APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1993, p. 1176–1180
0099-2240/93/041176-05$02.00/0

Vol. 59, No. 4

Isolation and Characterization of a Mo^{6+}-Reducing Bacterium

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Received 11 September 1992/Accepted 21 January 1993

A Mo^{6+}-reducing bacterium (strain 48), which grew on medium supplemented with 200 mM Mo^{6+}, was isolated from stream water obtained from Chengkau, Malaysia. The chemical properties of strain 48 conform to the characteristics of *Enterobacter cloacae*. Under anaerobic conditions in the glucose-yeast extract medium containing phosphate ion (2.9 mM) and Mo^{6+} (10 mM), the bacterium reduced Mo^{6+} to form molybdenum blue. Approximately 27% of Mo^{6+} added to the medium was reduced after 28 h of cultivation. The reduction of Mo^{6+} with glucose as an electron donor was strongly inhibited by iodoacetic acid, sodium fluoride, and sodium cyanide, suggesting an involvement of the glycolytic pathway and electron transport in Mo^{6+} reduction. NADH and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine served as electron donors for Mo^{6+} reduction. When NADH was used as an electron donor, at first cytochrome *b* in the cell extract was reduced, and then molybdenum blue was formed. Sodium cyanide strongly inhibited Mo^{6+} reduction by NADH (5 mM) but not the reduction of cytochrome *b* in the cell extract, suggesting that the reduced component of the electron transport system after cytochrome *b* serves as an electron donor for Mo^{6+} reduction. Both ferric and stannous ions strongly enhanced the activity of Mo^{6+} reduction by NADH.

It has been known that bacteria have the abilities not only to oxidize metal ions but also to reduce them. The reduction of metal ions by microorganisms has an important role not only in the biological system but also in the cycling of metals in the environment. One of the most important examples for metal reduction in the biological system is Fe^{3+} reduction. Ferrochelatase is the terminal enzyme of heme biosynthesis and catalyzes the insertion of Fe^{2+} into the protoporphyrin nucleus. To supply Fe^{2+} as a substrate for ferrochelatase, the Fe^{3+} incorporated into the cells must be reduced. The reduction of iron was observed with cell extracts of *Spirillum itersonii*, *Escherichia coli*, and *Paracoccus denitrificans* (3). The membrane-bound Fe^{3+} reductase, which reduces Fe^{3+} with NADH or succinate, was found in *S. itersonii*, and the effects of respiratory inhibitors suggested that reduction of Fe^{3+} occurs at one or more sites on the respiratory chain before cytochrome *c* (3, 7). The ferric reductases found in *Azotobacter vinelandii* and *Pseudomonas aeruginosa* use NADH as an electron donor for the reduction of Fe^{3+}. These enzymes were found to be located in the cytoplasm (2, 4).

The reduction of Fe^{3+}, Mn^{4+}, Cu^{2+}, Cr^{6+}, and Se^{6+} by bacteria has been reported (1–5, 7, 9–14, 18, 19, 24, 27, 28). In some bacteria the detoxification of toxic metal ions is done by reducing them. The enzymatic reduction of ionic Hg^{2+} to highly volatile Hg^0 has been known to be the most important detoxification mechanism for mercury-resistant bacteria against the toxic Hg^{2+} (17). Chromate is also toxic for most organisms. Recently, Ohtake showed that washed cells of *Enterobacter cloacae* reduced chromate under anaerobic conditions and that the chromate reductase activity was associated with the membrane fraction of the cells (27, 28).

Molybdenum ion is one of the most important metals for biocatalyst activity, such as nitrogenase, nitrate reductase, and sulfite reductase. There have been few reports on microbial reduction and oxidation of Mo. Recently, it was found that in the presence of phosphate ions, the iron-oxidizing chemolithotrophic bacterium *Thiobacillus ferrooxidans* reduces Mo^{6+} with elemental sulfur as an electron donor to produce molybdenum blue (26). In *T. ferrooxidans*, a hydrogen sulfide ferric ion oxidoreductase catalyzes the reduction of not only Fe^{3+} but also Mo^{6+} and Cu^{2+}, with hydrogen sulfide as an electron donor (18, 20, 22, 23, 25, 26). Molybdenum blue thus formed was oxidized enzymatically by molybdenum oxidase in this bacterium (21). However, there have been no reports on the enzymatic reduction of molybdenum by heterotrophic bacteria.

