Fine Structure of Selected Marine Pseudomonads and Achromobacters

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The fine structure of more than 20 marine pseudomonads and more than 15 achromobacters was examined. Under the conditions extant, clear differences between members of these two groups were seen. The pseudomonads displayed the characteristic gram-negative morphology: the cell wall was irregularly undulant and the cytoplasmic membrane more nearly planar, ribonucleoprotein (RNP) particles were loosely packed throughout the periphery of the cytoplasm, and the deoxyribonucleic acid (DNA) was axially disposed. Cell division appeared to be by constriction. Some strains characteristically produced evaginations or blebs of the cell wall. Occasionally, thick, densely stained ring structures were seen which are possibly analogous to mesosomes. In contrast, the achromobacters demonstrated a regularly undulant outer cell wall element and a planar inner wall. The cytoplasmic membrane was thin and not readily observed. RNP particles were densely stained and tightly packed in the cytoplasm; the DNA was most often lobate in disposition. Cellular division was mediated by the formation of a septum which consisted of the cytoplasmic membrane and the inner element of the cell wall. Mesosomes were observed in all of the strains examined. Dense inclusion bodies were also seen in many strains.

The majority of the aerobic and heterotrophic bacteria isolated from the marine environment during the past 100 years have been identified as gram-negative, asporogenous rods from two major groups, the pseudomonads and the achromobacters. Numerous studies of the physiology, biochemistry, and light microscope morphology have been performed on these two groups. Information concerning their fine structure, however, is extremely limited. Only a few strains of marine pseudomonads have been examined with an electron microscope (4–6, 11, 20), and none of the marine achromobacters has been investigated. On the basis of his examination of a very few strains (pseudomonads), Brown (5) concluded that the fine structure of most gram-negative marine bacteria was similar to that of terrigenous forms and that a separation between organisms from these two environments could not be made on the basis of differences in fine structure. However, the few strains examined represented so limited a variety of types that such general conclusions appear unwarranted at present.

In recent years, the attempts of many investi-

gators to correlate structure with function have stimulated additional interest in the fine structure of bacteria. For example, McCarthy (16) demonstrated by combined electron microscopy and biochemical methods that magnesium starvation of growing Escherichia coli produced a drastic reduction in the number of ribonucleoprotein (RNP) particles present in the cytoplasm.

The highly saline milieu in which they exist renders marine bacteria of particular interest with regard to the correlation of structure with function. Such an environment might be expected to favor the appearance of structural adaptations to accommodate to problems of ion exchange and balance. This expectation is mitigated, however, by the fact that some isolates require high levels of seawater salts for growth and for the prevention of cell lysis, whereas others, equally capable of growth in seawater and taxonomically similar to the isolates with high salt requirements, require levels of salts on the order of those estimated for some terrestrial bacteria (17).

The purpose of this study was to examine the fine structure of a relatively large number of heterotrophic bacterial isolates from marine sediments, including both seawater "requirers" and "nonrequirers", in order more clearly to establish

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the morphological variation within individual groups and between different groups of pseudomonads and achromobacters. This study, incidentally, provides the first comparison of fine structure between pseudomonads and achromobacters.

A taxonomic examination of 366 aerobic and heterotrophic bacterial strains, isolated from sediments off the Oregon and Washington coast, revealed that pseudomonads and achromobacters constituted over 80% of all isolates examined (W. J. Wiebe, Ph.D Thesis, Univ. of Washington, Seattle, 1965). Provisionally, based on the general descriptions in *Bergey's Manual* and the specific studies on marine bacteria by Shewan (22), Colwell (Ph.D. Thesis, Univ. of Washington, Seattle, 1961), and Liston (Ph.D Thesis, Aberdeen Univ., Scotland 1957), most of the pseudomonads were placed in the genus *Pseudomonas* and the achromobacters in the genus *Achromobacter*. Results of an Adansonian (numerical taxonomic) analysis, utilizing 117 coded characters (including morphological, physiological, and biochemical criteria), demonstrated a clear separation between these two groups (Wiebe and Liston, *unpublished data*). Species designations within either genus have not been assigned at this time, but the *Pseudomonas* strains were divided on the basis of the numerical analysis into several subgroups. The considerable overlap between some groups indicates that a more extensive examination is necessary before meaningful species epithets can, or should, be applied.

MATERIALS AND METHODS

Isolation of cultures. The cultures used in this study were isolated during a series of cruises on the University of Washington College of Fisheries research vessel M. V. Commando, which were conducted by the Exploratory and Gear Research Division of the Bureau of Commercial Fisheries in Seattle, Wash., under the direction of D. Alverson. Sediment samples were obtained by use of a small geological corer at all stations except Point Harrington and Buoy 8. At these two stations, a Smith-MacIntyre grab (23) was used, since the sandy sediment in the region was not adequately penetrated or retained by the corer. Upon retrieval of the samples, the upper sediment layer and immediately overlying water were placed in a sterile container for quantitative analysis. Agarplate viable-count procedures were performed as soon as feasible and never more than a few hours after sampling. The following medium was used: 0.1% peptone (Difco), 0.1% yeast extract (Difco), artificial seawater (15), and 1.1% agar; the medium was adjusted to pH 7.4 with 1 N NaOH. (The medium is abbreviated SWA.) After incubation at 0 to 2 C for up to 1 month, colonies were selected by a random designation method from the viable-count plates, streaked on homologous agar, and subsequently passaged

three times to insure purity of individual clones. Cultures were stored on SWA slants at 0 to 2 C. The complete methods are presented elsewhere (Wiebe and Liston, *in press*).

Classification procedures. The morphology, physiology, and biochemistry of the representative isolates were examined, and the data were analyzed by numerical taxonomic procedures (13, 24, 25).

Growth of cells for electron microscopy. Strains were grown in the seawater medium (SWA) without agar at 10 C on a roller rotator (Cole-Parmer Instrument & Equipment Co., Chicago, Ill.) for 24 hr, passaged, and reincubated at 10 C for 24 hr.

