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Bilophococcus magnetotacticus gen. nov. sp. nov., a motile, magnetic coccus

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Abstract. The morphological, biochemical, and magnetotactic properties of a single magnetic bacterium are reported. Although this bacterium has not been cultured axenically, the unusual magnetotactic behavior has allowed the collection of cell material of sufficient quantity and purity to allow characterization. The results indicate that this organism represents a new genus of colorless, sulfur-depositing bacteria, albeit of uncertain affiliation. The name proposed for this new genus/species, *Bilophococcus magnetotacticus*, reflects the most distinctive traits of morphology, motility, and magnetic mineral formation. Classification is based on type descriptive material.

Introduction

The magnetic bacteria constitute a diversity of morphological types distributed in aquatic sediments throughout the world (Blakemore 1982; Moench & Konetzka 1978). Their stable magnetic dipole moment and ability to display magnetotaxis result from the biogenic precipitation of iron as crystalline magnetite in enveloped, electron-dense, cytoplasmic inclusions called magnetosomes (Balkwill et al. 1980; Blakemore 1982; Mann et al. 1984; Moench & Konetzka 1978; Towe & Moench 1981). Thus far, only *Aquaspirillum magnetotacticum* has been cultured axenically in a chemically defined medium (Blakemore et al. 1979; Maratea & Blakemore 1981). Another morphological type, a magnetic coccus, has been obtained in sufficient purity by use of its magnetotactic properties to allow characterization. This magnetic coccus is a Gram-negative, spherical bacterium that is easily identified by its distinctive morphology and magnetic properties (Moench & Konetzka 1978). The purpose of this paper is to extend the characterization of this magnetic coccus and propose it as a new bacterial genus of uncertain affiliation.

Materials and methods

Magnetic cocci were enriched and isolated magnetically from reconstructed wastewater aeration basin environments as described previously (Moench & Konetzka 1978). These environments were produced by layering the bottom of a plastic tub, 74 × 58 × 30 cm, with black, sulfurous mud from the Winston-Thomas Wastewater Treatment Facility aeration basin (Bloomington, Indiana), and the tub was filled with secondary treated influent to approximately 12 cm above the sediments. This basin underwent a biological succession and developed a thick blanket of duckweed (*Lemna* sp.) on the surface and magnetic cocci in the water column and sediments. This pond was kept in a rooftop greenhouse and maintained throughout the year with regular additions of water to counteract evaporation and occasional additions of influent and mud. It served as a permanent source of organisms free of the seasonal disruptions which reduce the natural populations during the fall and winter months.

Lemna sp. (duckweed, an aquatic surface macophyte) were isolated axenically by immersing fronds in 1.75% sodium hypochlorite solution (1/3 dilution of household bleach) for 45–90 sec, rinsing in sterile water, and transfering to 20 ml of HTS (Hutner's-Sucrose (0.25%)-Tryptose (0.06%)) medium. After 2 days of incubation in the dark at room temperature the fronds were put into a continuous fluorescent light incubator at 27° C. Once adapted to HTS, the *Lemna* sp. was kept as stocks on HTS agar (0.8% agar) in 50 ml flasks.

Phase and electron micrographs were produced as described previously (Moench & Konetzka 1978).

Catalase activity was determined using a microtest (Clarke & Rowan 1969) where hydrogen peroxide and a suspension of cells were drawn up into a capillary tube together and the formation of bubbles observed. *Pseudomonas aeruginosa* X and *Escherichia coli* 1485 grown overnight in 1% tryptone broth were used as positive controls.

Growth media were formulated using Hutner's salt medium (C. Miller, pers. comm.) as a common base. The component solutions in grams/liter consisted of:

Solution *A:* $ZnSO_4$, 6.6; H_3BO_3, 1.4; $Na_2MoO_4 \cdot 2H_2O$, 2.5; $CuSO_4$, 0.39; $CoCl_2$, 0.012; $MnCl_2 \cdot 4H_2O$; five drops of 5 N HCl were added and the solution slowly heated to dissolve the salts.