In this work, isolation and characterization of the molybdenum-resistant bacterium that reduces Mo^{6+} with glucose as an electron donor is presented. The results suggest that the reduced component of electron transport system after cytochrome *b* serves as an electron donor for Mo^{6+} reduction. To our knowledge, this is the first report on Mo^{6+} reduction by a heterotrophic bacterium.

MATERIALS AND METHODS

Microorganism, media, and conditions of cultivation. The bacterium used in this study was isolated from a stream in Chengkau, Malaysia. The method for the isolation of the Mo^{6+}-reducing bacterium was as follows. Stream water was incubated under anaerobic conditions at 30°C in a 2.9 mM phosphate medium (pH 7.0) containing glucose (1%), yeast extract (0.05%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4 \cdot 7H_2O$ (0.05%), Na_2HPO_4 (0.05%), NaCl (0.5%), and $Na_2MoO_4 \cdot 2H_2O$ (0.242%). When bacteria grew on this medium, the culture medium turned blue. This is because of the formation of molybdenum blue, i.e., Mo^{6+} was reduced by microorganisms in the medium and the Mo^{5+} thus formed made a complex with phosphate ion to form molybdenum blue. Aliquots of the culture medium were spread over agar plates

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containing 2.9 mM phosphate medium. A deep-blue colony appearing on the plate was isolated. The isolate (strain 48) was preserved on an agar plate containing 2.9 mM phosphate medium and used throughout this study. A 100 mM phosphate medium (pH 7.0) was used for the large-scale production of strain 48 cells. The only difference between 100 mM phosphate medium and 2.9 mM phosphate medium is the phosphate concentration.

Preparation of cell extract. Strain 48 was grown on 100 mM phosphate medium under anaerobic conditions at 30°C for 24 h. Washed, intact cells were prepared by washing the harvested cells with salts solution (pH 7.0) containing $(NH_4)_2SO_4$ (0.3%), $MgSO_4 \cdot 7H_2O$ (0.05%), and NaCl (0.5%). Washed, intact cells were disrupted by sonic oscillation (20 kHz) for 30 min and centrifuged at 12,000 × *g* for 20 min. The supernatant fraction (cell extract) was further centrifuged at 105,000 × *g* for 60 min to obtain a supernatant fraction (cytosol fraction) and a precipitated fraction (membrane fraction).

Mo^{6+} reductase activity. The activity of Mo^{6+} reductase was determined by measuring the amount of molybdenum blue formed in the reaction mixture. Although the cells growing in 100 mM phosphate medium did not produce molybdenum blue, cells and cell extracts contained active enzyme. Therefore, we used cells grown in 100 mM phosphate medium for preparation of all intact cells and cell extracts. The reaction mixture contained intact cells (5 mg of protein) or cell extract (20 mg of protein), salts solution (8 ml), Na_2MoO_4 (50 μmol), Na_2HPO_4 (1 μmol), and glucose (100 μmol). The total volume was 10 ml, and the gas phase of the reaction vessel was nitrogen gas. When NADH was used as an electron donor, the reaction mixture contained the cell extract or the cytosol fraction (20 mg of protein), 1 ml of salts solution (pH 7.0), Na_2MoO_4 (200 μmol), Na_2HPO_4 (10 μmol), and NADH (4 μmol). Total volume was 2.0 ml. When *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was used as an electron donor, 100 μmol of sodium ascorbate was added to the reaction mixture. The reaction was carried out in a 1-cm light path cuvette. The amount of molybdenum blue in the reaction mixture was determined from a standard curve by measuring A_{710} (26). A good linearity was obtained between the concentration of Mo^{6+} (0.1 to 1.5 μmol of Mo^{6+}), which was reduced chemically by $SnCl_2 \cdot 2H_2O$ in the presence of phosphate ion, and the developed blue color.

Other chemical methods. DNA base composition was determined by a method described previously (6). The protein content was determined by the biuret method (8), with crystalline bovine serum albumin as the reference protein.

