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The Effect of Nickel on a Marine Bacterium, *Arthrobacter marinus* sp.nov.

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SUMMARY

An unbalanced growth situation, resulting in large, plasmolysed spheroplasts (megalomorphs) produced by the addition of nickel to the growth medium of a marine bacterium, *Arthrobacter marinus* sp.nov., is described. The effect of increasing nickel concentrations was gradual. Under the cultural conditions used, a slight effect was noted on size in 1×10^{-4}M-$NiCl_2$, but in 4×10^{-4}M-$NiCl_2$ the cells formed megalomorphs attaining a size of 10 to 15 μm. Cell division did not occur in 5×10^{-4}M-nickel. The lag phase of the culture increased from about 3 h. without added nickel to more than 70 h. at 4×10^{-4}M-nickel. Concurrently, the maximum population decreased as the nickel concentration was increased. The classification of the marine bacterium is described and the organism is designated as *A. marinus* sp.nov.

INTRODUCTION

There are few criteria which distinguish marine from fresh-water bacteria. The ability of marine bacteria to survive and grow in the ocean is their most salient feature (Jones, 1968). The presence of heavy metals in the oceans is one of the factors limiting the survival of terrestrial bacteria in the marine environment (Scarpino & Pramer, 1962; Jones, 1964). In the course of a survey to compare marine and fresh-water bacteria for their tolerance to nickel ions, an unusual morphological response was noted in several of the marine micro-organisms (Wirsen, 1966). The most unusual of these organisms and its megalomorphic response to these metal ions is described in this paper.

METHODS

Organisms and growth media

Arthrobacter marinus sp.nov. was isolated from a littoral water sample at Woods Hole, Massachusetts. The unusual morphological response was observed initially in cultures grown on a modified marine medium, 2216E, of Oppenheimer & ZoBell (1952) with 1×10^{-3}M-Ni^{2+} as $NiCl_2$ (Wirsen, 1966). Subsequent investigations were performed on a stock culture not previously grown in the presence of added nickel. Stock cultures were transferred every 6 weeks to fresh slants of marine agar 2216 (Difco Laboratory, Detroit, Michigan, U.S.A.), incubated at ambient temperature (23 to 27°) for 24 h. and stored at 4 to 6°. This organism has been deposited in the American Type Culture Collection as ATCC25374.

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The type species of the genus, *Arthrobacter globiformis* ATCC 8010, was obtained and the stock culture maintained as above on slants of trypticase soy agar (Baltimore Biological Laboratory (B.B.L.), Baltimore, Maryland, U.S.A.).

The basal medium used in demonstrating the megalomorphic response contained Bacto-peptone 0·5 g.; Bacto-yeast extract 0·5 g.; ferric ammonium citrate 0·01 g.; 75% synthetic sea water (Lyman & Fleming, 1940), 1000 ml. The final pH was 7·8 to 8·0. A 1·0M aqueous solution of reagent grade $NiCl_2$ (Mallinckrodt Chemical Works, St Louis, Missouri, U.S.A.) was prepared and the desired dilution added to the basal medium prior to sterilization by autoclaving at 121° for 15 min.

Identification of organism

Cultures of *Arthrobacter marinus* and *A. globiformis* were plated on their respective growth media, and four single colony isolates of *A. marinus* and two of the type species were isolated, transferred to agar slants and maintained for use in the subsequent identification procedures. Cultural characteristics were determined using the criteria in the Society for American Bacteriologist's *Manual of Microbiological Methods* (1957), Cowan & Steel (1965) and Skerman (1967). Growth cycle pleomorphism was observed and photographed by phase contrast microscopy. Hucker's modification of the Gram stain and the malachite green spore stain were used. Flagella were demonstrated by electron microscopy after shadow casting with tungstic oxide.

Growth characteristics and colony morphology were determined by inoculation to: extract agar (B.B.L.), extract broth (B.B.L.), tryptose-glucose-extract agar (Difco) and potato–dextrose agar (B.B.L.), all prepared in 75% synthetic sea water, and marine agar prepared in distilled water. Trypticase soy agar–soil extract (TSA-soil extract) was prepared using trypticase soy agar (B.B.L.), with 50% soil extract (Pringsheim, 1964) and 50% distilled water. Nicotine agar (Sguros, 1955) and thiotone broth (Blankenship & Doetsch, 1961) were prepared both in 75% synthetic sea water and in distilled water.

