Use of Aromatic Compounds for Growth and Isolation of Zoogloea

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Nine Zoogloea strains, were examined for their ability to utilize 35 aromatic compounds. Benzoate, m-toluate, and p-toluate, as well as phenol, o-cresol, m-cresol, and p-cresol, were utilized by eight strains. These strains exhibited meta cleavage of catechol and of methyl-substituted catechols. With the exception of L-tyrosine, none of the aromatic compounds tested supported growth of Z. ramigera ATCC 19623. A medium containing sodium m-toluate was used to isolate 37 zoogloea-forming bacteria from various polluted environments. The isolates were identified as strains of Zoogloea.

Although the literature is replete with reference to the appearance of Zoogloea in wastewaters, only recently have intensive studies been undertaken on axenic cultures of these bacteria (6, 7, 10, 12, 23, 24). Generally, these studies have revealed the need for extensive characterization of the genus Zoogloea in order that the taxonomy of this group may be clarified. Zoogloea strains have been shown to utilize sodium benzoate and sodium m-toluate as sole carbon and energy sources (22, 24). The present investigation concerns the ability of Zoogloea strains to utilize a variety of aromatic compounds and is a sequel to earlier work on the nutritional characterization of these bacteria (22–24).

Zoogloea-forming bacteria, including Z. ramigera, have been considered by some to be functionally important in aerobic, biological wastewater treatment (3–5). Dias and Bhat (8) characterized 319 bacteria from activated sludge isolated on sewage agar and identified 126 of them as Zoogloea strains. Further studies are needed to elucidate the role of Zoogloea in polluted waters. However, as stated by Crabtree and McCoy (6), past workers have experienced difficulty in isolating this organism. Conventional bacteriological isolation techniques generally have not been satisfactory for rapid isolation. These bacteria grow slowly, produce indistinct, punctiform, nonpigmented colonies, and often become overgrown by other microorganisms on primary isolation media. Most workers have employed the uncertain and time-consuming dispersion-serial dilution technique of Butterfield (3) or some modification of this technique (5, 10, 12, 14, 18, 25). Crabtree and McCoy (6) isolated Z. ramigera I-16-M, a floc-forming, nonzoogloea bacterium, from an enrichment culture by using a spread-plating procedure. Unz and Dondero (23) used the technique of micromanipulation to dissect individually bacterial cells from wastewater zoogloea while observing the cells with a microscope. In this fashion, they obtained 147 Zoogloea strains, albeit with considerable effort.

It seemed, therefore, desirable to have a simple method for the isolation of Zoogloea strains. In this study, certain of the aromatic compounds found to support good growth of Zoogloea strains were tried as primary carbon sources in isolation media for these organisms.

MATERIALS AND METHODS

Bacterial cultures. Z. ramigera I-16-M (ATCC 19623) and the following Zoogloea strains originally isolated by Unz and Dondero (23) were used in studies on aromatic compounds and as reference cultures to aid in the identification of freshly isolated bacteria: strains 9, 13, 21 (ATCC 19122), 106 (ATCC 19544), 201 (ATCC 19325), 216 (ATCC 19123), 235 (ATCC 19324), and 238 (ATCC 19173). Cultures were transferred monthly on a Casitone-yeast autolysate (CY) medium which contained in 1 liter of distilled water: 5.0 g of Casitone (Difco) and 1.0 g of yeast autolysate (Albimi Laboratories, Flushing, N.Y.) Cultures were incubated at 20 C.

