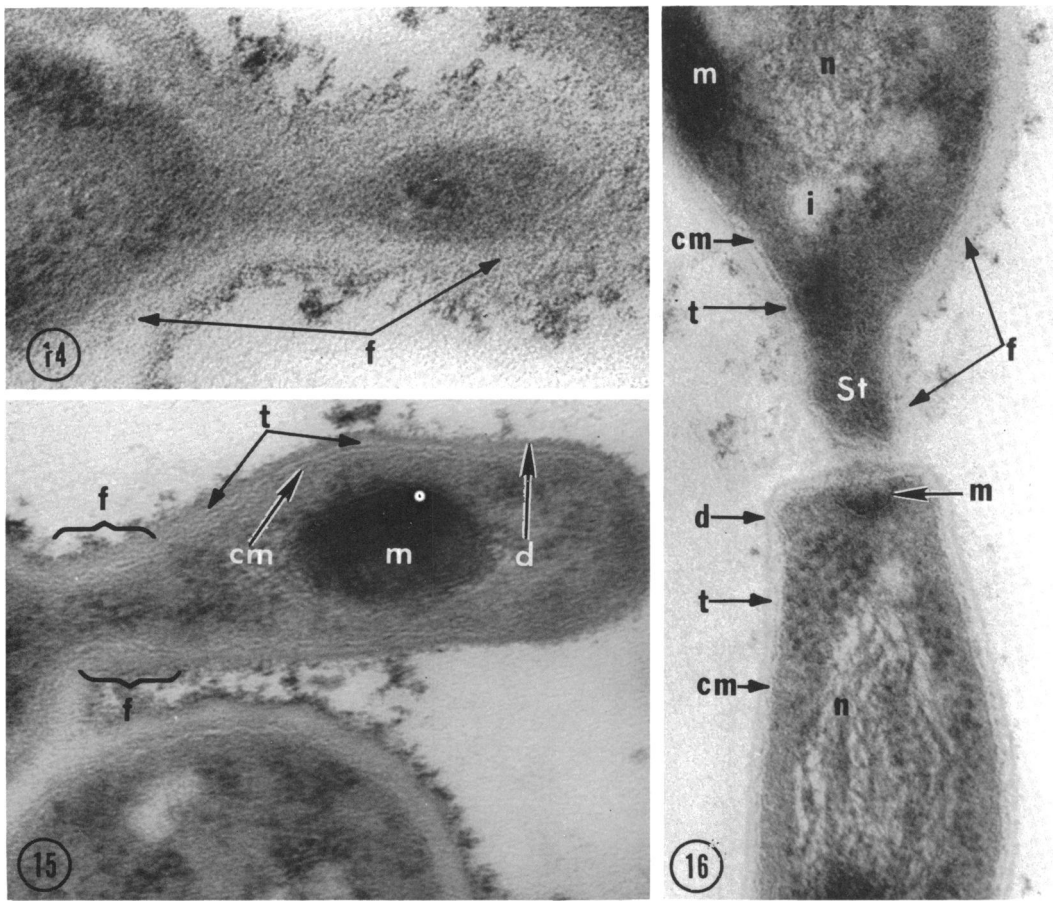

FIG. 14. *Tangential section through young bud during morphogenesis of C-form to R-form, showing fibrous structure of outer wall layer (f). Note that layer covers entire surface of bud.* ×*140,000.*

FIG. 15. *Fibrous outer layer (f) extends only as far as short stalked region between growing bud and C-form. Note dense (d) and transparent (t) layers of bud cell wall.* ×*78,300.*

FIG. 16. *Mature R-form apparently detached from C-form. Note that the latter appears intact and that septum on stalk (St) is complete. Fibrous layer (f) extends only as far as stalk.* ×*90,000.*

tion of disaggregated coccoid cells or intact C-forms was observed in slide cultures. Our results suggest that the C-form is incapable of multiplying in CB medium and eventually lyses.

