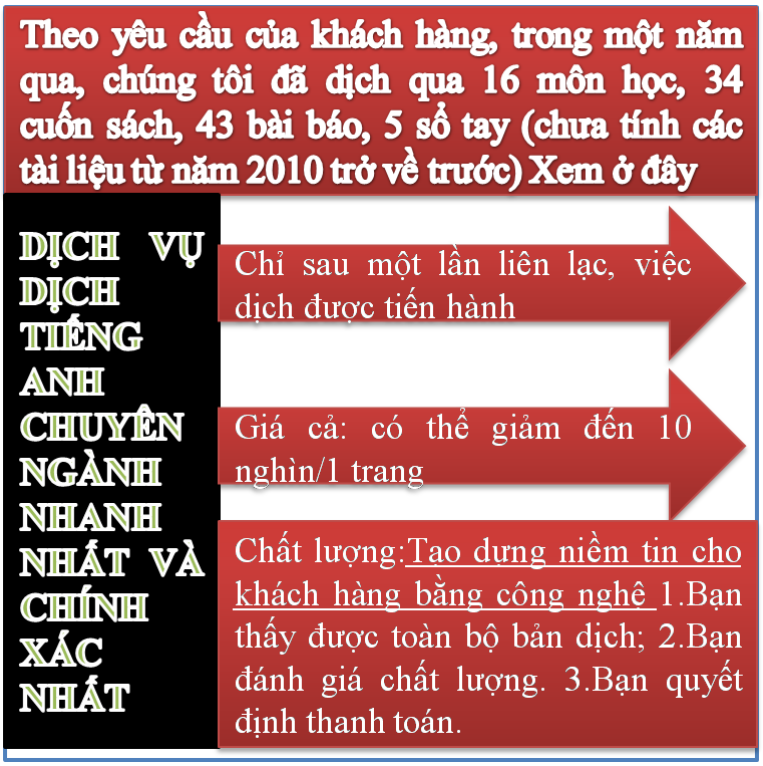

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Enumeration, Isolation, and Characterization of Beggiatoa from Freshwater Sediments An accurate most-probable-number enumeration method was developed for counting the number of Beggiatoa trichomes from various freshwater sediments. The medium consisted of extracted hay, diluted soil extract, 0.05% acetate, and 15 to 35 U of catalase per ml. The same enrichment medium, but without the acetate, was the best enrichment medium from which to obtain pure cultures because it supported good growth of the beggiatoas without allowing them to be overgrown by other bacteria. A total of 32 strains of Beggiatoa were isolated from seven different freshwater habitats and partially characterized. The strains were separated into five groups based on several preliminary characteristics. Four of the groups contained cells with trichomes of approximately the same diameter (1.5 to 2.7 /IIn) and may be Beggiatoa leptomitiformis or an unnamed species. The fifth group appeared to be Beggiatoa alba. With the exception of three strains, all of the strains deposited sulfur in the presence of hydrogen sulfide, and all strains grew heterotrophically and deposited poly-/?-hydroxybutyrate and volutin when grown on acetate supplemented with low concentrations of other organic nutrientsề Thin sections of sulfur-	Đếm, phân lập và xác định tính chất của Beggiatoa sống trong trầm tích nước ngọt Chúng tôi xây dựng một phương pháp chính xác, được gọi là phương pháp đếm số có xác suất cao nhất để đếm số trichome của Beggiatoa trong các trầm tích nước ngọt khác nhau. Môi trường bao gồm chiết suất cỏ khô, chiết xuất đất pha loãng, 0.05% acetate, và 15 đến 35 U catalase trên mỗi ml. Một môi trường giàu dưỡng chất tương tự, nhưng không có acetate là môi trường tốt nhất để thu được các chủng thuần vì nó giúp beggiatoas tăng trưởng rất tốt và không bị lấn át bởi các vi khuẩn khác. Tổng cộng 32 chủng Beggiatoa được phân lập từ bảy môi trường sống nước ngọt khác nhau và được xác định một số đặc tính. Các chủng được chia thành năm nhóm dựa trên một số đặc điểm sơ bộ. Bốn nhóm chứa các tế bào có trichome đường kính gần giống nhau (1.5 đến 2.7 …) và có thể là Beggiatoa leptomitiformis hoặc một loài không tên. Nhóm thứ năm có vẻ là Beggiatoa alba. Ngoại trừ ba chủng, tất cả các chủng làm kết tủa lưu huỳnh khi có hidro sunfua, và tất cả phát triển dị dưỡng và gây kết tủa poly-beta-hydroxybutyrate và volutin khi tăng trưởng trên acetate được bổ sung các dưỡng chất hữu cơ khác ở nồng độ thấp. Các phần mỏng trichome chứa lưu huỳnh	

bearing trichomes indicated that the sulfur granules were external to the cytoplasmic membrane and that they were surrounded by an additional membrane.	cho thấy rằng các hạt lưu huỳnh nằm bên ngoài màng tế bào chất và chúng được bao quanh bởi một màng nữa.	
Beggiatoa is a filamentous gliding bacterium capable of oxidizing sulfide to elemental sulfur, which it deposits in its cells (14, 36). When the sulfide is depleted, the bacterium further oxi-dizes the deposited sulfur to sulfate, which is then released to the environment (28). The ecol-ogy, taxonomy, physiology, and many other as-pects of Beggiatoa biology are poorly under-stood.	Vi khuẩn trượt dạng sợi có khả năng oxy hoá sunfua thành lưu huỳnh nguyên tố, và vật chất này lắng tụ trong tế bào của nó (14, 36). Khi sunfua cạn kiệt, vi khuẩn tiếp tục oxy hoá lưu huỳnh thành sunfat, rồi sau đó giải phóng sunfat ra môi trường (28). Các đặc tính sinh thái học, phân loại học và sinh lý học, cũng như nhiều khía cạnh khác về đặc tính sinh học của Beggiatoa vẫn chưa được nghiên cứu nhiều.	
Interest in the organism has been sporadic, perhaps because of the difficulty of isolating and maintaining cultures in the laboratory. Recently, interest in Beggiatoa has been revived because of the fine work of Pitts et alệ (25) and of Joshi and Hollis (12), who suggested that Beggiatoa and rice plants may occur together in a mutu-alistic association in which the bacterium oxidizes H2S in the root zone, thus protecting the plant from the toxic effects of H2S, and the plant roots excrete catalase, which decomposes the toxic peroxides produced by the bacterium during its metabolism.	[redacted]	
We have seen Beggiatoa in close association with the root zone of the marsh grass Spartina alterniflora (unpublished results), and J. Charba has seen it in close		

association with the roots of water hyacinths (personal communication). It is possible that Beggiatoa plays an important role in plant health in the entire flooded-soil/plant ecosystem.

