Neotype Strain of *Zoogloea ramigera* Itzigsohn

Request for an Opinion

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The neotype strain of *Zoogloea ramigera* Itzigsohn is strain I-16-M (= ATCC 19623 = NCIB 10340 = NCTC 10482). However, this strain is not suitable as a neotype, primarily because of its inability to form the true zoogloea cited by Itzigsohn in his original description of this organism. It is requested, therefore, that the Judicial Commission issue an Opinion rejecting strain I-16-M as the neotype of *Z. ramigera* and replacing it with strain 106 (= ATCC 19544).

The systematics of the genus *Zoogloea* and its species are encumbered by nomenclatural problems which have been reviewed by Zvirbulis and Hatt (45). Furthermore, the determination of the taxonomic position of *Zoogloea ramigera* Itzigsohn (24), the type species of the genus, has been complicated by (i) reports that *Z. ramigera* may not be a valid species but rather a growth form of several unrelated bacteria (20, 22), (ii) the dubious purity of cultures claimed by individuals to be *Z. ramigera* [e.g., the "saltpeter fungus" of Stutzer and Hartleb (36), and Soriano's strain cited by Wattie (42)], and (iii) insufficient physiological, biochemical, and nutritional data to accurately characterize and distinguish the species.

Since its discovery and brief description by Itzigsohn, *Z. ramigera* has been identified primarily from the characteristic tree-like zoogloea which are frequently observed in polluted environments. However, some workers have not made clear the distinction between zoogloea formation and the less specific property of flocculation in describing the growth habit of their cultures (31, 32, 37, 42). Crabtree et al. (10, 12) isolated and described a nonencapsulated, nonzoogloea bacterium which they named *Z. ramigera* strain I-16-M. Crabtree and McCoy (11) proposed that *Z. ramigera* strain I-16-M be designated the neotype strain of *Z. ramigera* Itzigsohn. Recently, Angelbeck and Kirsch (2) described two non-slimeforming strains of *Z. ramigera*, one of which is strain I-16-M. Friedman and Dugan (17) suggested that *Z. ramigera* strain I-16-M

may be a *Pseudomonas* species. It may be inferred from these reports that *Z. ramigera* strain I-16-M does not conform to the description of *Z. ramigera* as originally given by Itzigsohn (24). As such, the wisdom in designating a nonzoogloea bacterium as the neotype for *Z. ramigera* seems questionable. In view of these developments, a study was undertaken to compare the properties of *Z. ramigera* strain I-16-M with those of *Zoogloea* strain 106, a typical zoogloea isolate which was obtained by microdissection of a natural branched zoogloea (39).

MATERIALS AND METHODS

Zoogloea strain 106 (ATCC 19544) and *Z. ramigera* strain I-16-M (ATCC 19623) were received from the American Type Culture Collection. The procedures employed in the original isolation of these strains were described previously (11, 39). The technique used by Unz and Dondero (39) for the isolation of 147 *Zoogloea* strains, including *Zoogloea* strain 106, was an important factor in establishing the relationship between the bacteria and source material. The direct isolation method permitted cultures to be obtained which indisputably originated from natural zoogloea. In the isolation work of Unz and Dondero (39), a washed zoogloea was transferred with a clean capillary pipette to a thin layer of clarified isolation medium located on a clean, alcohol-disinfected cover slip. The cover slip was mounted with the medium side down on a moist chamber. The isolations were made while under observation with a microscope at 440 × magnification. Bacterial cells were individually teased from the zoogloea with a glass microneedle fixed in a micromanipulator. The single cells were drawn to a sterile, marked area on the surface of the medium. The moist chamber and cover slip were incubated at 28 C

in a covered glass container humidified with dampened blotting paper. Microcolonies which formed during incubation were transferred to 3 ml of liquid isolation medium with the aid of sterile capillary pipettes. The liquid cultures were checked for purity by conventional streak plating.

