An MPN Method for the Enumeration of Iron-Reducing Bacteria

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Abstract

A simple medium for the enumeration of iron-reducing bacteria, containing a readily available iron source, ferric EDTA, was developed. Three cultures of iron-reducing bacteria were used to evaluate various media compositions: *Shewanella putrefaciens* 200 (NCIB 12577), *Shewanella putrefaciens* ESSO 4-1 (NCIB 12580) and a mixed culture of iron-reducing bacteria. Thirteen different media were prepared using various combinations of carbon source(s) in a basal salts medium containing ferric EDTA. Significant iron reduction was observed in all media, but the medium containing 1.5 g/L peptone as the carbon source was considered to be superior to all of the other media for supporting iron reduction.

INTRODUCTION

The objective of this study was to develop a simple, rapid medium for the enumeration of iron-reducing bacteria in natural habitats. Iron-reducing bacteria (IRB) are a significant biological component of passive systems used to treat acid mine drainage. IRB may also play a role in mobilizing other metals in anaerobic environments by reducing and dissolving iron oxyhydroxides releasing both iron and other metals such as Cu, Zn, Ni and As that may be adsorbed to the iron oxides. A large number of microorganisms are capable of reducing ferric iron and are widely distributed in many environments (Lovley, 1987, 1991). Ferric iron is the most abundant electron acceptor in most soils (Ponnamperuma, 1972). Ethanol, propanol, pyruvate, lactate, butanol, acetate, formate, hydrogen and BTEX compounds have been shown to be electron donors for bacterial iron reducing activity (Lovley *et al*., 1989; Roden and Lovley, 1993). Biological iron reduction has been shown to occur with a number of oxidized or partially oxidized iron compounds such as magnetite (Kostka and Nealson, 1995) and various Fe(III) oxyhydroxides (Lovley and Phillips,1988). The rate of bacterial reduction of various electron acceptors was in the following order: ferric citrate > amorphous Fe(III) oxyhydroxides > more crystalline Fe oxides. Increasing the bioavailability of iron through chelation allows the anaerobic biodegradation rates of aromatic hydrocarbons to approach the degradation rates obtained under aerobic conditions (Lovley *et al.*, 1994, 1996). Present media for culturing and enumerating iron-reducing bacteria from natural environments are complex, difficult to prepare and growth rates are generally slow. The medium most commonly used contains amorphous Fe(III) oxyhydroxides as an electron acceptor (Lovley and Phillips, 1988). A simpler medium containing a readily available iron source such as ferric EDTA would find wide application for the enumeration of iron-reducing bacteria.

MATERIALS and METHODS

(a) Cultures

Two pure cultures of iron-reducing bacteria were used to initially evaluate various media compositions. They were *Shewanella putrefaciens* 200 (NCIB 12577) and *Shewanella putrefaciens* ESSO 4-1 (NCIB 12580) designated cultures A and B respectively. A mixed culture of iron-reducing bacteria was also used (supplied by M. Francis, NOVA Chemicals Corporation) and designated as culture C. The mixed culture was

initially isolated anaerobically on BTX compounds using amorphous iron oxyhydroxides as electron acceptor (Francis and Gould, in preparation).

(b) Preliminary Study: Evaluation of Various Carbon Sources

Thirteen different media were prepared using various combinations of carbon source(s) (Table 1) in a basal medium. The basal medium consisted of the following constituents in g/L: $NaHCO_3$, 2.5; NH_4Cl, 1.5; NaH_2PO_4, 0.6; $CaCl_2.2H_2O$, 0.1; KCl, 0.1; $MgCl_2.6H_2O$, 0.1; $MnCl_2.4H_2O$, 0.005; $Na_2MoO_4.2H_2O$, 0.001 and ferric EDTA, sodium salt, hydrate 1.84.

The final medium was adjusted to pH 7.0 and boiled under an N_2 atmosphere for 20 minutes. Additional water was added initially to compensate for evaporative losses during boiling. The medium was then removed from the heat, allowed to cool and transferred to an anaerobic glove chamber (95%N_2-5%H_2 atmosphere) and 9 mL of medium were dispensed into each 20 mL serum bottle. The serum bottles were stoppered with rubber septa and sealed with aluminum-crimped caps. The bottles containing the media were autoclaved and then placed in the anaerobic glove chamber to cool. One mL of inoculum was then added to each bottle using a sterile syringe and needle. The inocula were grown anaerobically in sealed vials containing nutrient broth. Each of the three inoculum cultures were placed on 10% Trypticase Soy Agar (TSA) (Gould *et al.*, 1985) and enumerated in order to determine the inoculum size that was added to each bottle. The inoculum size for culture A was 1.27 X 10^6 Colony Forming Units (CFU), for culture B was 1.39 X 10^5 CFU and for culture C was 3.47 X 10^4 CFU. For each medium, 72 bottles of medium were inoculated; triplicates for each of eight sampling times and three different cultures. Three bottles of each medium were not inoculated and were analyzed for background Fe(II) present at the beginning of the experiment.

