FUEL RESEARCH INSTITUTE OF SOUTH AFRICA.

TECHNICAL MEMORANDUM NO. 27 OF 1966.

BACTERIAL OXIDATION OF PYRITES IN SOUTH AFRICAN COAL MINES.

PART I

BY:

P. C. VAN ZYL.

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I. Isolation and Identification of Four Chemosynthetic Autotrophic Bacteria Associated with Acid Mine Drainage.

INTRODUCTION:

The occurrence of sulphuric acid in coal mine drainage is the result of the oxidation of pyrites associated with the coal. Powell and Parr[1] suggested, as early as 1919, that bacteria or some catalytic agent hastened the sulphur oxidation reaction in coal mines. They arrived at this conclusion after it was found that fresh coal in sterilized flasks, produced acid faster when it was inocculated with well-oxidized coal than the coal in flasks that were not inocculated. Davidson[2] in 1931, and Carpenter and Herndon[3] in 1933, consistently found higher acid formation in certain unsterilized coal samples than in sterilized samples of the same coal. A bacterium was isolated and identified as Thiobacillus thio-oxidans. Conclusive evidence of the presence of Thiobacillus thio-oxidans in acid mine water was obtained by Hinkle et al at the West Virginia University Engineering Experiment Station[4,5]. During this period a new bacterium, responsible for the oxidation of ferrous to ferric iron in acid mine water, was also isolated[4,6].

The ..../

The role of Thiobacillus thio-oxidans in the formation of acid from coal pyrites is still uncertain. Leathen, Braley and McIntyre[7] reported that Thiobacillus thio-oxidans was unable to enhance the oxidation of any iron disulfide with the exception of museum-grade marcasite. On the other hand, Temple and Delchamps[8] and Temple and Koehler[9] reported the oxidation of marcasite and certain pyrite-containing-concretions by this bacterium.

The enhancement of acid formation from pyritic materials by iron-oxidizing bacteria, was first demonstrated by Leathen, Braley and McIntyre[10]. Their findings were confirmed by Temple and Koehler[9]. They also demonstrated that iron oxidizing bacteria enhanced the formation of acid from pure samples of pyrites when the particle size was small enough. Silverman, Rogoff and Wender[11] reported that iron oxidizing bacteria were unable to enhance the oxidation of pure pyrites consisting of large crystals, even when it was ground to minus 325 mesh.

The chemical oxidation of pyrites associated with South African coals was invested by Teichmann[12]. He found that the chemical oxidation of pyrites in a stream of moist oxygen at 105°C was very slow, and that the increase in acidity during wet oxidation at room temperature was very small. Lategan[13] investigated the rates of oxidation of Witbank coals and could find no evidence of sulphate formation. In spite of these findings, pyrites are oxidized at a relatively high rate under certain conditions. This is indicated by the large amounts of sulphuric acid and iron sulphate present in the water drainage of some of the coal mines in the Witbank and Natal areas.

The present investigation was undertaken to determine whether bacteria, similar to those described in the literature, are also present in South African coal mines, and if so, to what extent they influence the oxidation of pyrites and the formation of acid mine drainage.

Bacteria ..../

Bacteria isolated from acidic mine and acidic leaching waters.

Up to the present time many iron and sulphur oxidizing bacteria have been isolated from acid mine waters and acidic leaching solutions. Only four distinct species were, however, described in the literature. They belong to a small group of chemolithotrophic bacteria of the family Thiobacteriaceae. This group of bacteria is enique in that they require only inorganic compounds for growth and multiplication. They obtain energy from the oxidation of sulphur and/or ferrous iron and synthesize their cell material from carbon dioxide. Nitrogen and other elements are obtained from dissolved inorganic salts.

The four bacteria identified and classified as distinct species are the following:

(a) Thiobacillus thio-oxidans is a well-known sulphur oxidizing bacterium and was first isolated by Waksman and Joffe[14] from soil composted with sulphur. It oxidizes sulphur and thiosulphates to sulphuric acid, thrives at a pH between 2 and 4 and can survive under extreme acid conditions. It was repeatedly isolated from acid mine waters[9].

(b) Thiobacillus ferro-oxidans was first isolated from acid mine water by Colmer, Temple and Hinkle[15]. It was stated to be able to oxidize thiosulphate to sulphate and ferrous to ferric iron under acid conditions, but to be unable to oxidize sulphur. As a result of its similarity to T. thio-oxidans it was assigned to the genus Thiobacillus by Temple and Colmer[16] and the specific name Thiobacillus ferro-oxidans was suggested to indicate its ability to oxidize iron.

(c) Ferrobacillus ferro-oxidans was isolated from acid mine water by Leathen and Braley[17,18]. This bacterium can also oxidize ferrous to ferric iron under acid conditions, but differs from

T. ferro-oxidans ..../

T. ferro-oxidans in that it is unable to oxidize thiosulphate. It is differentiated from T. thio-oxidans as a result of its ability to oxidize ferrous iron and its inability to oxidize thio-sulphate or sulphur.