Stock cultures of *Zoogloea* strain 106 and *Z. ramigera* strain I-16-M were maintained at 20 C in CY medium which contains 5.0 g of Casitone (Difco) and 1.0 g of yeast autolysate (Albimi Laboratories, Flushing, N.Y.) per liter. *Zoogloea* strain 106 would not grow in the arginine medium of Crabtree and McCoy (11). Inocula for differential tests were prepared by harvesting 48-hr-old CY-medium shake cultures and washing centrifuged cells three times in distilled water. The washed cells were adjusted to an optical density (OD) of 0.1 at 500 nm. The standard inoculum for all tests, except the nutritional tests, was 0.1 ml. Carbon and nitrogen sources were tested by using 0.1 ml of one-tenth strength OD 0.1₅₀₀ cell suspensions.

The characterizations made of the two *Zoogloea* strains along with references describing the procedures employed are presented in Table 1.

RESULTS

The results of a comparative diagnostic study of *Zoogloea* strain 106 and *Z. ramigera* strain I-16-M are presented in Table 1. In some cases, the results obtained with *Z. ramigera* strain I-16-M were contradictory to those reported by Crabtree and McCoy (11). Some of these discrepancies may be accounted for in the test procedures used. Certain tests conducted by Crabtree and McCoy (11) were not repeated in this investigation, and the reader is referred to the aforementioned publication for further details on *Z. ramigera* strain I-16-M.

Characteristic of *Zoogloea* strain 106 is the variability of its growth habit in laboratory culture. After original isolation, *Zoogloea* strain 106 grew almost entirely in flocculent form in CY or CGY (CY plus glycerol, 5 g/liter) medium and produced thick, slippery pellicles in static culture. Repeated subculturing resulted in the gradual shift in growth habit from the flocculent to the dispersed cell form. However, after 8 years of subculturing, periodically and unpredictably our cultures of *Zoogloea* strain 106 may produce copious flocculent masses with little turbidity, both in static and shaken cultures. Very infrequently, pellets of branched zoogloeoas form in shake cultures, although amorphous zoogloeoas are common.

Other evidence of cultural change is the increased capacity of *Zoogloea* strain 106 to grow on standard nutrient agar (Difco). When first isolated, the organism grew poorly on nutrient agar, and 3-day-old colonies were flat, punctiform, lobate, and translucent with some

grey coloration in well-separated colonies. The nutrient agar colonies of *Zoogloea* strain 106 described in Table 1 were grown by using a stock culture which had been repeatedly carried in the CY medium. The colonies are relatively large, and it is probable that the strain is so well acclimated to the amino acids-rich CY medium that it is now able to grow well on nutrient agar. No changes have been noted in the appearance of CY agar colonies after 8 years of subculture. Other observed properties of *Zoogloea* strain 106 have not changed over years of subculturing.

DISCUSSION

Bacterial flocculation should not be confused with zoogloea formation. [As used in this paper, the term "floc" refers to a fluffy, flaky, or granular aggregation of microorganisms in suspension ("floc" is not a synonym of "zoogloea"); "zoogloea" refers to a clearly defined gelatinous matrix in which bacteria are embedded, and "zoogloeoal floc" refers to a floc composed of bacterial zoogloeoas.] McKinney and Horwood (31) stated that bacteria other than *Z. ramigera* are capable of floc formation, and their work prompted others to isolate and describe floc-forming bacteria. Unfortunately, little attention has been paid to the distinction between ordinary aggregation of cells and the formation of zoogloeoas. It is a simple matter to demonstrate the zoogloeoal nature of activated sludge particles with the aid of the microscope and cytological reagents. It is not so easy to determine which of the bacteria in the activated sludge floc contribute to the production of the zoogloeoal matrix and by what means. McKinney and Weichlein (32) could not attest to the presence of zoogloeoal matrix in flocs of their diverse activation sludge isolates and used the term "pseudozoogloeoal" to describe the condition of the bacterial flocs. Kiuchi et al. (25, 26) have demonstrated the floc-forming capabilities of two activated sludge bacteria, *Pseudomonas stutzeri* and *Alcaligenes faecalis*. However, their published photographs do not show the flocculated bacteria to be zoogloeoal, and they did not include tests to demonstrate the presence or absence of extracellular matrix. That these workers were able to demonstrate flocculation by employing mixtures of pure-culture floc-forming bacteria, non-floc-forming bacteria, or both, does not in any way contribute to the establishment of a relationship between these bacteria and *Z. ramigera*.

