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Detection of *Thiobacillus ferrooxidans* in Acid Mine Environments by Indirect Fluorescent Antibody Staining

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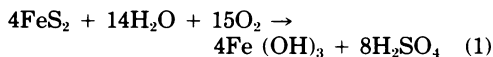
An indirect fluorescent antibody (FA) staining technique was developed for the rapid detection of *Thiobacillus ferrooxidans*. The specificity of the FA stain for *T. ferrooxidans* was demonstrated with both laboratory and environmental samples. Coal refuse examined by scanning electron microscopy exhibited a rough, porous surface, which was characteristically covered by water-soluble crystals. Significant numbers of *T. ferrooxidans* were detected on the exterior refuse surface by FA staining, whereas none were detected in the refuse pores. A positive correlation between numbers of *T. ferrooxidans* and acid production in coal refuse in the laboratory was demonstrated with the FA technique.

Among the natural sources of energy available in the United States are the vast coal fields throughout the Appalachian region. The mining of this coal presents a number of problems, among which is the production of acid mine drainage. It has been estimated that mine drainage contributes 4 million tons of sulfuric acid per year to 11,000 miles of waterways (17) in addition to high concentrations of dissolved iron and other metal ions. These pollutants result primarily from the oxidation of pyritic minerals that have been exposed to oxygen and water. The pyritic materials occur naturally in several forms, with the chalcopyrite and iron pyrite being two of the most prevalent.

In iron pyrite (FeS_2), both iron and sulfur are in the reduced state and, consequently, both are oxidizable. The oxidation of iron pyrite in the presence of water results in the production of sulfuric acid and ferric hydroxide in accordance with equation 1.



$$4FeS_2 + 14H_2O + 15O_2 \rightarrow 4Fe(OH)_3 + 8H_2SO_4 \quad (1)$$

In the environment, these oxidative processes occur in coal refuse, which is composed of shale, clay, low-grade coal, and the pyritic minerals found in intimate contact with coal. Due to this close association, the pyritic materials are mined along with the coal, separated, and piled on the surface as part of the refuse. The water that characteristically drains from refuse often has a pH of less than 3 and results in formation of a yellow-brown ferric hydroxide precipitate known as "yellow boy," which is deposited on stream beds. An influx of this type of drainage into a normal waterway can drastically alter the ecology of the waterway (3, 10, 14, 15, 19, 28).

Although the reaction shown in equation 1 will occur abiotically, it has been shown in the laboratory that acidophilic species of *Thiobacillus*, particularly *Thiobacillus ferrooxidans*, are capable of oxidizing reduced iron and sulfur with concomitant production of acid and ferric hydroxide (16) at rates far in excess of the abiotic rates (25). Furthermore, acidophilic thiobacilli appear to be the predominant bacteria in acid coal mine drainage (27). Thus, the question of the relative importance of chemical versus bacterial oxidation of pyrite in the environment arises, and it becomes pertinent to evaluate the role of *Thiobacillus* in acid production, because it may be possible to inhibit the metabolic production of acid produced by acidophilic thiobacilli (9).

Belly and Brock (2) developed a technique for evaluating $^{14}CO_2$ fixation in coal refuse and indirectly equated the CO_2 fixation with chemoautotrophic activity of acidophilic thiobacilli in coal refuse. Since this technique measures total $^{14}CO_2$ uptake, it is impossible to equate CO_2 fixation directly with numbers of acid-producing species of bacteria, because many bacteria are present in refuse that are incapable of oxidizing either Fe^{2+} or S^{2-} (27) but may be capable of fixing CO_2.

However, fluorescent antibody (FA) staining techniques, originally developed by clinical immunologists (7, 8), have been used by microbial ecologists to both identify and quantitate a variety of microorganisms in the environment. Schmidt et al. (20, 21) have used FA staining to study fungi in soil, whereas other researchers

have used the FA technique to study bacteria in both soil and water (4, 11, 13, 18). FA staining also has been used in conjunction with autoradiography to individually identify metabolically active bacteria (12).

To our knowledge, the FA technique has not as yet been applied to the study of acidophilic thiobacilli in coal refuse.