(d) Ferrobacillus sulfo-oxidans is another iron oxidizing bacterium. It was isolated from acid mine water by Kinsel[19]. In addition to iron, this bacterium can also oxidize sulphur, and this characteristic distinguishes it from the other two iron oxidizers, viz. T. ferro-oxidans and F. Ferro-oxidans. The utilization of iron as an alternate energy source distinguishes it from T. thio-oxidans.

Other autotrophic iron oxidizing bacteria isolated from acid mine and leaching waters.

Ashmead[20] isolated, from acid mine waters in Scotland, an organism similar to T. ferro-oxidans. Beck[21] isolated, from acidic leaching waters in Bingham Canyon, a bacterium that could oxidize iron, sulphur and to a minor extent, thiosulphate. This bacterium was not identified but was described as morphologically identical to other previously described autotrophic iron-oxidizing Pseudomonales. Bryner and Jameson[22] isolated a bacterium that could oxidize ferrous iron, sulphur and metallic sulfides, and suggested that the strain was similar to T. ferro-oxidans. Razzel and Trussell[23] described the isolation of an iron oxidizing Thiobacillus which they considered as an isolate of the T. ferro-oxidans. Unz and Lundgren[24] isolated a bacterium similar to the one described by Beck[21].

THE PRESENT CLASSIFICATION.

A certain amount of uncertainty exists regarding the present classification and the characteristics which are used to distinguish between these bacteria.

Leathen .../

Leathen et al[10,25], questioned the validity of classifing Thiobacillus ferro-oxidans as a pure species. They suggested that the oxidation of iron and thiosulphate by a single culture, might be due to the combined activities of Thiobacillus thio-oxidans and Ferrobacillus ferro-oxidans, or to a purely chemical reaction causing a decomposition of the thiosulphate. Leathen and Braley[26] drew attention to the inadvisability of using thiosulphate under acid conditions in bacteriological media to characterize these bacteria. Addition of an acid inocculum to such a medium causes a decomposition of the thiosulphate to form sulphurous acid and free sulphur. The turbidity caused by the free sulphur can easily be mistaken for bacterial growth. On the other hand, if bacterial growth does occur, it is not certain whether the free sulphur or the thiosulphate in the medium is oxidized by the bacteria. Colmer[27] reisolated Thiobacillus ferro-oxidans, and again demonstrated its ability to oxidize both iron and thiosulphate on solid media. He also confirmed that the turbidity in liquid thiosulphate was due to bacterial growth and not due to the chemical decomposition of thiosulphate, as suggested by Leathen et al.

Unz and Lundgren[24] made a comparative study of the nutritional requirements of T. thio-oxidans, T. ferro-oxidans and F. Ferro-oxidans, and found justification for questioning the validity of the current classification. The cultures of T. ferro-oxidans and F. ferro-oxidans used by them could both oxidize sulphur and thiosulphate, and the only difference between the two organisms appeared to be in the degree to which each could utilize thiosulphate. They suggested as a result of their work that: (i) the genus Ferrobacillus be abolished as a generic category, (ii) the species Ferrobacillus ferro-oxidans be placed in the genus Thiobacillus and identified as a strain of Thiobacillus ferro-oxidans and (iii) Thiobacillus ferro-oxidans be recognized to be able to oxidize elemental sulphur.

Leathen ..../

Leathen was cited in a reference to have confirmed that F. ferro-oxidans could oxidize elemental sulphur. The strain of F.ferro-oxidans used by Silverman, Rogoff and Wender[11] was found to be able to oxidize elemental sulphur and ferrous iron.

The species Ferrobacillus sulfo-oxidans was not included in the above mentioned study of Uns and Lundgren, and its position in the new classification suggested by them is still uncertain.

Experimental Work.

The preparation of the media used in this investigation is described in the Appendix.

The bacteria were isolated from two samples, A and B, which were collected from a mine yielding acid mine water.

Sample A was a water sample taken aseptically in a sump from which acid drainage was pumped to the surface. This water was clear and had a pH of 2.8. Twenty-four hours after the sample had been taken the water had a brown colour due to the oxidation of ferrous to ferric iron.

Sample B was a mud sample taken in a worked out section of the mine. A band of mud 4 to 5 inches wide and 2 to 3 inches deep stretched along the wall of one of the sections of the mine. The water oozing from this band of mud left a trail of amber and yellow iron oxides as it ran down the wall and on to the floor of the mine. The pH of this sample was 1.7.

From samples A and B four cultures, I, II, III and IV, were prepared as follows:

CULTURE I. A liquid medium containing precipitated sulphur as oxidizable substrate was inoculated with 5.0 ml of sample A.

CULTURE II. A liquid medium containing ferrous sulphate was inoculated with 5.0 ml of sample A.

CULTURE III .../

CULTURE III. A liquid medium containing sulphur was inoculated with 3 to 5 gram of sample B.
CULTURE IV. A liquid medium containing ferrous sulphate was inoculated with 3 to 5 gram of sample B.

