Antonie van Leeuwenhoek **72:** 127–134, 1997.

Thiobacillus sp. W5, the dominant autotroph oxidizing sulfide to sulfur in a reactor for aerobic treatment of sulfidic wastes

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Received 18 July 1996; accepted in final form 4 December 1996

Key words: chemolithoautotrophic, floating filters, sulfide, sulfur, thiobacilli

Abstract

The floating filter technique was successfully adapted for the isolation of the dominant, chemolithoautotrophic, sulfide-oxidizing bacterium from a sulfur-producing reactor after conventional isolation techniques had failed. The inoculated polycarbonate filters, floating on mineral medium, were incubated under gaseous hydrogen sulfide at non-toxic levels. This technique gave 200-fold higher recoveries than conventional isolation techniques. Viable counts on the filters, making up 15% of the total count, appeared to be all of the same species. Chemostat cultures of the new isolate had a very high sulfur-forming capacity, converting almost all hydrogen sulfide in the medium to elemental sulfur under high sulfide loads (27.5 mmol l^{-1} h^{-1}) and fully aerobic conditions. This behaviour closely resembled that of the microbial community in the sulfur-producing reactor. Moreover, similar protein patterns were obtained by electrophoresis of cell-free extracts from the isolate and the mixed culture. It has therefore been concluded that this isolate represents the dominant sulfide-oxidizing population in the reactor. The isolate has been shown to be a new *Thiobacillus* species, related to *Thiobacillus neapolitanus*. In view of the general confusion currently surrounding the taxonomy of the thiobacilli, a new species has not been formally created. Instead, the isolate has been given the working name *Thiobacillus* sp. W5.

Introduction

Sulfide is produced by many anthropogenic activities, and is one of the factors disturbing the natural sulfur cycle. Sulfide in waste water must be treated because it is toxic at low concentrations (Maximum Acceptable Concentration 10 mg m^{-3}), is corrosive, has a high oxygen demand and a very unpleasant odour (threshold value 0.005-0.20 mg m^{-3}). It can be removed by chemical methods (e.g. precipitation, chemical oxidation or air-stripping), but these are expensive, and may generate other wastes. In addition, an increasing need to decrease sulfate emission has led to the development of new treatment techniques. Several years ago, a biotechnological process able to convert sulfide almost stoichiometrically to elemental sulfur was described (Buisman et al. 1989). The major advantages of this process were the low operational costs, the possibility of re-using the biologically-formed sulfur, and a low sulfate discharge, thereby preventing the boosting of the sulfur cycle. Research has indicated that biologically-formed sulfur, which can be removed from the process water by sedimentation, is a better substrate in bioleaching processes than commercially-available flour of sulfur (Tichy et al. 1994).

The composition of a microbial community in a biological reactor system is determined by the operating conditions of the reactor system and the composition of the influent. The fact that the influent of sulfide-converting reactors is dominated by inorganic reduced sulfur compounds, and contains very little organic material gives a selective advantage to obligately chemolithoautotrophic, sulfide-oxidizing bacteria. Based on preliminary work by Stefess (1993), and a model developed by Gottschal & Kuenen (1980), the microbial community in the reactor should contain obligately autotrophic colourless sulfur bacteria. In order to gain detailed insight into the mechanisms of

sulfur formation, it is necessary to study pure cultures of representative bacteria where different variables can be controlled. Understanding these mechanisms should allow the development of a model in which the occurrence of sulfur formation can be predicted. This model can then be used for process control and optimization. Moreover, such experiments may explain why these bacteria, under certain conditions (e.g. oxygen limitation and high sulfide loads), only partially oxidize sulfide to sulfur instead of completely oxidizing it to sulfate. To obtain a representative pure culture, the dominant organism from the sulfur-producing reactor system had to be isolated. However, standard isolation techniques are not always the most suitable for these types of sample. Direct culture of obligate autotrophs from mixed bacterial communities on agar or agarose can fail because of the interference of heterotrophs growing on trace amounts of soluble organic material in the solidifying agents (Kuenen & Tuovinen 1981). Furthermore, it has been observed that the presence of organic material can inhibit obligately autotrophic growth (c.f. Matin 1978). The use of serial dilutions in inorganic liquid media often results in the selection of the bacterial strain with the highest growth rate combined with a high viability under laboratory conditions. An additional problem is that the growth of microorganisms on sulfide, which is chemically labile in the presence of oxygen and potentially toxic to bacteria, requires special cultivation techniques. This paper describes the isolation and characterization of the dominant bacterium from a mixed microbial community in a sulfur-producing laboratory-scale reactor using a modification of a floating filter method that had been successfully used with *Thiobacillus ferrooxidans* cultures (de Bruyn et al. 1990).

