

Bacterial dissimilatory MnO₂ reduction at extremely haloalkaline conditions

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Abstract A possibility of dissimilatory MnO₂ reduction at extremely high salt and pH was studied in sediments from hypersaline alkaline lakes in Kulunda Steppe (Altai, Russia). Experiments with anaerobic sediment slurries demonstrated a relatively rapid reduction of colloidal MnO₂ in the presence of acetate and formate as electron donor at in situ conditions (i.e., pH 10 and a salt content from 0.6 to 4 M total Na⁺). All reduced Mn at these conditions remained in the solid phase. A single, stable enrichment culture was obtained from the slurries consistently reducing MnO₂ at pH 10 and 0.6 M total Na⁺ with formate. A pure culture of a haloalkaliphilic Mn-reducing bacterium obtained from the positive enrichment was phylogenetically closely related to the anaerobic haloalkaliphilic *Bacillus arseniciselenatis* isolated from Mono Lake (CA, USA). *Bacillus* sp. strain AMnr1 was obligately anaerobic, able to grow either by glucose fermentation, or respiring few nonfermentable substrates by using MnO₂ as the electron acceptor. Optimal growth by dissimilatory

MnO₂ reduction was achieved with glycerol as electron donor at pH 9.5–10 and salt content between 0.4 and 0.8 M total Na⁺.

Keywords Haloalkaliphilic · MnO₂-reducing bacteria · Soda lakes · *Bacillus*

Introduction

Dissimilatory Mn(IV) reduction to Mn(II) is an important electron sink in anoxic habitats, such as freshwater and marine sediments (Burdige 1993; Nelson and Saffarini 1994; Thamdrup 2000; De Schamphelaire et al. 2007). Natural Mn cycling consists of the oxidation of soluble Mn(II) to insoluble MnO₂ with a tendency for spontaneous oxidation at increasing pH and reduction of MnO₂ to Mn(II) with spontaneous reaction at acidic pH values (Tebo et al. 2004). At neutral pH conditions, bacteria can accelerate both reactions of the Mn cycling. In particular, MnO₂ can be used as an electron acceptor by specialized metal-reducing Proteobacteria, such as *Geobacter* and *Shewanella*, in a dissimilatory respiration process (Lovley et al. 2004; Lovley 2006; Nealson and Scott 2006). Usually, these organisms utilize both Fe and Mn oxides as electron acceptors. Dissimilatory MnO₂ reduction has also been demonstrated for several Gram-positive bacteria, such as *Bacillus infernus* (Boone et al. 1995) and *Bacillus subtterraneus* (Kanso et al. 2002).

Apart from the fact of a fast spontaneous oxidation of Mn(II), almost nothing is known on the possibility of the opposite reaction at highly alkaline conditions, which must be mainly biological in the absence of strong reductants, such as sulfide. Soda lakes are haloalkaline habitats where such process may be catalyzed by extremophilic

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prokaryotes. Until now, however, only a single iron-reducing Epsilonproteobacterium from a soda lake, *Geoalkalibacter ferrireducens*, with a potential for dissimilatory MnO₂ reduction at alkaline conditions has been described, but its Mn reduction was not explored in details (Zavarzina et al. 2006).

In the current study the active biological reduction of MnO₂ was demonstrated for sediments from hypersaline soda lakes and the properties of an obligately anaerobic haloalkaliphilic *Bacillus* sp. AMnr1 specialized on MnO₂ reduction are described.

Methods

Samples

Surface sediment samples (0–10 cm depth) were obtained from 10 hypersaline soda lakes in southeastern Kulunda Steppe (Altai, Russia) in 2008. The pH of the brines varied from 9.8 to 10.6, the total salt concentration from 60 to 400 g l⁻¹, and the total soluble carbonate alkalinity from 0.3 to 3 M. Sediments from individual lakes were mixed 1:5 with their brines, pooled together in equal volumes and their fine colloidal fraction was separated from the coarse fraction by several steps of low-speed centrifugation. This procedure also removed the soluble sulfide.

MnO₂ reduction in soda lake sediments

Twenty milliliter of the fine sediment fraction was mixed with 80 ml of sodium carbonate buffer (pH 10) containing 0.1 g l⁻¹ K₂HPO₄ and 0.1 g l⁻¹ of NH₄Cl in 120 ml serum bottles. The buffer contained 0.6, 2, and 4 M total Na⁺ as a mixture of sodium carbonate/bicarbonate. A total of 10 mM acetate or 25 mM formate were used as electron donors and 20 mM of freshly prepared colloidal MnO₂ (prepared by reduction of KMnO₄ by H₂O₂) as electron acceptor. Subsequently, the bottles were sealed with butyl rubber stoppers and made anoxic by five cycles of evacuation flushing with argon. Controls included sediments without electron donor and with heat-sterilized sediments. The bottles were incubated at 30°C statically with occasional mixing. MnO₂ reduction was monitored by color change and by the analysis of Mn(II).