All the following media were prepared in 75% synthetic sea water and after inoculation, incubated at 24 to 27°. Oxidative or fermentative capacity was determined by using Hugh & Leifson medium (1953) containing glucose. The utilization of the carbohydrates, glucose, sucrose, galactose, lactose, mannitol, mannose, maltose, inositol, glycerol, ribose, xylose, sorbitol, arabinose, rhamnose, salicin and dextrin was determined by two methods: phenol red broth (Difco) with 0·5% carbohydrate, and secondly in a synthetic basal medium composed of NH_4NO_3, 0·1%; K_2HPO_4, 0·002%; phenol red, 0·002%; asparagine, 20 μg./ml. and supplemented with carbohydrate, 1·0%. Cellulose utilization was demonstrated by placing strips of Whatman no. 2 filter paper (W & R Balston Ltd) in the above synthetic basal medium without the phenol red indicator. Simmon's citrate medium (B.B.L.) and Koser's citrate broth (Difco) were used to demonstrate citrate utilization. The medium of Dubos & Miller (1937) prepared both in 75% synthetic sea water and in distilled water was used to determine the utilization of creatinine and creatine as sole source of nitrogen and/or carbon. For testing the utilization of creatinine and creatine as sole carbon source, 0·5% $(NH_4)_2SO_4$ was added to the media. Growth in media containing NH_4Cl or KNO_3 0·1%, glucose 1·0% and K_2HPO_4 0·005% was used to test for the utilization of ammonium and nitrate ions as sole source of nitrogen. The ability to utilize atmospheric nitrogen as sole source of nitrogen was determined in a medium composed of

mannitol, 1·5%; $MgSO_4.6H_2O$, 0·02%; K_2HPO_4, 0·02%; NaCl, 0·01%; $CaSO_4.2H_2O$, 0·01%; $CaCO_3$, 0·5%, and Noble agar (Difco), 1·5% in distilled water.

Nitrate reduction was determined in trypticase soy broth (B.B.L.) containing 0·1% KNO_3 using the Griess–Ilosvay reagent; indole production in trypticase soy broth using Kovac's reagent; H_2S production on lead acetate agar (Difco) slants and extract agar with 0·1% cysteine and 0·02% $FeSO_4.7H_2O$; acetylmethylcarbinol production in MR–VP medium (B.B.L.) using Barritt's method (1936). Urease production was tested in Bacto-urea broth (Difco) prepared both in 75% synthetic sea water and in distilled water; catalase with 3% H_2O_2 and oxidase by Kovac's oxidase reagent. Hydrolysis of gelatin was determined on extract agar with 0·4% gelatin (Difco) by flooding the plates with acid mercuric chloride solution and by liquifaction of stab cultures in extract broth containing 12% gelatin. Incubation was at 20° and at 25°. Starch hydrolysis was determined on starch agar (Difco) by flooding plates with iodine solution.

The growth response to temperature was determined in a medium consisting of peptone, 0·05%; yeast extract, 0·05%; $MgCl_2.6H_2O$, 0·3%; $CaCO_3$, 0·06%; KCl, 0·04%; and NaCl, 7·02% in distilled water.

Base composition of DNA

Deoxyribonucleic acid (DNA) was isolated by the method of Marmur (1961) and the guanosine + cytosine (G + C) molar concentration determined by the method of Wang & Hashagen (1964). Fish sperm DNA (Sigma Chemical Co., St Louis, Missouri, U.S.A.) was used as control. The 5′-monophosphates of thymidine and the deoxy forms of guanosine, adenosine and cytodine were used in preparing the standards.

RESULTS

Characteristic proportion of the isolate, Arthrobacter marinus sp.nov.

A striking characteristic of the genus *Arthrobacter* is its extreme morphological variability during growth. This was characterized originally by Conn & Dimmick (1947) as, 'Varied, with a tendency to go through a more or less definite cycle, the most characteristic features of which are Gram-negative rods in young cultures and Gram-positive coccoid forms (arthrospores) in old cultures.' Skerman (1967) definitely stated, 'Ultimately the rods transform completely to cocci,' and considered the transformation of the cocci through rods to cocci again as a prime feature of the genus.

The marine organism isolated by us exhibited this cyclic growth pattern and therefore met the principal requirement for the definition of the genus. In the stationary phase the bacteria were single and coccoid in shape, measuring 0·6 to 0·8 μm. × 1·0 μm. (Pl. 1, fig. 1). When the coccoid organisms were inoculated onto marine agar they elongated into large rods about 1·2 to 1·5 μm. × 2·0 to 4·0 μm. during the logarithmic phase of growth (Pl. 1, fig. 2). As the culture reached the early stationary phase the cells gradually became smaller, ranging from about 1·0 to 1·5 μm. × 2·0 μm. With further incubation the sizeand shape of the cell completed the cycle, becoming coccoid in about 5 days (Pl. 1, fig. 3). The rods were often slightly bent and appeared singly, more often in pairs, and occasionally in short chains of three to four cells. The daughter cells of dividing rods were often in V formation, a characteristic commonly seen in coryneform bacteria (Starr & Kuhn, 1962).

Motility, observed in hanging drop preparations, was most evident in cells from the logarithmic (rod-shaped) phase of growth. Electron photomicrographs showed the presence of a single, subpolar flagellum consisting of two thin very long strands (Pl. 1, fig. 4, 5). The position of the flagellum is similar to that found in the 'degenerate peritrichous' group described by Conn, Wolfe & Ford (1940).

