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Identification of *Zoogloea* species and the Relationship to Zoogloeal Matrix and Floc Formation

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Three floc-forming, gram-negative, polarly flagellated rods were isolated and characterized. Our isolates were compared to four similar floc-forming organisms previously isolated in another laboratory and classified as two species of *Zoogloea*, one of *Pseudomonas*, and as one unidentified gram-negative rod. Possession of zoogloeal matrix or flocculent growth habit was examined in relation to growth and biochemical patterns of the bacteria. A possible relationship of *Zoogloea* to other gelatinous matrix-producing bacteria is also discussed.

Research was initiated for the purpose of examining the functional role of the gelatinous matrix which surrounds, and is characteristic of, the bacteria classified in the genus *Zoogloea* (*Bergey's Manual*, 7th ed.). At the beginning of this study, no *Zoogloea* isolates were available from culture collections or from laboratories which had reported on *Zoogloea*. We isolated bacteria from water samples, and an isolate was characterized and identified as *Zoogloea ramigera* according to published descriptions (*Bergey's Manual;* 1, 3, 9, 16).

During our investigation into the nature of the zoogloeal matrix surrounding our isolate 115, other investigators reported that *Z. ramigera* did not possess a capsule or a recognizable slime layer. On the basis of this description, these investigators requested recognition of their culture as the neotype of *Z. ramigera* Itzigsohn (6). This report necessitated a reappraisal of the identification of our isolates and prompted a more thorough study of the taxonomic status of *Zoogloea* in general. Re-examination of cell morphology, particularly with reference to capsule or zoogloeal matrix formation, led to a study of the literature pertaining to those zoogloeal-forming bacteria which are not generally recognized as belonging to the genus *Zoogloea*.

The purpose of this report is to establish that a new isolate identified as *Z. ramigera*, on the basis of published descriptions, does possess a clearly recognizable gelatinous matrix. There appears to be no reason not to classify the same organism in the genus *Siderocapsa*, as it is presently described in the literature. The relationship of *Zoogloea* to gelatinous matrix-producing genera of the *Siderocapsaceae* is therefore considered. Furthermore, the formation of a zoogloeal matrix by a bacterial strain is not synonomous with floc formation, since many species of bacteria are known to form flocs, but produce no gelatinous matrix (12).

MATERIALS AND METHODS

Isolation of organisms. Isolations were made from zoogloeal masses on limestone taken from organically polluted water. Material scraped from the limestone was placed into 0.05% Proteose Peptone-yeast extract broth (PPYE, Difco). The resulting suspension was exposed to ultrasound (Branson, 20-kc output) until the clumps dispersed. The treated samples were serially diluted in PPYE and were incubated at 28 C. After 3 days, the highest dilutions showing growth (10^{-7} to 10^{-10}) were streaked onto PPYE agar and Tryptone Glucose Extract Agar (TGE, Difco). When sufficient growth occurred, colonies of different morphological types were transferred to PPYE in duplicate. One tube was placed in an incubator at 28 C, and the other was placed on a rotary shaker (60 rev/min) at 24 ± 1 C. Incubation was continued until growth was observed. Those tubes containing a pellicle (stationary) or a floc (shaker) were selected, and the entire isolation procedure was repeated until pure cultures were obtained.

Characterization of organisms. Cells were inoculated into 100 ml of 0.05% PPYE or 0.5% Trypticase Soy Broth (BBL) and were grown on a rotary shaker at 24 ± 1 C for 48 hr. They were washed twice with 0.3 M phosphate buffer (*p*H 7.0) and were resuspended in 10 ml of the same buffer.

Casein hydrolysate (0.2% enzymatic, Difco) containing 16 μg of bromothymol blue indicator per ml served as the basal medium for carbohydrate utilization studies. Carbohydrates were added to the basal medium as dry material to give either 1% (w/v) solutions or as sterile differential discs (1 disc per 7 ml,

Difco). Sugars other than those added on discs were sterilized by autoclave, except for cellobiose, fructose, glucose, and sorbose, which were sterilized by membrane filtration. Alcohols were prepared in 1% concentrations. Crystal violet was added to TGE to give a final concentration of 0.001% for use as a differential growth medium. All other media were prepared according to standard methods (15). Tubes were inoculated with 0.1 ml of a cell suspension, and results were read after 24, 48, and 120 hr.

