New Medium for Isolating Iron-Oxidizing and Heterotrophic Acidophilic Bacteria from Acid Mine Drainage

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A new solid medium is described for growing iron and heterotrophic bacteria from acid mine drainage (AMD). Examination of AMD from five states revealed several kinds of colonies of iron-oxidizing bacteria: (i) smooth, (ii) smooth with secondary growth sectors or branching, (iii) star-shaped, (iv) radiating lobe, and (v) flat-rough. All AMD samples yielded whitish colonies that could not use ferrous iron, sulfur, or hydrogen, nor could they grow on nutrient agar, brain heart infusion agar, or Trypticase soy agar. Glucose and sucrose supported growth if the sugar-salts medium was at pH 3.0. The new iron medium has several advantages over others: (i) easy preparation, (ii) rapid growth, (iii) larger colonies, (iv) differentiation of colony morphology, and (v) detection of a new group of heterotrophic acidophilic bacteria.

Several media have been developed to culture iron-oxidizing bacteria on a solid surface. Colmer et al. (1) used filter-sterilized natural acidic mine water and 3% agar. Leathen et al. (3) developed an inorganic medium by using silica gel in combination with inorganic salts in isolating Ferrobacillus ferrooxidans. Kinsel (2) prepared a medium containing inorganic salts and Freeda agar for isolating a sulfur-oxidizing iron bacterium, F. sulfooxidans. Tuovinen and Kelly (6) used Japanese agar as the gelling agent in their iron-salts medium for testing growth of iron-oxidizing cells on various membrane filters.

All these media supported growth of iron-oxidizing bacteria, although it appears that the colonies were small, slow-growing, and undifferentiated. Also, the preparation of silica gel medium can be time consuming and unpredictable. There is a need for an easily prepared, reliable medium capable of differentiating colony types of iron-oxidizing bacteria. This report describes (i) the preparation of such a medium for iron oxidizers, (ii) the types of colonies that several mine drainage samples have yielded, and (iii) the types and nature of several apparently heterotrophic isolates that also grow on the medium.

MATERIALS AND METHODS

Mine drainage samples. Five different samples of acid mine drainage (AMD) were studied. Most of the samples were obtained from field personnel of the U.S. Environmental Protection Agency through the Industrial Waste Treatment Research Laboratory.

A brief description of each of the samples is given in Table 1.

Growth of iron-oxidizing bacteria. Samples were shipped unrefrigerated in plastic containers. The reaction of the sample was determined and the presence or absence of a precipitate was noted. The sample was used as a 10% inoculum into 9K medium of Silverman and Lungren (5). Each culture was incubated at ambient temperature in Erlenmeyer flasks on a rotary shaker at 200 rpm until the yellow medium was oxidized to a reddish-brown color. Cultures were either streaked directly on iron-salts-purified agar (ISP), to obtain isolated colonies, or serially diluted (10-fold) in salts solution at pH 3.0, and 0.1 ml of each dilution was spread with a bent glass rod on the ISP medium. Inoculated plates were incubated at 28 C and examined daily for orange-brown colonies. No percentage of recovery was determined with the new medium.

Media. ISP medium is prepared by combining three separately sterilized solutions: (solution A) FeSO₄·7H₂O (33.4 g/liter) at 300 ml adjusted to pH 2.5 with 6 M H₂SO₄, stirred until almost colorless, filtered sterilized, and brought to ambient temperature before use; (solution B) basal salts [6.0 g of (NH₄)₂SO₄ per liter, 0.2 g of KCl per liter, 1.0 g of MgSO₄·7H₂O per liter, 0.02 g of Ca(NO₃)₂ per liter] at 550 ml adjusted to pH 3.0 and autoclaved at 121 C for 15 min in a 1-liter flask; (solution C) Purified Agar L28 (7.0 g, Oxoid product of Flow Laboratories, Rockville, Md.) added to 150 ml of distilled water, soaked for 15 min, and autoclaved at 121 C for 15 min in a 1-liter flask. Solutions B and C were removed from the autoclave and allowed to cool for 5 min at ambient temperature, and solution B was added to C with gentle mixing. Solution A was added to this combination and mixed well. The mixture then was poured into petri dishes to about one-half the depth of the bottom dish.

