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Isolation and study of two strains of *Leptospirillum*-like bacteria from a natural mixed population cultured on a cobaltiferous pyrite substrate

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Abstract

Two strains of *Leptospirillum*-like bacteria, L6 and L8, have been isolated from a mixed inoculum, also containing *Thiobacillus ferrooxidans* and *T. thiooxidans*, cultured for one year with a colbaltiferous pyrite as energy substrate in a 100 l continuous bioleaching laboratory unit. Several physiological properties of the strains are described. The vibrio-shaped microorganisms grew at pH values lower than 1.3. Their growth rate was maximum between 2.5 and 8.0 g l^{1} ferrous iron. The optimal growth temperature was 37.5° C. Ferric iron had a stimulative effect on bacterial development up to 8 g l^{-1}, and growth was as rapid at 14 g l^{-1} ferric iron as at 8 g l^{-1}. The negative influence of cobalt on the final cell concentration was observed at 0.5 g l^{-1}, but the growth rate was not affected up to 2 g l^{-1}. The G + C content of strains L8 is 55.6 mol%.

Introduction

The mesophilic bacteria principally involved in the oxidation of sulfides are *Thiobacillus ferrooxidans, T. thiooxidans* and *Leptospirillum ferrooxidans*. The rod-shaped bacteria of the genus *Thiobacillus* has already been extensively studied when *L. ferrooxidans* was described by Markosyan in 1972. Since this first observation, similar vibrio or coiled microorganisms have been detected in several mining environments. Such *Leptospirillum*-like bacteria were isolated in samples from uranium mines, coal spoil heaps, and copper acid drainages (Norris 1983; Harrison & Norris 1985; Sand et al. 1992). Some properties of those vibrio-shaped strains have been defined. Pure cultures of the iron-oxidizing bacterium *Leptospirillum* cannot oxidize elemental sulfur or inorganic sulfur compounds such as thiosulfate or tetrathionate. On the other hand, pyrite can be used as energy substrate (Merretig et al. 1989; Sand et al. 1992). Furthermore, pyrite materials seem to favour the growth of *Leptospirillum*: enrichment cultures of *Leptospirillum*-like bacteria have been obtained with pyrite (Norris 1983). Helle & Onken (1988) noted that *Leptospirillum*-like bacteria overgrew *T. ferrooxidans* during the continuous bioleaching of a pyrite by a mixed culture.

The present paper describes the main characteristics of two *Leptospirillum*-like bacterial strains that belong to a mixed population originally sampled in a sulfide ore mine. Strains of *T. ferrooxidans* and *T. thiooxidans* were isolated from this inoculum, which had been used for one year to bioleach an arsenopyrite concentrate (Collinet & Morin 1990). When the culture was transferred to cobaltiferous pyrite substrate, *Leptospirillum*-like bacteria were observed. After several months of continuous biological oxidation of pyrite, the vibrio-shaped microorganisms were the dominant component of the population. The medium from which the two *Leptospirillum*-like bacterial strains were isolated possessed certain specific properties: in addition to the high ferric iron concentrations and low pH values, which are typically obtained during the growth on pyrite, some cobalt had been solubilized. The behaviour of the *Leptospirillum*-like bacteria towards ferric iron, pH, ferrous iron, cobalt, and temperature were determined so as to explain why *Leptospirillum* is favoured as compared with *T. ferrooxidans* when pyrite is the energy sub-

strate. We compared the characteristics of those strains with other *Leptospirillum*-like bacteria that originated from environments where natural oxidation of sulfides occurs.

Materials and methods

Origin of microorganisms

The indigenous inoculum was initially sampled by BRGM (Bureau de Recherches Géologiques et Minières) in sulfide ore mines. It has been successively cultured on arsenopyrite, then on cobaltiferous pyrite, in a continuous bioleaching unit composed of four agitated and aerated reactors in cascade. Collinet & Morin (1990) isolated strains of *T. ferrooxidans* and *T. thiooxidans* from this mixed population when arsenopyrite was used as energy substrate.

