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（メタンを基質とした嫌気的微生物集積系から得られた2種の新
規鉄還元細菌の分離と性状解析）

２０１３

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PREAMBLE

In partial fulfillment of the requirements stipulated under the Science of Biological Resources Course in The United Graduate School of Agricultural Science, Gifu University, this documentation, a compilation of data garnered over a duration of more than 3 years of extensive research, is hereby, submitted along with two of my scientific publications, as fulfillment for the Doctor of Philosophy (PhD) in Agricultural Science, majoring in Environmental Microbial Engineering.

This documentation shall be referred to as the *PhD Thesis* hereafter, comprises of **Chapter 1** (Introduction), **Chapter 2** (anaerobic enrichment culture), **Chapter 3** (isolation and characterization of an isolate strain OSK6^T), **Chapter 4** (isolation and characterization of an isolate strain OSK2A^T) followed by **Chapter 5** (Overall conclusion).

Chapter 1, ***Introduction section***, is a compilation of numerous published literatures relevant to the background of this research, and the objectives of the PhD thesis.

Chapter 2 contains the full description of the establishment of an ***anaerobic microbial enrichment culture***, which was inoculated with lotus field mud and, later used as the inoculums source for isolation of two novel strains of bacteria described in the subsequent chapters.

Chapter 3 provides a detailed documentation of the methodologies and subsequent data generated during the ***isolation and characterization of strain OSK6^T***, a novel species under the genus *Geobacter*, a distinguished group of species capable of dissimilatory Fe(III) reduction.

Chapter 4 describes in full details the findings obtained on the **isolation and characterization of a novel subspecies of *Geobacter sulfurreducens***, capable of generating current in microbial fuel cell (MFC) studies and is one of the well studied bacterial species to date, with an established complete genome.

Chapter 5 is the ***General Conclusion*** of the PhD Thesis, a complete and overall summary of all the chapters.

MY SUCCESS IS THROUGH THE SWEAT OF MY FATHER AND THE PAIN OF MY MOTHER, THROUGH GOD, IMPOSSIBLE IS NOTHING.

ABSTRACT

Enclosed herein is my *PhD thesis*, a detailed summary of the bits and pieces of lengthy researches and tedious experimental procedures, conducted in the isolation and characterization of two novel strains of *Geobacter* species, from an anaerobic microbial enrichment, inoculated with mud from lotus field and induced with methane.

Initial research focused on the possibility of discovering or constructing a consortium or clutch of microbes presumably syntrophs, capable of oxidizing methane anaerobically (AOM), coupling to Fe(III) reduction and later, targeted the isolation of *Geobacter* strains for use in microbial fuel cell (MFC) studies.

The initial strategy was, establishing of an anaerobic microbial enrichment culture which was regularly supplemented with 20 mM amorphous Fe(III) hydroxide at minimum, and a consistent daily adlib supply of CH_4. Bio-samples for observation of microbial activities were monitored over time.

Mud samples used to inoculate the anaerobic microbial enrichment chamber, were collected in the summer of June 2009, from a lotus field in Japan (Aichi Prefecture), since wetlands such as rice fields and lotus fields are major anthropogenic sources for methane production and emission in the world. Wetlands are also habitats for methanogens and methanotrophs; producers of biological methane and microbes that utilize for respiration with oxygen as the terminal electron acceptor respectively.

In chapter 2, the study revealed that over the course of the anaerobic microbial enrichment cultivation, heterotrophic bacteria such as *Acidaminobacter* bacteria were the most dominant microbes within the anaerobic enrichment culture, which are not uncommon considering the richness in organic matter content of the sample source. But, presence of Fe(III)-reducing bacteria, sulfate reducers and oxidizers were also detected. Active Fe(III)

reduction was also determined, reaching a high of 28 mM Fe(II). This coincides with the highest average difference of 1.8 mM methane measured between the inlet and outlet, in the same time period, indicating a possible occurrence of AOM. However, isotopic incubations with $^{13}C\text{-}CH_4$ in batch cultures remained inconclusive. For future studies, an improved anaerobic enrichment culture with high quality analyses using proper high tech analytical equipments such as GC-IRMS is central to the study of AOM.

In chapter 3, after almost a year of cultivation, isolation of a novel Fe(III)-reducing bacterium from the microbial enrichment described in chapter 2, published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) as *Geobacter luticola* OSK6[T] (127) is described. Strain OSK6[T] was isolated in deep gellan gum tubes and purified in the six well-plate method. It utilizes acetate for respiration with Fe(III) as the terminal electron acceptor (TEA). Strain OSK6[T] also grows with other TEAs such as Fe(III)-NTA, ferric citrate, amorphous Fe(III) hydroxide, and nitrate, but not with fumarate, malate or sulfate. With Fe(III)-NTA as the TEA, the isolate metabolized acetate, lactate, pyruvate, and succinate during respiration. Analysis of the near full-length 16S rRNA gene sequence revealed that strain OSK6[T] is closely related to *G. daltonii* and *G. toluenoxydans* with 95.6% similarity. Morphology, physiology and chemotaxonomic analyses of strain OSK6[T] are described in this chapter.

In chapter 4, another novel strain of Fe(III)-reducing bacterium, described as a novel subspecies belonging to the genus *Geobacter*, named as *Geobacter sulfurreducens* subsp. *ethanolicus* OSK2A[T], in reference to its ability to utilize ethanol as substrate for growth in comparison to its closest relative, published in the Journal of General and Applied Microbiology (JGAM) is reported. This was the second *Geobacter* species to be isolated in Japanese soils from the microbial enrichment reported in chapter 2. Similar to *Geobacter luticola* OSK6[T], the novel isolate was initially isolated in deep gellan gum tubes and further

purified in roll tubes with agar. It is spherical in shape and red in color when grown in solid medium. Morphological studies showed that strain OSK2A^T is a Gram-negative, motile, rod-shaped bacterium. It strives at 20-40 °C, pH 6.0-8.1 and tolerates up to 1 % NaCl. 16S rRNA gene sequencing showed 99.6% similarity to *Geobacter sulfurreducens* strain PCA^T and far lower similarity to other *Geobacter* species. G+C content of the genomic DNA, chemotaxonomic studies and DNA-DNA hybridization also corroborated that the strain OSK2A^T should be classified as a novel subspecies of *Geobacter sulfurreducens*.

Chapter 5 entails the summation of every finding garnered throughout the conduction of the PhD thesis which in reality commenced in the final year of my two years of Master of Science (MSc.) studies; the establishment of the anaerobic microbial enrichment culture detailed in Chapter 2. Bibliography/references and Acknowledgement brings up the rear of this PhD thesis.

Successes, trials, tribulations, difficulties and all other challenges experienced during the duration of this thesis, has given us further insights in the isolation of Fe(III) reducing bacteria. For a guaranteed success, any future microbial enrichment intended for cultivation, isolation and characterization of Fe(III)-reducing bacteria which could be used for model studies on terrestrial AOM, must be implemented in an improved anaerobic reactor.

CHAPTER 1 Introduction

Advancing our understanding about microbes and their biological functions and processes is vital to our understanding of the environment that we live in since, microbes are a major component of the ecosystem and play a pivotal role in the recycling of minerals and survival of mankind.

One of the most used strategy by scientists' world over, to advance our current knowledge on microbes is, to enrich and isolate microbes of special interest to them. Model studies were then devised based on their understanding of these isolates. This has led to many beneficial findings such as amalgamating mitigation factors against climate change, discoveries of new bioremediation techniques, bio-fuel production, bio-fuel cells and many more.

Indeed, our advanced understanding of microbes garnered over the decades from models based on microbial isolates has tremendously helped the advancement of biological science in general.

One of the imaging fields in science is the utilization of microbes to generate current or electricity by exploiting microbial normal respiration processes. This phenomenon is scientifically referred to as the microbial fuel cells (MFCs). These microbial genera involved in MFC are ubiquitous and are also present in tropical conditions, which could potentially become a viable solution for provision of electricity amongst poor island states including Solomon Islands (my country of origin). Not surprisingly, such studies are made possible through our advanced understanding of microbes that inhabit/dwell in the environment that we share.

But the efficiencies of such newly found science still eludes us so, in order to further enhance and expand our basic but important knowledge on the potential use of microbes for

the benefit of mankind, more studies and isolation of beneficial microbes must continue to be pursued.

My PhD thesis is aimed to achieve just that; to provide new details and insights on beneficial microbes that may help scientists to discover and formulate new ideas and technologies for the advancement of science and engineering, consequently improving our approaches towards sustaining our environment.

To achieve the intended purpose of this research, enrichment and isolation of beneficial bacteria that could strive in the presence of methane coupling to Fe(III) reduction, for potential use in model studies on MFCs, was initiated.

The use of methane and Fe(III) in the initial enrichment of the isolates were based from previously published studies on methane as a sole carbon substrate for anaerobes in anoxic environment, and the ubiquitous presence of Fe(III) mineral on earth with high concentrations reported on sub-surface and surface environment. Fe(III) oxides also have a higher affinity as a terminal electron acceptor in microbial respiration.

On the other hand, methane has scant information and most evidences on the oxidation of methane in anoxic environments were circumstantial observations with exceptions to AOM coupling to either nitrate/nitrite or sulfate. These were the only two AOM processes widely accepted as scientifically authentic, with numerous evidences dotted across the globe.

Based on thermodynamic calculations, methane and Fe(III) oxides are supposed to be able to support microbial respiration as a sole carbon source and terminal electron acceptor, respectively. Therefore, based on this prediction in addition to the many previous studies, I embarked on trying to discover the microbial evidences reported herein.

1.1 Methane – its properties, sources, uses and potency as a greenhouse gas (GHG)

Methane (CH_4) is a hydrocarbon and contains four hydrogen carbon covalently bond to a carbon as a tetrahedron (Fig. 1.1). Its melting and boiling point are -183 and -161.5 °C, respectively, explaining why methane is gaseous at room temperatures. Methane is a non-polar molecule and therefore only slightly soluble in water (136). It is a colorless and odorless gas at room temperature and the simplest alkane (8), primarily produced in the natural ecosystem by microorganisms in a process called methanogenesis, a biological process by which microorganisms belonging mainly to the domain Archaea produce methane from the decomposition of organic compounds in several geo-chemical and anthropogenic sources (9, 32, 49, 73, 80, 89–90, 104, 115, 132, 134, 140–141).
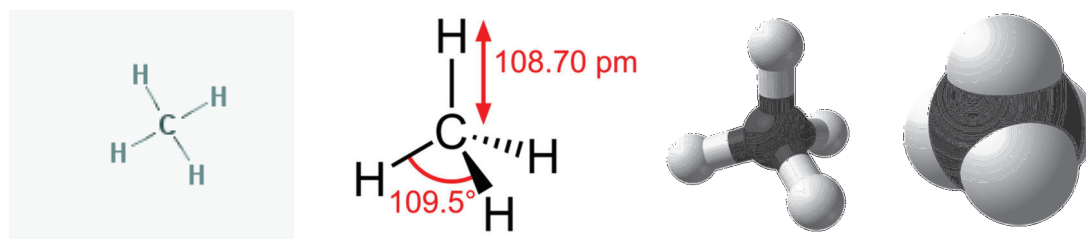
Fig. 1.1 Different representations of the methane molecule (123).

These sites are completely anaerobic environments, including gas hydrants and seeps in deep ocean floors, ponds, lakes and rivers, paddy fields (rice & lotus), pipelines and biogas recycling plants as well as digestive tracts of ruminants, humans and termites (Fig. 1.2 & Table 1.1). Almost all of the methane produced within these sites is lost to the environment and humans utilize just a slight fraction of methane that is naturally produced globally. Nowadays, it is common for methane from anaerobic digester tanks to be harvested and

utilized as gas for cooking, vehicle fuel and in some cases to supplement electricity supply, particularly in developed nations that could afford to construct such expensive facilities.
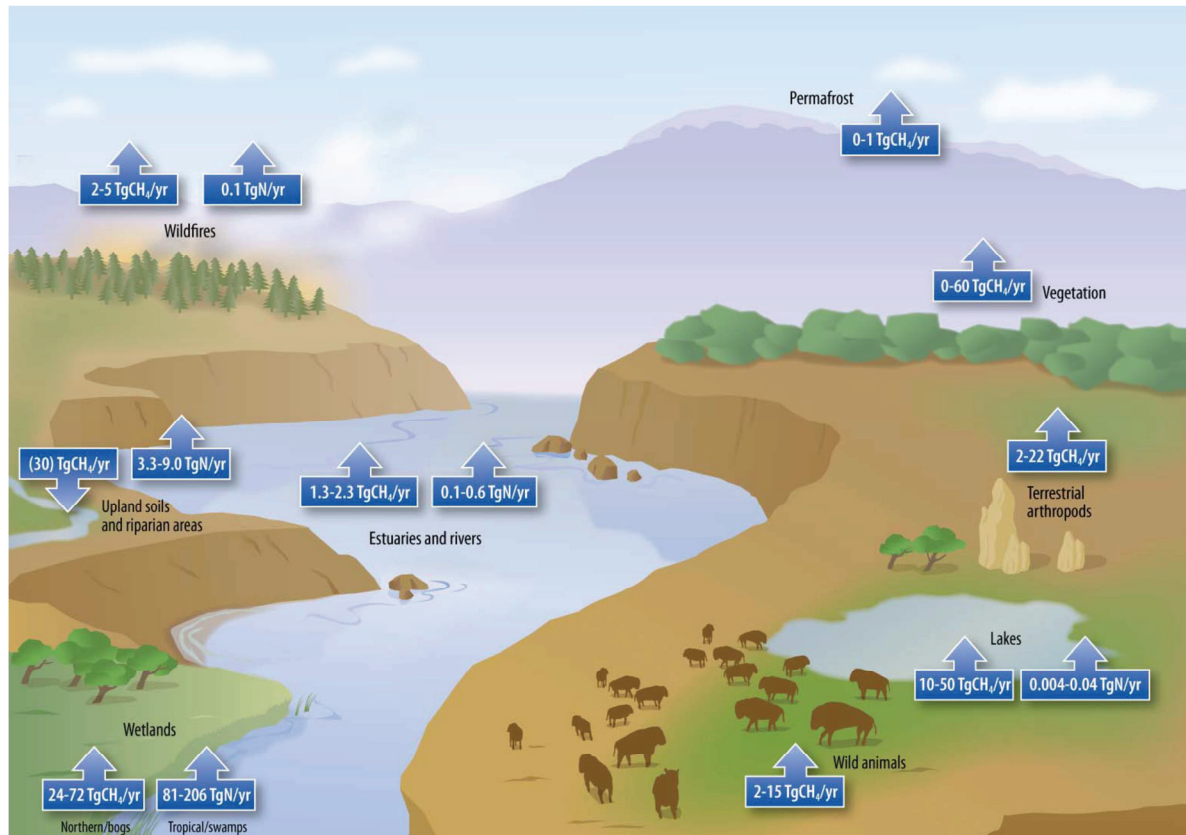
Fig. 1.2 Estimated annual emission of methane from terrestrial sources (adopted from EPA, 2010).

Recently increased efforts to harvest methane as a liquefied natural gas are ongoing in developed nations. However, large quantity of methane from some of these natural environment are produced unabated in large quantity, and are mostly out of reach for human usage due to the difficulties associated with harvesting thus, escapes directly into the atmosphere, eventually contributing to global warming.

Methane emitted into the atmosphere is an important radiative trace gas as, it is at least twenty (20) times more potent than carbon dioxide (CO_2) as a heat trapping gas (67), accounting for about 20% of the green house effect (17, 123, 135), and takes longer time to be removed completely from the atmosphere hence, constitute as a major greenhouse gas (GHG) and therefore posing a greater risk to global warming. Furthermore, Saint Louis *et al*

(94) estimated that 7% of anthropogenic global warming equivalents come from methane emitted from man–made reservoirs alone. For instance, paddy fields alone constitute as the major man-made reservoir for methane emissions (35), with about 90% of the methane produced, escaping into the atmosphere (100). Similar levels of methane emissions maybe possible for lotus fields since both have similar farming characteristics. From current estimates and trends, methane emission is forecasted to increase, attributed largely to increased human activity (Table 1.1 & IPCC, 2007).

However, microbes do oxidize methane as a substrate for growth either aerobically with oxygen as the terminal electron acceptor or anaerobically (AOM) with sulfate, nitrate/nitrite and manganese/iron as terminal electron acceptors. The oxidation of methane acts as very important methane sinks in preventing the direct escape of methane into the atmosphere thus, potentially contributes indirectly to the reduction of global warming.

Table 1.1 Current global methane emissions from natural sources (Source; EPA, 2010). NA, not available.

Methane (Tg CH_4/Year)			
Source			$\delta^{13}C$ (‰)[c]
	Emissions estimate	**Range**	
Wetlands.	170.3	41.5-139	-60.45
Upland soils and riparian areas.	-30	NA	
Oceans, estuaries and rivers.	9.1	2.3-15.6	-58
Permafrost.	0.5	0-1	
Lakes.	30	10-50	-53.8
Gas hydrates.		2-9	-62.5
Terrestrial and marine geologic sources.		42-64	-41.8
Wildfires.		2-5	-25
Vegetation.		Not a source or 20-60	NA
Terrestrial. arthropods.	20	2-22	-63
Wild animals.	8	2-15	-60.5
All natural sources.	208		-57
All sources to the atmosphere (anthropogenic and natural).	566	503-610	-54.5
Natural sources as a percent of the total.	37%		NA

1.1.1 Anaerobic oxidation of methane (AOM)
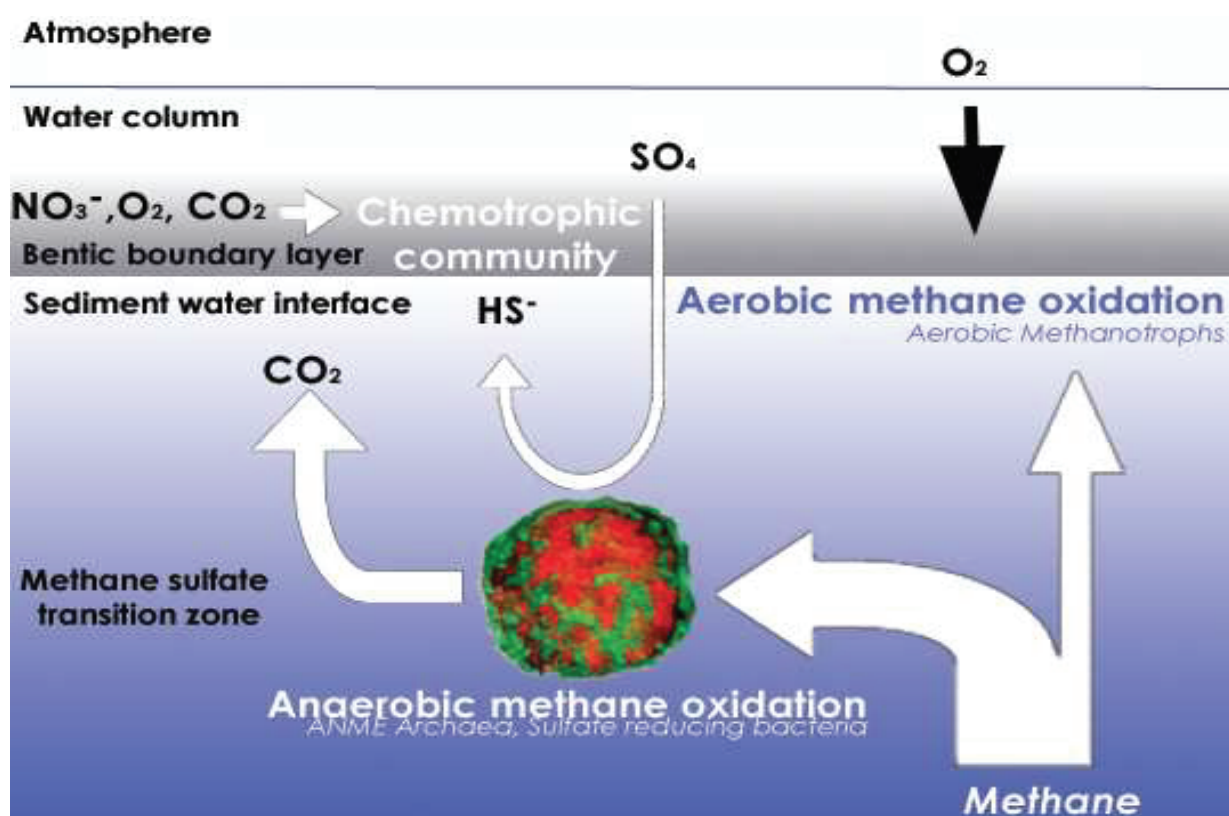
Fig. 1.3 Flow diagram of AOM in marine sediments (47).