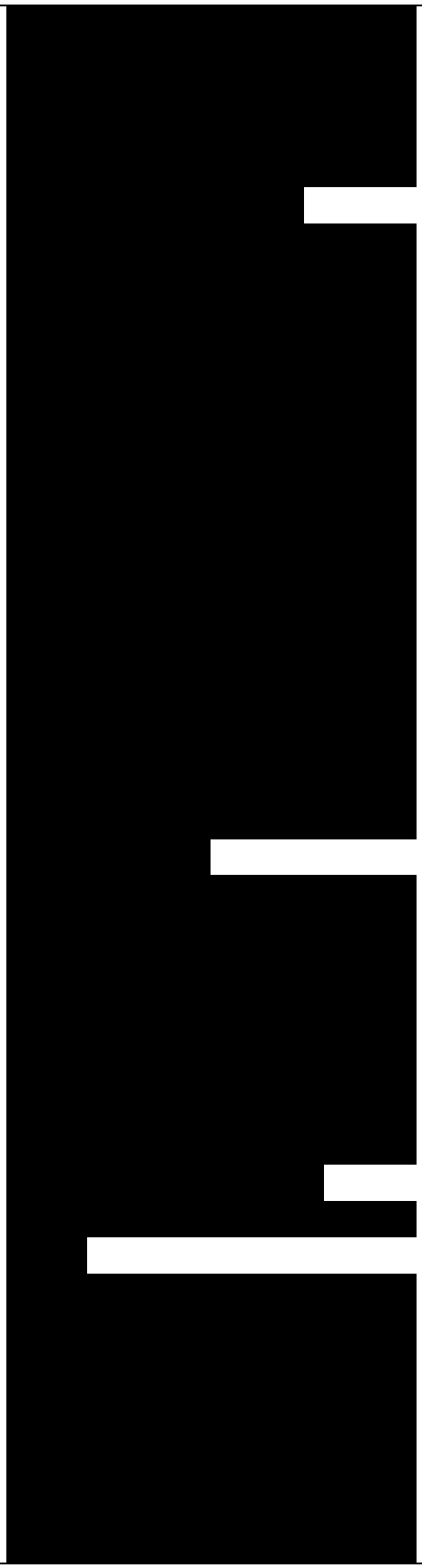
Several techniques for the production of en-richment cultures of Beggiatoa from nature have appeared, the most recent by Joshi and Hollis (11). All are based on the techniques originally described by Cataldi (5), in which extracted hay (EH) is a prime ingredient.

Be-cause of the probable ecological significance of Beggiatoa in the flooded-soil habitat, it would be valuable to know which enrichment tech-niques work the best, and if any of them can be adapted for the enumeration of Beggiatoa in its habitat.

In this paper we present our attempts to de-velop methods for the enrichment, enumeration, and isolation of Beggiatoa from nature, as well as the preliminary results of our attempts to characterize 32 isolated strains in some of theù morphological and physiological features.

MATERIALS AND METHODS

Media. All media for the enrichment of Beggiatoa were based on the method of Cataldi (5) and on the various modifications of her technique as used by others. An ingredient common to all of the enrichment media was hay, or grass, which was extracted at least



five times for 30 min each in boiling water, with two rinses in cold tap water between each extraction (EH). When needed, a soil extract (SE) was prepared by mixing approximately 500 g of black, sulfide-containing mud with 1 liter of tap water, allowing the coarse particles to settle out, and then filtering the supernatant fluid through Whatman no. 2 filter paper contained in a Buchner funnel. For diluted soil extract (DSE), the SE was diluted 1:2 with tap water.	[redacted]	
Pringsheim (14) basal salt solution as modified by w. Koch (personal communication) consisted of, per liter, 5 ml of a trace element solution (14), 20 ml of a saturated CaS04 solution, 0.00045 g of NH4C1, 0.001 g of K2HPO4, and 0.0001 g of MgS04- 7H2O.	[redacted]	
BP medium consisted of the following ingredients: basal salt solution, 0.05% sodium acetate, 0.05% nutrient broth (DIFCO Laboratories, Detroit, Mich.), and 1.0% agar. Filter-sterilized catalase to give a final concentration of 15 to 35 u/ml (3) was added before pouring into plates.	[redacted]	
MP medium consisted of the	[redacted]	

following ingredients: basal salt solution, 0.0001% sodium acetate, 0.03% Na2S, and lé0% agar. The Na2S was autoclaved sepa-rately and added to the medium before plates were poured.

Microcyclus-Spirosoma agar has been described previously (19). Nutrient agar was obtained from Difco.

Evaluation of MPN techniques. To determine which medium would yield the best results for a most-probable-number determination (MPN) of Beggiatoa in sediments, sediment samples were inoculated in duplicate into five sets of tubes (three dilutions per set) containing media that had been used successfully for simple enrichment by others (5, 9,11, 35) or media with various modifications that seemed appropriate to us.

The three media that gave the highest counts, plus one new medium suggested by the results, were reexamined in quadruplicate for theừ abilities to provide suitable MPN results with additional sediment samples.