Good growth, as indicated by a decrease in the pH of the sulphur flasks or the precipitation of ferric oxides in the iron flasks, was observed in all four cultures after incubation for 12 to 18 days at room temperature. The four cultures were maintained by inoculating 1.0 ml of the old culture into fresh media of the same composition every 12 to 14 days.

Eight successive transfers were thus made before growth on solid media was attempted. The plating out on solid media consisted of placing 0.1 ml of suitable dilutions of the culture on the surface of the solid medium and then spreading it out with a sterile platinum loop. After incubation for 10 to 16 days at room temperature, the plates were examined under a stereomicroscope. With the conventional illuminating system of the microscope, i.e. directly from below, or with reflected light from above, some of the colonies were hardly or not at all visable. The best resolution was obtained by placing two lamps in front of the microscope and reflecting the two beams of light with a mirror placed beneath the stage, obliquely through the plates.

Single colonies were removed from the solid media with a platinum needle and transferred to liquid media. After growth in liquid media, cultures were again plated out and examined under the microscope. This process was repeated until only one colony-type could be observed to grow on solid media. Pure cultures growing in sulphur media were tested for iron oxidation and vice versa. Isolates having the same morphological and physiological characteristics were regarded as isolates of the same bacteria and treated as such.

Growth .../

Growth on solid iron media was difficult compared with the growth on thiosulphate agar and consequently all the isolations were made from the latter. Culture IV (Ferrous iron medium inoculated with sample B) did not grow on any of the solid media and the method of single colony isolation could not be adopted in this instance.

Results:

Isolation and Identification.

By a combination of enrichment culture and single colony isolation on thiosulphate agar, four isolates were obtained in pure culture. These four isolates were identified as four species previously described in the literature[14,15,16,17,18,19].

ISOLATE No 1. Single colonies of this bacterium were isolated from a mixture of colonies obtained when culture I was plated out on thiosulphate agar. The same colonies were also observed to grow when culture III was plated out on thiosulphate agar. Culture III was apparently a pure culture as only the one colony type was observed to grow. Young colonies growing on thiosulphate agar were round, raised, smooth and glistening with a smooth margin (Figure 1). In older colonies a characteristic white precipitate (probably sulphur) was formed in the centre of the colony. Viewed under the microscope this precipitate appeared black when illuminated from below (Figure 2). The single cells were found to be rods 0.4 to 0.5 μ wide and 0.8 to 1.5 μ long. The Gram stain was negative. When this bacterium was transferred to a liquid iron medium no growth was obtained. From the characteristics described above this isolate was identified as Thiobacillus thio-oxidans[14].

ISOLATE No. 2. Single colonies of this bacterium were isolated when both culture I and culture II were plated out on thiosulphate agar. Both iron and sulphur were oxidized when the ability of this organism to do so was tested. Single cells were rods, 0.5 μ wide and 1.0 to 1.5 μ long and they stained Gram negative. The colonies

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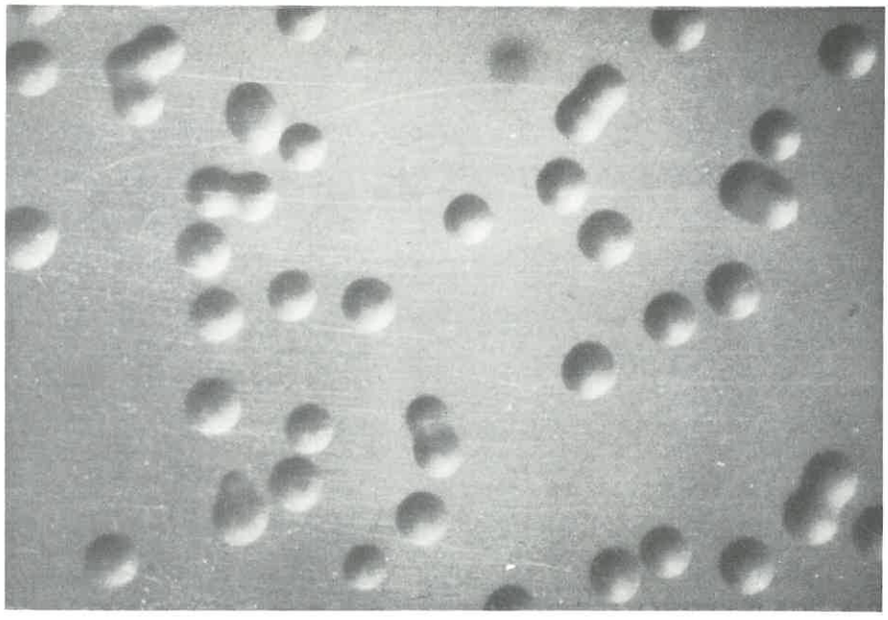

Figure 1. Photomicrograph (16x) of colonies of Thiobacillus thio-oxidans growing on thiosulphate agar; 8 days old culture.