Methane has been well documented to aerobically oxidize to CO_2 by Methanotrophic bacteria identified as Type I methanotrophs belonging to γ-proteobacteria, Type II methanotrophs of α-proteobacteria and type X methanotrophs belonging to genus *Methylococcus* (γ-proteobacteria) (11, 14,40, 75, 116). These methane oxidizing methanotrophic bacteria are found in methane producing sites such as rice fields, ponds, sediments, lakes, landfills and rivers (34, 88, 107, 131, 133), amongst others. The aerobic oxidation of methane provides energy for growth of these microbes and in the process, abate the direct escape of methane into the atmosphere, as methane is being oxidized to CO_2. However, since large junk of methane is produced in anaerobic sites such as, gas hydrants and gas seeps in the deep ocean floors instead of aerobic sites or near water surfaces, it is believed that there is an alternative process other than aerobic oxidation of methane (Fig. 1.3), at which methane is oxidized, referred to as anaerobic oxidation of methane (AOM).

AOM is an anaerobic microbial process by which microorganisms in anoxic environment utilize methane as a substrate for growth. The responsible microorganisms are usually present in microcosms either as clusters or aggregates (Fig. 1.7) (10, 77, 81–82, 84, 96, 123–124) and individual communities (30). Several decades ago it was first discovered that methane disappeared long before it came into contact with oxygen (44, 69, 89, 139, 127). First direct evidences of AOM being regulated by microorganisms was discovered around the same period (42–43). These findings gave rise to the phenomena of AOM, which to this day is still poorly understood although great stride has been made in discovering its occurrence, with most studies being done on sediments collected from the marine environment, which consist mainly of methane hydrates, carbonates and methane seeps all located deep in the ocean floor. In marine sites, cold temperatures and anaerobic oxidation of methane (AOM) acts as very important sinks in trapping methane gas from escaping directly into the atmosphere, and is estimated to remove more than half of the methane arising from marine sediments (42). In this process, methane is anaerobically oxidized to CO_2, which is coupled to the reduction of sulfate, mediated by obligate anaerobic microorganism (Fig. 1.4, Fig. 1.5). AOM therefore represents a potential mechanistic constraint on global warming but despite the global significance of AOM and considerable effort to identify the exact mechanisms and organism(s) involved in marine sediment AOM (10, 43, 121), much about the process and responsible organisms remains unclear, and little is known about the occurrence and importance of the process in non-marine systems/environments.

Difficulty in understanding AOM is due to the difficulty in culturing of the responsible microbes (84), their slow doubling period (77) and absence of isolates. Current proposed mechanisms are largely based on circumstantial evidences (120) therefore, obtaining an isolate capable of AOM is paramount to identifying the bio-chemical mechanisms and intermediates involved in AOM. Over the past years, several investigations

have revealed the process of AOM but so far, only two processes involving AOM has been widely reported with the third AOM still lacking information.

1.1.2 Anaerobic oxidation of methane coupling to sulfate reduction

The first discovered and widely reported AOM (equation 1 or Fig. 1.4, Fig. 1.5) is coupled to sulfate (SO_4^{2-}) reduction (10, 43, 50, 121), carried out by a consortium of archaea oxidizing microbes known as *Anaerobic Methanotrophs* (ANME I, II, III) (Fig 1.6) and sulfate reducing bacteria (SRB) of the *Desulfosarcina/Desulfococcus* (DSS) branch of the Deltaproteobacteria (10, 53, 72, 81).

1.1.3 Anaerobic oxidation of methane coupling to nitrate/nitrite reduction

The second mostly widely reported AOM process discovered is coupled to NO_3^-/NO_2^- reduction (equation 2) carried out solely by '*Candidatus* Methylomirabillis oxyfera', bacteria of a novel phylum (Fig 1.8) in a proposed novel pathway (Fig. 1.9c) whereby oxygen is produced from nitrite reduction, which oxidized methane (29–30, 87). Nitrate/nitrite dependent AOM has since been reported for several other different sites and seems to be a widespread occurrence in terrestrial environments (24, 28, 45–46).

1.1.4 Anaerobic oxidation of methane coupling to Mn(IV) oxide and Fe(III) reduction

The third and the least reported AOM process is coupled to Mn(IV) and Fe(III) reduction and has so far been reported for a lone marine sediment site (7) with very limited supporting citations available. No further clear evidences exist on this process although attempts were made (23, 105). Although it is an energy yielding process (equation 3) which could support microbial growth (120) no new evidences have been found for both terrestrial marine environment other than the report from Beal *et al*., (7).
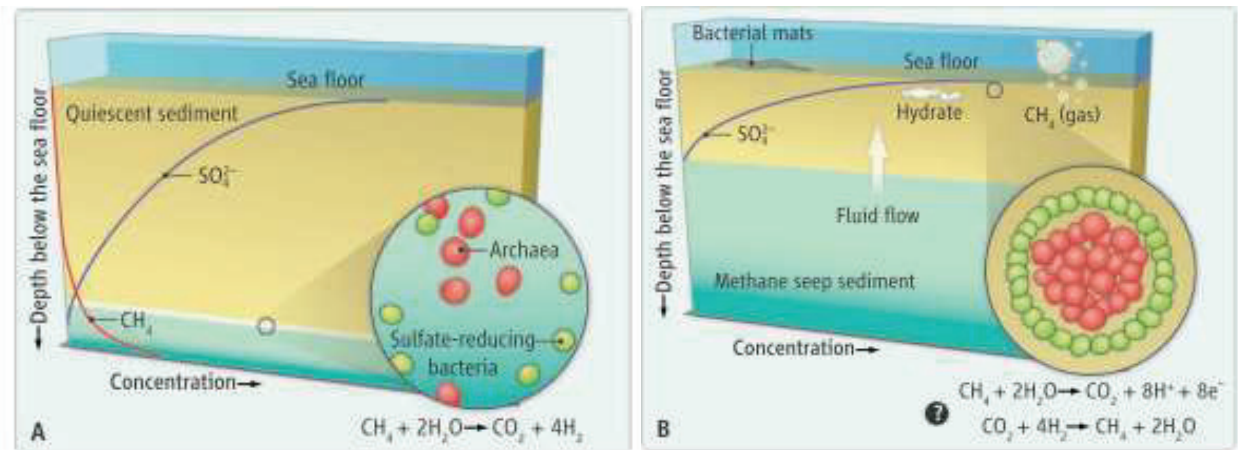
Fig. 1.4 Proposed pathways for anaerobic methane oxidation coupled to sulfate reduction in quiescent ocean sediments (A) and dynamic methane seeps (B) (3).

$$CH_4 + SO_4^{2-} + H^+ \longrightarrow CO_2 + HS^- + 2H_2O$$

$$\Delta G°' = -21 kJ/mol \qquad (1)$$

$$5CH_4 + 8NO_3^- + 8H^+ \longrightarrow 5CO_2 + 4N_2 + 14H_2O$$

$$\Delta G°' = -765 kJ/mol \qquad (2)$$
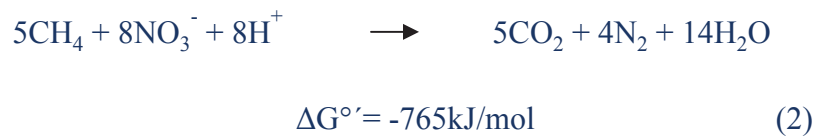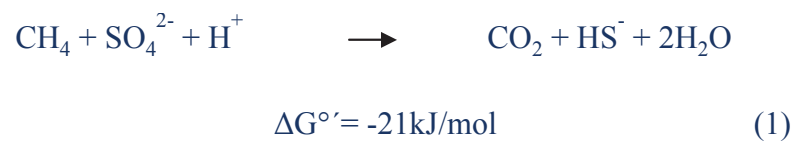
Fig. 1.5 Chemical equations and energy yield for anaerobic oxidation of methane coupling to sulfate (1) and nitrate (2) reduction at standard room temperature condition.
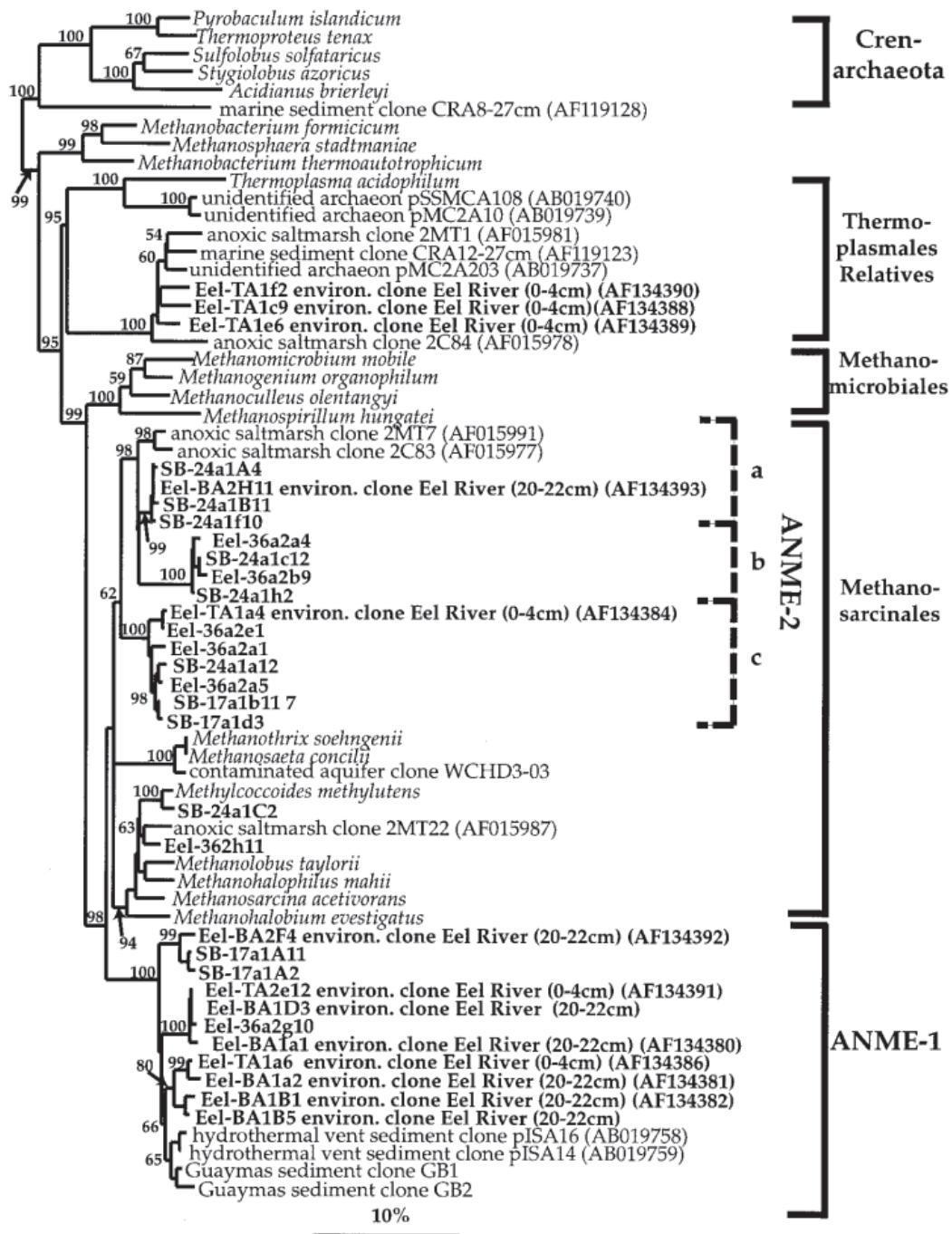
Fig. 1.6 Phylogenetic tree showing relationships of 16S rDNA archaeal clone sequences from Santa Barbara Basin and Eel River Basin seep sites (in boldface) to selected cultural and environmental euryarchaeotal sequences in the database (81).
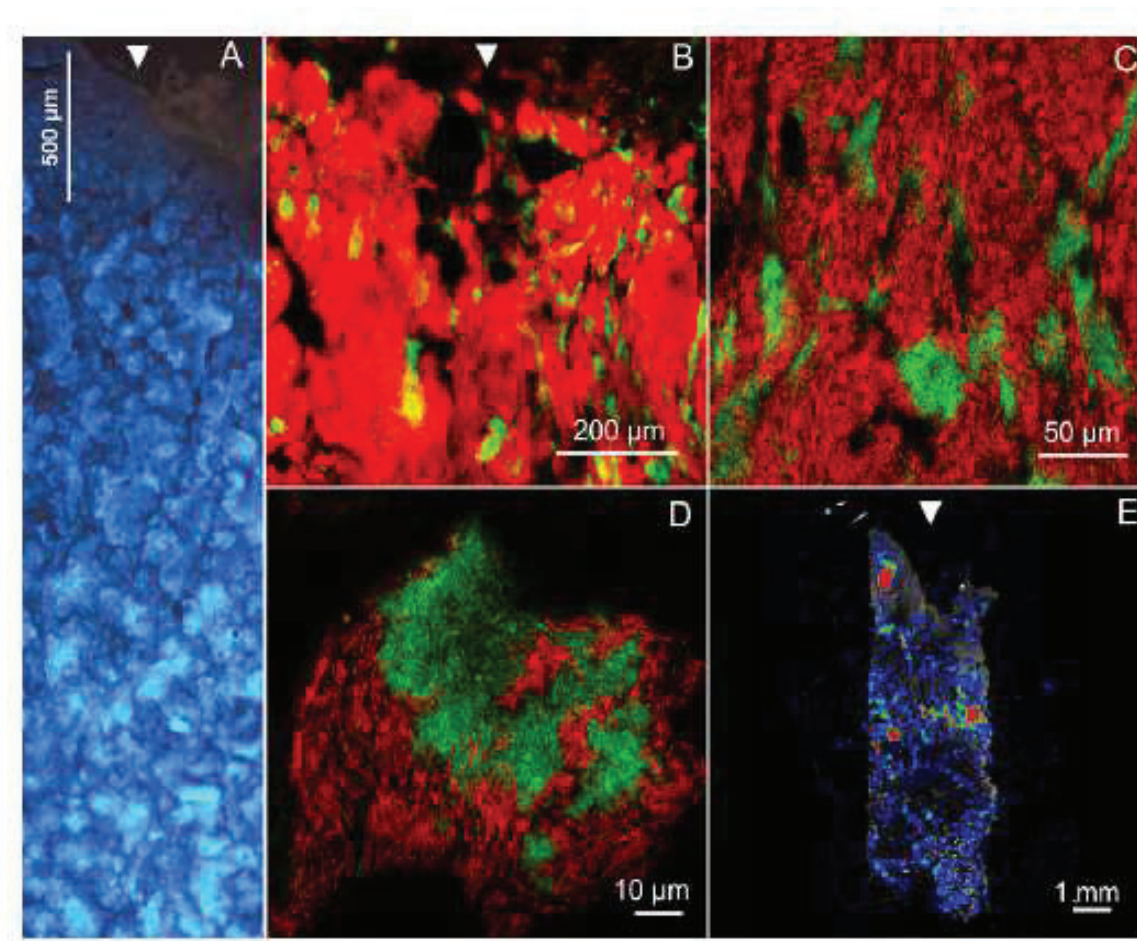
Fig. 1.7 Fluorescent image showing a thin section of the pink mat. (A) A thin section of mat stained with DAPI. (B) Archaea of the cluster ANME-I were targeted with a red fluorescent group-specific oligonucleotide probe. The SRB were targeted with a probe specific for a cluster of δ-proteobacteria in the Desulfosarcina/Desulfococcus group and fluoresce green. (C) Microcolonies of SRB are surrounded by bulk ANME-I cell clusters. (D) ANME-I cells have a unique rectangular shape. SRB are small coccoid cells. Single SRB cells are dispersed throughout the ANME-I cell clusters. (E) beta-imager micrograph of a thin section of mat incubated with $^{13}CH_4$ (23).
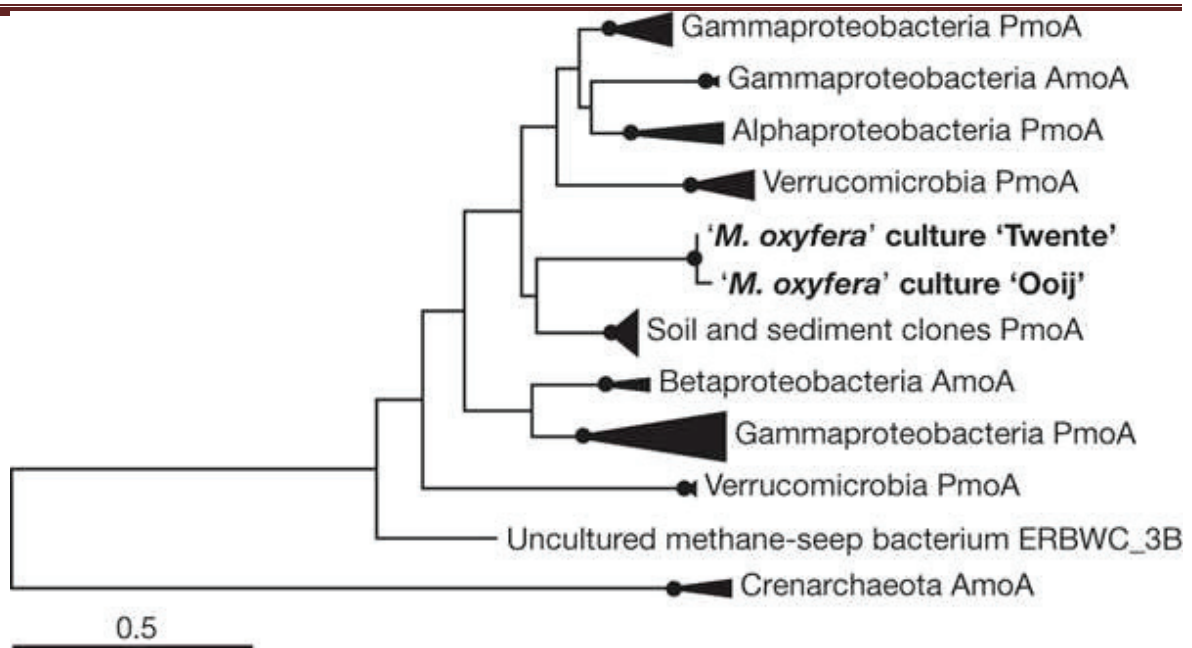
Fig. 1.8 Phylogeny of '*Methylomirabillis oxyfera*' pmoA protein sequences. Methylomirabillis is reported to be solely responsible for the process of AOM coupling to nitrate reduction (30).
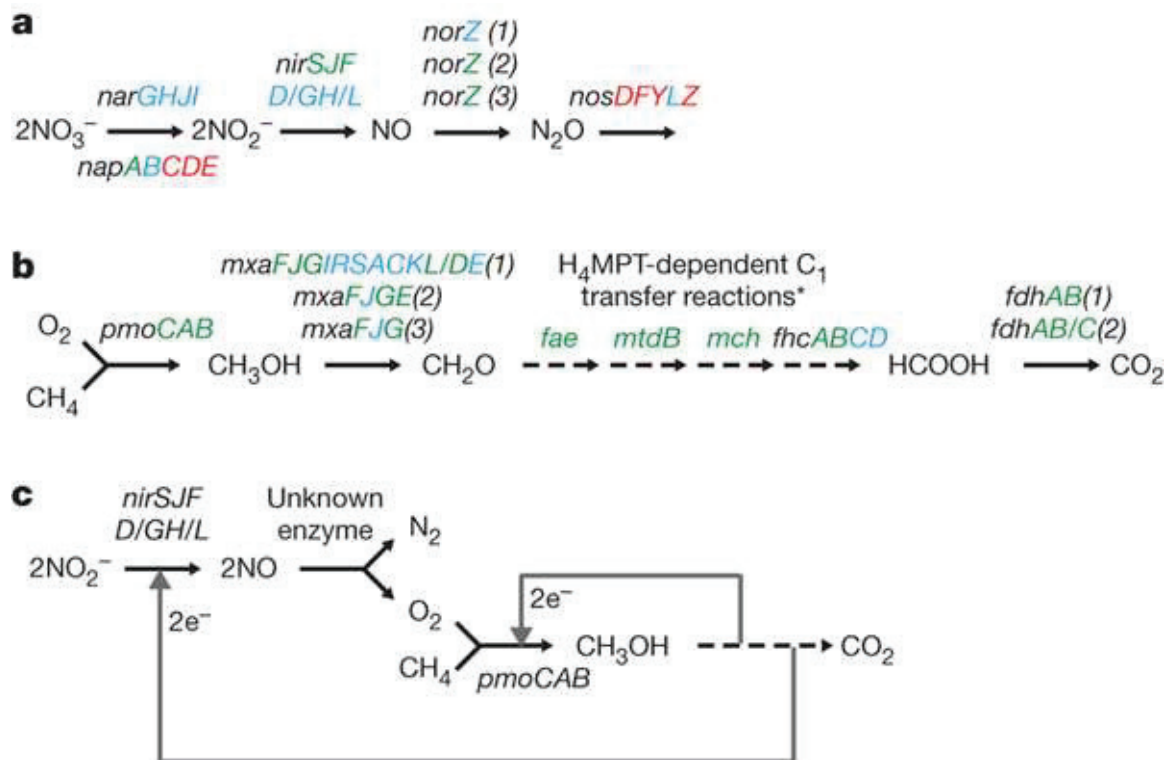
Fig. 1.9 Significant pathways of '*Methylomirabillis oxyfera*'. Cannonical pathways of denitrification (a), aerobic methane oxidation (b) and proposed pathway of methane oxidation with nitrite (c) (30).