Electron microscopy techniques. After the second period of growth at 10 C, the cells were initially fixed in 0.1% osmium tetroxide for 30 min, centrifuged slowly, and then fixed in 1.0% osmium tetroxide by the Kellenberger-Ryter method (13) for 16 hr at room temperature. In addition to the standard ingredients, 0.25 ml of 0.1 M MgCl₂ was added to each 25 ml of fixative. After fixation, the cells were washed twice (1 hr each wash) in 0.5% uranyl acetate in the modified Kellenberger buffer. Dehydration was performed in the following sequence: ethyl alcohol—50, 70, 85, 95, 100, and 100%, each for 20 min; propylene oxide—two changes of 15 min each. The cells were infiltrated and embedded in Epon by the method of Luft (14), by use of no. 0 gelatin capsules and a temperature of 60 C for 24 hr. Ultrathin sections were cut with both glass and diamond knives in Sorvall Porter-Blum MT-1 and MT-2 ultramicrotomes. Sections were floated on distilled water, collected on collodion-coated, 200 mesh copper grids, stained with saturated aqueous uranyl acetate, for 1 to 3 hr at room temperature, after the method of Watson (29), or with 0.2 to 0.4% lead citrate and saturated aqueous uranyl acetate, following the method of Venable and Coggeshall (28), and examined in an RCA EMU-2D electron microscope, fitted with a 0.015-inch externally centerable (Canalco) condenser aperture and an approximately 50-μ aperture in the standard objective pole piece.

RESULTS AND DISCUSSION

Pseudomonas. Most of the more than 20 marine strains of the genus *Pseudomonas* examined displayed the characteristic fine structural features of terrestrial gram-negative rods (Fig. 1). The cell wall is irregularly undulant, and the cytoplasmic membrane is more nearly planar. Ribonucleoprotein (RNP) particles are densely stained and loosely packed throughout the periphery of the cytoplasmic matrix in a seemingly random fashion. The deoxyribonucelic acid (DNA) is axially disposed. It consists of a fine fibrillar network of strands approximately 20 A in diameter. In some strains, it tended to condense into thick strands, and in others it remained evenly dispersed. Dividing cells (Fig. 2) displayed a constrictive or pinching off mode of division. Although this mode of division in the past has been advanced as characteristic of gram-negative bacteria, Steed and
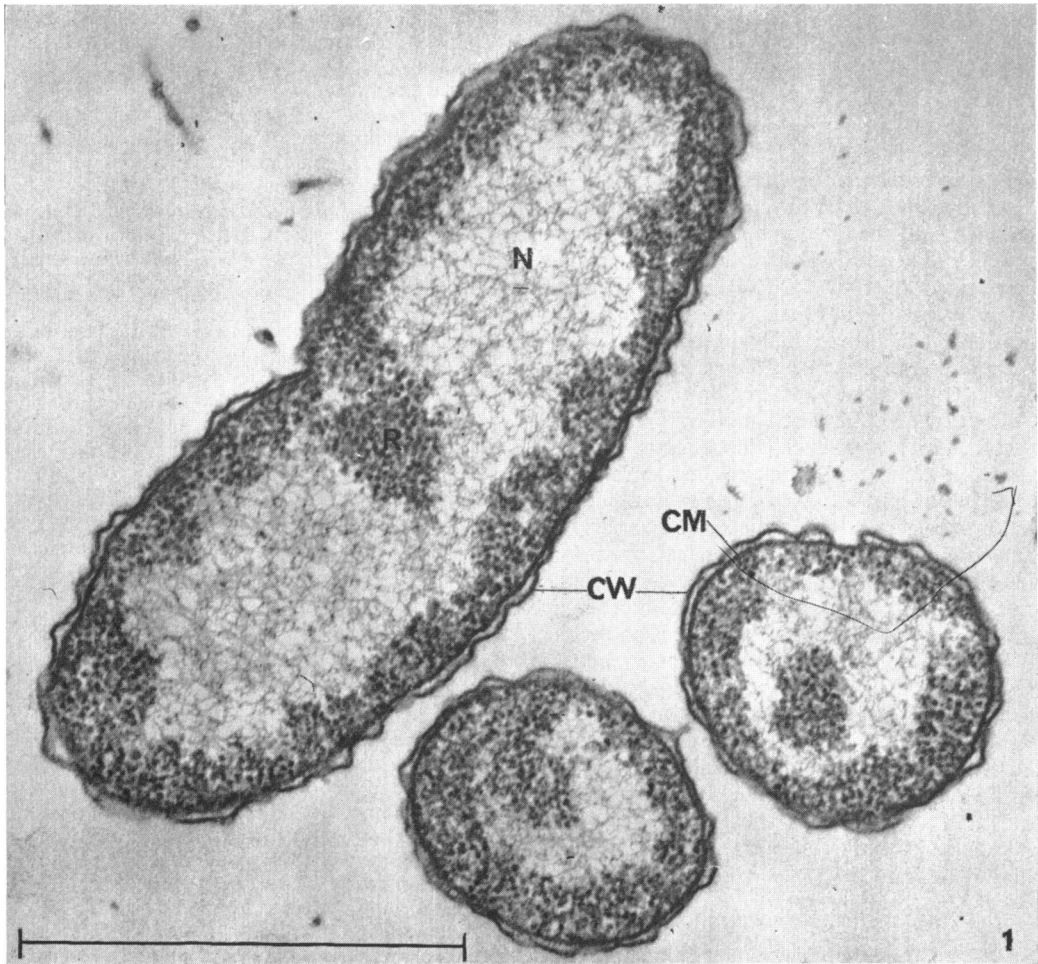
FIG. 1. *Characteristic cytology of the marine Pseudomonas spp. Cell wall (CW), cytoplasaic membrane (CM), RNP particles (R), and the axially oriented nuclear region (N) are seen. Pseudomonas strain 6-150-8.* × *57,300.*