Solution *B:* Ferric-EDTA, (1) heat to dissolve 17.9 g sodium ethylenediaminetetra-acetate (EDTA) and 3.23 g KOH in 186 ml distilled water, and (2) dissolve 13.7 g $FeSO_4 \cdot 7H_2O$ in 364 ml distilled water, and then mix the two solutions and bubble with air overnight.

Solution *C:* $Ca(NO_3)_2 \cdot 4HO_2O$, 35.4; disodium EDTA, 56.7; K_2HPO_4, 40.0; NH_4NO_3, 20.0; KOH, 22.4.

Solution *D:* $MgSO_4$ 7 $7H_2O$, 50.0.

The final Hutner's salt medium (H) was formulated by combining solutions, A, B, C, and D in the order given and the ratio: 10:1:10:10 ml per liter of glass distilled water. The pH was adjusted to 6.0–6.5 with KOH before autoclaving. The autoclaved medium showed a fine white precipitant which went into solution upon cooling and standing.

MPSS:H medium was a 0.1% succinate-0.5% peptone medium combined 1:5 with H. HOX medium consisted of H combined with filter sterilized aeration basin water, OX (i.e. oxidation pond water), in a ratio 1:2.5. NB:H was standard nutrient broth (8g/L)(NB) combined with H (1:10). Pep:H was 0.5% peptone broth (Pep) combined with H (1:10). Thioglycollate:H was 0.29% Difco thiglycollate combined with H (1:4).

The vitamin solution (VIT) was formulated in mg/L as follows: folic acid, 10; riboflavin, 10; nicotinic acid, 10; biotin, 1; thiamine, 5; para-aminobenzoic acid, 5; calcium pentothenate, 5; pyridoxine, 25; and B_{12}, 1. The solution was filtered sterilized and stored refrigerated. One ml was added to 10 ml of medium.

Poly-β-hydroxybutyrate (PHB) was assayed by the method of Law & Slepecky (1961) using *Bacillus megaterium* as a positive control and *E. coli* as the negative control. The control cultures were grown for 24 h at 30° C with shaking in the medium of Macrae & Wilkinson (1958). The PHB was extracted in boiling chloroform and converted to crotonic acid by heating in concentrated sulfuric acid. The absorbance of the crotonic acid was measured from 210–280 nm with a Cary Model 14 recording spectrophotometer. The PHB concentration was determined from the absorption maximum at 235 nm. Protein was determined by the method of Lowry et al. (1951).

Sulfur was quantified using flask combustion in dilute hydrogen peroxide followed by colorimetric titration of precipitated barium (Midwest Microlab, Ltd, Indianapolis, IN). Samples of magnetically collected whole cells were concentrated by centrifugation for 10 min at 12 100 g at 2° C, washed twice in distilled water, and lyophillized for shipping and analysis. Sulfur was released from whole cells by resuspending a cell paste of magnetic cocci in 0.02 ml of 20% (w/v) sodium lauryl sulfate for 30 min at 37° C. After cell lysis occurred a whitish material was concentrated by low speed centrifugation at 3020 g for 5 min. This material was washed at least 10 times with distilled water. Microscopic examination of this material revealed refractile globules identical in size range and appearance to those found within intact cells. The percent of sulfur was calculated based on the initial concentration of whole cells.

Results

Magnetic bacteria are found ubiquitously in and above freshwater and coastal marine sediments. Using a specific enrichment procedure and magnetic techniques one coccoid cell type having a distinctive morphology (Figs. 1, 2) was collected (Moench & Konetzka 1978). Numerous and varied attempts to cultivate this organism axenically have been unsuccessful. These attempts included the media in this report as well as other aerobic and gradient techniques on defined and complex media (Moench 1978).