For all three AOM processes, isolation of the responsible AOM microbes have not been elucidated to date and only few successes from countless attempts had been made, to cultivate the responsible microbes in the laboratory as a consortium growing in adjacent to each other, displaying syntrophism (36, 77), but with a very slow doubling period of 7 months.

On the other hand, Fe(III)-reducing microbes belonging to genus *Geobacter* and *Shewanella* have been proposed as potential bacteria capable of AOM in terrestrial environment (25, 74, 106) but with no direct evidence to date.

1.2 Fe(III) reduction – role/importance in the environment

Iron is the fourth most abundant mineral on the earth crust and is ubiquitous in the environment as Fe oxides (113). It is a very important trace element for biological processes and is part of hemoglobin in living organisms. It is also a major water pollutant and its presence in contaminated water makes it difficult for designing of effective and cheaper water treatment facilities. Of all Fe oxides, ferrihydrite is the least stable with the least crystallized or unstable structures, making it more favorable in bio-chemical reactions with its large surface area (97). There are two forms of ferrihydrite; a 6-line & 2-line ferrihydrite based on their differing but somewhat flexible x-ray diffraction (XRD) peak. In this study the 2-line ferrihydrite which is referred to as ferrihydrite or amorphous Fe(III) hydroxide was utilized. It is not commercially available and was therefore synthesized in the laboratory according to methods previously described (98). In the wet form it is soluble in aqueous solutions but may form other Fe(III) hydroxides such as goethite and hematite during storage over longer period and even when sterilization techniques such as autoclaving is done (99). Freeze-drying or lyophilizing to remove moisture from synthesized ferrihydrite is a better option however, dried ferrihydrite is difficult to dissolve and insoluble in neutral pH aqueous solutions. Thus, most studies involving microbial reduction of ferrihydrite has been

conducted in the wet form (57, 62, 112) and the same for this study. An alternative is to harvest microbially produced ferrihydrite for those who have access to such vital apparatus in their laboratory (37).

1.2.1 Carbon cycling with Fe(III) reduction in terrestrial environment

Iron is also utilized by Fe(III)-reducing microbes as the terminal electron acceptor in the decomposition of organic matter in terrestrial environment. Different Fe(III) oxides provide the ochre to the brown color of soils in temperate zones or the red color of tropical zones (22). Environmental sites such as oxygenated lake water contain low soluble ferric hydroxides, which are mainly found as constituents of living organisms or in complexes of their biological origin and may precipitate as Fe(III) hydroxides once degradation of the organic residues occurred. As a result, it accumulated in the sediment to high concentrations, averaging at around 1 - 5 % of the sediment dry matter (20 mM to 100 mM) (110). With these high concentrations iron is probably the most important electron acceptor for microorganisms under anoxic conditions especially in water logged soils, natural wetlands, paddy fields and fresh water lake sediments, compared to oxygen (around 0.3 mM), nitrate (<0.1 mM), or sulfate (0.2 mM in fresh water lakes) as reported by Straub *et al.,* (111). However, Fe(III) oxides are only poorly soluble at neutral pH with concentrations of soluble Fe^{3+} $\leq 10^{-9}$ M (54). Hence, microbial reduction of Fe(III) oxides under these conditions has to cope with a practically insoluble electron acceptor (37). Reduction of ferric iron hydroxides leads to release of Fe^{2+} ions and the formation of ferrous carbonate (siderite). In anoxic habitats, Fe(III) oxides and humic substances are wide spread, and Fe(III) iron and humic acid reducing microorganisms probably play an importantly role in the oxidation of organic matter and therefore contributes to the global cycling of metals and carbon.

1.2.2 Major Fe(III)-reducing microbes in terrestrial environment

Dissimilatory reduction of Fe(III) oxide by Fe(III)-reducing bacteria plays an important role in organic matter mineralization (57–60, 62, 66, 119, 130) and contributes to the global cycling of metals and carbon. The process is predominated by *Geobacter* species (4, 18, 59, 62, 64, 108).

1.2.3 Timeline in the discovery of *Geobacter* species

The genus *Geobacter* was established by Lovley *et al*. (61) with *Geobacter metallireducens* GS-15^T isolated from the Potomac River, the first species to be described under the genus followed by *Geobacter sulfurreducens* PCA^T (13). *Geobacter* species have been found to be metabolically very versatile and are capable of not only Fe(III) reduction but also degradation of aromatic contaminants (55, 68, 85, 138). Potential applications for bioremediation in uranium-contaminated aquifers (2, 5, 79, 103) as well as electrogenic activity on microbial fuel cells (59, 92, 109) have also been reported for the genus *Geobacter*. With their versatility, *Geobacter* species play an important role in the protection of groundwater resources as well as oxidation of organic matter in anoxic freshwater environments with Fe(III) as the sole electron acceptor (59). In addition, *G. sulfurreducens* PCA^T, the most closest relative to the novel strain OSK2A^T is one of the most studied *Geobacter* species as a model organism on various researches such as biochemical and molecular studies on respiratory mechanisms of iron (III) oxide (52, 56, 64, 93), development of genetic manipulation techniques (1, 21, 83, 125), and the first genome analysis within the genus *Geobacter* (71).

1.2.4 Relationship between microbial Fe(III) reduction and methane in terrestrial environment

Major part of this study wass to isolate and characterize novel Fe(III)-reducing bacteria and determine if these bacteria contain the capacity to utilize methane as a substrate

for growth or vice versa. As depicted in equation 3, thermodynamically methane is a potential electron donor in the reduction of Fe(III) oxides and far favorable over AOM coupled to sulfate reduction. Yet no consortium let alone isolates, capable of facilitating equation 3, has been discovered or reported. *Geobacter* species on the other hand is the far most dominant bacteria in Fe(III)-reducing environment and has been found to decompose organic matter along with Fe(III) reduction. Not only do *Geobacter* species capable of organic matter decomposition but also their ability to utilize aromatic and hydrocarbon contaminants makes them a very versatile group. It is their versatility that drives my interest to investigate *Geobacter* species as a potential model for studies on utilization of methane as a substrate for growth coupling to Fe(III) reduction. Since paddy fields are some of the most important anthropogenic sources of methane emission, and the conduciveness of such sites as habitats for *Geobacter* species, I descended to retrieve mud samples from a lotus field to investigate the hypothesis that Fe(III)-reducing microbes may possesses the ability to utilize methane as a substrate for growth.

1.3 Aims and Objective

Microbial Fe(III)-reduction in terrestrial environment plays a major role in the global recycling of metals and carbon. *Geobacter* species are the most predominant bacteria in such environment and play an important role in a diversity of natural environments including decomposition of organic matter in tandem with Fe(III) reduction. *Geobacter* species can be easily cultured and studies on their physiology and the whole genome sequence analyses for *Geobacter sulfurreducens* revealed their versatility and has since become the bacteria of choice amongst the microbial Fe(III)-reducing communities for various researches on bioremediation techniques, electrical generation in microbial fuel cells, biochemical and molecular studies on respiratory mechanisms of Fe(III) oxide and development of genetic manipulation techniques. These studies underlay the importance of *Geobacter* species on the geochemistry of anaerobic soils and sediments including paddy fields like rice and lotus fields.

Given its abundance as well as its poorly crystallized nature, Fe oxide is widely used by Fe(III)-reducing bacteria in the decomposition of organic matter and the Fe(III)-reducing microbes may possess the ability to degrade methane. Thermodynamic calculations shown in equation 3, strongly suggests that AOM coupling to Fe(III) hydroxide reduction is energetically more favorable for growth of responsible microbes, compared to equation 1 hence it is a question of when and where rather than a question of possibility.

This study was carried out to establish a microbial Fe(III)-reducing enrichment whereby novel Fe(III)-reducing isolates are isolated and characterized. It was also to investigate the reduction of amorphous Fe(III) hydroxide by either an Fe(III)-reducing community (consortium) or Fe(III)-reducing isolates supplemented with methane as a substrate. Monitoring the bacterial community composition of an established anaerobic

microbial enrichment to determine any shift in bacterial community under constant methane supplementation may provide any indication in the activity of Fe(III)-reducing bacteria.

Geobacter species are ubiquitous in terrestrial environment just as Fe oxides are in soils. The growing number of validly published type strains of the genus *Geobacter* is a clear testament to its widespread. By constructing an anaerobic microbial enrichment with mud from a paddy field such as lotus mud which is a major anthropogenic source for methane emission thus a habitat for methanogens, an in situ environment replicating lotus fields whereby methanogens and potential anaerobic methane utilizing microbes interact is envisage. The expected outcome of this work is to show that, Fe(III)-reducing isolates continuously induced with methane in a microbial enrichment will, display characteristics of methane degradation in a proposed pathway depicted in Fig 1.10.

$$CH_4 + 8Fe(OH)_3 + 7HCO_3^- + 7H^+ \longrightarrow 8FeCO_3 + 21H_2O$$

$$\Delta G°' = -286kJ/mol \qquad\qquad (3)$$
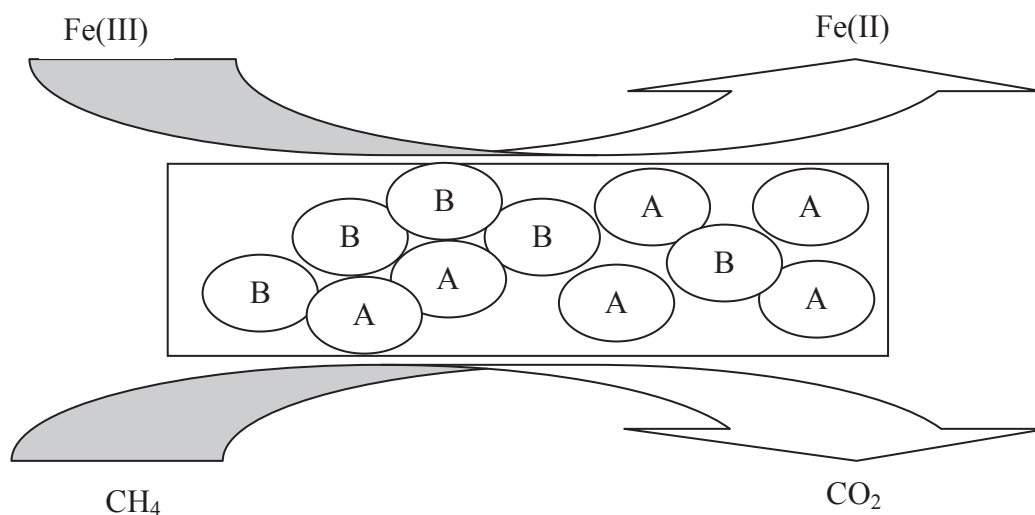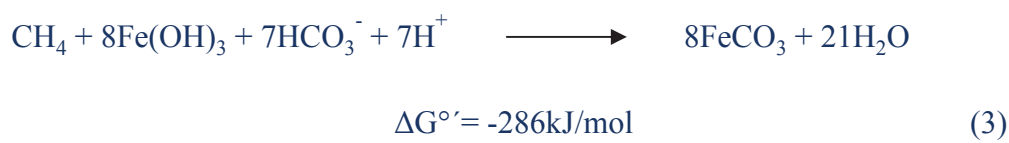
Fig. 1.10 Proposed pathway for AOM coupling to Fe(III) reduction in a probable consortium of (A) archaea and (B) Fe(III-reducing bacteria.

The long term overall aim of this study was to isolate and characterize Fe(III)-reducing bacteria that could be utilized in future model studies for determining the utilization

of methane as a substrate for growth more so, anaerobic oxidation of methane coupling to the

reduction of Fe(III).

STRATEGY:

```
                      ┌─────────────────────────┐
                      │  Sampling of lotus field │
                      └─────────────────────────┘
                                   │
                                   ▼
┌─────────────────┐   ┌─────────────────────┐   ┌─────────────────────┐
│ Screening of     │◄──│  Enrichment of Fe(III)-│──►│ Isolation of Fe(III)- │
│ microbial        │   │  reducing bacteria   │   │ reducing bacteria    │
│ activity         │   └─────────────────────┘   └─────────────────────┘
└─────────────────┘           │        │                    │
        │          │          │        ▼                    ▼
        ▼          │          │   ┌───────────────┐  ┌─────────────────────┐
┌─────────────┐    │          │   │ Batch         │  │ Characterization of  │
│ Fe(III)      │    │          │   │ incubations   │  │ novel Fe(III)-reducing│
│ reduction    │    │          │   └───────────────┘  │ bacteria             │
└─────────────┘    │          │          │            └─────────────────────┘
        │          │          │          ▼
        ▼          │          │   ┌─────────────────────┐
┌─────────────────┐│          │   │ Isotope ¹³CH₄        │
│ Measurement of  ││          │   │ incubations         │
│ methane         ││          │   └─────────────────────┘
│ concentrations  ││          │
└─────────────────┘          ▼
              ┌─────────────────────┐
              │ Molecular analysis  │
              └─────────────────────┘
```
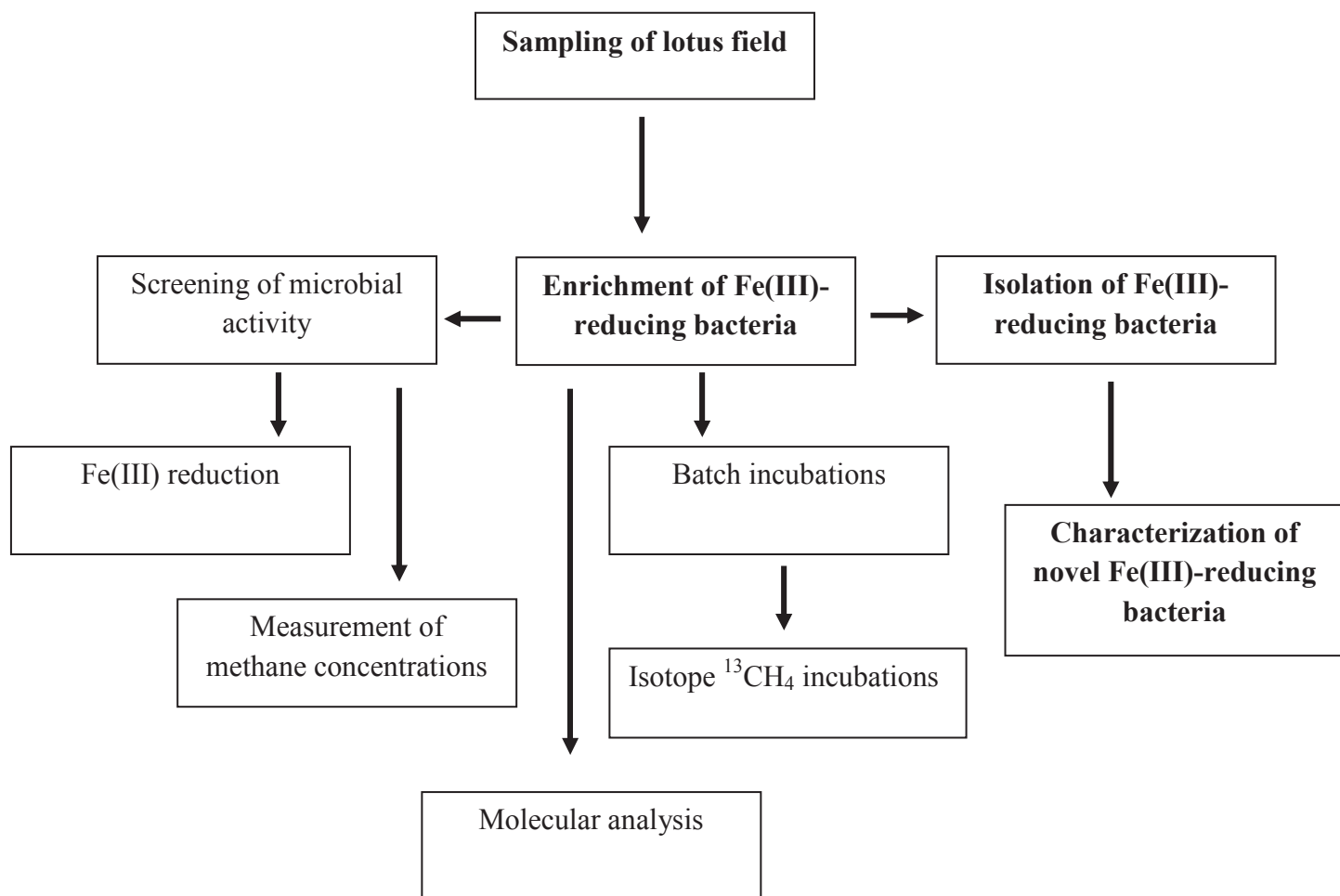
Fig. 1.11 Schematic diagram of the strategy used in this study.