Ultrastructure of the R-form. Motile R-forms had polar tufts of one to four flagella, three being the common number observed (Fig. 20, 21). Examination of sections confirmed that the most striking difference between the C-form and R-form was in cell wall structure (Fig. 22). R-form cell walls had an inner transparent membranous layer (10 to 12.5 nm) and an outer dense diffuse layer (7.5 to 10 nm). The outer cementing layer of the C-form was absent. Cells accumulated large quantities of storage inclusions which appeared to be more transparent than those found in the C-form. Composition of the inclusion is unknown. Large mesosomes were common; their function is unknown, but it may be speculated that they play a role in budding. The R-form multiplies exclusively by the budding process. Buds developed as polar to subpolar outgrowths. Mother and daughter cells were joined by a slender stalk (Fig. 23) which was easily distinguished by phase-contrast microscopy as a transparent region between cells (Fig. 4, 5). A septum formed on the stalk upon maturation of the bud (Fig. 24, 25). Parent cells retained the stalks after detachment of buds (Fig. 23). Short chains of two to three buds were sometimes formed. The considerable variation in size and shape noted in populations of actively growing R-forms may be a direct consequence of budding reproduction. Daughter cells were consistently

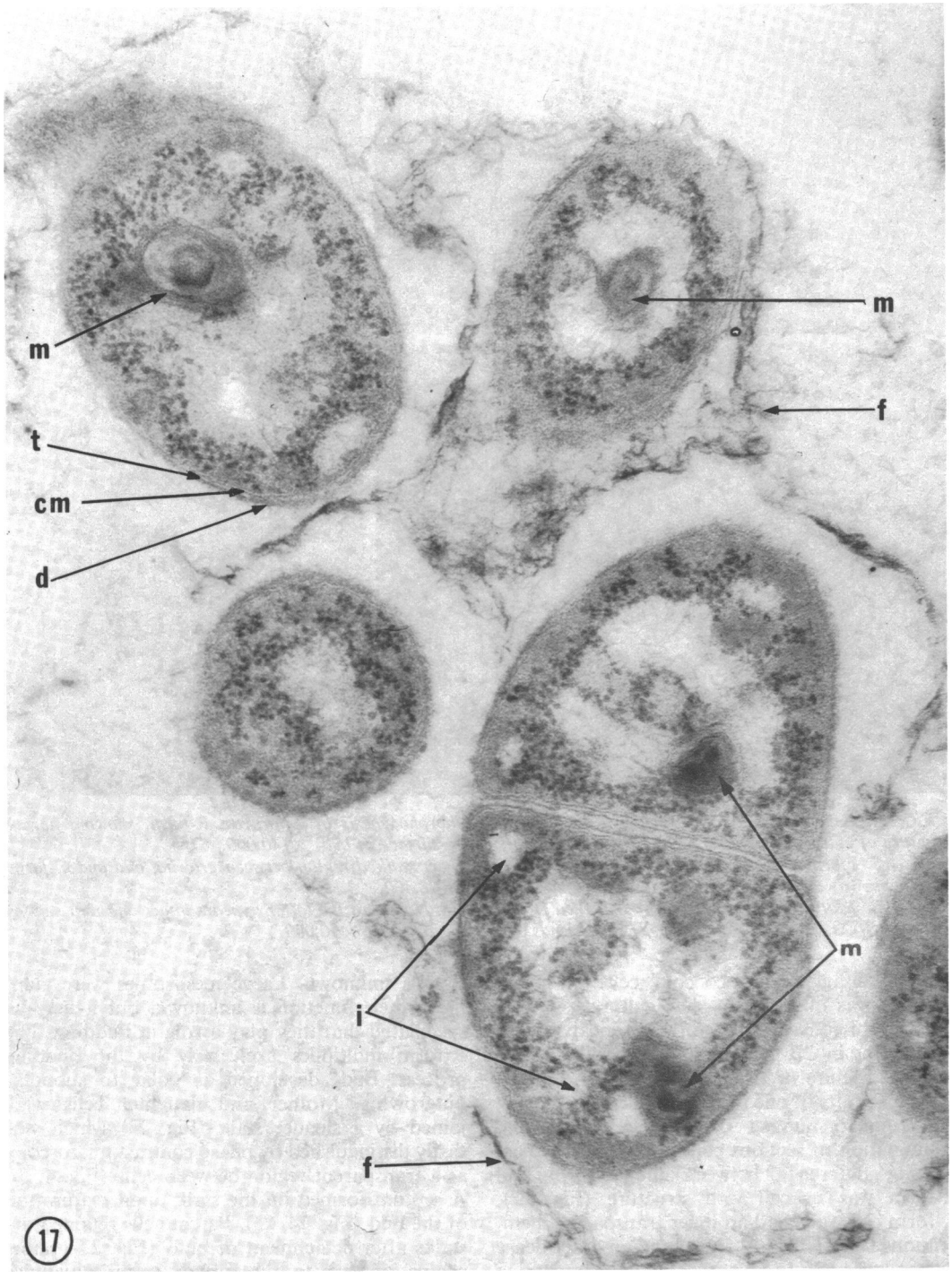

FIG. 17. *Disaggregation of C-form apparently caused by degradation of outer fibrous layer (f). Cells are not lysed. Dense (d) and transparent (t) cell-wall layers are visible. Note well-defined ribosomes.* ×*69,600.*