Materials and methods

Sample and organisms

The samples from the laboratory-scale sulfur-forming continuously stirred tank reactor (Buisman et al. 1989), which was inoculated with sludge from a sulfur-forming reactor system in Eerbeek (The Netherlands), were collected at 4 °C. The sulfur flocs were mechanically disrupted using a Potter Elvejhem homogenizer. *Thiobacillus neapolitanus*^{T} (LMD 80.58) was used as a reference organism and was obtained from the Delft Culture Collection.

Media for isolation methods and continuous growth in the chemostat

Batch mineral medium (pH 7.5) contained (g l^{-1}): NH_4Cl 0.4; KH_2PO_4 4.0; K_2HPO_4 4.0; $mgSO_4.7H_2O$ 0.8; Na2S2O3.5H2O 2.5 or 7.5 and 2 ml l^{-1} of a trace element solution according to Vishniac & Santer (1957), except that it contained 2.2 g l^{-1} $ZnSO_4.4H_2O$. Rich medium (pH 7.0), for heterotrophic growth, contained (g l^{-1}): sodium lactate 1.0; glucose 2.0; yeast extract 5.0; peptone 5.0 and K_2HPO_4 0.5. Solid media were prepared by the addition of 1.5% Bacto-agar (Difco Laboratories) or 0.8% agarose (Merck) to the liquid media. Where H_2S was the required substrate, plates and liquid cultures in Erlenmeyer flasks were incubated in a desiccator as described below for the modified floating filter technique. Continuous cultivation under sulfide limitation was performed as described before (Visser et al. 1997).

Transient-state experiments

Short-term sulfur formation was studied using transient-state experiments which were described in detail by Stefess et al. (1996). The experiments described in this study were performed at a dissolved oxygen tension of 50% air-saturation, 25 °C, pH 7.5 and 20 min intervals.

Modification of the floating filter method

Diluted samples were aseptically filtered through 0.2 mm polycarbonate membrane filters (Poretics corporation) with a diameter of 25 mm and placed in sterile tissue culture clusters with 6 wells of 35 mm (Costar) containing the mineral medium described for autotrophic batch growth. These were then placed in a desiccator (C) through which a hydrogen sulfide-containing gas was pumped (Figure 1). The hydrogen sulfide was generated by pumping a 20 mM sodium sulfide solution (A) into a vessel containing a 0.18 M sulfuric acid (B) at a rate of 1.4 ml h^{-1}. Auto-oxidation of the sulfide in the sulfide-reservoir was prevented by passing nitrogen through the headspace. Because of the low pH in vessel B, the sulfide equilibrium ($S^{2-} \leftrightarrow HS^- \leftrightarrow H_2S$) shifted towards H_2S. The sulfuric acid solution was sparged with air of 0.7 l h^{-1} thereby carrying hydrogen sulfide to the desiccator. Due to efficient stripping of hydrogen sulfide by the gas stream, sulfide did not accumulate in vessel B. The hydrogen sulfide-containing air was diluted with 1.3 l h^{-1} gas from

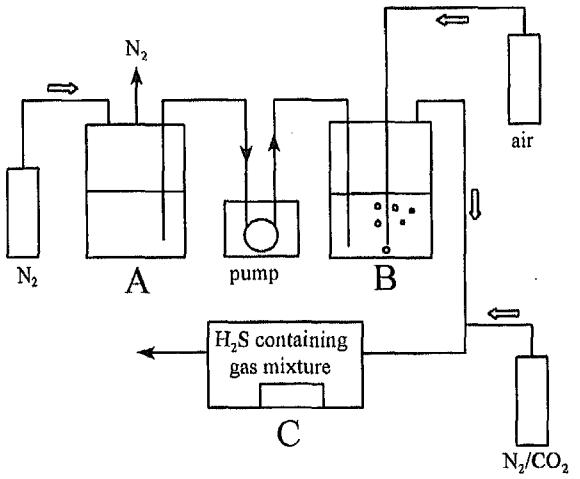

Figure 1. The experimental set-up for the isolation of sulfide-oxidizing bacteria using the floating filter technique. A-sodium sulfide solution, B-dilute sulfuric acid, C-desiccator containing floating polycarbonate filters on liquid mineral medium. The open arrows indicate the direction of the gasflow.