Three isolates, designated as 115, C-3, and I-16-M, were examined in various defined growth media for the purpose of comparing flocculation, zoogloeal matrix formation, and growth. The media that were used were designated as A, B, and C. Medium A was the arginine medium of Crabtree et al. (5); this medium was varied by adding (A1) folic acid (10 mμg/ml) or (A2) folic acid (10 mμg/ml) plus glucose (0.5%). Medium B was the ammonium nitrate medium of Crabtree (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1966); it contained NH_4NO_3, 0.1%; $MgSO_4 \cdot 7HOH$, 0.02%; K_2HPO_4, 0.2%; KH_2PO_4, 0.1%; and glucose, 0.5%. This medium was varied by adding either (B1) vitamin B_{12} (1.5 mμg/ml) or (B2) B_{12} (1.5 mμg/ml) plus folic acid (10 mμg/ml) plus biotin (2.0 mμg/ml). Medium C was the Casamino Acids medium of Crabtree (Ph.D. Thesis, 1966); it contained Vitamin Free Casamino Acids, 0.1% (Difco); $MgSO_4 \cdot 7HOH$, 0.02%; K_2HPO_4, 0.2%; KH_2PO_4, 0.1%; B_{12}, 1.5 mμg/ml; biotin, 2.0 mμg/ml; this medium was varied by adding either (C1) folic acid (10 mμg/ml) or (C2) folic acid (10 mμg/ml) plus glucose (0.5%).

RESULTS

Characterization of isolates. The four floc-forming isolates obtained from Crabtree, which were identified as *Z. ramigera*, *Z. filipendula*, *Pseudomonas denitrificans*, and one unidentified species, as well as three of our floc-forming isolates, were examined with standard techniques for identification and characterization. Tables 1, 2, and 3 list the results of biochemical reactions. Crystal violet decolorization is listed as a differential criterion for classification, because it was observed that some of the isolates behaved differently when grown on crystal violet agar. Isolate 115 decolorized crystal violet; C-1, C-3, and C-22-4 failed to develop colonies on the medium; and I-16-M, P-8-4, and P-95-5 adsorbed the dye to produce violet colonies.

The *p*H indicator bromothymol blue is a very sensitive acid indicator, and it should be stressed that the values recorded in Tables 1, 2, and 3 as VSA (very slightly acid) are not significantly acid but merely represent a change from the inital *p*H (7.2) of the medium toward acidity. Therefore, the difference between values recorded as + and as VSA is probably not highly significant.

Comparison of organisms. All isolates were gram-negative, polarly flagellated rods which flocculated and contained sudanophilic granules under certain growth conditions. None of the Crabtree isolates (I-16-M, P-8-4, P-95-5, C-22-4) has been shown to produce a zoogloeal matrix or capsule. Strain 115 of our isolates had been shown to possess a zoogloeal matrix, whereas C-1 and C-3 have not.

Isolate I-16-M has been recommended by Crabtree and McCoy as a neotype *Z. ramigera* (6). Comparisons of isolate I-16-M to P-8-4, P-95-5, and C-22-4 have been made by Crabtree and will not be further considered in this paper

TABLE 1. *Biochemical reactions of floc-forming bacteria*[a]

Organism	Litmus milk	Methyl red	Nutrient gelatin	Urease	Indole	Citrate	NO_3 reduced	$NO_3 \downarrow N_2$	Casein hydrolysis	Starch hydrolysis	Crystal violet decolorized	Catalase
Zoogloea ramigera												
115	Slightly alkaline	−	−	+	−	−	+	−	+	+	+	+
C-1	Alkaline	−	−	−	−	+	+	−	−	−	NG	+
C-3	Acid	+	+	−	+	+	−	−	+	+	NG	+[b]
Z. ramigera												
I-16-M	Slightly alkaline	−	−	−	−	−	+	−	−	−	−	+
Z. filipendula												
P-8-4	Slightly alkaline	−	−	+	−	−	+	−	−	−	−	+
Pseudomonas denitrificans												
P-95-5	Slightly alkaline	−	−	−	−	−	+	+	−	−	−	+
C-22-4	Slightly alkaline	−	−	−	−	−	+	−	−	−	NG	+

[a] All organisms were cytochrome oxidase positive. None produced sulfide. Reactions are indicated as follows: −, negative response; +, positive response; NG, no growth.

[b] Very slight.