Isolation of Leptospirillum*-like bacteria*

The indigenous mixed culture was sampled from the continuous bioleaching reactors loaded with cobaltiferous pyrite. This bacterial population was subcultured ten times in the liquid medium of Silverman and Lundgren (1959) containing 9 g l^{-1} ferrous iron as energy substrate (9K medium), to which 8 g l^{-1} ferric iron were added to make it selective. The pH of the cultures was adjusted to 1.7 with H_2SO_4. *Leptospirillum*-like bacteria were isolated by spreading 50 μl of culture, appropriately diluted in 2 g l^{-1} H_2SO_4, on plates containing the *Thiobacillus* Solid Medium 1 (TSM1). This medium, developed by Visca et al. (1989) in order to obtain quantitative yields of *T. ferrooxidans* colonies, contains 4 g l^{-1} ferrous iron as energy substrate. It was prepared with agarose Sigma low EEO. The plates were incubated at 30° C for 10 days.

Characterization of Leptospirillum*-like strains*

Several energy substrates were tested for their ability to support growth. The following chemicals were added to 50 ml of basal salt medium, i.e. the medium of Silverman and Lundgren (1959) but without ferrous iron, in 100 ml Erlenmeyer flasks: 10 g l^{-1} pyrite, 5 g l^{-1} elemental sulfur, 5 g l^{-1} sodium sulfite, or 5 g l^{-1} sodium thiosulfate. Flasks were placed at 35° C on a 100 rpm reciprocal agitating table.

The influence of pH, ferric iron, ferrous iron, and cobalt were tested at 35° C in 250 ml Erlenmeyer flasks containing 50 ml of medium and placed on a 100 rpm reciprocal agitating table. The standard liquid medium was 9K medium containing 9 g l^{-1} ferrous iron as energy substrate, and 8 g l^{-1} ferric iron. Culture pH was adjusted to 1.7 with H_2SO_4. The concentration of ferric iron was varied from 0 to 24 g l^{-1} when the influence of this ion was studied. For the experiments concerning the influence of pH, ferric iron, and cobalt, the medium contained 9 g l^{-1} ferrous iron. In order to study the influence of ferrous iron, the concentration of this ion was varied from 2 to 14 g l^{-1}. The tolerance to cobalt was tested at 0.5, 1.0, 2.0, and 5.0 g l^{-1}. Ferrous, ferric iron, and cobalt were introduced into the medium as sulfates. Influence of pH was tested between 0.9 and 2.0. The influence of temperature was tested in air-lift tubes containing 200 ml of standard liquid medium. Air was provided through a glass pipe, immersed in the culture, which delivered the gas at the bottom of the tubes. Experiments at 20, 30, 35, 37.5, 40, and 45° C were performed simultaneously in six different thermostated baths.

Liquid media were sterilized by filtration at 0.22 μm and elemental sulfur by heating at 100° C for one hour for three days. Pyrite was autoclaved in the basal salt medium, at 110° C for 30 min.

Analytical methods

Bacteria were enumerated using a Thoma counting cell under an optical microscope (× 400). Ferrous iron and total iron were analyzed by (1) a colorimetric method using orthophenantroline, and (2) a volumetric method with potassium dichromate (Charlot 1966). Cobalt was determined by atomic absorption spectrophotometry (Varian, SpectrAA-300).

DNA base composition

DNA was extracted by the technique of Visuvanathan et al. (1989) for strain L8. The mol % G+C of the DNA was determined at the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Braunschweig, Germany, using the methods described by Mesbah et al. (1989) and Tamaoka & Komagata (1984).

