

Immunofluorescence Approach to the Study of the Ecology of *Thermoplasma acidophilum* In Coal Refuse Material

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Received for publication 25 March 1974

Specific immunofluorescence staining was applied to the study of the localization, distribution, and growth of *Thermoplasma acidophilum* in its natural habitat, the coal refuse pile. Different antigenic groups of *T. acidophilum* could be isolated from the same refuse pile, and the same antigenic groups were isolated from piles from different geographical areas. No correlation could be established between the antigenic groups and the pH or temperature of the habitats. Brightly fluorescing cells of *T. acidophilum* were detected on microscope slides buried in contact with the coal refuse material or immersed in the water in the stream draining a refuse pile. *T. acidophilum* grew when inoculated into either coal refuse material and/or an aqueous extract of coal refuse when incubated at its optimal temperature of 55 C, but not when incubated at room temperature or 37 C. The coal refuse pile appears to be a primary habitat for *T. acidophilum*.

Thermoplasma acidophilum is a thermophilic, acidophilic microorganism lacking a cell wall and has been classified with the mycoplasmas (9). The first isolate came from portions of a coal refuse pile which had undergone self-heating (9), but many more strains have since been isolated from similar habitats from different regions of the United States (4). All isolates have similar temperature and pH characteristics (4).

In the few years since *Thermoplasma* was first reported, some progress has been made in understanding the structural features which enable this unusual organism to withstand the physical and chemical extremes of such a harsh environment. Belly and Brock (3) and Smith et al. (16) have reported on the extreme cellular stability of *Thermoplasma* to such factors as high temperature, low pH, and chemical surfactants. Langworthy et al. (13) have demonstrated the presence of unusual ether linkages in the membrane lipids of *Thermoplasma*. The antigenic characteristics of the membranes of *Thermoplasma* have been studied by immunofluorescence-adsorption (4) and immunodiffusion-adsorption techniques (Bohlool and Brock, *Infect. Immunity*, in press).

Very little information is, however, available on ecological features of this organism in its natural habitat, and the origin and source of

this unique organism in its natural habitat, the self-heated coal-refuse pile, have not been established. The immunofluorescence approach provides a useful tool for in situ studies of specific microorganisms directly in nature, and immunofluorescence has been applied successfully to the study of bacteria and fungi in soil (5-7, 10-12, 14) and of bacteria in water (11). This study was undertaken to investigate the applicability of immunofluorescence to the study of occurrence, distribution, and growth characteristics of *Thermoplasma* in coal refuse material and refuse pile drainage water.

MATERIALS AND METHODS

Habitats. All coal refuse material, drainage water, and field slides were from a coal refuse pile at New Hope, Indiana (9), and were provided through the courtesy of M. Tansey, Indiana University, Bloomington.

Organisms. The *Thermoplasma* isolates used for the preparation of the fluorescent antibodies have been characterized previously (4). Iron-oxidizing bacteria were isolated from coal refuse material by serial dilution into the 9K medium of Silverman and Lundgren (15). Several unidentified heterotrophic organisms were isolated from coal refuse material and tested for cross-reaction with the fluorescent antibody (FA). Agar plates of the basal salts-yeast extract medium, at pH 2.0 and 4.0, were prepared by the procedure of Brock et al. (8) and used for the isolation of heterotrophic organisms from thermal and acidic coal refuse materials. Colonies which developed on these plates after 7 to 10 days at 55 C were transferred

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to liquid medium with the same composition and allowed to grow at 55 C. Several other heterotrophs from coal refuse were obtained from R. T. Belly, who had isolated them on malt extract agar, rose bengal agar, and basal salts medium (1, 8) supplemented with 0.1% yeast extract and 0.1% glucose at pH 6.0. *Mycoplasma laidlawii* strain V was obtained from the laboratory of R. P. Hanson (University of Wisconsin, Madison) and grown in PPLO medium at 37 C for 5 days.

Immunofluorescence procedure. Membrane fractions of *T. acidophilum* were isolated and used as the antigen in New Zealand White rabbits for the production of antisera. The details of antigen and antibody preparation, the specificity of the antisera, and the FA procedures have been reported previously (4).