Murray (26) recently demonstrated that, under specific physiological conditions, the majority of cells of a strain of *E. coli* and a strain of *Spirillum serpens*, both gram-negative organisms, divided by the initial formation of a transverse septum. The authors suggested that this division process, which is similar to that observed for gram-positive bacteria (7), may in fact represent the actual mode of division and that the constrictive division so often observed in electron micrographs of gram-negative bacteria represents an artifact of the fixation procedures. However, since both types of division were observed by them in the same preparation, this question would appear to remain unresolved. Furthermore, using the Kellenberger-Ryter technique (12), they saw transverse septum formation in *E. coli* only when the cells were

grown at supraoptimal (45 C) and suboptimal (30 C) temperatures. In *S. serpens*, transverse septa were observed when the cells were grown at 45 C and fixed according to the Kellenberger-Ryter (12) procedure. At 30 C, *S. serpens* showed septum formation only when fixed in a 1:5 to 1:6 dilution of the Kellenberger-Ryter buffer. The use of temperatures well below and above the optimal (37 C) could result in unbalanced growth, which might induce the formation of atypical membranes, or, as suggested by Steed and Murray (26), such conditions might act to stabilize the very rapidly assembled septum and prolong its appearance. In our studies, some bacterial strains also displayed transverse septum formation under specific physiological conditions, although, again, the constrictive mode of division was also
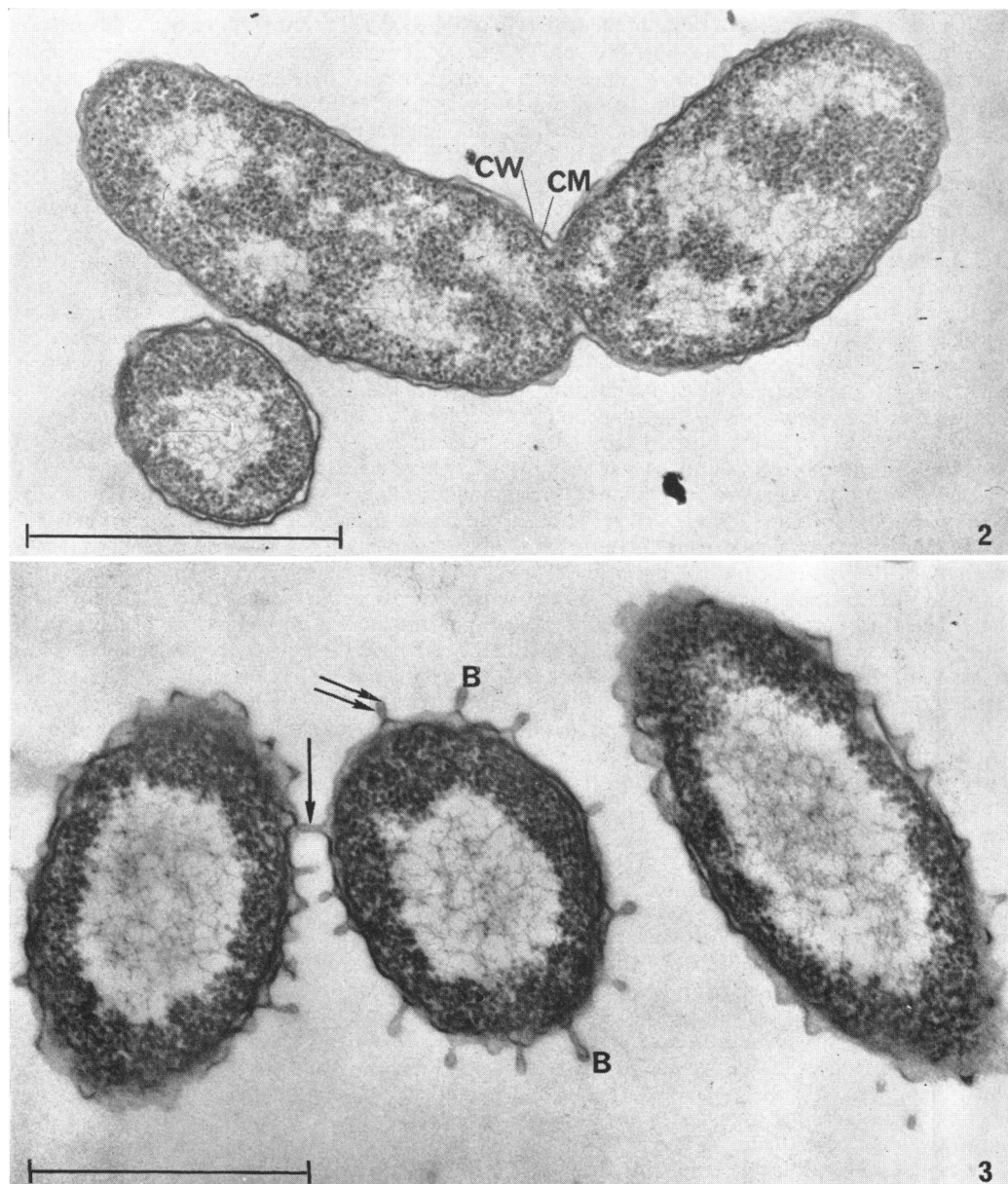
CW
CM
2

B
B
3

FIG. 2. *Dividing cell. The typical constriction type of division is seen. Note that the cytoplasmic membrane (CM) slightly precedes the cell wall (CW) in this process. Pseudomonas strain 5-000-2.* × *41,800.*

FIG. 3. *Formation of bleblike evagination (B) of the cell wall. Some blebs (arrow) appear to be continuous with the cell wall while, in other cases (double arrow), the nature of this relationship is not clear. Pseudomonas strain 5-000-13.* × *36,000.*

observed in the same preparations. This work will be reported in a separate paper.

