SHORT COMMUNICATIONS

Thermophilic *Thiobacillus*-type Bacteria from Icelandic Thermal Areas

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INTRODUCTION

Thermophilic bacteria which oxidize reduced inorganic sulphur compounds, sulphur and ferrous iron have been isolated from various sources (Kaplan, 1956; Egorova & Deriugina, 1963; Schwartz & Schwartz, 1965; Williams & Hoare, 1972) and all can be placed in the genus *Thiobacillus* on the basis of their structure and physiology. We have isolated three strains of thermophilic bacteria which are similar to mesophilic thiobacilli both morphologically and in their ability to grow on thiosulphate, sulphur, ferrous iron or sulphide minerals over a wide range of pH values. These bacteria were isolated from samples collected by Williams & Pask-Hughes (1973) from the steam vents and mud springs of south-western Iceland.

METHODS

Isolation and growth of bacteria. Twenty samples of water or of material taken from hot springs were inoculated into the ferrous iron medium of Silverman & Lundgren (1959), with the iron decreased to 2 g Fe^{2+} l^{-1} and the pH at 3.5, and into the thiosulphate medium S of Postgate (1966) at pH 4.8 and 7.5. Incubation temperatures were 60 and 75 °C. In later growth tests these media were supplemented with 0.02 % (w/v) yeast extract and 0.2 % (v/v) trace metal solution (Tuovinen & Kelly, 1973). For growth on sulphide minerals, Silverman & Lundgren (1959) medium without FeSO_4 was used as a basal salts medium. Growth on sulphur was tested in the sulphur medium S of Postgate (1966).

For initial isolation, cultures were kept stationary but subsequently 100 ml cultures in 250 ml conical flasks were shaken at 200 rev. min^{-1} in a rotary incubator (Gallenkamp). Evaporation losses were replenished with water before sampling for analysis, and when samples were removed from batch cultures an equal volume of basal salts medium was added.

Determinations of the rate of pyrite oxidation were carried out using similar methods to those described for the initial isolation. Supplemented basal salts medium (96 ml) plus pyrite was inoculated with 4 ml of an active culture of the isolate grown on pyrite. Non-biological oxidation of pyrite was followed in a duplicate culture after adding 0.01 % Panacide (BDH) to kill the bacteria.

Iron estimation. Ferrous iron, in the supernatant solution of samples which had been allowed to settle for 5 h, was determined colorimetrically using 2,2'-bipyridyl (Grat-Cabanac, 1951). At 50 and 60 °C some of the ferric iron produced in cultures containing potassium salts was precipitated as potassium jarosite [$\text{KFe}_3(\text{SO}_4)_2(\text{OH})_6$] even at pH 1.2. Therefore, samples for ferric iron analyses were treated with conc. HCl to dissolve the precipitate. The

ferric iron was then reduced by $\text{NH}_2\text{OH} \cdot \text{HCl}$ and estimated as ferrous iron using the method above.

Pyrite. A commercial grade of Spanish lump pyrite with only 10 % impurities was crushed and wet ground to a particle size of $< 75 \mu\text{m}$ (< 200 mesh). Chemical analysis of the pyrite gave (% w/w): Fe, 41.8; S, 48.1; As, 0.53; Ca, 0.18; Cu, 0.65; Pb, 0.67; Zn, 1.25; CO_2 , 0.60; SiO_2 , 3.75; O_2 (excess over that combined with carbon and silicon), 2.50.

RESULTS

Bacteria which grew on thiosulphate medium S at pH 7.5 and at 60 °C were isolated from 11 of the 20 samples and a further six isolates from the same 11 samples grew at 75 °C. Bacteria from another sample grew only at 75 °C. On the same medium at pH 4.8, isolates were obtained from five samples at 60 °C but from only two at 75 °C. On ferrous iron medium at pH 3.5 bacteria which grew at 60 °C were isolated from four samples, but no bacteria were found which grew at 75 °C on this medium.