Each tube (25 by 250 mm) in the MPN series received approximately 0.5 g of EH, 50 ml of the liquid medium to be tested, and a

sediment inoculum known to contain Beggiatoa. The tubes were incubated for 2 weeks at room temperature (approximately 22°C) and were then examined macroscopically for the presence of Beggiatoa by looking for the "fluff ball" tufts of colonies, characteristic of Beggiatoa (9) or for mat formation supported by Beggiatoa filaments. Confir-mation of Beggiatoa presence, and an estimate of the degree of contamination by other bacteria in presumptively positive tubes, was made by phase microscopy. The MPN obtained with each medium was determined from a standard table (18). To determine whether the MPN procedures that resulted in the highest counts would be accurate for the enumeration of Beggiatoa from sediments, a pure culture of Beggiatoa was grown and divided into four aliquots. One aliquot was used for a dùrect microscopic count in a hemacytometer to determine the number of trichomes present. The viable population in the second aliquot was determined with a plate count on BP medium; another aliquot was divided, and the beggiatoas were enumerated by the MPN technique with the media chosen on the basis of the experiments described above. The fourth aliquot was inoculated into a	[redacted]	

black sulfide-emitting sediment which was then stirred, divided, and assayed with the various MPN media to determine which one(s) gave the highest counts and what percentage of the initial inoculum was recovered with each technique. These procedures were carried out in triplicate using Beggiatoa isolate B14LD. Enrichments for the isolation of Beggiatoa. The same media as described above were placed into 160-ml sterile prescription bottles and were inoculated with 1 to 2 g of sulfide-containing sediments as described by Joshi and Hollis (11) except that the bottles were autoclaved before use to hold down fungal contamination. Cycloheximide was added to a final concentration of 40 mg/ml to some cultures to reduce fungal and protozoan contamination. After about 1 week of incubation at room temperature, the enrichments were examined by phase microscopy for the presence of Beggiatoa and for the degree of contamination by other microbes. For enrichment of Beggiatoa strains from an s. alterniflora-containing salt marsh, a medium consisting of EH, DSE prepared from mud obtained at the collection site, and filter-sterilized catalase was used.	[redacted]	

The salinity was adjusted to 0, 20, 30, 35,40,45, 50, 55, 60, or 65% of synthetic sea water (Seven Seas Marine Mix, Utility Chemical Co., Patterson, NǎJ.) because the salinity of that site, near Leesville, La., was about one-half that of sea water (W. Patrick, personal communication).	[redacted]	
Isolation of Beggiatoa. Beggiatoas were isolated from enrichment media using a modification of Pringsheim's technique (27) for the isolation of fila-mentous gliding organisms. Tufts of filaments from enrichment cultures were transferred with sharp- pointed forceps through four washes in sterile basal salt solution made with tap water. A final wash con-sisted of a 5-min soak in the same solution with 100 Ư of catalase added per ml. The washed tufts of filaments were dried by absorption of the excess water onto an agar plate. Some of the partially dried filaments were then placed onto a freshly poured plate of either BP or MP medium.	[redacted]	
After 2 to 4 days of incubation at 28 to 35°c, the cultures were observed with a dissecting microscope, and isolated filaments were picked up by cutting out a	[redacted]	

block of agar beneath a trichome and transferring it to a fresh plate of the same medium. Occasionally, isolation attempts could be made as early as 8 to 10 h after inoculation, but usually the filaments had not glided far enough away from the contaminants by that time, and attempts at 4 days proved best.	[redacted]	
To determine the optimum concentration of agar in the isolation medium, concentrations of 0.8, 1.0, 1.2, 1.4,1.6, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5% were includedė A range of temperatures, including 17, 23, 28, 35, and 45°c, was tested. Concentrations of 0.001, 0.01, 0ǎ05, 0.1,0.5, and 1.0% sodium acetate and/or nutrient broth were added to determine the optimal concentration of each nutrient. The plates were inoculated with washed filaments from enrichment cultures, and they were examined after 2 to 4 days with a dissecting microscope to determine the level of contamination around the filaments and the ability of the filaments to glide away from the contaminants. This was repeated with several pure cultures after they were isolated.	[redacted]	
Use of inhibitors. The antibiotic sensitivity of Beggiatoa strains was assayed to determine whether they would be useful as aids in the isolation of Beggiatoa. Antibiotic disks were placed on the surface of BP medium in a petri dish, and then	[redacted]	

another layer of BP medium was added to just cover the disks. The plates were incubated for 6 to 8 h to allow the anti-biotics to diffuse, and then a washed tuft of filaments was placed on the agar above each disk. They were examined periodically with a dissecting microscope to determine the viability of the filaments, the degree of contamination, and the ability of the filaments to glide away from the contaminants.

Sodium azide was incorporated into the medium at concentrations ranging from 0.001 to 0.5% to determine if it would be an aid in facilitating isolation of Beggia- toa by reducing the level of contaminants.

Physiological characterization of isolated strains. Beggiatoa cultures were stab inoculated into semisolid (0.2% agar) medium under three conditions: MP medium, BP medium, and BP medium with a sterile petrolatum overlay to provide anaerobic conditions. After 2, 4, 6, 8, and 12 days of incubation at 28°c, the growth and position of the growth were recorded.

Gelatin and casein hydrolysis were assayed according to Pringsheim's methods (27). Catalase production was assayed by adding 3% hydrogen peroxide onto actively metabolizing cultures of Beggiatoa and observing for bubble formation. Cytochrome oxidase was assayed by flooding plates of 48-h Beggiatoa cultures with a 1% aqueous solution of N,N,N',N'-tetramethyl- p-phenylenediamine dihydrochloride (Eastman Kodak Co., Rochester, N.Y.) and observing for the rapid formation of a purple color. The effect of cyanide and sodium dodecyl sulfate (SDS) on Beggwtoa was assayed on plates of BP medium (without catalase) which contained 0.01 or 0.05% filter-sterilized KCN or SDS, respectively.	[redacted]	
The deposition of sulfur in trichomes grown in the presence of H2S was demonstrated by a modification of the methods of Skerman et ală (38) and of Skerman (37). One drop of cell suspension and 1 drop of reagent-grade pyridine (Mallinckrodt Chemical Works, St. Louis, Mo.) were mixed on a slide, and the suspension was sealed with a cover	[redacted]	

slip and petrolatum. Positive results were recorded if the granules disappeared from the cells and if rhombic or monoclinic crystals formed external to the cells as viewed by phase microscopy. Controls using trichomes grown on BP medium, and therefore without sulfur granules, were used.	[redacted]	
Poly-beta-hydroxybutyrate (PHB) and volutin were stained for light microscopy using Sudan Black Ð and methylene blue, respectively. Electron microscopy was used to verify the inclusions in a representative strain, B15LD.	[redacted]	
Electron microscopy. A modified Ryter-Kellen- berger (33) technique was used for the thin sections. Plates containing 96-h trichomes grown on BP medium were flooded with 0.05% OsȮ4 in 0.1 M Veronal acetate buffer at pH 6.0 for 20 min. The trichomes were then scraped off the agar surface and transferred to 0.1 M Veronal acetate-buffered 1% 0s04 for 16 h at room temperature. The trichomes were then rinsed in Veronal acetate buffer, postfixed with 0.1 M Veronal acetate-buffered 0.5% uranyl acetate for 2 h, and dehydrated with 25, 50, 75, and 90% and two changes of 100%	[redacted]	

ethanol, followed by two washes in 100% propylene oxide.