This paper describes the development of an indirect FA stain, specific for *T. ferrooxidans*, and the use of this technique to detect both the presence of, and to evaluate the role of, this bacterium in acid production in coal refuse.

MATERIALS AND METHODS

Culture methods. *T. ferrooxidans* were cultivated under forced aeration in the "9K" medium previously described by Silverman and Lundgren (24). After 5 days of incubation at 23 ± 2°C, cells were harvested using a Sharples supercentrifuge (The Sharples Corp., Philadelphia, Pa.) at 43,000 × *g*. The cell paste was suspended in pH 3 H_2SO_4 and allowed to stand overnight at 4°C to precipitate residual iron. The cells were separated by centrifugation for 15 min at 1,000 × *g* in a Sorvall RC2-B centrifuge to further remove residual iron and were stored in pH 3 H_2SO_4 at 4°C until needed.

Immunological methods. *T. ferrooxidans* cells to be used as an antigen source were washed three times in 0.85% saline solution and suspended in Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) to a density of 2×10^{-3} g of cells (dry weight) per ml of Hanks balanced salt solution. This cell suspension then was mixed 1:1 with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Equal quantities of the adjuvant-cell emulsion were injected subcutaneously at four sites into 4.5-kg white New Zealand rabbits so that each rabbit received 5 ml of the preparation. The injections were repeated after 14 and 21 days, and on day 28 the rabbits were bled by cardiac puncture. The titer of the antibody directed against *T. ferrooxidans* was determined by complement fixation tests (6). Normal rabbit serum served as a control and to confirm that no antibody against *T. ferrooxidans* was present in normal rabbit serum. In all instances, the titers of the immune sera were greater than 1:500 and were considered satisfactory. The immunoglobulin G (IgG) portion of the pooled antisera was separated using the method described by Baumstark et al. (1) and concentrated to approximately 25 mg of protein per ml with a continuous-flow beaker 80 hollow fiber device (Bio-Rad Laboratories, Richmond, Calif.). The concentrated IgG was divided into 10-ml aliquots and stored at −20°C until needed.

At the time of use, aliquots of IgG were thawed and cross-adsorbed with whole-cell preparations of *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Thiobacillus novellus*, and *Escherichia coli* until cross-reactivity was removed. The IgG then was diluted with phosphate buffer (1.5 g of KH_2PO_4 plus 9.1 g of Na_2HPO_4 per liter adjusted to pH 7.4 with 1 N NaOH) to the greatest dilution that would give +4 fluorescence (defined below) when staining pure cultures of *T. ferrooxidans*. This adsorbed IgG was tested for cross-reactivity with 23 isolates of bacteria (Table 2). Table 1 lists homologous control reactions of anti-*T. ferrooxidans* IgG.

FA staining procedures. Environmental samples examined by using the FA technique were prepared by heat-fixing the samples to glass microscope slides. A 0.05-ml volume of bovine serum albumin (BSA) at a concentration of 20 mg/ml of 0.1 M phosphate buffer (pH 7.4) or a commercially prepared rhodamine isothiocynate (RITC)-conjugated BSA (Microbiological Associates, Bethesda, Md.) was applied to the smear, which was placed in a humidity jar for 30 min. The smear then was thoroughly washed with 0.1 M phosphate-saline buffer (8.5 g of NaCl per liter of phosphate buffer), and 0.05 ml of the working dilution of anti-*T. ferrooxidans* IgG was applied. The smear was incubated as previously described, after which it was again washed with phosphate-saline buffer. A 0.05-ml volume of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Microbiological Associates, Bethesda, Md.) was applied to the smear. After another 30 min of incubation and washing with this phosphate-buffered saline, the smear was blotted, and a drop of phosphate-saline-buffered glycerol (1:9) was applied. The smear was covered with a cover slip and examined with a Zeiss Universal microscope equipped for ultraviolet (UV) epiillumination. The intensity of the fluorescence was classified from (−) to +4, with (−) representing no visible fluorescence and +4 indicating intense fluorescence.