CHEMICALS. Commercially available, reagent grade chemicals were used in all experiments. Aromatic carbon sources (Table 1) were prepared in
**Table 1. Utilization of aromatic compounds by nine Zoogloea strains**

| Substrates                        | Conc (mg/liter) | No. of strains utilizing substrate |
|-----------------------------------|-----------------|-----------------------------------|
| Aromatic acids                    |                 |                                   |
| Sodium benzoate                   | 300             | 8                                 |
| Sodium o-toluolate                | 300             | 0                                 |
| Sodium m-toluolate                | 300             | 8                                 |
| Sodium p-toluolate                | 300             | 0                                 |
| Sodium o-phthalate                | 300             | 0                                 |
| Sodium p-phthalate                | 300             | 0                                 |
| Sodium mandelate                  | 300             | 0                                 |
| Sodium p-hydroxy benzoate         | 300             | 1                                 |
| Sodium salicylate                 | 300             | 0                                 |
| Sodium 2,3-cresocate              | 300             | 0                                 |
| Phenolic compounds                |                 |                                   |
| Phenol                            | 200             | 8                                 |
| o-Cresol                          | 200             | 8                                 |
| m-Cresol                          | 200             | 8                                 |
| p-Cresol                          | 200             | 8                                 |
| 3,4-Xylenol                       | 300             | 0                                 |
| 3,5-Xylenol                       | 300             | 1                                 |
| Chlorinated aromatic compounds    |                 |                                   |
| Sodium o-chlorobenzoate           | 100             | 0                                 |
| Sodium m-chlorobenzoate           | 100             | 0                                 |
| Sodium p-chlorobenzoate           | 100             | 0                                 |
| o-Chlorophenol                    | 100             | 0                                 |
| p-Chlorophenol                    | 100             | 2                                 |
| Nitrogen-containing aromatic acids|                 |                                   |
| Pyridine                          | 200             | 2                                 |
| Aniline                           | 300             | 4                                 |
| Sodium anthranilate               | 300             | 0                                 |
| Sodium p-nitrobenzoate            | 300             | 2                                 |
| Sodium o-nitrobenzoate            | 300             | 1                                 |
| Benzonitrile                      | 300             | 0                                 |
| Mandelonitrile                    | 300             | 1                                 |
| Benzaldehyde cyanohydrin          | 300             | 1                                 |
| Aromatic amino acids              |                 |                                   |
| L-Tryptophan                      | 200             | 0                                 |
| L-Phenylalanine                   | 200             | 0                                 |
| L-Tyrosine                        | 200             | 1                                 |
| 3,5-Diido-L-tyrosine              | 200             | 0                                 |

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stock solutions, sterilized by filtration through 0.45-
µm membrane filters (Millipore Corp., Bedford,
Mass.), and aseptically transferred to the basal me-
dium as needed.

**Basal medium.** The basal medium was a modifi-
cation of that described by Unz and Donders (24) and contained in 1 liter of distilled water: MgSO3•7H2O, 0.200 g; CaCl2, 0.002 g; K2HPO4, 0.100 g; (NH4)2SO4, 0.375 g; yeast autolysate, 0.010 g; and vitamin B12, 10-6 g. Concentrated K2HPO4 solution and the rest of the basal medium were heat-
sterilized separately and combined after cooling final

pH, 7.2. The yeast autolysate was included in the basal medium as a vitamin source, and, alone, did not support growth of Zoogloea strains in the concentration used.

**Screening procedure.** Utilization of aromatic compounds by Zoogloea strains was determined on a growth-no growth basis. Test tubes containing 4.9 ml of complete media and the basal medium (no carbon source) received 0.1 ml of a washed suspension [optical density at 500 nm (OD660), 0.1] of logarithmic-phase cells grown in shaken CY medium. All strains were able to grow in the basal medium supplemented with 200 mg of sodium lactate per ml, which was included in all experiments as a check on the viability of cultures. Tubes were incubated at 24°C in a 45° inclined position on a reciprocal shaker (80 strokes/min). For 1 month, tubes of media containing carbon sources were compared at intervals with a tube of the basal medium for evidence of growth.

**Growth measurements.** Zoogloea strains were grown in 2 liters of the culture medium on a reciprocal shaker at 24°C. Cells were harvested at the end of logarithmic-growth phase by centrifugation, washed twice in distilled water, and dried to constant weight at 105°C.