The fixed and dehydrated trichomes were embedded in Epon 812 plastic (21) and then sectioned on an LKB Ultrotome (LKB Inc., Stockholm, Sweden) using a diamond knife. The thin sections were picked up on 300-mesh copper grids and stained with uranyl acetate (40) and then lead citrate (31).
All thin-section micrographs were obtained using an RCA EMU-2 electron microscope at 50 kV. Cells containing sulfur granules were exposed to MP medium for 4 h prior to fixation.

Both puff balls and surface colonies of Beggiatoa were viewed by scanning electron microscopy. The puff balls were prepared from an axenic liquid culture of strain B15LD grown in a static liquid BP medium. They were fixed for 2 h in 1 M Veronal acetate- buffered 3% glutaraldehyde.

The samples were then dehydrated with 25, 50, 75, and 90% and two changes of 100% ethanol, and then they were critical-point dried with 100% acetone as the transition solvent. The surface colonies of

Beggiatoa were prepared by fixing strain B12LD, grown on MP medium, with 4% osmium vapors for 24 h at room temperature. Small blocks of agar containing the trichomes were cut out of the plates, carefully rinsed with distilled water, and then dehydrated with acidified 2,2-dimethyoxypropane (23). They were then critical-point dried with 100% acetone as the transition solvent. The scanning electron microscope samples were coated with 15 to 20 nm of gold-palladium using a Hummer I Sputter Coater (Technics, Inc., Alexandria, Va.) and were viewed on a Hitachi S-500 scanning electron microscope. Chemicals. Cycloheximide and fungal catalase were obtained from the Sigma Chemical Co., St. Louis, Mo. The latter was always filter sterilized and then added to the sterile media in an amount sufficient to give 15 to 35 U/ml. Additional procedures. Measurements of fila-ment size were obtained with a Filar micrometer. A Gillet and Sibert microscope equipped with a Nikon AFM camera attachment was used	[redacted]	

for phase and bright-field observations and photomicrography.

RESULTS

Enumeration of Beggiatoa Using the pres-ence of puff balls (Fig. 1) or mats followed by microscopic confirmation (Fig. 2), a preliminary screening of the various enrichment media as possible media for MPN techniques showed that four media gave significantly higher MPN results (Table l)ê The best results were obtained with a medium consisting of EH, DSE, and 0.1% acetate.

However, this medium was badly overgrown by contaminating bacteria and so was not suitable as an enrichment medium from which to attempt the isolation of Beggiatoa. The addition of catalase enhanced some media (cf. stream water versus stream water plus catalase, or DSE versus DSE plus catalase) without

Fig. 1. Typical appearance of a Beggiatoa fluff ball from an enrichment culture. Bar, 100 ịurn.

Fig. 2. Typical appearance of Beggiatoa from an enrichment culture. The granules consist of sulfur (S) and PHB (P). Bar, 10 fim.

appearing to stimulate the growth of contaminating bacteria, so three of the above media were reexamined; the fourth medium, consisting of EH, DSE, 0.1% acetate, and catalase, was used. Using the highest MPN as a sole criterion, a medium of EH, DSE, 0.05% acetate, and catalase was the best (Table 2).

The three media that appeared to be the best were then examined for theŭr ability to recover a known number of trichomes from sediments inoculated with a pure culture (Table 3). A plate count of a pure culture of the inoculum yielded 3.4 X 104 trichomes per ml. The same culture yielded 1.6 X 104 to 3.7 X 104 trichomes per ml when counted with the three MPN media. After inoculation of the culture into a nonsterile sediment and correcting for the dilution and for the

TABLE 1. Preliminary comparison of various enrichment media for the enumeration of Beggiatoa

TABLE 2. Comparison of the four best media for the enrichment and enumeration of Beggiatoa

All media contained EH and about 50 ml of the appropriate liquid medium.

Average of four replicates each.

TABLE 3. Evaluation of three media for their abilities to recover Beggiatoa inoculated into a sediment
All MPN media contained EH, DSE, 15 to 35 U of catalase per ml, and the amount of acetate indicated.

Results shown are after adjustment for dilution of the culture and for the background Beggiatoa population in the sediment.

……
background Beggiatoa population, recoveries ranged from 1.5 X 104 to 3.2 X 104 trichomes per ml. In each case the medium with no acetate gave the poorest results, with 44 to 47% recovery, and the medium composed of EH, DSE, 0.05% acetate, and catalase (SACH medium) gave the highest recovery rates, with 94 to 109% of the viable count being recovered. Increasing the acetate concentration to 0.1% resulted in a decrease in the recovery rate.

The SACH medium was used to enumerate the beggiatoas from a variety of flooded sedi-ments in the

Baton Rouge area. Typical results (Table 4) ranged from 11 to 95 trichomes per g of wet sediment.