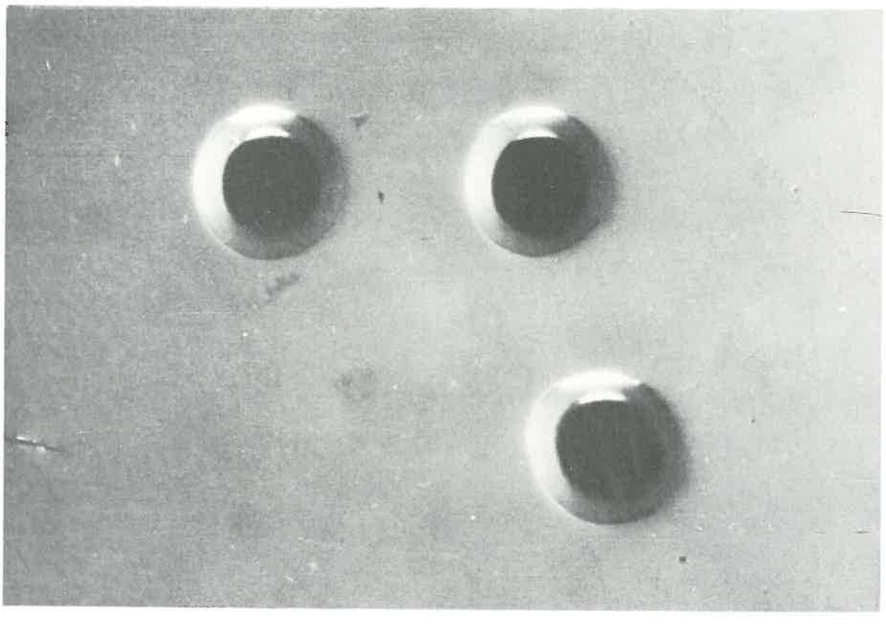

Figure 2. Photomicrograph (16x) of colonies of Thiobacillus thio-oxidans growing on thiosulphate agar; 16 days old.

that developed on thiosulphate agar were large and very flat with an irrigular margin. In the older colonies a precipitate was formed in the centre which gave them a characteristic frosty appearance (Figure 3). Except for the oxidation of sulphur, all the other characteristics indicated that this bacterium was a strain of T. ferro-oxidans. Unz and Lundgren[24], however, reported that T. ferro-oxidans could oxidize sulphur.

ISOLATE No. 3. Very small colonies of this bacterium (Figure 4) were observed to grow together with those of T. ferro-oxidans when culture II was plated out on thiosulphate agar. These colonies were difficult to observe in a mixed culture, but when they were once detected, they could never be overlooked again. By transferring single colonies to an iron medium a pure isolate was eventully obtained. It was found that this isolate could also oxidize sulphur without any difficulty to adapt itself. Colonies on thiosulphate agar of sulphur grown cells resembled those of iron cultured cells except that the latter were usually somewhat smaller. (Refer to Figures 4 and 5). The ability of this organism to oxidize both iron and sulphur indicated that it might be an isolate of F. sulfo-oxidans. This was, however, not supported by the appearance of the colonies growing on thiosulphate agar which was quite different from those obtained by Kinsel[19]. It was, however, found that the condition of the surface of the agar had a remarkable influence on the shape of the colonies. A relatively wet or rough surface resulted in the development of irregular colonies. The micro-photograph in Figure 6 was taken of a section where the surface of the agar was damaged when the culture was spread out with the platinum loop. The microphoto-graphs in Figures 5 and 6 were taken on two different sections of the same Petri dish. Thus there was no doubt that this bacterium was an isolate of Ferro-bacillus sulfo-oxidans[19].

ISOLATE No. 4.

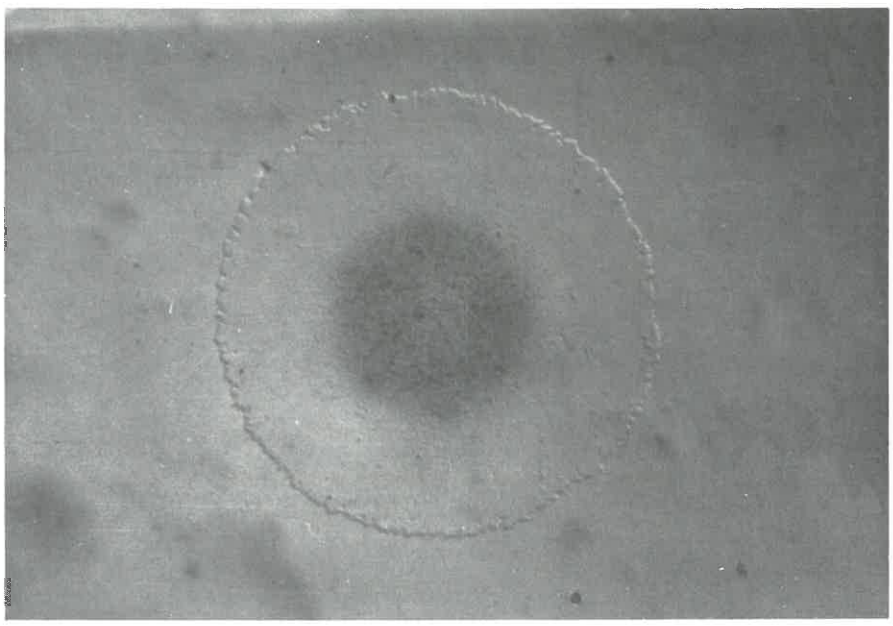

Figure 3. Photomicrograph (16x) of colonies of Thiobacillus ferro-oxidans growing on thiosulphate agar; 16 days old.