Generation times were determined from growth curves constructed from direct-count data obtained with a Petroff-Hausser counting chamber.

**Sources.** Sources of primary inocula used in isolation work are given in Table 4.

**Isolation procedure.** The primary isolation was carried out in the basal medium supplemented with an aromatic carbon source and agar [1.0% (w/v) final concentration]. Soils and feces were suspended in equal volumes of sterile, distilled water and streaked directly on plates. Wastewaters and wastewater sludges were streaked directly on plates. Incubation was carried out at 20, 28, and 37°C. Single colonies of suspected Zoogloea species were picked from plates, incubated for 5 to 7 days, and individually suspended in tubes containing 5 ml of CY medium. Tubes were agitated on a test tube vibrator for 10 min to dis-
integrate partially the colony. A loopful of the suspension was streaked on fresh isolation medium and incubated as before. Resulting colonies were checked for purity by streaking on primary isolation medium and on solid CY medium.

**Chemical and diagnostic procedures.** Meta cleavage of aromatic compounds was determined by using a modification of the method of Stanier et al. (21) in which a liquid medium containing sodium benzoate was used.
instead of a solid medium. Spectroscopic analyses were performed on centrifuged (cell-free) culture fluids by using a Beckman model DB spectrophotometer equipped with an ultraviolet light source (Beckman Instruments, Inc., Fullerton, Calif.). Cell nitrogen was determined by the semimicro-Kjeldahl method of McKenzie and Wallace (17). Zoogloal formation was determined by treating wet mounts of microbial flocs with skim milk (9) and microscopically observing for a sharply defined matrix boundary against the skim milk background (Fig. 1). Cells were measured from photomicrographs. Staining reactions (Hucker’s modification of the Gram stain and Burdon’s test for sudanophilicity), litmus milk changes, Koser citrate utilization, indole formation, and nitrate reduction were determined by using standard procedures (20). Catalase was determined by treating 3 ml of concentrated cells with 2 drops of 30% H2O2. Oxidase, urea hydrolysis, H2S production (peptone-cysteine sulfate medium and the lead acetate paper method), acid and gas production from carbohydrates, starch hydrolysis, and gelatin hydrolysis were determined by test procedures of Skerman (19) and media formulations of Unz and Dondono (23). Crystal violet decolorization was tested by using the method of Friedman and Dugan (12).

RESULTS

Aromatic compounds. The results of studies on the utilization of aromatic compounds by Zoogloeae strains are summarized in Table 1. All strains of Unz and Dondono (23) utilized benzoate, m-toluate, p-toluate, phenol, o-cresol, m-cresol, and p-cresol. In addition, certain strains grew on other aromatic compounds. Z. ramigera I-16-M did not utilize any of the aromatic compounds tested, except L-tyrosine.

The strains occasionally formed zoogloal flocs in media containing sodium benzoate or sodium m-toluate, but growth on aromatic compounds was usually in dispersed-cell form. Early stationary-phase cells obtained on sodium benzoate or sodium lactate were filled with highly refractile, sudanophilic granules which presumably were poly-β-hydroxybutyric acid (23). Strains growing on sodium benzoate, sodium m-toluate, and o-cresol produced a yellow-green color in culture media after 2 days and a chocolate brown color in 7 days. The yellow-green color formed when washed cells were incubated with catechol or methyl-substituted catechols. A spectroscopic analysis of the culture fluids revealed absorption peaks between 375 and 385 nm at pH 7.1 and between 310 and 320 nm at pH 2.5 (Table 2). Spectra of the sodium m-toluate-, o-cresol-, and 3-methyl catechol-containing culture fluids were similar to that of the meta cleavage product of 3-methyl catechol (2). Spectra of sodium benzoate or catechol culture fluids were similar to that of 2-hydroxymuconic semialdehyde (16).