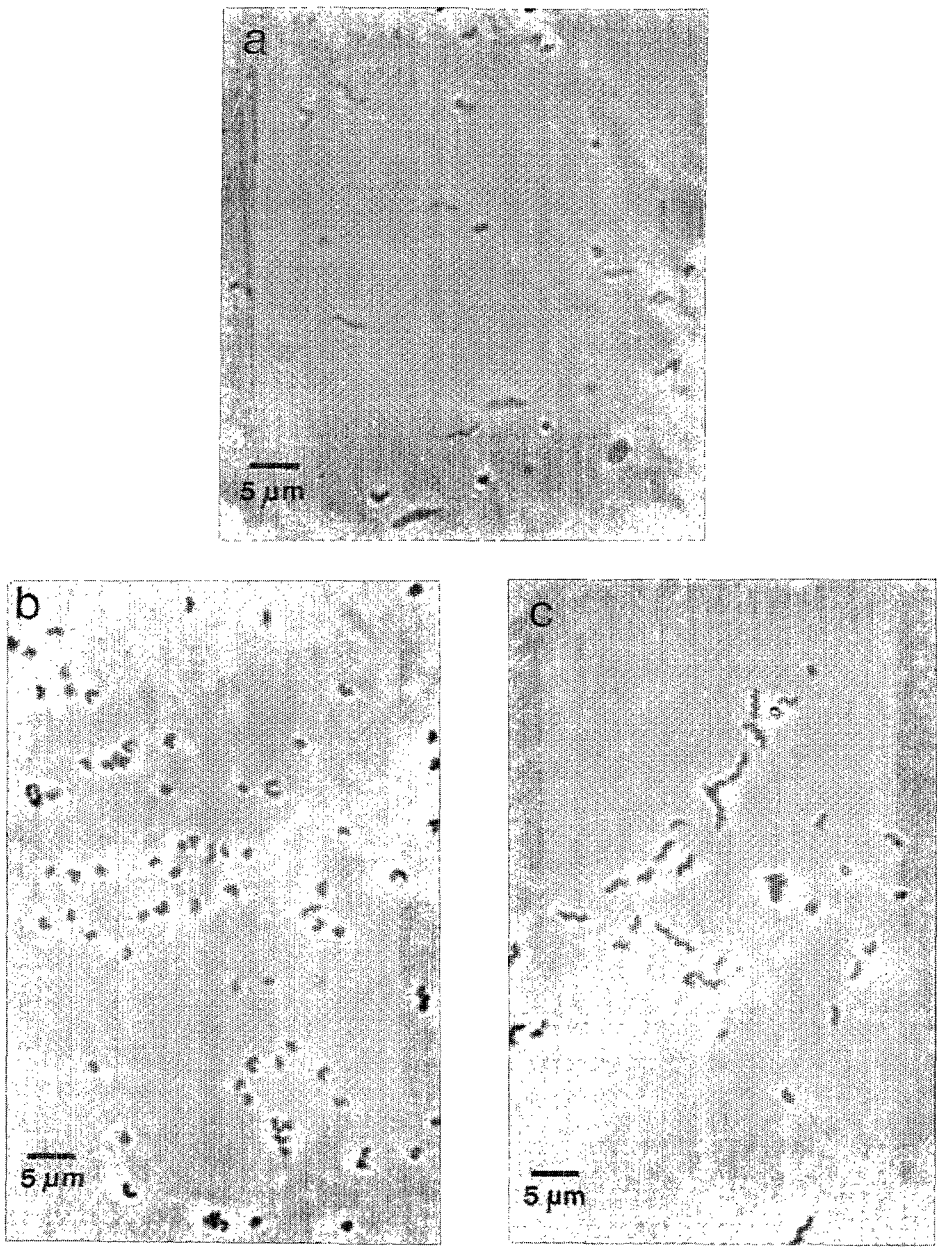


Fig. 1. Observations of the cultures by optical microscopy. *(a)* mixed population provided by BRGM: both rod-shaped and vibrio-shaped bacteria can be seen in this picture; *(b)* strain L8 of *Leptospirillum*-like bacterium isolated from the mixed culture provided by BRGM incubated at 35° C; *(c)* strain L8 incubated at 25° C.

Results and discussion

Isolation of Leptospirillum-*like bacteria*

The indigenous mixed inoculum provided by BRGM had been cultured for one year in a continuous bioleaching unit loaded with a cobaltiferous pyrite substrate. The pulp contained small bacilli, 1.5 μm in length and 1.0 μm in diameter, vibrio-shaped bacteria, 1.0 to 1.5 μm in diameter, and some long stretched spirals, 3.0 μm in length and 0.5 μm in diameter (Fig. 1a). The rod-shaped bacteria were less numerous than vibrio and helicoïdal microorganisms, which presented some similitude with the mesophilic, acidophilic,

autotrophic, and iron-oxidizing bacterium *L. ferrooxidans*.