Scanning electron microscopy. Coal refuse samples were fixed to specimen stubs, gold coated, and shadowed at a 20° angle. The specimens then were viewed at various magnifications in a Mini scanning electron microscope (International Scientific Instruments, Palo Alto, Calif.).

Examination of refuse by FA staining. Individual pieces of coal refuse were thoroughly washed with pH 3 H_2SO_4 solution. The supernatant from these washings were decanted aseptically and then

TABLE 1. *Results of controls testing the specificity of the goat anti-T. ferrooxidans IgG*

Antigen	Reagent for:		Result
	Step 1	Step 2[a]	
T. ferrooxidans	Saline	FITC-conjugated goat anti-*T. ferrooxidans* IgG	(−)
T. ferrooxidans	Normal serum	FITC-conjugated goat anti-*T. ferrooxidans* IgG	(−)
T. ferrooxidans	Adsorbed specific antiserum	FITC/conjugated goat anti-*T. ferrooxidans* IgG	(−)
T. ferrooxidans	Specific antiserum	FITC-conjugated goat anti-*T. ferrooxidans* IgG	+4

[a] FITC, Fluorescein isothiocyanate.

TABLE 2. *FA staining reactions of a variety of bacterial isolates*

Isolate[a]	FA staining reaction[b]
1. *Alcaligenes faecalis* (9)	–
2. *Bacillus megaterium* (125)	–
3. *Enterobacter aerogenes* (7)	–
4. *Escherichia coli* (455)	–
5. *Flavobacterium suaveolans* (40)	–/+
6. *Micrococcus flava* (190)	–
7. *Pseudomonas aeruginosa* (167)	–
8. *Salmonella enteritidis* (218)	–
9. *Serratia marcescens* (234)	–
10. *Shigella flexneri* (82)	–
11. *Streptococcus faecalis* (48)	–
12. *Thiobacillus ferrooxidans* (207)[c]	+4
13. *Thiobacillus ferrooxidans* (207)[d]	+3
14. *Thiobacillus novellus* (407)	–
15. *Thiobacillus thiooxidans* ATCC 19377	–
16. *Xanthomonas campestris* (108)	–/+
17. *Pseudomonas* (229)	–
18. Gram (+) heterotroph	–
19. Gram (–) heterotroph 1	–
20. Gram (–) heterotroph 2	–
21. Gram (–) heterotroph 3 22. Gram (–)	–
heterotroph 4	–
23. Gram (–) heterotroph 5	–

[a] Isolates 17 through 23 were isolated directly from pH 2.8 acid coal mine drainage and partially characterized. Numbers in parentheses are Ohio State University culture collection numbers except where otherwise designated.

[b] Staining reactions were classified from (–) to +4, with (–) being no detectable staining and +4 being intense staining. –/+ denotes very slight staining.

[c] Grown with iron energy source ("9K" medium).

[d] Grown with reduced sulfur energy source ("9K" salts and reduced S).

concentrated by centrifugation. After being washed, the undissolved refuse was ground in a mortar with a pestle, and the resulting powder was further disrupted by ultrasound (Branson Sonic Power) in an attempt to release any *T. ferrooxidans* that may have been present in the internal refuse pores (see Fig. 11). The powders that resulted after ultrasound exposure were thoroughly washed with pH 3.0 H_2SO_4 and decanted. The decanted liquid was centrifuged at 10,000 × *g* to concentrate any suspended cells, which were then resuspended into a total volume of 0.1 ml. A 100-μl amount of this suspension was placed on Bellco antibody plaque assay slides (Bellco Glass, Inc., Vineland, N.J.), allowed to air dry, and heat fixed. A control containing *T. ferrooxidans* was prepared in a similar manner. The smears were stained by the previously described staining technique, and the number of cells per field was enumerated.

Comparison of acid production versus *T. ferrooxidans* numbers in refuse. Two-hundred-gram portions of refuse, which had been washed by pouring 10 liters of distilled water over the refuse on a Buchner funnel, were placed in each of six 2-liter Erlenmeyer flasks, and 1 liter of distilled water was added to each flask. Three of the flasks were autoclaved for 45 min at 20 lb/in^2 and served as sterile controls. The six flasks were incubated as standing cultures at 23 ± 2°C. Samples were periodically removed from each of the flasks and examined both for numbers of *T. ferrooxidans* by the above described staining technique and for titratable acidity.