Less frequently observed was a variant form of the characteristic *Pseudomonas* rod just described. The difference in the fine structure in these strains is the occurrence of bleblike evaginations of the cell wall (Fig. 3). The length of the blebs appeared to vary from strain to strain and there was some length variation within a single strain. These strains regularly produced blebs under growth and

fixation conditions identical to those of the strains which did not form blebs. (The described results were consistent for two, or in some cases, three embeddings.) Comparison between the cell wall fine structure of these two types suggests that the outer visible layer of the double-layered cell wall is most often involved in bleb formation, although the entire cell wall appears to be involved at times. In addition to the "constitutive" bleb-producing strains, other strains produced similar evagination only under specific physiological and nutritional conditions. In these cases, bleb formation clearly represents a phenotypic variation of the wall morphology. Thus, the occurrence of blebs appears to be a phenotypically stable feature in some strains and an "inducible" phenomenon in others. It should be emphasized that bleb formers and nonformers were fixed simultaneously by use of the same reagents and procedures. What function, if any, this structural modification of the cell wall perfoms in the overall economy of the cell is at present uncertain. The increased surface area produced by the formation of the blebs may be of some importance in the ecology of these microorganisms in terms of either physical attachment of cells to particle surfaces, particularly small and irregularly shaped particles, or as an increased surface area for the initial acquisition of substrates. Whether such structures actually exist in growing cells in situ, however, remains to be examined, and the possibility that this phenomenon is an artifact of preservation cannot be eliminated by this study. Nevertheless, it is clear that there are differences in the cell wall composition of closely related *Pseudomonas* spp., because not all strains have as yet been induced to produce blebs and because different strains produce blebs under different growth conditions.

Structures similar to blebs have been observed for several gram-negative bacteria. Bayer (2) suggested that the evaginations of the cell wall which he observed subsequent to osmotically shocking *E. coli* resulted from the escape of cytoplasm through pores (sites of growth) in the mucopeptide cell wall layer, expanding the overlying, more elastic, outer wall layer. Since, in our study, the initial fixation was performed under approximately isotonic conditions, osmotic shock would appear to be an unlikely explanation of the results. Furthermore, as seen by comparing Fig. 4a with Fig. 4b, when the total ionic concentration during fixation was increased by the addition of 1.0 M NaCl to the buffer (Fig. 4b), bleb formation was neither enhanced nor completely suppressed. However, the cell shape was affected, and the RNP particles appeared more densely packed. The latter seems due to the expansion of the nuclear areas to occupy a larger proportion of the total cell volume, with a concomitant reduction in cytosomal volume. Studies in the elucidation of the chemical nature of blebs and their possible function *vis-à-vis* attachment and nutrient acquisition by bacteria should prove of interest.

Clearly, recognizable peripheral bodies (7) or mesosomes (9) were not observed in any of the *Pseudomonas* strains examined under standard conditions. Occasionally, a thick, densely stained ring structure, similar to that seen in *Cytophaga marinoflava* by Valentine and Chapman (27), was observed. Such structures were rarely seen, however, and are not easily equated with the characteristic mesosome structure. When mesosomes have been seen in the gram-negative bacteria, as illustrated by Steed and Murray (26), they are usually manifest as infoldings of the unit membrane and are generally much simpler in structure than those observed for gram-positive bacteria. Within several groups of gram-negative bacteria, for example, the *Caulobacteriaceae*, large mesosomes, as well as, in this case, a membranous organelle located at the juncture of the stalk and the cell, have been observed (22). These, however, represent the exception in the gram-negative bacteria rather than the rule.

In general, the present observations support the conclusion of Brown (5). Of course, there may be other morphological forms of marine *Pseudomonas*, and the morphology, which we observe for strains grown in vitro, may not represent the actual morphology of cells in situ.