For immunofluorescence staining of natural samples, microscope slides were buried in contact with the coal refuse material for 7 to 15 days, either in the field or in the laboratory under the desired conditions. The air-dried contact slides were, prior to the application of FA, fixed in 4% formaldehyde in Allen basal salts medium (1) for 15 min, washed in two changes of distilled water for 15 min, and air-dried. Immunofluorescent staining was carried out by the procedure developed for soil contact slides (6), using a rhodamine-gelatin conjugate to overcome nonspecific adsorption (5).

The procedure for the enumeration of specific soil organisms on nonfluorescent membrane filters by immunofluorescent staining has been reported (7). In the present study, cells in liquid suspensions were fixed for 15 min by the addition of formaldehyde (4% final concentration). After filtration of the appropriate sample volume, washing of filters with 50 to 100 ml of prefiltered, distilled water, they were stained with the desired FA as described (7). In all FA reactions, a *Rhizobium japonicum* FA was used as the negative control (6).

Growth studies. For growth studies of *T. acidophilum* added to coal refuse material, the cells from a 7-day-old culture of isolate 124-1 were harvested on 0.25- μ m membrane filters and washed with the basal salts medium at pH 2.0. To 100-g portions of the self-heated coal refuse material in 250-ml flasks was added 1 ml of the washed-cell suspension to a final concentration of approximately 10^8 cells per g.

The flasks were incubated at the desired temperatures, and samples were taken at intervals for FA enumeration on membrane filters. The FA counts were made on the formalin-fixed supernatant of a 1:10 dilution of the coal refuse material made in the basal salts medium (pH 2.0).

For preparation of a coal refuse extract, a 1:1 mixture of coal refuse and distilled water was incubated at 60 C with occasional mixing. The particulate fraction was removed by centrifugation ($3,000 \times g$, 10 min), and the extract was used for growth studies. To 100 ml of the extract in 250-ml flasks, 1 ml of a washed cell suspension was added to a final density of approximately 10^8 cells per ml, and the flasks were incubated at the desired temperatures. Counts of the number of cells per milliliter were carried out at different times by using the membrane filter FA procedure.

RESULTS

Habitat. Coal refuse piles are widely distributed in the coal-mining regions of eastern and central United States. Such piles are created by the discard of the sulfur-rich materials (primarily pyrite-rich rock) removed from the crude coal by separation processes. If such piles are not covered, acidic conditions develop within a few years due to the development and activity of iron-oxidizing bacteria (3). In many cases, these piles undergo a spontaneous combustion process and may catch on fire. A burning coal refuse pile is, of course, too hot for the development of living organisms. However, some piles do not ignite into flame but merely smolder, and temperatures from ambient to 70 to 80 C may develop. *T. acidophilum* is found in portions of coal refuse piles which have temperatures around 55 C (4, 9). Smoldering coal refuse piles are not always obvious in the field but can frequently be recognized in rainy weather by the steam rising from them. Often such piles elicit a sulfurous odor, presumably due to the exhalation of gaseous sulfur compounds such as sulfur dioxide or hydrogen sulfide. In the study of Belly et al. (2), 32 coal refuse piles in Pennsylvania and Indiana were sampled, and *T. acidophilum* was isolated from 20 different piles; a total of 52 separate isolates was obtained. Some of these isolates were used in the present study.

The field work reported in the present paper was done at one coal refuse pile, the New Hope South pile at the abandoned Friar Tuck mine near Dugger, Ind. This habitat was the source of the first *T. acidophilum* isolate, and the pile has been under study for 5 years in this laboratory. During that time, the pile has smoldered continuously, and *T. acidophilum* has been isolated routinely. In addition to the smoldering refuse itself, an additional habitat at New Hope is a warm acidic stream which flows continuously from the west side of the pile. This stream generally has a temperature of 35 to 37 C at its source, and the water, rich in ferrous iron, has a pH of 2. The water from this stream was used in some of the studies reported below.