a N_2 / CO_2 cylinder (97.5% / 2.5%) because carbon dioxide enrichment can promote autotrophic growth, whereas high oxygen concentrations can inhibit aerobic autotrophic growth. The composition of the gas stream reaching the desiccator was as follows: 7.3% O_2 / 1.6% CO_2 /91% N_2 / ± 300 ppm H_2S. The concentration in the liquid phase in the desiccator could be calculated using the Henry equation ($H_{H2S} = 4.89 \times 10^9$ Pa at 20 °C) and the pH of the liquid medium ($H_2S \leftrightarrow HS^- + H^+$; $K_a = 3.0 \times 10^{-7}$). The concentration of hydrogen sulfide in the gas stream could easily be regulated by changing the concentration of the sulfide solution in the reservoir, changing the pumping rate or the gas flow. The inoculated polycarbonate filters were incubated at room temperature.

Sulfur formation

The production of intermediary sulfur from sulfide was investigated by monitoring an increase of extinction at 430 nm in a Hitachi double beam spectrophotometer (model 100-60) after a pulse of 200 μM sulfide to a cell suspension (Hazeu et al. 1988).

Biological Oxygen Monitoring and distribution of the bacteria in the sample

Substrate-dependent oxygen uptake rates were measured polarographically at 25 °C with a Clark-type oxygen electrode. In order to minimize auto-oxidation, or toxic effects, substrates were added at the lowest concentrations to give the maximum biological oxidation rate (25 μM sodium sulfide, 25 μM sodium thiosulfate and 40 μM sulfur dissolved in acetone). The Biological Oxygen Monitor was used to estimate the percentages of freely-suspended and attached sulfide-oxidizing biomass in the sulfur-containing sample. The maximum sulfide-dependant oxygen-uptake ($V_{max,sulfide}$) of the complete samples represented the activity of the total (suspended and attached) biomass. Separation of suspended cells and biomass attached to the sulfur flocs was established by overnight sedimentation of the sulfur flocs at 4 °C. The maximum sulfide-dependant oxygen-uptake ($V_{max,sulfide}$) of the supernatant, thus represented the activity of the freely-suspended biomass. The $V_{max,sulfide}$ of the attached biomass was calculated by subtracting the $V_{max,sulfide}$ of the supernatant from the $V_{max,sulfide}$ activity of the complete sample.

Analysis of sulfur compounds

Sulfide in gas streams was removed by passing it through a zinc acetate solution to precipitate as ZnS, and then analyzed by the methylene blue method (Trüper & Schlegel 1964). Sulfide in medium reservoirs was measured by iodometric titration (Rand et al. 1985). Samples from transient-state cultures were filtered over 0.45 μm nitrocellulose filters (Schleicher and Schüll) and immediately washed with cold water to remove water-soluble inorganic reduced sulfur components. The sulfur was extracted overnight in acetone and analyzed by cyanolysis (Bartlett & Skoog 1954). The filtrate was used for determining thiosulfate and tetrathionate colorimetrically after cyanolysis (Kelly 1969), and for determining the concentration of sulfate by ion-exchange HPLC (Machery-Nagel anion exchange column, Nucleosil). The eluent was 0.04 M sodium salicylate (pH 4.0), and a refractive index detector (Waters 410) was used. Sulfite was semiquantitatively determined with commercially-available dipsticks (Merck).

Protein determination and protein electrophoresis

The protein content of whole cells was measured by a modified biuret method (Verduyn et al. 1990). These values were used for the calculation of specific rates. Cell-free extracts were prepared by disrupting cell paste in a French pressure cell, after which the suspen-

sion was centrifuged at 10.000 g for 30 min to remove cell debris and sulfur. The total protein pattern was obtained under denaturing conditions by SDS/PAGE (Laemmli 1970) using a 12% polyacrylamide gel and Mini Protean equipment (Bio-Rad). Gels were stained for protein with Coomassie Brilliant Blue G250.