Achromobacter. The fine structure of more than 15 strains of *Achromobacter* was examined. Strains in this group displayed several dissimilarities to the *Pseudomonas* (Fig. 5), although they retained the multiple-layer gram-negative cell wall. In contrast to the irregular undulations of the entire wall in *Pseudomonas*, the cell wall of the *Achromobacter* displayed a very regularly undulant outer layer and a dense planar inner layer. This inner layer, in many preparations, assumes such prominence as to constitute one of the most striking morphological differences between these genera. The RNP particles were densely stained and tightly packed in the cytoplasm. The DNA was typically disposed in a lobate fashion, although in some strains it was axially disposed. Mesosomes, often simple in structure (M, Fig. 5), but occasionally complex, were observed in all strains under at least one condition of growth and fixation. An example of the complex type of mesosome is shown at M in Fig. 6. In this cell, the mesosome is located at the site of division and forms a grapelike cluster of spheres when the cell is sectioned in a longitudinal plane.
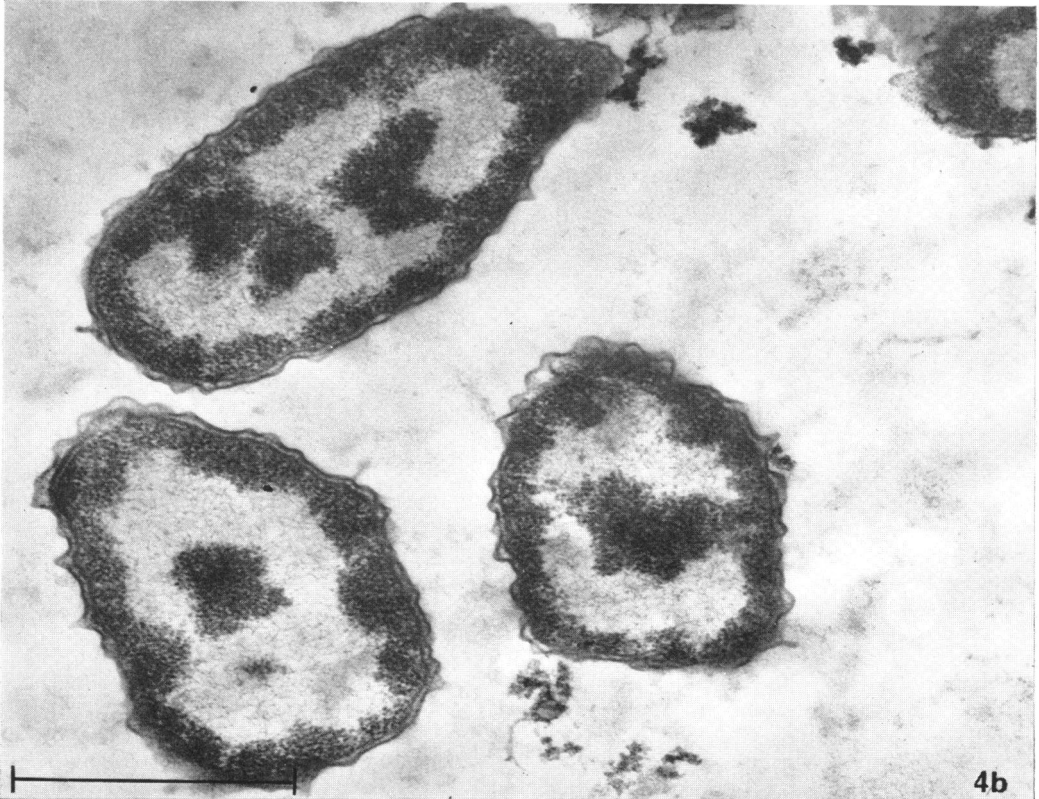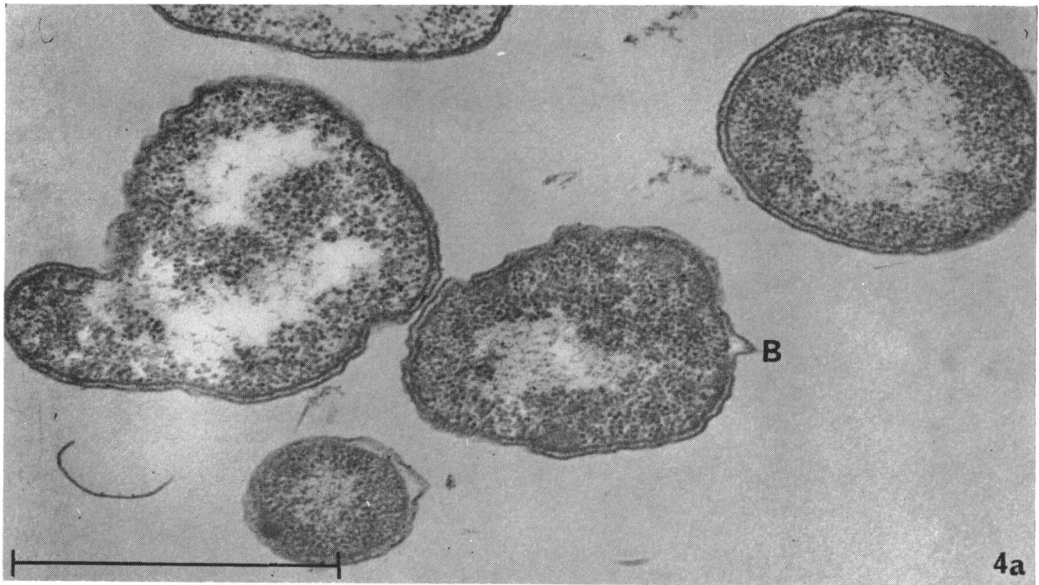
FIG. 4a. *Cells fixed in Kellenberger-Ryter buffer without added NaCl. Cell shape is in some cases altered, but there was little evidence of lysis, and occasional blebs (B) could be seen. Pseudomonas strain 8-B8-15.* $\times$ *41,800.*
FIG. 4b. *Cells fixed in Kellenberger-Ryter buffer in 1 M NaCl. Cell shape, as in Fig. 4a, is affected, but the formation of blebs is not completely suppressed. Pseudomonas strain 8-B8-15.* $\times$ *36,000.*
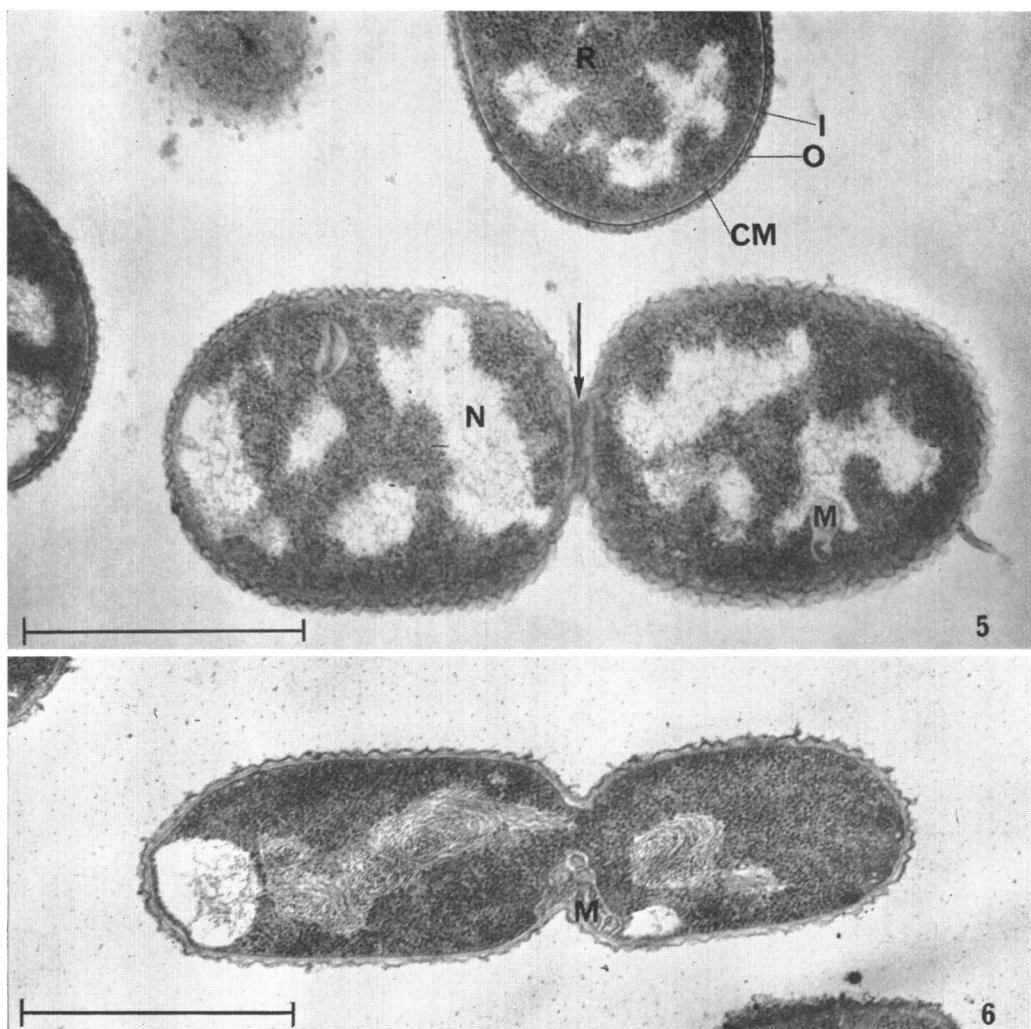
FIG. 5. *Characteristic morphology of the marine Achromobacter spp. The outer element* (O) *and the inner element* (I) *of the cell wall, the cytoplasmic membrane* (CM), *RNP particles* (R), *mesosome* (M), *lobate, electronlucent nuclear region* (N), *and thickened region at the site of division* (arrow) *are seen. Achromobacter strain 5-100-11.* × *36,000.*