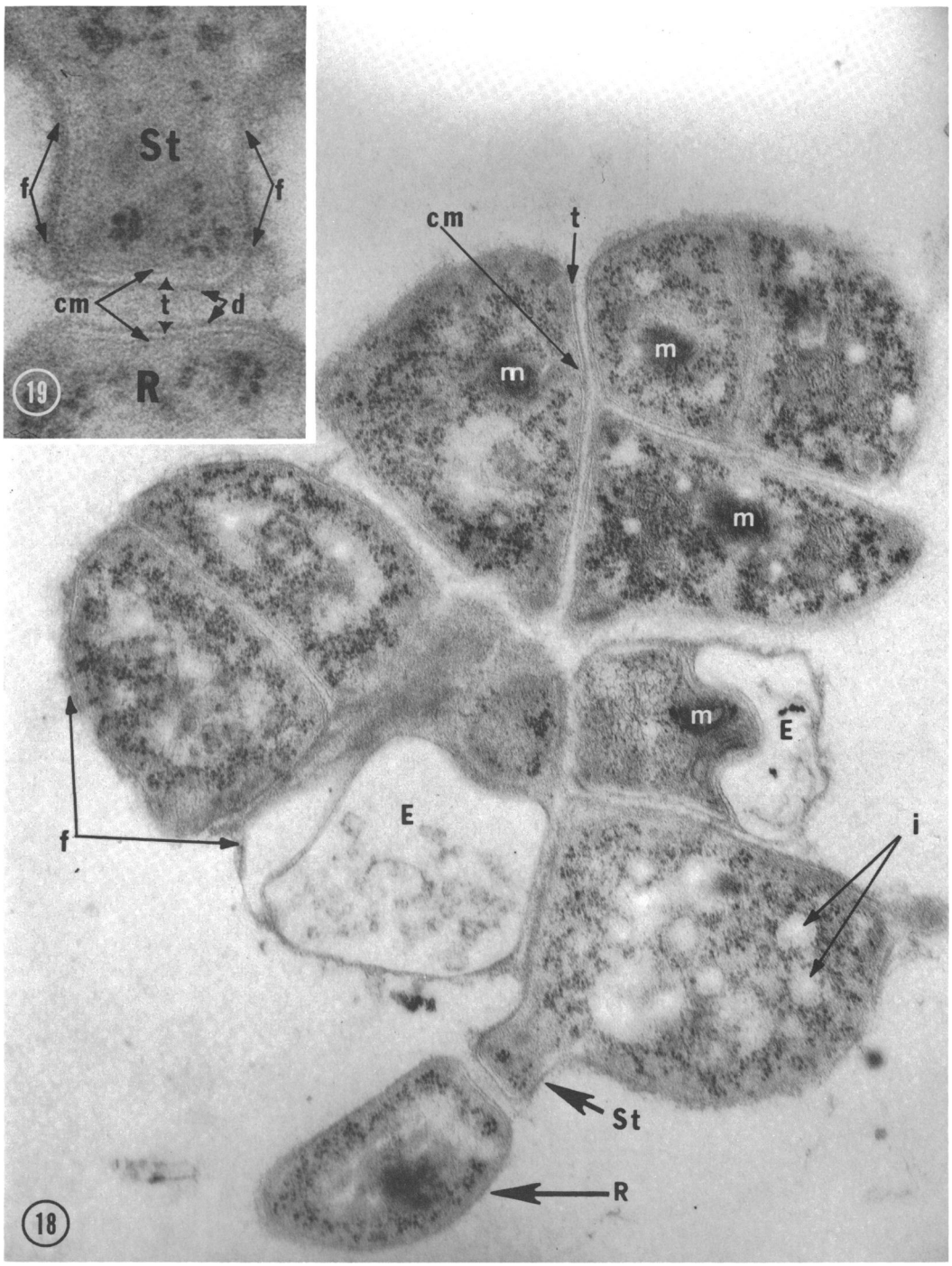

FIG. 18. *C-form in process of disaggregation. Only traces of fibrous layer (f) are present and coccoid cells are beginning to separate. Note degenerate cells (E) and distinct ribosomes.* ×*43,000.*

