ZOOGLOEA RAMIGERA ITZIGSOHN,
IDENTIFICATION AND DESCRIPTION¹

Request for an Opinion as to the status of the
generic name Zoogloea

K. Crabtree and Elizabeth McCoy

SUMMARY: The problem of identity and nomenclature of Zoogloea ramigera Itzigsohn has been presented. A new strain I-16-M has been described in terms of modern systematic bacteriology and it is proposed for designation as the neotype strain. An Opinion as to validity of the generic name Zoogloea Cohn is requested, and, if found invalid, designation of Zoogloea Itzigsohn as a nomen conservandum.

- - - - -

Introduction

Floc formation in sewage, particularly activated sludge, is so distinctive that there has been a tendency to attribute it to a special microorganism. Zoogloea ramigera was presumed to be that organism, although from time to time it has been questioned whether other floc-formers are not also involved (Wattie 1943, McKinney and Horwood 1952, and Jasewicz and Porges 1956). Conflicting statements concerning the properties of Z. ramigera have added to the confusion to the point that some investigators have concluded that Z. ramigera is not a true species but a growth form of any of several species capable of floc formation in sewage (Hawkes 1963, and Eckenfelder and O'Connor 1961). In this concept "Z. ramigera" is the composite of floc-formers in natural sewage flocs. To a bacteriologist such use of a

¹ Contribution from the University of Wisconsin, published with the approval of the Director of the Wisconsin Agricultural Experiment Station, and Engineering Experiment Station, Madison, Wisconsin 53706.

This investigation was supported in part by the Division of Water Supply and Water Pollution Control, and Public Health Service grant WP 00061 from the National Institutes of Health.

species name is not permissible and especially so because of earlier application of the name to pure cultures as representing the species.

This confusion is understandable when it is realized that Zoogloea ramigera Itzigsohn was proposed in 1867, that its description was emended by Blösch (1918) who may or may not have had a pure culture, and that later possibly pure cultures of Butterfield (1935), Butterfield et al. (1937), and Wattie (1943) were not preserved and thus cannot now be studied. Recent studies by Rich (1955), Dugan and Lundgren (1960) and Dias and Bhat (1964) involve new isolates by these investigators. While the general behavior of their isolates implies that they had Z. ramigera, certain discrepancies imply that at least some cultures were impure. All recent authors remark upon the difficulty of isolation, and it is readily understandable when one considers the firm and adhesive character of the flocs.

Our interest in Z. ramigera resulted from the need to obtain it for purposes of research on activated sludge and from the conviction that it was timely to investigate the taxonomy and nomenclature of the genus and its type species. When our requests for cultures of Z. ramigera to several investigators and culture collections went unfilled, it was necessary to begin with sewage flocs, to accumulate a representative series of isolates, and to study them in terms of modern systematic bacteriology.

Materials and Methods

Since pure-culture representatives are essential to the characterization of Z. ramigera, special effort was expended on the original choice of sewage flocs and on critical purification of the isolates. Activated sludges were obtained from five sewage-treatment plants including purely domestic, domestic-industrial from a large city, and dairy-industrial from a cheese factory. The flocs were gently washed in several changes of sterile phosphate buffer (pH 7.2), then collected by sedimentation and decantation to free them from floating cells and debris. Such washed flocs were homogenized for 2 min in a Waring Blendor and used to inoculate arginine broth (arginine 0.05%, K_2HPO_4 0.2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%; vitamin B_{12} and biotin at 2 $\mu g/ml$ were added after it was found that they, of all vitamins tested, were required). Penicillin G (2 to 5 units/ml) was added at the time of inoculation to inhibit Gram-positive contaminants. Cultures were grown in baffled Erlenmeyer flasks

at 30°C on a rotary shaker at 200 rpm for 12-24 hr. Under these conditions growth was turbid, without conspicuous flocs; nevertheless the cells were again homogenized in the Waring Blendor before plating. Suitable dilutions were plated on BBL Trypticase Soy (0.5%) agar by the spreading technique. Colonies were examined under a low power binocular microscope to ascertain morphological uniformity and isolation from neighboring colonies. For each isolate a typical colony (cf Fig. 1) was selected and a portion taken to inoculate arginine broth and another to inoculate the flocculation test medium (the same arginine broth + 5% glucose). If flocculation occurred in the medium with glucose and not in the plain broth, the purification procedures were repeated several times, always from the dispersed growth in the arginine broth to insure isolated cells from which to derive the succeeding colonies. The final isolates were maintained on Trypticase Soy (0.5%) agar slants with transfer every 6 months.