FIG. 6. *Complex mesosome* (M) *is seen at the site of division. Achromobacter strain 5-100-13.* × *35,200.*

The regular nature of the outer cell wall undulations is more easily observed in Fig. 7. In this negatively stained preparation, the undulations appear as a "warty" surface contour. Sections of these cells would be expected to yield the wall profiles observed. Every strain of *Achromobacter* examined displayed this type of cell wall, but none of the *Pseudomonas* strains demonstrated this feature. Several strains of bacteria, provisionally classified as *Achromobacter* sp. by J. S. Lee (Oregon State University, Corvallis) and isolated by him from marine fish, were also examined. These strains also displayed the regularly undulant outer cell wall.

The cell walls of several other groups of microorganisms also have been shown to be more or less regularly undulant, the most similar being *Neisseria meningitidis* (10) and, at least in some micrographs, the caulobacters, particularly *Asticcacaulis excentricus* (21). A preliminary examination of the DNA homology between *N. meningitidis* and two strains of *Achromobacter* sp. used in this study has shown a low relationship (D. Kingsbury, *personal communication*). *Caulobacter* strains are easily differentiated from *Achromobacter* by the formation of a stalk. *Micrococcus cryophilus* (19) also displays a regularly undulant cell wall, but it is a gram-positive bacterium and
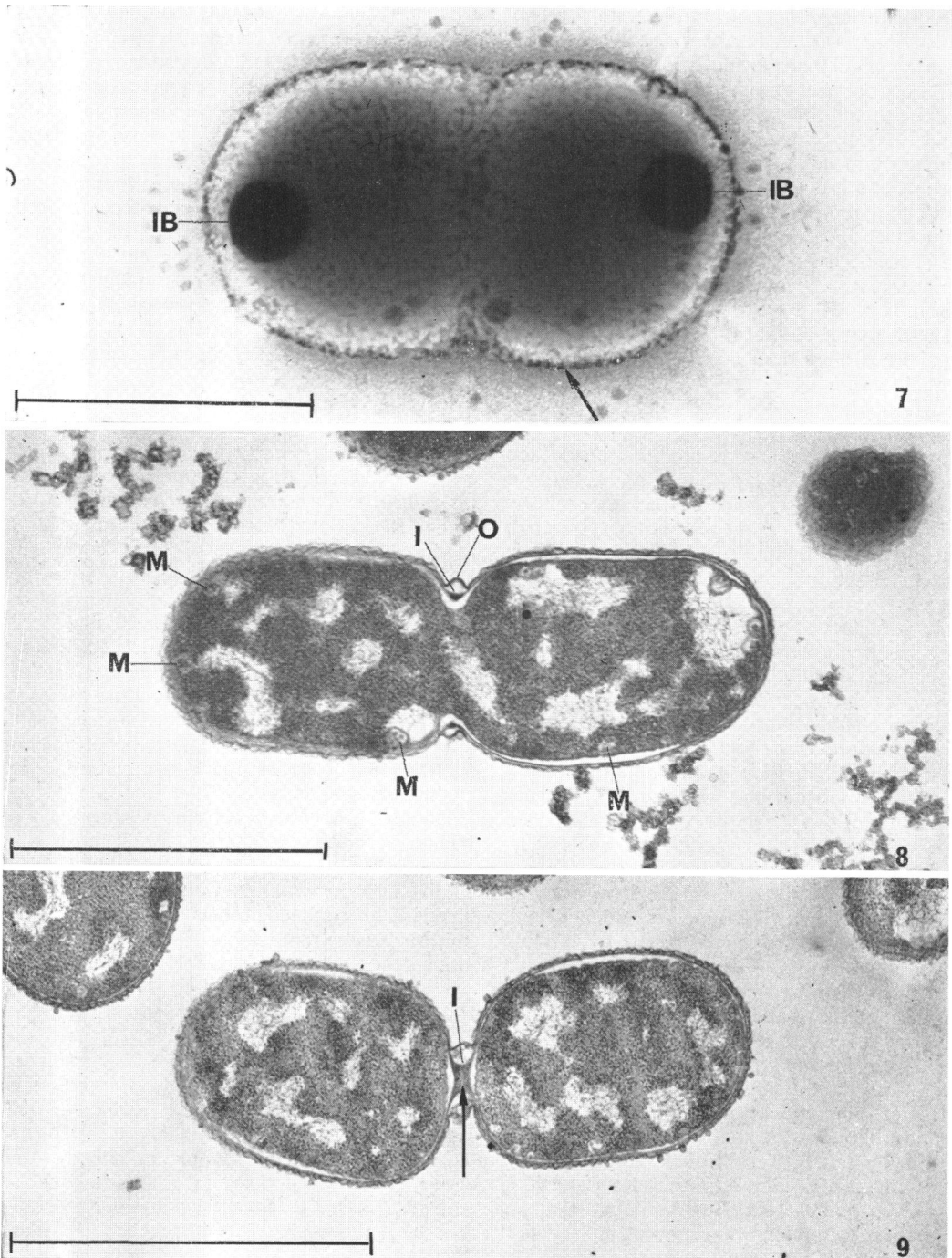
FIG. 7. *Negatively stained Achromobacter. The "warty" cell surface (arrow) and two dense inclusion bodies at the cell poles (IB) can be seen. Achromobacter strain 5-100-2.* × *41,800.*