The branched zoogloeoal colonies originally described by Itzigsohn (24) have been historically the important taxonomic characteristic of

TABLE 1. *Characteristics of Zoogloea strain 106 and Z. ramigera strain I-16-M*

Test	Procedure	Characteristics	
		<i>Zoogloea</i> strain 106	<i>Z. ramigera</i> strain I-16-M
Cell shape (2 days)	CY medium; phase contrast	Rods, slightly curved or straight with rounded or slightly tapered ends	Rods, straight with rounded, blunt, or tapered ends
Cell size (2 days)	CY medium; cells measured from photographs	1.1 to 1.3 μ m by 2.5 to 3.5 μ m	0.6 to 0.9 μ m by 1.5 to 2.5 μ m
Motility	Wet mount	Active	Active
Flagella	Electron microscopy	Single monopolar flagellum (Fig. 1)	One to several flagella attached laterally to cell (Fig. 2)
Capsules	Skim milk (14)	Capsules not easily demonstrated in dispersed cells; readily seen with small groups of cells (Fig. 3)	Capsules not found associated with cells in any form
Spores	Staining (35)	Absent	Absent
	Heat (19)	Absent	Absent
Gram reaction	Hucker's modification (35)	Negative	Negative
Growth habit (3 days)			
Stationary	CY medium	Dispersed cells with flocculent sediment; membranous surface film	Dispersed cells with pellicle; occasionally lacy network of cells attached to sides of tube
Shaken	CY medium	Dispersed cells with some floc	Dispersed cells with floc
Zoogloea formation	Skim milk (14); india ink	Branched and amorphous zoogloea (Fig. 4-6)	Absent (Fig. 7)
Nutrient gelatin			
Plates	Reference 35	Colonies round, 1 to 2 mm, dull white, liquefaction in 3-4 weeks	Colonies round, 1 to 2 mm, translucent or dirty white; no liquefaction
Stab	Reference 35	Good surface growth; crateriform liquefaction	Good growth at surface and along path of stab; no liquefaction
Gelatin agar	Unz and Dondero (39)	Growth and liquefaction	Growth; no liquefaction
Agar slant	CY medium	Translucent, effuse, dry and tough	Creamy white to straw-colored, filiform, dry, wrinkled, tough, and leathery
Agar colonies (3 days)	CY medium	Punctiform or circular with entire or undulate edges, flat or slightly raised, nonpigmented, glistening, usually not more than 2 mm in diameter, granular by reflected light (Fig. 8)	Circular, straw-colored centers, glistening, raised, entire or undulate edges
	Standard nutrient agar (Difco)	Well-separated colonies circular, approx 1 to 2 mm in diameter, entire edges, raised and sometimes umbonate, cream or grey-colored (depending on size of colony), glistening	Well-separated colonies circular, 2 to 3 mm in diameter, entire or undulate edges, convex, some colonies have apical sinus, straw-colored, glistening

TABLE 1. *Characteristics of Zoogloea strain 106 and Z. ramigera strain I-16-M (Cont'd)*