FIG. 19. *Enlargement of area in which a mature R-form (R) has apparently detached. Note completed septum on stalked region (St) of C-form. Cell walls of C- and R-forms are identical except for remnants of fibrous layer (f) in the former.* ×*114,000.*

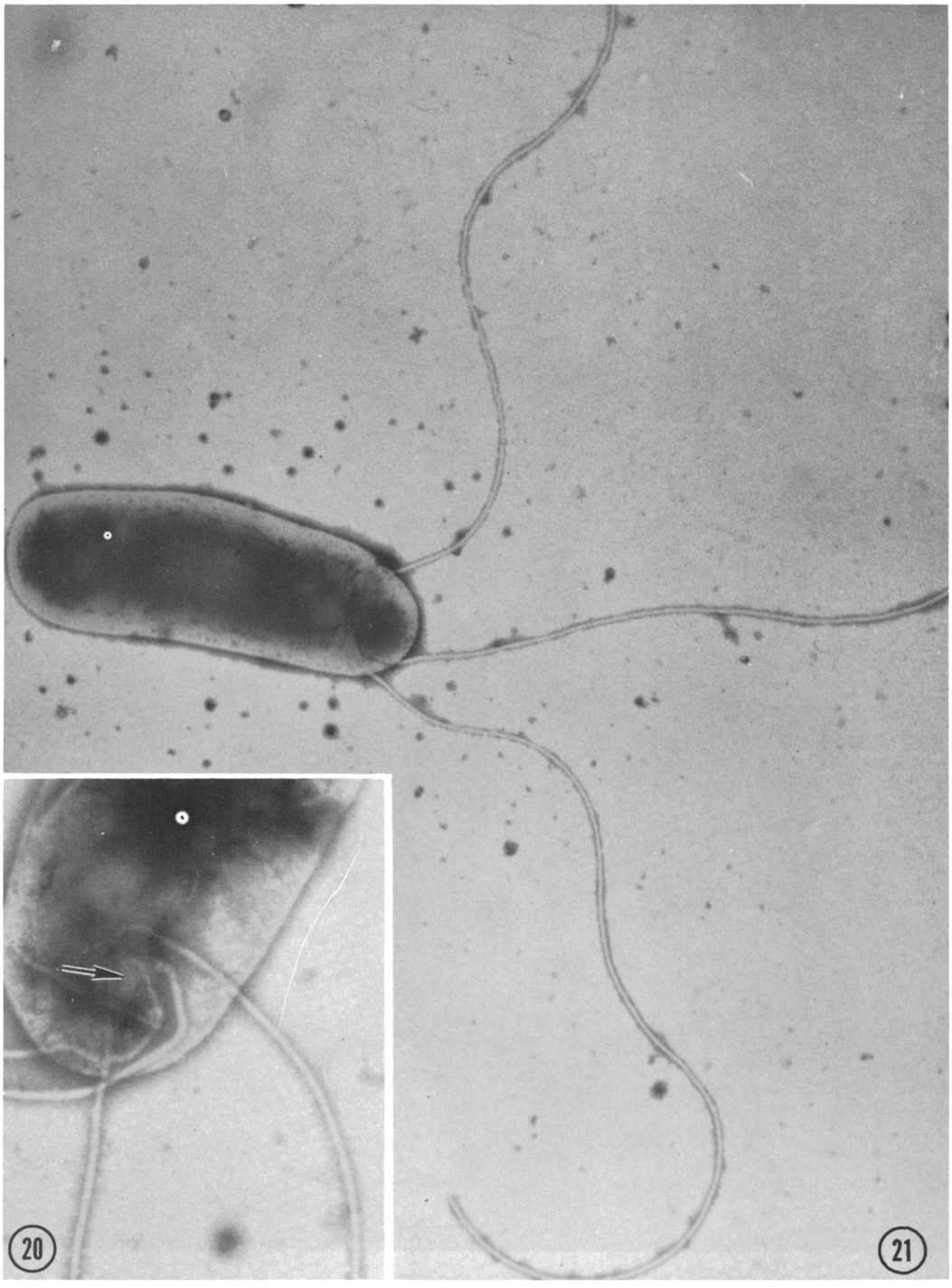

FIG. 20. *Autolysed 20-hr-old R-form showing polar insertion of four flagella. Note hooked flagella termini and basal area (arrow). Negatively stained with 2% potassium phosphotungstate. ×69,200.*

