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Reduction of molybdate by sulfate-reducing bacteria

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Abstract Molybdate is an essential trace element required by biological systems including the anaerobic sulfate-reducing bacteria (SRB); however, detrimental consequences may occur if molybdate is present in high concentrations in the environment. While molybdate is a structural analog of sulfate and inhibits sulfate respiration of SRB, little information is available concerning the effect of molybdate on pure cultures. We followed the growth of *Desulfovibrio gigas* ATCC 19364, *Desulfovibrio vulgaris* Hildenborough, *Desulfovibrio desulfuricans* DSM 642, and *D. desulfuricans* DSM 27774 in media containing sub-lethal levels of molybdate and observed a red-brown color in the culture fluid. Spectral analysis of the culture fluid revealed absorption peaks at 467, 395 and 314 nm and this color is proposed to be a molybdate–sulfide complex. Reduction of molybdate with the formation of molybdate disulfide occurs in the periplasm *D. gigas* and *D. desulfuricans* DSM 642. From these results we suggest that the occurrence of poorly crystalline Mo-sulfides in black shale may be a result from SRB reduction and selective enrichment of Mo in paleo-seawater.

Keywords Molybdate · Molybdenum disulfide · Transition metals · Dissimilatory metal reduction · Sulfate-reducing bacteria

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Introduction

Sulfate-reducing bacteria (SRB) are anaerobic chemolithotrophic bacteria that characteristically derive energy for growth by coupling electron transport from electron donors to the reduction of sulfate by a process known as dissimilatory sulfate reduction. From recent reviews (Barton et al. 2003; Bruschi et al. 2007) it is apparent that SRB also have the capability of diverting electrons to several oxidized metals including Fe(III), $Cr(VI)O_4^{2-}$, $U(VI)O_4^{2-}$, $Mn(IV)O_2$, $Te(VI)O_4^{2-}$, and $Se(VI)O_4^{2-}$. Large quantities of these oxidized metals are reduced by SRB and this process is termed dissimilatory metal reduction. Unlike the reduction of sulfate which is a cytoplasmic activity, the reduction of metals occurs at

the surface of the cells with most of the reduced metal released into the extracellular fluid. Transition metals, including molybdenum, are important to SRB for the synthesis of enzymes involved in redox reactions (Moura et al. 2007; Barton and Fauque 2009); however, at elevated levels molybdate it is an inhibitor of sulfate metabolism and toxic to SRB.

Anaerobic sulfidogenic bacteria of the genus *Desulfovibrio* are capable of removing molybdate from the extracellular chemical environment through a reduction process. Previously, it was reported that at sub-inhibitory concentrations, molybdate, Mo(VI), was converted to Mo(IV) by *D. desulfuricans* DSM 642 with the production of insoluble MoS_2 (Tucker et al. 1997). This paper presents molybdate metabolism by SRB, reports the conditions required for Mo(VI) reduction, and discusses a possible role of SRB for enrichment of reduced Mo in natural black shale environments containing clay minerals, organic matter and sulfide minerals.

Molybdoproteins present in SRB

Although there had not been a systematic analysis of molybdoenzymes present in SRB, several molybdoproteins have been isolated. The NapA segment of the periplasmic nitrate reductase from *D. desulfuricans* ATCC 27774 contains Mo and has been extensively studied (Moura et al. 2007). Unique molybdoproteins of unknown physiological activity have been isolated from *Desulfovibrio africanus* (Hatchikian and Bruschi 1979) and *Desulfovibrio salexigens* (Czechowski et al. 1986). Additionally, the aldehyde dehydrogenase from *D. gigas* and several strains of *D. desulfuricans* has been purified and characterized (Moura and Barata 1994; Rebelo et al. 2000; Moura et al. 2004).

Enzymes demonstrated in SRB that are characterized as molybdoproteins in other bacteria include: carbon monoxide dehydrogenase in *D. vulgaris* Hildenborough (Voordouw 2002), ethylbenzene dehydrogenase in SRB strain EbS7 (Kniemeyer et al. 2003), dimethyl sulfoxide reductase in *D. desulfuricans* strain PA2805 (Jonkers et al. 1996), and formate dehydrogenase in *Desulfovibrio alaskensis* (Brondino et al. 2004). Several strains of *Desulfovibrio* including *D. vulgaris* Hildenborough, *D. gigas*, and *D. desulfuricans* display nitrogenase activity (Postgate and Kent 1985; Lespinat et al. 1987).