Results

Cultures possessing the growth characteristics of Z. ramigera were successfully isolated from each of the sewage treatment plants. Later (1965) Dr. Claude Vezina used our method and obtained a culture from the plant at St. Eustache, Montreal, Canada. It proved to be typical of Z. ramigera in our tests. Of our 12 isolates which were thoroughly studied, five were Z. ramigera, three were Z. filipendula, two Pseudomonas denitrificans and two were unidentified pseudomonads. Details of the properties of these floc-formers and defense of the decisions on their identification will be published elsewhere (now in manuscript). Suffice it to say here that at the time of isolation from sewage all appeared similar and gave the required "flocculation test" in pure culture. If full bacteriological characterization had not been done, they would have been passed as "Z. ramigera" and in that sense the literature is right that sewage flocs are, or may be, made up of more than one kind of floc-forming bacteria. On the contrary, it is not right to assume, as some have done, that the test of floc formation is diagnostic for Z. ramigera. Some of the confusion as to the properties of Z. ramigera may well have resulted from this assumption.

The five isolates of Z. ramigera were remarkably homogeneous and may well be epitomized by the description of one of them, strain I-16-M, as follows:

Gram-negative, non-sporeforming rods with round, blunt or pointed ends, 0.5 to 1.0 by 1.0 to 3.0 μ . Pleomorphic with age.

Young cells actively motile with a long straight flagellum at one or both poles (Fig. 2).

Capsules not demonstrable (nigrosin, India ink, Leifson, Anthony, Tyler stains). No slime on K gluconate (Haynes test).

Old cells usually distended by sudanophilic granules of poly- β hydroxybutyric acid polymer (Fig. 3). Age of culture and nature of medium influence amount of PHB and extent of flocculation (Crabtree *et al.*, 1965).

In natural habitat (natural waters, sewage) cells occur in irregular "starlike" aggregates with "fingerlike" projections (Figs. 4, 5).

In liquid laboratory media flocculation is influenced by carbon/nitrogen ratio; growth flocculent with ratio $> 10/1$ and dispersed with $< 5/1$. Fig. 4 shows flocs grown in arginine broth plus 0.5% glucose.

Gelatin colonies: Circular, creamy to straw color, with small, distinctive sinus at apex of the colony (72 hr).

Gelatin stab: Good growth at surface, no liquefaction.

Gelatin agar streak: Good growth, no hydrolysis.

Agar slant: Creamy white to straw color, filiform but later spreading with lobate edge, dry, wrinkled, tough and leathery.

Agar colonies: At 72 hr usually 2 to 3 mm in diameter, tan to straw color, undulate, dry, wrinkled, tough, leathery with distinctive small sinus at the apex (Fig. 1). Entire colony movable with push by needle.

Broth (arginine with glucose; standing tube): Fragile pellicle, lacy tapelike flocs adhere to inner sidewall and eventually extend to bottom (Fig. 6). Broth clear.

Broth (as above, shake flask): Irregular star clusters; addition of oxidizable carbohydrate stimulates rapid, complete flocculation. Broth clear.

Coagulated egg white: Not hydrolyzed.

Arginine stab: Arginine dihydrolase (Thornley test).

Tyrosine agar: Rapid clearing of tyrosine, melanin pigment.

Caseinate agar: Good growth, no hydrolysis.

Starch agar: Good growth, no hydrolysis.

Litmus milk: Slight reduction, alkaline, no peptonization.

Potato slant: Semimoist, dirty white to light brown.

Catalase: Positive.

Cytochromeoxidase: Positive (Gaby and Hadley test).

Indole: Negative.

Methyl red: Negative.

Acetylmethylcarbinol: Negative.

Citrate: Not utilized.

Ammonia: Produced from peptone and amino acids.

Nitrates: Not reduced to nitrites or N_2 gas.

Urea: Not hydrolyzed.

Acetamide: Not decomposed (Buhlman et al. test).

Hydrogen sulfide: Not produced (Kligler agar).

Carbohydrates: Not fermented but actively oxidized:

glucose, arabinose, xylose, fructose, galactose, mannose, sorbose, rhamnose, fucose, maltose, sucrose, lactose, trehalose, cellobiose, salicin, ethanol, glycerol, mannitol, sorbitol. Not oxidized: dulcitol, melibiose, melezitose, raffinose, glycogen, starch (soluble), inulin, and cellulose.