Isolation and cultivation of MnO₂-reducing bacteria

Sediment samples that showed MnO₂ reduction were sub-cultured at 1:100 dilution using the same sodium carbonate/bicarbonate buffer as a mineral base supplemented with 0.5 g l⁻¹ K₂HPO₄, 4 mM NH₄Cl, 1 mM MgSO₄, 20 mg l⁻¹ of yeast extract, and 1 ml l⁻¹ of trace metal

solution (Pfennig and Lippert 1966). Acetate (20 mM) or formate (50 mM) was used as the electron donor. In case of visible MnO₂ reduction, the cultures were plated into 1% (w/v) soft agar media containing up to 2 M total Na⁺ and 10 mM colloidal MnO₂ using agar-shake technique. The plates were incubated in closed jars under argon with an oxygen-scavenging catalyzer for 3–4 weeks. Colonies with clearing of the brown background were placed in 12 ml serum bottles containing 10 ml liquid medium of the same composition used for enrichment and made anoxic by evacuation–argon flushing. Tests for utilization of different electron donors and acceptors were done in 30 ml serum bottles containing 20 ml carbonate medium at pH 10 and 0.6 M total Na⁺. Electron donors (with MnO₂ as acceptor) were used at 20 mM final concentration. Electron acceptors (with glycerol as donor) were used at the following concentrations: ferrihydrite at 20 mM; nitrate, sulfate, thiosulfate, fumarate, and sulfur at 10 mM; nitrite, sulfite, arsenate, arsenite, selenate, selenite, and AQDS at 5 mM. Aerobic growth with glycerol was tested at 2% O₂ in the gas phase. To test the possibility of fermentation, the electron acceptor was omitted.

The pH dependence of growth and MnO₂ reduction by resting cells was examined at 0.6 M Na⁺, using the following filter-sterilized buffers: for pH 6–8, 0.1 M HEPES/NaHCO₃/NaCl; for pH 8.5–11.0, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. The final pH values were considered as a suitable range for growth and activity. The influence of salt concentration on growth and activity of MnO₂ reducing bacteria was tested with sodium carbonate/bicarbonate buffer containing 0.2–2.0 M of total Na⁺ at pH 10.

Analytical procedures

Because of using highly alkaline carbonate media, routine Mn(II) analysis was a challenge, since in that case reduced manganese precipitated together with MnO₂ and needed to be released before it could be detected by colorimetric assay according to Goto et al. (1961). According to literature data (such as Schnell et al. 1998), addition of up to 0.5 M HCl does not lead to dissolution or chemical reduction of MnO₂ and, therefore, could be used to separate MnCO₃ and MnO₂ in the solid phase. Our experiments showed that this is true only in the absence of reducing organic compounds. If compounds, usually used as carbon source, such as sugars, organic acids, and glycerol, are present during acidic treatment, a substantial part of the colloidal MnO₂ is immediately reduced making the analysis impossible. Another problem with Mn at high pH is that Mn(II), in contrast to neutral conditions, is rapidly oxidized spontaneously. Therefore, handling of the samples taken from the anoxic cultures must be rapid. The protocol

for sample preparation developed for alkaline conditions is as follows: the culture is homogenized by vigorous shaking and 0.1 ml sample is rapidly centrifuged in 1.5 ml Eppendorf tube. The supernatant is carefully withdrawn and replaced by 1 ml of 0.5 M acetate buffer, pH 3.5. This treatment released Mn(II) in the form of MnCO_3 into the solution. After thorough vortexing the remaining solid containing coarse fraction of MnO_2 is removed by centrifugation and the remaining colloidal MnO_2 in the supernatant is removed by filtration through 13 mm Acrodisc filters (Pall, USA) with 0.2 μm pore size. The final solution is used for Mn(II) detection by the formaldoxime method.

Cell protein was measured after alkaline hydrolysis by the Lowry method (Lowry et al. 1951) after removal of MnO_2 by low-speed centrifugation. Cell-free extract was obtained by sonication and the cell membranes were separated from the soluble fraction by ultracentrifugation at $144,000\times g$ for 2 h. Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). Cytochrome composition in cell-free extracts and cell fractions was analyzed spectroscopically using UV–visible diode array HP 8453 spectrophotometer (Amsterdam, NL). Cellular fatty acids were extracted from the freeze-dried cells (grown at pH 10, 0.6 M Na^+ and 30°C with glucose) by acidic methanol and analyzed by GC–MS according to Zhilina et al. (1997).

