Fluorescent-Antibody Study of Natural Finger-Like Zoogloae

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Fluorescent-antibody techniques using Zoogloea ramigera 106 antiserum were used to study fresh activated sludge flocs and finger-like zoogloae in the microbial film that developed over stored samples of activated sludge. Few cells in fresh activated sludge reacted positively with the fluorescein-labeled antiserum. Finger-like zoogloae containing reactive cells were readily observed in the microbial film layer over stored activated sludge. Certain of the natural finger-like projections were entirely composed of cells that reacted positively to the labeled Z. ramigera 106 antiserum, whereas other projections were devoid of reactive cells.

Although microdissection is an ideal method for the study of natural zoogloal projections, it is too difficult and time consuming for routine study. In this work one of the zoogloea-producing organisms isolated by Unz and Dondero (12, 13) was used as the antigen for the preparation of fluorescent antibody. This preparation was then used to study fresh, activated sludge flocs and the microbial film that developed over stored, activated sludge.

MATERIALS AND METHODS

Bacterial cultures. The bacteria used in this study and their sources were as follows: (i) Zoogloea strains 9, 13, 21 (ATCC 19122), 106 (ATCC 19544), 201 (ATCC 19325), 216 (ATCC 19123), 235 (ATCC 19324), and 239 (ATCC 19173), all of which were isolated by Unz and Dondero (12); (ii) Zoogloea ramigera 115, isolated by Friedman and Dugan (3) and kindly supplied by P. R. Dugan, The Ohio State University, Columbus; (iii) Z. ramigera 1-16-M (ATCC 16293), isolated by Crabtree et al. (2); (iv) 34 Zoogloea strains isolated by Unz and Farrah (14); and (v) bacteria from the stock culture collection of the Department of Microbiology, The Pennsylvania State University. Cultures were transferred monthly on a Casitone-yeast autolysate medium containing per liter of distilled water, 5.0 g of Casitone (Difco) and 1.0 g of yeast autolysate (Charles Pfizer and Co., Inc., New York).

Preparation of inoculum and serum production. Z. ramigera 106 was cultured in a medium containing, per liter of distilled water: (NH₄)₂SO₄, 0.264 g; K₂HPO₄, 0.087 g; MgSO₄, 0.120 g; CaSO₄, 0.136 g; sodium lactate, 1.0 g; Casitone, 0.1 g; and yeast autolysate, 0.02 g. Incubation was at 20 C on a gyratory shaker for 48 h. Z. ramigera 106 cells along with extracellular material were washed twice in distilled water and suspended in 50 ml of distilled water, and the suspension was adjusted to pH 10.0 by using 1 N NaOH. The suspension was boiled for 3 min.
and cooled, the pH was readjusted to 10.0, and the suspension was boiled for another 3 min. Finally, cells were centrifuged at 27,000 × g for 10 min and washed twice in distilled water. Microscopic examination of treated cells in wet mounts with India ink revealed clean preparations of cells devoid of exocellular gelatinous substances. A quantity of the cells equal to 1.0 mg of Kjeldahl nitrogen as determined according to the procedures of McKenzie and Wallace (7) was mixed with an equal volume of mineral oil and used as the inoculum for production of antiserum. Domestic rabbits were given 1-ml subcutaneous injections in each flank. After 1 month, rabbits were given intravenous injections consisting of 1 ml of the cell preparation equal to 0.2 mg of Kjeldahl nitrogen per ml. One week after intravenous injections, antiserum was obtained by bleeding the animals from the heart.