RESULTS AND DISCUSSION

Morphology and physiological characteristics of the Mo^{6+}-reducing bacterium. Strain 48 was a gram-negative, nonspore-forming, rod-shaped bacterium that had an optimum pH for growth of 7.0. The main biochemical characteristics of strain 48 were the same as those of *E. cloacae* (Table 1). The mean G + C content of the DNA was 51.7 mol% for strain 48. The following organic compounds were assimilated by strain 48: glycerol, erythritol, L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-glucoside, *N*-acetylglucosamine, arbutine, esculine, salicine, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch, gentiobiose, D-turanose, D-lyxose, gluconate, 2-ketogluconate, acetate, succinate, fumarate. DL-lactate, DL-glycerate, DL-malate, pyruvate, 2-ketoglutarate, aconitate, citrate, phenylacetate, DL-alanine, L-serine, L-threonine, L-cysteine, L-histidine, L-aspartate, L-glutamate, L-ornithine, L-proline, diaminobutane, and glucosamine. The G + C contents of the DNA are 52 to 54, 57, 53 to 58, 53 to 54, and 60 mol% for *E. cloacae*, *Enterobacter sakazaki*, *Enterobacter agglomerans*, *Enterobacter aerogenes*, and *Enterobacter gergoviae*, respectively (16), supporting the hypothesis that strain 48 is *E. cloacae*.

TABLE 1. Morphological and biochemical characteristics of strain 48

Test or characteristic	Strain 48	*E. cloacae*
Gram stain	−	−
Morphology	Rod	Rod
Motility	+	+
Yellow pigment	−	−
Catalase	+	+
Oxidase	−	−
Gas from glucose at 37°C	+	+
Citrate utilization	+	+
Indole	−	−
Voges-Proskauer	+	+
Methyl red	−	−
Nitrate reductase	−	+
β-Galactosidase	+	+
Decarboxylase		
Ornithine	+	+
Arginine	+	+
Lysine	−	−
G + C content (mol%)	51.7	52 to 54

Growth of strain 48 on 2.9 and 100 mM phosphate media. When strain 48 grew under anaerobic conditions on a 2.9 mM phosphate medium containing glucose, salts, yeast extract, 10 mM Mo^{6+}, and 2.9 mM phosphate ion, the color of the medium turned blue (Fig. 1). The amount of molybdenum blue produced increased concomitantly with cell growth. Approximately 27% of Mo^{6+} added to the medium was reduced to form molybdenum blue at 28 h of cultivation (Fig. 1). After 28 h of cultivation, the concentrations of both

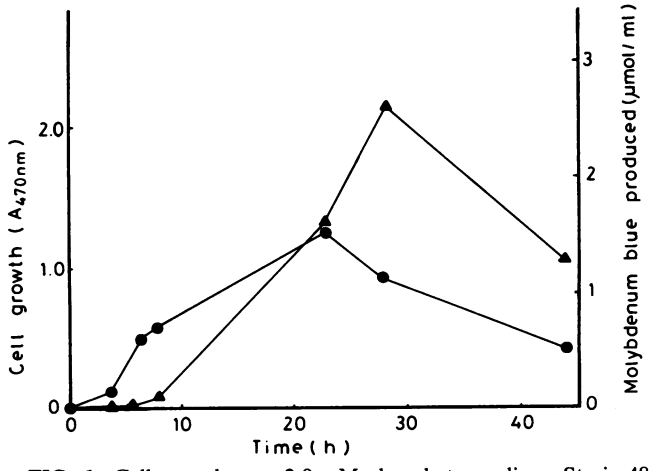

FIG. 1. Cell growth on a 2.9 mM phosphate medium. Strain 48 was grown on glucose-yeast extract-salts medium containing 2.9 mM sodium phosphate and 10 mM sodium molybdate under anaerobic conditions. Symbols: ●, cell growth; ▲, concentration of molybdenum blue.