In order to characterize this organism in lieu of pure cultures it became paramount to establish that the presence of contaminating organisms was sufficiently low as to not contribute in any significant way to the results obtained. To establish a working level of purity three criteria were examined: morphological, bacteriological, and molecular. Morphological purity was determined by examining collections of cells using phase contrast microscopy. The magnetic coccus in question is rather large and always has one or more refractile bodies at the anterior end of the cell (leading edge during swimming) and a darkened area at the end posterior (Fig. 1). When swimming it exhibits a side to side wobbling motion which is unlike the translational motion in any of the motile organisms I have studied (eg. *Pseudomonas* spp., *E. coli, Thiovulum* spp., *Salmonella* spp., and *Proteus* spp.) nor am I familiar with any reports in the literature of a similar motility pattern. Furthermore, when fixed with formaldehyde, the cells can still be rotated in place using a magnet. When

Table 1. Growth of non-specific contaminants in ferrous sulfide culture tubes.

	Growth			
	Light		Dark	
	High	Low	High	Low
MPSS:H + CO_2	+	−	+	−
HOX + VIT + CO_2	+	−	−	−
HOX + Lactate (0.2%)	+	−	+	−
HOX + CO_2 + Lactate (0.2%)	−	−	+	−
NB:H	+	−	+	+
Pep:H	+	−	+	+
Thioglycollate:H	+	+	+	+

+ = growth; − = no growth.
Where designated 100% CO_2 was bubbled through the medium for 5 seconds. The high inocula were $8.3 \times 10^6 - 1.7 \times 10^7$ cells/10 ml/tube. Low incula were $8.3 \times 10^2 - 1.7 \times 10^3$ cells/10 ml/tube. Tubes were incubated at room temperature for 30 days and examined microscopically at 500×.

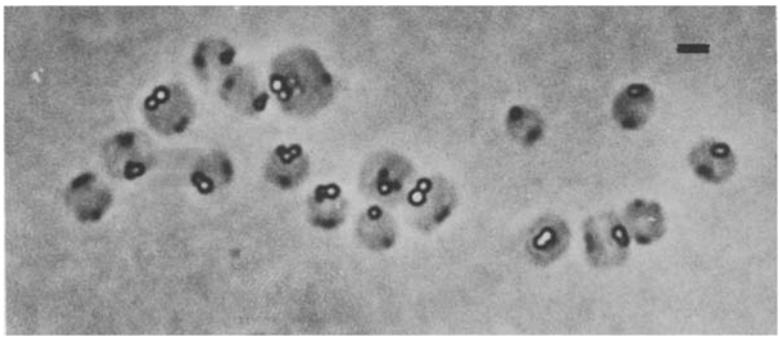

Fig. 1. Phase micrograph of *Bilophococcus magnetotacticus* showing refractile, sulfur inclusions and smaller dark magnetosomal regions. Bar = 1.0 μm.

Table 2. Growth of non-specific contaminants in simulated aeration basin culture Tubes.

	Growth	
	Low	High
	Inoculation	Inoculation
No *Lemna*		
Old mud[a]	−	+
New mud[b]	−	+
Lemna blanket[c]		
Old mud	−	+
New mud	−	+
Old mud + FeS	−	+
New mud + FeS	−	+
Fresh *Lemna*[d]		
Old mud	−	+
New mud	−	+
Old mud + FeS	−	+
New mud + FeS	−	+

[a] Two week old sterilized oxidation pond mud.

[b] Freshly sterilized oxidation pond mud.

[c] *Lemna* blanket was 17 days old when the magnetic cocci were inoculated. Medium pH was 6.7–7.3.

[d] 'Fresh *Lemna*' means both the *Lemna* and the magnetic cocci were inoculated into the sugar tubes the same day.

+ = growth; − = no growth.

The low inoculum was 7.1×10^3 magnetic cocci/tube and the high inoculum was 7.1×10^7/tube. The tubes were incubated in a continuous light incubator at 27°C. Tubes were monitored microscopically over 35 days.

