

A Combined Immunofluorescence-DNA-Fluorescence Staining Technique for Enumeration of *Thiobacillus ferrooxidans* in a Population of Acidophilic Bacteria

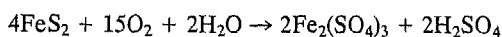
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An antiserum raised against whole cells of *Thiobacillus ferrooxidans* was allowed to react with a variety of acidophilic and nonacidophilic bacteria in an enzyme-linked immunosorbent assay and an indirect immunofluorescence assay. Both experiments demonstrated that the antiserum was specific at the species level. This preparation was used to evaluate the role of *T. ferrooxidans* in the microbial desulfurization process. Leaching experiments were performed, and the numbers of *T. ferrooxidans* cells and other bacteria were estimated by using a combined immunofluorescence-DNA-fluorescence staining technique that was adapted for this purpose. Nonsterile coal samples inoculated with *T. ferrooxidans* yielded high concentrations of soluble iron after 16 days. After this period, however, *T. ferrooxidans* cells could no longer be detected by the immunofluorescence assay, whereas the DNA-fluorescence staining procedure demonstrated a large number of microorganisms on the coal particles. These results indicate that *T. ferrooxidans* is removed by competition with different acidophilic microorganisms that were originally present on the coal.

Acid rain causes considerable environmental and economic hazards to many industrialized countries. It is not only harmful to the life of plants and animals but it also affects buildings and monuments. A major cause is sulfur dioxide released during the combustion of sulfur-containing fuels, such as coal. To reduce the emission of sulfur dioxide attempts have been made to remove sulfur from coal before combustion. Physical, chemical, and microbiological methods have been suggested. A recently completed feasibility study demonstrates that the microbial desulfurization process is a realistic option (4). In this process microorganisms are used that can oxidize pyrite, the main inorganic sulfur compound in coal, by the following equation:



An organism that is supposed to play a prominent role in this desulfurization process is the chemolithotrophic bacterium *Thiobacillus ferrooxidans*. This aerobic and acidophilic organism grows at a temperature of about 20 to 35°C and at a pH of between 1.5 and 4. It uses ferrous iron and reduced sulfur as sources of energy and carbon dioxide as a source of carbon. Much research has been devoted to the physiology of this species (9, 12, 19). *T. ferrooxidans* can be isolated from acid mine drainage waters and sulfidic ores and coals that have been exposed to air and moisture. It may be accompanied by a variety of other acidophilic organisms such as *T. thiooxidans*, *T. acidophilus*, *Leptospirillum ferrooxidans*, and *Acidiphilium cryptum*.

Results of several studies (5, 18, 19) have suggested that mixed cultures of acidophilic bacteria are more efficient in the leaching of pyrite than are pure bacterial cultures. A determination of the number in which individual species occur in the coal samples and their in situ localization may be helpful in assessing their role in the desulfurization process.

Microorganisms can be visualized in soils and other natural environments by epifluorescence microscopy (incident illumination) after they are labeled with fluorescent dyes such as acridine orange (6), rhodamine 123 (17), or fluorescamine (20). Recently, Huber et al. (11) have described a modified DNA-fluorescence staining procedure for the detection of the microflora present on coal particles.

For the recognition of individual bacterial taxa in mixtures of microorganisms, immunological methods, such as immunofluorescence, are well suited. This technique has been successfully applied by many investigators in the fields of microbial ecology and geomicrobiology for the detection and characterization of specific microorganisms in their natural environments. Fungi (22) and various bacteria (10) have been detected in soils, water (2, 7, 21), and coal refuse material (1, 3).

In this report we describe a combined immunofluorescence-DNA-fluorescence staining procedure for the in situ localization of *T. ferrooxidans* cells in a heterogeneous population of microorganisms that are responsible for the oxidation of pyrite in coal.