RESULTS

The specificity of the FA stain when processed with 23 isolates of bacteria is shown in Table 2. The stain cross-reacted very slightly with only two species, while giving +4 fluorescence with iron-grown *T. ferrooxidans* and +3 fluorescence with sulfur-grown *T. ferrooxidans*. The FA stain did not react with *T. thiooxidans*, another acidophilic *Thiobacillus* commonly found in the acid mine environment.

Both nonspecific fluorescence (i.e., the adsorption of fluorescent-conjugated compounds by mechanisms other than immunological reactions) and autofluorescence (i.e., the natural fluorescence of compounds upon exposure to UV illumination) proved to be a problem in the acid mine samples. It had been previously reported that an RITC-conjugated gelatin counterstain could be used to suppress background fluorescence (5). BSA was found to be preferable to gelatin during this investigation, since BSA is less viscous. Also, RITC-conjugated BSA was commercially available, whereas RITC-conjugated gelatin was not. It was found that unconjugated BSA was sufficient when only nonspecific fluorescence was a problem, but RITC-conjugated BSA was necessary to combat autofluorescence. Fluorescein isothiocyanate-stained cells were readily apparent against both the dark background provided by the unconjugated BSA and the red background provided by the RITC-conjugated BSA.

Figures 1 through 6 are photomicrographs that demonstrate the specificity of the FA stain for *T. ferrooxidans*. Figure 1 is a phase-contrast photomicrograph of a smear containing a mixture of *T. ferrooxidans*, *Pseudomonas* 229, *E. coli*, *Salmonella enteritidis*, and *A. faecalis*. Figure 2 is the same field as in Fig. 1 but was photographed under UV illumination. The *T. ferrooxidans* in the field fluoresced brightly. Figure 3 is a phase-contrast photomicrograph of acid strip mine spoil which had been FA stained, whereas Fig. 4 shows the same field as Fig. 3 under UV illumination. The *T. ferrooxidans* cells in the field were easily detectable. Figure 5 is a phase-contrast photomicrograph of