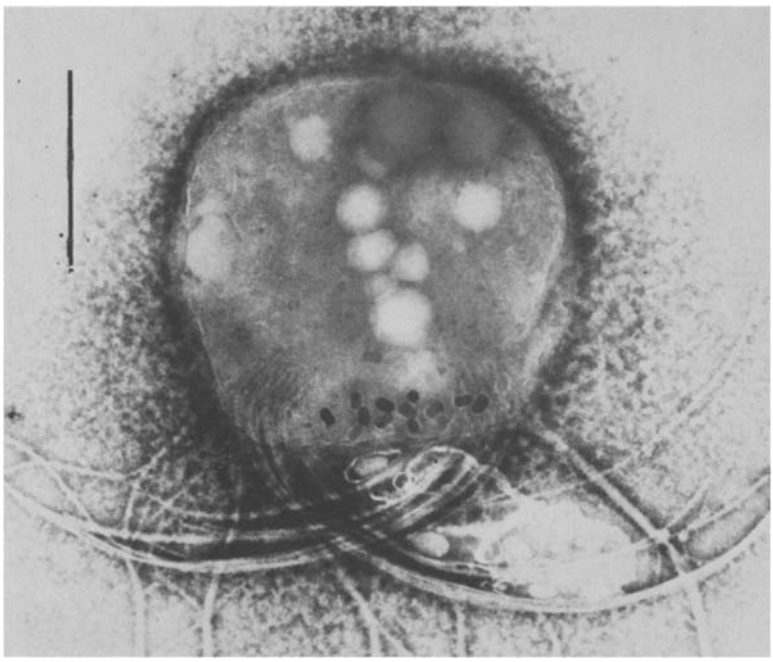

Fig. 2. Electron micrograph of negatively stained *B. magnetotacticus* showing two sulfur inclusions (darker circular inclusions) and the regularly shaped, electron dense magnetosomes between the two flagellar tufts. Bar = 1.0 μm.

examining microscopic fields of freshly collected or fixed samples, it is remarkably easy to detect the presence of contaminating organisms which do not fit the above description, including other magnetic cocci. Under phase microscopy, contaminating organisms, magnetic or otherwise, were rarely if ever detected visually indicating a conservative estimate of <1% contamination.

To assess the bacteriological contamination of these collections of magnetic cocci, cells were subjected to various growth conditions and the presence of contaminant growth was compared with direct counts of the coccoid bacterium to determine the percent contamination. In the first instance, high concentrations of magnetic cocci were plated on nutrient agar and incubated aerobically at 30° C. The magnetic cocci did not grow. The aerobic contamination was 0.05%.

In a second experiment, attempts were made to cultivate this magnetic coccus in various media under dark conditions, light conditions favorable to photosynthetic organisms, and above ferrous sulfide to establish an oxygen gradient. High and low concentrations of inocula were used. The low inocula were an attempt to have only magnetic cocci be present without potential contaminants. The results are shown in Table 1. For all the media listed,