The overall scenario of this study is illustrated in the figure above (Fig. 1.11) with the initial collection of mud samples from the lotus field and eventual construction of an anaerobic microbial reactor and finally isolation and characterization of two novel Fe(III)-reducing bacteria from the subsequent microbial enrichment.

Lotus roots are a local delicacy amongst the Japanese and lotus paddies are widespread throughout Japan. Paddy fields including rice and lotus fields are major sources of methane emission (a greenhouse gas) and are suitable habitats for microbial communities such as methanogens, methanotrophs and Fe(III)-reducing bacteria, amongst many others. Particularly, Fe(III)-reducing bacteria belonging to the genus *Geobacter* plays a major role in the carbon cycling in terrestrial environments, bioremediation and even production of electricity in microbial fuel cells. Their versatile characteristics in utilization of a range of aromatic hydrocarbons and whole genome analyses showed their potential suitability as candidates in model studies for utilization of methane oxidation. The overall strategy was to initially enrich microbial communities dominated by Fe(III)-reducing bacteria, induced with methane as an alternative growth substrate and supplemented with Fe(III) as the sole terminal electron acceptor. The subsequent anaerobic microbial enrichment is then used for isolation and characterization of *Geobacter* spp. Methane isotopic studies were also attempted in batch cultivations with inoculums from the anaerobic microbial enrichment. This study was divided into three major phases which are reported in subsequent separate chapters.

The first phase is documented in chapter 2 whereby an anaerobic microbial enrichment was established with mud from a lotus field in Japan as the inoculums source. Fe(III) and methane were supplied as sole terminal electron acceptor and substrate respectively, to stimulate growth of Fe(III)-reducing bacteria induced with methane. Monitoring of cultivation conditions, microbial activity and microbial compositions within the anaerobic enrichment was implemented throughout the incubation period. Fe(II)

production resulting from Fe(III)-reduction, methane concentrations between outlet and inlet, isotopic methane incubations and molecular analyses were all attempted in the first phase, described in chapter 2.

The second phase of this study was focused on the taxonomic characterization of a novel Fe(III)-reducing bacteria, described in chapter 3. Inoculums from the anaerobic microbial enrichment established with lotus field sediments (mud) were cultivated with gellan gum in deep tubes and red colored single colonies were purified. A novel Fe(III)-reducing bacterium was successfully isolated under the second phase and taxonomic characterizations identified the novel isolate, as belonging to the genus *Geobacter*, a versatile group of bacteria.

Under the third and final phase, similar to the second phase, another novel Fe(III)-reducing bacteria was initially isolated in deep gellan gum and finally purified in roll tubes with agar, described in chapter 4. Taxonomic characterization of the novel isolate identifies it as belonging to the genus *Geobacter* as a separate novel strain the isolate described in chapter 3.

CHAPTER 2 Anaerobic microbial enrichment of Fe(III)-reducing bacteria

2.1 Background

Terrestrial wet fields such as paddy fields including rice and lotus fields are major anthropogenic sources for methane emissions. Lotus roots known as Renkkon in Japanese dialect, is a favorite delicacy amongst the Japanese population, and is widely grown all over the country in irrigated paddy fields. Such environment is very conducive for methanogenesis and 90% of the methane produced through methanogenesis is emitted to the atmosphere. Since methane is 20 times more potent than carbon dioxide as a radiative gas, methane emission contributes significantly to global warming and so are paddy fields.

Iron as the fourth most abundant element on earth is ubiquitous in soils as Fe oxides, and has been reported to play a dominant role in the global cycling of metals and carbon through the microbial reduction of Fe oxides and decomposition of organic matter. *Geobacter* species are primarily the most dominant microbes in Fe(III)-reducing environment and are also widespread in many environments. Their versatility provides them as the best candidate for model studies on bioremediation and electrical generation in microbial fuel cells. With that in mind, enrichment of Fe(III)-reducing microbes induced with methane was established in an enrichment culture, to determine whether Fe(III)-reducing microbes can utilize methane as a substrate for their growth.

The microbial enrichment was established in an anaerobic glass reactor (Fig. 2.2), inoculated with mud collected from lotus field (Fig. 2.1). The anaerobic microbial enrichment was cultured in a basal medium (Table 2.1), amended with amorphous Fe(III) hydroxide regularly replenished at (>20 mM), as the electron acceptor. Methane was continuously supplied with a constant flow rate of 20 ml min^{-1} at 7.4 MPa. Fe(II) production was frequently monitored as direct evidence for Fe(III) reduction. Methane concentration between the inlet and outlet valve was measured constantly, to determine the differences in levels of

methane entering and emitting from the anaerobic microbial enrichment reactor. DGGE, clone library construction and RFLP analysis and eventual sequencing of both purified DGGE bands and clones were implemented to observe the bacterial profile and determine the dominant groups of microbes that composed the microbial enrichment. Overall, this chapter attempts to distinguish the relationship in the inducing of methane and the enriched microbes detected within the microbial enrichment culture.

2.2 Materials and methods

2.2.1 Sampling

Mud sediments were collected from a lotus field in Aichi prefecture, Japan, at a sediment depth of 30 cm below the water surface. pH of the sampling site was near neutral pH and water temperature was 30 °C. Mud samples were immediately transported to the laboratory in sterilized oxic sampling bottles and passed through micro-sieve sizes of 500 µm, 250 µm, 150 µm and 90 µm in the clean bench.

Fig. 2.1 Sampling site, Aichi prefecture, Japan.

2.2.2 Apparatus for microbial enrichment

The anaerobic reactor, which was used for the microbial enrichment is shown in (Fig. 2.2 & 2.3) along with its operational conditions. The glass reactor was made of silicon glass to prevent air penetration and or contamination, with an inner space where water with a maintained temperature at 30 °C was siphoned into to maintain the incubation temperature of the inner column where the actual microbial enrichment culture wass enclosed. The anaerobic reactor had a gas inlet opening located at its bottom (Fig. 2.2 & 2.3(13)) for inflow of methane gas through a control valve (Fig. 2.2 (9)) Another inlet and two outlets were located at the top of the anaerobic reactor. Inlet (labeled 6) was for automated supply of medium into the enrichment culture through a peristaltic pump (labeled 6) while outlet (labeled 7) was for sample collection. Another outlet valve positioned at the top of the glass reactor (not shown in the Fig.) was for collection of methane exiting the reactor.

2.2.3 Medium

A bicarbonate-buffered basal medium (Table 2.1) was degassed with deoxygenated N_2/CO_2 gas and autoclaved, stored in a glass medium bin, and continuously purged with filtered N_2/CO_2 gas. The stock medium prepared above was automatically added to the enrichment culture at regular intervals by timer hydraulic pumps, to maintain the amount of medium in the enrichment reactor. The culture in the anaerobic reactor was periodically replaced by the above degassed medium and continuously supplied with a gas mixture of CH_4/CO_2 (95:5, v/v). The anaerobic enrichment reactor was initially amended with approximately 100 mM poorly crystalline ferric oxide, hereafter referred to as amorphous Fe(III) hydroxide, as the terminal electron acceptor, prepared according to previous protocols (97), described in the preceding subsection. The anaerobic enrichment reactor was inoculated with 10% mud (w/v) and incubated in the dark at 30 °C.

2.2.4 Synthesis of amorphous Fe(III) hydroxide

Amorphous Fe(III) hydroxide was synthesized in the laboratory according to previously described method (97). 40 g of $Fe(NO_3)_3 \cdot 9H_2O$ was dissolved in 500 ml of distilled water and pH was adjusted to pH 7–8 using a Horiba pH meter (D-54) with approximately 340 ml of 1.0M KOH under vigorous stirring. The precipitates were washed with distilled water by centrifuging six times at 6000 rpm, 15 minutes at 4 °C to remove any potassium. The resulting precipitates were dissolved in the basal medium and manually added into the anaerobic reactor. The concentration of amorphous Fe(III) hydroxide within the enrichment culture was maintained above 20 mM.

2.2.5 Cultivation

70 g wet weight of lotus field mud as inoculums and 20 g weight wet of freshly prepared amorphous Fe(III) hydroxide, which was calculated to be 20 mM as the electron acceptor, was added to the anaerobic reactor. Methane was constantly supplied as the substrate with a consistent flow rate of 20 ml min^{-1} at 7.4 MPa. Incubation temperature was maintained at 30 °C with a water bath temperature (Fig. 2.2 (11)). Stock medium for replenishing the anaerobic medium in the column was constantly exchanged with N_2/CO_2 (80:20) at the flow rate of 20 ml per minute 7.4 MPa. Anaerobic medium was continuously supplied to the column by airtight tubes at flow rate of 25 ml for 10 minutes per day. Effluent was collected 20–24 hours later, after the influent has been automatically added to the column at the same flow rate. The column was designed such that an empty space is provided between the exterior and interior glass walls whereby, a cool bath is used to maintain the incubation temperature at 30°C. This was achieved by auto-pumping of water into the empty space and keeping the temperature of the water-filled space at 30°C throughout the duration of the experiment.
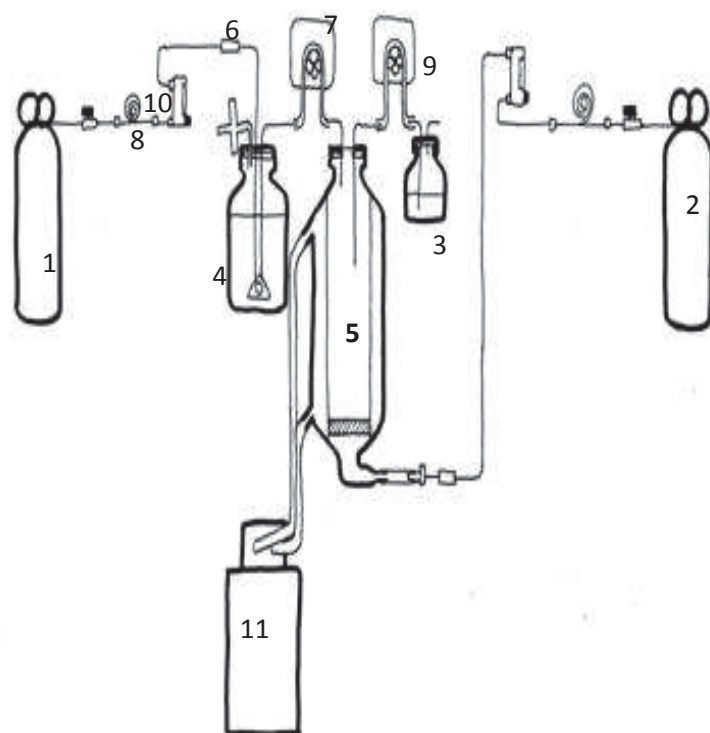
Fig. 2.2 A schematic diagram of the anaerobic column; 1; N$_2$/CO$_2$ gas supply, 2; CH$_4$ gas supply, 3; effluent bottle (sample collection for Fe assay), 4; medium feeder bottle, 5; enrichment column, 6; timer controlled inlet pump, 7; timer controlled outlet or effluent pump, 8; filter, 9; CH$_4$ gas flow rate control valve, 10; N$_2$/CO$_2$ gas flow rate control valve, 11; cool bath for temperature.
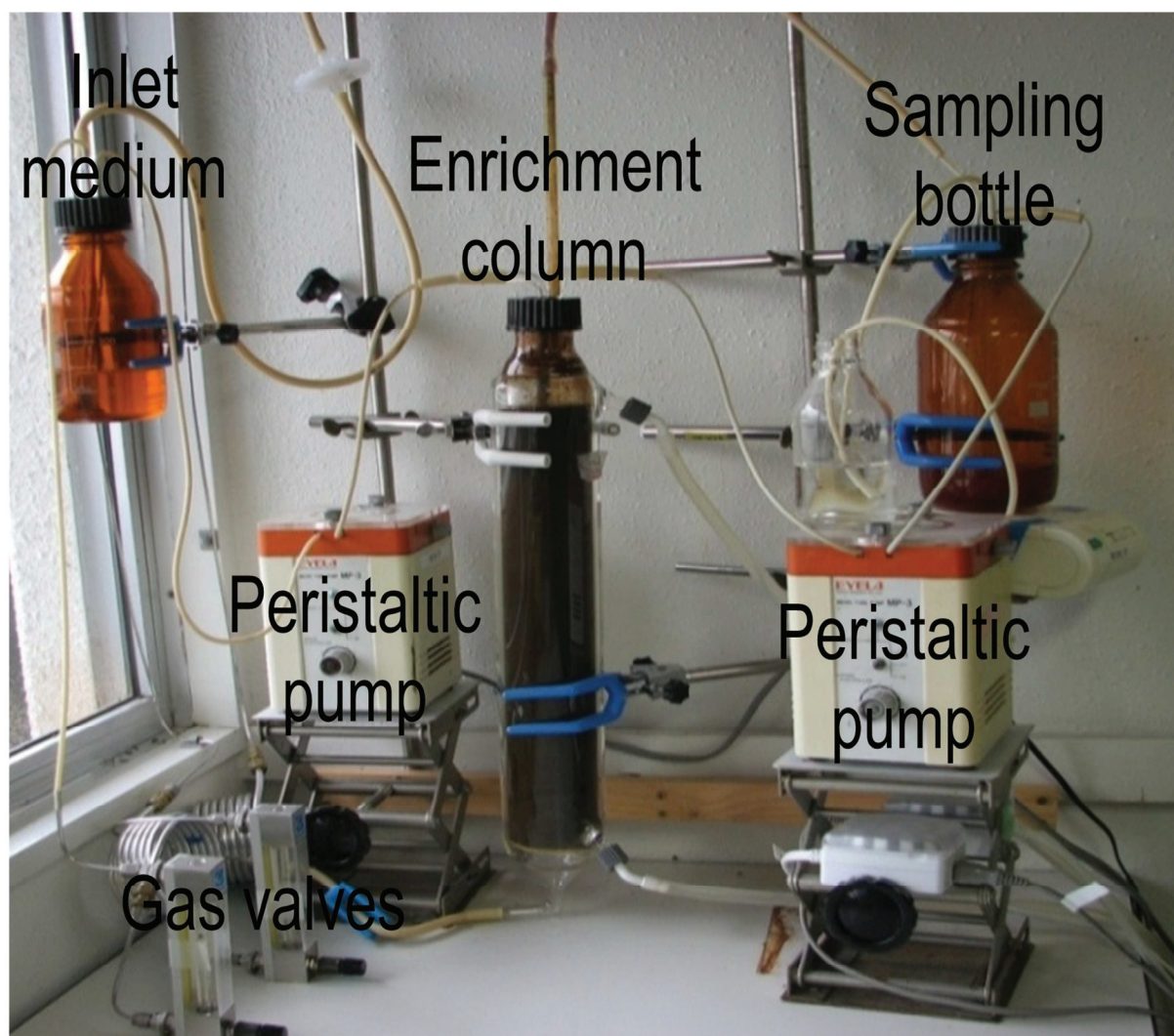
Fig. 2.3 A photograph of the anaerobic glass reactor for the enrichment of Fe(III)-reducing bacteria.

2.2.6 Screening of Microbial activity

2.2.6.1 Temperature, pH and ORP measurement

Anaerobic conditions of the microbial enrichment were regularly maintained by measuring the pH, oxidation-reduction potential (ORP) and temperature with a multi-purpose Horiba pH meter (D-54) and a Horiba ORP meter reader. This was periodically done simultaneously along with the Fe(II) and DNA samplings.

2.2.6.2 Sampling for Fe assay

Quantification of ferrous iron production (HCl – extractable Fe (II) in mud samples) was determined by spectrophotometer according to the ferrozine method (62), illustrated in Fig 2.4. Effluent was collected from the anaerobic column at 25 mL per day and kept in 0.5 M HCl under anaerobic condition. After 15 minutes in room temperature, the sample extract was added to ferrozine (1 g/L) in 50 mM HEPES (*N-2*-Hydroxyethylpiperazine-*N´-2*-ethanesulfonic acid) buffer at pH 7.0. After being mixed for few seconds, it was centrifuged at 13,000 rpm for 10 minutes in room temperature. Quantification of the Fe(II) amount was determined by measuring the absorbance of the filtrate at A_{562} using the UV-1240 Shimadzu Spectrophotometer. Ferric iron was determined after assaying for the iron total by following the same procedure as stated for ferrous quantification however; samples in hydrochloric acid were further diluted in hydroxylamine hydrochloride prior to addition with ferrozine. Ferric iron was then determined by subtracting the ferrous iron concentrations from total iron concentrations, as non-reduced ferrihydrite. Standard solutions were prepared from ferrous ethylenediammonium sulfate.

Sample diluted in HCl

Fe(II) check

HCl extracts in Ferrozine solution.

Fe-total check

HCl extracts in $H_2OH \cdot HCl$

$NH_2OH \cdot HCl$ extracts in ferrozine

Spectrophotometer quantification(OD_{562})
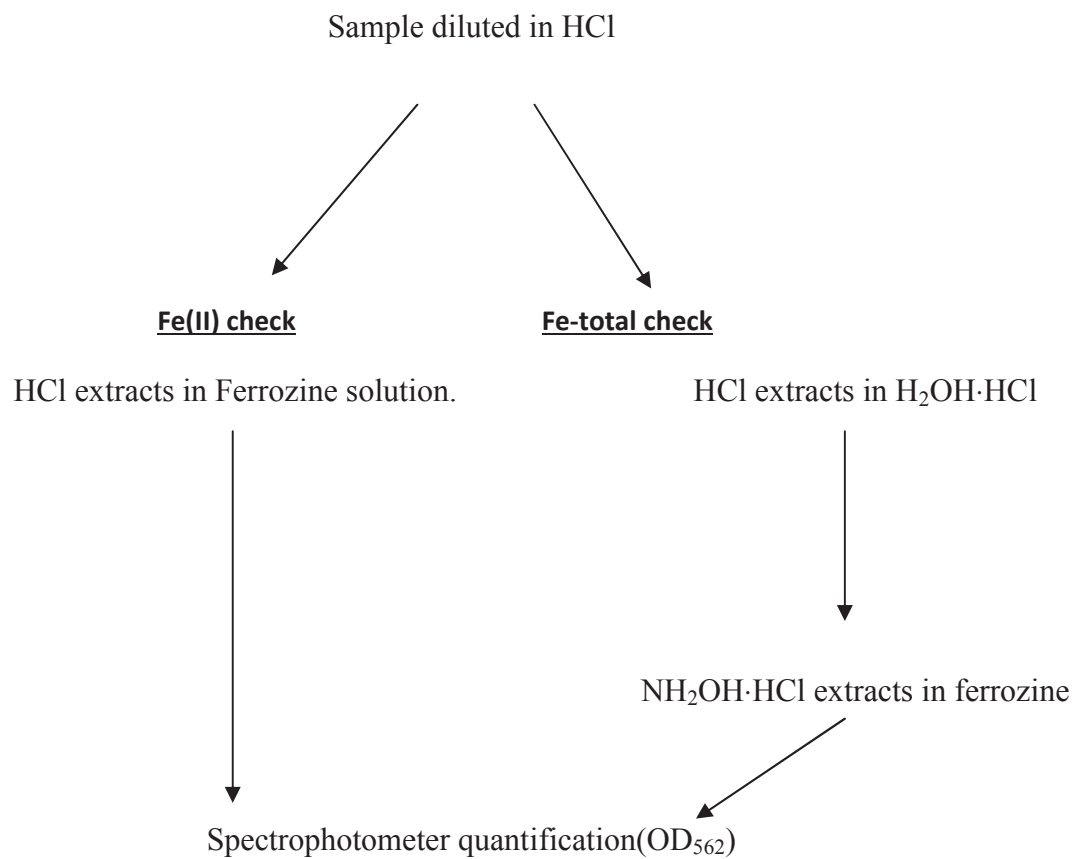
Fig. 2.4 Flow chart of the ferrozine protocol used for assaying of Fe(II) and Fe-total of both the batch and continuous culture.