Genetic and phylogenetic analysis

Isolation of the DNA and determination of the G + C content of the DNA was performed according to Marmur (1961) and Marmur and Doty (1962). For PCR, the genomic DNA was extracted from the cells using the Microbial DNA Extraction Kit (MoBio Laboratories, USA), following the manufacturer's instructions. The nearly complete 16S rRNA gene was obtained from pure cultures using general bacterial primers GM3 and GM4 (Schäfer and Muyzer 2001). The PCR product was purified from agarose using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced by the commercial company Macrogen (South Korea).

Results and discussion

Potential for biological MnO_2 reduction in hypersaline soda lake sediments

In contrast to heat-treated sediment samples and the sediment samples without electron donor, in native sediment slurries supplemented with formate or acetate active reduction of MnO_2 took place in sodium carbonate brines at pH 10 and salt content 0.6–2 M total Na^+ . At soda-saturating

Table 1 Dynamic of MnO_2 reduction in soda lake sediment slurries at pH 10

Electron donor	Salt, M Na^+	Incubation time (days)	Mn(II) mM
None	0.6	20	1.8
	2.0	20	1.5
	4.0	20	0.9
Formate, 25 mM	0.6	3	1.1
		8	3.0
		20	8.4
	2.0	3	1.5
		8	3.8
		20	5.6
	4.0	3	0.4
		8	1.8
		20	3.0
Acetate, 10 mM	2.0	3	2.4
		8	4.5
		20	8.8
	4.0	3	0.3
		8	2.0
		20	4.5
Formate (heat-killed control)	0.6	20	0.4

conditions (i.e., 4 M total Na^+) the process was evident as well, but with much lower reduction rates (Table 1). The reduction of MnO_2 was accompanied by the formation of a whitish layer (presumably of MnCO_3) on top of the sediments.

Enrichment and isolation of a pure culture

When sediment slurries actively reducing MnO_2 were used as an inoculum (1:100) in sodium carbonate medium at pH 10 with MnO_2 as electron acceptor and either formate or acetate as electron donor, the MnO_2 reduction rate decreased sharply at 2–4 M Na^+ as compared to the slurry experiments, and after a third transfer only the culture initiated at 0.6 M Na^+ with formate remained active. This culture reduced up to 10 mM MnO_2 within 3 weeks and a visible suspended cell growth appeared at the end of this period dominated by long spore-forming rods. After plating into soft agar with MnO_2 and formate, clearance zones appeared within 3 weeks of incubation around the tiny colonies that, after transfer to the liquid medium of the same composition, resulted in a pure culture reducing MnO_2 . The isolate was designated strain AMnr1. The bacterium is a Gram-positive motile rod with 1–2 subpolar flagella, 2–10 \times 0.4–0.5 μm , forming terminal round endospores (Supplemental Figure). The strain has been deposited in two culture collections, the DSMZ (Germany)

Table 2 Comparative properties of *Bacillus* AMnr1 (1) and *B. arseniciselenatis* (2)

Characteristic	1	2
Motility	+	–
Aerobic growth	–	–
Growth pH optimum	9.5–10	9.5
Salt tolerance (M Na ⁺)	1.0	2.0
Fermentative growth with	Glucose, sucrose	Fructose
Electron acceptors	(with glycerol)	(with lactate)
MnO ₂ , AQDS	+	nd
Selenate, arsenate	–	+
Nitrate, fumarate, Fe(III)	–	+
Electron donors	(with MnO ₂ or AQDS)	(with nitrate)
	Formate, glycerol, succinate	Lactate, malate, starch, citrate
Major fatty acids of the cellular polar lipids	16:1 ω 7c, 16:0, a15, 18:1 ω 7c, i17:1 ω 8, i15	nd
G + C content in the DNA (mol%)	36.9	40.0
Habitat	Soda lakes	Mono Lake
	(Kulunda Steppe, Altai)	(CA, USA)

nd Not determined

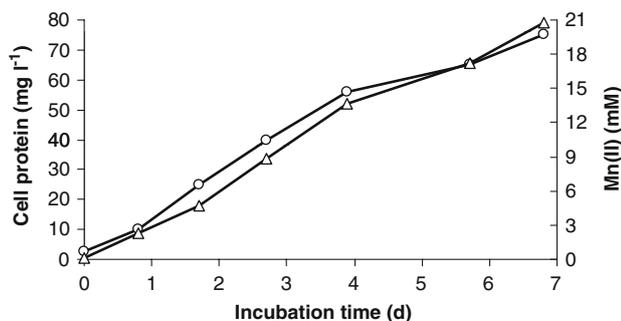


Fig. 1 Growth (open circles) and MnO₂ reduction (open triangles) in an anaerobic culture of strain AMnr1 with glycerol as electron donor at pH 10 and 0.6 M of total Na⁺. The results are mean from three independent experiments with deviation below 10%. The chemical MnO₂ reduction (uninoculated control) by glycerol did not occur

and the UNIQEM (Moscow) under the numbers DSM 22341 and U792, respectively.