16S rDNA sequence determination, DNA-DNA homology and ubiquinone content

16S rDNA sequence analysis was carried out at the Deutsche Sammlung von Mikroorganismen (DSM) according to Rainey et al. (1992) and Rainey & Stackebrandt (1993). The EMBL accession number for the 16S rDNA sequence of *Thiobacillus* sp. W5 reported in this paper is X97534. DNA-DNA hybridization studies and ubiquinone content determinations were also performed at the DSM according to de Ley et al. (1970).

Results and discussion

Sampling material from the sulfur-producing reactor system

The samples from the sulfur-producing laboratory scale reactor contained sulfur flocs. Because the composition of the microbial community is influenced by the presence of the sulfur flocs, for example by selective attachment of microorganisms, the distribution of the sulfide-oxidizing bacteria in the sample was investigated (see Materials and methods). The oxygen-uptake rate of the complete sample (suspended and attached biomass), without added sulfur compounds, was 286 $\pm$ 18 μmol O_2 l^{-1} min^{-1}. This oxygen uptake was due to the oxidation of sulfur in the sample. Because of the sulfur, the isolation procedure should be started immediately after sampling to prevent changes in the microbial community. The addition of sulfide, thiosulfate or extra sulfur did not increase oxygen uptake, indicating that the rate measured in the presence of the sulfur flocs was the maximum oxygen-uptake capacity (V_{max}) of the biomass. The freely-suspended bacteria showed no significant oxygen uptake without the addition of reduced sulfur compounds. Addition of sulfide or sulfur resulted in maximum oxygen uptake rates of 28 $\pm$ 3 μmol O_2 l^{-1} min^{-1} and 25 $\pm$ 4 μmol O_2 l^{-1} min^{-1}, respectively. The maximum oxygen consumption of the biomass attached to the sulfur flocs was therefore calculated to be 258 $\pm$ 21 μmol O_2 l^{-1} min^{-1}. Assuming that the specific maximum oxygen uptake rates of the free and attached cells were the same, it could be calculated that about 90% of the sulfide-oxidizing biomass was attached to the sulfur. Successful isolation procedures therefore depended on detaching the bacteria from the sulfur flocs. Indeed, mechanically disrupting the sulfur flocs caused an almost 8 fold increase from 1.8 $\pm$ 0.2 $\times$ 10^8 to 1.4 $\pm$ 0.3 $\times$ 10^9 cells ml^{-1}. This is in close agreement with the observations stated above.

Conventional isolation methods

Thiosulfate is commonly used as a substrate in obtaining pure cultures of obligately chemolithoautotrophic sulfide-oxidizers (Kuenen et al. 1992). It is relatively stable at neutral pH, is easily soluble and is non-toxic at higher concentrations. Streaking diluted samples on solid media containing different concentrations of thiosulfate (10 or 30mM) as the energy source and agar or agarose as the solidifying agents gave similar results. The number of colony forming units (CFU) obtained was, on average 2.6 $\pm$ 1.0 $\times$ 10^6 CFU ml^{-1}, representing 0.2% of the bacteria directly counted in the inoculum. However, approximately 80% of the isolates thus obtained could not oxidize reduced sulfur compounds, but were heterotrophs growing on the organic compounds in the agar and agarose. The plating efficiency for the obligate chemolithoautotrophs was therefore significantly lower than 0.2%. Tests for the presence of facultative chemolithotrophs were negative. When a dilution series in liquid thiosulfate-containing mineral medium was made, growth was observed up to the 10^5 dilution (equivalent to 1 - 9 $\times$ 10^5 cells ml^{-1} or 0.007 - 0.06% of the inoculum). Although these low counts indicated that the enrichment procedures were far from optimal, it was decided to isolate the organism present at the highest dilution. This culture, named Isolate W, was not able to grow on plates or in liquid media with organic substrates, and therefore appeared to be an obligately chemolithoautotrophic organism.