2.2.6.3 Measurement of inlet and outlet methane

Methane entering and exiting the anaerobic microbial enrichment reactor was collected over a 12h period in an anaerobic aluminum bag during each sampling time and analyzed by GC-TCD.

2.3.7 Molecular Microbial Community Analyses

2.3.7.1 DNA extraction

Samples for DNA were collected at fortnightly intervals by retrieving mud from the enrichment reactor with a long sterilized glass tube, centrifuged to collect the residues and preserved at −80°C until extraction. DNA extraction was done with the ISOIL Beads beating kit (Nippon Gene. Co.), as per protocol specified therein. Samples were mixed with lysis solution followed by beads beating. DNA molecules were purified by the addition of a purification solution and removal of other molecules such as proteins was achieved by adding chloroform to the extracts. DNA extracts were then washed with 70 % ethanol and ethanol was removed by evaporating with a Speed Vacuum. DNA extracts were then diluted in Tris-EDTA (TE) buffer and incubated at 55°C for one hour. DNA concentration was either determined by NanoDrop ND −1000 spectrophotometer (NanoDrop Technologies, Japan) or Qubit fluorometer and is either stored at 4°C or immediately used for analysis.

2.3.7.2 PCR amplification

Total SSU 16S rDNA PCR amplification was done for bacterial species using EUF10F; 5′-AGAGTTTGATCCTGGCTCAG-3′ as a forward primer and U1500R; 5′-GGYTACCTTGTTACGACTT-3′ as a reverse primer. 16S rDNA V3 PCR was also done to target the variable region 3 (V3) of the bacterial genome DNA with GC341F; 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGGCACGGGGGGGCCTACGGGAGGC-3′ as forward primer and 517R; 5′-ATTACCGCGGCTGCTGG- 3′ as reverse primers. All

preceding PCR amplifications were done with either a Biorad or Takahara thermal cycler.

16S rDNA PCR running conditions are summarized in Tables 2.2 and 2.3.

Table 2.2 Reaction Mixture for 16S rDNA PCR

Reagent	Volume (µL)
GoTaq, HS Green Master Mix, 2X	5
Forward primer (EU10F)	0.5
Reverse primer (U1500R)	0.5
Template DNA	1
ddH$_2$O	3
Total	10

Table 2.3 Amplification Conditions for 16S rDNA PCR

Conditions	Time
Initial denaturing temperature	95°C for 2 minutes
Denaturation	95°C for 15 seconds
Annealing	55°C for 30 seconds
Extension	72°C for 1 minute 30 seconds
Final Extension	72°C for 7 minutes 30 seconds
Total number of cycles	35 cycles

16S rDNA V3 PCR amplification running conditions are shown in Tables 2.4 and 2.5.

Table 2.4 Reaction Mixture for 16S rDNA V3 PCR

Reagent	Volume (μL)
GoTaq, HS Green Master Mix, 2X	5
Forward primer (12.5 μM GC341F)	0.5
Reverse primer (12.5 μM 517R)	0.5
Template DNA	1
ddH$_2$O	3
Total	10

Table 2.5 Amplification Conditions for 16S rDNA V3 PCR

Conditions	Time
Initial denaturing temperature	95°C for 2 minutes
Denaturation	95°C for 15 seconds
Annealing	50°C for 15 seconds
Extension	72°C for 30 seconds
Final Extension	72°C for 7 minutes
Total number of cycles	35 cycles

Tables 2.6 and 2.7, Cloning PCR amplification-running conditions.

Table 2.6 Reaction Mixtures for Cloning PCR

Reagent	Volume (µL)
GoTaq buffer	2.6 c
2.5 mM dNTP Mix	0.8
Forward primer, T3 (10 p/µL)	0.5
Reverse primer, T7 (10 pmol/µL)	0.5
Template (10 ng/µL)	1.0
milliQ H_2O	3
Total	10

Table 2.7 Amplification Conditions for Cloning PCR

Conditions	Time
Initial denaturing temperature	95°C for 2 minutes
Denaturation	95°C for 15 seconds
Annealing	55°C for 30 seconds
Extension	72°C for 1 minute 30 seconds
Final Extension	72°C for 7 minutes 30 seconds
Total number of cycles	35 cycles

2.3.7.3 Agarose gel electrophoresis

Electrophoresis of 16S rDNA PCR products were done with 0.8% agarose gel while electrophoresis of 16S rDNA V3 PCR products were done with 2.0 % agarose gel in 1× TAE buffer for both amplifications.

2.3.7.4 Cloning

Cloning was done with a TOPO TA Cloning Kit for sequencing (invitrogen) according to the procedures specified therein. 16S rDNA PCR amplification was done at several numbers of cycles and ligation was performed with a TOPO TA Cloning Kit for sequencing whereby, a ligation mixture of 2 µl of the PCR product, 1 µl of salt solution, 0.5µL of TOPO vector and 2.5 µl of MilliQ water was prepared. 5 µl of the ligation mixture was mixed with 50 µl of *E. coli* competent cells and 200 of SOC medium. The transformed cells were incubated at 37°C for one hour under continuous shaking at 600 rpm. 120 µl of the transformed cells were then streaked onto pre-warmed LB plate medium and incubated over night at 37°C. Single colonies were picked and diluted in 20 µl of MilliQ water. 1 µl of the diluted colonies were added to colony PCR cocktail (Table 2.6) and colony PCR amplification was performed according to PCR running conditions in Table 2.7.

PCR amplification of clones was performed with primers T3; 5′-AATTAACCCTCACTAAAGG-3′and T7; 5′-AATACGACTCACTATAGG-3′.

Cloning PCR conditions are shown in Table 2.6 and 2.7.

2.3.7.5 Denaturant gradient gel electrophoresis (DGGE)

The assembling of gradient gel including all other necessary preparations needed for a successful DGGE run as well as sample preparations were done according to procedures of Bio – Rad Laboratories © 1996.

2.3.7.5.1 Assembling of the Parallel Gradient Gel Sandwich.

All procedures were done on a clean working surface as follows:

The sandwich assembly was assembled and fixed onto the alignment slot and kept in 4°C, whilst reagent preparation was being done, to minimize the variance in temperature between the sandwich assembly and the reagent during casting.

Table 2.8 Composition of stock solutions for 0 % and 100 % denaturant.

Composition of 0 % Denaturant		Composition of 100 % Denaturant	
40% Acrylamide/Bis	15 ml	40 % Acrylamide/Bis	15 ml
50 X TAE buffer	1 ml	50 X TAE buffer	1 ml
Distil H_2O add to	100 ml	Formamide	40 ml
		Urea	42 g
		Distil H_2O add to 100 ml	100 ml

Table 2.9 Composition of High and Low Density solutions.

Reagent	Density	
	70%	30%
0%	6 ml	14 ml
100%	14 ml	6 ml
Dye solution	100 ml	-
10 % APS	180 ml	180 ml
TEMED	18 ml	18 ml

Stock solutions of 0 % and 100 % denaturant were prepared to a final gel percentage of 6 %, shown in Table 2.8. All of the above chemicals were mixed under room temperature and stored in 4°C. The $50 \times$ TAE buffer was added at 1 ml only, to achieve a final strength of $0.5 \times$ TAE buffer. A low and high-density solution with a final range of 30% - 70% (Table 2.9) was prepared from the stock solutions of Table 2.8. A homogenized solution of the low and high density solution was then separately cast into the sandwich assembly via two separate 30 ml syringes mounted on a gradient delivery system. It is strongly suggested that the density solutions should be cast in not more than $7 - 10$ minutes once 10% APS and TEMED were added to the above mixture. 10% APS was obtained by dissolving 0.1 g of ammonium persulfate in 1 ml elix/distil water. This was prepared on the same day of use.

2.3.7.5.2 Sample preparation

Bacterial 16S rDNA PCR amplification targeting the variable region 3 (V3) was done as previously discussed under subheading '*PCR amplification'* then diluted according to rates in Table 2.10 prior to addition into the wells. The dilution was done to obtain a final concentration of 100 ng/µl of DNA by estimating the thickness of PCR bands obtained during agarose gel electrophoresis.

Table 2.10 Composition of sample mixture added into the DGGE wells.

PCR product	Sterilized distilled water	$2 \times$ Loading buffer	Final volume
χ µl	10 - χ µl	10 µl	20 µl

Where χ = amount of your PCR products.

2.3.7.5.3 Preheating the Running Buffer

- The electrophoresis tank was filled up with $0.5 \times$ TAE buffer up to the "Run" mark and the sandwich assembly containing the already solidified gel was placed into the tank. The top part of the gasket was then filled with $0.5 \times$ TAE buffer.

- The temperature control module was then placed on top of the electrophoresis tank and the switch button for power; pump and heater were switched on.

- The temperature was set to 65 °C and allowed to preheat up to the desired temperature.

2.3.7.5.4 Electrophoresis

- Sample dilutions according to Table 2.10 was done when the preheating buffer reached 65 °C then, carefully loaded into the wells with tender care so as not to rapture the shape of the wells.

- After loading the samples, the running temperature was reset to 60 °C and appropriate electrical leads were then attached to the DC power supply.

- The voltage and current of the DC power supply were fixed at 80 volts and 400 amps respectively and electrophoresis was allowed to run for 8.5 hours.

2.3.7.5.5 Staining and image analyzing

After the completion of electrophoresis, the resulting gel was stained with cyber gold (0.5 µl of Cyber Gold with 5 ml $0.5 \times$ TAE buffer) for 30 minutes in the dark. The DGGE bands were then viewed under UV light on the Biorad GelDoc XR.

Table 2.1 Composition of the Amended Basal Medium

Compound	Final concentration (mM)	/l
NH_4Cl	10	0.535g
Na_2SO_4	1	0.142g
$NaHCO_3$	30	2.52g
$Fe(OH)_3$	40	0.001g
Yeast Extract	0.02%	0.2g
*Mineral solution	50 ml	50 ml
**Trace element solution	1 ml	1 ml
***Vitamin solution	1 ml	1 ml
****Se/W solution	1 ml	1 ml
Resazurin	1 ml	1 ml

*Mineral Stock Solution

Compound	g/l
KH_2PO_4	0.136
$MgCl_2$ $6H_2O$	0.203
$CaCl_2$ $2H_2O$	0.147

****Se/W Solution

Compound	g/l
$Na_2SeO_3 \cdot 5H_2O$	0.004
$Na_2WO_4 \cdot 2H_2O$	0.004

** Trace Element Solution

Compound	g/l
Nitrirotriacetic acid (NTA)	12.800
$FeCl_3 \cdot 6H_2O$	1.350
$MnCl_2 \cdot 4H_2O$	0.100
$CoCl_2 \cdot 6H_2O$	0.024
$CaCl_2 \cdot 2H_2O$	0.100
$ZnCl_2$	0.100
$CuCl_2 \cdot 2H_2O$	0.025
H_3BO_3	0.010
$Na_2MO_4 \cdot 2H_2O$	0.024
NaCl	1.000
$NiCl_2$	0.065

*** Vitamin Solution

Compound	mg/l
Biotin	2.000
Foric acid	2.000
Pyridoxine HCl	10.000
Thiamine HCl	5.000
Riboflavin	5.000
Nicotinic acid	5.000
D-Ca Pantothenate	5.000
Vitamin B_{12}	0.100
p-Aminobenzoic acid	5.000
Lipotic acid	5.000

2.4 Results and Discussion

2.4.1 Microbial Growth conditions

pH measurement of the anaerobic microbial enrichment showed consistent pH values of near-neutral or slightly basic (Table 2.11) indicating stable growth conditions particularly for growth of Fe(III)-reducing bacteria. Most physiological studies on optimum pH for Fe(III)-reducing bacteria such as those belonging to the genus *Geobacter* have been consistently reported within the same pH ranges observed for the anaerobic reactor. Inconsistencies and major fluctuations in pH measurements on the other hand, may have indicated an unstable anaerobic reactor. Temperature within the anaerobic reactor was also maintained at 30 °C with a water bath temperature controller, as is seen in the measurements (Table 2.11). Oxidation reduction potentials (ORP) range from -155 mV to -270 mM indicated very stable anaerobic conditions suitable for growth of anaerobes (Table 2.11). In all, temperature, pH and ORP measurements indicated optimum anaerobic culturing condition for anaerobes, which was maintained throughout the whole duration of the enrichment.

2.4.2 Microbial Fe(II) production and methane concentration

There were two methods of Fe extraction employed to determine the microbial Fe(III) reduction within the anaerobic reactor. From 0–459 days of incubation, Fe was extracted in HCl and Fe(II) production was determined by the ferrozine method whereas, from 466 and onwards, Fe was extracted by a combination of ammonium oxalate and HCl, referred to as oxalate-HCl, hereafter. Under the HCl extraction method alone, highest Fe(II) concentration was recorded at 340 days with 10 mM Fe(II) and for the oxalate-HCl extraction method, highest Fe(II) production was recorded at 495 days of incubation with 28 mM Fe(II). This pattern was observed over a period of 35 days (466d–501d) whereby Fe(II) production was recorded at its highest peak. Results showed that Fe extraction by a combination of oxalate

and HCl was more effective than extraction by HCl alone (Fig. 2.5). Unfortunately, after 495 days, Fe(II) production started to decrease and steadily declined until the microbial Fe(III) reducing activity within the anaerobic reactor was near-complete lose. There might be several contributing factors to the loss in the ability of the anaerobic enrichment culture to utilize Fe(III), as observed. The first plausible reason could have been due to the breakage of the anaerobic reactor at 414 days of cultivation, which might have allowed oxygen contamination thereby, losing its anaerobic condition. However, high Fe(II) production was still observed after the anaerobic reactor was broken. At 498 days of incubation, a new bottle of methane gas was obtained from the methane supplier and most presumably, there must had been an oxygen contamination coming from the methane stock because, the decline in Fe(II) production was first evident at 501 days (3 days after the methane stock was replaced). From a high of 28 mM Fe(II) it declined to 21 mM Fe(II) and steadily declined to a low point of 2 mM or less (Fig. 2.5). The observed loss in Fe(II) production within the anaerobic reactor was, suspected to be most probably due to oxygen contamination, most likely through oxygen contamination from the methane stock. This assumption was corroborated by the sudden appearance of aerobic bacteria in the bacterial community (Fig. 2.6), after the methane stock was replaced, as discussed in more details in the next section.

Measurement of the inlet and outlet methane concentration showed that methane exiting the anaerobic enrichment reactor was 1.8 mM less, than the methane inflow, but corresponding Fe(II) concentration do not correlate with the expected Fe(II) production assuming, that the difference in methane concentration was due to microbial utilization in the anaerobic reactor (Fig. 2.5). However, at this same period of methane measurement, Fe extraction to determine the Fe(II) production was done by HCl only, and not by oxalate-HCl, which was the far more effective extraction method. Thus, it could not be ascertained as to whether there was any occurrence of methane utilization or an absence of it. The difference in

methane concentration observed between the inlet and outlet flow of methane could be either way so, inoculums from the anaerobic reactor were used for isotope methane incubations in batch cultures, reported in the following section.

Although the inconvenient design of the anaerobic reactor may have contributed to lack of any concrete supporting data for AOM coupled to Fe(III) reduction, Fe(II) production observed by the oxalate-HCl extraction method indicated that the anaerobic reactor was suitable for cultivating Fe(III)-reducing microbes and if further improved, it could handle isotopic methane studies of Fe(III)-reducing bacterial communities in any laboratories more sufficiently.

2.4.3. Isotopic methane incubations

Inoculums from the microbial enrichment were cultivated in batch cultures at 30 °C with isotope ^{13}C-CH_4 as the sole substrate for growth. Amorphous Fe(III) hydroxide and Fe(III)-NTA were used as sole electron acceptors and resulting isotopic $^{13}CO_2$ was measured by GC-MS.　All incubations including kill controls and treatments did not show any production of isotope $^{13}CO_2$ over the 17 months of incubation (Table 2.12) except, mud cultures from rice field (positive control) which showed methanotrophic growth with ^{13}C-CH_4 that, yielded $^{13}CO_2$ (not included in data). GC-MS data showed no evidence of methane utilization by anaerobic microbial enrichment cultures cultivated in batch treatments. To date, there had been no successfully reported studies that show growth of methane utilizing anaerobes and, most studies have been conducted in enrichment incubations unlike this study, which was a major bottleneck in determining the possibility of utilization of methane by Fe(III)-reducing bacteria.　Further improvement to the existing anaerobic reactor to cater for isotopic studies in continuous cultures, would immensely help to directly observe the possibility of Fe(III)-reducing bacterial community, utilize methane as a potential substrate for growth.

Table 2.11 Temperature, pH and ORP measurements of the anaerobic column which was inoculated with Lotus field mud and added with amorphous Fe(III) hydroxide with constant supply of methane at a flow rate of 25 ml/10 min/day.

Days	Temperature (C)	pH	ORP (mV)
0	29.9	7.84	-155
7	29.5	7.90	-289
14	30.0	7.79	-287
21	30.3	7.80	-282
28	30.5	7.77	-252
42	30.4	7.78	-295
56	30.1	7.90	-250
70	29.9	7.92	-232
84	30.1	7.96	-237
98	29.9	8.03	-313
112	29.6	8.02	-266
126	30.0	8.03	-310
140	30.0	8.10	-234
154	30.0	8.01	-261
168	29.4	7.96	-286
182	29.9	8.04	-270

2.4.4 Microbial community composition and profile

Based on the results in Fig 2.5, DNA samples from specific incubation periods were selected for molecular analyses. The profile of the bacterial community in the anaerobic enrichment culture remained steady without any major shift in the microbial pattern. DNA bands were excised from DGGE samples of 354 and 424 days, and purified through sequential consecutive DGGE runs then sequenced. According to the 16S rDNA band patterns there were no changes in the bacterial community composition from 354 to 495 days. However, a significant shift in band patterns was observed from 508 days and onwards. This coincided with the new methane stock bottle, which was replaced and it was also the same period at which decline in Fe(II) production was observed.

Distinctive new 16S rDNA bands from the bacterial community profile were purified and sequenced. The most dominant 16S rDNA bands belonged to *Rhodococcous* spp. aerobic bacteria. This confirmed the occurrence of oxygen contamination within the anaerobic reactor which may have inhibited the growth of Fe(III)-reducers and instead favor the growth of aerobes. The aerobic condition within the anaerobic reactor may have also stimulated the abiotic production of Fe(III) from Fe(II) produced by the microbes, thus, reversing the desired process since, amorphous Fe(III) oxide is the least stable amongst all Fe oxides and can be easily converted into other forms abiotically, which is hard for microbial utilization.