Phylogenetic analysis of 16S rRNA gene sequence obtained from strain AMnr1 showed a close affiliation (99% sequence similarity) with the obligately anaerobic haloalkaliphilic *Bacillus arseniciselenatis* isolated from haloalkaline Mono Lake (Switzer Blum et al. 1998) for which dissimilatory MnO₂ reduction has not been demonstrated.

Metabolic properties of *Bacillus* sp. AMnr1

Strain AMnr1 showed an obligately anaerobic phenotype with a restricted catabolism. It grew by fermentation of glucose and sucrose and by respiration of a few

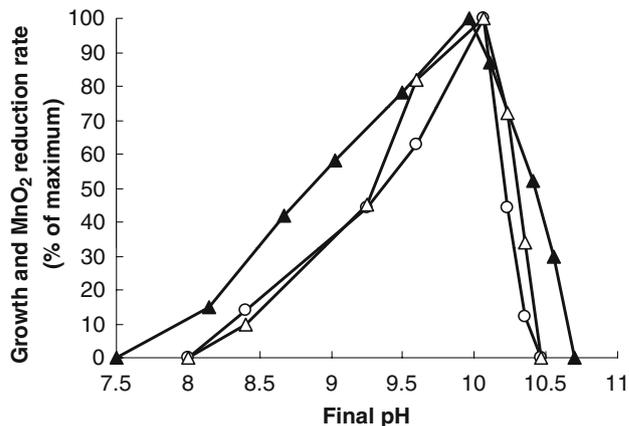


Fig. 2 Influence of pH at 0.6 M total Na⁺ on growth (open circles) and activity of MnO₂ reduction in culture (open triangles) and by resting cells (closed triangles) of strain AMnr1 with glycerol as electron donor. 100% for specific growth rate was 0.07 h⁻¹ and for specific activity of MnO₂ reduction–15 nmol (mg protein min)⁻¹

non-fermentable substrates using either MnO₂ or AQDS (anthraquinone disulfonate) as electron acceptor. Obviously, despite their close phylogenetic relation, strain AMnr1 is significantly different in its phenotype from *B. arseniciselenatis* (Table 2). Growth with H₂, either autotrophic or in presence of acetate as an additional carbon source, was not observed.

When grown with glucose, the presence of MnO₂ did not influence the growth kinetics and the final biomass yield of strain AMnr1, despite that up to 12 mM MnO₂ was reduced. In case of non-fermentable substrates, such as formate and glycerol, growth occurred only in the presence

Fig. 3 Visual presentation of the influence of pH on the activity of MnO₂ reduction with glycerol as electron donor in anaerobic culture of strain AMnr1. MnO₂ reduction is performed in the light colored cultures. The final pH values are indicated

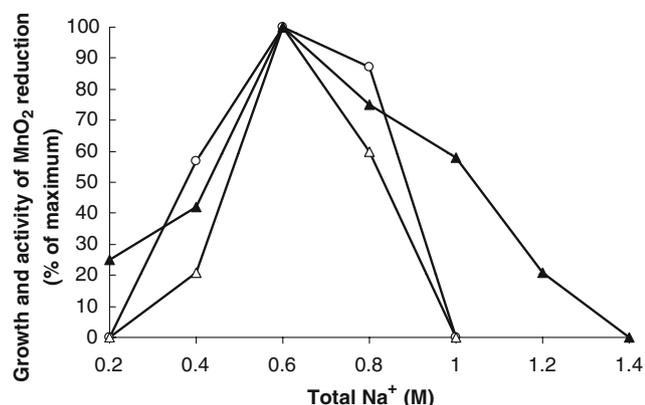


Fig. 4 Influence of sodium carbonate/bicarbonate concentration at pH 10 on growth (*open circles*) and activity of MnO₂ reduction in culture (*open triangles*) and by resting cells (*closed triangles*) of strain AMnr1 with glycerol as the electron donor. Average from two independent experiments

of an electron acceptor. The best growth with MnO₂ as an electron acceptor at pH 10 was observed with glycerol at a maximum specific growth rate of 0.017 h⁻¹ and with a complete reduction of 20 mM MnO₂ (Fig. 1).

