Nonautotrophic Thiobacillus in Acid Mine Water\textsuperscript{1}

PAULA S. MYERS\textsuperscript{2} AND W. N. MILLAR\textsuperscript{3}*

Division of Plant Sciences, West Virginia University, Morgantown, West Virginia 26506

Received for publication 30 June 1975

Nonautotrophic thiobacilli were isolated from the acidic water of a coal mine. Based on their mixotrophic physiology, the isolates are regarded as strains of \textit{Thiobacillus permetabolis}.

The detrimental environmental effects of acid mine drainage result, in part, from the oxidation of iron and sulfur compounds by autotrophic thiobacilli, notably \textit{Thiobacillus ferrooxidans} and \textit{T. thiooxidans} (2, 3). Although heterotrophic bacteria are not directly involved in these oxidations, they have been isolated from acid mine water (4, 7). While investigating the distribution of heterotrophic bacteria in mine water, we isolated bacteria that, though capable of heterotrophic growth, displayed enhanced growth in organic media supplemented with inorganic sulfur compounds. In the present study, these mixotrophic bacteria were examined for physiological and nutritional characteristics.

The acid (pH 2.6) mine water under investigation was the same as described previously (7). The nonautotrophic thiobacilli, grown in tryptone-yeast extract (TYE) medium (7) containing 1% sodium thiosulfate, were enumerated by three-tube most-probable-number (MPN) series after incubation for 21 days at 20°C. The organisms were present in water samples collected monthly over a 1-year period at a level of $10^6$ to $10^7$ cells/ml. They thus were numerically minor compared with the heterotrophic and iron-oxidizing chemolithotrophic bacteria reported in this ecosystem (7). For further investigation, two isolates, numbers 16 and 17, were selected.

Starkey's (11) basal salts solution containing 0.1, 0.5, or 1% sodium thiosulfate at pH values of 3.0 to 7.0 and several other thiosulfate-containing media (1, 6, 8, 12, 13) were evaluated for their ability to support autotrophic growth of the acid mine water isolates. In addition, sodium sulfite, sulfide, tetrasulfide, and elemental sulfur were investigated as possible inorganic energy sources in the above media. Autotrophic growth of isolates 16 and 17 with ferrous iron as the energy source was attempted in medium 9K of Silverman and Lundgren (9). These studies, using incubation temperatures of 20, 25, 30, and 40°C, were unsuccessful in demonstrating autotrophic growth by the mine water isolates.

Growth did occur, however, in TYE and was enhanced as much as fivefold by the addition of 1% sodium thiosulfate. Growth enhancement was also noted when elemental sulfur, sulfite, or sulfide was added to TYE; the pH decreased from 6.0 to 3.0 during growth in such mixotrophic media. During growth on solid mixotrophic media, elemental sulfur was not deposited.

The time course of thiosulfate oxidation by the isolates during growth in TYE containing 1% sodium thiosulfate was monitored by the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Oxidation of sulfur compounds by heterotrophically grown cells of isolate 17. Symbols: Endogenous, △; sodium tetrasulfate, ▲; sodium sulfite, O; sodium sulfide, ●; sodium thiosulfate, □.}
\end{figure}

\textsuperscript{1} Published as West Virginia University Experiment Station Scientific Paper no. 1324.

\textsuperscript{2} Present address: Bacteriology Department, West Virginia State Department of Health, South Charleston, W. V. 25305.

\textsuperscript{3} Present address: The Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind. 46206.
method of Sorbo (10). As the culture turbidity increased, the thiosulfate concentration decreased; thiosulfate could not be detected after 6 days. Further, the simultaneous increase in acidity suggested that sulfuric acid was the oxidation product.

The oxidation of sulfur compounds by the isolates was also demonstrated manometrically at 30 °C in a Warburg constant-volume respirometer (Fig. 1). The reaction vessels contained cells (1.76 mg of protein); tris(hydroxymethyl)aminomethane buffer at pH 7.0 (100 μmol); and sodium phosphate buffer at pH 7.0 (10 μmol). The side arms contained either Na$_2$S$_2$O$_4$$\cdot$5H$_2$O (10 μmol), Na$_2$SO$_3$ (10 μmol), Na$_2$S (5 μmol), or Na$_2$S$_2$O$_8$ (5 μmol). Whereas all the sulfur compounds were dissolved in 0.1 M tris(hydroxymethyl)aminomethane buffer, the sulfite and sulfide solutions also contained 5 mM disodium ethylenediaminetetraacetate to minimize auto-oxidation. The total volume of each reaction mixture was 3.0 ml. Because the isolates, grown either heterotrophically on TYE or mixotrophically on TYE containing soluble thiosulfate, oxidized thiosulfate, sulfite, and sulfide without a lag, it is concluded that the responsible enzymes are constitutive.