Attempts were made to adapt the SACH me-dium to the enumeration of Beggiatoa from the flooded sediments associated with the marsh grass S. alterniflora in salt marshes. SACH medium was used with salinities ranging from 0 to 65% that of sea water. The salinities that supported the best growth of those beggiatoas were 40 to 45% of that of sea water. Salinity of greater than 60% or less than 30% of that of sea water resulted in a reduction or complete inhibition of growth. Trichomes ranging from 3 to 35 jLim in width were observed in the MPN tubes from the

TABLE 4. Population of Beggiatoa trichomes in various flooded sediments from the Baton Rouge area

Sample site Trichomes per g (wet wt) of sediment

salt-marsh sediments (Figẽ 3)ẻ Attempts to enu-merate these organisms by the MPN technique developed for the freshwater strains were not reproducible due to the

growth of a white floc-forming bacterium which mimicked Beggiatoa and interfered with its growth. The addition of Na2S, vitamin Bi2 (27), or various concentrations of aoetate did not help significantly.	[redacted]	
Isolation of Beggiatoa The best medium for isolation purposes, because it contained the lowest level of contaminating bacteria with a reasonably high recovery of Beggiatoa, was a medium consisting of EH, DSE, and catalase (EDC medium). Enrichments using nonex-tracted hay or undiluted SE resulted in high contamination levels and low counts of Beggia- toa (data not shown).	[redacted]	
From any Beggiatoa-contaìiùng enrichment ùrom a freshwater sediment it was possible to isolate the organism, although it was easiest from EDC medium. With some of the initial isolates, the utility of antibacterial agents as selective agents was assessed.	[redacted]	
Used singly, nitro-furantoin, sulfathiazole, penicillin G, and triple sulfa appeared to inhibit many contaminants while leaving Beggiatoa unharmed (Table 5).	[redacted]	

Ampicillin, gentamicin, and polymyxin B either killed the beggiatoas or prevented theù gliding away from the contaminants.

Tetracycline, kan- amycin, and streptomycin offered minimal hope as selective agents. Those reagents that appeared to be useful when used singly were then tried in combination, with the combination of penicillin G plus nitrofurantoin and triple sulfa appearing to be the most promising. Subsequent attempts to isolate Beggiatoa from enrichments were made on MP, BP, and BP-plus-antibiotic

TABLE 5. Relative efficacy of various antibacterial chemicals to aid in the isolatwn of Beggiatoa
Results are graded from + to ++++ on the effec-tiveness of the agents to prevent growth of contami-nants while allowing the Beggiatoa to glide away from themẻ A negative sign indicates that Beggiatoa was killed.

media.

Thirty-two strains of Beggiatoa were isolated from seven different locations. No two strains from a single enrichment were kept if they

ap-peared to be similar on initial isolation. The best agar concentration for theù isolation was 1.0 to 1.2%, and the best nutrient concentrations were 0.0001% acetate (if 0.03% Na2S was supplied) or 0.05% acetate (if 0.01 to 0.5% nutrient broth was added). The best temperature for isolation was about 33°c, but the cutures did not survive past 3 to 4 days at that temperature, and the temper-ature was decreased to about 25°c at that time. The use of sodium azide in the medium at concentrations ranging from 0.0001 to 0.05% did not facilitate isolation. At concentrations of 0.0001 to 0.025% azide the contaminants were not sufficiently inhibited. Beggiatoa was not affected by these low concentrations, but increased concentrations first inhibited gliding and then inhibited the growth of the trichomes. Although our data with antibiotics indicated that theù incorporation into the media should be of help, it was found that isolating the beg-giatoas was relatively easy on the other media and that the antibiotics offered no significant advantage.	[redacted]	

Characteristics of the Beggiatoa isolates.

Thirty-two isolates were obtained, and the mor-phological and physiological characteristics that were shared by all of our strains are shown in Table 6. All strains were motile by gliding, were able to grow on both MP and BP media, stored volutin and PHB as noted previously by Pringsheim and Weissner (30) when grown het-erotrophically (Fig. 4), and grew in media made with freshwater but not with salt water. They were all oxidase and catalase negative and were stimulated by the presence of catalase in the medium. They failed to grow in the presence of 0.05% KCN or 0.05% SDS.

All strains except those designated as group B (Table 7) deposited sulfur when grown in the presence of Na2S.

The 32 strains tested were placed into five groups (Strohl and Larkin, Abstr. Annu. Meet. Am. Soc. MicrobiolÊ 1977, N84, p. 242) based upon physiological and morphological charac-teristics (Table 7). Many isolates formed spùral patterns when grown on an agar surface (Fig. 6A), but only

group A cells glided over themselves to produce three-dimensional "super-coiled balls," which etched into the agar and rotated in place (Fig. 5).

The group A strains were relatively fastidious and grew well with nutrient concentrations of less than 0ă05% but poorly on nutrient concentrations above 0.05%.

TABLE 6. Characteristics shared by all of the Beggiatoa isolates

Except for group B strains (Table 7), which grew well on MP medium but did not deposit sulfur.

Group B strains were similar in size and in some physiological characteristics to group A strains, although they did not deposit sulfur in the pres-ence of H2S and they did not normally form the supercoiled balls. Groups c and D were similar to each other but were differentiated on the basis of temperature relationships, trichome di-ameter, and the ability to grow on nutrient agar. The group E strains were different from each of the other strains. They had wider trichomes; the average length of theù trichomes was shorter; they grew dispersed in liquid culture under appropriate conditions; they

were sensitive to pen-icillin and insensitive to 0.01% SDS, KCN, and NaN3; they grew well on nutrient agar; and they all grew well at 0°c.	[redacted]	
Of 20 strains tested, 17 were viable after 6 weeks at 28°c in a semisolid medium which contained 0.03% Na2S as a hydrogen sulfide source and 0.0001% acetate. All of the strains tested grew on MP medium plates, and they were viable after 1 month when left at room temperature. Although some autolysis occurred (especially with group E strains), the autolysis was less and was slower to occur than when the strains were grown on BP medium.	[redacted]	
When grown on a medium composed of basal salt solution with acetate concentrations of 0.00001 to 0.05%, all of the strains except one from group c and one from group D grew poorly. The same medium, but with 0.03% Na2S, supported good growth of all of the strains, including those that did not deposit sulfur (group B).	[redacted]	

In liquid BP medium, group E strains grew best when the medium was shaken at 200 to 300 rpm.

In semisolid (0.2% agar) stab tubes of BP medium, growth began at approximately 4 to 5 mm below the surface, and in four of the five groups the growth took the form of a dense ring at that level.

The fifth group (A) grew dispersed at a depth of 5 to 40 mm from the surface. With Na2S (semisolid MP medium) the growth of the four ring-forming groups occurred farther from the surface, to depths of 20 to 45 mm. However, none of them grew under the strictly anaerobic conditions at the bottom of the tubes or under a petrolatum seal.