TABLE 2. *Reaction of floc-forming bacteria in sugar broth with bromothymol blue indicator*

Organism	Arabinose	Cellobiose	Fructose	Galactose	Glucose	Glycogen	Inulin	Lactose	Levulose	Maltose	Mannose	Melezitose	Melibiose	Raffinose	Rhamnose	Salicin	Sorbose	Sucrose	Xylose
Zoogloea ramigera																			
115	VSA[a]	VSA	VSA	SA[b]	+[c]	SA	+	VSA	VSA	SA	VSA	+	+	+	+	+	+	VSA	+
C-1	VSA	VSA	VSA	+	VSA	+	VSA	VSA	+	VSA	VSA	VSA	VSA	VSA	VSA	VSA	VSA	VSA	VSA
C-3[d]	A[e]	A	A	A	A	A	+	A	A	A	A	+	+	+	+	A	+	A	+
Z. ramigera I-16-M	A	+	VSA	VSA	VSA	+	+	+	VSA	SA	+	+	+	+	+	+	+	SA	VSA
Z. filipendula P-8-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas denitrificans																			
P-95-5	SA	+	SA	SA	+	+	+	+	SA	SA	VSA	+	+	+	+	+	+	VSA	SA
C-22-4	A	+	A	A	A	+	+	+	SA	VSA	SA	+	+	+	SA	+	+	SA	SA

[a] Very slight acid, blue green, *p*H 6.9 to 6.8.
[b] Slight acid, yellow green, *p*H 6.7 to 6.3.
[c] Growth but no acid, blue, *p*H 7.2 to 6.9.
[d] Acid was also produced in sufficient quantity to change bromocresol purple-yellow (*p*H 5.2). All other organisms grew without acid reaction in purple broth base.
[e] Acid, yellow, *p*H 6.2 to 6.0.

TABLE 3. *Reactions of floc-forming bacteria in alcohols and sugar alcohols with bromothymol blue indicator*[a]

Organism	Methanol (1%)	Ethyl alcohol (1%)	Propanol (1%)	n-Butyl alcohol (1%)	Glycerol (1%)	Inositol	Mannitol	Adonitol	Sorbitol	Dulcitol
Zoogloea ramigera										
115	+	+	+	±	+	+	+	+	+	+
C-1	+	VSA	VSA	+	VSA	VSA	VSA	VSA	VSA	VSA
C-3	+	+	+	+	A	+	A	+	+	+
Z. ramigera I-16-M	+	+	±	±	+	+	VSA	+	+	+
Z. filipendula P-8-4	+	VSA	VSA	+	+	+	+	+	+	+
Pseudomonas denitrificans										
P-95-5	+	+	+	+	VSA	SA	VSA	SA	SA	+
C-22-4	+	A	VSA	±	+	+	SA	+	SA	+

[a] Reactions are indicated as follows: +, growth but no acid, blue; ±, slight growth but no acid, blue; VSA, very slight acid, blue green; SA, slight acid, yellow green; A, acid yellow.

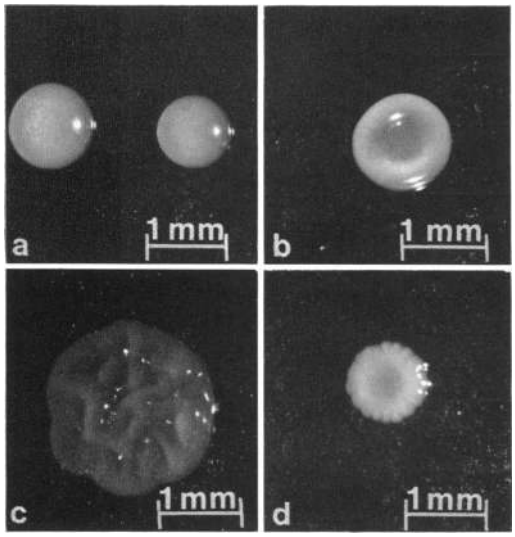

FIG. 1. *Colonies of Zoogloea ramigera isolates 115 and I-16-M after cultivation on TGE agar at 28 C. All colonies are straw colored and have a viscous appearance but are tough and leathery. (a) 115 after 24 hr, showing glistening appearance. (b) 115 after 30 hr, showing characteristic depressed center of "donut" shaped colony. (c) 115 after 40 hr, showing the rugose appearance. (d) I-16-M after 72 hr, showing the depressed center and a crenated periphery. I-16-M did not develop a completely rugose appearance.*

(Ph.D. Thesis, 1966). C-3 differed markedly from all other isolates with regard to biochemical reactions and was considered to be a flocculent *Pseudomonas* or *Aeromonas* species.