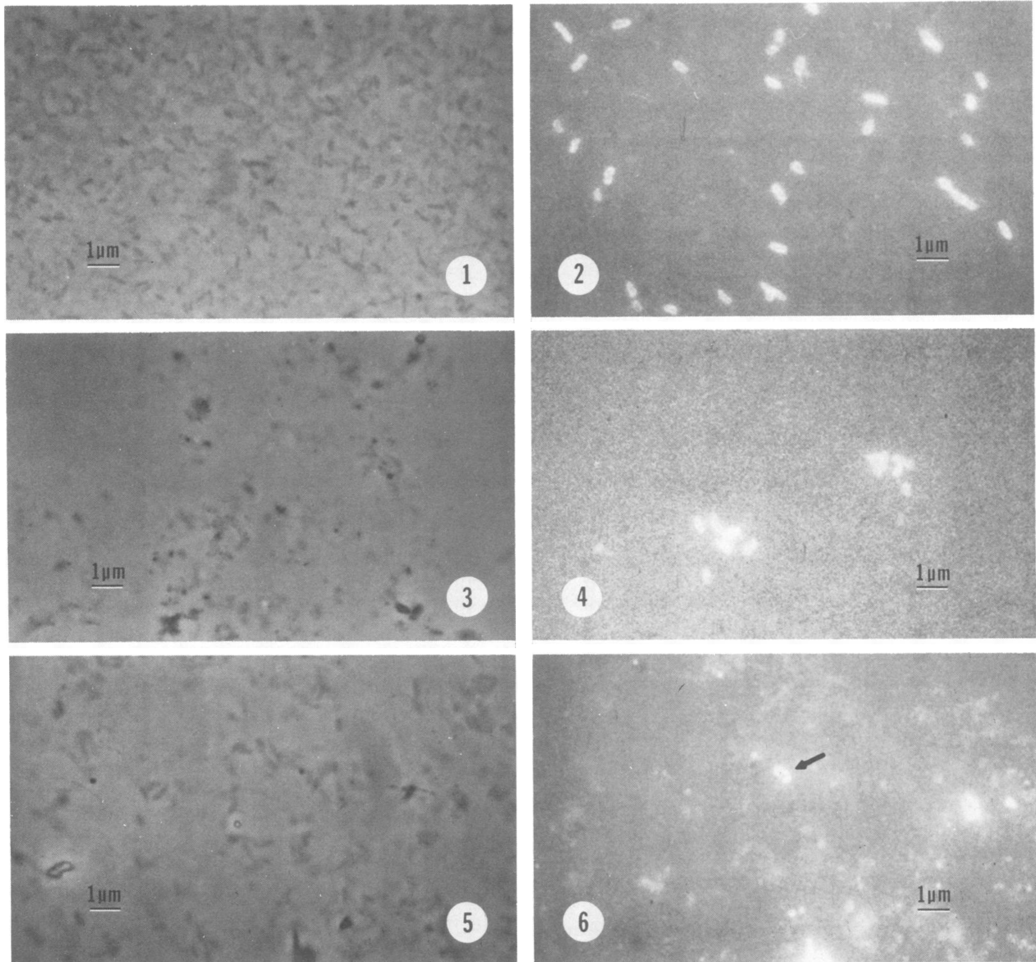


FIG. 1. *Phase-contrast photomicrographs of an FA-stained mixed culture of five different gram-negative bacteria, including T. ferrooxidans.*

FIG. 2. *Photomicrographs showing the same field as Fig. 1 under UV illumination.*

FIG. 3. *Phase-contrast photomicrograph of FA-stained acid strip mine spoil.*

FIG. 4. *Photomicrograph showing the same field as Fig. 3 under UV illumination.*

FIG. 5. *Phase-contrast photomicrograh of an FA-stained sample of acid copper mine drainage from Shasta County, Calif.*

FIG. 6. *Photomicrograph showing the same field as Fig. 5 under UV illumination. (Bright spots appear red in color photomicrographs, due to RITC-conjugated BSA, whereas the T. ferrooxidans cell appears yellow-green due to fluorescein isothiocyanate-conjugated IgG.)*

FA-stained bacteria present in acid copper mine drainage mailed to the laboratory from Shasta County, Calif. When the field in Fig. 5 was examined under UV illumination (Fig. 6), only one *T. ferrooxidans* cell was apparent.

Coal refuse viewed in a scanning electron microscope at relatively low magnifications was shown to superficially possess a rough, irregular surface (Fig. 7 and 8). At a higher magnification it became apparent that the refuse was covered by crystals (Fig. 9 and 10), which proved to be water soluble. When the soluble crystals were washed away with water, a number of pores were noted in the irregular refuse surface (Fig. 11). The size of *T. ferrooxidans*, shown for comparison in Fig. 12, would have allowed penetration of the pores by these cells.

Appreciable numbers of *T. ferrooxidans* were detected in the surface washings of the refuse, but none were detected in refuse that had been prewashed and then disrupted by grinding, fol-