Specificity of the Thermoplasma FA. The specificity of the FA prepared against isolates of *T. acidophilum* was tested by FA staining of various microorganisms. All 38 isolates of *T. acidophilum* from different coal refuse piles in various geographical locations reacted, but to varying degrees, with FA prepared against strain 122-1B3. By absorption studies, it was possible to define five serological groups based on reactivity with homologous and heterologous FA (4). Among the 33 unidentified heterotrophic isolates tested, only 3 rods reacted

faintly (1+ to 2+) with strain 122-1B3 FA. In addition, one chain-forming rod exhibited a strong (3+ to 4+) but anomalous reaction both with strain 122-1B3 FA and with an unrelated FA used as the negative control. This strain presumably binds some normal rabbit immunoglobulin. The chain length of this organism decreased upon subsequent transfers, and cultures acquired the ability to produce endospores after three to four transfers. The intensity of the nonspecific FA reaction of this isolate decreased gradually after each transfer and was reduced to zero after five to six transfers. Because of the morphological differences between this rod and *Thermoplasma*, the nonspecific staining reaction of this organism would not be expected to cause serious limitations to the in situ localization of *Thermoplasma* in natural samples.

None of the six iron-oxidizing rods isolated from different coal refuse material reacted with strains 122-1B3 or 124-1 or the negative FA. One strain of *M. laidlawii* was tested for cross-reactions with *Thermoplasma* antibodies and found to be nonreactive when stained with FA against strains 122-1B3, 124-1, or R8D55.

Geographical distribution of different antigenic groups of *Thermoplasma*. Table 1 is a survey of the geographical distribution of various *T. acidophilum* serological groups isolated in culture. This survey revealed that no correlation could be established between the antigenic groups and the geographical location

of the isolates. Different serological types could be isolated from the same refuse pile, but the same serological type could be found inhabiting piles from different geographical areas.

In situ detection and identification of *Thermoplasma* in coal refuse material. Both coal refuse material and drainage water from the coal refuse pile were tested with the FA for the presence of *Thermoplasma*. A few reactive cells could be found on microscope slides buried in contact with the coal refuse material either in the field or when incubated at 55 C in the laboratory, but the reactive cells, when found, occurred only singly and sparsely.

On the other hand, when tested with group I antiserum, reactive cells could be detected readily, and in high numbers, on microscope slides immersed in the coal refuse pile drainage water and incubated at 55 C. Brightly fluorescing cells were found colonizing the surface of the slides and particles attached to the slide.

Table 2 compares the relative abundance of reactive cells on contact slides from different sources. Large numbers of reactive cells could be found on slides from the drainage water incubated at 55 C but not at room temperature or 37 C, indicating that the cells were probably growing in the drainage water in the laboratory at 55 C. Field slides from the drainage channel (37 C at the source) harbored considerably more reactive cells than those at 37 C in the lab, but cell distribution was essentially random and no colonies were detected, suggesting that the cells

TABLE 1. Geographical distribution of antigenic groups of *Thermoplasma*

Serological group	Isolate	Habitat		
		Location	Temp (C)	pH
I	122-1B3 135-1	Dugger, Ind. Chartiers, Pa.	45-56	1.95
			32-55	3.51
II	97-2 107-1 124-1 133-1	Irwin, Pa. Clymer, Pa. Bentleyville, Pa. Chartiers, Pa.	35-62	3.42
			32-51	2.34
			37-57	3.44
			35-75	5.21
III	R8D55 110-1 115-1	Dugger, Ind. Gypsy, Pa. Carroltown, Pa.	53-78	1.61
			30-50	6.78
			32-62	2.33
IV	22-7 105-4 111-1 117-2	Dugger, Ind. Saltzburg, Pa. Carroltown, Pa. Ehrenfield, Pa.	37-52	1.85
			37-59	3.06
			39-65	4.05
			45-65	1.65
V	96-2 114-1 121-2	Baldwin, Pa. Carroltown, Pa. Lily, Pa.	30-55	3.23
			35-58	2.52
			45-65	2.75

TABLE 2. In situ detection and identification of *T. acidophilum* serological group I in coal refuse material and refuse pile drainage water^a

Source	Habitat temp (C)	pH	Incubation ^b	No. of reactive cells/100 fields	
Refuse pile drainage water	31-37	2.8	Lab: RT	10	
			37 C	20	
			55 C	1,000	
Refuse material	0-5 cm	42-49	3.3	Lab: 55 C	3
				Field	0
				Lab: 55 C	10
	5-10	49-55	2.5	55 C	3
	10-15	55-57	2.8	55 C	7
	15-20	57-57	2.6	55 C	0
	20-25	57-60	2.5	55 C	0
	70-75	50	2.3	55 C	0

^a Study was done at New Hope South Pile, Dugger, Ind., or on samples from this region incubated in the laboratory (as noted).