Test	Procedure	Characteristics	
		<i>Zoogloea</i> strain 106	<i>Z. ramigera</i> strain I-16-M
Arginine dihydrolase	Thornley (38)	Absent	Absent ^a
Tyrosine agar	Collins (9); CY medium base and 0.9% tyrosine	Growth; no clearing	Growth with clearing and appearance of brown pigment
Caseinate agar	Unz and Dondero (39)	Growth, hydrolysis	Growth, no hydrolysis
Starch agar	Skerman (34); half strength CY medium base	Growth, no hydrolysis	Growth, no hydrolysis
Potato slant	Collins (9)	No growth	Dull, brownish-grey growth
Litmus milk	Reference 35	No change	Reduced
Catalase	Skerman (34)	Present	Present
Oxidase	Skerman (34)	Present	Present
Peroxidase	Skerman (34)	Present	Present
Indole	Reference 35	Not produced	Not produced
Acetylmethylcarbinol	Skerman (34)	Not produced	Not produced
Koser citrate	Reference 35	No growth	No growth
Ammonia			
Casitone	CY medium	Not produced	Produced
Arginine	Skerman (34)	Not produced	Produced
Asparagine	Mineral salts medium and Nessler's reagent (40)	Produced	Produced
Nitrate	Reference 35	Reduced to nitrite and nitrogen gas	Not reduced
Acetamide	Buhlman et al. (5)	Not decomposed	Not decomposed
Trimethylamine	Wood and Baird (43)	Not produced	Not produced
Hydrogen sulfide	Kligler iron agar (Difco)	No growth	Growth; H ₂ S not formed
	Skerman (34): peptone- cysteine-sulfate medium tested with lead acetate paper	Growth; H ₂ S not produced	Growth; H ₂ S produced
Tributyrin	Skerman (34); CY medium base	Hydrolyzed	Hydrolyzed
Urea	Skerman (34); Casitone substituted for peptone and agar omitted	Hydrolyzed	Not hydrolyzed
Vibriostat 0/129 in peptone water	Collier et al. (8)	Growth inhibited by vibriostat 0/129	Growth not inhibited by vibriostat 0/129
Carbohydrates	Hugh and Leifson in Skerman (34); half- strength CY basal medium	No acid or gas from any carbohydrates tested	Acid formed oxidatively from arabinose, ethanol, rhamnose, ribose, xylose, fructose, glucose, glycerol, mannitol, sucrose. No acid formed from starch and inulin
Sole carbon sources	Unz and Dondero (40)	Utilizes malate, fumarate, pyruvate, acetate, pro- pionate, butyrate, myristate, palmitate, lactate, oxalacetate, α - hydroxybutyrate, citrate,	Utilizes acetate, pyruvate, malate, fumarate, oxalacetate. Does not utilize benzoate or <i>m</i> -toluate. [Crabtree and McCoy (11) report utilization of oxalate]

TABLE 1. *Characteristics of Zoogloea strain 106 and Z. ramigera strain I-16-M (Cont'd)*

Test	Procedure	Characteristics	
		<i>Zoogloea</i> strain 106	<i>Z. ramigera</i> strain I-16-M
Sole carbon and nitrogen sources	Unz and Dondero (40)	succinate, α -keto glutarate, acetaldehyde, ethanol, <i>n</i> -propanol, <i>n</i> -butanol, benzoate, <i>m</i> -toluate Utilizes asparagine, aspartate, glutamate, and cysteine	Utilizes asparagine, aspartate, glutamate, citrulline, ornithine, arginine, histidine, tyrosine
Inorganic nitrogen	Unz and Dondero (40)	Ammonia utilized	Ammonia utilized
Salt tolerance	CY basal medium	Growth in 0.5% but not 0.8% NaCl	Not tested. [Crabtree and McCoy (11) report growth in 3% but not 6.5% NaCl]
Optimum temperature	Unz and Dondero (40)	Growth most rapidly initiated at 28 C. Rate of cell division fastest near 37 C. Slow growth at 9 C and no growth at 45 C.	Not tested. [Crabtree and McCoy (11) report optimum temp to be 28–30 C, with slow growth at 10 C and no growth at 45 C]
Optimum pH	Unz and Dondero (40)	Growth most rapidly initiated at neutrality. No growth at pH 5.0 or 11.0.	Not tested. [Crabtree and McCoy (11) report the optimum pH to be 7.0–7.5 with no growth at 4.5 or 9.6]
Distinctive characters		Forms amorphous and branched zoogloes; nonamylolytic; proteolytic	Three to five cells frequently attached at poles to form star-like aggregates; flocs are compact but nonzoogloal. [Crabtree and McCoy (11) state the following distinctive characters for the strain: colonies smooth, convex with sinus at apex, tough and leathery; formation of lacy, tape-like growth in standing broth cultures, starlike flocs in shaken cultures; cells in flocs compactly arranged in "finger-like" projections, contain large amount of sudanophilic granules (polymer of β hydroxybutyric acid); inactive in fermentation of carbohydrates but very active in oxidative utilization. Non-proteolytic.]
Source		Trickling filter branched zoogloea	Crabtree and McCoy (11) state the source of their strain as flocs in sewage sludge (activated)
Habitat		Organically polluted waters and wastewater biological flocs	Crabtree and McCoy (11) report the habitat of their strain as natural waters containing decomposing organic matter, flocs of sewage, and some industrial wastes