Molybdate transport systems

The uptake of molybdate into cells and the formation of molybdopterin, the molybdenum cofactor, is well characterized (Mendel 2005). To satisfy the requirement for molybdenum, *D. vulgaris* Hildenborough has an active transport system that is similar to other bacteria. Analysis of the *D. vulgaris* Hildenborough genome reveals that genes for high affinity molybdate uptake by an ABC transporter are clustered in an operon. The periplasmic binding protein for molybdate is encoded on *modA*, the transmembrane permease lipoprotein is a product of *modB*, and the cytoplasmic ATP–binding protein is produced from *modC*. These genes (e.g., *modA*, *modB*, *modC*) are located at the gene locus of DVU0177, DVU0181 and DVU180, respectively. Several bacteria have an additional regulatory protein, ModE, which regulates the synthesis of the molybdate uptake operon. In the presence of molybdate, ModE binds to a specific sequence in the operator/promoter region of the operon of the ABC-molybdate transporter and interferes with the reading of the *modA*, *B*, *C*, and *D* genes (Grunden and Shanmugam 1997). This ModE regulator prevents synthesis of the proteins for the molybdate transporter system under conditions where molybdate uptake exceeds encorporation of molybdate into molybdopterin. In some nitrogen-fixing bacteria that do not have this tight couple between molybdate uptake and utilization, there appears to be a molybdenum storage protein that prevents accumulation of free intracellular levels of molybdate (Grunden and Shanmugam 1997).

Homeostasis of transition metals, including molybdate, is important because elevated levels of free metal ions would be toxic to the cell. How bacteria of the *Desulfovibrio* species achieve molybdenum homeostasis is not established but it may be expected to be similar to the metal regulatory systems used by other bacteria. Zn, Mn and Ni homeostasis in *Escherichia coli* is proposed to be under the regulation of the FUR system (Lee and Helmann 2007) and Fe homeostasis in *D. vulgaris* is proposed to be under the FUR system (He et al. 2006). Export of molybdate may be attributed to transporters similar to the

P-type ATPase established for metals in prokaryotes (Cooms and Barkay 2005); however, a P-type ATPase for molybdate has not been reported in SRB.

Molybdate inhibition of sulfate activation

Metabolism of sulfate by SRB and the role of molybdate as an inhibitor of sulfate metabolism is well established. ATP sulfurylase, also known as ATP sulfate adenylyltransferase, catalyzes the activation of sulfate according to the following reaction:

$$SO_4^{2-} + ATP + 2H^+ \rightarrow APS + PPi.$$

The reduction of APS (adenylyl sulfate) to bisulfite and AMP in SRB is attributed to APS reductase and the reaction is given as follows:

$$APS + H_2 \rightarrow HSO_3^- + AMP + H^+.$$

As reported by Peck (1959), MoO_4^{2-} inhibits ATP sulfurylase, the first enzyme in sulfate activation. The mechanism of inhibition by molybdate and other Group VI anions (SeO_4^{2-}, WO_4^{2-} and CrO_4^{2-}) is the formation of an unstable molecule equivalent to APS (Peck 1961). The result from using an anion that was a competitive inhibitor for sulfate in the ATP sulfurylase reaction was that energy was consumed but an appropriate electron acceptor was not generated.

Molybdate is a competitive inhibitor for the sulfate uptake system of enteric bacteria (Kredich 1987). In *D. vulgaris* Hildenborough, the uptake of sulfate is by the sulfate permease (SulP) family at the gene locus of DVU0053, DVU0279, DVU1999. Molybdate at elevated concentrations would prevent inhibit sulfate import in *D. vulgaris*. Newport and Nedwell (1988) propose that there are several sites of action for molybdate inhibition of SRB and provide evidence for molybdate inhibition of sulfate transport in several SRB including *Desulfovibrio* sp. For some time, chromate was used to control the growth of SRB in cooling tower waters and other environments but due to the high toxicity of chromate, molybdate was commonly employed to inhibit the production of H_2S from sulfate. To control the growth of SRB and generation of hydrogen sulfide by sulfidogenic bacteria, additions of 20 mM molybdate were effective in salt marsh sediments (Banat et al. 1981) and mangrove forest sediments (Lyimo et al. 2002). The addition of 3 mM molybdate to distillery wastes was found to inhibit production of H_2S from sulfate respiration for several days (Ranade et al. 1999).