MATERIALS AND METHODS

Cultures. The pure cultures used in this study are listed in Table 1. All cultivation methods used have been described by Kuenen and Tuovinen (14). *T. ferrooxidans* LMD 81.68.3G is a single cell isolate of *T. ferrooxidans* LMD 81.68 (ATCC 19859). The isolation was performed by the procedure described by Mackintosh (15). Apart from pure culture strains, a mixed culture of acidophilic pyrite-oxidizing bacteria obtained from a coal cleaning plant, as described by Kos et al. (13), was used. This mixed Born culture has been maintained for several years on a pyrite-rich coal and transferred every 2 months to a fresh suspension of coal in water.

Leaching experiments. Coal slurries with 16% pyrite, obtained from Maamba Coal Mine in Zambia, were incubated

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TABLE 1. Systematic specificity of the anti-*T. ferrooxidans* serum

Species	Strains		ELISA ^c	IFA ^{c,d}
	LMD ^a	ATCC ^b		
<i>T. ferrooxidans</i>	81.44	23270	+	+
<i>T. ferrooxidans</i>	81.45		+	+
<i>T. ferrooxidans</i>	81.66	13661	+	+
<i>T. ferrooxidans</i>	81.68	19859	+	+
<i>T. ferrooxidans</i>	81.68.3G ^e		+	+
<i>T. ferrooxidans</i>	81.69	21834	+	+
<i>T. ferrooxidans</i>	81.107		+	+
<i>T. neapolitanus</i>	80.58	23641	-	-
<i>Thiobacillus</i> sp. strain Q ^f	81.11		-	ND
<i>T. concretivorans</i>	81.54	19703	-	-
<i>T. thiooxidans</i>	81.55	8085	-	-
<i>T. organoparus</i>	81.76	27977	-	ND
<i>T. acidophilus</i>	84.12	27807	-	-
<i>L. ferrooxidans</i>	81.1	29047	+	ND
<i>L. ferrooxidans</i> ^g			-	ND
<i>A. cryptum</i>	82.2	33463	-	-
<i>Sulfolobus acidocaldarius</i>			-	ND
<i>Desulfovibrio desulfuricans</i>			-	ND
<i>Hyphomicrobium</i> sp. strain EG ^h	84.101		-	ND
Th 1 ⁱ	85.12		ND	-
LM ^j	85.11		ND	-
V b6 ^j	85.1		ND	-

^a Culture collection numbers of the Laboratory of Microbiology, Delft.

^b Numbers of the American Type Culture Collection, Rockville, Md.

^c Scoring is as follows: +, positive reaction; -, negative reaction; ND, not determined.

^d IFA, Indirect immunofluorescence assay.

^e Single-cell isolate of LMD 81.68 (ATCC 19859).

^f From Gottschal and Kuenen (8).

^g From Marsh and Norris (16).

^h From Suylen and Kuenen (in press).

ⁱ Thermophilic autotrophic isolates (iron and sulfur oxidizing) received from P. R. Norris, Warwick University, Coventry, United Kingdom.

^j Our own isolate (not identified) from coal slurry (acidophilic, iron oxidizing).

for 16 days at 30°C and pH 1.8 with a number of different acidophilic bacteria. Pyrite leaching was monitored by measuring the concentrations of pyrite and ferric iron by the procedure described by Kos et al. (13).

Preparation of the antiserum. A pure culture of *T. ferrooxidans* LMD 81.68.3G cells grown on ferrous iron was harvested by centrifugation. Subsequently, the cells were washed once with dilute sulfuric acid (pH 1.6) and three times with a 0.85% (wt/vol) NaCl solution.

A New Zealand white rabbit was immunized by the following schedule. A total of 10⁹ cells suspended in 0.5 ml of saline and thoroughly mixed with an equal amount of Freund complete adjuvant were injected subcutaneously. After 14-day intervals the animal was reimmunized with the same dose of bacteria emulsified in Freund incomplete adjuvant. The rabbit was bled from a puncture of the marginal ear vein 10 days after the third injection. The blood was allowed to coagulate for 30 min at 37°C and then overnight at 4°C. The serum was obtained from the clotted whole blood and frozen at -20°C in the presence of 0.02% (wt/vol) of the preservative sodium azide until needed.