(c) Fe(II) Analysis

A 0.1% (wt/wt) ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2-triazine)] solution was prepared as per Sorensen (1982) except that the pH of the ferrozine solution was not adjusted (pH 4.36). The standard curve for Fe(II) was identical at pH 4.36 and pH 7.0.

(d) MPN Procedure

In order to enumerate iron-reducing bacteria, 1 g of soil or sediment (or 1.0 mL of liquid) is added to each of 5 replicate serum bottles (each bottle contains 9 mL of medium #8, prepared as described in section (b) in the anaerobic chamber. The inoculated serum bottles are then diluted tenfold using 1 mL syringes into another series of 5 serum bottles. This is repeated until there are 10 to12 serial dilutions. The bottles are then incubated for 28 days in an anaerobic chamber and enumerated. To determine if iron reduction has taken place, 0.2 mL of ferrozine reagent are injected into each serum bottle with a purple color indicating a positive result. A Most Probable Number (MPN) table (Cochran, 1950) was used to determine the number of iron-reducing bacteria.

(e) Enumeration of Iron Reducers in Environmental Samples

A core was obtained from a tailings pond at a mine site to evaluate the iron reducers MPN medium that we developed. The field site, sampling protocols and the chemical properties of the core samples were described previously (Stichbury *et al.,* 2000). Heterotrophic bacteria were enumerated on 10%TSA (Gould *et al.*, 1985) and incubated at 25°C under aerobic conditions and the numbers of iron-reducing bacteria were determined by the MPN technique using medium #8.

RESULTS and DISCUSSION

Significant background levels of ferrous iron were observed in two of the media after autoclaving (Table 1). Both of the media (media #10 and #11) contained glucose which is a reducing sugar and likely responsible for the iron reduction that occurred in those media during autoclaving. Significant bacterial iron reduction was observed in all media (Table 2), including the basal medium alone, which can probably be attributed to the hydrogen in the sample headspace serving as an

electron donor. *Shewanella* spp. have been shown to couple iron reduction with the oxidation of hydrogen (Lovley, 1991). Organic material carried over in the inoculum could likely serve as a carbon source. The lower inoculum size observed from the enumeration of culture C could be due to some of the organisms not growing on 10% TSA under aerobic conditions. After twenty eight days medium #8 supported the most iron reduction for cultures A and B and the second most iron reduction for culture C (Table 2). Thus, medium #8 was chosen for all subsequent studies.

All of the cultures had a one-week lag period followed by exponential growth with iron reduction essentially complete after two weeks (data not shown). The original ferric iron concentration in the medium was 5 mM (280 mg/L). Ferric iron does not appear to be limiting because even in the most active cultures only 60% of the available iron was reduced (Table 2). For most of the core samples from the field site, the numbers of iron-reducers were 5 to 50 % of the heterotrophic numbers (Table 3). However, the numbers of iron reducers in the two samples from
the upper part of the core were one to two orders of magnitude higher than the numbers of heterotrophic bacteria (Table 3). The MPN technique tends to overestimate the numbers of bacteria. In some environments iron-reducing bacteria such as *Geobacter* sp. that are obligate anaerobes will predominate (Lovley, 1991). These anaerobic iron-reducing bacteria will not grow on a heterotrophic medium that is incubated under aerobic conditions.

Molecular techniques (Fry *et al*., 1997) and MPN enumeration of enrichment cultures (Cummings *et al*., 2000) have been used previously to evaluate environmental populations of iron-reducing bacteria. Molecular probes used to evaluate the microbial communities in two anaerobic aquifers indicated that some metal-reducing bacteria were present but did not provide quantitative information about the total population of iron-reducing bacteria (Fry *et al*., 1997). The medium used by Cummings *et al*. (2000) for enumeration of enrichment cultures contained amorphous iron oxyhydroxides as the electron acceptor and required six months of incubation before they could be enumerated.

CONCLUSIONS

The following procedure was adopted for the enumeration of iron-reducing bacteria.