Molybdate inhibition with pure cultures

At sublethal concentrations of molybdate, hydrogen sulfide production from sulfate by cells from SRB cultures was inhibited but at a partial level. As shown in Fig. 1, some difference was observed in the extent of inhibition when molybdate at 0.5–2 mM was added to reactions containing 20 mM sulfate. *D. gigas* and *D. vulgaris* were more sensitive to molybdate as a respiratory inhibitor than the two cultures of *D. desulfuricans*. Above 2 mM molybdate, H_2S production is completely inhibited in SRB cultures and the effective ratio of molybdate:sulfate is ~1:10.

Inhibition of SRB growth by the addition of molybdate to culture medium is reflected in Fig. 2. A liter of the culture medium adjusted to pH 7.0 contained: Na lactate, 4 ml of a 60% solution; NH_4Cl, 3 g; $MgSO_4 \cdot 7H_2O$, 2 g; Na_2SO_4, 4 g; K_2HPO_4, 0.5 g;

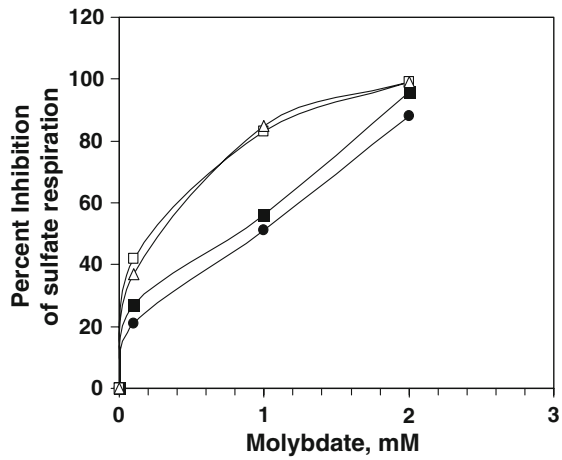

Fig. 1 Molybdate inhibition of sulfate respiration by SRB. Molybdate, 0.1 mM, was added to an anaerobic solution of $^{35}SO_4^{2-}$, 20 mM, containing 0.8 mg protein of bacterial cells according to the procedures of Newport and Nedwell (1988). After 60 min incubation at 35°C, citric acid was added to volatilize H_2S which was trapped in 10N NaOH. The amount of radiolabeled sulfate in the inhibited reactions was compared to test cultures receiving no molybdate and inhibition of sulfate respiration is expressed as a percentage. *Open squares* = *D. vulgaris*; *open triangles* = *D. gigas*; *solid circles* = *D. desulfuricans* DSM 27774; and *solid squares* = *D. desulfuricans* DSM 642

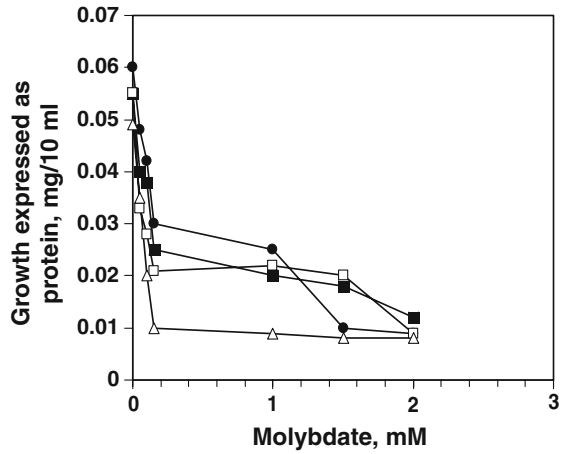

Fig. 2 Inhibition of growth by molybdate added to culture media. Growth at 3 days of incubation at 35°C is expressed as quantity of cell protein the 10 ml anaerobic culture at a range of concentrations of molybdate. Growth is expressed as mg protein/10 ml of growth media. *Open circles* = *D. vulgaris*; *open triangles* = *D. gigas*; *solid circles* = *D. desulfuricans* 27774; and *solid squares* = *D. desulfuricans* 642