Cytology. The typical appearance of a cell growing on MP medium is shown in Fig. 6. Sulfur granules appear as densely outlined granules when viewed by phase microscopy (Fig. 6A), or as refractile bodies when viewed by dark-field microscopy (Fig. 6B). The granules were dem-onstrated to be sulfur by pyridine extraction (Fig. 7). In thin sections, the sulfur granules were seen external to the cytoplasmic membrane in invaginated pockets of the membrane,

and they were enclosed within a membrane consisting of three dark and two electron-translucent layers (Fig. 8). The sulfur granules consisted of a washed-out space, as noted by Shively (36), which usually contained some electron-dense stringy material that may be proteinaceous. The cell wall appeared to be rather tight fitting around the cytoplasmic cylinder except in the area of a sulfur granule (compare Fig 8 with Fig. 9).

At the point where the cell wall is pulled away it can be seen that the wall is typical of gram-negative bacteria, but with an additional layer (Fig. 10). When grown on BP medium, large lipid storage vacuoles (PHB) were observed in most cells (Figă 9), and in some cells the lipid occupied much of the cytoplasmic space.

Whether the cells were grown in broth (Fig. 11) or on an agar surface (Fig. 12), the cells were connected by strands of extracellular slime. Moreover, on the agar surface, the cells left trails where the trichomes had glided (Fig. 12B). Cross walls are not seen in these scanning electron micrographs (Fig. 11, 12), indicating that the outer surface of

the Beggiatoa trichomes was continuous and did not invaginate at the septa between individual cells of a trichome.

DISCUSSION

Beggiatoa cannot always be enumerated directly from its habitat, and especially from sediments, as some bacteria can (iẽeẼ, Escherichia coli), because of the lack of a specific selective medium and because gliding cells may move about over the surface of an agar medium. A reasonable approach to solving this problem is to develop an MPN procedure using an enrichment medium in which Beggiatoa is favored over competing microorganisms, so that high recoveries may be expected.

The SACH medium provides such an enrichment, and by confirming presumptively positive tubes by phase microscopy an accurate enumeration of beggiatoa from freshwater envùronments may be obtained.

From brackish and marine environments, this medium does

not always provide reproducible results even though beggiatoas may grow in it; we are trying various modifications to overcome this problem.

In the SACH medium the presence of catalase was important, presumably because it decomposes the peroxide that Beggiatoa produces (3). The concentration of acetate also appeared to be critical, with 0.05% being the optimum concentration.

Our strains, with two exceptions, grew very poorly on a medium composed of 0.0001% acetate plus catalase. The addition of Na2S to this medium greatly stimulated growth, and the cells deposited sulfur. We have not investigated the mechanism of H2S stimulation.

The relative abundance of Beggiatoa in the sediments of southern Louisiana lakes and streams (Table 4) was not suprising. Lackey (15), Lackey et al. (17), and Pringsheim (28) indicated that Beggiatoa were present in large numbers in most of the habitats that were suited for them. Suitable conditions were governed by the available nutrients, the proper salt balance, the proper O2-H2S balance, a supply of CO2,

and slightly alkaline conditions. Pringsheim (28) noted that standing water and black mud were typical environments that nearly always produced thriving trichomes in enrichment cultures,

and Scotten and Stokes (35) indicated that Beg- giatoa were common in most lake and river sediments, sulfur springs, and marine habitats. Pitts et al. (25) and Joshi and Hollis (12) have found Beggiatoa in association with rice roots

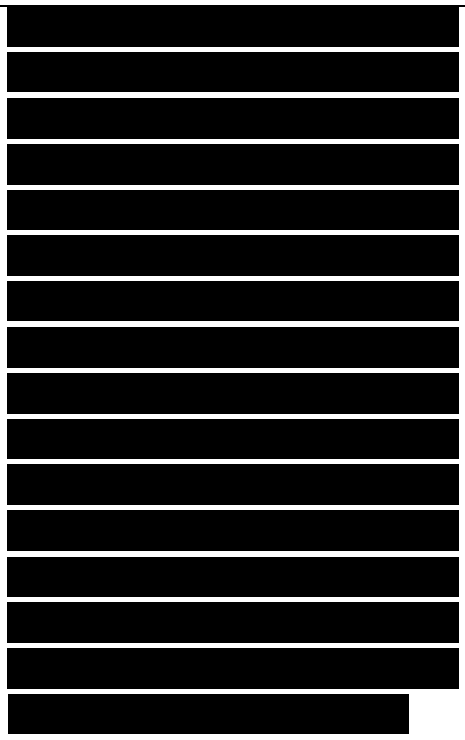


Fig. 5 Phase micrograph of strain B8GC (group A) growing on the surface of BP agar. Two of the trichomes have formed supercoiled balls, which rotate on the agar and etch the surface. The large rotating (but flat) colony was associated with many strains. Pringsheim (27) referred to the latter colony type as circuitans, or c, type of colony. Bar, 30 ịun.

under the annual flood-soil conditions of Louis-iana rice paddies. Whereas Beggiatoa was not found to be a good indicator of pollution (16), some researchers have reported the presence of Beggiatoa in polluted lakes or streams (26) and in activated sludge (8). The presence of the

larger forms of Beggiatoa in the Spartina salt marsh (trichome width up to 35 /xm) also corre-sponds to the reports by Lackey (15) and Lackey et al. (17), who reported that Beggiatoa mirab- ilis and Beggiatoa gigantea, with arbitrary tri- chome widths of 15 to 21 jbim and 26 to 55 /im, respectively (20), were found only in marine or brackish-water environments.	[redacted]	
SACH medium, with the acetate deleted and with or without cycloheximide added, gave ad-equate recovery of beggiatoas without heavy contamination by other bacteria; it was therefore used routinely as an enrichment medium from which to obtain pure cultures.	[redacted]	
Although our basic isolation procedures differ little from some of the procedures reported in the literature (5, 9, 27, 35), the wash in catalase and the blotting of the trichomes were factors which greatly enhanced isolation. By using those variations, along with optimal nutrient and agar concentrations and optimal temperature, iso-lates were routinely obtained from enrichment cultures. Strains were readily isolated by using MP medium with	[redacted]	