Isolates at pH 7.5

No obvious differences in morphology or rate of growth could be detected between isolates and only two, from Hveragerdi (11B) and from Hengill (58), were studied further. The bacteria from both sources grew well at 60 °C and isolate 58 also grew well at 75 °C (giving 10^8 to 10^9 cells ml^{-1}). Acid was produced in both media; shaken cultures at 60 °C reached a minimum pH of 4.8 in 5 days on thiosulphate medium S and a pH of 4.6 after 6 days on sulphur medium S, after the organism had been adapted to the growth conditions by subculturing several times. At 75 °C on these media, a minimum pH of 5.4 was attained after 7 days. Growth slightly improved when thiosulphate medium S was supplemented with 0.2 % trace metal solution, but 0.02 % yeast extract had no effect. Isolate 58 also showed sparse growth at 60 °C on medium S with the sulphur or thiosulphate replaced by natural chalcopyrite (CuFeS_2). Growth was only slightly improved by the addition of 0.2 % trace metal solution and 0.02 % yeast extract.

In all liquid cultures the bacteria of both isolates were long, thin, non-motile rods 4 to 6×0.3 to $0.4 \mu\text{m}$. They normally occurred singly but in ageing cultures were often seen as long chains frequently clustered around mineral particles.

Isolate 11B grew well at 60 °C on thiosulphate medium S at pH 7.5 solidified with 0.5 % (w/v) Ionagar no. 2 (Oxoid). White translucent umbonate colonies, 1 to 2 mm diam., with an irregular edge, were produced in 3 days. In contrast, isolate 58 showed only meagre growth on the solid medium: after 10 days incubation, needle-shaped crystals, which were not analysed, appeared on the surface of agar media and after a further 10 days minute white colonies formed around these crystals. The bacteria in these colonies were of similar appearance to those grown in liquid culture.

Isolates at pH 4.8

Growth of all the bacteria isolated on thiosulphate medium S at pH 4.8 was poor and was only slightly improved by the addition of 0.02 % yeast extract and 0.2 % trace metal solution. Attempts to increase growth by incubating the bacteria in sealed bottles containing air supplemented with approximately 1 % (v/v) CO_2 were unsuccessful. The bacteria were similar in appearance to acidophilic thiobacilli and were short non-motile rods 1.3 to $2.0 \times 0.5 \mu\text{m}$. Many of the organisms occurred in pairs.

Fig. 1. Bacterial oxidation of pyrite at 50 °C. Basal salts medium (96 ml) containing 0.02 % yeast extract, 0.2 % trace metal solution and pyrite was inoculated with 4 ml of an active culture. The initial quantities of pyrite used were (g): ▽, 1; △, 2; □, 3. ○, Sterile control with 3 g pyrite and 0.01 % Panacide. Filled symbols show the final pH values of representative cultures. Before analysis precipitated iron was dissolved in HCl.

Isolates on ferrous iron medium at pH 3.5

The growth of the bacteria isolated from four samples at 60 °C was very poor. Sparse growth was also obtained on Silverman & Lundgren (1959) medium in which the ferrous iron was replaced by 0.5 % (w/v) pyrite. The bacteria isolated from sample 21B (Hveragerdi) grew slightly better on pyrite and only this isolate was studied further. Growth was slightly improved when both culture media were supplemented with 0.02 % yeast extract and 0.2 % trace metal solution, and was further improved when the incubation temperature was decreased to 50 °C. After several subcultures on the supplemented pyrite medium at 50 °C, growth improved markedly but repeated subculturing of the bacteria on ferrous iron media did not improve growth in this medium nor did subculturing from the pyrite-grown cultures. Optimum growth on pyrite was obtained when 0.02 to 0.03 % yeast extract was added as a medium supplement, but 0.1 % yeast extract inhibited bacterial growth. In the absence of pyrite, no growth occurred on the basal salts medium containing 0.02 % yeast extract. When trace elements were omitted from the pyrite medium subcultures did not grow so well, but once cultures were established they grew unimpaired. Hence in all subsequent work 0.02 % yeast extract and 0.2 % trace metal solution was added to the culture medium.

One g pyrite in 100 ml culture was almost completely oxidized in 5 days and the acid produced caused a fall in pH from 3.28 to 1.4. Larger quantities of pyrite were also readily oxidized initially but bacterial activity almost ceased after about 5 days when a pH value of 1.2 to 1.3 was attained, and the bacteria quickly became non-viable (Fig. 1). The routine cultures contained only 0.5 g pyrite in 100 ml culture medium and subcultures were made every 3 to 4 days: this prevented bacterial death from excessively low pH.