$FeSO_4 \cdot 7H_2O$, 4 mg; yeast extract, 1 g. To this growth medium, $Na_2MoO_4 \cdot 2H_2O$ was added at concentrations ranging from 0.01 to 5 mM. While *D. gigas* was more sensitive to molybdate in comparison to *D. vulgaris*, *D. desulfuricans* 27774 and *D. desulfuricans* 642, none of the cultures grew when more than 5 mM was added to the growth medium. The ratio of molybdate:sulfate to inhibit growth of SRB is ~1:5.

Reduction of molybdate

Several bacterial species have been reported to reduce molybdate, Mo(VI), to molybdenum blue which has a mean oxidation state between +5 and +6 for Mo (Williams and da Silva 2002). Cells of *Serratia marcescens* in the presence of sucrose and ammonium sulfate reduced molybdate to molybdenum blue, a polymer of phosphate and molybdate, which has maximum absorption at 865 and 700 nm (Shukor et al. 2008). Anaerobically grown cells of *E. coli* will reduce molybdate in the presence of phosphate and glucose to produce molybdenum blue (Campbell et al. 1985).

Reduction of molybdate by cultures of SRB is by a process that does not involve the production of molybdenum blue. Tucker et al. (1997) indicate that *D. desulfuricans* DSM 624 reduced molybdate to the mineral molybdenite (MoS_2). Reduction of Mo(VI) by *D. desulfuricans* was demonstrated to be an enzymatic process requiring viable bacterial cells plus an electron donor of either lactate or H_2. With the addition of 1–3 mM molybdate to the culture medium, reduction of Mo(VI) coincided with sulfate reduction and production of H_2S. Columns constructed with *D. desulfuricans* immobilized in acrylamide simultaneously reduced molybdate, chromate, selenate and uranyl ions when lactate and sulfate was in the feed solution along with the oxyanions (Tucker et al. 1998). In these experiments dealing with MoS_2, formation there was no indication of molybdenum blue being formed but the culture media containing molybdate had a red-brown color (Tucker et al. 1997).

In a distinctly different experiment, Chen et al. (1998) added elemental molybdenum to a culture of *D. desulfuricans* ATCC 7757 and they observed the formation of an orange color which had adsorption peaks at 314, 396, and 468 nm. Through a series of experiments, they determined that in the appropriate chemical environment, sulfide reduced $Mo(VI)O_4^{2-}$ to Mo(V)-S producing the orange solution and that Mo(V)-S is analogous to Mo(V)-cysteine and Mo(V)-thiocyanate. Using X-ray photoelectron spectroscopy, Chen et al. (1998) demonstrated that molybdenum disulfide was produced by *D. desulfuricans* in media amended with elemental molybdenum.

When we added 0.1 mM Na_2MoO_4 to spent media of a SRB culture, the media developed a red-brown color similar to the tubes with molybdate in the growth media. A spectral review of the spent culture medium to which molybdate was added revealed peaks at 314, 396, and 468 nm which is in agreement to that reported by Chen et al. (1998). The difference in color reported by Chen et al. (1998) and that reported here could be attributed to concentration of the molybate-sulfide complex. By means of verifying the presence of molybdate-sulfide complex, absorbancy of the cultures was determined at various periods of growth (Fig. 3). At 5 days of growth, the color intensity in all four SRB cultures was greater than at 3 days and at 7 days. When the cultures were stored at room temperature, this red-brown color of the culture fluid remained for many months.

