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14 August 1970

A Thermophilic, Acidophilic Mycoplasma Isolated from a Coal Refuse Pile

Abstract. *A thermophilic, acidophilic procaryote lacking a cell wall has been isolated from a coal refuse pile which had undergone self-heating. Electron micrographs, chemical assays for hexosamine, and the inability of vancomycin to inhibit growth confirm the lack of a cell wall. The apparent ability of the organism to reproduce by budding and the low guanine plus cytosine content of its DNA indicate a relation to the mycoplasmas. The temperature optimum of the organism is 59°C, and growth occurs over a range of 45° to 62°C. No growth occurs at 37°C or at 65°C. The optimum* pH *for growth is between 1 and 2, and growth occurs between* pH *0.96 and 3.5 but does not occur at* pH *0.35 and only poorly at* pH *4.0. We propose to call this organism* Thermoplasma acidophila. *The existence of this organism extends considerably the range of habitats in which mycoplasma may occur.*

Free-living procaryotes which lack cell walls and grow as spheres or pleomorphic filaments are generally called mycoplasmas. In recent years, interest in these organisms has increased greatly due to the fact that they have been shown to be associated with a wide variety of disease syndromes in animals and humans (*1*). We report the isolation and characterization of a very unusual microorganism, clearly related to the mycoplasmas, which grows only at low *p*H and moderately high temperatures. The existence of this unusual organism forces a considerable enlargement of the idea of a mycoplasma (*1*).

The organism was isolated from a burning coal refuse pile at the Friar Tuck mine in southwestern Indiana. The temperature of the refuse pile from which the organism was isolated was 56°C, and the *p*H (1 : 1 mixture in water) was 1.96.

Approximately 0.5 g of black refuse from this site was inoculated into 5.0 ml of a medium containing 0.02 percent $(NH_4)_2SO_4$, 0.05 percent $MgSO_4$, 0.025 percent $CaCl_2 \cdot 2H_2O$, 0.3 percent KH_2PO_4, and 0.1 percent yeast extract (Difco). The *p*H was adjusted to either 2 or 3 with 10*N* H_2SO_4. Glucose was added after autoclaving to yield a final concentration of 1.0 percent. The tubes were incubated at 55°C. After about 2 weeks, the medium was slightly turbid. Dilutions were made into fresh medium and turbidity was detected by 3 days. Two more transfers resulted in microscopically pure cultures. Two isolates were obtained, 122-1B2 and 122-1B3 by enrichment at *p*H 2 and 3, respectively. Another isolate, 3-24, was obtained 6 months later from a different location in the same pile.

Table 1. The effect of antibiotics on the growth of *Thermoplasma acidophila*. The antibiotic was added to the indicated concentration in standard medium [0.02 percent $(NH_4)_2SO_4$, 0.05 percent $MgSO_4$, 0.025 percent $CaCl_2 \cdot 2H_2O$, 0.3 percent KH_2PO_4, 0.1 percent yeast extract, and 1.0 percent glucose] at *p*H 3. The cultures were examined after 5 days at 56°C. Isolate 104-1A is a spore-forming rod which is thermophilic and acidophilic and is used as a control to show that the antibiotics are active under the extreme conditions used.

Isolate	Minimum inhibitory concentration (μg/ml)	
	Novobiocin	Vancomycin
122-1B2	0.1	> 5000
122-1B3	0.1	> 5000
104-1A	10	50

Morphologically the isolates are pleomorphic spheres which, by phase microscopic examination, appear to reproduce by budding. Although a sphere appears to be the basic structural unit, filamentous structures are often seen, particularly in young cultures. The cells vary in diameter from about 0.3 to 2 μm. In keeping with the small size of some of the cells, it was demonstrated that viable units are able to pass through 0.45 μm membrane filters.

Electron micrographs reveal the relatively simple structure of a procaryotic organism. Nuclear material is dispersed throughout the cell with no evidence of a limiting nuclear membrane (Fig. 1A, left). No indication of membraneous organelles within the cytoplasm is seen. Unlike bacterial cells, however, these isolates lack a rigid cell wall and are separated from the surrounding environment by only a double membrane (Fig. 1A, right). A freeze-etch preparation of the organism shows in more detail the structure of the membrane (Fig. 1B). The outer membrane contains particles (arrows) which are about 20 nm in diameter (*2*).