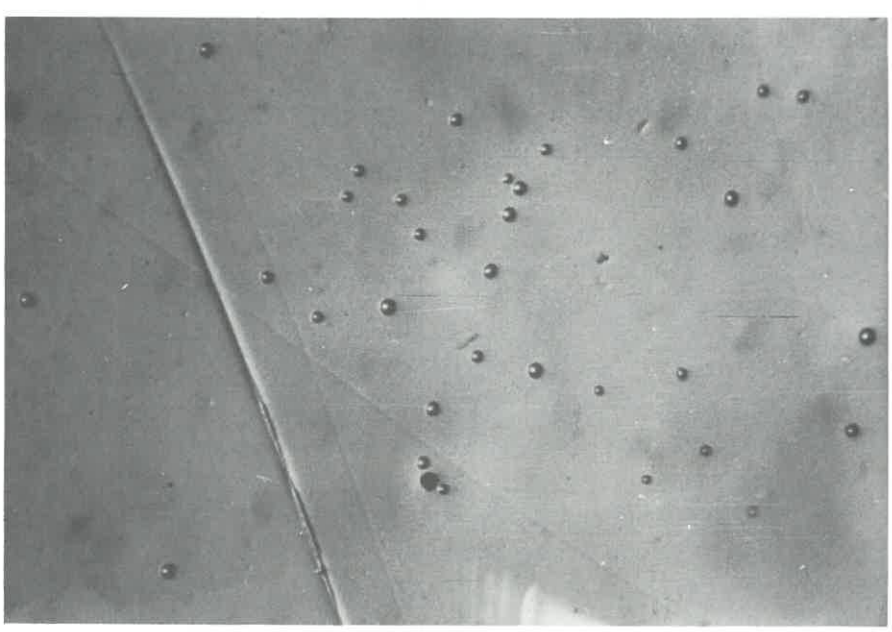

Figure 4. Photomicrograph (16x) of colonies of iron cultured cells of Ferrobacillus sulfo-oxidans growing on thiosulphate agar; 16 days old.

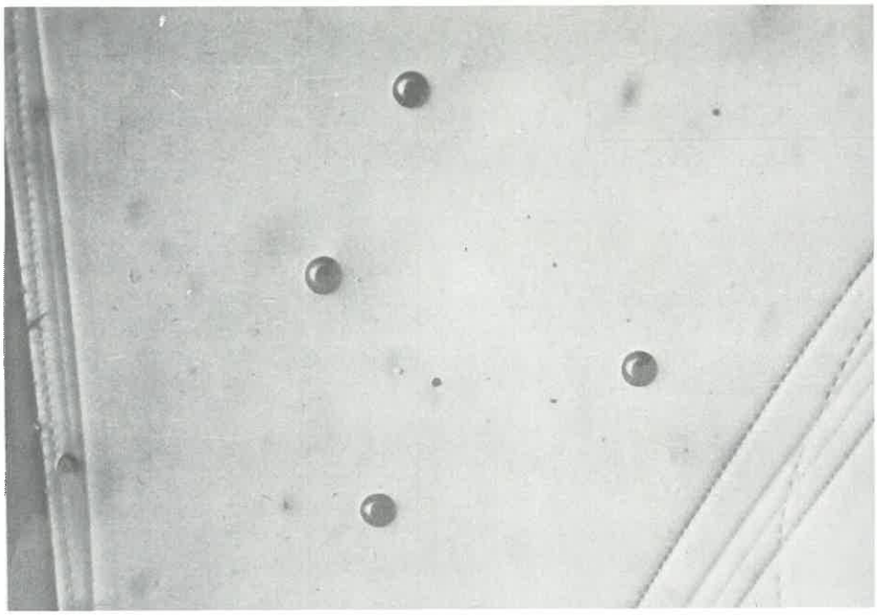

Figure 5. Photomicrograph (16x) of colonies of sulphur cultured cells of Ferrobacillus sulfo-oxidans growing on thiosulphate agar; 16 days old. The surface of the agar was very smooth.