Samples of pulp were used to inoculate liquid media. When the medium did not contain ferric iron, the proportion of bacilli in the mixed population increased. The vibrio forms were entirely eliminated after a few subcultures. In order specifically to inhibit the development of *T. ferrooxidans*, which is the main iron-oxidizing rod-shaped bacterium, 8 g l^{-1} ferric iron were added to the 9K medium, which also contained 9 g l^{-1} ferrous iron as sole energy substrate. Collinet & Morin (1990) showed that the growth of a *T. ferrooxidans* strain isolated from the same inoculum was markedly reduced by 10 g l^{-1} ferric iron. *Leptospirillum ferrooxidans* is known to be less affected by ferric iron than *Thiobacillus* species (Norris et al. 1987; Johnson 1991). The *Leptospirillum*-like bacteria became the major morphological type after several successive passages in 9K medium containing 8 g l^{-1} ferric iron. Afterwards, this medium was used as standard liquid medium.

The culture resulting from this enrichment step was used for the isolation on solid medium TSM1 containing ferrous iron as sole energy substrate. Some red-brown colonies, 0.5 to 1.0 mm in diameter, surrounded by a large red halo of oxidized ferrous iron, were obtained. Several colonies were sampled and used to inoculate the standard liquid medium. Nevertheless, the results of spreading the bacterial suspension on solid medium TSM1, which is normally used for the iron-oxidizer *T. ferrooxidans*, were not reproducible in terms of counts and growth of *Leptospirillum*-like bacteria, and seemed to be closely dependent upon the physiological state of the inoculum. The same phenomenon was mentioned by Johnson et al. (1987) who developed the Iron and Tryptone Soya Broth solid medium (FeTSB) for the simultaneous isolation and enumeration of iron-oxidizing bacteria and acidophilic bacteria. Two isolated strains, L6 and L8, were chosen for the study.

Morphology

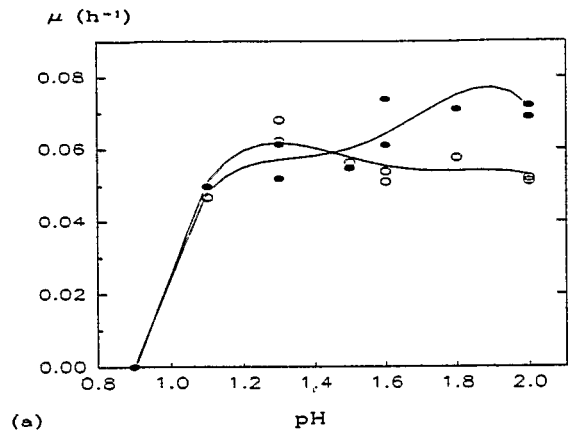
Strains L6 and L8 stained Gram negative. They exhibited some polymorphism: cells were vibrio-shaped under optimal conditions (Fig. 1b); spiral forms appeared at sub-optimal temperature (Fig. 1c); small coccoid cells, which tended to aggregate, were also occasionally observed. At the beginning of the exponential growth phase, bacteria were very motile. All those characteristics correspond to the descriptions of *L. ferrooxidans* (Balashova et al. 1975; Pivovarova et al. 1981; Harrison & Norris 1985).

Physiology

Several energy substrates which are likely to support the growth of sulfur- and iron-oxidizing bacteria were inoculated with strains L6 and L8. The two strains showed the same characteristics. They were able to grow when ferrous iron or pyrite was added to the basal salt medium. With elemental sulfur, the inoculum did not multiply, however, the bacteria remained observable for 10 days at 35° C. In the presence of thiosulfate or sulfite, no growth occurred and the bacteria disappeared. The results of these tests are in agreement with the substrate specificity of *L. ferrooxidans* (Sand et al. 1992).