Enzyme-linked immunosorbent assay. A suspension (100 µl) of bacteria (10⁷ cells per ml) suspended in 50 mM carbonate buffer (pH 9.6) was added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated at 4°C overnight. To saturate the remaining binding sites on the plastic, 3% (wt/vol) bovine serum

albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS; pH 7.4) was applied over 30 min at room temperature. The samples were then incubated for 1 h at room temperature with 100 µl of antiserum and diluted 1,000 times with PBS containing 0.1% (wt/vol) BSA and 0.05% (vol/vol) Tween 20 (pH 7.4; Sigma). Then, the microtiter plate was rinsed five times with PBS-0.05% (vol/vol) Tween 20. Subsequently, the samples were incubated with 100 µl of goat-anti-rabbit immunoglobulin G (whole molecule), alkaline phosphatase conjugate (Sigma; 1 h; room temperature; dilution, 1/1,000 in PBS-0.1% [wt/vol] BSA-0.05% [vol/vol] Tween 20). The microtiter plate was rinsed at least five times with PBS-0.05% (vol/vol) Tween 20 to remove free conjugate. Thereafter, the samples were incubated with 100 µl of *p*-nitrophenylphosphate (Boehringer GmbH, Mannheim, Federal Republic of Germany; 0.5 mg/ml; 10% [vol/vol] diethanolamine [pH 9.8]) as a substrate. After incubation for 30 min at 37°C in the dark, the phosphatase reaction was arrested by adding 100 µl of 3 N NaOH to the wells. The absorbance of the stained solution was measured at 405 nm with a photometer (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). This experiment was carried out several times, and a preimmune serum of the same rabbit was used as a blank in each experiment.

Combined immunofluorescence-DNA fluorescence. A similar technique, which is used for the detection of B cells, has been described by van Rood et al. (23). Bacterial cell suspensions or leached coal particles were treated as follows. Cells were centrifuged and then washed with dilute H₂SO₄ (pH 1.6) and subsequently with distilled water to remove culture medium and ferric iron-containing precipitates. Finally, the materials were suspended in distilled water. Portions (5 µl) of these suspensions were applied to holes of a microprint stock slide (Cel-Line Associates, Inc.). The slide was air dried, and the preparations were fixed by gentle heating. Each sample was incubated for 30 min with 15 µl of 1% (wt/vol) BSA diluted in Tris-buffered saline (TBS; pH 7.4) to prevent nonspecific adsorption of the antibodies to the slide or the coal particles and thus reduce background fluorescence. All incubations were carried out at room temperature in a humid chamber. Subsequently, the samples were incubated with 15 µl of anti-*T. ferrooxidans* serum or preimmune serum as described above. The slide was rinsed three times for 10 min in TBS-0.05% (vol/vol) Tween 20. Thereafter, 15 µl of goat-anti-rabbit-immunoglobulin-fluorescein isothiocyanate (FITC) (Nordic Laboratories; dilution, 1/10 in TBS-0.1% [wt/vol] BSA-0.05% [vol/vol] Tween 20) was added to each sample and incubated again for 1 h. To remove unbound conjugate, the slide was rinsed twice in TBS-0.05% (vol/vol) Tween 20 for 10 min and once in TBS for 10 min. A TBS solution containing 2.3% (wt/vol) 1,4-diazabicyclo-[2,2,2]-octane, 1 µg of ethidium bromide per ml, and 5% (wt/vol) EDTA was added with the dual purpose of reducing the fading of the fluorochrome and of staining the bacterial DNA. The specimen was covered with a cover slip and examined with a phase-contrast epifluorescence microscope (Olympus) with filters for FITC and ethidium bromide. Photomicrographs were made on Kodak Tri-X black and white film and 3M Colorslide 640-T film.