FIG. 8. *Cell initiating division. The outer element (O) of the cell wall has evaginated, while the inner wall element (I) has thickened and is proceeding centrally. Note several simple mesosomes (M) in this cell. In this case, none is at the division site. Achromobacter strain 5-100-2.* × *44,000.*

FIG. 9. *Further progression in division. The inner wall element (I) has thickened greatly and a fissure (arrow) is seen in the center, indicating that separation is about to occur. Achromobacter strain 5-100-2.* × *51,000.*

is readily distinguishable from the gram-negative genus *Achromobacter*. Furthermore, the periodicity of the undulations is much longer. Occasionally, electron micrographs of other organisms, e.g., *E. coli* and *Simonsiella* sp. (26) show similar undulations, but there does not appear to be any consistency of this structure from section to section in these groups, whereas the *Achromobacter* gives highly reproducible results. Again, as in the case of bleb formation for the *Pseudomonas*, the role of this structure, while of potential ecological significance to the microorganism, remains unknown.

The regular occurrence of *Achromobacter* cells in pairs has often been utilized as an important determinative characteristic of the group. (Although single cells are observed by light and electron microscopy, the predominant cell arrangement is pairs.) As the predominance of the paired state might be expected to be related to a modification of the cell wall at the site of cellular division, special attention was paid to this area. The inner layer of the wall and the cytoplasmic membrane, at the site of division, invaginate, whereas the outer wall layer evaginates, after an initial invagination (Fig. 8). A following step in the sequence is shown in Fig. 9. The inner wall layer at the site of division has thickened, and a fissure, probably indicating that the cells are nearing physical separation, has developed. Mesosomes are often observed (Fig. 10) in association with this division process. In this cell, a channel, reminiscent of a plasmodesma, remains between the two daughter cells, although the division process is nearly complete. Such structural connections are rarely seen, possibly because of the small area involved and the short duration of this connection. The final resolution of this structure is thought to be represented in Fig. 11, in which only a ring of outer wall remains between the cells, which are now almost at the stage of complete separation. It might be suggested that this ring of outer wall material represents a fortuitously located bleb. The fact that this ring is several orders of magnitude larger than any bleb on the cells involved in division tends to rule out this possiblity. It is also unlikely that a bleb would be found precisely at this locus of one cell which bore just this relationship to another cell. It should be pointed out, however, that blebbing and ring formation may represent different degrees of a basically similar process involving the outer wall layer. All strains of *Achromobacter* displayed some cells undergoing this division sequence, but cells were also observed which showed the constrictive or pinching-off type of division. Under low nutrient conditions of standard seawater broth (SWB), division by constriction was infrequently observed. Interestingly, although pairs of cells were common, chains of cells were not, and cells in pairs were seldom seen initiating another division cycle. This suggests, perhaps, that this cell wall structural modification and its dissolution may be involved in the initiation of the cell division process, in the persistence of cell pairs, and in the final separation of daughter cells. In connecton with the last event, the thickened inner wall is apparently reassimilated by the cell or is in some other manner modified since only very rarely were scars observed. Such scars resembled those in budding yeasts (1). In cell pairs which showed constriction, resembling the constrictive mode of division, the nearly completed stage of division was never observed. Constriction, then, may represent an alternate, but real, mode of division, an initial state in the division process, or it may be an artifact of fixation, as tentatively suggested by Steed and Murray (26). It should be noted that the thickened inner wall and ring structure were commonly found in the *Achromobacter* and never observed in the *Pseudomonas* strains. A similarly thickened inner cell wall at the site of division has been observed in at least two other groups: *A. excentricus* (21) and the genus *Chlorobium* (8). Apparently, in these cases, the thickened inner wall also disappears after division (as described for the *Achromobacter*) since no "scar" marks were seen in any of the micrographs.

Several *Achromobacter* strains produced dense inclusions in log-phase cultures. Examples are shown in Fig. 12 and in the negatively stained cell in Fig. 7. The granules were produced under standard growing conditions in this strain. In another strain, more thoroughly examined, the size and frequency of the granules were dependent upon the temperature of growth and on the medium (Wiebe and Chapman, *submitted for publication*). Similar inclusions have been observed in other gram-negative genera (8, 18). For example, Martinez (18) examined the granules of the genus *Spirillum* and concluded that they were composed primarily of poly-β-hydroxybutyrate. Cohen-Bazire et al. (8), in a fine structure study of the green bacteria, called the inclusions poly-metaphosphate granules. Further studies of the granules of *Achromobacter* are in progress. None of the *Pseudomonas* strains produced granules under any of our culture conditions.