Preparation and use of fluorescein-labeled antibody. Serum globulins were fractionated and buffered according to the procedure of Romano and Gason (10). Buffered globulins were mixed with 0.015 mg of fluorescein isothiocyanate per mg of protein as suggested by Olson (8). The mixture was allowed to react overnight at 5°C under continuous mechanical stirring followed by dialysis against buffered saline consisting of 0.15 M sodium chloride and 0.1 M potassium phosphate, pH 7.2. Dialysis was continued until no fluorescein was observed in the dialysate. The labeled globulin was passed through a 0.45-μm membrane filter and frozen. Labeled globulin was used in studies of cross-reactions with various known strains of Zoogloea and other axenic cultures of bacteria as well as unknown bacteria isolated from activated sludge. The bacteria were isolated on Casitone-yeast autolysate agar medium and activated sludge agar prepared according to the method of Prakasam and Dondoro (9). Bacteria were spread on microscope slides, air dried, fixed in ethanol, and permitted to react with antiserum for 15 min at 28°C. After incubation, slides were washed with buffered saline and covered with mounting fluid consisting of equal parts of glycerol and buffered saline. Slides were examined by dark-field microscopy, using a Zeiss universal microscope equipped with an Osram HBO 500-W ultraviolet light source, UG 5 exciter filter, and 47/65 barrier filter. A cross-reaction with Z. ramigera 115 was eliminated by adsorbing labeled antiserum with Z. ramigera cells. The resulting highly specific Z. ramigera 106 antiserum was used to study fresh, activated sludge flocs and the microbial film that formed at the surface of settled activated sludge stored in beakers at 28°C for 48 h.

RESULTS

Five of eight Zoogloea strains originally isolated by micromanipulation from natural finger-like zoogloea fluoresced when stained with fluorescein-labeled Zoogloea ramigera antiserum. Only 3 of 34 Zoogloea strains isolated by streaking wastewater samples on sodium m-toluate medium expressed a similar reaction. A slight reaction took place between the labeled serum and Z. ramigera 115; however, absorption of the labeled serum with cells of Z. ramigera 115 eliminated the cross-reaction but did not alter the intense fluorescence observed with directly isolated Zoogloea strains. No cross-reaction took place between the labeled antiserum and Z. ramigera I-16-M, Streploccocus faecalis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, P. fluorescens, or P. putida. In addition, specificity studies were conducted on 71 axenic cultures of unidentified bacteria obtained from mixed liquor and scums formed at the surface of mixed liquor stored in glass beakers. These bacteria were isolated on Casitone-yeast autolysate and activated sludge extract agars and included 59 gram-negative rods, 9 gram-positive rods, and 3 gram-positive cocci. None of the 71 unidentified cultures reacted with labeled Z. ramigera antiserum (Table 1).

The purified Z. ramigera 106 labeled antiserum reacted with the bacteria within certain natural, bacterial zoogloae obtained from the scums of stored, mixed liquors. The affinity of the labeled serum for the bacteria within the zoogloae was apparent when treated smears

### Table 1. Specificity of Zoogloea ramigera 106 antiserum in fluorescent antibody tests with axenic bacteria

| Bacteria                        | No. of strains | No. strains showing intense fluorescence | Percent of strains showing intense fluorescence |
|--------------------------------|----------------|----------------------------------------|-----------------------------------------------|
| Zoogloea strains (direct isolates) | 34             | 3                                      | 9                                             |
| Zoogloea strains (indirect isolates) | 8              | 5                                      | 63                                            |
| Z. ramigera 115                  | 1              | 0                                      | 0                                             |
| Z. ramigera I-16-M               | 1              | 0                                      | 0                                             |
| Streptococcus faecalis           | 1              | 0                                      | 0                                             |
| Staphylococcus aureus            | 1              | 0                                      | 0                                             |
| Escherichia coli                 | 1              | 0                                      | 0                                             |
| Proteus vulgaris                 | 1              | 0                                      | 0                                             |
| Pseudomonas aeruginosa           | 3              | 0                                      | 0                                             |
| Pseudomonas fluorescens          | 1              | 0                                      | 0                                             |
| Pseudomonas putida               | 1              | 0                                      | 0                                             |
| Unidentified isolates            | 71             | 0                                      | 0                                             |

*Isolated by Unz and Dondoro (12).*  
*Isolated by Unz and Farrah (14).*  
*Slight initial fluorescence was observed which was eliminated by absorbing conjugated Z. ramigera 106 antiserum with Z. ramigera 115.*  
*Bacteria isolated from mixed liquor and the scum formed at the surface of mixed liquor stored in beakers at 28°C for 48 h.*
were examined by tungsten (Fig. 1A) and ultraviolet light (Fig. 1B). Only bacteria that reacted with the antiserum were visible by ultraviolet light, whereas many types of cells were seen around the zoogloeae under tungsten light. The majority of fluorescing cells were observed to be arranged in finger-like projections. In another series of exposures with labeled antiserum-treated samples, filamentous bacteria and the bacteria of finger-like zoogloeae were visible by tungsten light (Fig. 2A); however, only the cells within zoogloeae were apparent by ultraviolet illumination (Fig. 2B). At no time were cells arranged in filaments observed to react with the *Z. ramigera* 106 antiserum.