RESULTS

To determine the specificity of the antiserum, the antiserum preparation was allowed to react with a series of acidophilic and nonacidophilic bacteria in an enzyme-linked



FIG. 1. Phase-contrast and fluorescence photomicrographs of an artificial mixture of three different acidophilic bacteria, *T. ferrooxidans*, *T. concretivorus*, and *A. cryptum*, treated by the combined immunofluorescence–DNA-fluorescence staining technique. (A) Phase-contrast micrograph of the mixture; (B) photomicrograph of the same field as shown in panel A, with UV illumination and filters for ethidium bromide. Note that all bacteria visualized by phase-contrast microscopy are stained with the ethidium bromide fluorochrome. (C) Photomicrograph of the same field shown in panel A, with UV illumination and filters for the FITC conjugate. *T. ferrooxidans* cells are stained green. Note that the other bacteria are stained red by the ethidium bromide staining and can be visualized simultaneously with the FITC staining at this filter combination.

immunosorbent assay (ELISA) and an indirect immunofluorescence assay (Table 1). Strong reactions were obtained with all the tested strains of *T. ferrooxidans*. Apart from *T. ferrooxidans* a number of species were tested that are supposed to belong to the normal microflora of microbial leaching systems: the autotrophic organisms *T. thiooxidans* and *L. ferrooxidans*, the facultative heterotrophs *T. acidophilus* and *A. cryptum*, and the thermophilic pyrite-oxidizing bacterium of the genus *Sulfolobus*. No reactions were found with any one of the bacteria other than *T. ferrooxidans* and one of our strains of *L. ferrooxidans*. We established in subsequent studies, however, that this bacterium had the same physiological properties as *T. ferrooxidans* (i.e., it was able to use reduced sulfur compounds) and that it obviously had been mislabeled.

The possibility of whether the reactivity of *T. ferrooxidans* with the antiserum depended on the conditions under which the bacteria were cultivated was investigated. Cells of *T. ferrooxidans* LMD 18.68.3G were grown on five different substrates (viz., ferrous iron, tetrathionate, thiosulfate, iron pyrite, and elemental sulfur), and the reactivity with the antiserum was tested. Positive reactions of equal intensity occurred with all the cultures that were tested.

ELISA is a rapid and reliable assay for screening a large number of different antigens, but it is not suited for the localization or enumeration of individual bacteria in natural environments such as coal slurries or soils. In the figures it is demonstrated that the combined application of DNA-fluorescence and immunofluorescence staining gives excellent results. Figure 1A shows a phase-contrast photomicrograph of an artificial mixture of different acidophilic bacteria (*T. ferrooxidans*, *T. concretivorus*, and *A. cryptum*). Figure 1B shows the same mixture but visualized by epifluorescence microscopy with filters specific for the ethidium bromide fluorochrome. Note that all bacteria that were visualized by phase-contrast microscopy are also stained with ethidium bromide. The immunofluorescence staining beautifully singled out the cells of *T. ferrooxidans* in this mixed population (Fig. 1C).

This technique was used to estimate the abundance of *T. ferrooxidans* among mixed populations of acidophilic bacteria in coal slurries. Coal samples were inoculated with

different bacterial populations. Fractions of the coal suspensions were taken at intervals, over a period of 16 days. Pyrite leaching was monitored by measuring the concentration of solubilized iron, and finally, the numbers of bacteria were estimated by the combined immunofluorescence–DNA-fluorescence staining procedure described above. The results of the leaching experiments are shown in Fig. 2. A low level of pyrite removal was obtained when *T. ferrooxidans*