The Gram reaction varied with growth conditions. In very young cultures from marine agar slants, the rods were Gram-negative. In older cultures the cocci appeared to be Gram-positive, but on closer examination many of the cells clearly have a Gram-negative cytoplasm with large positively-stained granules. These granules are evident in the phase contrast photomicrographs (Pl. 1, fig. 3) and appear to be similar to those observed by Jensen (1960). Two week old cultures from TSA–soil extract and marine agar slants stained with malachite green showed no signs of spores.

Cultural characteristics

Agar slant: growth was luxuriant, filiform, glistening, butyrous (extract, potato–dextrose agar) becoming viscid on ageing (marine, TSA–soil extract agar) or viscid (tryptose–glucose extract agar), ivory or cream, opaque, marine agar) or translucent (potato–dextrose agar).

Agar plate: colonies are circular, 2·0 to 5·0 mm. in diameter depending on the medium, convex, entire margin, smooth with the colony consistency and colour as above.

Broth: slight surface ring, dense uniform turbidity with a viscid sediment.

No pigment was formed on the synthetic sea water–nicotine agar. Cells of normal morphology occurred in the thiotone broth prepared with synthetic sea water. The organism failed to grow in both media prepared with distilled water.

The following tests were negative: hydrolysis of gelatin and starch, nitrate reduction, indole, acetylmethylcarbinol and urease production and oxidase. Positive results were obtained in H_2S production, citrate utilization and catalase. The culture was weakly oxidative in the Hugh & Leifson medium.

Good growth occurred in litmus milk with reduction of the litmus. The milk slowly cleared without coagulation, clearing being complete in three weeks with the production of an abundant viscid growth.

No growth occurred in creatinine, creatine, cellulose or nitrogen-free media, indicating that the organism could neither utilize creatinine, creatine or cellulose as sole source of carbon, nor utilize creatinine, creatine or atmospheric nitrogen as sole source of nitrogen. Growth occurred in the inorganic nitrogen media demonstrating the ability to utilize nitrate and ammonium ions as a nitrogen source and that growth factors are not required by the organism.

The growth temperature range was from 6° (the lowest temperature used) to 40°. Most rapid growth occurred at 40°, with a maximum population at 25°. No growth was obtained at 42°.

The G+C molar composition of *Arthrobacter marinus*, *A. globiformis*, fish sperm and calf thymus DNA, using the indirect method of Wang & Hashagen (1964) are presented in Table 1. The G+C value of 63·3 for *A. marinus* is in accord with the range of values found for the type species of *A. globiformis*.

In phenol red broth base with 0·5% carbohydrate the culture produced acid but no gas from glucose, galactose and sucrose, but weak acid from mannitol. An alkaline

reaction occurred in arabinose, xylose, glycerol, lactose, and dextrin. No pH change occurred in the other carbohydrates. In the synthetic basal medium with 1% carbohydrate, acid but no gas was produced in glucose, galactose, sucrose, glycerol, maltose, mannose, mannitol, inositol and ribose; there was slow acid production in lactose. No pH change occurred in the other carbohydrates.

Table 1. *Guanosine plus cytosine (G+C) percent molarity of Arthrobacter marinus DNA using the method of Wang & Hashagen (1964)*

Sample	G+C (% M)	Previously published data
Arthrobacter marinus	63·3	—
Arthrobacter globiformis	64·3	62 to 64 (1)
Calf thymus DNA	40·7	40 (2)
Fish sperm DNA	41·9	—

References: (1) Marmur, Falkow & Mandel, 1963; (2) Schildkraut, Marmur & Doty, 1962.

The use of phenol broth medium for detecting acid production from carbohydrates was unsatisfactory as the alkaline reaction, presumably from the breakdown of peptone, masked the acid produced from the carbohydrate. After 48 h. incubation, at ambient temperature (24 to 27°) the phenol broth medium showed acid reactions in three of the 16 carbohydrates tested, whereas 10 acid reactions occurred in the synthetic basal medium with *Arthrobacter marinus*. The type species, *A. globiformis*, produced acid reactions from 14 carbohydrates in the synthetic basal medium and none in the phenol broth medium during the same time interval. At the end of 1 week, the number of positive reactions in the synthetic basal medium increased by only one carbohydrate for each organism: lactose for *A. marinus* and ribose for *A. globiformis*. The synthetic medium, once acid, did not revert to a neutral or alkaline reaction as had occurred with the peptone containing medium. Conn & Dimmick (1948) found the same problem with the peptone medium and used a synthetic medium to characterise the species *A. simplex*.

Arthrobacter marinus has a definite inorganic requirement which is easily satisfied by the addition of sea water. ZoBell & Upham (1944) studied several biochemical reactions of marine bacteria in both fresh- and sea-water media and noted quantitative but no marked qualitative differences in the results. Sea water must be used in the media.