FIG. 21. *R-form (16-hr culture) with polar tuft of three flagella. Negatively stained with 0.02% potassium phosphotungstate. ×45,400.*

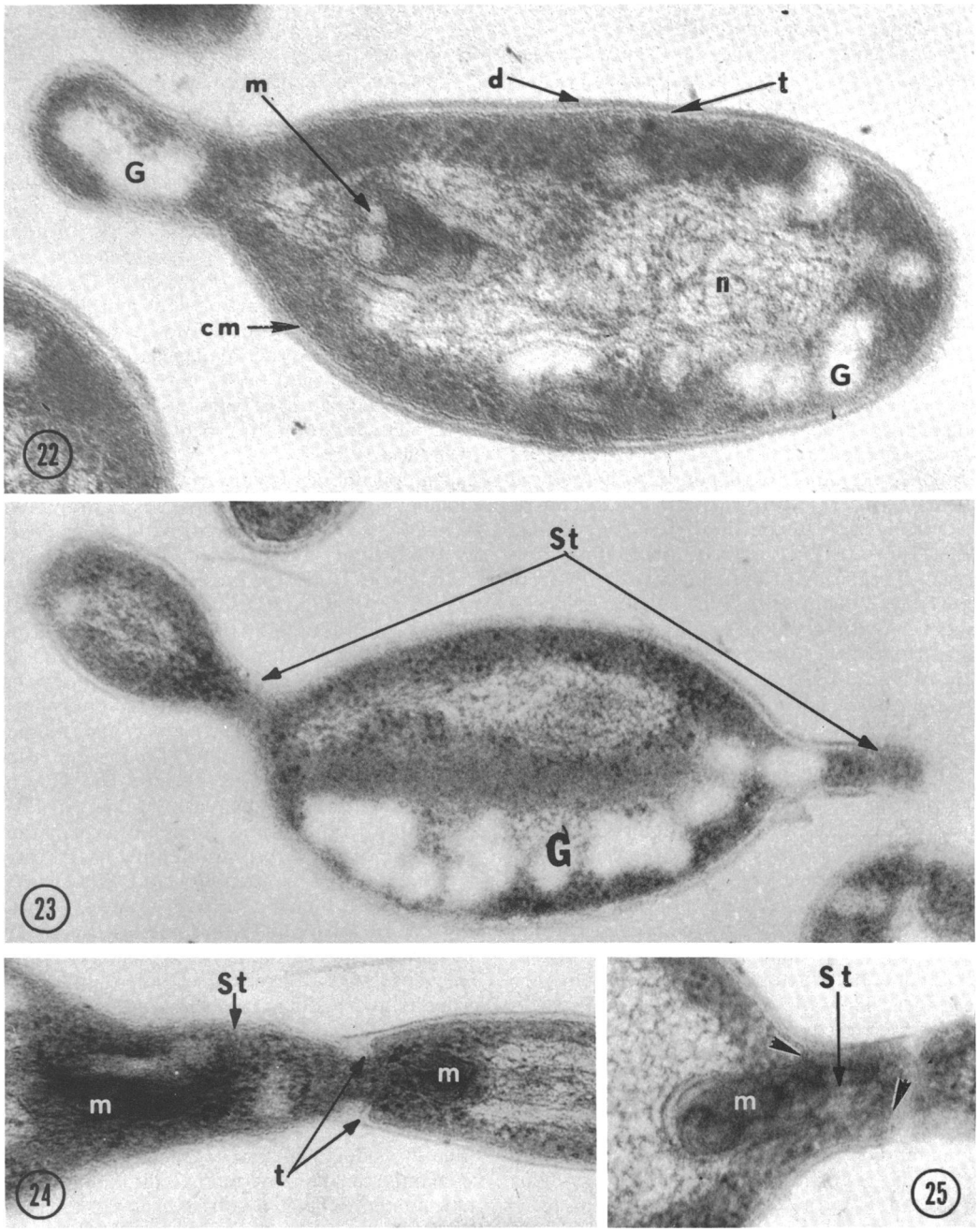

FIG. 22. *General features of R-form (24-hr culture). Dense cytoplasm contains transparent inclusions (G), large mesosome (m), and fibrillar nucleoplasm (n), and is bound by cytoplasmic membrane (cm) and cell wall consisting of dense outer layer (d) and transparent inner layer (t). Note beginning stage of budding. ×82,300.*