To estimate the rate of oxidation of ferrous iron at pH 2.5 and 3.4 by the poorly-growing bacteria in stationary culture, inoculated and sterile media containing 2 g Fe^{2+} l^{-1} were incubated at 50 °C for 23 days. Although bacterial growth was observed in the inoculated media, the amount of ferrous iron oxidized in both media at both pH values was approxi-

mately 35 % indicating that there was little difference between the biological and the chemical rates of iron oxidation.

The pyrite-grown bacteria readily grew in thiosulphate medium S and sulphur medium S at pH 3.5 and 50 °C when both media were supplemented with yeast extract and trace metals. In sulphur medium, acid was produced and within 6 days the pH value of the culture had fallen to 1.48. Good growth also occurred on the thiosulphate medium at pH 4.0 and 5.0 but at pH 6.0 growth was much slower. In the absence of yeast extract and trace metals growth on thiosulphate and sulphur media was extremely poor.

The morphology of the bacteria when growing well on pyrite and sulphur media was similar to that of acidophilic thiobacilli. The bacteria were non-motile rods 1.3 to 2.0 × 0.4 to 0.6 µm. In actively growing cultures the bacteria occurred singly, but in older more acidic cultures they were in short chains. On thiosulphate medium the bacteria were shorter and broader (1.2 to 1.5 × 0.5 to 0.7 µm). In ferrous iron medium the morphology changed considerably and the bacteria were much larger, 2.0 to 5.0 × 0.6 to 1.0 µm, occurring predominantly in short chains.

The bacteria did not grow on ferrous iron medium solidified with 0.5 % Ionagar no. 2.

DISCUSSION

The bacterial distribution in Icelandic hot spring waters supports the suggestion (Brock & Darland, 1970) that classical bacteria cannot tolerate both high temperature and high acidity, although the morphologically unusual *Sulfolobus* and *Thermoplasma* species seem to be exceptions to this (Brock *et al.*, 1972; Brierley & Brierley, 1973; Darland *et al.*, 1970).

The rapid bacterial oxidation of pyrite by the mesophilic *Thiobacillus ferrooxidans* depends partly on the reoxidation of ferrous iron produced by the chemical oxidation of pyrite, but the thermophilic bacteria which eventually grew so readily on pyrite continued to show poor growth on dissolved ferrous iron. At 50 °C appreciable chemical oxidation of pyrite may occur at comparatively low pH values and the thermophilic bacteria could be growing on the sulphur released in this reaction. Alternatively insoluble pyrite or copper sulphides could be the natural substrates of the bacteria since Arnorsson, Hawkes & Tooms (1967) have reported the presence of these minerals in the area from which the bacteria were obtained.

The rapid growth of the pyrite-oxidizing bacteria on thiosulphate medium at 50 °C over a fairly wide pH range was also unexpected since the bacteria were isolated from a sample which failed to grow in thiosulphate medium at pH 4.8. Whether any of the other bacteria obtained by enrichment culture on thiosulphate medium at pH 4.8 would have grown on pyrite or ferrous iron media was not determined because of the continued poor growth of these bacteria. (In retrospect, a pyrite medium should have been one of the enrichment media used.)

The cultural characteristics of all of the organisms suggest that they be classified in the genus *Thiobacillus*, with the possible exception of the isolate obtained on thiosulphate medium at pH 4.8 which grew poorly, although it was morphologically similar to the acidophilic thiobacilli.

The isolates carried out similar chemical reactions to the genera *Thiobacillus* and *Sulfolobus*. The isolates at pH 7.6 and 3.5 had overlapping pH ranges which would allow sulphur compounds to be progressively converted to sulphuric acid, in the same way as the mesophilic species *T. thioparus* and *T. thiooxidans* can produce acid, even in initially alkaline environments. One of the alkaline isolates also grew slowly on chalcopyrite, indicating that

sulphide minerals could be slowly oxidized under alkaline conditions at temperatures much higher than have been reported previously for this reaction. The excellent growth of the acidophilic isolate on pyrite at 50 °C at pH 3.5 and below suggests that this organism is as important geochemically in its thermal habitat as is *T. ferrooxidans* at lower temperatures. The organism isolated by Brierley (1966) is the only other known thermophile which grows on mineral sulphides (Brierley & Murr, 1973). Whether the two organisms are confined to the areas from which they were isolated or whether they are more widespread and there is competition between them is unknown.

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