Sulfur formation by Isolate W

Isolate W was grown in a chemostat under sulfide limitation. Short-term transient-state experiments, designed to measure sulfur production by growing cell suspensions under increasing sulfide loads (Stefess et al. 1996), were then carried out with these cells. Sulfur production started at a sulfide load of 14 mmol l^{-1} h^{-1} (Figure 2a). Measurements were terminated at

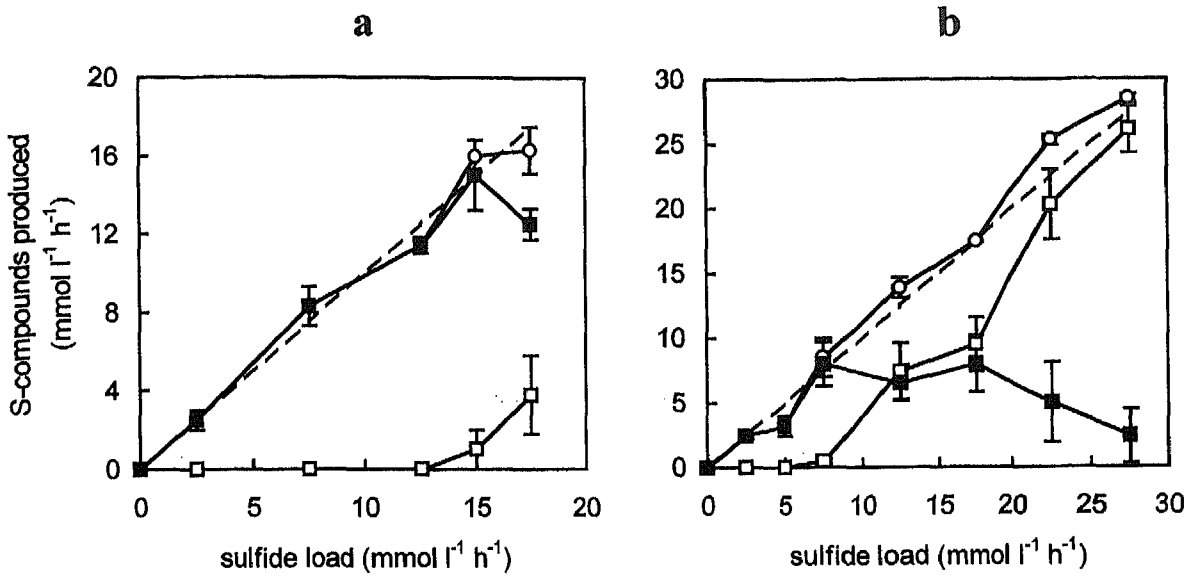

Figure 2. Rates of product formation from sulfide by Isolate W (a) and Isolate W5 (b) during transient-state experiments at different sulfide loads at 50% air saturation. The sulfur balance (○) was calculated from the production rates of sulfur (□) and sulfate (■). The dashed line represents the theoretical 100% S-recovery.

a sulfide load of 18 mmol l^{-1} h^{-1} because a further increase in the sulfide load resulted in the accumulation of sulfide in the medium and off-gas. Other sulfur compounds, such as thiosulfate, tetrathionate and sulfite, were not detected during the experiment. Under these conditions, Isolate W was thus capable of converting a maximum of 20% of the incoming sulfide to elemental sulfur, while the rest was oxidized to sulfate. This is in sharp contrast to the conversion capacity of the biomass in the sulfur-forming reactor system, which converted all of the incoming sulfide to elemental sulfur (Buisman et al. 1991). It was therefore concluded that conventional isolation methods had not resulted in the isolation of a sufficiently representative colourless sulfur bacterium from the sulfur-producing reactor system. It became obvious that other isolation methods were necessary.

Floating filters

During the isolation experiments using floating polycarbonate filters, the concentration of hydrogen sulfide in the gas flow was maintained at ± 300 ppm, which is equivalent to a dissolved total sulfide concentration of approximately 5 μM. This is well below the inhibitory concentrations of 150 μM to 1 mM reported for thiobacilli (Hirayama & Vetter 1989). A few days after inoculation, small, transparent, circular colonies could be observed on the filters. Enumeration showed 2.0 ± 0.4 × 10^8 CFU ml^{-1}, which is almost 15% of the total count. This is exceptionally high for (semi)natural samples, and shows that the efficiency of the floating filter method for the isolation of obligately chemolithoautotrophic sulfide-oxidizing bacteria was at least 200-fold higher than the efficiency of the conventional techniques. In order to obtain pure cultures, ten colonies (isolates W1-10) were repeatedly transferred to new floating filters. The colony morphologies of these ten strains were identical, but different from Isolate W. The isolates grew rapidly in liquid mineral medium with gaseous sulfide and on agar under the same H_2S-atmosphere. However, the plating efficiencies were lower on the agar plates than on the polycarbonate filters, indicating possible inhibition by components in the agar. The isolates did not grow in thiosulfate-containing mineral media, even though suspended cells were able to oxidize thiosulfate at roughly the same rate as they could oxidize sulfide. This inability to grow on thiosulfate under these conditions might be related to the redox potential of the environment (Sokolova & Karavaiko 1968), but this remains to be confirmed. The use of hydrogen sulfide rather than thiosulfate as a substrate for these isolation