FIG. 23. *Bud development at end of stalk (St) on 72-hr-old R-form. Note stalk after detachment of bud. ×61,200.*

FIG. 24. *Septum formation on stalk (St). Note invagination of inner transparent wall layer (t) and densely stained mesosomes (m). Parent cell is on left. ×68,000.*

FIG. 25. *Stalk with completed septum. Note attachment (arrowheads) of mesosome (m) to cytoplasmic membrane. Parent cell is on left. ×68,000.*

smaller than parent cells, a situation analogous to that reported in *Rhodopseudomonas* (17).

Ultrastructure of R-form to C-form morphogenesis. R-form cells (20-hr inoculum) used in these experiments were ultrastructurally identical to that shown in Fig. 22 except that few showed signs of budding. They were enlarged and contained granular storage inclusions 8 hr after transfer to TYB (Fig. 26). Also evident was the fibrous outer cell wall layer. Dense amorphous material was commonly observed and may have originated from the growth medium. This material adhered to the cell surface, suggesting that the outer layer is of an adhesive nature. Formation of one to three transverse septa was noted in cells sampled at 20 hr (Fig. 27). At 30 hr, septation had begun along the longitudinal axis of the compartmentalized cell (Fig. 28). Increase in cell mass is reflected in the OD_{660} (Fig. 2, curve D) during this period. Thus, fission initially followed a definite pattern with septation first occurring transversely and then along the longitudinal plane. The initial cell aggregates resulting from continued septation of the differentiating R-form were typically elongated (Fig. 29). In advanced stages, septation occurred in all planes (Fig. 30). The carbon replica shows that 48-hr cell aggregates are extensively septated and are two cell layers thick. The first C-forms arose by separation of these aggregates (Fig. 29, 30).

DISCUSSION

Geodermatophilus represents still another example of microbial diversity. The organism can be cultured in two major morphological forms. Our present evidence suggests that morphogenesis is controlled by a single unidentified nutritional factor. This allows us to speculate on the morphology of the organism in nature. Ecological niches containing factor M would be selective for the C-form. How M works is unknown. Purification and characterization of this factor is in progress. It is known that the C-form differentiates to the R-form in its absence. We have never observed more than one bud formed per coccoid cell during this process. Furthermore, the C-form is apparently reproductively dormant after giving rise to the R-form and eventually lyses. Thus C-form to R-form differentiation cannot technically be regarded as a means of reproduction since only one cell capable of reproduction is formed. There seems to be no obvious reason for the behavior exhibited by the C-form during this stage since it does not represent a resting stage in the growth cycle. One possibility which explains our observations is that factor M represents a growth requirement for the C-form. If so, differentiation to the R-form in absence of M may be considered as a survival mechanism on the part of the organism.

This study has confirmed and extended a report on the morphogenetic growth cycle of *Geodermatophilus* (Ishiguro and Fletcher, Bacteriol. Proc., p. 24, 1966). We have shown also striking similarities in the ultrastructures of this organism and *Dermatophilus* (3), thus providing support for Luedemann's (8) taxonomic work. In addition, resemblances with *Corynebacterium ovis* (4) and isolates from leprosy cases (2, 6) are noteworthy. Disregarding obvious morphological differences, C- and R-forms can be distinguished by at least three criteria: flagellation, mode of multiplication, and cell envelope structure. Apparent differences in types of storage inclusions awaits confirmation by chemical identification of these structures.