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Table 1. Samples used

| Sample name | Location | pH | Ferric iron precipitate |
|-------------|----------|----|-------------------------|
| Borehole AMD | Christopher Coal Co. mine no. 93 in W. Va. | 2.87 | Yes |
| Utah AMD | Copper leaching operation of Kennecott Copper Corp., Salt Lake City, Utah. | 2.50 | Yes |
| Horton AMD | Obtained by Appalachian permit no. 2442-70s no. 1 near Horton, Ky. | 2.50 | No |
| Pennsylvania AMD | Drainage of Kittanning Run near Altoona, Pa. | 2.80 | Yes |
| Lake Hope AMD | Mine complex 47 | 2.68 | Yes |

AMD samples were cultured in the iron broth medium 9K of Silverman and Lundgren (5). The use of elemental sulfur as an energy source was tested by adding 100 ml of sterile basal salts of medium 9K to 1 g of sterile precipitated sulfur. The sulfur was sterilized by autoclaving with flowing steam for 1 h on each of 3 successive days. Common laboratory media, such as nutrient agar, brain heart infusion agar, and Trypticase soy agar, were commercially supplied. A glucose-salts-yeast extract (GSYE) agar was developed for growing and maintaining the heterotrophic acidophiles observed on ISP medium from the AMD. GSYE agar was prepared by combining the following separately autoclaved solutions: (i) 25 ml of a 20% glucose solution, (ii) 750 ml of the salts-yeast extract solution (1.0 g of \((\text{NH}_4\text{)}_2\text{SO}_4\) per liter, 0.1 g of KCl per liter, 1.0 g of \(\text{K}_2\text{HPO}_4\) per liter, 0.2 g of \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) per liter, and 0.5 g of yeast extract per liter; the solution is adjusted to pH 2.8 with 6 M sulfuric acid), and (iii) 225 ml of distilled water containing 15.0 g of agar (Difco). The same size flask used for the salts solution should also be used for the agar solution, and solution (ii) should be added to solution (iii) after autoclaving. Solution (i) is added to the salts-yeast extract-agar solution to complete the medium. Yeast extract is not essential for growth of the heterotrophic acidophiles and may be eliminated to give a glucose-salts minimal agar medium that still yields good growth. A GSYE broth may be prepared by adding solution (i) to 975 ml of solution (ii). Autotrophic use of hydrogen by the heterotrophic-like isolates was tested under an atmosphere of 55% hydrogen, 24% nitrogen, 5% oxygen, and 5% carbon dioxide using a medium prepared by combining autoclaved solutions of 75 ml of the salts solution of GSYE medium (minus the yeast extract) at pH 3.0 plus 25 ml of distilled water containing 0.7 g of purified agar. Incubation was at 30°C.

**RESULTS**

Types of iron colonies. The AMD samples from five different states revealed several types of colonies on ISP medium. The most frequently observed type is generally circular (angular colonies have been observed) with an entire margin, a smooth surface, and a papilla-like protrusion in the center (Fig. 1). Growth begins with a very small clear colony and subsequent deposition of yellow-orange iron oxide in the center. In time all of the colony becomes orange and then dark brown with a yellow-orange band just outside the brown colony. An interesting feature of some smooth colonies is the development of sectors that emanate from a point within the colony. Figure 2A shows a smooth colony with several fan-like sectors. Sectors seem to enlarge by waves of growth that extend from one side of the sector to the other (arrow Fig. 2A). The growth of sectors may completely engulf a colony (Fig. 2B). When this stage has been reached a band of almost transparent growth may surround all or part of the colony. The band turns whitish nearest the colony and may contain hair-like projections. The white part of the band will turn orange and then brown (Fig. 3A). The wave-like nature of the sectors is more evident in this photomicrograph. Figure 3B shows a branched smooth colony to the left of the photomicrograph, which is just starting to lay down the hair-like projections (bottom). Part of the colorless band of growth can also be seen (arrow). An older colony that has become surrounded by the growth of the hair-like projections is at the right of the photomicrograph. Another aspect of the secondary growth of some smooth colonies is the formation of fine whitish spreading growth coming from a portion of the colony. Figure 3C shows a branched colony with white flat growth spreading away from the colony. As this growth proceeds away from the colony, it is nearly transparent (arrow), but aging turns it white with a granular appearance that becomes orange and

**FIG. 1. Smooth type colony of ISP medium showing papilla in center (white spots) and yellow-orange area surrounding colony (shown as white). Bar indicates 0.4 mm.**
then brown. The colorless band of growth surrounding the colony is visible.

Another type of colony observed is roughly star-shaped and has four to five arms extending from its center (Fig. 4A). The colony seems to be in the plane of the surface of the agar. A form of secondary growth may also occur with star-shaped colonies (Fig. 4B). A part of one of the arms of the star (arrow) is still visible.