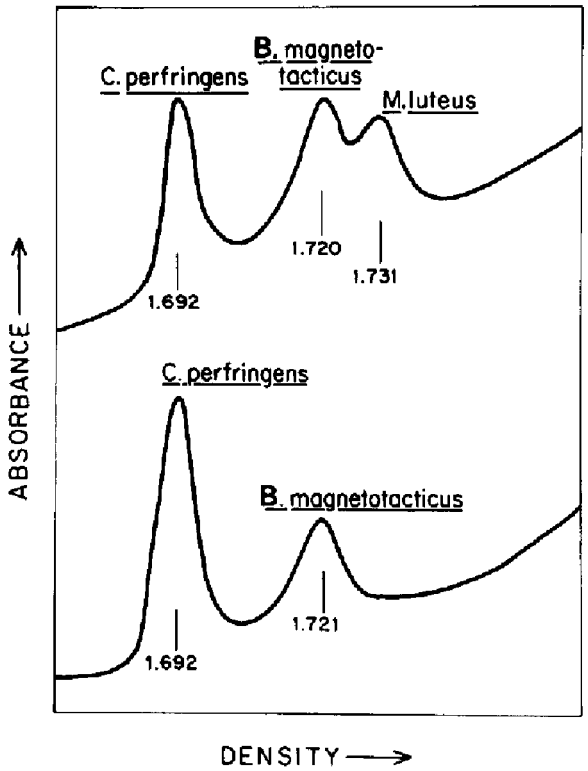

Fig. 3. Microdensitometer tracings of magnetic coccoid DNA samples equilibrated in CsCl density gradients. DNA from Clostridium perfringens and Micrococcus luteus were used as markers.

contaminants were capable of growth under these conditions. Then the magnetic cocci were diluted sufficiently to form the low inoculum. Contaminating growth was not observed in 10 of 14 tubes. The result indicates that one in 8×10^2 cells is not a magnetic coccus,or 0.13% contamination. A simple Most Probable Number treatment (APHA, 1981) of the tubes under the light and dark low inoculum conditions gives 0.019% to 0.079% contamination, respectively.

A third and similar experiment established the level of contamination in collections of these magnetic cells through the colonization of simulated aeration pond culture systems as shown in Table 2. Whereas all the high inoculum tubes became contaminated, the low inoculum tubes exhibited no contamination. The maximum probable contamination in the low inoculum conditions was 0.014%.

Contamination was also assessed by a molecular criterion through the isolation of DNA from pooled collections of magnetic cocci. Analytical CsCl

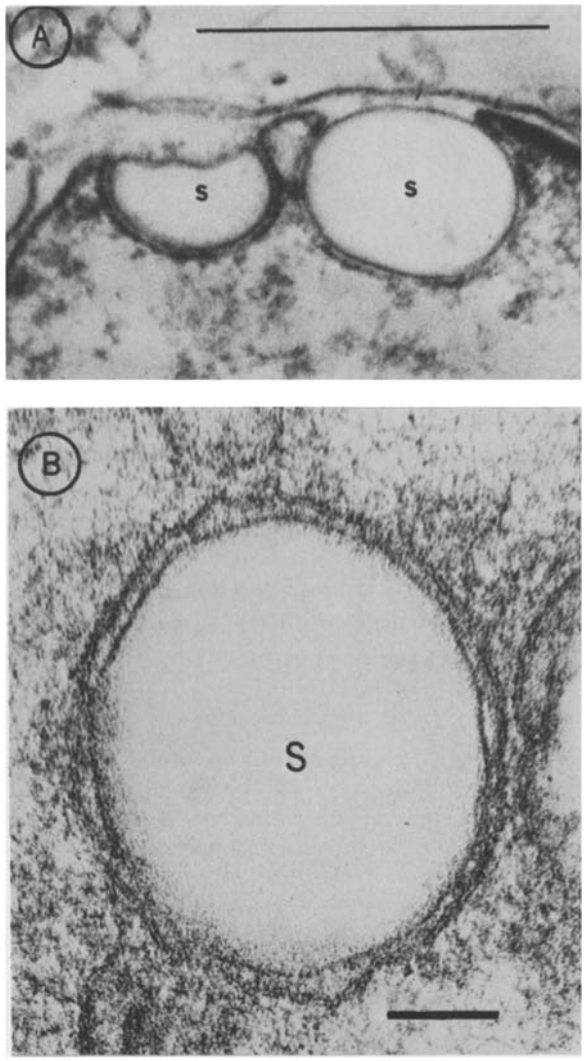

Fig. 4. (A) Electron micrograph thin section of sulfur inclusions (S) lying between the cell wall and an invagination of the cytoplasmic membrane. Bar = 0.5 μm. *(B)* Thin section of a sulfur inclusion body (S) surrounded by a unit membrane. Bar = 0.1 μm.

centrifugation produced a single peak (Fig. 3) corresponding to a guanine-cytosine content of 61.7%.