or without antibiotic disks. The nature of Beggiatoa nutrition has been the center of controversy for several years. Al-though Keil (13) and Bavendamm (2) reportedly cultured autotrophic strains of Beggiatoa which oxidized H2S to sulfur for energy-yielding purposes, there is some question as to the validity of theừ results (39). Kowallik and Pringsheim (14) and Pringsheim and Kowallik (29) reported the facultatively autotrophic growth of 5 of theứ 14 strains, and 2 strains which could be coaxed to autotrophic growth if pregrown mixotrophi- cally. Pringsheim later stated (28) that none of the strains could grow autotrophically but that they deposited sulfur when in autotrophic, non-growth conditions. He further stated that all of his strains requừed small amounts of organic materials for growth, which demonstrated theừ mixotrophy. Cataldi (5) and Faust and Wolfe (9) reported the isolation of Beggiatoa	[redacted]	

strains that grew heterotrophicaUy on media containing low amounts of organic nutrients. Faust and Wotfe (9) showed that theὺr strains could tolerate low levels of H2S, and they observed that the cells deposited sulfur under those conditions;

theὺr strains did not grow autotrophicaUy. Cataldi (5) attempted to grow her strains autotrophically, but she neglected to include H2S as an energy source and was unsuccessful. Scotten and Stokes (35) isolated three strains which requὺred H2S and an organic substrate such as acetate for growth, which was perhaps an indication of obligate mixotrophy by those strains.

The other two strains they studied, those supplied by Pringsheim (27), grew well in the presence of H2S, but like those of Faust and Wolfe (9) they didn't reqinre H2S for growth.

Burton and Mor- ita (3) studied a strain which deposited sulfur in the presence of H2S but which also grew heter- otrophically on acetate or other organic acids and yeast extract. We have not yet been able to grow any of our strains autotrophically.

It does not seem likely that our hay

enrich-ments, or those of Cataldi (5) or Faust and Wolfe (9), selected against autotrophic strains. The hay enrichments, with or without the addition of H2S-eminating mud, should have provided an envùronment similar to that in which beggiatoas are found, i.e., low levels of organic material, sulfide, and oxygen.

The presence of catalase in our enrichments should be a stimulus to any autotrophic strains present. Moreover, in these enrichments, Pringsheim (14, 27) and we have noted that the trichomes contained sulfur granules, and upon isolation some of Pringsheim's strains were mixotrophic.

It is possible that the isolation techniques, instead of the enrichment techniques, may have selected for the heterotrophic strains over au- totrophic strains, if the latter exist. Pringsheim (27), Faust and Wolfe (9), Maier and Murray (22), Scotten and Stokes (35), and we have all used heterotrophic media for the primary isolation of Beggiatoa from enrichment cultures. Ca- taldi (5) used a medium deficient in nutrients

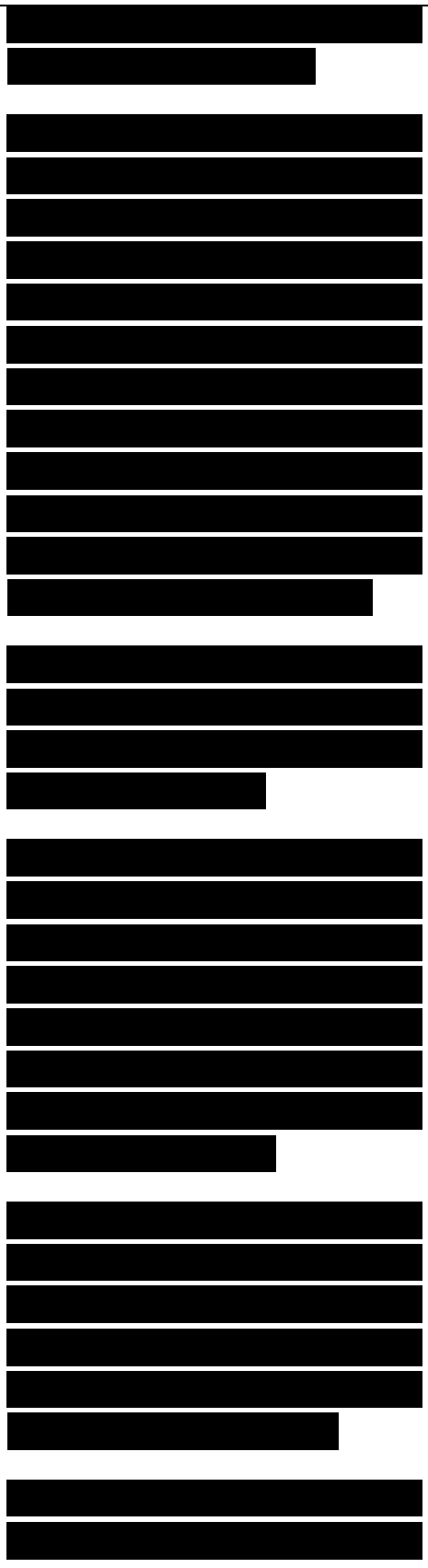


Fig. 8. Thin sections of strain B15LD grown on MP medium. (A) A sulfur granule (S), the invaginated cytoplasmic membrane (CM), and the sulfur granule membrane (SM) are seen. Bar, 1 Įun. (B) A high- magnification micrograph of the sulfur membrane

shows that it consists of three electron-dense layers (arrows). The invagination of the cytoplasmic membrane is apparent (CM). Bar, 0.1 fim.

Fig. 9. Thin section of strain B15LD grown on BP medium. The large inclusions are PHB. Bar, 1 jtim.

Fig. 10. A high magnification of strain B15LD grown on MP medium, showing the various layers of the cell envelope' the cytoplasmic membrane (CM), the peptidoglycan layer (PL), the unit membrane (UM), and the outer layer (OL). Bar, 0.1 įun.

Fig. 11. Scanning electron micrograph of strain B15LD from broth culture. The cells are bound in slime (S), and the septa (P) are poorly defined. Bar, 1 Įim.

and containing no energy source. She retrieved her trichomes after

short periods of time, relying on the endogenous metabolism of the organisms for gliding and minimal growth.

The use of the MP medium with a low (10~4%) acetate concentration, or modifications of this medium, may allow the isolation of autotrophic strains in the future, if such strains exist.