In summary, the fine structure of the marine *Pseudomonas* spp. studied differs sufficiently from that of the marine *Achromobacter* spp. to suggest
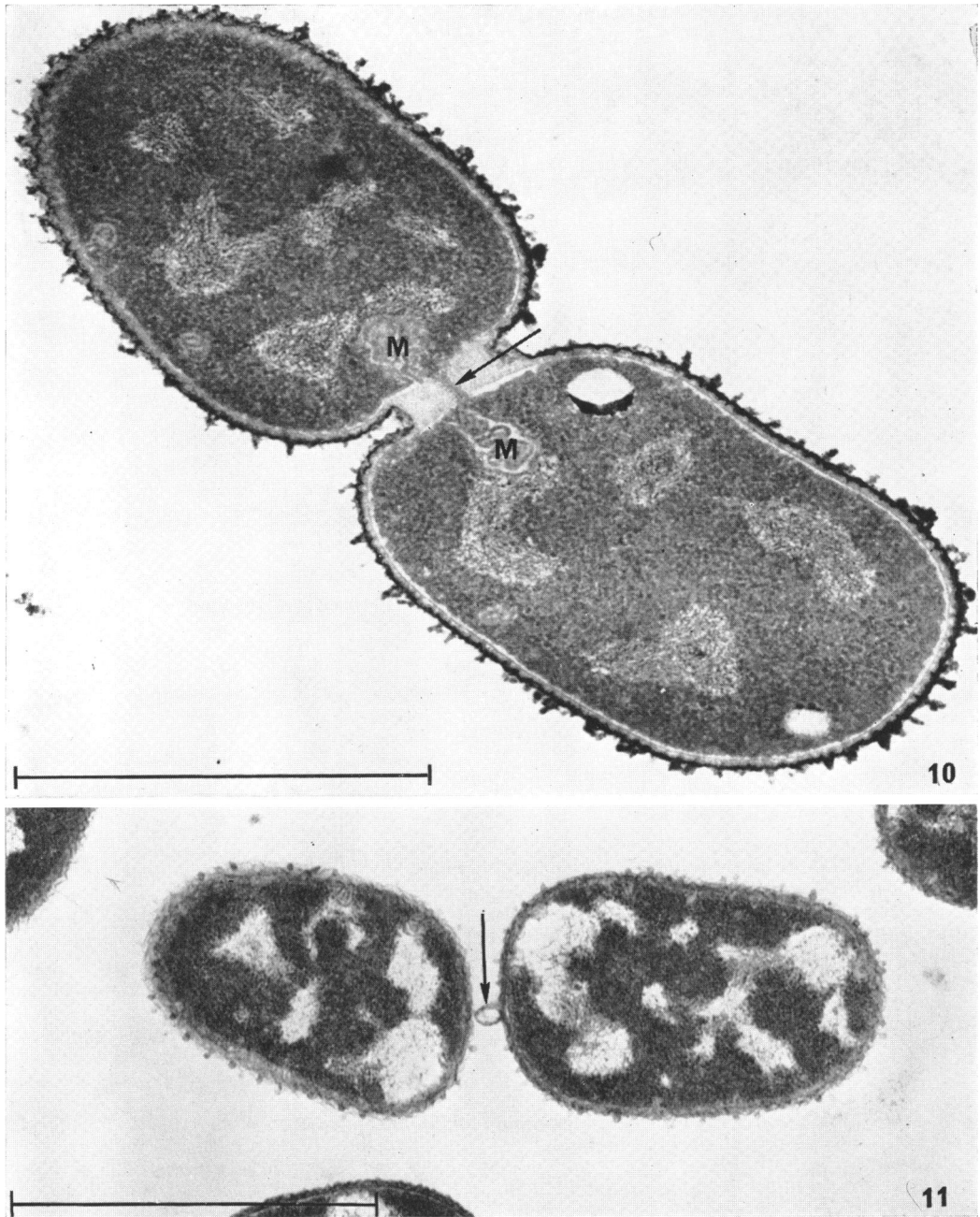
FIG. 10. *Stage similar to that in Fig. 9. In this cell, complex mesosomes (M) are seen at each side of the division site. A connection (arrow) between them may be observed. Achromobacter strain 5-150-4.* X *57,300.*

FIG. 11. *Final stage of division. A small ring-like structure (arrow) is seen at the site of division. Achromobacter strain 5-100-2.* X *51,000.*
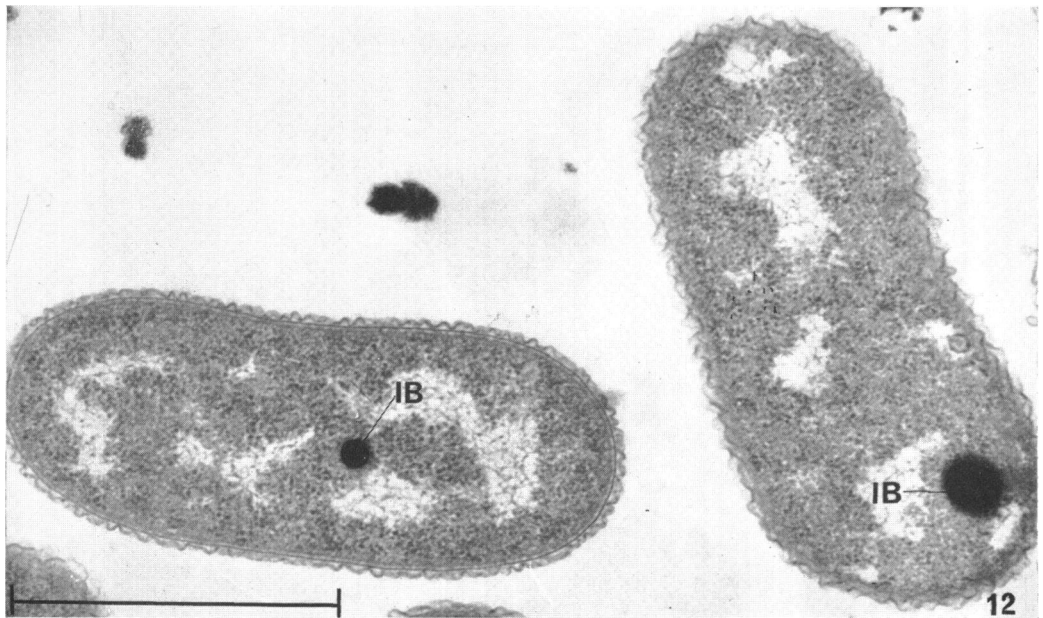
FIG. 12. *Achromobacter sp. with inclusion bodies (IB). Also seen is the "warty" surface structure of the cell on the right. Achromobacter strain 5-150-12.* × *41,800.*

that a separation of the two groups on strictly morphological grounds may be made. Such a separation would be consistent with results of an Adansonian taxonomic analysis.

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