^a Result not in agreement with that reported by Crabtree and McCoy (11).

Fig. 1. Single, monopolar flagellum of *Zoogloea* strain 106. Casitone-yeast autolysate liquid medium. 28 C, 24 hr. Electron micrograph.

Fig. 2. Lateral flagella of *Zoogloea ramigera* strain I-16-M. Casitone-yeast autolysate liquid medium. 28 C, 24 hr. Electron micrograph.

Fig. 3. Capsules of *Zoogloea* strain 106. Casitone-yeast autolysate liquid medium. 28 C, 6 days. Skim milk-treated wet mount. Phase contrast.

Fig. 4. Branched zoogloea. *Zoogloea* strain 106. Lactate-mineral salts liquid medium. 28 C, 60 hr. Skim milk-treated wet mount. Phase contrast.

Fig. 5. Branched zoogloea with gelatinous matrix clearly defined. Note small branches beginning to develop from large zoogloea structure (left center). Lactate-mineral salts liquid medium. 28 C, 60 hr. Skim milk-treated wet mount. Phase contrast.

Fig. 6. Amorphous zoogloea. *Zoogloea* strain 106. Casitone-yeast autolysate liquid medium. 28 C, 72 hr. Skim milk-treated wet mount. Phase contrast.

Fig. 7. Compact, nonzoogloal floc. *Zoogloea ramigera* strain I-16-M. Halo around floc is an optical artifact. Casitone-yeast autolysate liquid medium. 28 C, 72 hr. Skim milk-treated wet mount. Phase contrast.

Fig. 8. Granular, slightly raised colonies of *Zoogloea* strain 106. Casitone-yeast autolysate agar medium. 28 C, 72 hr. Reflected light.

Z. ramigera (3, 6, 27, 28, 36, 44). In recent publications, reference is made to the presence of *Z. ramigera* in mixed cultures (1, 16), and Varma and Reid (41) indiscriminately used *Z. ramigera* synonymously with the biological slimes of a trickling filter. Unz and Dondero (39) succeeded in isolating bacteria from branched zoogloes and showed that the pre-

dominant bacteria of these zoogloes fitted the description of *Zoogloea*. However, these same workers cautioned against relying upon colonial characteristics alone in the identification of *Z. ramigera*. For example, *Thiodendron mucosum*, a sulfuretum described by Lackey and Lackey (30), closely resembles *Z. ramigera* in colonial morphology. Rubenchik (33) illustrated *Azotobacter chroococcum* in peculiar branching colonies. Unz and Dondero (39) observed that 37 of their 147 *Zoogloea* strains formed amorphous but not branched zoogloes in broth culture, although the two types of zoogloea-forming bacteria proved to be physiologically similar. Furthermore, the interpretation of branching in zoogloes is highly subjective. Crabtree and McCoy (11) and Friedman and Dugan (17) published photographs of the so called "finger-like projections" which they considered characteristic of *Z. ramigera*. I have been unable to confirm their observations after numerous examinations of their cultures [*Z. ramigera* strain I-16-M of Crabtree and McCoy (11) and *Z. ramigera* strain 115 of Friedman and Dugan (17)]. I have noted both temporary and permanent loss of branched zoogloea-forming ability in my *Zoogloea* strains, and freshly isolated *Zoogloea* strains that formed branched zoogloes in CY broth media did so much more profusely and dependably than did strains which had been subcultured 50 times. Nevertheless, the strains are zoogloea-forming. The detection of zoogloes, along with physiological, nutritional, and other morphological characteristics, is required for the accurate identification of *Zoogloea* species.