Amino acids and derivatives: Utilized as sole source of nitrogen and carbon in salts medium with vitamin B_{12} : arginine, aspartic, ornithine, citrulline, glutamic, glutamine, tyrosine and histidine.

Organic compounds: Utilized as carbon source: acetate, pyruvate, malate, fumarate, and oxalate. Utilized as nitrogen and carbon source: pantothenate, glucosamine, adenine, uracil and thiamine.

Inorganic nitrogen: Ammonia utilized as sole source provided that vitamin B_{12} requirement is met; biotin stimulatory.

Salt tolerance: Growth with 3% but not 6.5% NaCl.

Optimum temperature: 28-30°C. Slow growth at 10°C, none at 45°C.

Optimum pH: 7.0-7.5. No growth at pH 4.5 or 9.6.

Distinctive characters: Colonies smooth, convex with sinus at apex, tough and leathery; formation of lacy, tapelike growth in standing broth cultures, starlike flocs in shaken cultures; cells in flocs compactly arranged in "fingerlike" projections, contain large amount of sudanophilic granules (polymer of β hydroxybutyric acid); inactive in fermentation of carbohydrates but very active in oxidative utilization. Nonproteolytic.

Source: Isolated from/flocs in sewage sludge (activated).

Habitat: Natural waters containing decomposing organic matter, flocs of sewage and some industrial wastes.

- Figure 1. Colony with typical sinus at apex. 72 hr.
- Figure 2. Electron micrograph of cell with long straight flagellum. Uranyl acetate stained.
- Figure 3. Cells with sudanophilic granules of poly- β -hydroxybutyric acid. Grown with glucose excess (0.5%) in arginine broth. Phase microscopy of Sudan Black stained preparation. Magnification 2700X.
- Figure 4. Starlike flocs, grown in flocculation broth. Magnification 17X.
- Figure 5A. Electron micrograph of cells in a microfloc showing compact cellular arrangement. Uranyl acetate stained.
- 5B. Phase contrast preparation showing fingerlike projection at periphery of floc and several detached clusters of cells. Wet mount, unstained. Magnification 830X.
- Figure 6. Lacy tapelike growth in standing broth cultures. 3 wk.

Discussion of nomenclature of the genus Zoogloea and its species.

Zoogloea termo Cohn 1854 is the name of the species of the original monotypic genus Zoogloea but this name was later discarded by Cohn as representing a growth form. Z. termo was described as "Cellulae minimae, bacilliformes, hyalinae, gelatina hyalina in massas mucosas globosas, uvaformes, mox membranaceae [sic] consociatae, dein singulae elapsae, per aquam vacillantes." Later another species was added, namely Z. ramigera Itzigsohn 1867, and in recent times it has been often assumed to be the type species of the genus Zoogloea. Based upon Z. ramigera the genus is defined as containing: "Rod-shaped cells embedded in gelatinous matrix. Free-floating forms found in fresh water that contains organic matter. Occur as compact masses or as branched forms. Cells may become detached and motile in which case they are monotrichous" (Bergey's Manual, 7th Ed. 1957). One other species, Z. filipendula Beger 1928, is also included in Bergey's Manual, 7th Ed. However, two species named earlier are not, namely, Z. pulmonis equi Bollinger 1870 and Z. beigeliana Eberth 1873.

The validity of the generic name Zoogloea Cohn may well be questioned. Cohn definitely abandoned it in 1872, believing it to refer to a growth form of Bacterium termo Dujardin. Presumably for that reason Billroth (1874) suggested Coccolgia or Gliacoccus but neither of these names has come into use; in fact all names proposed by Billroth are rejected as not validly published (Opinion 25, Judicial Commission). Winogradskyia was proposed by Trevisan (1889) for the placement of Itzigsohn's Z. ramigera, but Winogradskyia is a later homonym and has been declared invalid on that basis (Opinion 13, Judicial Commission).

Zoogloea Cohn continued to be used by investigators in the sewage field. However, it was not recognized in Bergey's Manual until the 5th Ed. (1939). Its inclusion then probably resulted from the isolation and re-study of Z. ramigera by Butterfield (1935) and Butterfield et al. (1937). In the present Bergey's Manual (7th, 1957) the description of Z. ramigera is based upon the emendation by Blösch (1918) with citations of Butterfield and later workers. The second species Z. filipendula Beger 1928, was not recognized until the 7th edition (1957) of the Manual.