A clone library was constructed from DNA samples obtained from 495 days of incubation and RFLP method was applied to distinguish the different number of clones in the clone library. According to the clone library, the bacterial community within the anaerobic reactor was dominated by heterotrophic bacteria followed by Fe(III)-reducers and sulfate reducers (Fig. 2.6). Presence of sulfate oxidizers was also detected in the clone library however, their role in the observed Fe(III)-reduction was ambiguous. *Geobacter* spp. were

the dominant Fe(III)-reducers detected in the anaerobic reactor and are known as versatile group of microorganisms.
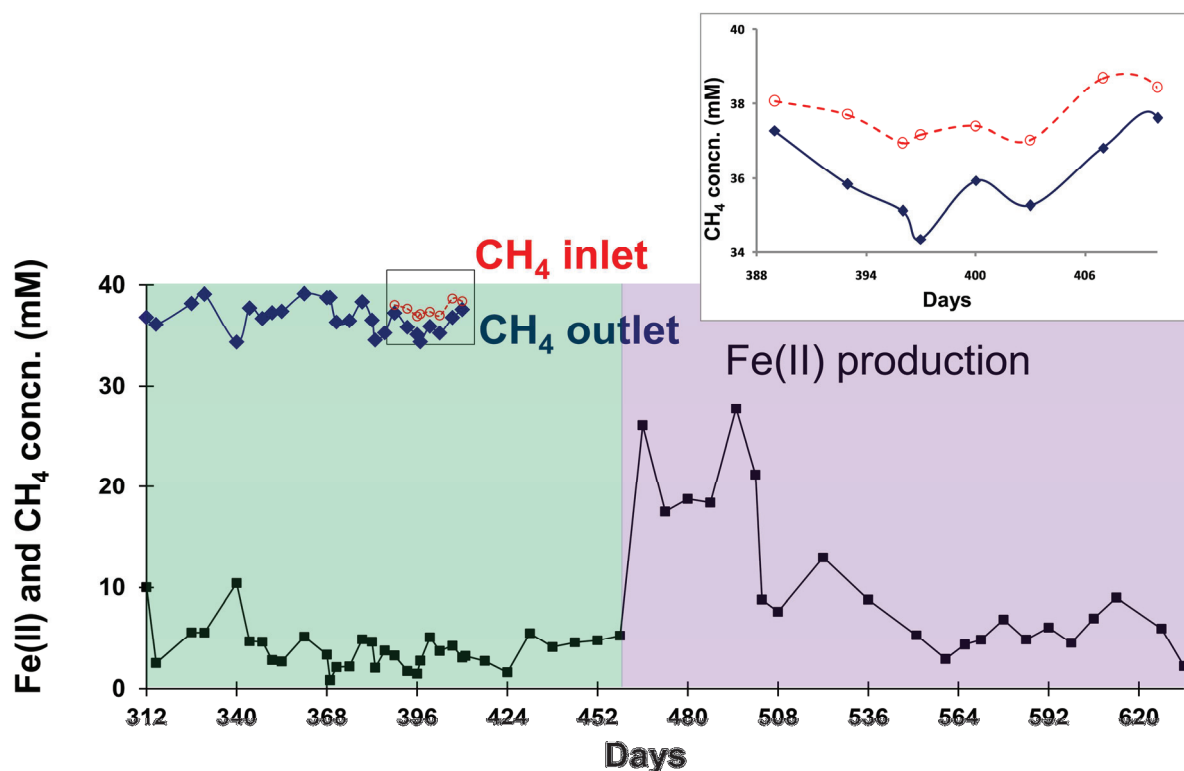
Fig. 2.5 Fe(II) production observed within the anaerobic microbial enrichment column and differences in methane concentration between the inlet and outlet flow. Inlet methane (open circles), outlet methane (filled diamond), Fe(II) concentration (filled square). All measurements were done in replicates. Enlarged insert on the top right showed the measurements of methane concentrations.

Table 2.12 Isotopic ^{13}C-CH$_4$ incubations with inoculums from the anaerobic microbial enrichment. A–D, treatments.

	0 Days			2 months			3 months			17 months		
	^{13}CH$_4$ (mM)	Fe(II) (mM)	^{13}CO$_2$/^{12}CO$_2$ (%)	^{13}CH$_4$ (mM)	Fe(II) (mM)	^{13}CO$_2$/^{12}CO$_2$ (%)	^{13}CH$_4$ (mM)	Fe(II) (mM)	^{13}CO$_2$/^{12}CO$_2$ (%)	^{13}CH$_4$ (mM)	Fe(II) (mM)	^{13}CO$_2$/^{12}CO$_2$ (%)
A	25.55	5.46	1.17	22.85	5.53	1.20	21.62	5.03	1.05	19.43	4.98	0.56
B	0.55	5.56	1.17	0	5.36	1.17	0	5.31	1.19	0	6.11	0.85
C	24.69	5.25	1.18	23.30	5.86	1.06	20.97	5.87	0.85	21.30	7.86	0
D	23.30	5.39	1.18	23.13	6.06	1.14	20.25	5.09	1	19.74	5.61	0

A, kill control; B, no addition of ^{13}CH$_4$; C, ^{13}CH$_4$ & Fe(III)-NTA; D, ^{13}CH$_4$ & Fe(OH)$_3$. Data presented are average values of replicates obtained from 0 – 17 months incubation period.
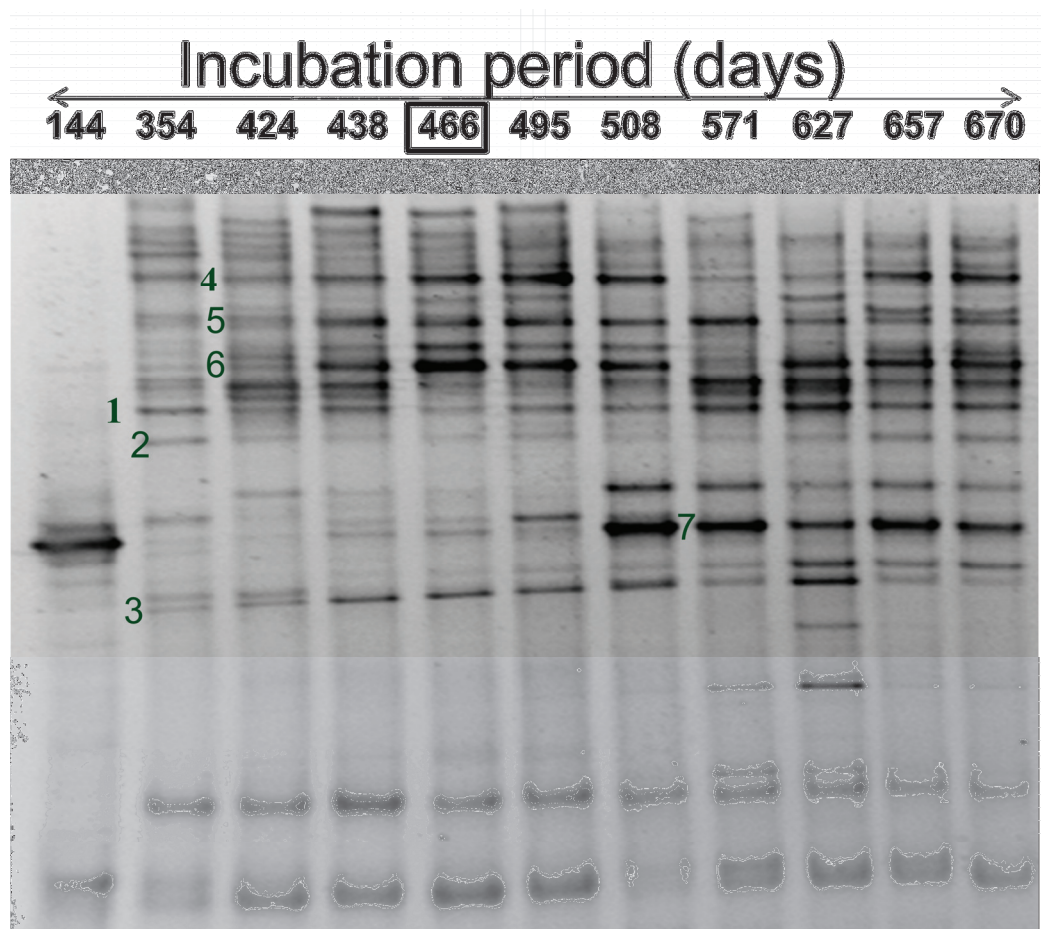
Fig. 2.6 Bacterial community profile of the anaerobic microbial enrichment column as observed byDGGE.

Table 2.13 BLAST affiliations of purified 16S rDNA bands displayed in Fig. 2.6.

Band number	Taxonomic name	% identities
1	*Geothrix* sp.	88
2	**Uncultured soil bacteria**	84
3	*Acidobacteria* sp.	85
4	*Geobacter* sp.	96
5	*Acidaminobacter* sp.	100
6	*Ferrimicrobium* sp.	96
7	*Rhodococcus* sp.	100

2.4.4.1 Cloning/RFLP

Table 2.14 Number of clones constructed from 16S rDNA obtained from 466 days of incubation

Ribotype	Ratio of clones (%, n=103)	Affiliation by RDP classifier (≥80% confidence)	Affiliation by Blast (% identity)
1a	26	*Acidaminobacter*	*Acidaminobacter hydrogenoformans* (97.3)
2b	8	*Geobacter*	*Geobacter grbicium* (98.1)
2?b	2	*Geobacter*	*Geobacter grbicium* (98.9)
3e	4	*Acidobacteria*	*Acidobacteria* bacterium (85)
3a'	2	*Betaproteobacteria*	*Propionivibrio pelophilus* (92.7)
2c	2	*Clostridium*	*Clostridium* sp. CYP5 (99.5)
8c	2	*Petrimonas*	*Petrimonas sulfuriphila* (99.8)
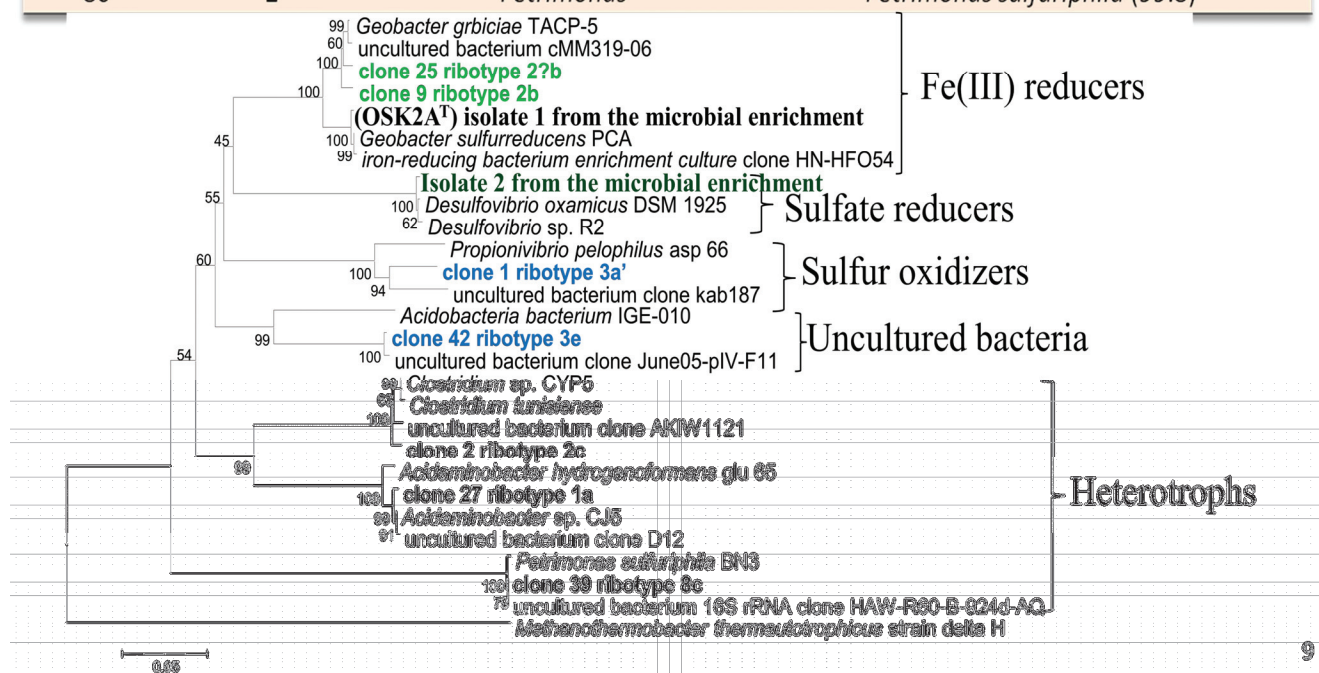
Fig. 2.7 Clone library and RFLP analyses of the microbial community in the anaerobic enrichment column. DNA samples were collected at 466 days of incubation.

The clone library was constructed from 16S rDNA and a total of 103 clones were obtained. RFLP was then applied to differentiate the various clones which were classified into

55

separate ribotypes. Ribotypes with two or more percentages of clones were sequenced (Table 2.14) and a phylogenetic tree was constructed (Fig. 2.7) to show the positions of each clones obtained from the anaerobic enrichment reactor. Table 2.14 showed the different ribotypes with two or higher percentages of clones while, ribotypes with less than two percentages of clones were omitted.

Heterotrophic bacteria were the most dominant within the anaerobic microbial enrichment with 16S rDNA clones identified as, belonging to *Acidominabacter hydrogenoformans* with 97.3% similarity, according to the basic local alignment search tool (BLAST) affiliation (Fig. 2.7). Other heterotrophic bacteria such as *Clostridium* and *Petrimonas* were also present with two percentages each, from the total percentages of 16S rDNA clones obtained from the anaerobic enrichment reactor.

Fe(III)-reducers belonging to the genus *Geobacter* were the second most dominant group detected within the anaerobic microbial enrichment (Table 2.14 & Fig. 2.7). Presence of sulfate reducers and uncultured bacteria were also detected. Although the anaerobic reactor was only supplemented with Fe(III) as the sole terminal electron acceptor, to stimulate the growth of Fe(III)-reducing bacteria, it was no surprise that the microbial community within the anaerobic enrichment was so diverse due to the source of inoculums (obtained from lotus field).

Paddy fields are well known habitats for diverse microbial communities and this was reflected in the microbial community composition of the anaerobic reactor. The heterotrophic bacteria within the anaerobic enrichment must have been the major decomposers of dissolved organic matter contained within the mud samples, and in the process produced acetate whereby Fe(III)-reducing bacteria utilized for their growth in the dissimilatory reduction of Fe(III). On the

other hand, both the heterotrophic and Fe(III)-reducing bacteria might have played a role in the possible anaerobic oxidation of methane as depicted in Fig 2.5.

2.5 Summary

Findings in this chapter revealed that over the course of the microbial enrichment cultivation, heterotrophic bacteria were the most dominant group and presence of Fe(III)-reducing bacteria, sulfate reducers and oxidizers were also detected (Fig. 2.7 & Table 1.14).

Active Fe(III) reduction was observed, reaching a high of 28 mM Fe(II) and an average difference of 1.8 mM methane was measured between the inlet and outlet methane flow (Fig. 2.5).

The microbial activity seen in the active Fe(III) reduction suddenly declined steadily to an all-time low of 2 mM Fe(II) accompanied with a major shift in the microbial community profile of the enrichment culture as observed by the DGGE profile (Fig. 2.6 & Table 2.13). This coincides with the replacement of methane gas bottles that were used to supply methane to the enrichment culture. An abundance of aerobic microbe belonging to the *Rhodococcus* genus was detected in the same duration when decline in Fe(II) production (Fig 2.6 & Table 2.13) was observed, indicating an occurrence of oxygen contamination within the anaerobic reactor. Abiotic Fe(III) production may have occurred in parallel with Fe(III) reduction since Fe(II) easily reconverts to Fe(III) in the presence of oxygen, resulting in low concentration of Fe(II) observed.

Although methane concentrations showed an average difference of 1.8 mM methane between the inlet and outlet, isotopic methane incubations in batch cultures showed no production of isotopic carbon dioxide (Table 2.12), indicating no occurrence of AOM in the batch cultures, which were inoculated with inoculums from the enrichment culture.

These results are inexplicit requiring further studies to characterize such microbial ecosystem induced with methane but, with an improved and properly designed anaerobic reactor with a capacity to cater for isotopic methane studies in the near future, as well as for easy handling during experimental procedures.

CHAPTER 3 Isolation and characterization of *Geobacter luticola* strain OSK6[T] sp., nov.

3.1 Background

Fe(III)-reducing bacteria play an important role in organic matter mineralization (59–60, 63, 66, 130) and *Geobacter* species have often been detected as the predominant Fe(III)-reducing bacteria found in most terrestrial environments (4, 18). Ever since the establishment of the genus *Geobacter*, species belonging to this genus have been found to be a very metabolically versatile group of microorganisms that are capable of not only Fe(III) reduction but also degradation of aromatic contaminants (90, 55, 68, 138). Potential applications for bioremediation in uranium-contaminated aquifers (5, 2, 103) as well as electrogenic activity on microbial fuel cells (59) have also been reported for the genus *Geobacter*. With their versatility, *Geobacter* species play an important role in the protection of groundwater resources as well as oxidation of organic matter in anoxic freshwater environments with Fe(III) as the sole electron acceptor (59).

Although this group appears to be versatile, amongst the 17 species of the genus *Geobacter* published to date, only four species were reported to be capable of utilizing nitrate as an electron acceptor, namely, *G. argillaceus* (102), *G. lovleyi* (114), *G. metallireducens* (61), and *G. thiogenes* (27, 78). The newly discovered strain adds to that list of nitrate utilizers.

A novel species named *Geobacter luticola* OSK6[T], an iron (III)-reducing bacterium, capable of growth by nitrate respiration was isolated from lotus field in Japan (Fig. 3.1). Strain OSK6[T] has been deposited in the Japan Collection of Microbes (JCM) with the identification number 17780[T] and in DSMZ with identification number 24905[T].

Sample source; Lotus Field

Anaerobic column enrichment

Acetate + Fe(III)-NTA
10^{-5} dilution

Inoculums obtained at 424 days

Deep gellan gum

Colony pick up

6 well plate isolation

Red colony pick up

Liquid medium (basal medium)

Liquid medium (basal medium)

6 well plate isolation

Colony pick up

Liquid medium (NIH medium)

DNA extraction

DNA extraction

Sequencing of 16S rRNA gene

Fig. 3.1 Overall background of chapter 3.

3.2 Materials and Methods

3.2.1 Medium

The composition of the bicarbonate-buffered basal medium for enrichment is described in chapter 2 (Table 2.1). Medium preparation and cultivation condition of the enrichment is as previously described in chapter 2.

3.2.2 Isolation

The enrichment culture was serially diluted and the dilutions were inoculated into deep gellan gum tubes with the basal medium containing acetate (10 mM), Fe(III)-NTA (10 mM), and gellan gum (0.1%, w/v). Single colonies formed on the deep gellan gum tube after 2 months of incubation at 30 °C were picked up and purified with the same medium using the six-well plate method as previously described (76). Purity was checked by microscopic uniformity of the isolate obtained from liquid culture, as well as observation that no growth occurred when the liquid culture was inoculated into an anaerobic heterotrophic medium (NIH thioglycolate broth, Difco) amended with pyruvate (20 mM).

3.2.3 Morphology

Morphological features were determined by scanning electron microscopy (SEM) and phase contrast microscopy. The culture for SEM was grown at the stationary phase in the basal medium with acetate (10 mM), amorphous Fe(III) hydroxide (ca. 100 mM), and pieces of a glass plate (about 5×5 mm^2). The glass plates were removed from the culture after 14 days of incubation, and immersed in PBS containing 2% glutaraldehyde for 2 h at room temperature, followed by dehydration through a stepwise increase in the concentration of ethanol (50, 60, 70, 80, 90, 95, and 99.5%, 30 min at each concentration followed by 100% overnight at 4 °C). The plates were placed on a paper filter at room temperature to remove ethanol completely, and

immediately coated with OsO_4 in an osmium coater (Neoc-ST, MEIWAFORSIS). The OsO_4-coated plates were observed with SEM at 5 kV (S-4800, Hitachi). The motility of strain OSK6[T] was observed using culture grown on acetate (10 mM) and amorphous Fe(III) hydroxide (ca. 100 mM) and examined using a phase contrast microscope.