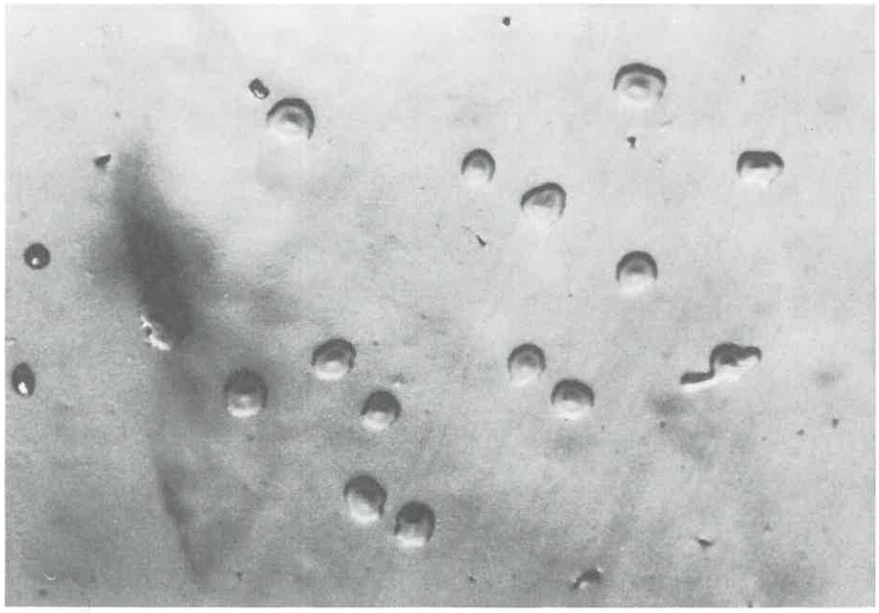

Figure 6. Photomicrograph (16x) of colonies of sulphur cultured cells of Ferrobacillus sulfo-oxidans growing on thiosulphate agar; 16 days old. The surface of the agar was rough as a result of damage by the platinum loop used for spreading out the culture.

ISOLATE No. 4. When culture IV was plated out on thiosulphate agar no colonies developed. Numerous attempts to grow this culture, on either iron agar or iron silica gel plates, were without success. Transfers to liquid sulphur media did not result in any growth. Centrifuge concentrated and washed cells of this culture stained Gram negative and the rods were somewhat smaller than those of the previously described isolates (0.4 μ wide and 0.6 to 1.0 μ long). As a result of the absence of the two previously described iron oxidizers in culture IV, it was regarded as a pure culture. The inability to grow on sulphur and the absence of growth on thiosulphate agar were sufficient proof to identify this bacterium as an isolate of Ferrobacillus ferro-oxidans.

Growth of the pure cultures on iron agar and iron silica gel media.

Growth on these media were again investigated with the pure cultures to determine its value as an additional method to distinguish between the iron oxidizers.

In spite of numerous attempts no success was obtained by growing any of the three iron oxidizing bacteria on iron silica gel plates.

Attempts to grow F. ferro-oxidans on an iron agar medium were continuously unsuccessful. Growth of T. ferro-oxidans and F. sulfo-oxidans on iron agar media was very disappointing. The colonies of these bacteria were either totally darkened by the oxidized iron or so small and insignificant, that differentiation was practically impossible. Microphotographs of the growth of T. ferro-oxidans and F. sulfo-oxidans on iron agar are shown in Figures 7 and 8.

DISCUSSION ..../

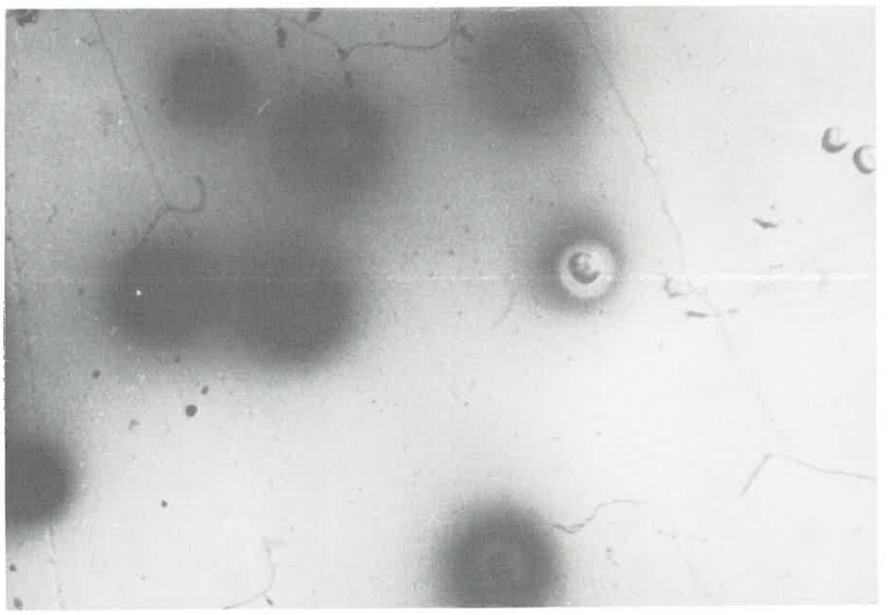

Figure 7. Photomicrograph (40x) of colonies of Thiobacillus ferro-oxidans growing on iron agar medium; 21 days old.