The influence of pH was studied in the range from 0.9 to 2.0. It was not possible to study higher pH conditions because the ferric iron introduced into the standard liquid medium precipitated between pH 2.3 and pH 2.6. The logarithmic growth rate was not greatly affected when the initial pH reached values as low as 1.1 (Fig. 2a). The growth rate of strain L8 increased with initial pH up to 1.8, whereas the maximum growth rate of strain L6 was obtained at pH 1.3. On the other hand, the final cell yield on ferrous iron increased with the initial pH for the two strains (Fig. 2b). Regardless of pH values, the final cell concentration was slightly higher with L6 than with L8. Collinet & Morin (1990) observed that the growth of *T. ferrooxidans* was strongly inhibited when the pH was lower than 1.4. The present results, in accordance with previously published data (Merretig et al. 1989; Sand et al. 1992) suggest that *Leptospirillum*-like bacteria are more resistant to very low pH than *Thiobacillus* species.

The influence of ferrous iron as sole energy substrate was studied in the range from 2 to 14 g l^{-1}. Figure 3a shows that the growth rate was optimal between 2.5 and 8.0 g l^{-1}. At higher concentrations, ferrous iron produced a progressive inhibitory effect with affected both growth rate and cell yield. The final cell density reached its maximum value at 2.5 g l^{-1} for L8 and at 3.2 g l^{-1} for L6 (Fig. 3b). A few data were published about the influence of ferrous iron concentration on *Leptospirillum*-like bacteria. Four strains of *L. ferrooxidans* exhibited their exponential growth rates between 0.0124 and 0.0289 h^{-1} in the presence of 2.8 g l^{-1} ferrous iron, at 30° C (Harrison & Norris 1985). The optimal ferrous iron concentrations were 8 g l^{-1} and



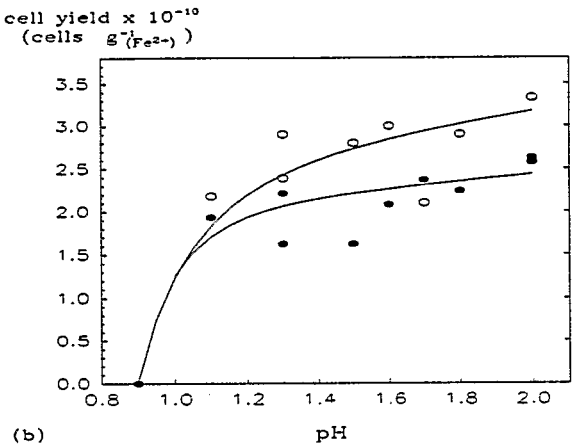
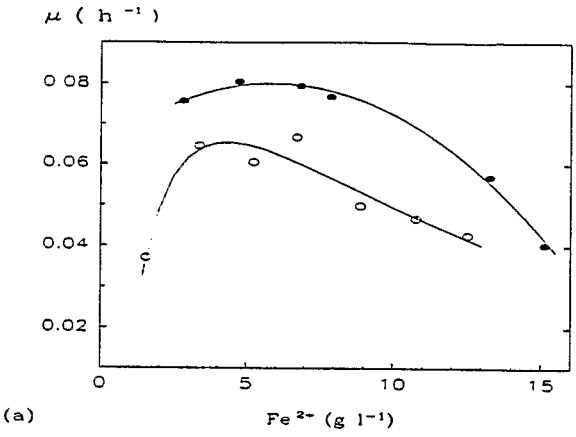


Fig. 2. Influence of pH on the growth of strains L6 and L8. *(a)* growth rate; *(b)* cell yield. Symbols: (o) strain L6; (•) strain L8.



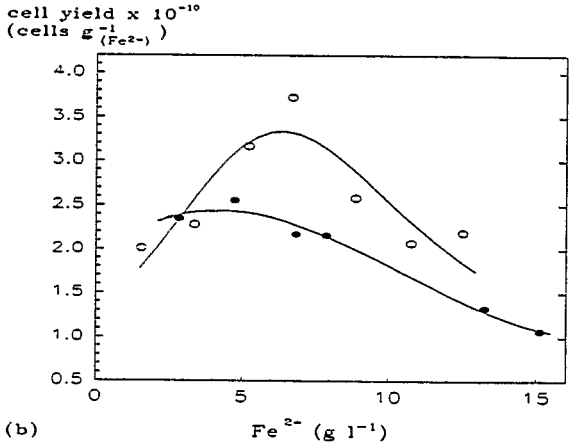


Fig. 3. Influence of ferrous iron concentration on the growth of strains L6 and L8. *(a)* growth rate; *(b)* cell yield. Symbols: (o) strain L6; (•) strain L8.