The failure to detect a cell wall with the electron microscope is in keeping with several pieces of indirect evidence. The addition of sodium lauryl sulfate to a culture of the organism results in a very rapid cell lysis, with a decrease in optical density at 540 nm of about 80 percent occurring within 30 seconds. We were unable to demonstrate the existence of hexosamine by the Elson-Morgan assay for amino sugars (*3*). Finally, the isolates are insensitive to the antibiotic vancomycin at concentrations as high as 5 mg/ml (Table 1). Since this antibiotic is a specific inhibitor of cell wall synthesis, blocking the addition of the muramic acid–lipid complex to an acceptor (*4*), the inability of this antibiotic to inhibit growth suggests that a cell wall is not essential for cell growth. However, the isolates were inhibited by novobiocin, an antibiotic that inhibits mycoplasmas, at a concentration of approximately 0.1 μg/ml. Since the antibiotic tests were performed under rather severe conditions, *p*H 3 and 56°C, it was necessary to have a control which showed that the antibiotics were indeed active under these conditions. We used another acidophilic thermophile, isolate 104-1A, a spore-forming rod which grows at *p*H 3 and 56°C. The inhibition of this organism by both antibiotics showed that the antibiotics were active. Because

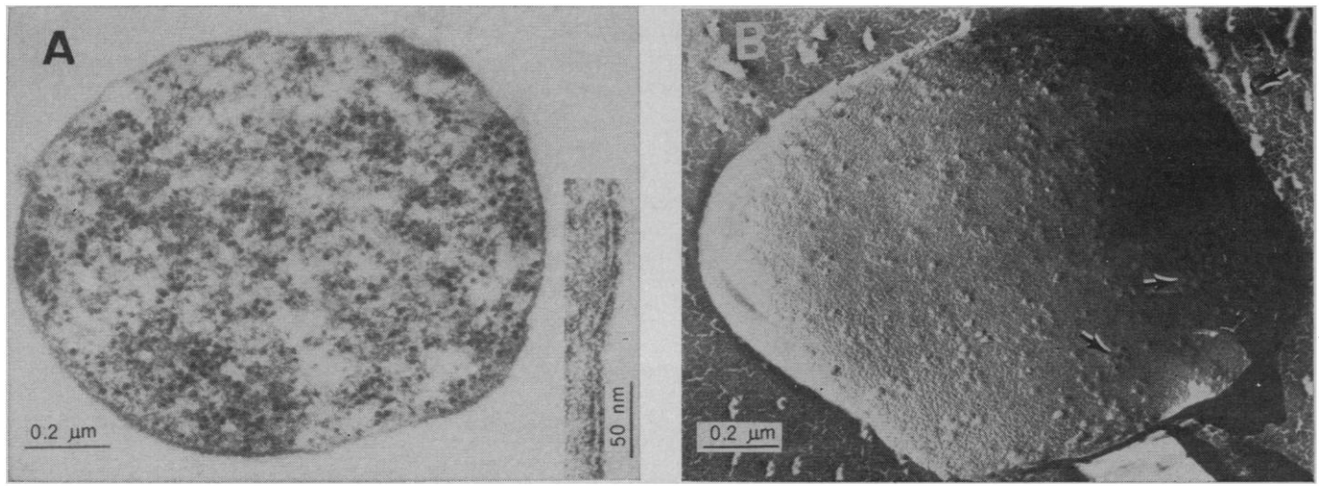


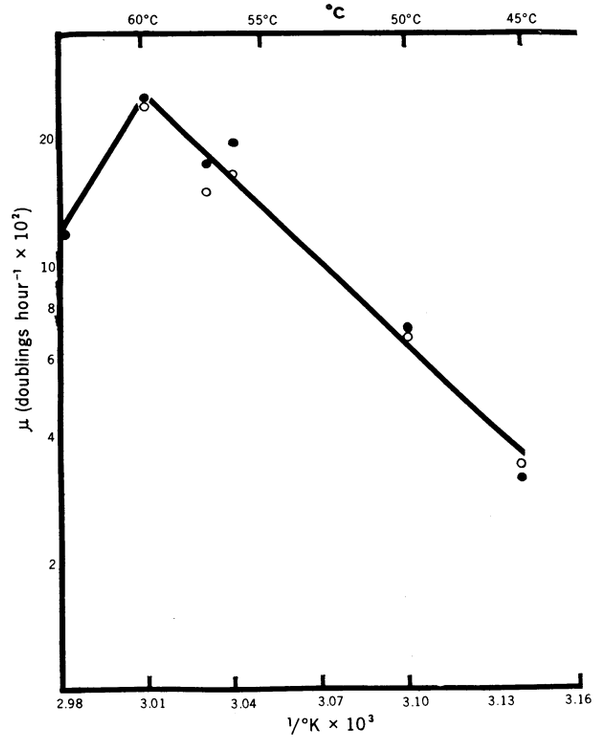
Fig. 1. (A) At the left is shown a thin section through cells of isolate 122-1B2. At the right is shown an enlargement of the membrane indicating its tripartite nature and the lack of any visible cell wall. The membrane is approximately 10 to 12 nm thick. (B) Freeze-etching of isolate 122-1B2. Enclosed arrow indicates the direction of the shadow.