Growth response of Arthrobacter marinus to nickel concentrations

The growth response of *Arthrobacter marinus* to nickel was determined by the addition of increasing concentrations of $NiCl_2$ (1×10^{-4} to 5×10^{-4}M) to the basal medium. This resulted in an extension of the lag phase from about 3 h. in the unsupplemented control to over 70 h. in the presence of 4×10^{-4}M-nickel (Fig. 1). At a concentration of 5×10^{-4}M-nickel the culture did not grow during a period of 144 h. The slope of the line during the logarithmic phase of growth decreased with each increase in the nickel concentration, resulting in an increased time required to reach maximum extinction (growth) at the higher nickel concentrations. Since the light transmittance depends on the size and shape of the particles, as well as on their

numbers, the values are relative and are presented to show the initiation of growth, general shape of the curve, and the time to reach maximum extinction at each nickel concentration. The occurrence of the maximum extinction coincided with the maximum population value of the culture. The populations normally associated with the maximum extinction are presented in Table 2.

The formation of the megalomorphic cells was a graded response and dependent upon the concentration of nickel, being most evident at 4×10^{-4}M-nickel in the basal medium. Plate 2 presents photomicrographs comparing the maximum size of megalomorphs from cultures with the various nickel concentrations with bacteria from

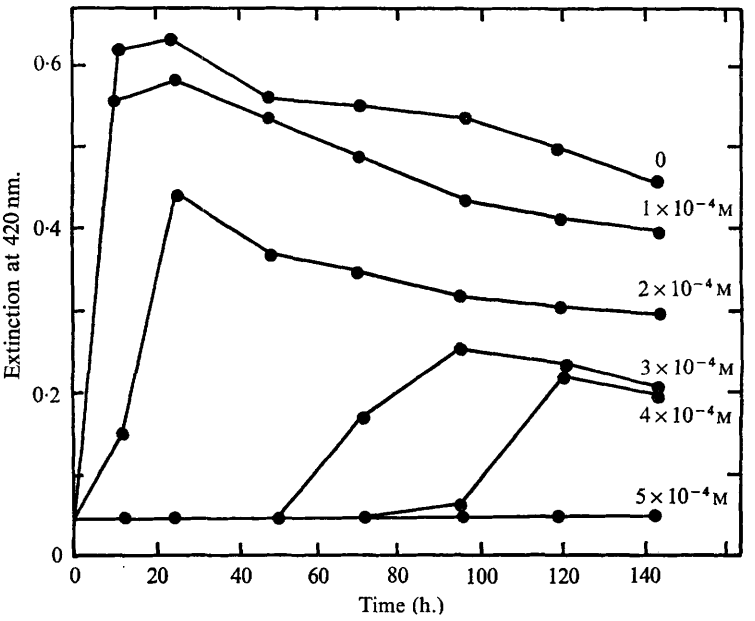

Fig. 1. Growth response of *Arthrobacter marinus* to nickel. Increasing molar concentrations of nickel were added to the basal medium inoculated with an 18 h. culture and shaken at 300 rev./min. at 25°. Samples taken at intervals and extinction read in a Bausch and Lomb Spectrophotometer at 420 nm.

Table 2. *Plate count populations and the size of Arthrobacter marinus cells in various concentrations of nickel at 25°*

Concentration of Ni (M)	Plate count (cells/ml.)	Maximum average size (μm.)
No addition	5 to 8×10^8	2×4
1×10^{-4}	3 to 6×10^8	2×4
2×10^{-4}	3 to 6×10^7	2 to 3×4 to 6
3×10^{-4}	3 to 5×10^6	7 to 10 in diam.
4×10^{-4}	4 to 7×10^5	10 to 15 in diam.

the control culture. The most dramatic change in morphology occurred between 2×10^{-4} and 3×10^{-4}M-nickel concentrations. Considerable vacuolation of the megalomorphs developed which increased with time and nickel concentration. The shape of the cells were generally round to oval, with cytoplasm pushed over to one side of the

cell in a crescent shape. As the cell became older, the cytoplasm became more vacuolated and the shape more irregular. In the stationary phase culture, the cells became smaller but retained the vacuoles and crescent-shaped cytoplasm.

When cells were inoculated into the basal medium containing 4×10^{-4}M-nickel and samples were removed periodically for observation by phase contrast microscopy, the majority (> 95%) were observed to have undergone transformation to megalomorphs. Plate 3 shows the course of this enlargement in comparison with the original inoculum (Pl. 3, fig. 12). After 1·5 h. incubation, the cells were somewhat larger and more oval-shaped than the control logarithmic phase rod. In some instances, a small vacuole was formed at the end of the cell. An occasional cell was seen to bulge in the middle as if the newly formed cell wall had weakened and was unable to retain the rod shape. The cells at this stage have an even phase density (Pl. 3, fig. 13). After 2·5 h. incubation, the nickel-treated cells became larger with a wide variation in phase density within the cell (Pl. 3, fig. 14). A tremendous change in size occurred during the subsequent 2 h. incubation period. During this time the cells were still somewhat oval, over half the volume appearing as vacuoles, and the size increased by about 30 volumes (Pl. 3, fig. 15) in relation to the control. After 6·5 h. incubation, the cells had increased to 70 volumes, and became multivacuolated (Pl. 3, fig. 16). At 10 h. incubation the cells were 3·5 times larger than at 6·5 h., multivacuolated and more irregular in shape (Pl. 3, fig. 17). These cells after 10 h. incubation in 4×10^{-4}M-nickel, were about 250 volumes larger than the untreated normal size rod with approximately 40% of the cell volume being crescent-shaped cytoplasm against the cell wall and 60% as a vacuole. The megalomorphic cells formed after 4·5 h. incubation were osmotically sensitive when placed in distilled water: the protoplasts burst, leaving the cell walls intact and resembling hollow spheres.