experiments clearly favoured a great proportion of the microbial community.

Oxidation capacities and sulfur-formation of batch-grown floating filter isolates

The oxidation capacities of the isolates for sulfide, thiosulfate and sulfur were 0.98 ± 0.06, 0.91 ± 0.04 and 0.88 ± 0.05 μmol O_2 (mg protein)$^{-1}$ min^{-1}, respectively. Preliminary estimates of sulfur-formation rates of the isolates were made with a semi-quantitative spectrophotometric method (Hazeu et al. 1988), with *Thiobacillus neapolitanus* as a reference organism. All floating filter isolates produced sulfur at specific rates of 1.4 Absorption Units (mg protein)$^{-1}$ min^{-1}, which was approximately 2.5 times faster than *T. neapolitanus*.

Because all of the floating filter isolates appeared to be identical, only one of them, Isolate W5, was selected for more quantitative assessment and grown in a sulfide-limited chemostat at a D=0.05 h^{-1}. Subsequent transient-state experiments showed that Isolate W5 started to produce sulfur at a sulfide load of 8 mmol l^{-1} h^{-1}. Further increasing the sulfide load gave increased sulfur-production rates, while the sulfate-production rates decreased. At a sulfide load of 27.5 mmol l^{-1} h^{-1}, almost all incoming sulfide was converted to sulfur (Figure 2b). Thiosulfate, tetrathionate and sulfite could not be detected. Sulfide loss occurred when the sulfide load was higher than 27.5 mmol l^{-1} h^{-1}. This is in good agreement with the behaviour of the microbial community in the sulfur-producing reactor system. The results showed that Isolates W and W5 differed in their sulfur-producing capacity. Stefess et al. (1996) also observed different sulfur-producing capacties with different strains. It thus seems that each species must be separately evaluated.

Identification of floating filter Isolate W5

Isolate W5 did not grow on organic substrates and did not show substrate-dependent oxygen uptake when the following substrates were tested (0.1 mm): glucose, acetate, pyruvate, citrate, malate, formate, methanol, formamide and dimethyl sulfoxide. It was therefore concluded that the organism was an obligate chemolithoautotroph. The isolate was a short, gram-negative rod (0.5 × 1.0-1.5 μm), with a single polar flagellum. It was oxidase and catalase positive. It did not denitrify and could not oxidize thiocyanate. The optimum temperature for growth was 25–30 °C, while growth did not occur above 42 °C and below 12 °C. Growth occurred above pH 3 and below pH 9 with an optimum between pH 7 - 7.5. The physiological properties and morphology corresponded with the taxonomic description of the genus *Thiobacillus* (Kelly and Harrison 1989; Kuenen et al. 1992). Isolate W5 contained ubiquinone 8 (Q-8), which is a marker of obligately autotrophic *Thiobacillus* species (Katayama-Fujimura et al. 1982). Its G+C-content was 56 ± 0.5 mol%, which is comparable to the G+C-content of *T. neapolitanus* (52 - 56 mol%) and *Thiobacillus capsulatus* (54.5 mol%) (Kuenen et al. 1992).

The 16S rDNA analysis (1446 bp) of Isolate W5 corresponded closely with that of *T. neapolitanus* (99.7 % homology). This together with the more 'classical' features of Isolate W5 (e.g. morphology, physiology, chemotaxonomy) clearly indicates that, under the taxonomic description presented in the current taxonomic handbooks, the isolate should be placed in the genus *Thiobacillus*, and that it is closely related to *T. neapolitanus*. *Thiobacillus neapolitanus* has a position at the borderline between the β- and γ-subgroups of the *Proteobacteria* (Lane et al. 1992), indicating a distant relationship to the type species of the genus, *Thiobacillus thioparus*. The use of 16S rDNA for taxonomy of the genus *Thiobacillus* has revealed that strains from the genus appear to be spread over the various α- and β-subgroups of the *Proteobacteria*. This implies that the genus will have to be redefined.