^b Lab, Laboratory; RT, room temperature.

may have resulted from passive attachment of the larger number of cells encountering the surface of the slides in the flowing channel.

Field slides from the refuse material were frequently covered with a thick deposit, making cell detection difficult. Few positive cells could be found on slides from the refuse material incubated at 55 C. In samples from depths below 20 cm, there was no evidence of the presence of *Thermoplasma*. Contact slides from most of the treatments occasionally contained small numbers of cross-reacting rods when stained with *Thermoplasma* FA. Because of their distinct rod-shaped morphology, the presence of these reactive cells did not in any way interfere with the detection of *Thermoplasma*.

Growth of inoculated *Thermoplasma* in coal refuse material and in a water extract of the refuse material. *Thermoplasma* cells more than doubled their numbers in 4 days when inoculated into coal refuse material and incubated at 55 C (Fig. 1). There was a cessation of growth after 4 days either due to nutrient or moisture limitations. The cells grew much better in the aqueous extract of the same refuse material, indicating moisture and/or nutrient availability as limiting factors for the growth of *Thermoplasma* in the particulate refuse mate-

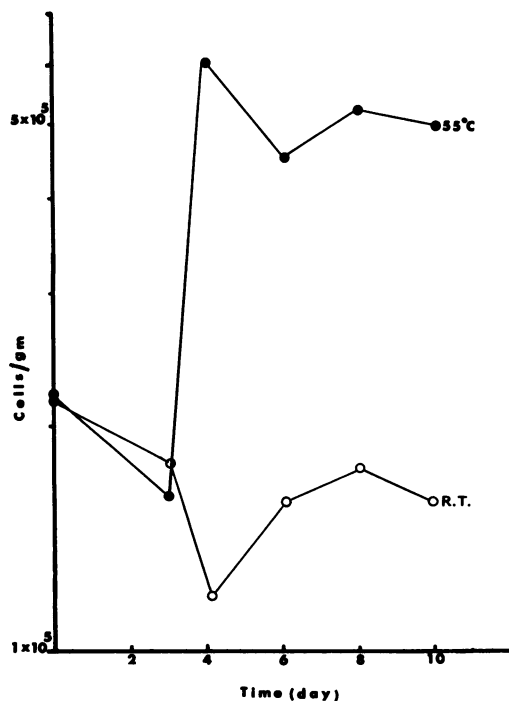


FIG. 1. Growth of *T. acidophilum* 124-1 in coal refuse material from the New Hope pile. RT, Room temperature.

rial. *Thermoplasma* cells added to the aqueous extract grew readily at 55 C but not at room temperature or 37 C (Fig. 2). At 55 C, the initial inoculum more than tripled in numbers in 4 days but remained unchanged at room temperature or 37 C.

DISCUSSION

Our survey of the geographical distribution of *Thermoplasma* serological types indicates that there may be a diversity of *Thermoplasma* types within the same coal refuse pile, since different serological groups could be isolated from the same pile and the same serological groups could be isolated from piles from different geographical areas.

The frequency with which *T. acidophilum* has been isolated from coal refuse piles that have undergone self-heating (4) indicates that this organism has become well adapted to the unusual conditions of the habitat. However, considering the relatively recent origin and man-made nature of coal refuse piles, it is doubtful that they are the primary and natural habitats of this organism. The questions that stem from lack of knowledge and the source and origin of *Thermoplasma* are of both evolutionary and ecological significance.