of its acid lability, penicillin, another specific inhibitor of cell wall synthesis, could not be used. The organism was also not inhibited by cycloheximide, a specific inhibitor of eucaryotes.

The thermophilic nature of the isolates is seen in Fig. 2 in which the growth rate in doublings per hour is plotted against the reciprocal of the absolute temperature. The optimum temperature for growth is about 59°C with a doubling time of about 4 hours. No growth occurs at 65° or 37°C.

The effect of *p*H on the growth of one of the isolates, 122-1B2, is seen in Fig. 3. No growth occurred at *p*H 0.35, and growth was very slow at *p*H 4. The organism grew well between *p*H 0.96 and 3.0, the optimum *p*H being about 1 to 2. The *p*H did not vary (within 0.2 unit) throughout this experiment.

We have been unable to grow the isolates on a defined medium and have used the medium described above for routine culture. The yield of cells after 48 hours of growth is roughly proportional to the concentration of yeast extract at concentrations less than 0.2 percent. At concentrations higher than



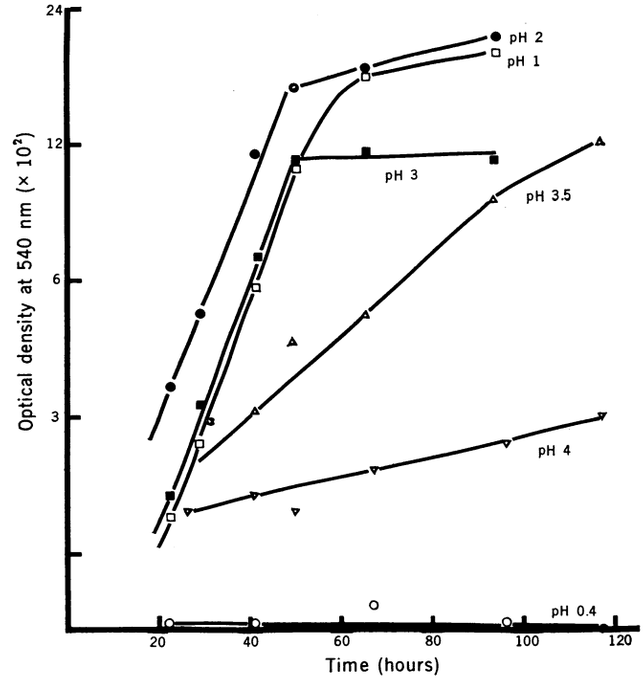


Fig. 2 (left). Arrhenius plot of the log of the growth rate (μ) versus the reciprocal of the absolute temperature. Cultures were grown without aeration in the standard growth medium at *p*H 2. Growth was followed by measuring the optical density at 540 nm in a Spectronic 20. Open circles, isolate 122-1B2; closed circles, isolate 122-1B3. Fig. 3 (right). Effect of *p*H on the growth of isolate 122-1B2. The organism was grown at 55°C in the standard growth medium. Growth was followed by measuring the optical density at 540 nm.