Despite the great 16S rDNA homology between Isolate W5 and *T. neapolitanus*, there was little DNA-DNA homology (51.8%). In the context of the recommendations of the ad hoc committee on reconciliation of approaches to bacterial systematics (Wayne et al. 1987), and recent other publications (Coates et al. 1996; Fox et al. 1992; Martinez-Murcia et al. 1992), it is clear that an apparently close match in 16S rDNA analysis is not sufficient to indicate species identity, but that at least 70% DNA homology is required. Because the taxonomy of the genus *Thiobacillus* is currently confused (Kelly & Harrison 1989; Lane et al. 1992), the situation would not be helped by the creation of yet another new species. It has therefore been decided to give this isolate the working name *Thiobacillus* sp. W5. It has been deposited in the Delft Culture Collection under this name (LMD 94.73).

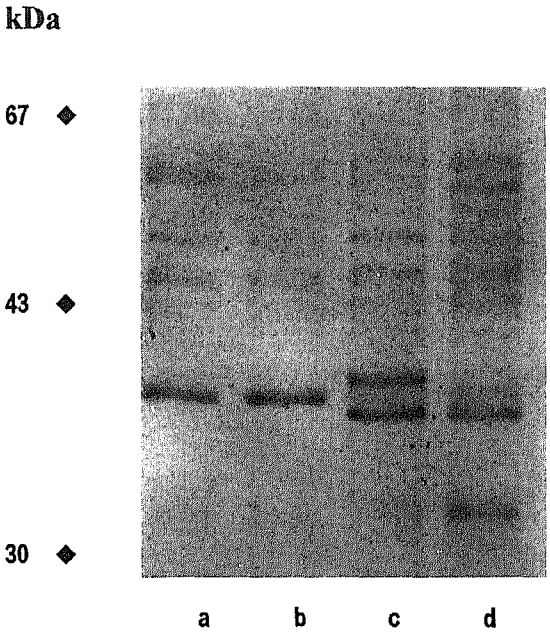

Figure 3. Protein pattern of total cell protein on a denaturing 12% polyacrylamide gel from (a) mixed microbial community (b) *Thiobacillus* sp. W5 (c) *T. neapolitanus* (d) Isolate W. All organisms were grown on sulfide and the protein loads were approximately equal.

Importance of Thiobacillus sp. *W5 in the bioreactor community*

As indicated earlier, the new isolates from the floating filters made up 15% of the total direct counts under the microscope. Fifteen percent is an unusually high recovery for samples from such a reactor system. Combined with the fact that all colonies appeared to be identical, this high recovery makes it probable that *Thiobacillus* sp. W5 is the dominant sulfide-oxidizing bacterium. Moreover, the sulfur-producing capacity of *Thiobacillus* sp. W5 resembled that of the microbial community in the reactor. Comparison of the protein patterns from pure and mixed cultures, generated by gel electrophoresis, provided support for the dominance of *Thiobacillus* sp. W5 in the community (Figure 3). This method has been used extensively for the identification and classification of bacteria (Kersters & de Ley 1980). It clearly showed an unidentified major protein band at 38 kDa in the original sample from the reactor and in *Thiobacillus* sp. W5. The protein patterns obtained with *T. Neapolitanus* and Isolate W differed. Having isolated the dominant organism from the sulfur-producing reactor, the way is now open to study in detail the mechanisms involved in the formation of elemental sulfur by partial oxidation of sulfide.

Acknowledgements

We thank Dr. Ir. Albert Janssen (Agricultural University of Wageningen) for enabling us to obtain samples from the sulphur-producing reactor system. We also thank Anke de Bruyn for her help with the floating filters and Robert Krösing for technical assistance. This work (Project IMB 91208) was financially supported by the IOP on Environmental Biotechnology which is financed by the Ministry of Economic Affairs and the Ministry of Housing, Physical Planning and the Environment.

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