Isolates C-1, I-16-M, and 115 differed in at least two biochemical characteristics. Of these three isolates, only I-16-M produced a significant amount of acid from any of the carbon sources examined. I-16-M produced acid from arabinose, presumably via an oxidative pathway.

Isolate 115, which formed a typical gelatinous or zoogloeal matrix, also differed from I-16-M in ability to degrade urea, starch, casein, and crystal violet dye. The colonial morphology of isolate 115 was quite similar to that of I-16-M after 24 hr of incubation. However, the colonies of isolate 115 developed a rugose appearance upon further incubation (40 to 72 hr), whereas the colonial appearance of isolate I-16-M remained the same. The colonies shown in Fig. 1 are typical of the colonial morphology of the two *Z. ramigera* isolates when cultivated on TGE agar plates at 28 C. Figure 1a is a photograph of isolate 115 taken after 24 hr of incubation. Colonies glistened and appeared viscous but were tough and leathery. Figure 1b shows a colony of isolate 115 after 30 hr of incubation; a typical "donut" shaped colony with a depressed center can be seen. Figure 1c shows the rugose appearance of the 115 colony after 40 hr of incubation. For comparative purposes, Fig. 1d shows a colony of *Z. ramigera* I-16-M after 72 hr. The depressed center was present and the colony periphery was crenated. However, the entire colony did not develop the wrinkled or rugose appearance of isolate 115.

Figure 2a illustrates the zoogloeal matrix and fingerlike projections formed by cells of isolate 115 when cultivated in the arginine medium of Crabtree. Figure 2b shows cells of isolate 115 which were stained by the Maneval technique and then photographed through a phase-contrast microscope; the photograph suggests that the floc was comprised of individual packets of cells. Figure 2c shows packets similar to those shown in 2b. The cells were stained by the Maneval technique and were photographed through a light micro-

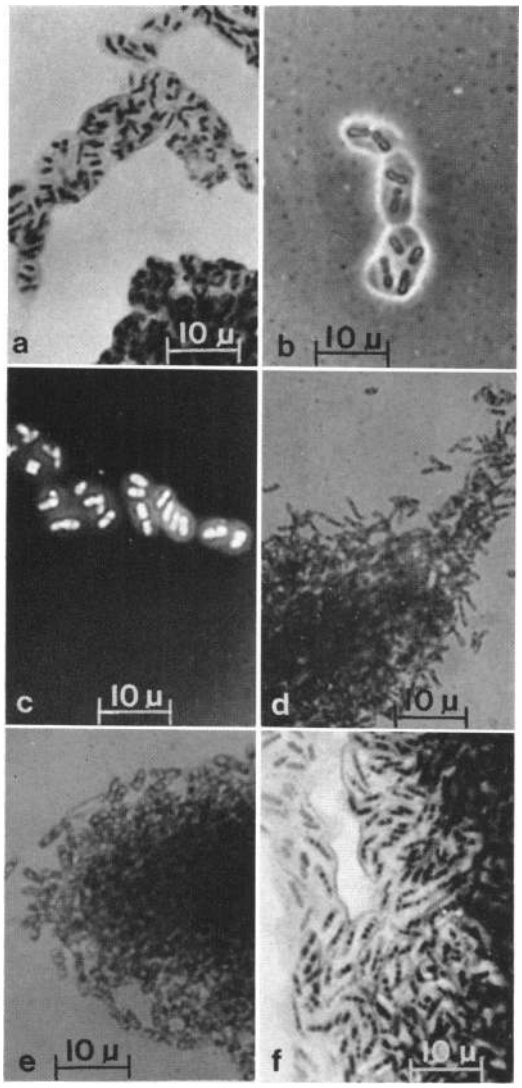

FIG. 2. *Photomicrographs of stained flocs of Zoogloea ramigera isolates 115 and I-16-M and Pseudomonas C-3. (a) 115 floc stained with 1% aqueous crystal violet; fingerlike projections composed of cells embedded within a zoogloeal matrix are shown. (b) 115 floc under phase contrast microscopy after being negatively stained by the Maneval technique; the cells are embedded in generally globular aggregates which adhere together. (c) 115 floc negatively stained by the Maneval method and photographed through a light microscope. (d) I-16-M floc stained with crystal violet; the lack of extracellular matrix and the granular appearance of the cells can be seen. (e) C-3 floc stained with crystal violet; the lack of extracellular matrix and the granular appearance of the cells can be seen. (f) 115 floc stained with crystal violet after cultivation on medium B2; the granular appearance of the cells, as compared to Fig. 2a, is shown. The extracellular matrix is also present.*