It can be seen from the above history that there are several nomenclatural problems. First the validity of the generic name, Zoogloea Cohn and of the species name Z. termo

Cohn are questionable. The description of Z. termo, hence of the genus Zoogloea, is so inadequate that its application is uncertain. Accordingly, these names may be regarded as illegitimate (Rule 24f). Further, in view of Cohn's 1872 abandonment, the continued use of Zoogloea would seem to violate Rule 12c(1).

However, the name Zoogloea has persisted in usage and it would now be very difficult to displace it. Perhaps it could be treated as a nomen nudum until the time of Itzigsohn's proposal of Z. ramigera in 1867. Z. ramigera is recognizable from the original description and the emendation by Blösch (1918), and it is correctly cited in Bergey's Manual (7th Ed.). If the nomenclature of Zoogloea can be accepted as Zoogloea Itzigsohn non Cohn, and if Z. ramigera Itzigsohn emend. Blösch is an acceptable type species, part of the problems are resolved.

There remains, however, the lack of original type strain or, in fact, of any historical strain of the early period. For that reason the present authors have at considerable effort isolated and described a new strain, I-16-M, which they propose as the neotype. This strain has been deposited with the American Type Culture Collection (ATCC 19623).

In conclusion, it is hereby requested that the Judicial Commission rule on the status of the generic name Zoogloea Itzigsohn and the species name Z. ramigera Itzigsohn, and that Zoogloea Cohn be placed in the list of nomina rejicienda. It is further proposed that strain I-16-M, whose history of isolation and description accompany this request, be designated as the neotype of Z. ramigera Itzigsohn.

REFERENCES

- Bergey, D.H. 1939. Manual of Determinative Bacteriology. 5th Ed. Williams and Wilkins, Baltimore, Md.
- Bergey's Manual of Determinative Bacteriology. 1957. 7th Ed. Breed, R.S., E.G.D. Murray, and N.R. Smith, eds. Williams and Wilkins, Baltimore, Md.
- Blösch, M. 1918. Beitrag zur Untersuchung über die Zoogloea ramigera (Itzigsohn) auf Grund von Reinkulturen. Zentr. Bakt. 2 Abt. 48:44-62.
- Butterfield, C.T. 1935. Studies of sewage purification. II. A zooglea-forming bacterium isolated from activated sludge. U.S. Pub. Health Rept. 50:671-684.

- _____, C. C. Ruckhoft, and P. D. McNamee. 1937. Studies of sewage purification. VI. Biochemical oxidation by sludges developed by pure cultures of bacteria isolated from activated sludge. U.S. Pub. Health Rept. 52:387-412.
- Crabtree, K., E. McCoy, W. C. Boyle, and G. A. Rohlich. 1965. Isolation, identification, and metabolic role of the sudanophilic granules of Zoogloea ramigera. Appl. Microbiol. 13:218-226.
- Dias, F. F. and J. V. Bhat. 1964. Microbiology of activated sludge. I. Dominant bacteria. Appl. Microbiol. 12:412-417.
- Dugan, P. R. and D. G. Lundgren. 1960. Isolation of the floc-forming organism Zoogloea ramigera and its culture in complex and synthetic media. Appl. Microbiol. 8:357-361.
- Eckenfelder, W. W. Jr., and D. J. O'Connor. 1961. Biological Waste Treatment. Pergamon Press, Oxford.
- Hawkes, H. A. 1963. The Ecology of Waste Water Treatment. Pergamon Press, The Macmillan Co. New York.
- International Code of Nomenclature of Bacteria and Viruses. 1958. Edit. Board Internatl. Com. Bact. Nomenclature. Iowa State College Press, Ames, Iowa.
- Jasewicz, L., and N. Porges. 1956. Biochemical oxidation of dairy wastes. VI. Isolation and study of sludge organism. Jour. Water Poll. Cont. Fed. 28:1130-1136.
- McKinney, R. E., and M. P. Horwood. 1952. Fundamental approach to the activated sludge process. I. Floc-producing bacteria. Jour. Water Poll. Cont. Fed. 24:117-123.
- Rich, L. 1955. Respiration studies on the organic nitrogen preferences of Zoogloea ramigera. Appl. Microbiol. 3:20-25.
- Wattie, E. 1943. Cultural characteristics of zoogloea-forming bacteria isolated from activated sludge and trickling filters. U.S. Pub. Health Rept. 57:1519-1533.