The megalomorphic cells, when returned to the basal medium without nickel, resumed division and rapidly produced normal size rods.

DISCUSSION

The morphological, cultural, physiological and G+C molar composition of this recently isolated marine micro-organism indicates a close relationship to the genus *Arthrobacter*. The isolate also exhibits properties that clearly distinguish it from species of the genus previously described by Lochhead (1957). Its characteristics also differ significantly from the published descriptions of *A. crystallopoietes* (Ensign & Rittenberg, 1963), *A. duodecadis*, *A. flavescens* (Lochhead, 1958), *A. atrocyaneus* (Kuhn & Starr, 1960), *A. polychromogenes* (Schippers-Lammertse, Muijsers & Klatser-Oedekerk, 1963), *A. viscosus* (Gasdorf, Benedict, Cadmus, Anderson & Jackson, 1965), and *A. ramosus* (Jensen, 1960). The genus has been divided into two groups on the basis of utilization of ammonium and nitrate salts and the use of citrate as the sole sources of nitrogen and carbon, respectively. This marine organism is most closely related to the nonchromogenic members of the first group, which are able to utilize these compounds. The isolate therefore appears to be a new species for which the name *Arthrobacter marinus* is proposed because of its original isolation from the marine environment.

The genus *Arthrobacter* has a propensity for an unusual growth response under conventional methods of culture. They are characterized, and stain Gram-negative

in the logarithmic phase of growth; however, their cell walls have the chemical composition of Gram-positive cells (Cummings & Harris, 1959; Gillespie, 1963; Krulwick, Ensign, Tipper & Strominger, 1967). The change of Gram reaction of *A. globiformis* from Gram-negative in the logarithmic phase of growth to Gram-positive in the late stationary phase is reported to be the result of an alteration in cell wall synthesis during the logarithmic phase of growth (Gillespie, 1963).

Wahlin & Almaden (1939) applied the term 'megalomorph' to those cells which were abnormally long and frequently swollen. The formation of such cells can be induced by a variety of conditions such as magnesium deficiency (Webb, 1949), deficiency in growth factors (Chaplin & Lochhead, 1956; Chan, 1964), metals (Rosenberg, Renshaw, Van Camp, Hartwick & Drobnik, 1967; Sobek & Talburt, 1968), antibiotics (Kantor & Deering, 1968), temperature (Terry, Gaffer & Sagers, 1966), and pressure (ZoBell & Cobet, 1962).

The morphological changes observed in *Arthrobacter marinus* when grown in the presence of Ni^{2+} appear to involve cell division mechanisms rather than metabolic growth processes. There is a continuation of cellular growth with cessation of multiplication, resulting in enlarged cells. The shape of the bacterial cell is determined by the mechanical rigidity of the cell wall (Salton, 1964). During balanced growth the plasma membrane is in contact with the cell wall and both expand and change shape. The maximum size a cell may attain is determined by the space within the cell wall. The osmotic pressure within the protoplast is at least as high as the surrounding medium so that the cell membrane is pressing against the cell wall with a turgor pressure (Mitchell & Moyle, 1956). If the cell wall is weakened, its protective action is lost and the protoplast is forced to regulate its own volume unless protected by osmotic support in the medium. Otherwise the cell will burst (Weibull, 1953; McQuillen, 1960).

An osmotic irregularity is seen to develop in the megalomorph as a result of nickel stress. The cells grown in the absence of nickel are seen to have normal morphology, whereas in the same medium with 4×10^{-4}M-nickel the cell wall expands away from the protoplast to produce the appearance of being plasmolysed (Pl. 3, fig. 16, 17). Hence the cell appears to have become hypotonic in relation to the exterior environment, resulting in shrinkage of the cell membrane away from the wall. A second possibility is that the cell wall synthesizing system has become uncontrolled with an unregulated production of cell wall material. This may, at the same time, disrupt the relationship between the cell wall and the cytoplasmic membrane needed for cell division. Because of diffusion problems associated with increased size the synthesis of the protoplast may lag behind, with the result that the cell membrane retracts from the wall.

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EXPLANATION OF PLATES

PLATE 1

Cellular development of normal cells of *Arthrobacter marinus* in the basal medium at 25°. Photomicrographs by phase contrast. × 2500.

Fig. 1. Inoculum, 18 h. culture from surface of slant of marine agar medium 2216.

Fig. 2. Logarithmic phase, 5 h. after inoculation.

Fig. 3. Late stationary phase, 8 days after inoculation.