Present within every cell are one to three bright refractile bodies which are always located anteriorly as shown in both phase contrast (Fig. 1) and electron (Fig. 2) micrographs. Positive staining of these bodies with Sudan Black B led Moench & Konetzka (1978) to suggest these bodies might be PHB storage

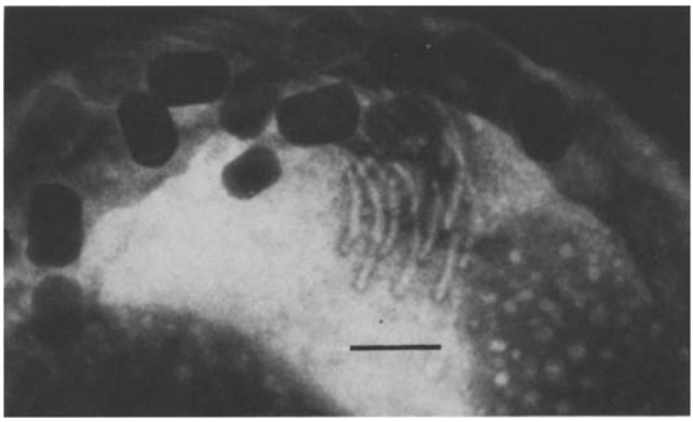

Fig. 5. Electron micrograph of flagella inserting into the cell surface to form a circular tuft. Magnetosomes are present. Bar ± 0.1 μm.

material. This conclusion was determined to be erroneous when biochemical analyses of whole cells to confirm the presence of PHB failed to detect this material even in concentrations of magnetic coccoid protein 28 times greater

Table 3. Compilation of general characteristics of *Bilophococcus magnetotacticus*.

Characteristics [a,b,c]
Habitat – aquatic, above and within sediments
Gram – negative
Coccus – 1.6 ± 0.2 μm
Motility – continuous forward motion – 69 ± 16 μms^{-1}
Flagellar tufts – two adjacent – 10–15 flagella/basal disk/tuft
Polar membrane in flagellar region
(%) Fe dry weight – 3.8 ± 0.3%
Magnetosomes
Single domain magnetite crystals (i.e. $(FeO)_2FeO_2$)
Hexagonal prisms – 99.3 ± 8.7 × 62.3 ± 6.1 nm
Enveloped – 15–40/cell
Localized in flagellar region
Cell division – binary along longitudinal plane
(%) Sulfur dry weight – 1.8–9.9%
Catalase negative
Mol % G + C of DNA – 61.7 ± 0.7 (Bd)

[a] Mann et al. 1984.
[b] Moench & Konetzka 1978.
[c] Towe & Moench 1981.

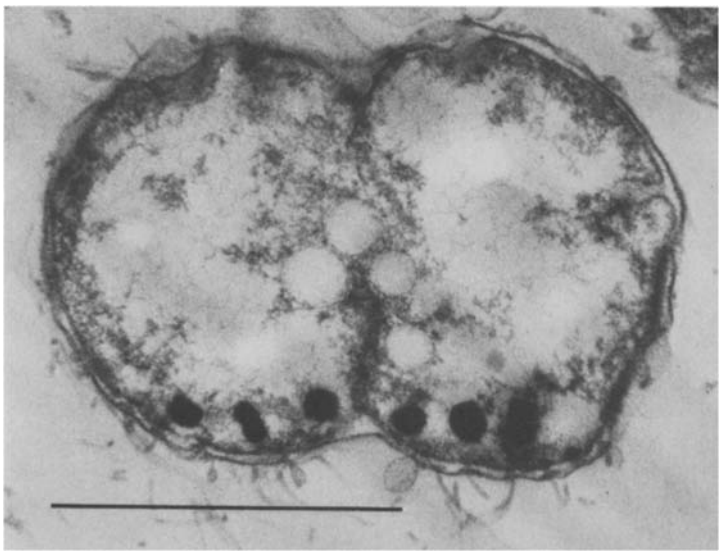

Fig. 6. Thin section of a dividing cell showing beginning septum formation to partition the cell. Bar = 0.5 μm.