3.2.4 Physiology

Unless otherwise stated, the same composition of bicarbonate-buffered basal medium used for the anaerobic enrichment was used for all physiological characterizations in batch cultures. All growth tests were performed in parallel with both reference strains *G. daltonii* FRC-32[T] (85) and *G. toluenoxydans* TMJ1[T] (55). Studies on growth ranges and optima for temperature, pH, and NaCl concentration were performed with the basal medium containing acetate (10 mM) and nitrate (10 mM) since strain OSK6[T] does not grow on fumarate, and with Fe(III) giving a strong background turbidity due to its dark color, making it difficult to distinguish cell growth from the background turbidity by OD_{560} measurements. Studies on all growth ranges and optima for strain OSK6[T] and both reference strains were performed in duplicates with growth monitored at OD_{560}.

3.2.4.1 Temperature optimum and range

Optimum temperatures for growth were tested at 4, 10, 20, 25, 30, 37, 40, 45, and 50 °C with 10 mM acetate and 10 mM nitrate in duplicates.

3.2.4.2 NaCl tolerance

Growth with NaCl was tested at 0, 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5% at near neutral pH with 10 mM acetate and 20 mM nitrate as electron donor and acceptor, respectively and incubated at 30 °C.

3.2.4.3 pH range optimum and range

Likewise, optimal pH growth tests were carried out in vials containing basal medium with acetate and nitrate, but without sodium bicarbonate. The non-bicarbonate basal medium was prepared in the same way as that of all other phenotypic characterizations described above, but with N_2/CO_2 gas replaced by N_2 gas, and supplemented with buffers (MES-NaOH, 5.0–6.0; PIPES-NaOH, 6.0–7.0; HEPES-NaOH, 7.0–7.5; Tris-HCl, 8.0–11) at a final concentration of 10 mM to give the desired pH values. Incubations were examined at 30 °C.

3.2.5 Substrates and Electron acceptor utilization

Utilization of electron donors was examined at 30 °C in the presence of 10 mM Fe(III)-NTA with the following substrates (concentrations are shown in parenthesis and are in mM, unless otherwise stated): formate (10), propionate (10), butyrate (10), pyruvate (10), lactate (10), fumarate (10), succinate (10), ethanol (10), butanol (10), glucose (10), phenol (1), benzoate (1), toluene (1), methanol (5), H_2 (ca. 62 kPa), and CH_4 (ca. 62 kPa); while utilization of electron acceptors was examined with nitrate (20), malate (20), sulfate (20), ferric citrate (30), amorphous Fe(III) hydroxide (50), and fumarate (40) in the presence of acetate (10). Fe(II) production was assayed by the ferrozine method (62) as previously described in chapter 2, as an indication of electron donor utilization. Utilization of electron acceptors was determined by the increase of absorbance at 560 nm due to turbidity resulting from microbial growth. Utilizations of substrates, which are distinguishable to strain OSK6[T] from the closely related strains, were further tested with *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T], purchased from JCM (JCM 15807[T]) and DSMZ (DSM 19350[T]), respectively. The type of nitrate respiration (*i.e.*, denitrification and ammonification) was examined in the presence of acetate (12.5 mM) and $Na^{15}NO_3$ (20 mM) using a GC-MS (GCMS-QP2010, Shimadzu) equipped with a GS-GASPRO column (ID 0.32

mm × 60 m, Agilent) according to previously described method (33). *G. metallireducens* GS-15^T, which reduces nitrate by ammonification, was purchased from DSMZ and used as a reference strain.

3.2.6 Isotopic methane incubation

Strain OSK6^T was also cultured with isotopic $^{13}CH_4$ at 30 °C in batch incubations with Fe(III)-NTA as the sole terminal electron acceptor.

3.2.7 Chemo-taxonomic analysis

Both respiratory quinones and fatty acids were performed by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. Cells of strain OSK6^T as well as reference strains were cultured on acetate (30 mM) and Fe(III) citrate (50 mM) until early stationary phases for chemotaxonomic analyses.

3.2.7.1 Respiratory Quinones

Analyses of respiratory quinones were carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany.

3.2.7.2 Fatty acids

Cellular fatty acids were carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. *G. daltonii* FRC-32^T and *G. toluenoxydans* TMJ1^T were used as references for fatty acid analyses.

3.2.8 Molecular analyses

3.2.8.1 16S rRNA gene analysis

The 16S rRNA gene of strain OSK6^T was amplified with EU10F and 1492R primer pairs and sequenced according to previously described procedures (76). The 16S rRNA gene sequence was aligned with reference sequences using the online alignment tool SINA (86). The aligned

sequence was imported into the ARB-Silva reference database (Silva106), and the alignment was manually edited using tools in ARB (65). Similarities of 16S rRNA gene sequences between the strain OSK6^T and its closely related species were calculated by ARB.

3.2.8.2 Phylogenetic tree construction

A phylogenetic tree was then constructed using the neighbor-joining method (95) with Jukes and Canter correction implemented in ARB (51). Bootstrap re-sampling analyses were performed with the neighbor-joining and maximum-likelihood methods to evaluate the reliabilities of tree topologies using MEGA 5.0 and PHYML 3.0.1 (38, 118), respectively, in accordance with Hall (39).

3.2.8.3 GC Content

Cells were disrupted by beads beating to extract genomic DNA. The G + C content of the genomic DNA were determined by HPLC according to previously described procedures (117).

3.3 Results and discussion

Strain OSK6^T was isolated from an anaerobic enrichment culture inoculated with mud sediment from a lotus field, and continuously supplied with methane as an electron donor and amorphous Fe(III) hydroxide as an electron acceptor. Cultures of strain OSK6^T after repetitive isolations of colonies exhibited no growth in the medium for heterotrophic anaerobes and uniformity in cell morphology under microscopic observation, showing that strain OSK6^T was eventually purified

3.3.1 Morphology

The new isolate is a strictly anaerobic, Gram-negative, and straight rod-shaped bacterium, 0.6–1.9 μm long and 0.2–0.4 μm wide (Fig. 3.2). Motility and a flagellum were found when

strain OSK6^T grew on acetate and amorphous Fe(III) hydroxide. Similar to *G. metallireducens*

GS-15^T (15), strain OSK6^T was immotile when grown with soluble Fe(III) as electron acceptor.
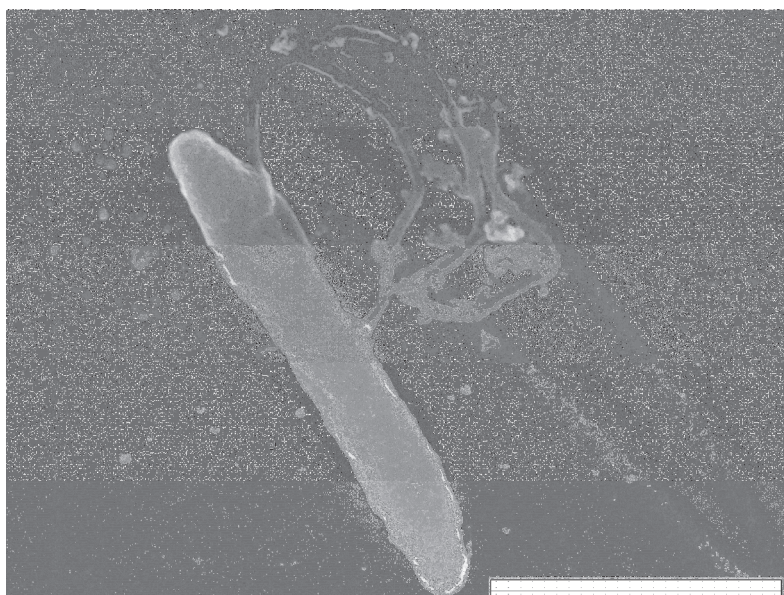
Fig. 3.2 Morphology of strain OSK6^T observed by scanning electron microscopy. Strain OSK6^T was grown with 10 mM acetate as electron donor and about 100 mM amorphous Fe(III) hydroxide as electron acceptor. Cells were harvested at the stationary phase, then prepared for SEM observation (5.0 kV, S4800, Hitachi). Bar indicates 1 µm.
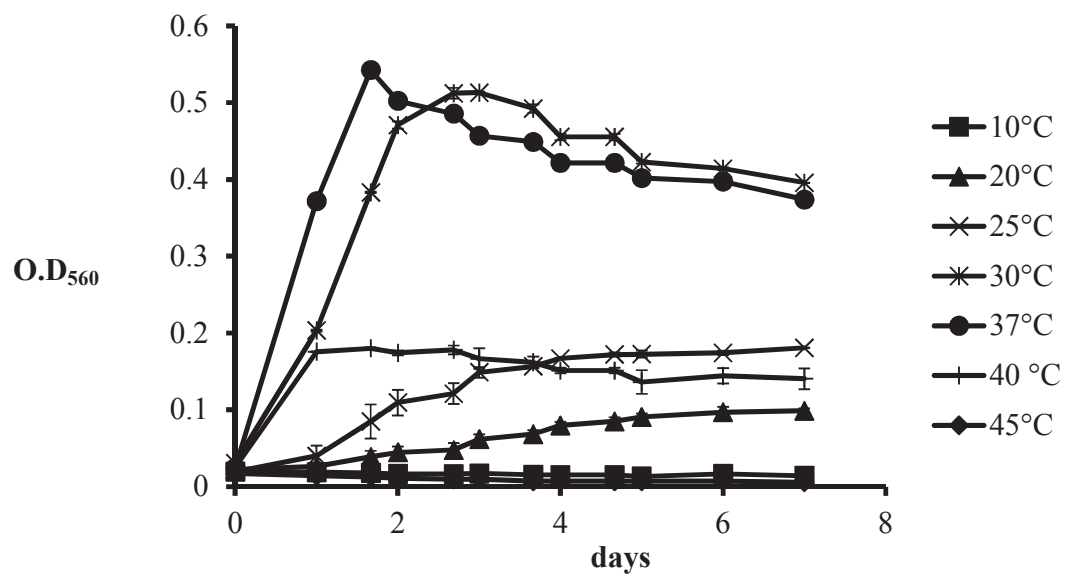
Fig. 3.3 Growth range of strain OSK6^T in various temperatures.
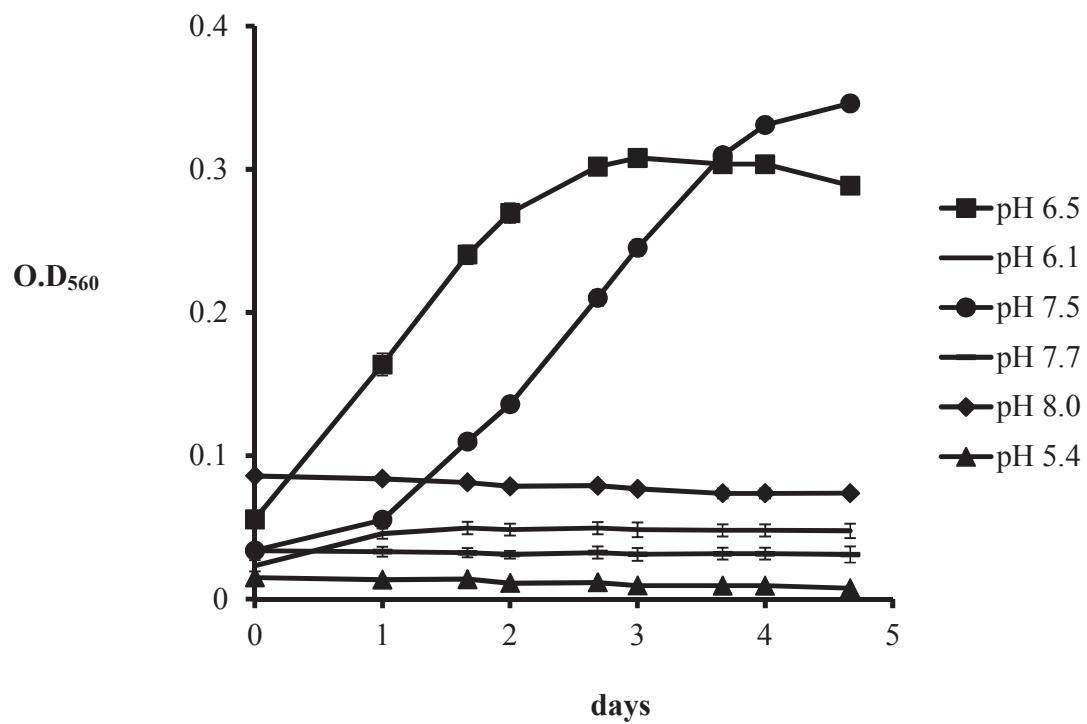
Fig. 3.4 Growth of strain OSK6^T in various pH values.
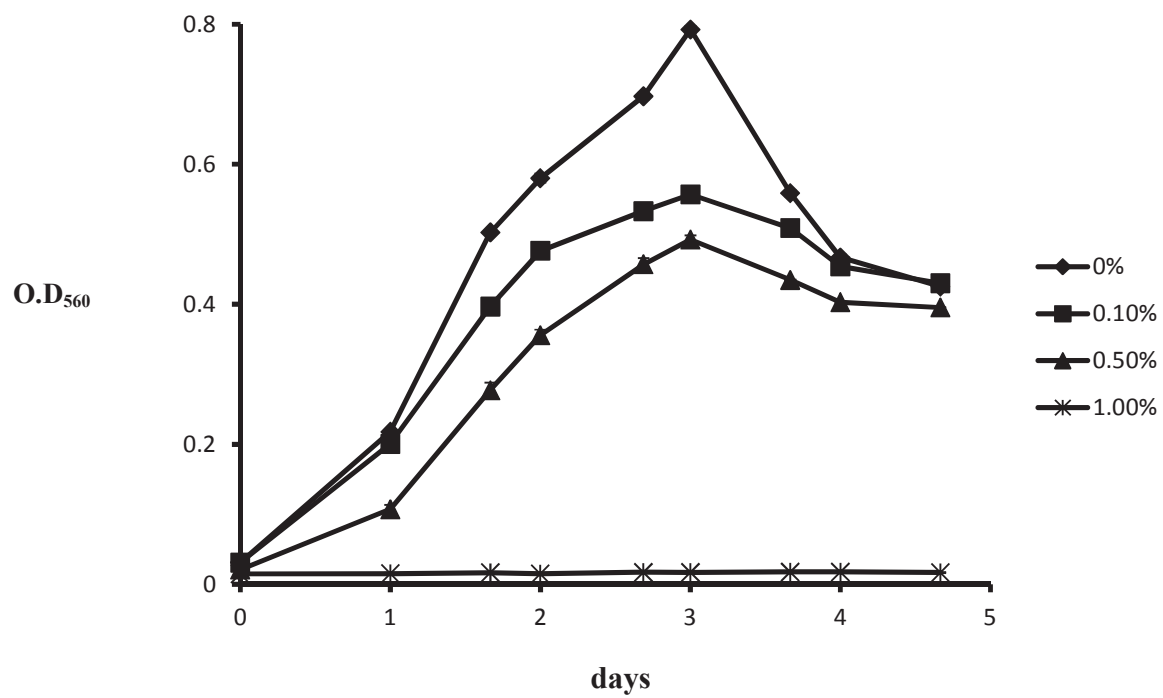
Fig. 3.5 Growth of strain OSK6[T] in various NaCl concentrations (%).

3.3.2 Physiology

3.3.2.1 Temperature optimum and range

Optimal temperature for growth of strain OSK6^T was observed at 30–37 °C with no growth below 20 °C or above 40 °C (Fig. 3.3).

3.3.2.2 pH optimum and range

The pH range for growth of strain OSK6^T was observed at 6.5–7.5 (Fig. 3.4).

3.3.2.3 NaCl tolerance

Strain OSK6^T grows best in the absence of NaCl but can tolerate the presence of 0.5% NaCl (Fig. 3.5).

3.3.3 Substrates and Electron acceptor utilization

As is commonly reported for all species belonging to the genus *Geobacter*, strain OSK6^T conserved energy for growth by coupling the reduction of ferric oxides (Fe(III)-NTA, Fe(III) citrate, and amorphous Fe(III) hydroxide) to the oxidation of acetate, as shown by the production of ferrous iron accompanied by increases in cell number and protein concentration (Fig. 3.6).

The novel strain utilized acetate, lactate, pyruvate, and succinate as electron donors with Fe(III)-NTA as the electron acceptor, but not toluene, H_2, formate, fumarate, propionate, benzoate, butyrate, butanol, phenol, methanol, ethanol, glucose, and methane. Amorphous Fe(III) hydroxide, Fe(III) citrate, and nitrate were reduced with acetate as electron donor, whereas fumarate, malate, and sulfate were not utilized as electron acceptors (Table 3.1).

Measurements of OD_{560} showed that the nitrate respiration by strain OSK6^T was a growth-yielding process (Fig. 3.7). When isotope $^{15}NO_3^-$ was used as an electron acceptor, it was reduced to $^{15}N_2O$, but not to $^{15}N_2$. This indicated an incomplete denitrification process (6, 33)