**Fig. 1.** Natural, finger-like, bacterial zoogloeae treated with *Zoogloea ramigera* 106 antiserum. Sample obtained from scum layer which developed on the surface of mixed liquor stored in a beaker for 48 h at 28 C. Dark-field condenser with: (A) tungsten; (B) ultraviolet light. Bar equals 10 μm.
Although all of the cells in certain natural finger-like zoogloeae reacted with the labeled antiserum, relatively few reactive cells were seen in antiserum-treated stored, activated sludge flocs (Fig. 3). It may be that the serum failed to penetrate the flocs and make contact with susceptible cells or that the flocs were opaque to the ultraviolet illumination. In certain cases, activated sludge flocs which did not initiate formation of finger-like zoogloeae did contain small clumps of cells and single cells that reacted with the labeled antiserum (Fig. 4).

**Fig. 2.** Natural, finger-like, bacterial zoogloeae and filamentous bacteria treated with Zoogloea ramigera 106 conjugated antiserum. Sample obtained from scum layer which developed on the surface of mixed liquor stored in a beaker for 48 h at 28°C. Dark-field condenser with: (A) tungsten light; (B) ultraviolet light. Bar equals 10 μm.
Fig. 3. Bifurcate, finger-like, bacterial zoogloea and an activated sludge floc treated with Zoogloea ramigera 106 conjugated antiserum. Sample obtained from scum layer which developed on the surface of mixed liquor stored in a beaker for 72 h at 28 C. Dark-field condenser with: (A) tungsten light; (B) ultraviolet light. Bar equals 10 μm.

Few reactive cells were observed in fresh activated sludge flocs, and these were found as single cells or in small clumps rather than in the finger-like projections found in stored activated sludge samples. Disruption of the flocs before staining might permit observation of cells obscured by nonfluorescing material within the sludge flocs.

Certain natural, finger-like zoogloeae did not react with Z. ramigera 106 antiserum although they were similar in morphology to those zoogloeae that did react. It was noted that when
antiserum positively reacted with a finger-like zoogloeae, all visible cells in the zoogloeae were involved in the reaction. In contrast, zoogloeae not reactive with the labeled antiserum contained no fluorescing cells under ultraviolet examination. There were a large number of zoogloeae that did not react with the labeled antiserum. In slide tests with zoogloeae developed in scums formed at the surface of mixed liquors collected from the State College and University Park, Pa., wastewater treatment plants, only 30 and 54%, respectively, of the...
zoogloeae counted demonstrated a reaction with Z. ramigera 106 labeled antiserum (Table 2).

**DISCUSSION**

In recent years organisms with different morphological and physiological characteristics have been described as Zoogloea ramigera (2, 3, 12, 14). The taxonomic status of the genus Zoogloea has been previously discussed (11, 15). As shown in this work, the organisms within certain natural, finger-like zoogloal projections are antigenically related to Z. ramigera 106, supporting the view of Unz (11) that this organism should be accepted as the neotype strain of Zoogloea ramigera Itzigsohn.

The fact that certain bacteria within natural zoogloae did not react with Z. ramigera antiserum may indicate that organisms other than Zoogloea spp. comprise these natural zoogloae or that different antigenic varieties of Zoogloea exist in nature. Since antigenic heterogeneity was observed among directly isolated Zoogloea strains, the latter possibility seems more likely. To more definitively establish the nature of those bacteria within natural zoogloae that did not react with Z. ramigera 106 antiserum, fluorescein-labeled serum should be produced against other Zoogloea strains such as Z. ramigera 115, Z. ramigera I-16-M, and those direct and indirect Zoogloea isolates that did not react with the Z. ramigera 106 antiserum (Table 1).