The strains of Beggiatoa studied by Cataldi (5), Faust and Wolfe (9), Scotten and Stokes (35), Burton and Morita (3), and Pringsheim (27) had trichome widths of about 1.0 to 3.0 fxm. For the most part, they were consistently shown to have similar characteristics, although the studies were limited. Faust and Wolfe's strains (9) appeared to be similar to our group c strains (Table 7) in trichome width, trichome length, sensitivity to cysteine (unpublished data), sen-sitivity to high concentrations of H2S and gen-eral morphology and growth patterns.

Scotten and Stokes's strains numbered 10 to 15 (35) were similar in size to our group A, B, c, and D strains, but our strains did not requùre H2S for growth as did strains 10 to 15, so they may have been different physiologically.

Pringsheim sep-arated his strains into eight subgroups, based mostly on trichome size, hormogonia production, nitrogen sources, and salinity sensitivity or requùrements (27) ǎ Because his groupings were based on characteristics different from the ones reported here, comparisons are hard to make Kowallik and Pringsheim (14) noted that theù strains numbered 5, 7, and 9 did not deposit sulfur in axenic culture and had trichome widths of 1.5 to 2.5 /AIĨ1. They may be similar to our group B strains, which are the same size, do not deposit sulfur, and share similar morphological characteristics with Kowallik and Pringsheim's (14) strains. Pringsheim's other strains had sev-eral characteristics that were shared by several of our strains, but none of ours appears to match identically with the strains. We also used Pringsheim's distinction of colony morphology between c type (circuitans) or L type (linguiformis) colonies (27) as a separating characteristic. Although colonies were subject to some changes due to nutritional or environmental conditions, they retained the same basic morphological formation.		

It is probable that our groups c and D and those strains studied by other investigators (3, 9, 14, 22, 27, 35) were strains of the species Beggiatoa leptomitiformis. They all had many similar characteristics, including trichome width of about 1.0 to 3.0 jiim, which corresponds closely to the 1 to 2 jxm described in Bergey's Manual (20). Our group E strains appear to constitute

Fig. 12. (A) Low- and (B) high-magnification scanning electron micrographs of strain B12LD grown on solid MP medium. There are no visible septa, and slime trails may be seen etched into the agar in (B) (arrow) Bars in (A) and (B), 10 ịun.

the species B. alba in the size (3 to 5 jLim). Further studies of the group E strains, along with similar strains from other locations, may allow us to create a well-defined species in the future. The group A strains appeared different from any of those in the recent literature, and they may, after further studies are completed, be found to constitute a new species.

The group B strains, along with Pringsheim's strains 5, 7, and 9, may be variants of B. leptomitiformis that are not able to produce sulfur granules under laboratory conditions. Because they were similar to other beggiatoas in characteristics such as resistance to and stimulation by sulfides, and in growth characteristics, they probably should not be considered as Vitreoscilla.	[redacted]	
Burton et al. (4) found that theù strain of Beggiatoa did not contain a cytochrome system, and our strains do not contain a cytochrome oxidase. Other apochloric gliding bacteria such as Vitreoscilla, Saprospira, and Leucothrix contain cytochrome systems (41). Dietrich and Big- gens (6) found that a 1.2 mM (0.078%) cyanide concentration was requùred to inhibit the electron transport to oxygen in Saprospira grandis and Vitreoscilla sp. by 73 and 88%, respectively. The sensitivity of Beggiatoa to 0ė01 to 0.05% KCN is similar to the sensitivities exhibited by these other filamentous gliding bacteria. This, plus the sensitivity of Beggiatoa to concentrations of above 0.025% azide, may indicate that some type of electron transport system is present in some beggiatoas.	[redacted]	

Moreover, other bacteria that oxidize reduced sulfur compounds, such as the thiobacilli (1), the purple and green sulfur bacteria (32), and perhaps the cyanobacteria (10), couple the oxidation to electron transport via one or more cytochromes.	[redacted]	
Our observations of the fine structure of the ceil envelope confirm those of Maier and Murray (22), who observed a cytoplasmic membrane, a dense peptidoglycan layer, a dense-light-dense membrane-like layer, and an outer layer which appeared single under heterotrophic conditions and double in the wild type. They also noticed that the outer layer did not take part in septa-tion, and this is substantiated by our thin sections and scanning electron micrographs.	[redacted]	
That the deposition of sulfur occurs external to the cytoplasmic membrane in Beggiatoa has been fairly well documented (22; J.V. Tredway, J. D. Lee, and s. D. Burton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, N69, p. 240). However, the membrane enclosing the granules in Beggiatoa has not been observed before, although Maier and Murray (22) saw a dense material attached to the inner	[redacted]	

surface of the cytoplasmic membrane around the sulfur granule.

This membrane is morphologically different from that seen in thin sections of purified sulfur granules obtained from Chromatium (24). Schmidt et alÊ (34) reported that the intact, isolated sulfur granules were composed of 93.5% sulfur, 5.2% protein, 0.55% lipid, and 0.047% bacteriochloro- phyll.

The sulfur granule membrane in Chro- matium consisted almost entirely of protein (34), and it was 2.5 to 3.0 nm thick (24).

The presence of this trilinear membrane (12 nm thick) may indicate that there is a specialized structure which functions in the oxidation and storage of sulfur compounds in Beggiatoa. This in turn would indicate that the oxidation of sulfur by Beggiatoa has a normal and beneficial physiological function, and it would differentiate the deposition of sulfur by Beggiatoa from the sulfur deposited by Sphaerotilus natans, Spirillum, and other bacteria under stressed conditions which kill them (22, 38).

Slime production and the

production of trails on the agar surface by Beggiatoa were not un- expected. Pringsheim (27) observed that Beg- giatoa and Vitreoscilla produced trails, whereas the other apochloric flexibacteria did not. The production of slime by other gliding bacteria has been well documented, and in some instances it has been associated with their gliding motility (7). …… …… ……	Sự tạo chất nhờn và sự tạo đường rãnh trên bề mặt thạch bởi Beggiatoa là điều khá bất ngờ. Pringsheim (27) thấy rằng Beg-giatoa và Vitreoscilla tạo ra các rãnh, trong khi đó các vi khuẩn apochloric flexibacteria không có khả năng đó. Việc tạo ra chất nhờn bởi các vi khuẩn khác đã được ghi nhận khá chi tiết và đầy đủ, và trong một số trường hợp, điều đó có liên quan đến chuyển động trượt của chúng (7).	