As reported in Fig. 4, growth of *D. vulgaris* Hildenborough in media containing a sublethal concentration of molybdate resulted in the culture

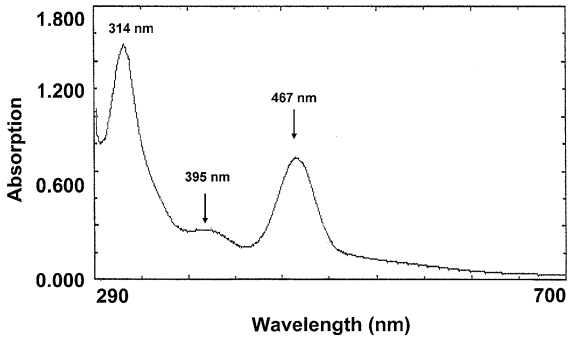

Fig. 3 Scan of centrifugate of the growth medium with *D. vulgaris* growing for 3 days in media containing 0.1 mM sodium molybdate. Absorption peaks are as indicated

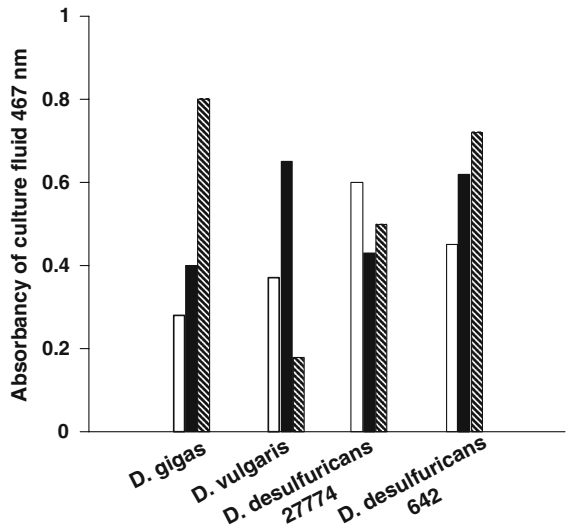

Fig. 4 Intensity of red-brown color in culture centrifugate with four different SRB cultures growing in the presence of 0.1 mM sodium molybdate. *Open bar* is at 3 days, *solid bar* is 5 days, and *striped bar* is 7 days of growth. Absorption of the culture centrifugate was measured at 467 nm

media becoming red-brown and three peaks characteristic of reduced molybdenum were observed. Other cultures of SRB (*D. desulfuricans* 27774, *D. desulfuricans* 642 and *D. gigas*) also produced the same color when grown in molybdate-containing media. The red-brown color in the culture tubes required metabolic activity because the color did not appear until after growth was observed. However, the red-brown color could be produced if 0.1 mM sodium molybdate was added to autoclaved growth medium along with 10 mM sodium sulfide. Once formed, the red-brown color persisted as long as the tube was sealed and there was no evidence for production of MoS_2 by non-biological reactions.

Cell associated reduction of molybdate

Observation of thin sections of SRB by high resolution electron microscopy indicates that molybdate was reduced at the periphery of the cells. Cultures of *D. gigas* and *D. desulfuricans* 642 were grown in lactate-sulfate medium containing 0.1 mM sodium molybdate. After 5 days of growth, bacterial cells were removed by centrifugation and bacterial cells were sectioned as described earlier (Tomei et al. 1995). Thin sections of the unstained bacteria were examined by electron microscopy and as presented in Fig. 5, electron dense material was deposited in the periplasm of *D. desulfuricans* and with *D. gigas* it was dispersed intermittently on the surface of the cell. Energy dispersive X-ray analysis (EDX) of these deposits (Fig. 5a, c) revealed that both sulfur and molybdenum were in considerable abundance. Analysis of the metal deposit outside of the cell of *D. gigas* is given in Fig. 6. In both cultures, there was considerable accumulation of extracellular molybdenum sulfide.

Mo sulfide in black shale

The Lower Cambrian black shale in Guizhlou and Hunan areas of southern China contains significant amount of Mo, As, and sulfide minerals (Fan 1983). Additionally, Mo and sulfides are closely associated with organic matter of kerogen. Transmission electron microscopy (TEM) results show pyrite microcrystals and Mo–As–S-bearing carbon (kerogen) (Fig. 7a). High-resolution TEM image shows that Mo-rich areas are Mo-sulfide (molybdenite) layers that form poorly crystalline structures in organic carbon matrix (Fig. 7b). X-ray energy-dispersive spectra (EDS) indicate composition from the pyrite (Fig. 7c) and the Mo-rich area (Fig. 7d). Black shale is very unique because of its high Mo concentration (Li and Gao 2000) and one possible mechanism for enriching Mo from paleo-seawater is the involvement of SRB.