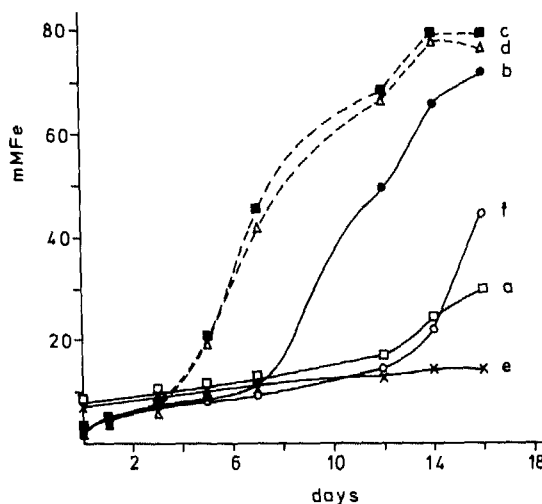


FIG. 2. Leaching results of coal samples incubated with *T. ferrooxidans*, the Born culture, or both. Bacteria were cultured in a 5% coal slurry with 16% pyrite (obtained from the Maamba Coal Mine, Zambia) at 30°C, and pH 1.8 for 16 days. Pyrite leaching was determined at different intervals by measuring the yield of pyrite and ferric iron. Curve a, leaching results of a sterilized coal sample incubated with a pure culture of *T. ferrooxidans*; curve b, leaching results of a nonsterilized coal slurry inoculated with the same culture as for curve a; curve c, results of the pyrite oxidation in a nonsterilized coal sample incubated with the Born culture; curve d, results of a nonsterilized sample incubated with the Born culture and a pure culture of *T. ferrooxidans*; curves e and f, leaching results of a sterilized and a nonsterilized coal sample, respectively, without extra added cells.

TABLE 2. Combined immunofluorescence–DNA-fluorescence staining of coal samples taken from the leaching experiments

Coal	Inoculum	Ethidium bromide ^{a,b}	FITC ^{a,c}
Sterile	<i>T. ferrooxidans</i>	+++	+++
Nonsterile	<i>T. ferrooxidans</i>	+++	+
Nonsterile	Born culture	+++	–
Nonsterile	Born culture + <i>T. ferrooxidans</i>	+++	+

^a Scoring is as follows: –, no fluorescent bacteria; +, few fluorescent bacteria; + + +, many fluorescent bacteria.

^b Results of ethidium bromide staining (DNA-fluorescence staining).

^c Results of FITC staining (immunofluorescence staining).

was added to a sterilized coal sample (Fig. 2, curve a). An uninoculated sterilized sample (Fig. 2, curve e) did not show any significant leaching. Curve b (Fig. 2) represents the leaching result of a nonsterilized coal sample inoculated with *T. ferrooxidans*. In this experiment the oxidation of pyrite started earlier, and the pyrite removal at day 16 was much higher than that without the extra added cells (Fig. 2, curve f). Two nonsterilized coal samples gave rise to high percentages of pyrite removal. One was incubated with the Born culture, a mixture of acidophilic bacteria (Fig. 2, curve c); the other one was incubated with the same mixed culture, but in addition it was incubated with a pure culture of *T. ferrooxidans* (Fig. 2, curve d). The percentage of pyrite removal of the last two experiments showed no significant difference.

The results of the microscopic observations of the dual fluorescence staining of samples from the leaching experiments taken after 16 days are summarized in Table 2.

DISCUSSION

It is generally assumed that in microbial ecosystems that are responsible for the leaching of metal ores and coal, *T. ferrooxidans* plays a dominant role. Evidence has been presented, however, that suggests that this species may be accompanied by a variety of other acidophilic organisms, such as *T. thiooxidans*, *T. acidophilus*, *A. cryptum*, and other less well characterized species (18). Some of these bacteria are obligate autotrophs, others are facultative autotrophs, and still others are oligotrophic heterotrophs. Although speculations have been put forward regarding microbial interactions within these consortia (9), consistent evidence on the exact role of the different microorganisms is still lacking.

It was the purpose of this paper to determine, by optical means, the relative abundance of *T. ferrooxidans* in coal slurries that undergo microbial leaching of pyrite. Such studies are fraught with difficulties, because strong adhesive forces between the microorganisms and solid particles preclude direct observations of the bacteria by transmitted light. It has now been demonstrated that fluorescence microscopy offers a better prospect for observing the bacteria.