6 g l^{-1} for two *Leptospirillum*-like bacterial strains (Sand et al. 1992).

The effect of temperature was studied between 20 and 45° C. The maximum exponential growth rate was observed at 37.5° C (Fig. 4). A higher growth rate was observed at 40° C compared to 30° C. Growth rate decreased at higher temperature. The optimal growth temperature of *L. ferrooxidans* was 30° C (Markosyan 1972). Other experiments with *L. ferrooxidans* and *Leptospirillum*-like bacteria have given optimal growth temperatures ranging from 28 to 35° C (Harrison & Norris 1985; Sand et al. 1992). At 35° C, *L. ferrooxidans* and *Thiobacillus* showed equivalent growth rates, but at 20° C, the growth rate of *L. ferrooxidans* was much lower than that of *Thiobacillus*. Thus, *L. ferrooxidans* cannot grow in mixed culture with *Thiobacillus* unless the temperature is sufficiently high (Sand et al. 1992). The optimal temperature for the oxidation of sulfides by the strains of *T. ferrooxidans* and *T. thiooxidans* which composed the mixed population used by BRGM was 32° C (Collinet-Latil 1989). Above 35° C, the development of the mixed culture was affected. The metabolism of strains L6 and L8 reached its maximum activity when the medium was maintained at 37.5° C, that is slightly higher than the optimum growth temperature of thiobacilli and *L. ferrooxidans*. Recently, a moderately thermophilic strain of *Leptospirillum* was isolated (Barrett et al. 1993). This new species, *Leptospirillum thermoferrooxidans*, grows optimally between 45 and 50° C, which is higher than the optimum temperature range for L6 and L8.

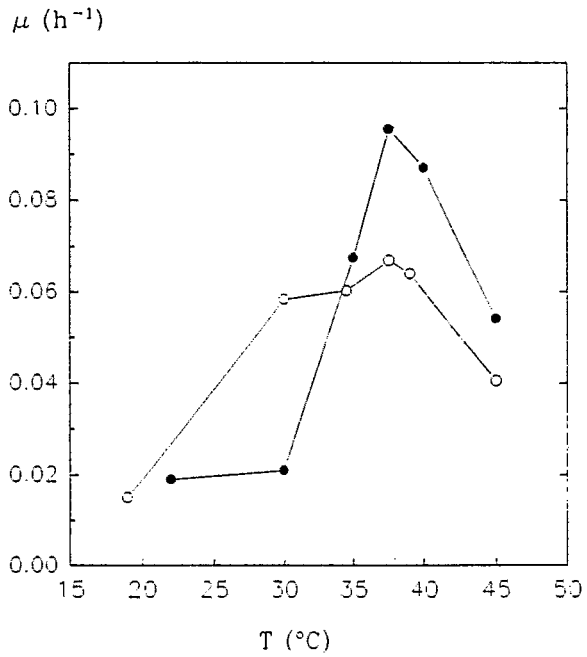


Fig. 4. Influence of temperature on the growth rate of strains L6 and L8. Symbols (○) strain L6; (●) strain L8.

Influence of ferric iron

Strains L6 and L8 were cultured at several initial ferric iron concentrations. Bacterial growth was improved by ferric iron up to 8 g l^{-1} (Fig. 5). This positive effect was observed with the two strains, but was more marked with L6. For the highest ferric iron concentration, i.e. 24 g l^{-1}, the lag time of L6 was extended (Fig. 5a). The same phenomenon was observed for L8 at 14 g l^{-1}, and this strain was fully inhibited at 24 g l^{-1} (Fig. 5b). However, for L6 and L8, the growth at 14 g l^{-1} was as rapid as the growth at 8 g l^{-1}. Collinet & Morin (1990) showed that ferric iron had a negative effect on the growth of thiobacilli starting at 5 g l^{-1}, and the oxidation of pyrite by the mixed population was slowed down at 9 g l^{-1} (Morin et al. 1993). Although *L. ferrooxidans* is known to be less affected by ferric iron than thiobacilli, a competitive inhibition of *Leptospirillum* by ferric iron has been mentioned (Norris et al. 1987). Strains L6 and L8 were usually cultured in presence of 8 g l^{-1} ferric iron. This could explain why their growth was only affected at higher concentrations. However, ferric iron seems to have a stimulating effect on the growth up to 8 g l^{-1}.