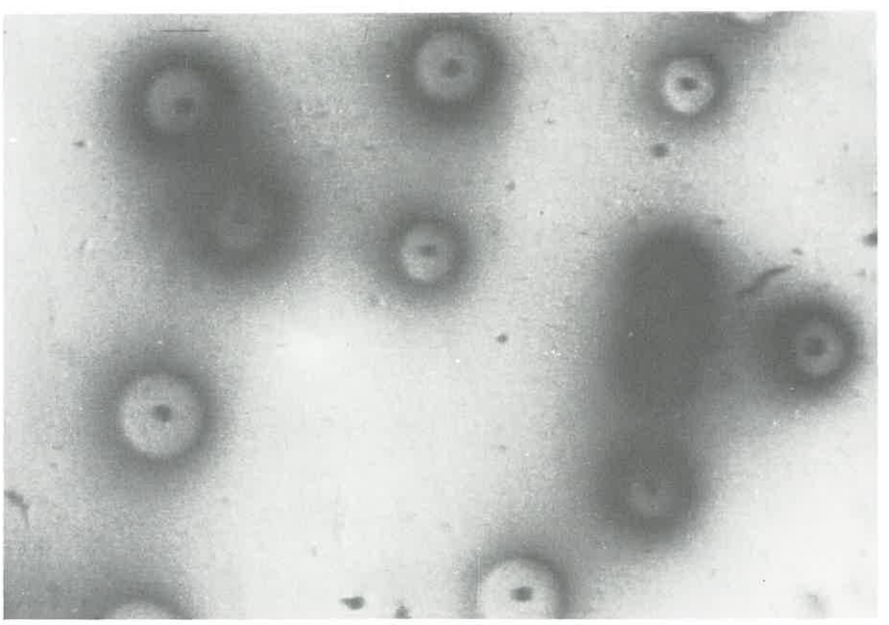

Figure 8. Photomicrograph (40x) of colonies of Ferrobacillus sulfo-oxidans growing on iron agar medium; 21 days old.

DISCUSSION OF RESULTS.

The method of single colony isolation from growth on thiosulphate agar, presented no difficulty in isolating T.thio-oxidans, T. ferro-oxidans and F. sulfo-oxidans. In the present study the isolation of F. ferro-oxidans was also readily achieved in spite of the fact that this bacterium could not be grown on any of the solid media. Sample B contained only the two very different species viz. T. thio-oxidans and F. ferro-oxidans. These two bacteria were easily separated by enrichment culture in selective media.

The oxidation of elemental sulphur by the bacterium identified as T. ferro-oxidans in this investigation confirmed similar findings by Unz and Lundgren[24], and by a number of other workers[21,22,23]. The relative large colony growth of T. ferro-oxidans on thiosulphate agar, seemed to indicate that this bacterium could also oxidize thiosulphate. However, as a result of the decomposition of thiosulphate in acid media to form sulphur[26], its use in liquid media is precluded and it was not used in this investigation.

The isolate of F. ferro-oxidans obtained in the present investigation could not oxidize sulphur and growth on thiosulphate agar was never obtained. These findings confirmed those of Leathen et al[17,18] but were at variance with those of Unz and Lundgren[24]. The culture of F. ferro-oxidans used by Unz and Lundgren could oxidize sulphur, and very large colonies, up to 6 mm in diameter, developed when sulphur cultured cells were smeared over a thiosulphate agar plate. The size of these colonies was surprising as they had found that growth of this organism in liquid thiosulphate had been difficult and undependable. The colonies described by them as rough, snow white, flat and irrigular, were very similar in appearance and size to the growth obtained in the present study from T. ferro-oxidans. This raises the question whether their culture of F. ferro-oxidans was not perhaps at that stage, contaminated with T. ferro-oxidans.

The .../

The abolishment of the genus Ferrobacillus on the grounds that F. ferro-oxidans can oxidize sulphur is therefore not justified. This genus also accommodates the species F. sulfo-oxidans. The similarity of T.ferro-oxidans and F. sulfo-oxidans both morphologically and physiologically, does not justify their classification as two different species. On the other hand, the difference in the colonies of the two organisms growing on thiosulphate agar are quite remarkable (refer to Figures 3, 4 and 5). It was also found that by subculturing a mixture of these bacteria in iron media, an enrichment of F.sulfo-oxidans was obtained, and after approximately 12 months T.ferro-oxidans was completely eliminated. This indicates that these bacteria may have very different generation times. Further research may reveal more striking differences which will justify their classification as two different species, and it is suggested that the present classification be retained.

SUMMARY .../

SUMMARY:

Four chemosynthetic autotrophic bacteria were isolated from acid drainage in a South African coal mine. The characteristics of these bacteria correspond with those of the original isolates of the species T.thio-oxidans, T.ferro-oxidans, F.ferro-oxidans and F.sulpho-oxidans.