Two forms of what might be described as a radiating lobe colony are shown in Fig. 5A and B. These colonies contain four to six radiating lobes or arms of growth derived from the center of the colony.

The fifth type of iron colony observed is designated a flat-rough variety. Figure 6A is an example of a young flat-rough colony. Growth occurs with an irregular outward spreading of a thin, grey-white, sheet-like mass of cells. When growth starts, the cell mass is almost transparent. As the cell mass becomes larger, the older cells in the center turn a yellow-orange then brown. Figure 6B shows a later stage of growth; the surface is rough and most of the colony is dark brown.

Iron broth (9K medium) cultures of AMD samples may be streaked on ISP medium to obtain isolated iron colonies. A freshly oxidized iron broth culture streaked onto ISP medium will usually yield macroscopically visible oxidized (yellow-orange in color) areas of growth in 3 to 4 days at 28°C incubation. The colonies are very small at this stage, but can easily be viewed with a dissecting microscope. More colonies will appear daily and almost all cells capable of growing on the medium will be visible after about 1.5 weeks of incubation. Colonies never become very large, and only some reach 2 to 3 mm after 4 to 5 weeks of incubation. Dilutions of iron broth cultures in salts solution and spread on ISP medium will not show colonies for about 10 to 12 days. Thickly poured plates (about one-half or more depth of petri dish) may be incubated and will continue to produce growth for up to 7 weeks at 28°C. Iron colonies are hard masses of oxidized iron, and subculturing requires removal of the entire colony. The colony is transferred to the glass wall of a flask or screw-cap tube containing 9K medium and crushed against the wall with a glass stirring rod (ca. 8 mm in diameter). The 9K medium is used to wash the crushed colony from the wall of the vessel. The flask or tube should be incubated on a rotary shaker at 28°C, but incubation on a shaker at ambient temperature will yield an oxidized culture. Incubation should be continued for about 2 weeks if the medium has not become orange-brown to red-brown before then. A tube-shaking device (4) on a rotary shaker was used to cultivate iron cells in small quantities (5 ml) of 9K medium.

Examination of AMD samples. AMD from five states was examined to determine the types of colonies ISP medium would yield. Both the borehole (W. Va.) and Utah AMD samples produced all of the kinds of iron colonies described above. The Kittanning Run (Pa.), the Hortan (Ky.), and the Lake Hope (Ohio) AMD samples yielded only smooth colony types.

Non-iron-oxidizing colonies. All of the AMD samples examined in this study, as well as four of the others, have yielded grey-white, non-iron-oxidizing colonies. The colonies have different morphologies (Fig. 7B, C, D, and E), and contain cells that are gram-negative short rods. Twenty-one isolates were obtained from several AMD samples. All can utilize glucose and will grow well on GSYE agar. They did not grow on ferrous iron (9K medium), sulfur, hydrogen, nutrient agar, brain heart infusion agar, or Trypticase soy agar. Adjustment of the reaction of the latter three conventional media to pH 4.0 did not yield growth, nor did GSYE broth at pH 6.9. Growth occurs on glucose or sucrose (0.5%) basal salts medium at pH 3.3.

Some of the grey-white colonies produce clear...
Fig. 3. Examples of secondary growth. (A) Old iron colony showing many sectors and granular type of growth (grey area). Note wave-like growth. Bar indicates 0.8 mm. (B) Left: Branched sectored colony with developing hair-like projections and clear margin (arrow). Right: Older colony with hair-like projections and clear margin. Bar indicates 0.5 mm. (C) Greatly branched and sectored colony with surrounding clear margin and spreading sheet-like growth (arrow). Bar indicates 0.4 mm.

Fig. 4. Star-shaped colonies. (A) Star-shaped colony in plane of agar. A small bulb-like growth is in center of colony (white spot). Bar indicates 0.5 mm. (B) Star-shaped colony almost covered with secondary growth. One of the arms of the colony is still visible (arrow). Bar indicates 0.6 mm.
areas when the background is oxidized iron from growing iron colonies. Figure 7A shows two clear areas (arrows) around non-iron-oxidizing colonies in a background of yellow-orange (appears white in the figure) iron oxidation. The black spots are iron-oxidizing colonies.