No absorption maxima, characteristic of

![Fig. 1. Fingered zoogloea showing gelatinous matrix clearly defined against a skim-milk background. Z. ramigera 106. Sodium lactate-mineral salts medium, 28 C, 60 hr. Phase-contrast.](http://aem.asm.org/Downloaded-from)
meta cleavage, were found in scanning culture fluids containing p-toluolate, phenol, m-cresol, and p-cresol as substrates. However, cells grown on these compounds meta-cleaved catechol, and the yellow-green color rapidly developed in culture fluids. Absorption maxima for all uninoculated control media were in the range of 250 to 290 nm.

The aromatic compounds found to support the growth of eight of the nine strains were evaluated in growth yield experiments. The results of a typical experiment with Z. ramigera 106 are given in Table 3. Cells were harvested in the declining growth phase as determined from growth curves. The wide carbon-to-nitrogen ratios obtained with cells cultured in sodium benzoate and sodium lactate apparently resulted from accumulation of intracellular sudanophilic granules as well as production of extracellular matrix.

**Isolation of Zoogloea strains.** Initially, four carbon sources were tested in the isolation media in the following concentrations (milligrams per liter): sodium benzoate, 500; sodium m-toluolate, 500; phenol, 200; and o-cresol, 200. They were inoculated with a loopful of the scum layer which developed in 24 to 48 hr on the surface of beakers of settled activated sludge. The scum layers were largely composed of fingered bacterial zoogloea similar in appearance to those described by Amin and Ganapati (1).

Sodium benzoate was found to be insufficiently restrictive to the growth of many microorganisms, resulting in difficulty in locating colonies which might be purified to yield Zoogloea strains. One zoogloea-forming bacterium was isolated on sodium benzoate. Phenol and o-cresol visibly supported growth of fewer numbers of microorganisms than did sodium benzoate, and colonies which developed were very small and difficult to manipulate. Two zoogloea-forming bacteria were isolated on o-cresol, and none was isolated on phenol. Sodium m-toluolate did not support growth of as many microorganisms as did sodium benzoate; with experience, it was possible to recognize colonies which could be purified to yield zoogloea bacteria. Incubation temperature did not influence the results of isolation. Based on the results of preliminary experimentation, the sodium m-toluolate medium was used in remaining isolation work, and all plates were incubated as 28 C. After 2 to 3 days, punctiform colonies of various microorganisms appeared, and a diffusible yellow-green color indicative of meta cleavage of the aromatic ring developed along the path of heaviest inoculation. In 5 to 6 days, large (2 to 3 mm), raised or convex, glistening, tough, cohesive colonies developed which were selected for purification (Fig. 2). These colonies were usually the largest present and became brownish or developed dark brown centers if incubated for a total of 7 to 10 days. The tenacious colonies were difficult to pick, and reisolation was usually necessary to ascertain purity.

Thirty-seven zoogloea-forming bacteria were isolated from several sources on the sodium m-toluolate medium (Table 4). The initial inexperience in selecting and purifying appropriate colonies is reflected in the relatively low percentage of zoogloea-forming bacteria isolated from mixed liquor and settled activated sludge. Besides wastewater, zoogloea-forming bacteria were isolated from the waters of a

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**Table 2. Absorption spectra of culture fluids of Zoogloea ramigera 106 grown on certain aromatic compounds**

| Substrate          | Inoculum                              | Absorption maximum (nm) | pH 7.1 | pH 2.5 |
|--------------------|---------------------------------------|-------------------------|--------|--------|
| Sodium benzoate    | Casitone-yeast autolysate-grown cells |                         |        |        |
| Sodium m-toluolate | Casitone-yeast autolysate-grown cells | 375-380                 | 315-320|        |
| o-Cresol           | Casitone-yeast autolysate-grown cells | 385-390                 | 310-315|        |
| Catechol           | Sodium benzoate autolysate-grown cells| 375-380                 | 315-320|        |
| 3-Methylcatechol   | Sodium m-toluolate autolysate-grown cells| 385-390           | 310-316|        |
| 4-Methylcatechol   | Sodium m-toluolate autolysate-grown cells| 382-385           | 310-316|        |