scope. *Z. ramigera* I-16-M is shown in Fig. 2d. No extracellular material was observable around isolate I-16-M when it was grown on the same medium as isolate 115 (Fig. 2a, b, c). Intracellular granules previously reported to be polyhydroxybutyrate were present (5). The morphological characteristics of isolate C-3 are shown in Fig. 2e. No extracellular material was observed, but large quantities of intracellular material accumulated. Continued synthesis of intracellular granular material caused pleomorphism. Since the granule synthesis was related to nutrient and growth conditions, cell size and shape were not desirable characteristics for identification purposes. Figure 2f is a photograph of isolate 115 cultured in medium B2; when compared to Fig. 2a it shows the difference in cell morphology due to nutrients.

Utilization of defined media. Further comparisons among isolates I-16-M, 115, and C-3 were made with the defined media A, B, and C (*see* Materials and Methods). All of the defined medium variations examined supported flocculent growth of the three isolates, although flocculation was not quantitatively consistent. For example, isolates 115 and I-16-M gave the greatest amount of flocculent growth when the media were supplemented with glucose and vitamins. Isolate C-3 produced more flocculent growth in the arginine medium (A) when no supplement was added, but it did flocculate in the supplemented medium. The Casamino Acids medium (C) allowed more rapid growth of the bacteria. The medium which promoted the most flocculent growth was (A) supplemented with glucose and B_{12}. This medium was used in all subsequent studies. It should also be noted that the cultures grew well in the presence of ammonium ions (medium B); this is contrary to the data of Rich (14).

DISCUSSION

The generic name *Zoogloea* is derived from Greek roots and literally translates as "living glue." It seems apparent that descriptions in the literature were based upon microscopic observations of the gelatinous material or zoogloeal matrix and that this was the unique characteristic of the organisms upon which classification resided. Cultures which do not possess a gelatinous matrix may flocculate but are not necessarily *Zoogloea* bacteria. That is, "floc formation" and "zoogloeal growths" are not synonymous and may not even be directly related phenomena. We prefer to consider zoogloeal formation as the production of a highly polymerized exocellular material analogous to a capsule, and flocculation as a clumping of cells probably resulting from cell surface attractions which may or may not involve

physiochemical influences of capsular material.

Cell size and shape, as well as gelatinous material production, vary considerably with culture media, incubation temperature, and, in liquid culture media, perhaps with oxygen tension. However, it should be stated that oxygen tension varies with temperature, and one influence is not exclusive of the other in our test system. Cell size and shape, within limits, are therefore not adequate criteria for characterizing *Zoogloea* species. However, cell size and floc shape are the primary criteria used to differentiate *Z. ramigera* from *Z. filipendula*, the only *Zoogloea* species recognized in *Bergey's Manual*. Comparisons of the Crabtree isolate I-16-M to our isolates, by use of identical procedures, show that somewhat similar patterns of carbohydrate reactions exist among cultures which do produce a gelatinous matrix (115) and those which do not (I-16-M). It is also apparent that cells which flocculate and appear similar morphologically under most growth conditions do not have similar biochemical reaction patterns (C-3 and 115). Isolate C-3 appears to be a *Pseudomonas* or *Aeromonas* species which flocculates. Isolate 115, by virtue of the presence of a zoogloeal matrix, is a *Zoogloea* species according to recognized systems of classification (*Bergey's Manual*). By comparison of isolate 115 to I-16-M, one must conclude that I-16-M is either a nonzoogloeal producing *Zoogloea*, as reported by Crabtree and McCoy (4), or a *Pseudomonas* species.

The presence of a zoogloeal matrix appears to be the only valid criterion for distinguishing between a *Zoogloea* species and a nonfluorescing *Pseudomonas* species. However, production of zoogloeal matrix is related to growth conditions. Therefore, the absence of matrix is not an adequate criterion for identification, particularly when culture conditions are not accurately described. This is the situation in much of the older literature, and it probably contributes to the confusion which exists with regard to classification of these bacteria.