Isolates 16 and 17 grew in a mineral salts medium (6) supplemented with appropriate single organic sources (Table 1). Similarly, certain amino acids could serve as sole sources of carbon and nitrogen when incorporated into this mineral salts solution prepared without NH$_4$Cl (Table 2). It was found (Tables 1 and 2) that a wider range of sugars, polyalcohols, organic acids, and amino acids supported mixotrophic growth than supported heterotrophic growth. A decrease in pH from 6.0 to 5.0 occurred in those defined mixotrophic media that supported growth.

Because in mixotrophic media the oxidation of inorganic sulfur compounds, as evidenced by pH decreases, is accompanied by greater cell yields, we suggest that isolates 16 and 17 are *Thiobacillus* species. This designation is compatible with the guanine plus cytosine content found for the deoxyribonucleic acid of isolate 17; the average value of 65.0 mol% as determined by the cesium chloride density gradient method falls within the range of 51 to 68 mol% reported for the genus (5).

Among the thiobacilli, the acid mine water isolates resemble most closely the soil inhabitant *T. perometabolis* (6). This latter organism,

### Table 1. Evaluation of sugars, polyalcohols, and organic acids to support growth of isolates 16 and 17

| Substrate | Heterotrophic | Mixotrophic |
|-----------|---------------|-------------|
| Arabinose | - | + |
| Cellulbiose | - | - |
| Dulcitol | - | + |
| Fructose | + | + |
| Galactose | - | + |
| Glucose | + | + |
| Glycerol | - | - |
| Inositol | - | + |
| Lactose | + | + |
| Maltose | + | - |
| Mannose | - | - |
| Mannitol | - | - |
| Raffinose | - | - |
| Ribose | + | - |
| Sorbitol | - | - |
| Succrose | + | + |
| Xylose | - | + |
| Acetate | - | - |
| α-Ketoglutarate | + | + |
| Benzate | - | - |
| Citrate | - | - |
| Lactate | + | + |
| Malate | + | + |
| Oxalate | - | + |
| Oxalacetate | + | + |
| Pyruvate | + | + |
| Succinate | + | + |

- a - Compound did not support growth; +, compound did support growth.

### Table 2. Evaluation of nitrogen-containing compounds to support growth of isolates 16 and 17

| Substrate | Heterotrophic | Mixotrophic |
|-----------|---------------|-------------|
| Alanine | + | + |
| Aspartate | + | + |
| Asparagine | - | - |
| Arginine | - | - |
| Adenine | - | - |
| Cysteine | - | - |
| Cytosine | - | - |
| Glutamate | + | - |
| Glycine | - | - |
| Histidine | - | - |
| Hydroxyproline | - | - |
| Isoleucine | - | - |
| Leucine | - | - |
| Lysine | + | + |
| Methionine | - | - |
| Ornithine | + | + |
| Phenylalanine | + | + |
| Proline | + | + |
| Serine | - | - |
| Threonine | - | - |
| Tryptophan | - | - |
| Thymine | - | - |
| Uracil | - | - |
| Valine | - | - |

- a, b See Table 1.
although incapable of autotrophic growth, achieves optimal growth by the simultaneous utilization of sulfur compounds and organic substrates. Based on its similar mixotrophic physiology, isolate 17 has been deposited with the American Type Culture Collection as *T. perometabolis* 27793. This strain differs from the type strain of *T. perometabolis* in that it is nonmotile, does not deposit elemental sulfur during growth on mixotrophic media, does not oxidize sodium tetrathionate, and is capable of heterotrophic growth with single organic compounds.

We thank Manley Mandel of the M. D. Anderson Hospital, Houston, Tex., for determining the deoxyribonucleic acid base compositions.

Portions of this investigation were supported by National Science Foundation grant GB-27518. P. S. M. was the recipient of a West Virginia University Foundation Fellowship.

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