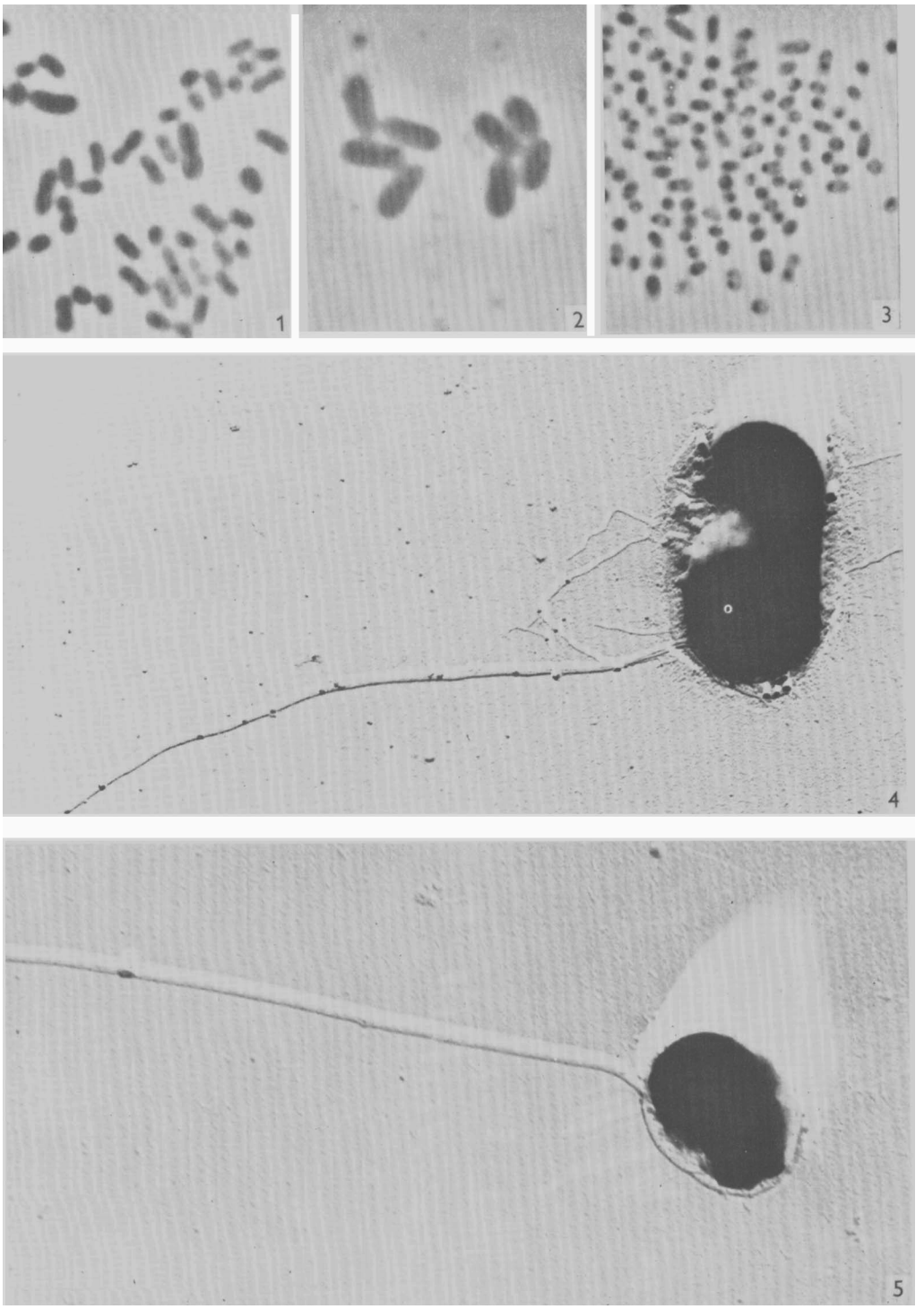
Fig. 4 and 5. Electron micrograph of flagellum. Cells taken 12 h. after inoculation and shadow cast with tungstic oxide at an angle of 30 degrees and 24 Å thick. × 10,000.

PLATE 2

Morphology of cells grown in the presence of increasing concentrations of nickel added to the basal medium. Cultures were incubated at 300 rev./min. at 25°. Photomicrographs by phase contrast. × 2500.

Fig. 6. Inoculum, 18 h. culture from surface of slant of marine agar medium 2216.

Fig. 7. Culture after 24 h. incubation in 1×10^{-4} M-nickel.



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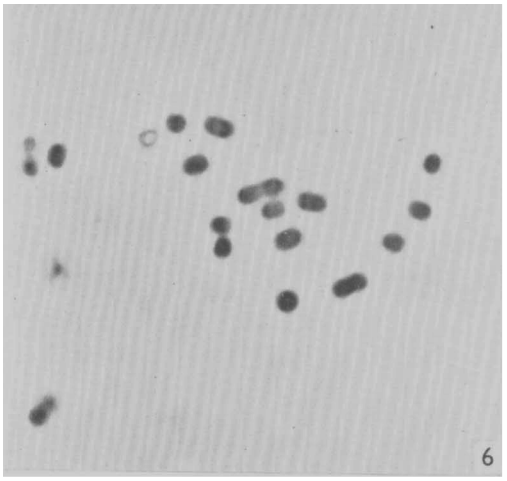