than the *B. megaterium* positive control. Analysis of whole cells for sulfur indicated that the cells contained 9.9% sulfur by cellular dry weight. Analysis of the isolated refractile bodies yielded 1.8% sulfur by cellular dry weight, the difference presumably reflects the loss of material during the isolation and purification procedure. Thin-sectioned electron micrographs through the anterior portion of the cell reveal the sulfur inclusions within invaginations of the cytoplasmic membrane as shown in Fig. 4A. The unit membrane enclosing one inclusion is shown more clearly in Fig. 4B.

This magnetic coccus is motile by means of two flagellar tufts (Fig. 2). The positioning of these tufts results in a side to side wobble as the cell moves forward in an applied magnetic field. Each tuft is composed of numerous flagella inserted into a circular area on the cell surface (Fig. 5).

Localized in the flagellar region are biogenic ferromagnetic crystals distributed in a randomly dispersed pattern (Figs 2, 5). These crystals or magnetosomes have been isolated and found to be parallelepipeds with a hexagonal prism habit composed of magnetite as determined by electron diffraction and high resolution transmission electron microscopy (Mann et al. 1984; Towe & Moench 1981). During cell division the magnetosomes, sulfur inclusions, and one flagellar tuft are believed to be partitioned to each daughter cell by the formation of a septum along the longitudinal plane (Fig. 6).

Catalase activity was not detected in this bacterium. The general characteristics are summarized in Table 3.

Discussion

Although different types of magnetic cocci have been reported (Blakemore 1982; Moench & Konetzka 1978) the magnetic coccus which is the subject of this paper appears to be unique. The enrichment conditions used to proliferate this bacterium are unusual in that they resulted in high concentrations of cells in the water column of a simulated aqueous environment in addition to the sediments. The situation resulted in the isolation by magnetic force of an essentially pure population of organisms (Moench & Konetzka 1978). In spite of numerous attempts this magnetic coccus has not been isolated in axenic culture, and therefore, there is no type culture. Accordingly, Rule 18a of the Code (Lapage et al. 1975) is invoked, "a description, preserved specimen, or an illustration may serve as the type".

In proposing a new genus and species great care should be taken. Because Rule 18a is invoked the question of the purity of the cell material studied must be firmly established. Two considerations need to be addressed. First, is the organism being proposed for taxonomic status descriptively distinctive so as to avoid overlap or confusion with any other known genera or species? Second, is the material used for characterization sufficiently pure such that any other organisms that might be present would only insignificantly contribute to assay results?

To address the first point, the magnetic coccus presented here is morphologically distinct in comparison to other magnetic cocci that I have studied or have been reported in the literature (Blakemore 1982). The positioning of the sulfur globules, the arrangement and numeration of the flagellar tufts and subsequent wobbling motility, the magnetosomes with their unique hexagonal prismatic crystalline structure and positioning are all attributes which are unique when taken collectively.

These unique attributes have been used to ascertain microscopically that these collections of magnetic cocci consist of one distinct magnetic organism, and that there is less than 1% contamination by any other bacteria, magnetic or non-magnetic. Inoculation of growth media that support the growth of aerobic, microaerophilic, and anerobic organisms both photosynthetic and non-photosynthetic yield levels of contamination ranging from 0.014–0.13%. Based on these two microscopical and bacteriological evaluations there appears to be no significant presence of contaminants.