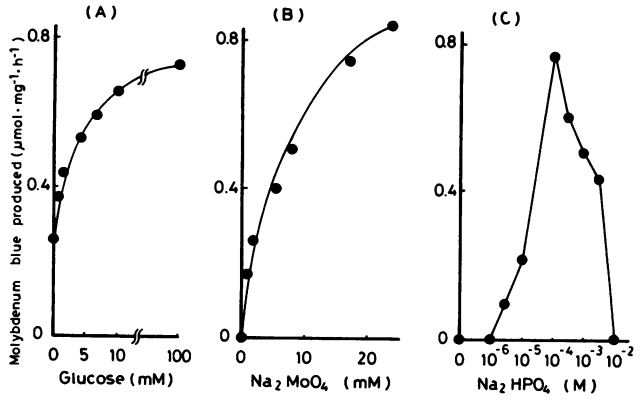

FIG. 2. Effects of glucose (A), sodium molybdate (B), and sodium phosphate (C) concentrations on the activity of molybdenum blue formation by washed, intact cells of strain 48.

TABLE 2. Reduction of Mo^{6+} by various sugars as an electron donor

Sugar	Molybdenum blue produced[a] (μmol · mg^{-1} · 18 h^{-1})
Sucrose	0.91
Glucose	0.80
Mannose	0.67
Lactose	0.65
Maltose	0.61
Galactose	0.57
Fructose	0.45
Sorbitol	0.37
Raffinose	0.37
Ribose	0.12
Xylose	0.08
Arabinose	0.02

[a] The reduction of Mo^{6+} was studied under anaerobic conditions with strain 48 cell extract with sugar as an electron donor. The rate of molybdenum reduction was linear, in most cases, from 9 to 18 h, and the maximum amount of molybdenum blue formed after 18 h is shown.

cells and molybdenum blue in the medium decreased. This was because of the complexation of molybdenum blue to the cells and precipitation of cell-molybdenum blue aggregates. Strain 48 grew just as well in 100 mM phosphate medium, but molybdenum blue was not formed.

Properties of molybdenum reductase. Although the cells growing in 100 mM phosphate medium did not produce molybdenum blue, cells and cell extracts contained active enzyme, i.e., specific activities of molybdenum reductase of the cells grown in 2.9 and 100 mM phosphate media containing 10 mM Mo^{6+} were 0.67 and 0.65 μmol of molybdenum blue produced per mg of protein per h, respectively. The properties of molybdenum reductase were studied with the washed, intact cells grown in a 100 mM phosphate medium. The 2.9 mM phosphate medium was not adequate for the medium to obtain cells, because when strain 48 grew on this medium, a portion of the cells aggregated with each other and precipitated at the bottom of the culture flask, to which a part of molybdenum blue attached (Fig. 1). However, this flocculation of cells was not observed in the cells grown on a 100 mM phosphate medium. The molybdenum reductase activity (0.24 μmol of molybdenum blue produced per mg of protein per h) was present in the cells grown on a 100 mM phosphate medium without Mo^{6+}. However, the specific activity of molybdenum reductase in strain 48 increased concomitantly with the increment of Mo^{6+} in 100 mM phosphate medium.

When the reduction of Mo^{6+} was studied with washed, intact cells with glucose as an electron donor, Mo^{6+} was reduced under anaerobic conditions but not under aerobic conditions (data not shown). Molybdenum reductase had a pH optimum of 7.0 (data not shown), and the activity increased in proportion to the concentration of glucose, Mo^{6+}, and phosphate ion added to the reaction mixture (Fig. 2). However, phosphate ion concentration greater than 0.5 mM markedly inhibited Mo^{6+}-reducing activity of the cells. This explained why strain 48 did not produce molybdenum blue in the 100 mM phosphate medium.

The reduction of Mo^{6+} by cell extract was supported by glucose, fructose, galactose, mannose, maltose, sucrose, lactose, raffinose, and sorbitol as electron donors (Table 2). Xylose, ribose, and arabinose were not effective as electron donors. A long lag phase (approximately 8 h) was needed for the start of Mo^{6+} reduction, suggesting that the metabolite(s) of sugars was used as an actual electron donor for Mo^{6+} reductase, because the metabolism of sugars into reductant must have been limited by other substrates or cofactors, etc., in the cell extract.

The reduction of Mo^{6+} was strongly inhibited by 1 mM iodoacetic acid and 10 mM sodium fluoride, respectively (Fig. 3). These are known inhibitors of glyceraldehyde 3-phosphate dehydrogenase and phosphopyruvate hydratase, respectively, suggesting that a glycolytic pathway was involved in Mo^{6+} reduction. The Mo^{6+} reduction was also strongly inhibited by 5 mM sodium cyanide, which suggested an involvement of the electron transport system in Mo^{6+} reduction.