Zoogloea strain 106 differs from *Z. ramigera* strain I-16-M in certain aspects of morphology and in the ability to (i) form amorphous and branched zoogloes, (ii) denitrify, (iii) hydrolyze urea, (iv) hydrolyze gelatin, and (v) hydrolyze casein; and in the inability to (i) oxidatively decompose carbohydrates with acid production, (ii) form even traces of H_2S from peptone, (iii) decompose tyrosine, (iv) produce ammonia from arginine, (v) grow on potato, (vi) reduce litmus milk, and (vii) grow in peptone broth containing $10 \mu g$ of 2,4 diamin-6,7 diisopropylpteridine (vibriostat 0/129) per ml. In general, past workers have not reported acid production by the action of *Z. ramigera* on carbohydrates (6, 7, 15, 21, 31). Wattie (42) observed slight acid production by her zoogloal bacteria on several sugars; however, she carefully avoided naming the isolates *Z. ramigera*. In addition, there is little past evidence for proteolytic or lipolytic activity by *Zoogloea* species, although Ganapati et al. (18) isolated

22 *Zoogloea* strains that could hydrolyze tributyrin, and Unz and Dondero (39) showed gelatin hydrolysis by 134 *Zoogloea* strains. Denitrification (2, 4, 39) and urea hydrolysis (4, 13, 39) have been demonstrated with *Zoogloea* strains.

The uncertainties about the taxonomy of *Zoogloea* need to be resolved in order that accurate identification of *Zoogloea* species can be possible. Two species of *Zoogloea* are listed in the seventh edition of *Bergey's Manual of Determinative Bacteriology*: *Z. ramigera* Itzigsohn and the less prominent *Zoogloea filipendula* Beger. *Z. ramigera* has been designated (29) as the type species of the genus *Zoogloea* Cohn, but this species has not been sufficiently well characterized to distinguish it from other nonfermentative, floc-forming bacteria. The lack of a type strain for *Z. ramigera* has created the need for a neotype strain which both conforms closely to the original description of *Z. ramigera* Itzigsohn and has been studied in depth with the aid of modern microbiological techniques. *Z. ramigera* strain I-16-M is not a zoogloea bacterium and, therefore, does not resemble the *Z. ramigera* originally described by Itzigsohn (24). *Zoogloea* strain 106 forms both amorphous and branched zoogloea, was directly isolated from a natural branched zoogloea similar in appearance to those illustrated by Koch (27) and Butterfield (6) and described by Itzigsohn (24), and has been fully characterized by pure-culture methods. Where comparison is possible, *Zoogloea* strain 106 bears resemblance to the *Z. ramigera* strain Z-1 of Butterfield and to the *Z. ramigera* Itzigsohn of Blösch (3), except in its inability to decompose cellulose and its ability to liquefy gelatin. Strain 106 is correctly identified as *Zoogloea ramigera* Itzigsohn.

For reasons already stated *Z. ramigera* strain I-16-M is considered by this author to be an objectionable choice as the neotype strain of *Z. ramigera* Itzigsohn. In this regard, an Opinion is requested of the Judicial Commission on the following proposal: That strain I-16-M be rejected as the neotype strain of *Zoogloea ramigera* Itzigsohn, and that strain 106, which was isolated directly from a natural, branched zoogloea similar in description to the zoogloea observed by Itzigsohn (24), and which was characterized in pure culture, be designated as the neotype strain of *Z. ramigera* Itzigsohn.

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