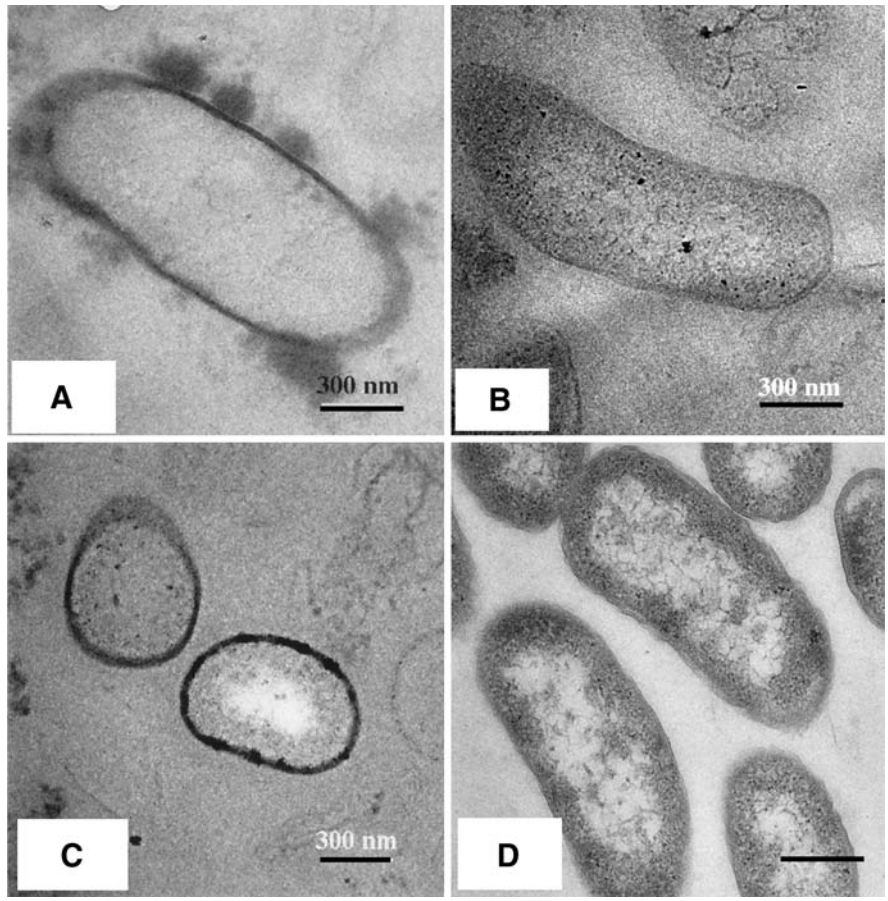

Fig. 5 Thin sections of cells of SRB grown in lactate sulfate medium. **a** *D. gigas* grown in medium containing 0.1 mM molybdate. **b** *D. gigas* grown in medium without added molybdate. **c** *D. desulfuricans* 642 grown in medium containing 0.1 mM molybdae. **d** *D. desulfuricans* 642 grown in medium without added molybdate. *Bar* is 300 microns

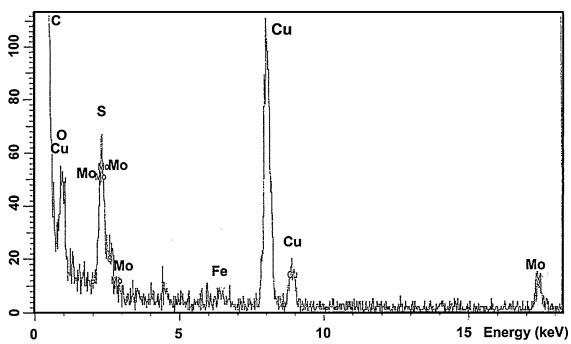

Fig. 6 Energy dispersive X-ray analysis of electron dense material on the surface of *D. gigas* in Fig. 5a. The abundance of Mo and S in the specimen is observed. The detection of copper is attributed to our use of copper grids to hold the specimen