**DISCUSSION**

A solid medium for the growth of iron-oxidizing cells is presented that seems to be superior to other media in several ways: (i) easy preparation, (ii) rapid growth, (iii) larger colonies, (iv) differentiation of colony morphology, and (v) detection of a new group of heterotrophic acidophilic bacteria. The ground work

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**Fig. 5.** Radiating lobe colonies. (A) Four colonies with various numbers of asymmetrical lobed arms. Bar indicates 0.6 mm. (B) Highly symmetrical six-lobed colony. Bar indicates 0.4 mm.

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**Fig. 6.** Stages in the development of flat-rough colonies. (A) Early stage of almost transparent cell mass. Whitish area in center is yellow-orange. Bar indicates 0.4 mm. (B) Mature flat-rough colony with dark brown granular surface, an outer edge of orange and a clear margin surrounding the colony. Bar indicates 0.4 mm.
for the development of ISP medium was done some years ago (H. L. Manning, M.S. thesis, Syracuse Univ., Syracuse, N.Y., 1964); refinements were necessary to ensure the reliability and predictability of the medium. Agar is thought to inhibit the growth of iron oxidizers and was the reason Leathen et al. (3) used silica gel to isolate *F. ferrooxidans*. Noble agar (Difco) and Freeda agar were tested in an iron-salts medium but were found unsatisfactory. Purified agar is the preferred agar because it produces a firm gel at low concentration (0.7%) and allows good growth of iron-oxidizing cells. To obtain gellation of the medium, the agar must be autoclaved in distilled water separately from the acidic (pH 3.0) salts solution. Autoclaving the agar in the acidic salts destroys the capacity of the medium to form a gel. The basal salts

**Fig. 7.** Non-iron-oxidizing colonies. (A) Clear zones (arrows) around grey-white colonies growing in area of oxidized iron on ISP medium. White region is oxidized iron caused by many iron colonies (black colonies). Bar indicates 1.1 mm. (B) Smooth-surfaced colonies grown on GSYE agar. Bar indicates 0.4 mm. (C) Flat-surfaced colony on GSYE agar. Bar indicates 0.5 mm. (D) Convoluted colony on GSYE agar. Bar indicates 0.5 mm. (E) Advanced stage of donut-shaped colony on GSYE agar. Bar indicates 0.4 mm.
solution of ISP medium is a modification of the salts used by Silverman and Lundgren (5) in their 9K medium. The major change involved the elimination of the potassium phosphate. The addition of even half the amount (0.025%) of the potassium phosphate in 9K medium (0.05%) is sufficient to prevent the growth of iron colonies. Apparently, the purified agar supplies enough phosphorus to support growth of the cells. The third solution of ISP medium contains the oxidizable energy source ferrous iron. The amounts of ferrous iron usable in ISP medium (1,000 or 2,000 μg/ml) are considerably less than that found in the widely used iron broth 9K medium (9,000 μg/ml).

The iron colonies on the media of other investigators resembled some colonies of this study, although only one type was described by each. The lobed colonies described by Colmer et al. (1) appear to be a form of secondary growth. The occurrence of secondary growth in colonies of iron bacteria seems to be both characteristic and unique and takes three forms: (i) sectors, (ii) granulation and formation of hair-like projections, and (iii) spreading of a sheet-like mass of cells. No other instances of these properties with other bacteria can be recalled. It is difficult to speculate what initiates the rapid growth of certain cells in a colony since secondary growth does not occur to all smooth or flat-rough colonies. Sectors seem to occur primarily in smooth colonies and spreading masses of cells from flat-rough colonies. Sectors appear to begin from a point source, i.e., from one or a few cells in a smooth colony. The point source is either from the center of a smooth colony (predominance of older cells) or the edge of a rough surface of a formerly smooth colony. They occur only with older colonies. Most sectors have an internal wave-like structure that resembles growth rings of a tree and seems to be the method of growth of the sector. It is tempting to speculate that these rings may correspond to the generation time of the cells.

The clear zones around some grey-white colonies that are growing in an area of oxidized iron (caused by adjacent colonies of iron bacteria) appear to be a dissolution of the oxidized iron by excretions of the colony. The excretions may have an acid reaction since acid will dissolve iron complexes, and growing cells of these colonies lower the pH (from 3 to 2) of glucose-salts medium. Exactly what the colonies are using for growth on ISP medium is not known, but several subcultures have been made on medium containing only distilled water and purified agar at pH 3.0. Their relationship to iron oxidizers is unknown, but may be symbiotic since cells increase in number during the oxidation of 9K medium by AMD. It is felt they are a new group of bacteria that requires a special type of solid medium to be detected. Much more work is needed to properly characterize and classify them. A separate report will cover the physiology of two strains of this new group.

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