Influence of pH and salt on the growth and activity of MnO₂ reduction in *Bacillus* sp. AMnr1

Anaerobic growth of strain AMnr1 with glycerol and MnO₂ was observed within a highly alkaline pH range from 8.3 to 10.3 with an optimum at 9.5–10, both for biomass growth and MnO₂ reduction rate (Figs. 2, 3). Washed cells that were pre-grown on glycerol and MnO₂ had a pH profile for MnO₂ reduction activity similar to growing cells with a slightly higher pH maximum of pH 10.5 (Fig. 2). At pH 10, anaerobic growth and MnO₂ reduction was possible within a very narrow and relatively low salt concentrations from 0.4 to 0.8 M Na⁺, while resting cells were still active up to 1.2 M Na⁺ (Fig. 4).

Cytochromes

The biomass of strain AMnr1 grown either by fermentation on glucose or by dissimilatory MnO₂ reduction with

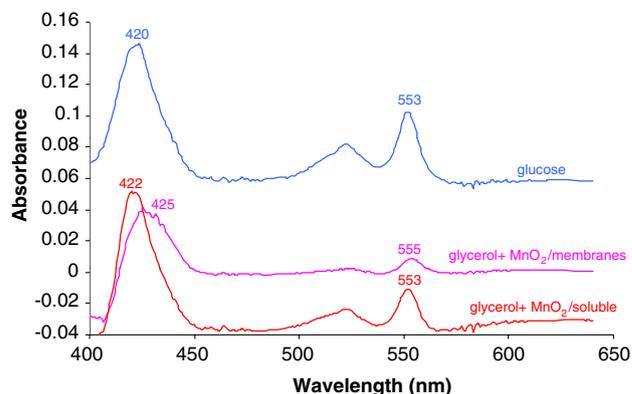


Fig. 5 Dithionite-reduced minus air-oxidized difference cytochrome spectra in strain AMnr1. Protein content: glucose (cell-free extract), 2 mg ml⁻¹; glycerol plus MnO₂ (soluble fraction), 1.5 mg ml⁻¹; membranes, glycerol plus MnO₂ (1.2 mg ml⁻¹). The membrane cytochromes were solubilized in 0.5% (w/v) β-D-dodecyl maltoside

glycerol had a distinct pink color due to a high concentration of the “soluble” cytochrome *c*₅₅₃. The membranes also contained a cytochrome with an alpha maximum at 555 nm which might be a cytochrome of *b*-type (Fig. 5). The “soluble” cytochrome *c*₅₅₃ might be loosely associated with the cell membrane or with the outer cell wall layer and released after sonication. In any case, to be involved in the reduction of the insoluble MnO₂, the cytochrome *c* must be located outside the cytoplasmic membrane, as, for example, the outer membrane cytochromes *c* Omc in Gram-negative Fe/Mn-reducer *Shewanella* (Myers and Myers 2003). Since the isolated bacterium cannot use other electron acceptors, it was impossible to make a direct comparison of cytochrome expression at different growth conditions. Comparison of the cells grown by fermentation of glucose and by MnO₂ reduction with glycerol showed that a main difference was in the membrane fraction.

In conclusion, the results demonstrated, for the first time, the possibility of an active biological MnO₂ reduction at extremely haloalkaline conditions in anoxic hypersaline soda lake sediments. However, the identity of microflora reducing MnO₂ in saturated soda brines remains unclear, since primary enrichments were inactivated upon removal of original sediment material by dilution. This might have either a biological reason (e.g., diluting out one of the

essential nutrients) or a physico-chemical reason (i.e., the necessity for a solid phase or for mineral interactions). A low salt-tolerant obligately alkaliphilic *Bacillus* sp. AMnr1 isolated from a MnO₂-reducing enrichment at pH 10 and salt concentration 0.6 M Na⁺ turned out to be a dissimilatory Mn-reducing specialist unable to utilize any other natural electron acceptors, including Fe(III), which is clearly unusual. On its properties, it resembled *Bacillus infernus*, a neutrophilic thermophilic anaerobe isolated as a MnO₂-reducer (Boone et al. 1995). Since Gram-positive bacteria lack an outer membrane and periplasm space, in which MnO₂-reducing cytochromes are resided in Gram-negative bacteria, their MnO₂ reduction system might be significantly different (especially for haloalkaliphiles) and therefore deserves further attention.

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