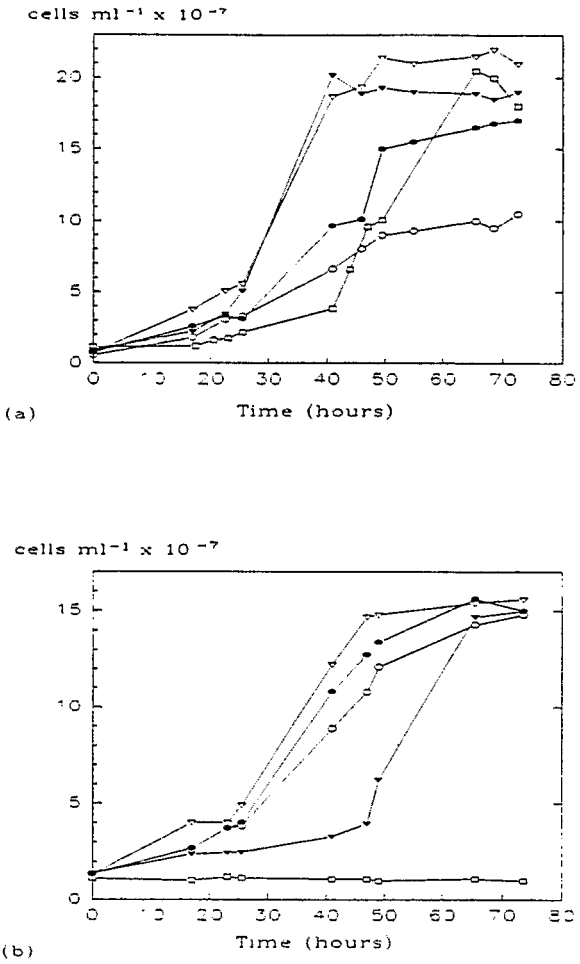


Fig. 5. Influence of ferric iron concentration on the growth of strains L6 and L8 *(a)* strain L6; *(b)* strain L8. Symbols for ferric iron concentrations: (○) 0 g l^{-1}; (●) 4 g l^{-1}; (▽) 8 g l^{-1}; (▼) 14 g l^{-1}; (□) 24 g l^{-1}

Influence of cobalt (Co^{2+})

Cobalt concentrations between 0.5 and 5.0 g l^{-1} were inhibitory (Fig. 6). Final cell density was more affected than growth rate. A decrease of the bacterial yield on ferrous iron was observed at 0.5 g l^{-1} (Fig. 6b). However, growth rate remained maximum up to 1.0 g l^{-1} for L8 and 2.0 g l^{-1} for L6 (Fig. 6a). When growing in a continuous way of bioleaching of a cobaltiferous ore, both bacteria could withstand 5.0 g l^{-1} cobalt (Morin et al. 1993). The mixed population was not inhibited by cobalt up to 15 g l^{-1}. Strains L6 and L8 were more affected by cobalt than the mixed population growing on cobaltiferous pyrite. A similar phenomenon was observed by Harrison & Norris (1985), who noted