Evidence obtained in this investigation confirms:

(a) the existence of a species similar to Ferrobacillus sulfo-oxidans;
(b) the ability of Thiobacillus ferro-oxidans to oxidize sulphur and
(c) the inability of Ferrobacillus ferro-oxidans to oxidize sulphur.

As a result of these findings it is suggested that the present classification be retained.

APPENDIX .../

APPENDIX:

Media used for growth and isolation of bacteria.

Liquid media.

For iron oxidation the medium 9K of Silverman and Lundgren[28] was used. This medium contained the following concentration of salts per litre of solution:

$(NH_4)_2 SO_4$: 3.0 g; KCl : 0.1 g; K_2HPO_4: 0.5 g;

$MgSO_4 \cdot 7H_2O$: 0.5 g; $Ca(NO_3)_2$: 0.01 g;

$FeSO_4 \cdot 7H_2O$: 44.2 g;

The pH of the solution was adjusted to 3.0 - 3.5 by adding 1.0 ml of a 10N sulphuric acid solution. The medium was made up by dissolving the basic salts in 700 ml of distilled water and the ferrous sulphate in 300 ml of distilled water. The two solutions were sterilized separately under 15 lbs steam pressure for 15 minutes and combined afterwards. The bacteria were grown in 250 ml conical flasks containing 100 ml of the medium.

For the oxidation of sulphur, the medium was made up as described for iron oxidation except that the ferrous sulphate was substituted with 10 gm per litre of finely powdered precipitated sulphur. The medium was sterilized in flowing steam for 30 minutes on three successive days.

Solid media.

Thiosulphate agar. The medium of Colmer, Temple and Hinkle[15] was used and had the following composition:

$(NH_4)_2 SO_4$ 0.2 g; KH_2PO_4 3.0g;

$MgSO_4 \cdot 7H_2O$ 0.1g; $CaCl_2$ 0.2 g;

$Na_2S_2O_3 \cdot 5H_2O$ 5.0g; Agar 15 g; distilled water 1000 ml. The thiosulphate was dissolved separately in double strength and was added to a double strength solution of the rest of the components after both solutions had been sterilized in the autoclave (121°C for 15 minutes) and

cooled .../

cooled to 50°C. Plates were poured at 50°C to a depth of 3 to 4 mm and to ensure a relative dry surface of the agar, the plates were only used 24 hours after pouring. The method of preparation described above together with the use of ion agar No. 2 gave a very clear medium.

Ferrous iron agar. The medium described by Leathen, McIntyre and Braley[29] was used and had the following composition:

$(NH_4)_2SO_4$ 0.15 g;

KCl 0.05g; $MgSO_4 \cdot 7H_2O$ 0.5 g; K_2HPO_4 0.05 g;

$Ca(NO_3)_2$ 0.01 g; $FeSO_4 \cdot 7H_2O$ 1.0 g; distilled water 1000 ml. The pH of the medium was adjusted to 3.5 to 4.0. The iron was added aseptically from a filter-sterilized 10 per cent solution to a solution of the rest of the components which had been sterilized in the autoclave under 15 lbs per square inch steam pressure for 15 minutes. Ion agar No. 2 (1.5 per cent) was used for solidification. Plates were poured as described above.

Ferrous iron silica gel. The medium described by Leathen, Kinsel and Braley[18] was made up as follows:
Solution A: $(NH_4)_2 SO_4$ 6.0 g; KCl 0.05 g;

$MgSO_4 \cdot 7H_2O$ 0.5 g; $Ca(NO_3)_2$ 0.01 g; distilled water 250 ml. Twenty-five ml aliquats of this solution were sterilized in the autoclave (121°C) for 15 minutes.

Solution B: A buffer solution containing 13.5 g of K_2HPO_4 in 100 ml distilled water was sterilized separately in the autoclave.

Solution C: Ten gram of $FeSO_4 \cdot 7H_2O$ was dissolved in 100 ml distilled water and filter-sterilized.

Solution D: Silicic acid was prepared by passing a solution of 15 g anhydrous meta sodium silicate in 100 ml of distilled water through an ion exchange column. The column was prepared by packing 500 gm of amberlite IR-120 (H) resin in a glass tube 2 inches wide and 24

inches .../

inches long. A filter disc fused in the bottom of the tube supported the resin and a stopcock was provided to regulate the flow. Throughput of the solution was 20 - 30 ml per minute and only the fraction having a pH of 2.0 was collected at the bottom. The resin was regenerated after use by washing with distilled water and then by passing through 1,500 ml of a 10 per cent hydrochloric acid solution. The resin was then again washed with distilled water until free from chloride ions.

To complete the preparation of the medium 25 ml of A, 1.0 ml of B, 1.0 ml of C and 75 ml of D were combined aseptically, poured into sterile Petri dishes, and set aside overnight for the gel to form.

(SIGNED) P. C. VAN ZYL.
SENIOR RESEARCH OFFICER.

PRETORIA.
22/7/66.

S. BOOYENS.
ASSISTANT RESEARCH OFFICER.

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