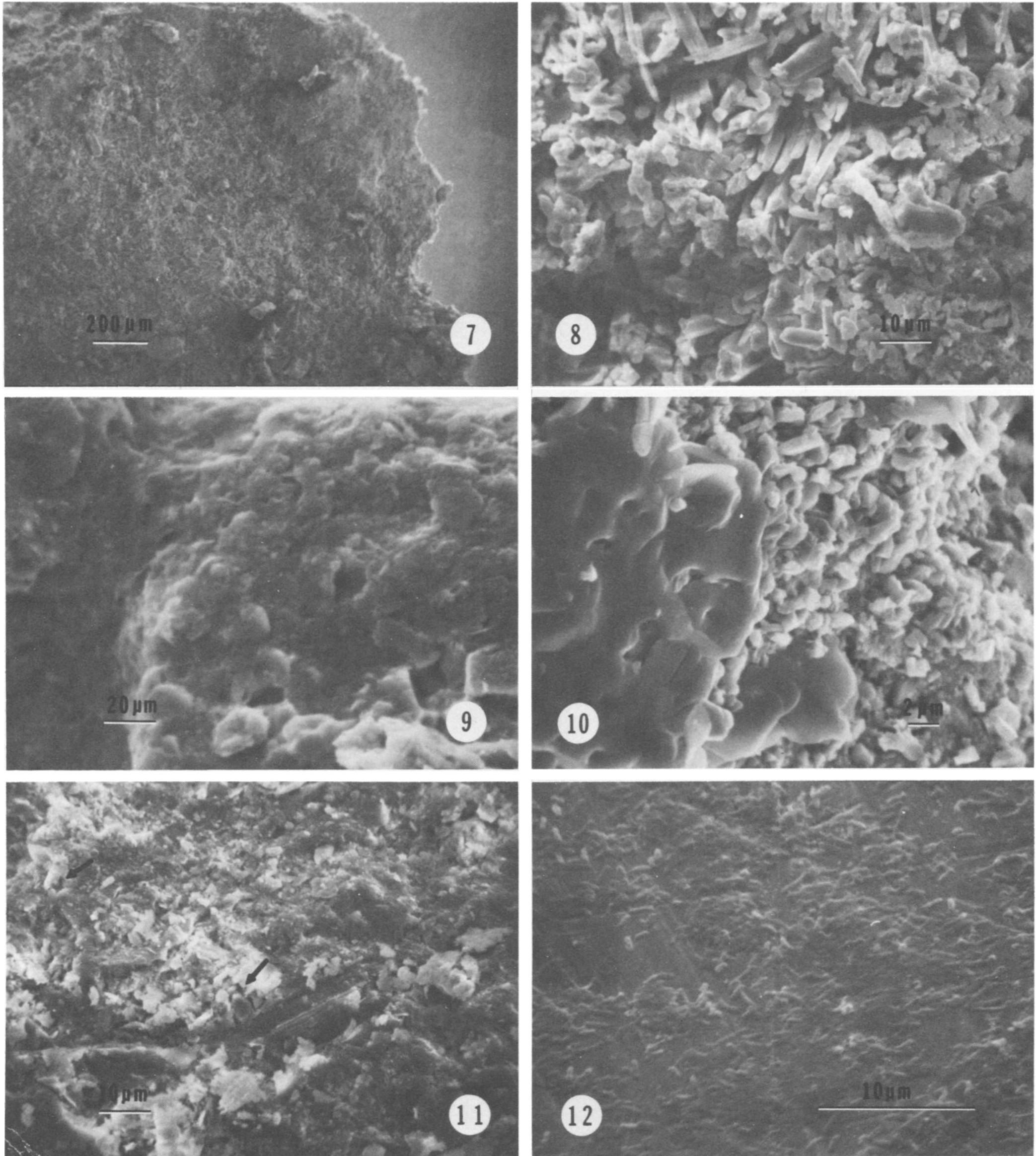


FIG. 7-10. *Scanning electron photomicrograph showing the refuse surface and associated crystal structure at various magnifications.*

FIG. 11. *Scanning electron photomicrograph showing the surface pores of washed refuse.*

FIG. 12. *Scanning electron micrograph showing the relative size of T. ferrooxidans cells in comparison to refuse pore size shown in Fig. 11.*

lowed by ultrasound. This indicates that bacteria may be present on the refuse surface but may not be present in the internal pores of the refuse materials, or that they were easily washed from the internal pores. The control demonstrated that the antigenic integrity of the *T. ferrooxidans* was unaffected by sonification.