Involvement of the electron transport system in Mo^{6+} reduction. The preceding results suggested that a reductant formed from sugar metabolism that could be transported by the cell cytochrome system was required for Mo^{6+} reduction. Therefore, the reduction of Mo^{6+} by NADH was studied in cell extracts. Just after the addition of NADH, the A_{568} increased concomitantly with the shift of the soret band from 420 to 430 nm, indicating that, probably, cytochrome *b* in the cell extract was reduced by NADH (Fig. 4). After the reduction of cytochrome *b* by NADH, A_{710} increased, indicating that molybdenum blue was formed in the reaction

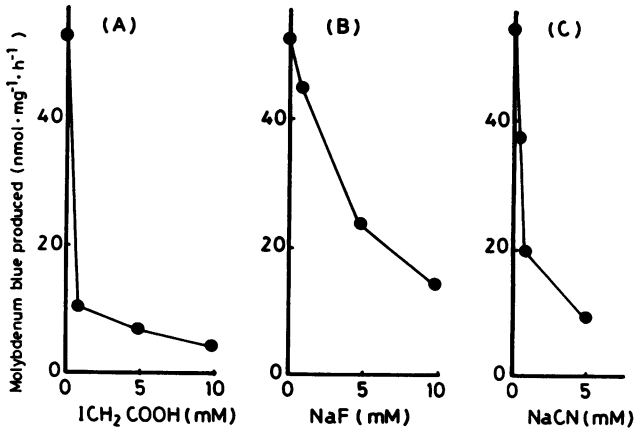

FIG. 3. Effects of idoacetic acid (A), sodium fluoride (B), and sodium cyanide (C) on Mo^{6+}-reducing activity. The reduction of Mo^{6+} was studied with cell extract and glucose as an electron donor.

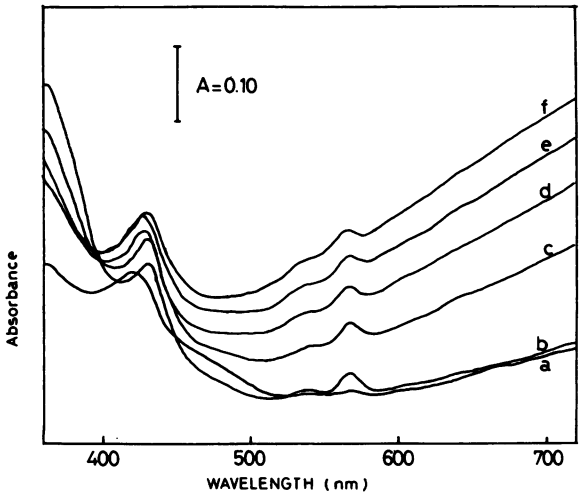

FIG. 4. Mo^{6+} reduction by NADH as an electron donor. The reduction of Mo^{6+} was studied with cell extract. a, spectrum of the complete reaction mixture lacking NADH; b to f, spectra of the complete reaction mixture just after the addition of NADH (b) or 1 min (c), 3 min (d), 6 min (e), and 10 min (f) after the addition of NADH.

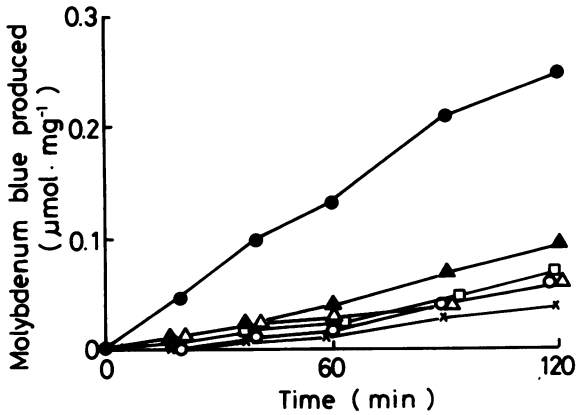

FIG. 5. Mo^{6+} reduction by ascorbate-reduced TMPD as an electron donor. Mo^{6+} reduction was studied with the cytosol fraction of strain 48. The TMPD in the reaction mixture was reduced with 10 mM sodium ascorbate. Results for molybdenum blue production in the complete reaction mixture without Fe^{3+} and Sn^{2+} (□), in the complete reaction mixture supplemented with 2 mM Fe^{3+} (●), and in the complete reaction mixture supplemented with 2 mM Sn^{2+} (▲); molybdenum blue production by the cytosol fraction boiled previously for 10 min (×); and molybdenum blue production by the cytosol fraction boiled previously for 10 min in the presence of 2 mM Fe^{3+} (○) or 2 mM Sn^{2+} (△) are shown.