The isolation of homogenous DNA from pooled collections of this magnetic coccus establishes two major points. First, it yields a mol% G + C value of 61.7 ± 0.7 for this magnetic coccus. Second, it indicates no other bacterium is present, magnetic or otherwise, with a different G + C content. It does not, however, preclude the possibility that there are other species of magnetic cocci present with identical morphological characteristics and the same G + C

content. The simplest explanation for the data at this time is that the DNA was released from a single species.

In the catalase negative determination the presence of contamination is a moot point since there was no reaction in spite of large numbers of motile magnetic cocci being present.

The presence of sulfur in the magnetic cocci is unequivocable. If there were low levels of sulfur containing contaminants such as photosynthetic organisms or other colorless sulfur bacteria in the collected population, then each of those cells would have to have unusually high sulfur contents (eg., 20% sulfur for 5% contamination to achieve a 2% sulfur by weight to account for the total sulfur yield). Such cells would be highly refractile and readily apparent during microscopic examination. No such organisms were ever detected in collections of these magnetic cocci.

The location of the sulfur in the anterior portion of the cell follows from examining the cells and noting that the refractile bodies of the appropriate size are nearly always seen to be located anteriorly (Figs 1, 2). Elementary analysis of these inclusions indicated sulfur. Thin sections of cells which captured the position of the magnetosomes and sulfur inclusions confirmed their placement at opposite sides of the cell. What is evident from the data is that these bacteria represent a single unique species that is not significantly contaminated by any other organism.

Microbial transformations of high concentrations of iron by bacteria, both magnetic and non-magnetic, have been reported (Freke & Tate 1961; Jones et al. 1976; Kirschvink 1983; Konetzka 1977). This magnetic coccus, like *Aquaspirillum magnetotacticum,* precipates iron intracellularly in the form of magnetosomes, morphologically distinct crystals of biogenic magnetite (Fe_3O_4) (Balkwill et al. 1980; Blakemore 1982; Frankel et al. 1983; Mann et al. 1984; Towe & Moench 1981) which in the future may play a role in taxonomic classifications.

This magnetic coccus has characteristics in common with many of the colorless sulfur bacteria including the presence of sulfur inclusions, the absence of catalse, and a micro-aerophilic habitat (La Riviere & Schmidt 1981). The difficulty surrounding the study and isolation of the organisms within the colorless sulfur bacterial group and the elucidation of their nutritional status in the absence of pure cultures are discussed by La Riviere & Schmidt (1981) and are fully applicable to this magnetic coccus. Organisms in this group are classified with numerous genera and are morphologically diverse (Buchanan & Ribbons 1974; La Riviere & Schmidt 1981). Based on these similarities and the distinctively unique flagellar arrangement and motility pattern of this bacterium, it is proposed as a new genus of colorless, sulfur-depositing bacteria. A description of this new genus/species follows:

Bilophococcus magnetotacticus (Bi·lo′·pho·coc′cus mag·ne·to·tac′ti·cus)

gen. nov., sp. nov.,; L. adv. *bis* twice, Gr. n. *lophos* a tuft, Gr. n. *kokkos* berry; Gr. n. *magnes* magnet, comb form magneto-; Gr. n. *taktikos* showing orientation or movement directed by an agent or force; N.L. mas. n. *bilophococcus* a doubly tufted spherical bacterium; N.L. mas. adj. *magnetotacticus* showing orientation in the presence of a magnet.

Spherical cells approximately 1.6 μm in diameter. Gram-negative. Strongly motile by means of two, adjacent flagellar tufts of 10–15 flagella each. Forward motion is continuous with a wobbly characteristic. In the region of the flagellar tufts a polar membrane underlies the cytoplasmic membrane. Iron is precipitated intracellularly as magnetite inclusions (magnetosomes). Magnetotaxis is exhibited. The cytoplasm contains sulfur. Catalase negative. Habitat is microaerobic, aquatic, sediments. Organism has not been grown in axenic culture. The mol % G + C of DNA is 61–62.

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