Huber et al. (11) used a modified 4,6-diamidino-2-phenylindole fluorescence staining procedure for visualization of the lithotrophic bacteria that are present on the surface of coal particles. Other investigators (1, 2, 7) have reported on the use of fluorescent antibodies for determining population levels of pyrite-oxidizing bacteria in acid mine drainage waters. It should be noted, however, that although these antibodies raised against *T. ferrooxidans* did not react with a number of other bacteria, no attempt was made to study their interaction with organisms that are closely re-

lated to *T. ferrooxidans* or with other species that are supposed to be present in the consortia responsible for the pyrite oxidation. Moreover, for the preparation of their antibodies, strains of *T. ferrooxidans* were used from which the purity, at that time, had not been thoroughly checked. It is now generally agreed that many culture collection strains of *T. ferrooxidans* are not pure. Harrison (9), for example, was able to isolate the oligotrophic heterotroph *A. cryptum* from a number of culture collection strains of *T. ferrooxidans*.

In this study a single-cell isolate was made of a *T. ferrooxidans* strain by the method of Mackintosh (15), and this was then used for the preparation of the antibodies. The specificity of these antibodies was investigated extensively. The reactivity was determined with a wide variety of microorganisms, including species that were closely related both in a systematic and ecological sense. Reactions were obtained exclusively with different strains of *T. ferrooxidans*, and in addition, it was found that growth of this species on different energy substrates did not affect the reactivity. Even cells of *T. ferrooxidans* that had been cultivated during a period of several years on reduced sulfur compounds gave positive results with the antiserum (W. Hazeu, personal communication). We conclude from these results that the antibody preparation used in our experiments was highly diagnostic for *T. ferrooxidans* at the species level.

The data presented in this report indicate that the composition of the microbial consortium present on the coal particles during pyrite leaching differs markedly from what is generally assumed. From the results of the leaching experiments it appears that significant numbers of *T. ferrooxidans* isolates can only be found in samples that are sterilized prior to inoculation with a pure culture of this species. In addition, a low number of *T. ferrooxidans* cells were found with nonsterilized samples that were inoculated with *T. ferrooxidans*. In nonsterilized samples to which *T. ferrooxidans* had been added, abundant microbial life was present, as revealed by ethidium bromide fluorescence microscopy. However, the immunofluorescence staining showed that the number of *T. ferrooxidans* cells was diminished. It is also of particular interest that after incubation of nonsterilized pyrite-containing coal samples with the Born culture no *T. ferrooxidans* cells could be detected, although enrichment of this culture in the ferrous iron media and with silica gel plates yielded strains that gave positive ELISA reactions (unpublished data). We therefore suggest that this traditional enrichment and isolation technique favors the growth of *T. ferrooxidans* and that as a result the role of this species in microbial pyrite leaching of coal has been overestimated in the past.

Our combined immunofluorescence–DNA-fluorescence staining technique has shown the presence of a microflora on coal that can compete effectively with *T. ferrooxidans*. The composition of this microbial mixture, and in particular the identity of the pyrite-oxidizing organisms, need further investigation.

Our results are very similar to those of Apel et al. (1). In contrast to what they expected, these investigators found low numbers of *T. ferrooxidans* cells in samples of an acid copper mine drainage. They suggest that *T. thiooxidans*, *T. perametalobus*, or an immunologically different strain of *T. ferrooxidans* is responsible for the acid production of the drainage.

We are presently producing antisera against other acidophiles, such as *T. thiooxidans*, *A. cryptum*, and *L. ferrooxidans*; and it is our purpose to use these preparations to

further analyze the composition of the microbial communities that are associated with acid-leaching systems. We suggest that immunofluorescence combined with DNA fluorescence will become a powerful technique not only for the study of microbial communities involved in microbial leaching but also in the study of other microbial systems.

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