mixture. When 10-min-boiled cell extract was used as an enzyme source, neither A_{568} nor A_{710} increased, indicating that the reduction of Mo^{6+} by NADH was catalyzed by enzyme. A long lag phase that was observed when sugar was used as an electron donor (Table 2) was not observed when NADH was used as an electron donor, suggesting that NADH was formed from sugar metabolism and used as an electron donor for Mo^{6+} reduction. Sodium cyanide completely inhibited the reduction of Mo^{6+} by NADH. Interestingly, it did not inhibit the reduction of cytochrome *b* in the cell extract by NADH (data not shown), suggesting that the reduced component(s) of electron transport system after cytochrome *b* was involved in Mo^{6+} reduction.

Enhancement of Mo^{6+} reduction by metal ions. The activity of Mo^{6+} reduction was also observed not only in cell extract but also in the cytosol fraction prepared from the cell extract of strain 48. The effect of metal ions on Mo^{6+} reductase activity was studied with the cytosol fraction. Ferric and stannous ions at 2 mM markedly enhanced Mo^{6+}-reducing activity approximately nine- and eightfold, respectively (Table 3). Nickel, zinc, and cobaltous ions slightly enhanced the activity. However, the Mo^{6+}-reducing activity was not activated by ferrous, silver, and manganese ions. Cupric and chromium ions markedly inhibited the activity of Mo^{6+} reduction.

TABLE 3. Effects of metal ion on Mo^{6+}-reducing activity

Metal ion (2 mM)	Molybdenum blue produced[a] (μmol · mg^{-1} · h^{-1})
Enzyme without metal ion	0.02
10-min-boiled enzyme	0.00
10-min-boiled enzyme with each metal ion[b]	0.00
Fe^{3+}	0.28
Sn^{2+}	0.25
Ni^{2+}	0.11
Zn^{2+}	0.10
Co^{2+}	0.08
Fe^{2+}	0.05
Ag^{2+}	0.04
Mn^{2+}	0.02
Cu^{2+}	0.00
Cr^{6+}	0.00

[a] Mo^{6+}-reducing activity was studied with the cytosol fraction of strain 48 and NADH as the electron donor.
[b] Mo^{6+} reduction with NADH, 10-min-boiled enzyme, and each metal ion shown.

In the presence of 2 mM Fe^{3+}, the Mo^{6+} in the reaction mixture was reduced with ascorbate-reduced TMPD (Fig. 5). Since TMPD is a well-known electron donor for cytochrome *o* or *d* terminal oxidase (15), there is a possibility that reduced cytochrome *o* or *d* is an actual electron donor for Mo^{6+} reductase. Stannous ion, which markedly enhanced the activity of Mo^{6+} reduction by NADH (Table 3), only slightly enhanced the activity of Mo^{6+} reduction by ascorbate-reduced TMPD as an electron donor.

Molybdenum reduction by *E. cloacae* 48 is a quite interesting phenomenon, not only on the scientific point of view but also on the application of this bacterium to the elimination of toxic heavy metals or Mo^{6+} from the environment. Data showed that a part of molybdenum ions added to the medium (approximately 15%) was removed from the culture medium as a precipitate by the action of this bacterium by the following mechanism: (i) attachment of a portion of the molybdenum blue to the cells, and (ii) flocculation and precipitation of a molybdenum blue-cell aggregate at the bottom of the culture flask. Strain 48 produced a sticky polymer outside the cells when it grew on 2.9 mM phosphate medium supplemented with 10 mM Mo^{6+}. Thus, the polymer production seems to be cause for strain 48 to make a flock and precipitate. Determination of the site in the cells at which molybdenum blue attached is important and now under investigation.

ACKNOWLEDGMENT

We greatly appreciate the help of the Japan International Cooperation Agency in carrying out this study.

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