Conclusion

$Mo(VI)O_4^{2-}$ reduction to $Mo(IV)S_2$ is facilitated by an environment containing sulfide with SRB having an important role in the process. The reduction with molybdate added to the culture media of SRB is similar to the reactions described for the production of MoS_2 following the addition of elemental $Mo_{(s)}$ to the culture media (Chen et al. 1998). The reduction of $Mo(VI)O_4^{2-}$ is a two step process. The initial step is a chemical reaction requiring a sulfide environment with the production of Mo(V) in the formation of some type of a molybdate-sulfide compound. This reduction of molybdate-sulfide appears to occur in the periplasm of cell and the reduced molybdenum sulfide accumulates extracellularly or at the surface of the cells. The process observed here with molybdate reduction may involve electron flow activities similar to that observed with U(VI) and Cr(VI) reduction in SRB (Barton et al. 2007, Bruschi et al. 2007) and other dissimilatory metal reducers. It appears that molybdate is not the molecule reduced directly by SRB but rather it is a molybdate-sulfide complex. The poorly crystalline Mo-sulfides in black

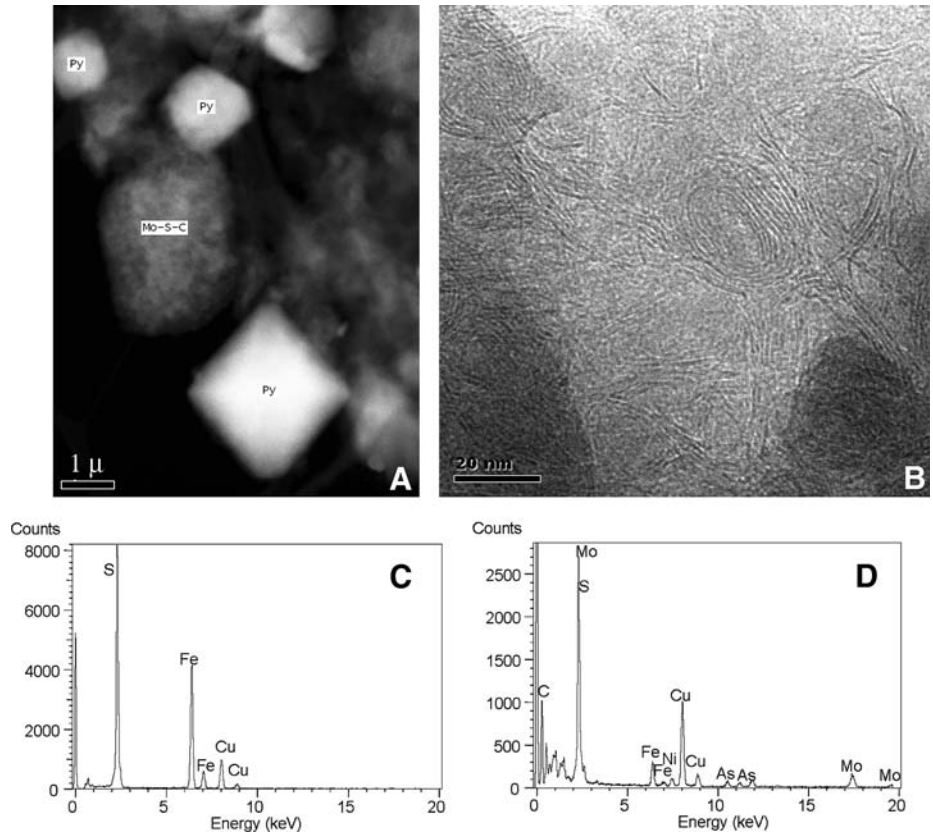

Fig. 7 **a** Z-contrast image of a kerogen-rich material showing pyrite and Mo–S-bearing organic matters. The image was obtained using a scanning transmission electron microscope operated at 200 KV; **b** High-resolution TEM image showing curved layers of MoS_2 within the kerogen matrix; **c** EDS spectrum from a pyrire crystals; **d** EDS spectrum from a MoS_2-rich area. All Cu peaks are from the TEM Cu-grid that hold the TEM specimen

shale could result from microbial reduction and selective enrichment of Mo in paleo-seawater via SRB.

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