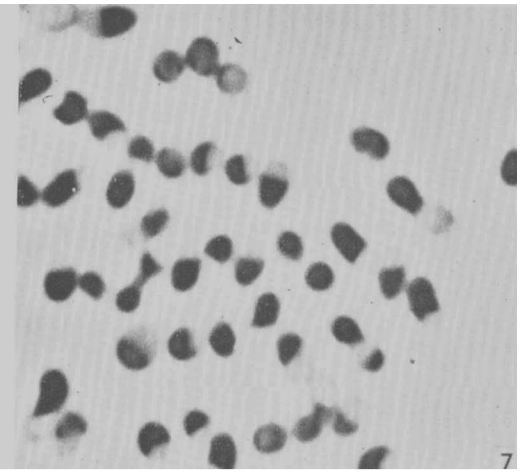

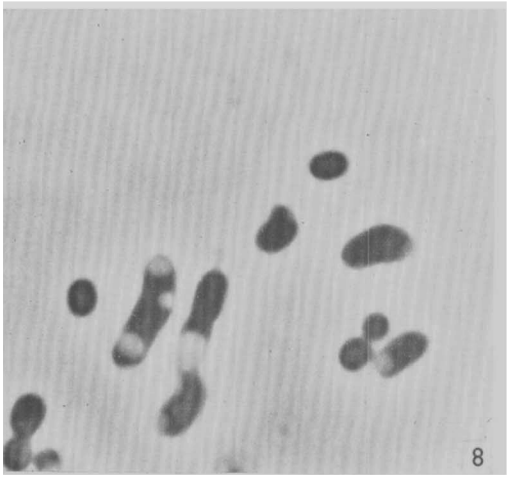

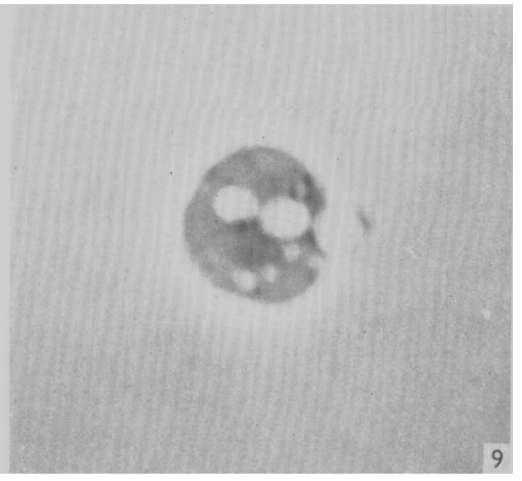

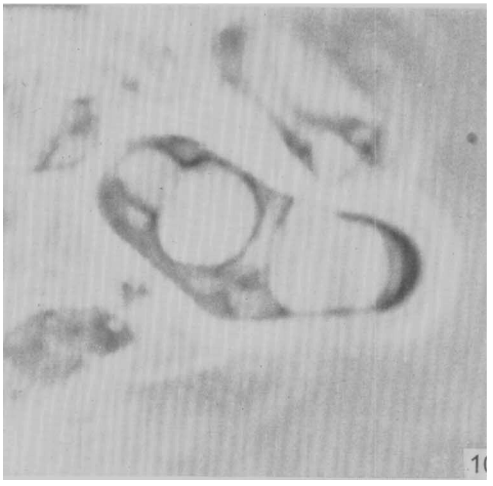

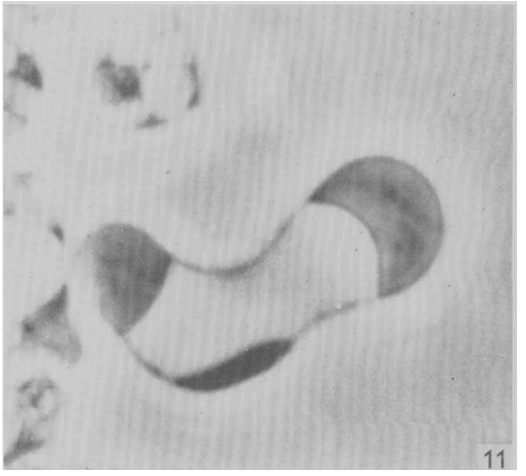

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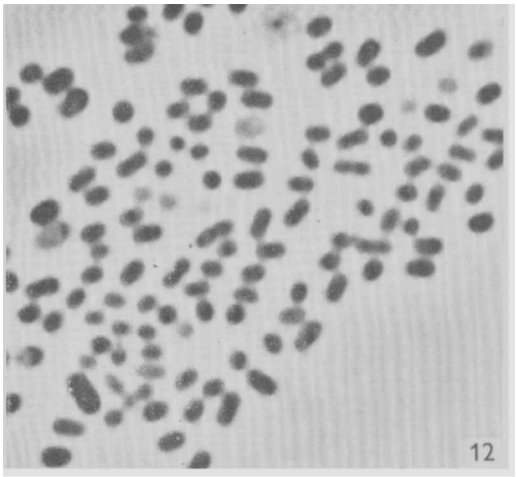