An interesting possibility of a natural habitat for *Thermoplasma* is a warm and acidic region of an animal host. Our preliminary attempts to establish *Thermoplasma* in the stomach of rabbits, an acidic environment (pH 1.0 to 1.5),

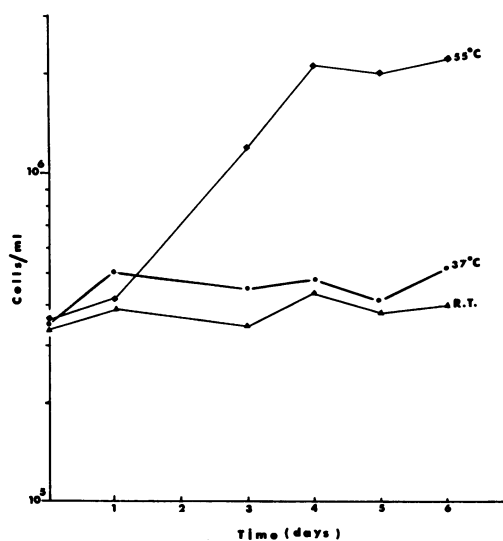


FIG. 2. Growth of *T. acidophilum* 124-1 in an aqueous extract of the coal refuse material from the New Hope pile. RT, Room temperature.

or to isolate this organism from untreated rabbit stomach were unsuccessful (unpublished data). The contents and the homogenates of the lining of stomachs of both control rabbits and rabbits force-fed with *Thermoplasma* contained a large variety of microorganisms when examined by phase-contrast and immunofluorescence microscopy. Although yeast-like organisms resembling *Saccharomycopsis guttulatus* predominated, a large number of small rods and spheres was present in all treatments. Immunofluorescence and culturing techniques failed to detect *Thermoplasma* in the force-fed rabbits over a 5-week period.

Although questions concerning the origin and natural habitat of *Thermoplasma* still remain unanswered, the thermal and acidic coal refuse piles seem to be suitable habitats for these organisms. Our immunofluorescence data indicated that *Thermoplasma* can be detected and identified directly on contact slides from coal refuse material. However, the population was very low in nature and never reached a high value even after laboratory incubation, probably due to water and/or nutrient availability in the particulate refuse material. In the water from the refuse pile drainage channel, on the other hand, *Thermoplasma* grew well and could be found in large numbers on immersion slides incubated at the proper temperature.

Growth studies indicated that both the coal refuse material and an aqueous extract of the same material could support the growth of inoculated *Thermoplasma* when incubated at the right temperature. *Thermoplasma* cells more than doubled their initial numbers when inoculated into the particulate refuse material and incubated at 55 C. This indicates that the coal refuse contains the necessary nutrients for the growth of *Thermoplasma*. However, perhaps growth takes place only to a limited extent, due to nutrient accessibility or moisture limitations in the particulate material. The cells grow much better when inoculated into an aqueous extract of the same coal refuse material and incubated at 55 C. This again lends support to the idea that moisture and nutrient accessibility may be factors limiting growth of *Thermoplasma* in the coal refuse piles.

The source of the nutrients for *T. acidophilum* is of interest. Attempts to culture this organism in a synthetic medium have so far failed (2, 8; K. M. and T. D. Brock, unpublished data; P. F. Smith, personal communication), and yeast extract is required for growth by all strains (2). Organic materials other than yeast extract obviously serve as nutrients in the coal refuse pile. Although the bulk of the coal

material has been removed, the coal refuse always contains coal fragments and other organic materials. It is unlikely that this complex high-molecular-weight material would serve directly as a source of nutrients for *T. acidophilum*, but the self-heating process will induce a series of pyrolytic reactions which should lead to the formation of lower-molecular-weight materials, some of which obviously serve as nutrients for *T. acidophilum*. Unfortunately, the organic geochemistry of coal and pyrolyzed coal is quite complex (18), and identification of relevant nutrients may be difficult. Our discovery that an aqueous extract of coal refuse will serve as a nutrient source for *T. acidophilum* may, however, be the first step leading to the discovery of the materials in coal refuse which support its growth. Indeed, the coal refuse material provides nutrients for the growth of a wide variety of microorganisms (17) and could be a source of new growth factors.

ACKNOWLEDGMENTS

This work was supported by a research contract from the U.S. Atomic Energy Commission (COO-2161-12).

The technical assistance of B. M. Thompson is gratefully acknowledged. We are indebted to M. Tansey for supplying the coal-refuse samples and to R. T. Belly for cultures and assistance in the rabbit colonization experiment.

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