Systematic and Applied Microbiology 38 (2015) 184–188

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Role of humic substances in promoting autotrophic growth in nitrate-dependent iron-oxidizing bacteria

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ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form 5 February 2015
Accepted 9 February 2015

Keywords:
Iron oxidation
Nitrate reduction
Autotrophy
Humic substance
Thiobacillus denitrificans

ABSTRACT

Nitrate-dependent iron oxidation was discovered in 1996 and has been reported from various environments ever since. To date, despite the widespread nature of this process, all attempts to cultivate chemolithoautotrophic nitrate-dependent iron oxidizers have been unsuccessful. The present study was focused on understanding the influence of natural chelating agents of iron, like humic substances, on the culturability, activity, and enumeration, of these microorganisms. Pure culture studies conducted with *Thiobacillus denitrificans* showed a constant increase in cell mass with a corresponding nitrate-dependent iron oxidation activity only when Fe(II) was provided together with humic substances, compared to no growth in control incubations without humic substances. The presence of a relatively strong chelating agent, such as EDTA, inhibited the growth of *Thiobacillus denitrificans*. It was concluded that complex formation between humic substances and iron was required for chemolithoautotrophic nitrate-dependent iron oxidation. Most probable number enumerations showed that numbers of chemolithoautotrophic nitrate-dependent iron-oxidizing bacteria were one to three orders of magnitude higher in the presence of humic substances compared to media without. Similar results were obtained when potential nitrate-dependent iron oxidation activity was determined in soil samples. In summary, this study showed that humic substances significantly enhanced the growth and activity of autotrophic nitrate-dependent iron-oxidizing microorganisms, probably by chelation of iron.

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Introduction

Nitrate contamination is prevalent among shallow aquifers in Europe and North America (http://www.eea.europa.eu/data-and-maps/figures/present-concentration-of-nitrate-in-groundwater-bodies-in-european-countries-2003), leading to public health concerns [44]. Reduction of nitrate to N_2 by denitrifying bacteria is an important process for nitrate attenuation in ground water aquifers [2], with autotrophic denitrification being the most prevalent process in these environments [31,32]. In comparison to organoheterotrophic denitrification, in which reduced organic carbon serves as both a carbon and an electron source, chemolithoautotrophic denitrification requires an inorganic electron donor (e.g., ferrous iron or thiosulfate), and CO_2 as a carbon source [38]. Fe(II) is produced in anaerobic regions of soils and aquifers, and could potentially serve as an electron donor for autotrophic denitrification in ground water aquifers [14]. However, further understanding of the process is hindered by the inability to culture the organisms involved.

The nitrate-dependent Fe(II) oxidation process was first reported by Straub et al. [38] in 1996. Subsequent studies have observed this process in various environments, such as lake sediments [39], marine environments [11], rice paddies [34], hydrothermal vents [15], peat bogs [21], and brackish lagoons [38], indicating its ubiquitous distribution in nature. Attempts to cultivate nitrate-dependent iron oxidizers resulted in the isolation and characterization of several microorganisms belonging to both *Archaea* [15], and *Bacteria* [43]. Among *Bacteria*, this physiological trait is prevalent in denitrifiers [5], and to date, most of the isolated organisms have belonged to the phylum *Proteobacteria*. However, a recent study reported nitrate-dependent Fe(II) oxidation in members of the phylum *Actinobacteria* [21].

Depending on the nature of the carbon source, nitrate-dependent Fe(II)-oxidizing organisms are classified as either autotrophs or heterotrophs. To date, all the available pure cultures have only been capable of chemolithoheterotrophic growth (e.g., using acetate as a carbon source). Although some geochemical

Abbreviations: HU, humic substances.

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http://dx.doi.org/10.1016/j.syapm.2015.02.009
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and microbial studies of natural environments have found evidence for chemolithoautotrophic nitrate-dependent Fe(II) oxidation [14,32], the ecology and physiology of this process is not well understood because of the lack of pure cultures. Since attempts to cultivate chemolithoautotrophic nitrate-dependent Fe(II) oxidizers using defined media have largely been unsuccessful, there is a lack of understanding of the factors required for their growth. The difficulties in isolating pure cultures have also been attributed to the relatively high redox potential (+100 to −100 mV) of the Fe(III)/Fe(II) couple [40], making chemolithoautotrophic growth energetically challenging [27].

The growth rate and adaptability of bacteria to an artificial medium depend on various factors, such as the composition of the medium, the chemical nature of nutrients, and the incubation conditions. Microbial communities in humic acid lakes, for example, were found to require humic substances (HU) for growth in defined media [25], indicating the importance of HU in culturing or enriching such microorganisms. Therefore, it was assumed that HU could play a significant role in the case of iron-oxidizing microorganisms, as both Fe(II) and Fe(III) are known to form chelate complexes with HU [23]. The chelation of iron by HU is a well reported phenomenon and its role in influencing the biological availability [19,20], mobility [4,35], reactivity [42], and speciation, between Fe(III)/Fe(II) [26,30] has been well documented for natural environments. Recent studies also showed that Fe(II) is seldom present in its free form in natural environments but is predominantly chelated to HU [6,46]. To date, this phenomenon has largely been ignored for isolation and cultivation of nitrate-dependent Fe(II)-oxidizing bacteria. Therefore, it was decided to investigate the role of humic substances in promoting chemolithoautotrophic growth of nitrate-dependent Fe(II)-oxidizing bacteria.