The medium consisted of the following constituents in g/L: $NaHCO_3$, 2.5; NH_4Cl, 1.5; NaH_2PO_4, 0.6; $CaCl_2.2H_2O$, 0.1; KCl, 0.1; $MgCl_2.6H_2O$, 0.1; $MnCl_2.4H_2O$, 0.005; $Na_2MoO_4.2H_2O$, 0.001; ferric EDTA, sodium salt, hydrate 1.84 and peptone 1.5. The final medium was adjusted to pH 7.0 and boiled under an N_2 atmosphere for 20 minutes. Additional water was added initially to compensate for evaporative losses during boiling. The medium was then removed from the heat, allowed to cool and transferred to an anaerobic glove chamber (95%N_2-5%H_2 atmosphere) and 9 mL of medium were dispensed into each 20 mL serum bottle. The serum bottles were stoppered with rubber septa and sealed with aluminum crimped caps. The bottles containing the media were autoclaved and then placed in the anaerobic glove chamber to cool.

In order to enumerate iron-reducing bacteria, 1 g of soil or sediment (or 1.0 mL of liquid) is added to each of 5 replicate serum bottles (each bottle contains 9 mL of medium #8, prepared as previously described) in the anaerobic chamber. The inoculated serum bottles are then diluted tenfold using 1 mL syringes into another series of 5 serum bottles. This is repeated until there are 10 to12 serial dilutions. The bottles are then incubated for 28 days in an anaerobic chamber and enumerated. To determine if iron reduction has taken place, 0.2 mL of ferrozine reagent are injected into each serum bottle with a purple color indicating a positive result. A Most Probable Number (MPN) table (Cochran, 1950) was used to determine the number of iron-reducing bacteria.

The medium reported in this paper is simple to prepare, gives high recoveries from environmental samples and can be enumerated in four weeks after inoculation. Iron reduction is essentially complete in two weeks and it may be possible to shorten the incubation time to two weeks.

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Table 1: Initial ferrous iron concentrations in the various media after autoclaving.

Medium No.	Carbon Source (g/L)	Fe(II) mg/L	Medium No.	Carbon Source (g/L)	Fe(II) mg/L
1	YE* (0.1) + acetate (1.5)	1.1	8	Peptone (1.5)	1.5
2	YE (0.1) + lactate (1.5)	1.4	9	YE (0.1) + peptone (0.8) + formate (0.2) + acetate (0.2) + lactate (0.2) + butyrate (0.2)	2.0
3	YE (0.1) + butyrate (1.5)	0.9			
4	YE (0.1) + formate (1.5)	1.2			
5	YE (0.1) + acetate (0.5) + lactate (0.5) + butyrate (0.5)	3.5	10	YE (0.1) + glucose (1.5)	9.7
			11	Glucose (1.5)	10.1
6	YE (0.1) + peptone (1.5)	2.4	12	YE (0.1)	1.2
7	YE (0.1) + peptone (1.5)	1.7	13	No carbon source	0.9

*YE – Yeast Extract

Table 2: Fe(II) concentrations in various media with three different bacterial cultures after 28 days incubation.

Medium No.	Fe(II) Concentration, mg/L		
	Culture A	Culture B	Culture C
1	115.2 (14.4)*	107.7 (5.5)	86.7 (5.2)
2	118.5 (3.5)	123.7 (5.5)	119.3 (5.8)
3	133.7 (3.4)	134.6 (2.5)	89.0 (2.7)
4	139.3 (2.8)	131.4 (1.1)	129.4 (9.4)
5	142.9 (13.1)	125.4 (9.8)	51.5 (1.1)
6	122.6 (5.1)	120.9 (7.1)	120.6 (1.9)
7	145.8 (7.3)	139.3 (1.6)	140.8 (5.6)
8	156.2 (10.5)	165.7 (12.0)	142.3 (8.8)
9	137.1 (9.7)	132.4 (4.0)	130.7 (2.4)
10	132.8 (2.1)	143.0 (9.4)	156.2 (7.8)
11	138.0 (1.0)	136.3 (10.3)	135.8 (5.4)
12	121.4 (10.3)	125.7 (0.9)	103.2 (5.9)
13	124.2 (8.7)	120.3 (7.9)	94.6 (5.6)

*Numbers in brackets are the standard deviations for the Fe(II) analyses (experiments in triplicate).

Table 3: Numbers of iron-reducing and heterotrophic bacteria in a core taken from a field site.

Depth, (m)	Heterotrophic bacteria (#/g)	Iron reducing bacteria (#/g)	Depth, (m)	Heterotrophic bacteria (#/g)	Iron reducing bacteria (#/g)
3.15	1.44×10^4	2.3×10^6	5.56	1.2×10^4	4.9×10^3
4.33	1.0×10^3	1.7×10^5	5.77	1.83×10^4	1.1×10^3
4.83	6.05×10^4	7.9×10^3	5.96	3.8×10^4	7.9×10^3
5.18	9.9×10^4	4.9×10^4	6.15	3.3×10^4	2.3×10^3
5.45	2.72×10^4	2.3×10^3	6.30	2.4×10^5	1.2×10^4