**Table 3. Growth of Zoogloea ramigera 106 on various aromatic compounds and sodium lactate**

| Substrate            | Generation time (hr) | Yield (mg dry wt/liter Kjeldahl nitrogen/ liter) | Ratio of dry wt to nitrogen of cell mass | Yield (mg of cell nitrogen/liter) |
|----------------------|----------------------|-----------------------------------------------|---------------------------------------|----------------------------------|
| Sodium benzoate      | 3                    | 21.1                                          | 1.18                                   | 18                               |
| Sodium m-toluolate   | 14                   | 11.4                                          | 0.85                                   | 13                               |
| Phenol               | 12                   | 3.5                                           | 0.30                                   | 12                               |
| o-Cresol             | 14                   | 3.7                                           | 0.27                                   | 14                               |
| m-Cresol             | 34                   | 2.4                                           | 0.20                                   | 12                               |
| p-Cresol             | 22                   | 2.1                                           | 0.19                                   | 11                               |
| Sodium lactate       | 5.5                  | 14.6                                          | 0.86                                   | 17                               |
| None                 | 5.5                  | 1.1                                           | 0.07                                   |                                   |
FIG. 2. Primary isolation plate with two colonies (arrows) indicative of Zoogloea species. Sodium-m-toluate medium, 28 C, 7 days.

TABLE 4. Isolation of zoogloea-forming bacteria from natural sources by using sodium m-toluate medium

| Source               | No. of samples processed | No. of colonies purified | No. of zoogloea-forming bacteria isolated | Per cent colonies yielding zoogloea-forming bacteria |
|----------------------|--------------------------|--------------------------|------------------------------------------|--------------------------------------------------|
| Raw sewage           | 2                        | 3                        | 2                                        | 67                                               |
| Settled sewage       | 2                        | 2                        | 2                                        | 100                                              |
| Mixed liquor         | 6                        | 20                       | 7                                        | 35                                               |
| Settled activated sludge | 9                      | 35                       | 12                                       | 34                                               |
| Scum*                | 5                        | 5                        | 4                                        | 80                                               |
| Trickling filter slime | 2                     | 5                        | 3                                        | 60                                               |
| Trickling filter effluent | 2                   | 8                        | 5                                        | 63                                               |
| Lakes                | 4                        | 0                        | 0                                        |                                                  |
| Spring               | 2                        | 0                        | 0                                        |                                                  |
| Unpolluted stream    | 1                        | 0                        | 0                                        |                                                  |
| Polluted stream      | 2                        | 4                        | 2                                        | 50                                               |
| Forest soil          | 6                        | 0                        | 0                                        |                                                  |
| Cultivated soil      | 4                        | 0                        | 0                                        |                                                  |
| Human feces          | 2                        | 0                        | 0                                        |                                                  |

*Settled activated sludge was incubated at 28 C for 24 hr. The scum layer which developed at the surface of the overlying water was used as inoculum.

DISCUSSION

With the exception of Z. ramigera I-16-M, all the strains studied utilized certain non-nitrogenous aromatic acids and phenolic substances. These compounds are present and discharge in certain industrial wastes and may thus become available as carbon sources for zoogloeads in polluted waters.
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TABLE 5. Response of Zoogloea strains to diagnostic tests