Materials and methods

Growth of Thiobacillus denitrificans

Pure culture studies were conducted using *Thiobacillus denitrificans*, since this bacterium is known for nitrate-dependent Fe(II) oxidation [38] and for containing all the genes required for autotrophic growth with nitrate and Fe(II) [3]. The basal medium contained (per liter): NH_4Cl (0.3 g), $MgSO_4.7H_2O$ (0.05 g), $CaCl_2$ (0.1 g), $MgCl_2$ (0.4 g), and KH_2PO_4 (0.3 g). After autoclaving and cooling the medium under a N_2 atmosphere, 1 ml of a trace element solution and 1 ml of a vitamin solution [45] were added. The cultivation of *T. denitrificans* was carried out under four different incubation conditions in order to address the role of Fe(II) chelation for growth. Thus, the media contained: (1) Fe(II) plus humic substances, (2) Fe(II) in its aqueous form without a chelating agent, (3) Fe(II) plus EDTA, and (4) Fe(II) plus both EDTA and humic substances. Control incubations were also carried out with no inoculation, no nitrate, and no Fe(II), for each set of incubation conditions. The concentrations of the additions were: nitrate (1 mM), $FeCl_2$ (2.5 mM), HU (600 mg L^{-1}), and EDTA (1.25 mM). Solutions of $FeCl_2$ were kept under a N_2 atmosphere in order to prevent autoxidation. HU were isolated from the southwest basin of Lake Grosse Fuchskuhle, according to the procedure described by the International Humic Acid Society [41], and they were sterilized by passing them through 0.2 μm membrane filters (Millipore, Darmstadt). The procedure ensured that low molecular weight substances were removed. The HU were kept in the dark to prevent photolytic degradation. All the media were prepared one day prior to inoculation and were left in an anaerobic glove box (Mecaplex, Grenchen, Switzerland) under 20% CO_2 and 80% N_2 to allow for equilibration of Fe(II) with the chelators.

The inoculum was prepared as follows: *T. denitrificans* (DSMZ 807) was initially subcultured on the recommended medium (DSM medium 113), which uses thiosulfate as an electron donor. Cells were harvested during logarithmic growth and washed in DSM 113 medium lacking thiosulfate. The procedure was repeated three times. Then, cells were re-suspended in 2 ml of the respective medium inside an anaerobic glove box, and inoculated into 100 ml of the medium. The headspace of the incubation bottles contained 20% CO_2 (as a sole carbon source), and 80% N_2. All incubations were undertaken in triplicate and incubated in the dark at 25 °C on a shaker (80 rpm).

The concentrations of Fe(II) and nitrate were measured at regular intervals using colorimetric assays described previously [16,36]. The increase in biomass was determined by measuring protein concentrations with the Bio-Rad protein assay kit, according to the manufacturer's instructions (Bio-Rad, Munich, Germany).

Potential rates of nitrate-dependent Fe(II) oxidation in natural environments

Potential rates of nitrate-dependent Fe(II) oxidation were measured with three different rice paddy soils originating from Verchelli (Italy), Changsha (China), and Fuyang (China), using the same media and control experiments as described above for the pure culture studies. The main characteristics of these soils have already been described in the literature [16,22,33,36,47], although they were not really relevant for the present study, since the soil inoculum was strongly diluted by the cultivation medium. Soil slurries were prepared by mixing a 1:1 ratio (wt/vol) of soil and deionized water in a 120 ml serum bottle, and they were left overnight at room temperature. Oxygen was removed using a vacuum manifold and by repeatedly flushing the headspace with N_2 gas. These serum bottles were transferred into an anaerobic chamber and 100 μl of the soil slurries were added to 10 ml of the media described above in 120 ml serum bottles. All the bottles were incubated at room temperature inside the anaerobic chamber and the concentration of Fe(II) was measured in the incubations as described above.

MPN quantification

The most probable number (MPN) method was used to enumerate potential autotrophic nitrate-dependent Fe(II)-oxidizing bacteria in the soils mentioned above, as well as in various additional soils and sediments. The composition of the media was as described above with 5 mM Fe(II), 5 mM nitrate, and 20% CO_2 in the head space as the sole carbon source. One set of incubations was with HU and the other without HU. Soil (1 g) or sediment (1 ml) was added to 9 ml of the medium and serially diluted in 25 ml test tubes. All the incubations were carried out in triplicate. The test tubes were incubated in the dark at 25 °C and were mixed by gently inverting once a day for a total incubation time of 2 weeks. Incubations were scored positive if the concentration of Fe(II) had decreased compared to the un-inoculated control. Fe(II) estimations were carried out using the ferrozine assay [36], and cell numbers were calculated according to standard MPN tables [1].

Results

Growth of Thiobacillus denitrificans

The concentration of Fe(II) in the cultures of *T. denitrificans* was monitored at regular intervals during incubation and it decreased only when the medium contained HU together with Fe(II) and nitrate (Fig. 1). Growth was also tested with different concentrations of HU as the sole substrate, but no growth was observed up

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