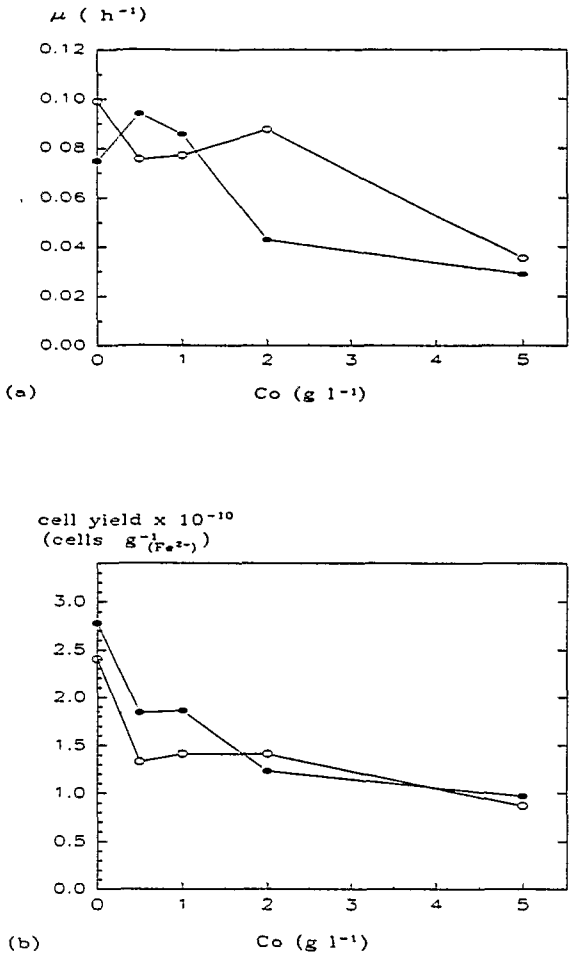


Fig. 6. Influence of cobalt on the growth of strains L6 and L8. *(a)* growth rate; *(b)* cell yield. Symbols: (o) strain L6; (•) strain L8.

that *L. ferrooxidans* was relatively sensitive to sudden exposure to copper while the *Leptospirillum*-like bacteria could withstand high copper concentrations during growth on minerals. They may have lost a major part of their cobalt resistance during the repeated passages in standard liquid medium, which did not contain cobalt.

DNA base composition

The guanine-plus-cytosine content of DNA from strain L8 determined in duplicate by HPLC was 55.6 mol %. Values of 51.7 mol % by the buoyant density method and 54 mol % by chromatography of hydrolysed DNA were obtained for *L. ferrooxidans* (Harrison & Norris 1985). The DNA base composition of various *Leptospirillum*-like bacteria have yielded guanine-plus-cytosine contents between 51 and 57 mol % (Harrison & Norris 1985; Sand et al. 1992). The result for strain L8 is situated within this range.

Conclusion

The mixed culture, originally sampled in a sulfide ore mine, was studied for the first time by Collinet-Latil (1989). Some strains of *T. ferrooxidans* and *T. thiooxidans* were isolated when the population was adapted to arsenopyrite, but no form of *Leptospirillum* was observed (Collinet & Morin 1990). The vibrio-shaped bacteria appeared during the bioleaching of pyrite. Such a phenomenon has already been described (Helle & Onken 1988). The present study shows that the *Leptospirillum*-like strains L6 and L8 could grow at pH values lower than 1.3, and were not affected by 14 g l^{-1} ferric iron. Thus the development of *Leptospirillum*-like bacteria could be favoured during the bioleaching of pyrite because large quantities of ferric iron and sulfuric acid are released by this sulfide.

The comparison of strains L6 and L8 with *L. ferrooxidans* strains described in the literature shows several common characteristics: morphology, substrate specificity, optimal ferrous iron concentration, and resistance to low pH. Nevertheless, the positive effect of ferric iron on the growth had never been mentioned. The relation between the behaviour of the strains towards ferric iron and their adaptation by subculturing in a ferric iron-containing medium remains to be studied. The optimum growth temperature, 37.5° C, is slightly higher than that of *L. ferrooxidans* and *Leptospirillum*-like bacteria described in the literature. Some *Leptospirillum*-like bacteria were present in the mixed population cultured on cobaltiferous pyrite when the bioleaching medium, whose pH was lower than 1.7, contained 5 g l^{-1} cobalt. Strains L6 and L8 were isolated and maintained in standard liquid medium without cobalt, at pH 1.7. The behaviour of strains L6 and L8 in the presence of cobalt shows that these strains are not always resistant to this element. On the other hand, the same bacteria can grow optimally at pH lower than 1.7 after several passages in standard liquid medium at pH 1.7. Unlike their resistance to cobalt, their ability to withstand low pH is independent of the bioleaching conditions.

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