| Test                          | Strains positive/total strains tested |
|-------------------------------|--------------------------------------|
|                               | Zoogloea strains isolated on sodium m-toluate medium | Zoogloea strains 21, 106, and 216 | Z. ramigera I-16-M |
| Zoogloea produced             | 3/3                                  | 0/1                                |
| Denitrification               | 3/3                                  | 0/1                                |
| Urease                       | 1/1                                  | 0/1                                |
| Catalase                     | 1/1                                  | 0/1                                |
| Oxidase                      | 1/1                                  | 0/1                                |
| Crystal violet decolorization | 0/3                                  | 0/1                                |
| Meta cleavage of catechols    | 0/1                                  | 0/1                                |
| Koser citrate                 | 0/1                                  | 0/1                                |
| Litmus milk reaction          | 0/1                                  | 0/1                                |
| Gelatin hydrolysis            | 0/3                                  | 0/1                                |
| Starch hydrolysis             | 0/3                                  | 0/1                                |
| H₂S (lead acetate)            | 0/3                                  | 0/1                                |
| H₂S (Kligler)                | 0/3                                  | 0/1                                |
| Indole                        | 1/1                                  | 0/1                                |
| Tyrosine agar                 | 1/1                                  | 0/1                                |
| Acid without gas from:        | 1/1                                  | 0/1                                |
| Glucose                      | 1/1                                  | 0/1                                |
| Galactose                    | 1/1                                  | 0/1                                |
| Sucrose                      | 1/1                                  | 0/1                                |
| Maltose                      | 1/1                                  | 0/1                                |
| Mannitol                     | 1/1                                  | 0/1                                |
| Lactose                      | 1/1                                  | 0/1                                |
| Xylose                       | 1/1                                  | 0/1                                |
| Arabinose                    | 1/1                                  | 0/1                                |

Spectroscopic analyses provided presumptive evidence that sodium benzoate, sodium m-toluate, and o-cresol are degraded by certain Zoogloea strains via meta cleavage of the aromatic ring (11, 16).

The salient properties of Zoogloea are Zoogloea formation, denitrification, urea hydrolysis, gelatin hydrolysis, and oxidase and catalase activities (23). With the exception of urea hydrolysis, nearly all of our zoogloea-forming bacteria possessed these characteristics. In addition, the zoogloea-forming bacteria did not utilize any of the carbohydrates, with the exception of five strains which produced acid from xylose. Unz and Dondero (23) found that 3 of 65 strains formed acid from xylose but that none utilized Koser citrate or produced H₂S. Twenty-one of our zoogloea-forming bacteria utilized Koser citrate, and six strains produced H₂S. Overall, the 40 Zoogloea isolates resembled the three Zoogloea strains of Unz and Dondero (23), including strain 106 which has been proposed to replace Z. ramigera I-16-M as the neotype strain of Z. ramigera Itzigsohn (22). All of the newly isolated zoogloea-forming bacteria may be properly identified as strains of Zoogloea. We suggest that the test for meta cleavage of catechols be included in future taxonomic studies of bacteria suspected to be Zoogloea species.

The sodium m-toluate medium proved satisfactory for convenient isolation from various wastewater sources. The organisms were distinguished by large colonies which were often tinted yellow-green by meta cleavage products or brown, depending on age. Friedman and Dugan (12) isolated a strain of Z. ramigera earmarked by colonies which decolorized crystal violet. None of our strains did.

It is obvious that the sodium m-toluate medium would not suffice for the isolation of zoogloea-forming bacteria unable to utilize the primary carbon source. Strains isolated in the present study appear different from cultures recently described by several other investigators (7, 12, 13). However, it is believed that the reference cultures of Unz and Dondero (23) are authentic in that they were isolated by micro-manipulation from fingered wastewater zoogloea similar in appearance to those originally described and named Z. ramigera by Itzigsohn (15).

It is anticipated that the sodium-m-toluate medium described in this report will prove valuable for the isolation of Zoogloea strains and, thereby, aid future physiological and ecological studies of these bacteria.

ACKNOWLEDGMENT

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ERRATUM

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Volume 23, no. 3, p. 528, column 2, line 9: Change "Zoogloea" to "zoogloea."
p. 528, column 2, line 12: Change the sentence, "The average width and length of cells were 1.0 and 1.0 ± 0.3 μm, respectively." to "The average width and length of cells were 1.0 and 2.0 ± 0.3 μm, respectively."
p. 529, column 1, line 6: Change the term "Zoogloea" preceding "formation" to "zoogloea."