Multiplication is exclusively by fission and by budding for C- and R-forms, respectively. We have adopted the definition of budding proposed by Whittenbury and McLee (17) since it clearly distinguishes this process from asymmetric growth and fission. The distinction is that buds are always produced at tips of slender tubes on the parent cells. R-form multiplication and C- to R-form morphogenesis obviously conform to this definition since both events involve short, stalked regions between daughter and mother cells. The term "germination" (8) for the latter process appears incorrect because the C-form remains intact and apparently viable, at least for a short period of time. A possible explanation for failure of the C-form to multiply has already been mentioned. Whittenbury and McLee (17) noted that all budding bacteria examined up to the time of their publication possessed complex membrane systems along the cell periphery. They suggested a direct correlation between this property and budding reproduction, since binary fission would have to result in a drastic reorganization of intracellular structure. *Geodermatophilus* represents an exception since peripheral membrane systems have not yet been detected. Finally, it should be noted that the stalked regions distinguish budding in this organism from hyphal branching in other actinomycetes, although it may not be difficult to observe how one could have evolved from the other. A similarity in these processes was suggested by Luedemann (8).

Difficulty was encountered in resolving C-form cell wall structure, and it may be more than coincidental that similar problems were found in *Dermatophilus* (3). Understanding of this structure was obtained largely from observations made on disaggregated C-forms. Our results

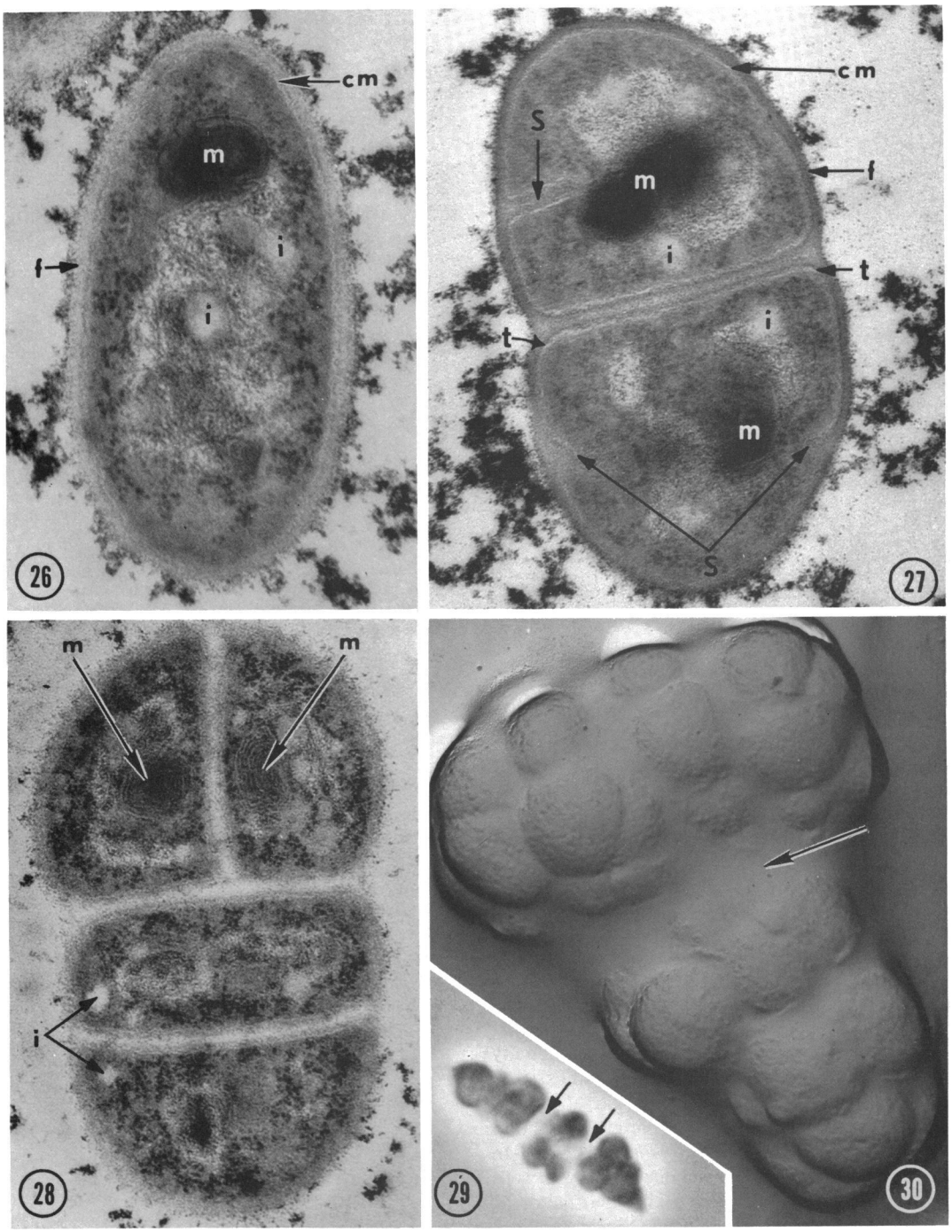

FIG. 26. *R-form differentiating to C-form after 8 hr in TYB medium. Note granular inclusions and fibrous outer layer (f). Dense amorphous material around cells was commonly observed during this stage.* ×*62,800.*