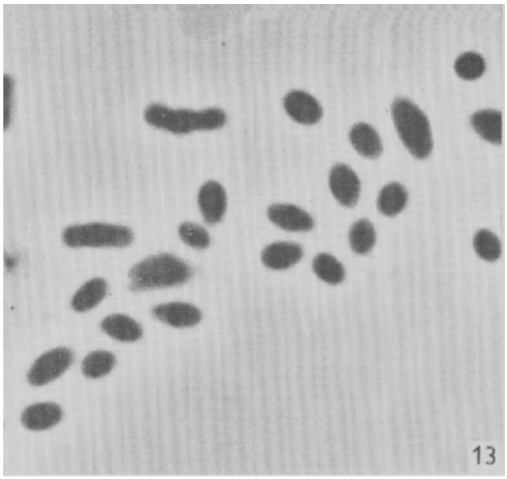

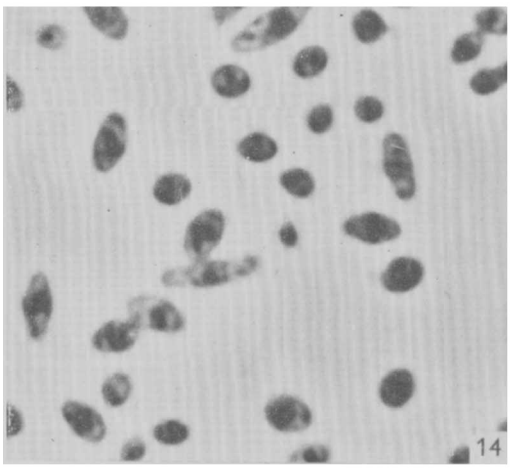

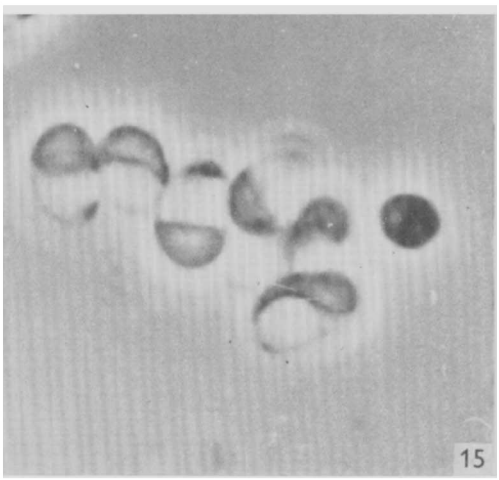

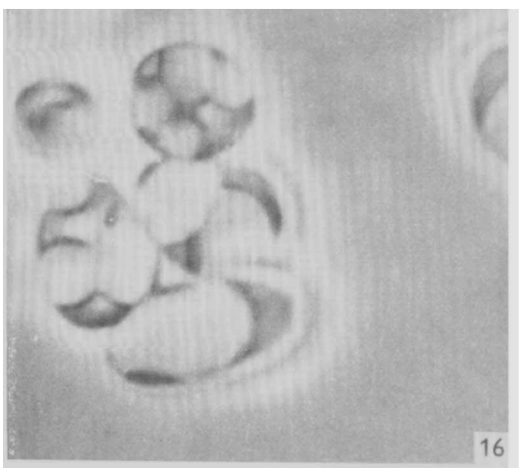

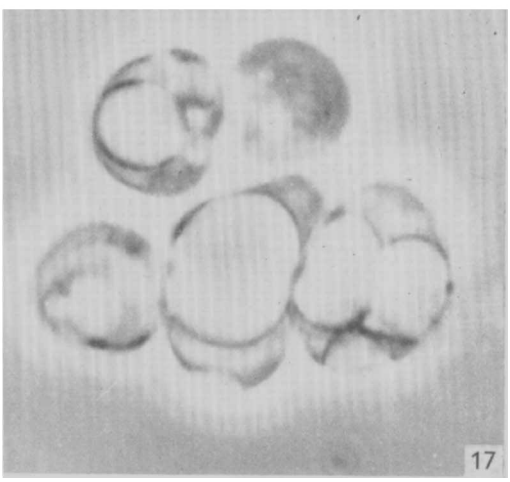

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Fig. 8. Culture after 30 h. incubation in 2×10^{-4}M-nickel.

Fig. 9. Culture after 96 h. incubation in 3×10^{-4}M-nickel.

Fig. 10 and 11. Culture after 120 h. incubation in 4×10^{-4}M-nickel.

PLATE 3

Morphological changes of a culture grown at 25° in basal medium containing 4×10^{-4}M-nickel. Wet mounts prepared at times shown and photomicrographs made by phase contrast. ×2500.

Fig. 12. Inoculum, 18 h. culture from slant of marine agar medium 2216.

Fig. 13. After 1·5 h. incubation.

Fig. 14. After 2·5 h. incubation.

Fig. 15. After 4·5 h. incubation.

Fig. 16. After 6·5 h. incubation.

Fig. 17. After 10 h. incubation.