0.5 percent, yeast extract is inhibitory. The isolates grow well on yeast extract, but are unable to grow on the ether-extractable fraction, suggesting that the growth factors are not lipoid in nature. Although glucose was added to the initial isolation medium, glucose, galactose, sucrose, ribose, or glycerol do not stimulate growth. We were unable to grow the isolate anaerobically in the yeast extract–glucose medium in a Brewer jar. Attempts to grow the isolates on solid media have failed, at least partly due to the difficulty of obtaining a good solid medium at 55°C with a *p*H of about 2.

The DNA base composition of isolate 122-1B2 was determined by buoyant density sedimentation in $CsCl_2$ and was 25 percent guanine plus cytosine content.

There seems little doubt that this organism is related to known members of the Mycoplasmatales. The absence of a rigid cell wall (*1*) and the low content of guanine and cytosine (*5*) support this notion. The unusual physiological properties, however, suggest that the relation may be rather distant and seems to warrant classification within a new genus and species. We propose the name *Thermoplasma acidophila.*

The very existence of *T. acidophila* broadens considerably the range of habitats in which "mycoplasma-like" organisms have been found. The only previously described saprophytic species of *Mycoplasma* is *M. laidlawii,* an organism that was originally isolated from sewage (*6*). However, the fact that this same organism has been isolated from a variety of animal hosts, including the oviducts of cattle where accidental contamination seems unlikely (*7*), indicates that the saprophytic nature of this species may be questionable (*1, 8*). Due to the unusual conditions needed for growth of *T. acidophila,* it seems unlikely that it can grow as a commensal or parasite on some host and probably represents a truly saprophytic member of the order Mycoplasmatales. In addition to its inherent interest, the habitat of this organism, a burning coal refuse pile, is of considerable interest. Because of the restricted distribution and unstable nature of such piles, it seems unlikely that they are the primary habitats of these organisms. Similar organisms have been isolated from acid hot springs of Yellowstone National Park (*9*), and it seems possible that such thermal springs are the primary habitat. The structural simplicity of these free-living mycoplasmas suggests that they might be homologs of a primordial organism, and hence the study of these organisms may provide some insight into aspects of the origin of life. A culture of *Thermoplasma acidophila* has been deposited with the American Type Culture Collection as ATCC 25905.

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31 July 1970; revised 16 September 1970 ■

Tongue Cooling: A New Reward for Thirsty Rodents

Abstract. *Thirsty rodents will persistently lick a stream of dry air pumped through a standard drinking tube. This air-licking is attenuated by experimental manipulations which reduce the evaporative cooling of the tongue and mouth produced by the airstream. This suggests that such cooling is itself an effective reward for thirsty rodents. We tested this hypothesis by presenting thirsty rodents with a piece of cold, dry metal. Different species spent from 9 to 40 percent of their session time licking the cold metal. When deprived of water hamsters reared from birth without access to drinking water licked cold metal in preference to metal maintained at room or body temperature. This preference was approximately equal to that of littermates reared normally. We conclude that tongue cooling is a primary reward for thirsty rodents.*

Rats deprived of water will lick persistently at a stream of air pumped through a standard drinking tube (*1*). This air-licking looks very much like drinking; the the experienced observer has difficulty distinguishing between these activities. Air-licking occurs at stable rates of up to 10,000 licks per hour and shows no tendency to extinguish, althought it fails to restore the animals' body fluid balance. In fact, air-licking intensifies the rats' thirst by causing loss of water through evaporation from the tongue each time it comes into contact with the airstream (*1*).

Air-licking is not restricted to rats. We have found that it can also be demonstrated in water-deprived mice, hamsters, and gerbils (*2*). Thus it seems to be a general characteristic of rodents deprived of water that they will lick a stream of air at least as persistently as they lick water. Furthermore, air-licking occurs not only when thirst is induced by lack of water, but also under other conditions that induce drinking of water. These include subcutaneous injections of hypertonic saline (*3*) and certain schedules of food delivery to animals that have been deprived of food (*4*). Thus, air-licking and drinking seem to be controlled by the same motivational variables. No one has yet reported an experimental manipulation that induces drinking of water but fails to elicit air-licking.

In view of the fact that air-licking