Although the biochemical reactions of isolate 115 and *Z. ramigera* I-16-M are similar, the organisms appear to be sufficiently different to conclude that they are different species. However, Unz has isolated 147 strains of floc-forming bacteria primarily on the basis of biochemical differences, all of which he considers to be *Zoogloea* species (Ph.D. Thesis, Rutgers, The State Univ., New Brunswick, N. J., 1965). This suggests that many strains of *Zoogloea* can be found in nature and leads us to conclude that both isolate I-16-M and isolate 115 are strains of *Z. ramigera*, particularly since there is no definitive basis for identifying them as strains of *Z. filipendula*. Indeed, the fact that Butterfield (3) and Wattie (16) did not find the same biochemical reactions using individual isolates of *Z. ramigera* is recognized in *Bergey's Manual*. Perhaps the taxonomic status of *Zoogloea* should undergo a complete revision. However, that is not the purpose of this report.

One further point we wish to make regards the observation that both isolates 115 and I-16-M deposit high concentrations of iron around the flocs when iron salts are added to the growth media. Since 115 has a gelatinous matrix around which iron is deposited, one must consider the possibility that it is a species of either the family *Siderocapsaceae* or the family *Caulobacteraceae*. A discussion of the relationships between iron-depositing bacteria and the gelatinous matrix of zoogloea producers is therefore warranted.

DeToni and Trevisan (7) used a gelatinous matrix as a criterion for differentiating subfamilies of *Coccaceae*. In subsequent classification of the same groups of bacteria, Winslow and Rogers (17) used the phrase "cells aggregated in groups, packets or zoogloea masses" for differentiating all known cocci. In 1917, Buchanan (2) suggested that the genus *Siderocapsa* might be sufficiently distinct from other cocci, and he presented a new key which included the tribe *Siderocapseae*. In 1929, Pribram (13) reported on bacterial classification and recognized both rod and coccoid forms of *Siderocapsaceae*. He used the term "zoogloea," although he did not recognize the genus *Zoogloea*. Drake (8), who recently presented a discussion of *Siderocapsa treubii*, considered the work of Hardman and Henrici (10) pertaining to occurrence, distribution, and growth of *Siderocapsa* in alkaline iron bearing waters.

With reference to a possible relationship between *Zoogloea* and species in the *Caulobacteraceae*, Henrici and Johnson (11) described the stalked iron-depositing bacterium *Nevskia* on the basis of lobose stalks composed of gum, forming zoogloea-like colonies. They published photomicrographs and drawings which show some similarity to photographs of isolate 115 (Fig. 2). However, there is no evidence to suggest a relationship between *Zoogloea* and *Nevskia*.

It is evident that zoogloeal-producing bacteria are common and ubiquitous in aquatic habitats. Classification of *Zoogloea* is presently based upon highly artificial criteria, such as presence of a gelatinous matrix, which is dependent upon growth conditions. Classification of *Siderocapsaceae* is also dependent upon artificial criteria, such as deposition of iron around the gelatinous matrix in the natural habitat. This is dependent upon the iron content of the natural water environment. Species of *Siderocapsaceae* are pres-

ently identified on the basis of growth on submerged slides. Few biochemical data for this genus are available because *Siderocapsa* cells have not been cultured on artificial media. We would like to suggest the possibility that past investigators would have considered the same bacterium a *Siderocapsa* if it was observed in iron bearing water, or a *Zoogloea* if it was isolated from water rich in organic substances but extremely low in iron content. *Z. filipendula* has been reported to deposit iron when in acid iron-bearing water (*Bergey's Manual*). Isolate 115 deposits copper, cobalt, nickel, and presumably other ions in the gelatinous matrix (*unpublished data*). If these cations had been present in sufficient concentrations in the environment, we would have considered this finding in a different manner.

It is possible to interpret Fig. 2a and b as a collection of generally spherical and ellipsoid shaped flocs, groups, packets, or zoogloeal masses, each containing several bacteria which are surrounded by a gelatinous matrix similar to the growth pattern now recognized for *Siderocapsa* species. Adherence of these globular, gelatinous masses would explain the makeup of what has historically been referred to as fingerlike projections in the literature on *Zoogloea*.

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