The observation that the cells within individual zoogloae were antigenically similar suggests that finger-like zoogloae represent the progeny of one or a few cells and are produced by multiplication of these cells rather than by aggregation of individual cells or packets of cells as has been suggested (4). Recent time lapse studies have shown that finger-like zoogloae develop from activated sludge flocs by a process of bacterial multiplication and movement within individual zoogloae rather than by aggregation of bacteria from a dispersed growth phase (S. R. Farrah, Ph.D. thesis).

No fluorescent cells were observed in trichomes resembling those produced by Sphaerotilus spp. It was previously proposed that Z. ramigera is a growth form of Sphaerotilus (5), but we have found no evidence to support this view.

The results obtained in this study were qualitative. However, in contrast to the numerous reactive cells found in scum layers over stored activated sludge, relatively few cells in fresh activated sludge reacted with labeled Z. ramigera 106 antiserum. This may indicate that Z. ramigera is a minor component of activated sludge or that the methods used in this study did not permit observation of all Z. ramigera cells present. Future work should attempt to enumerate Z. ramigera in sludge in order to assess the role of these organisms in biological waste treatment.

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**LITERATURE CITED**

1. Amin, P. M., and S. V. Ganapati. 1967. Occurrence of Zoogloea colonies and protozoans at different stages of sewage purification. Appl. Microbiol. 15:17–21.
2. 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.
3. Friedman, B. A., and P. R. Dugan. 1968. Identification of Zoogloea species and the relationship to zoogloal matrix and floc formation. J. Bacteriol. 95:1903–1909.
4. Friedman, B. A., P. R. Dugan, R. M. Pfister, and C. C. Remsen. 1968. Fine structure and composition of the zoogloal matrix surrounding Zoogloea ramigera. J. Bacteriol. 96:1328–1334.
5. Hynes, H. B. N. 1960. The biology of polluted waters. University Press, Liverpool.
6. Itzigsohn, H. 1968. Entwicklungsvorgänge von Zoogloea, Ocilloria, Synedra, Staurotria, Spirotaenia und Chroolepus, p. 30–31. Sitzungs-Berichte der Gesellschaft naturforschender Freunde zu Berlin.
7. McKenzie, H., and H. Wallace. 1954. The Kjeldahl determination of nitrogen: a critical study of digestion conditions-temperature, catalyst, and oxidizing agent. Aust. J. Chem. 7:55–71.
8. Olson, W. P. 1968. Maximal brightness and yield of fluorescent antibody. J. Bacteriol. 96:1170–1177.
9. Prakash, T. B. S., and N. C. Dondero. 1967. Aerobic heterotrophic bacterial populations of sewage and activated sludge. I. Enumeration. Appl. Microbiol. 15:461–467.

| Source of mixed liquor | No. of finger-like zoogloae observed | No. of finger-like zoogloae showing fluorescence | Percentage of finger-like zoogloae showing fluorescence |
|------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------------------------|
| State College, Pa., wastewater treatment plant | 23 | 7 | 30 |
| University Park, Pa., wastewater treatment plant | 110 | 54 | 49 |

*Scums formed at the surface of mixed liquor stored in beakers after 48 h at 28 C.*
10. Romano, A. H., and D. J. Geason. 1964. Pattern of sheath synthesis in Sphaerotilus natans. J. Bacteriol. 88:1140–1150.

11. Unz, R. F. 1971. Neotype strain of Zoogloea ramigera Itzigsohn. Request for an opinion. Int. J. Syst. Bacteriol. 21:91–99.

12. Unz, R. F., and N. C. Dondero. 1967. The predominant bacteria in natural zoogloal colonies. I. Isolation and identification. Can. J. Microbiol. 13:1671–1682.

13. Unz, R. F., and N. C. Dondero. 1967. The predominant bacteria in natural zoogloal colonies. II. Physiology and nutrition. Can. J. Microbiol. 13:1683–1694.

14. Unz, R. F., and S. R. Farrah. 1972. Use of aromatic compounds for growth and isolation of Zoogloea. Appl. Microbiol. 23:524–530.

15. Zvirbulis, E., and H. D. Hatt. 1967. Status of the generic name Zoogloea and its species. Int. J. Syst. Bacteriol. 17:11–21.