FIG. 27. *Differentiating R-form after 20 hr in TYB medium. Note transverse fission (S).* ×*51,900.*

FIG. 28. *Differentiating R-form after 30 hr in TYB medium. Note fission along longitudinal axis of cell.* ×*45,400.*

FIG. 29. *Differentiating R-form after 48 hr. Note that cell aggregate is elongated and is separating into three roughly equal fractions (arrows). Phase contrast,* ×*3,300.*

FIG. 30. *Carbon replica of 48-hr-old differentiating R-form. Elongated aggregate is two cell layers thick and apparently undergoing separation (arrow). Note uniform arrangement of cells.* ×*28,600.*

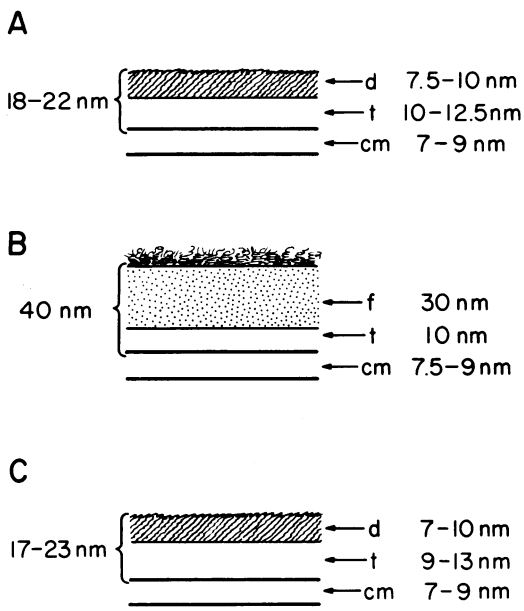

FIG. 31. *Diagram of cell envelope structures of (A) R-form, (B) C-form, and (C) disaggregated C-form, showing relationship of cell wall layers (d, t, f) and cytoplasmic membrane (cm).*

show that C-form and R-form cell walls are ultrastructurally identical except for the cementing layer in the former. A comparison of cell wall structure of each form is diagrammed in Fig. 31. The chemical structure of both cell walls is being examined; we have not yet found significant differences. However, in view of the ultrastructural findings, the possibility that the cementing layer was lost during preparation of C-form cell walls is being investigated. Degradation of this layer may undoubtedly occur under certain conditions, causing disaggregation of the C-form. Although this process is thought to be enzymatic, we have been unable to demonstrate such activity in cell-free extracts and in culture fluids. The cementing layer is similar in ultrastructural appearance, location, and apparently function to the capsule of *Dermatophilus* (3, 12). In *Geodermatophilus*, this layer occurs only in cells grown in TYB medium, suggesting that factor M plays an important role in its biosynthesis; it does not appear to be a simple capsule. It surrounds each coccoid cell as shown by transparent regions between cells and apparently has a certain degree of structural rigidity. In this respect, Roberts (12) has observed that the capsule of *Dermatophilus* possesses rigidity unusual for such structures. Further studies are required to determine whether these similarities in properties reflect a similarity in chemical structure.

Addendum. A report of a new budding bacterium isolated by Ahrens and Moll from Baltic Sea water appeared after this manuscript had been submitted for publication (1). Micrographs and description of morphogenesis of this isolate suggest that the organism is a species of *Geodermatophilus*.

ACKNOWLEDGMENTS

We thank B. V. Hall for use of the facilities of the Central Electron Microscope Laboratory and R. L. Uffen, B. C. McBride, S. C. Holt, and N. Pfennig for helpful discussions. We thank Judith Murphy for invaluable technical advice.

This work was supported by Public Health Service training grant GM-510 from the National Institute of General Medical Sciences and by National Science Foundation grant GB-8304.

LITERATURE CITED

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