The numbers of *T. ferrooxidans* from the refuse samples followed typical bacterial growth curves with time (a representative is shown in Fig. 13). The exponential phase of these curves corresponded to increases in titratable acidity in the unsterilized refuse samples, whereas over the same period acid production in the sterile controls was considerably less. No

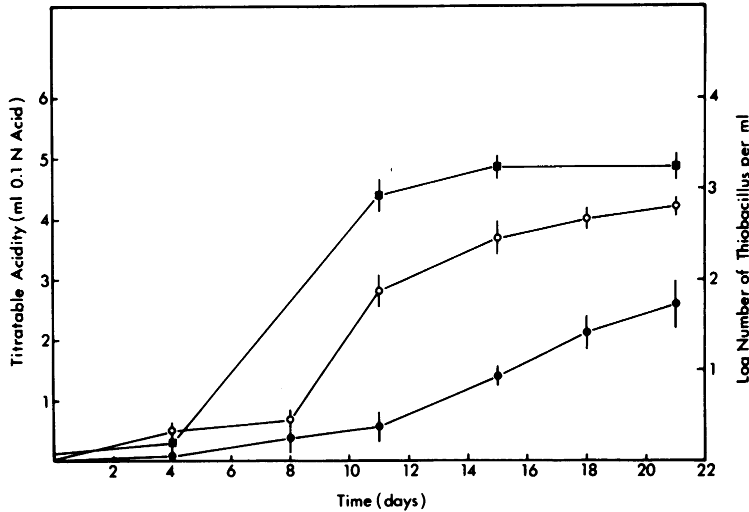


FIG. 13. *Acid production in sterilized (●) and unsterilized (○) refuse flasks versus time in days. Increase of FA-stained T. ferrooxidans in the nonsterile refuse is also plotted versus time (■).*

bacteria were detected in the sterile control samples during the course of the experiment.

DISCUSSION

The correlation between the acid production rate and the exponential growth phase of *T. ferrooxidans* in refuse suggests that bacteria are the dominant stimulus leading to acid formation from coal refuse in the environment. Refuse piles may be considered to be continuous-flow situations in which the population of *T. ferrooxidans* is constantly in a growth phase. This view is consistent with the report of Singer and Stumm (25), which indicated that the rate-determining step in the oxidation of pyrite, and the subsequent formation of acid, is the oxidation of ferrous to ferric iron. Smith et al. (26) reported that a high ferric-to-ferrous iron ratio is essential for high rates of acid production in refuse. When considering these factors, it can be concluded that *T. ferrooxidans* acts as a catalyst that accelerates the oxidation rate of ferrous to ferric iron and that pyrite is oxidized both directly via the metabolic activities of *Thiobacillus* species and indirectly via the abiotic oxidation of pyritic minerals by ferric iron, which was produced metabolically as suggested by Silverman (23). Thus, even though pyrite can be abiotically oxidized, inhibition of the metabolic activities of *T. ferrooxidans* and other acidophilic *Thiobacillus* species should decrease the rate of acid production in coal mine refuse.

The low density of *T. ferrooxidans* in the samples of acid copper mine drainage is surprising since it has been previously shown that this organism is capable of oxidizing chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), and covelite (CuS), substances reported to be the principal sources of acid in copper mine drainage (22). It is possible that either *T. thiooxidans*, which did not react with the FA stain, or an immunologically different strain of *T. ferrooxidans*, or a mixotroph such as *T. perometabolis*, is primarily responsible for acid production in the drainage tested from the copper mines. This again raises the possibility that there is more than one acidophilic iron-oxidizing *Thiobacillus* species. The above possibilities are consistent with previous work that demonstrated that numbers of acidophilic sulfur oxidizers exceed numbers of acidophilic iron oxidizers in certain environments (26). Presently, we are attempting to develop an FA stain specific for *T. thiooxidans* in response to the above considerations.

Direct FA staining of refuse particles did not reveal *T. ferrooxidans* cells directly on refuse surfaces. However, the cell density would have to be approximately 10^8 cells/g of refuse to anticipate one cell per microscope field. From the scanning electron micrographs, it was apparent that cells could also be situated behind surface crystals, etc. and thereby masked from view. Direct observation was further complicated by the high degree of autofluorescence seen in refuse, although this was controlled by the application of RITC-conjugated BSA and cells could be detected in control experiments in which relatively high nubers of *T. ferrooxidans* were added to the refuse.

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