instead of ammonification as observed in the nitrate respiration by *G. metallireducens* GS-15^T (61).
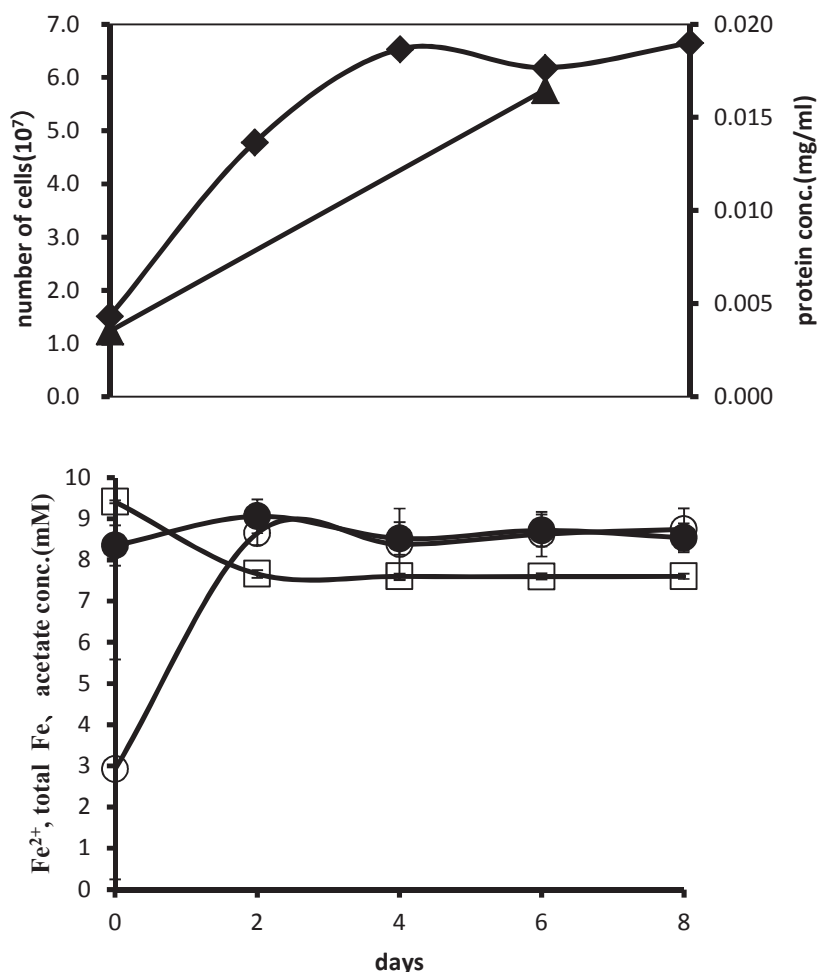
Fig. 3.6 Growth curve of strain OSK6^T with acetate as growth substrate and Fe(III)-NTA as electron acceptor. Strain OSK6^T conserved energy for growth by coupling the oxidation of acetate to the reduction of Fe^{3+}. (a) Microbial growth observed by cell numbers (filled diamond) and protein concentration (filled triangle) was monitored over time. (b) Oxidation of acetate and reduction of Fe(III)-NTA as indicated by the gradual decrease in acetate concentration (open square) and increase in Fe^{2+} concentration (open circles) over time while, Fe total (filled circle) remain constant. Values are means of three parallel replicate incubations and error bars represent standard deviations.
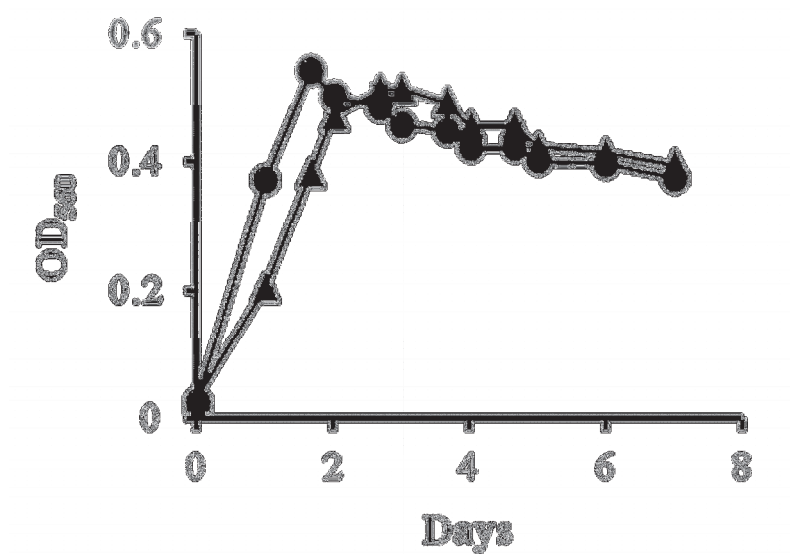
Fig. 3.7 Growth of strain OSK6[T] with acetate as growth substrate and nitrate as electron acceptor, observed at 30 °C (triangles) and 37 °C (circles). Utilization of nitrate by OSK6T is a denitrification process. Values are means of parallel duplicate experiments and error bars represent ranges of values.
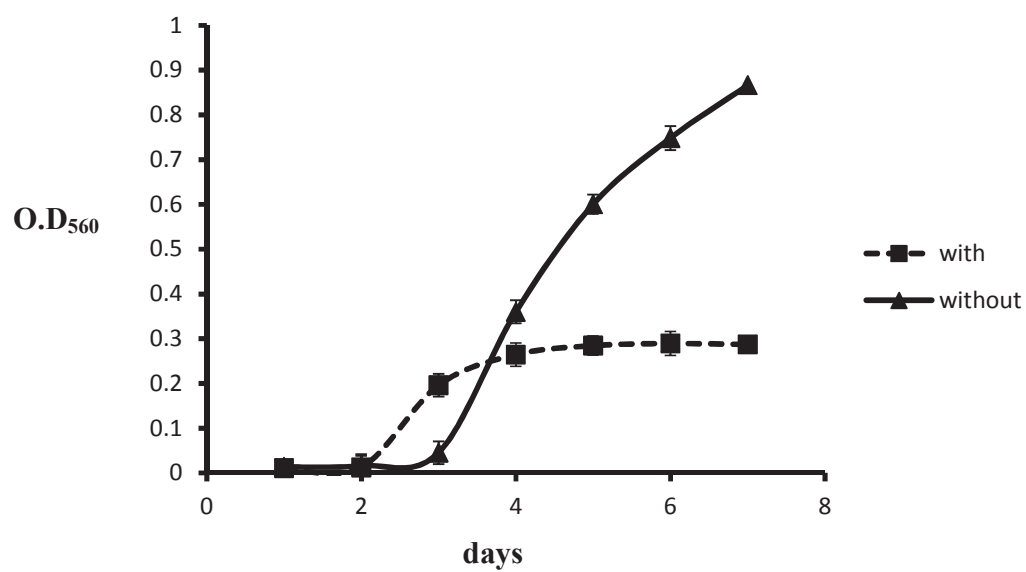
Fig. 3.8 Cultivation of strain OSK6^T either in presence and absence of Fe(III) citrate. Strain OSK6^T can grow with acetate and nitrate in absence of Fe(III) citrate.

In contrast to *G. metallireducens* GS-15[T] (101), strain OSK6[T] was able to grow on the medium containing acetate and nitrate without Fe amendment even after several passage cultures, indicating that growth of strain OSK6[T] by nitrate respiration is Fe-independent (Fig. 3.8).

Phenotypic characteristics of strain OSK6[T] showed that it has a very limited range of electron donor and electron acceptor utilizations and differs from its phylogenetically closest relatives (*G. daltonii* FRC-32[T] & *G. toluenoxydans* TMJ1[T]). It utilizes nitrate as an electron acceptor whereas *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T] do not. On the other hand, they utilize fumarate and malate as electron acceptors as previously described (55, 85), which are not utilized by strain OSK6[T]. In addition, strain OSK6[T] does not utilize toluene, benzoate, butyrate, and formate as electron donors, whereas both these other species do. However, strain OSK6[T] is capable of oxidizing lactate and succinate instead, whereas *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T] are not, in agreement with previous reports (55, 85). These findings in terms of substrate utilizations are similar to those of *G. pickeringii* G13[T] and *G. argillaceus* G12[T], isolated from a long-term enrichment culture of kaolin clays (102). Further comparison of phenotypic characteristics between strain OSK6[T] and closely affiliated *Geobacter* species is shown in Table 3.1.

Table 3.1 Physiological characteristics of strain OSK6^T and closely related species of the genus *Geobacter*: 1, strain OSK6^T (this study); 2, *G. daltonii* FRC-32^T (85); 3, *G. toluenoxydans* TMJ1^T (55, and this study); 4, *G. metallireducens* GS-15^T (61); 5, *G. argillaceus* G12^T (102); 6, *G. lovleyi* SZ^T (114); 7, *G. thiogenes* K1^T (78). All strains utilize acetate as an electron donor. +, positive; –, negative; NT, not tested.

Characteristics	1	2	3	4	5	6	7
DNA G + C content (mol %) †	59.7 (LC)	53 (G)	54.4 (LC)	56.6 (TM)	58 (TM)	56.7 (LC)	55.1 (LC)
Optimum temperature (°C)	30-37	30	25-32	30-35	30	35	30
pH range	6.5-7.5	6.7-7.3	6.6-7.0	6.7-7.0	6.2-6.8	6.5–7.2	6.5–7.0
Electron acceptor usage							
ferric citrate	+	+	+	+	+	+	–
amorphous Fe(III) hydroxide	+	+	+	+	+	+	NT
nitrate	+	–	–	+	+	+	+
fumarate	–	+	+	–	–	+	+
malate	–	+	+	NT	NT	+	+
Electron donor usage							
H$_2$	–	–	NT	–	–	+	+
formate	–	+	+	–	NT	NT	–
propionate	–	–	+	+	NT	NT	–
butyrate	–	+	+	+	+	NT	–
pyruvate	+	+	+	+	+	+	–
lactate	+	–	–	–	+	NT	–
succinate	+	–	–	NT	–	NT	–
benzoate	–	+	+	+	NT	NT	–
ethanol	–	NT	+*	+	+	NT	–
butanol	–	+	NT	+	+	NT	NT
phenol	–	NT	NT	+	NT	NT	NT
toluene	–	+	+	+	NT	NT	NT

†, determined by genome sequence (G), HPLC (LC), melting temperature (TM); *, determined in this study.

3.3.4 Isotopic methane incubation

Except methanotrophic growth of mud inoculums obtained from the rice field, no production of isotopic $^{13}CO_2$ neither Fe(III)-reduction was observed for all treatments including kill controls.

3.3.5 Chemotaxonomic characterization

Strain OSK6[T] contained MK-8, which is a typical respiratory quinone of the genus *Geobacter* (41, 55). Strain OSK6[T] has similar fatty acid composition with the two reference strains, such as 16:1 ω7*c* and 16:0 fatty acids as major constituents (more than 30 mol %); however, 16:1 ω5*c* and 18:1 ω7*c* fatty acids were only found in strain OSK6[T] as substantial components (≥ 1 mol %) (Table 3.2). In contrast, *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T] have 16:0 3OH, but strain OSK6[T] does not. Thus, fatty acid analyses clearly discriminates strain OSK6[T] from the closely related species.

Table 3.2 Fatty acid compositions of strain OSK6^T and closely related species of *Geobacter*.

Total lipid fatty acids (mol %)	OSK6^T	*G. daltonii* FRC-32^T	*G. toluenoxydans* TMJ1^T
16:1 ω7c	40.2	32.7	34.8
16:0	32.5	30.8	33.3
15:0 iso	9.19	11.4	11.3
14:0	6.28	8.75	4.75
18:1 ω7c	4.21	tr	tr
16:1 ω5c	2.12	tr	tr
13:1 at 12-13	1.01	5.47	4.47
16:0 3OH	tr	6.26	6.40
17:0 iso	tr	tr	1.24
16:0 10 methyl	-	1.04	-

All strains were cultivated with 30 mM acetate and 50 mM ferric citrate until early stationary phase. Fatty acid analyses were carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. One or more mol % of fatty acids are shown. tr, less than 1 mol %; -, not detected.
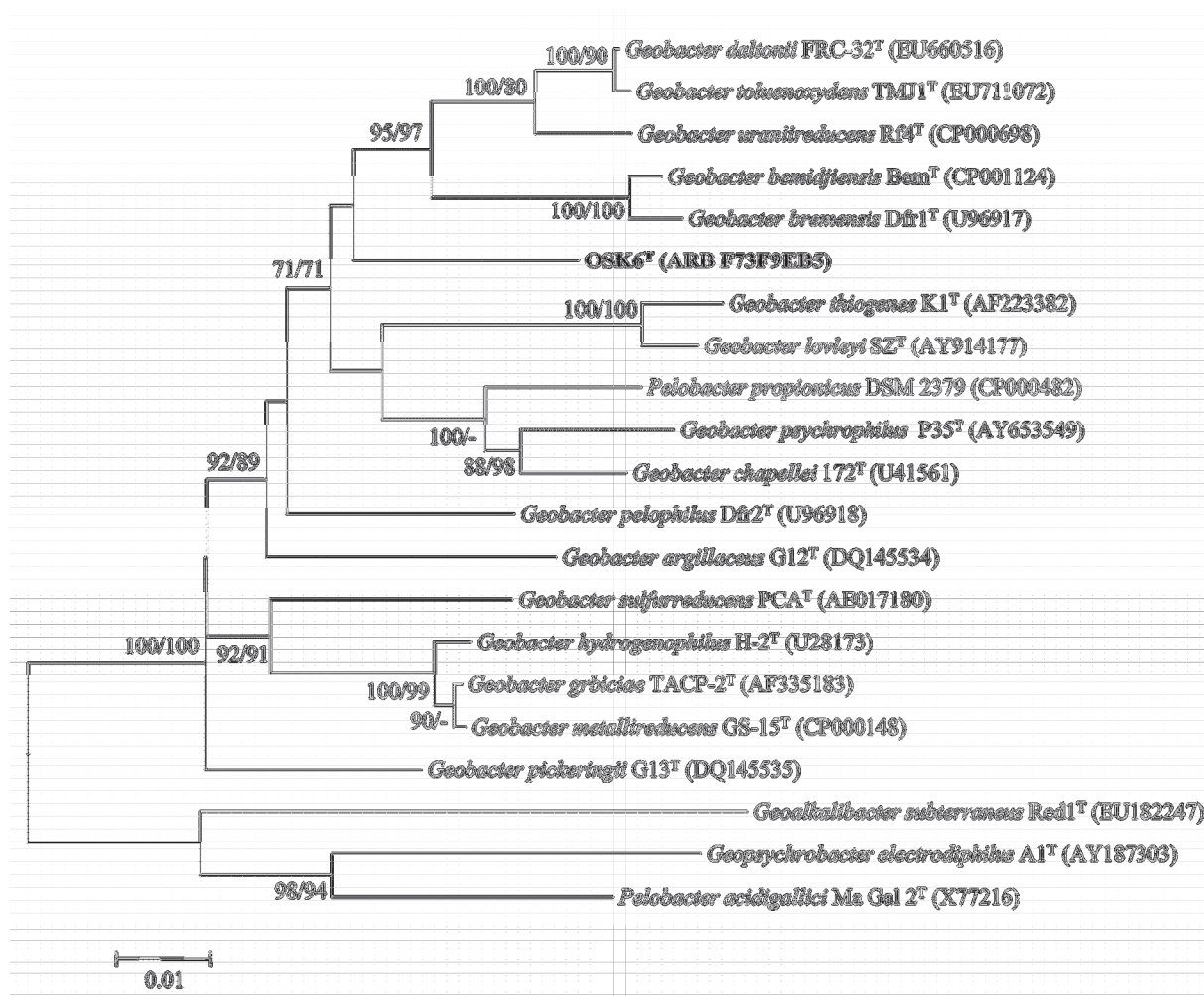
Fig. 3.9 Neighbor-joining phylogenetic tree constructed with Jukes-Cantor correction model based on 16S rRNA gene sequences, showing the position of strain OSK6[T] with its related taxa. The tree was rooted by the group of *Geoalkalibacter subterraneus* Red1[T], *Geopsychrobacter electrodiphilus* A1[T], and *Pelobacter acidigallici* Ma Gal 2[T]. Bootstrap values calculated by neighbor-joining (NJ) and maximum likelihood (ML) methods were obtained from 1000 replicates and are shown on the nodes of branching (NJ/ML), while values less than 70% are not shown. A hyphen indicates less than 70% reliability. Bars, 0.01 base substitutions per site.

3.3.6 16S rRNA gene analysis/phylogenetic tree

Analysis of the near full-length 16S rRNA gene sequence (1425 bp) as described above revealed that strain OSK6^T belongs to the genus *Geobacter* and is closely related to *G. daltonii* FRC-32^T and *G. toluenoxydans* TMJ1^T with 95.6% similarity. A phylogenetic tree of strain OSK6^T and its related strains showed that the new isolate is positioned as a member of the genus *Geobacter* (Fig. 3.9). Similarities among strain OSK6^T and type strains of species belonging to the genus *Geobacter* ranged from 92.9 to 95.6%.

3.3.7 GC Content

The G + C content of genomic DNA from strain OSK6^T was determined by HPLC and yielded 59.7 mol %, which was slightly higher than those of the two closely related species of the genus *Geobacter*, namely, *G. daltonii* FRC-32^T and *G. toluenoxydans* TMJ1^T, as determined by genome sequence and HPLC, respectively (Table 3.1).

3.4 Description of *Geobacter luticola* sp. nov.

Geobacter luticola (lu.ti' co.la. L. n. *lutum,* mud; L. suff. *-cola* (from L. n. incola), inhabitant, dweller; N. L. n. *luticola*, the mud dweller, the type strain of this species was isolated from mud of lotus field). Gram-negative, straight singular rods, 0.6–1.9 μm long and 0.2–0.4 μm wide, and motile with a flagellum. With Fe(III)-NTA as electron acceptor; acetate, lactate, pyruvate, and succinate are utilized as electron donors; H$_2$, formate, fumarate, propionate, benzoate, butyrate, butanol, methanol, ethanol, glucose, phenol, benzoate, toluene, and methane are not utilized as electron donors. With acetate as an electron donor, amorphous Fe(III) hydroxide, Fe(III) citrate, and nitrate are reduced as electron acceptors, but not sulfate, fumarate, or malate. Growth occurs at 20–40 °C with optimal temperature of 30–37 °C. Grows at pH 6.5–7.5. Grows best in absence of NaCl, but can tolerate up to 0.5% NaCl. Major respiratory quinone is MK-8. Contains 16:1

$\omega 7c$, 16:0, 15:0 iso, 14:0, 18:1 $\omega 7c$, 16:1 $\omega 5c$, and 13:1 at 12-13 fatty acids. The G + C content of genomic DNA of the type strain is 59.7 mol % as determined by HPLC.

The type strain OSK6[T] (= DSM 24905[T] = JCM 17780[T]) was isolated from a lotus field in Aichi prefecture, Japan.

3.5 Summary

A novel Fe(III)-reducing bacterium belonging to the genus *Geobacter* was isolated and characterized according to taxonomic criteria described by Tindall *et al*. (122). Comparative analysis of the phenotypic characteristics between strain OSK6[T] and its related taxa showed that the novel strain differs from *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T], with the abilities to utilize lactate and succinate as electron donors and nitrate as an electron acceptor, all of which are not utilized by the two closely related strains (Table 3.1). Total lipid fatty acid analyses further showed that strain OSK6[T] has distinguishable fatty acids (16:1 $\omega 5c$ and 18:1 $\omega 7c$) from the two relative strains (Table 3.2). In addition, the G + C content of genomic DNA also showed a value of 59.7 mol %, slightly higher than the closely related members of the genus *Geobacter*. Moreover, phylogenetic analyses based on the 16S rRNA gene sequence showed that strain OSK6[T] belongs to the genus *Geobacter* with a similarity of 95.6% to *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T], suggesting that strain OSK6[T] represents a new species of the genus *Geobacter*. On the basis of all these phenotypic and chemotaxonomic as well as phylogenetic traits of this study, strain OSK6[T] shares some common features but also differs from the most closely related *Geobacter* species, namely, *G. daltonii* FRC-32[T] and *G. toluenoxydans* TMJ1[T]; hence, a novel species belonging to the genus *Geobacter* as *G. luticola* sp. nov. was isolated and characterized. The results have been successfully published in the International Journal of Systematic and Evolutionary Microbiology.

CHAPTER 4 Isolation and characterization of *Geobacter sulfurreducens* subsp. *ethanolicus* subsp, nov., an ethanol-utilizing dissimilatory Fe(III)-reducing bacterium from lotus field.

4.1 Background

Dissimilatory reduction of Fe(III) oxides is an important process for oxidation of organic matter in terrestrial anoxic soils (57−58, 119). The process is predominated by *Geobacter* species (4, 18, 64, 108) thereby, contributing to the global cycling of metals and carbon (64). The genus *Geobacter* was established by Lovley *et al*. (61) with *Geobacter metallireducens* GS-15[T] isolated from the Potomac River, the first species to be described under the genus followed by *Geobacter sulfurreducens* PCA[T] (13). *G. sulfurreducens* PCA[T], is one of the most studied *Geobacter* species as a model organism on various researches such as biochemical and molecular studies on respiratory mechanisms of iron (III) oxide (52, 56, 64, 93, 138), development of genetic manipulation techniques (1, 21, 25, 68, 83), and the first genome analysis within the genus *Geobacter* (71).

A novel subspecies of *Geobacter sulfurreducens* strain OSK2A[T], capable of growth with ethanol as a substrate, was successfully isolated and characterized, forming the basis of this chapter. Findings in this chapter will supplement existing knowledge on the strain and add to the growing list of strains belonging to the genus *Geobacter*. *Geobacter sulfurreducens* OSK2A[T] was isolated according to the flow diagram depicted in Fig. 3.1.

4.2 Materials and Methods

4.2.1 Medium

The same medium composition and preparation reported in chapter 3 was used for isolation and characterization of strain OSK2A[T].

4.2.2 Isolation

Mud samples for enrichment were collected from lotus field and incubated in an anaerobic reactor described in chapter 2. Initial isolation procedures mentioned in chapter 3 were performed and further isolation was done in roll tube tubes with agar as the solidifying agent. Purity of the isolate was checked by microscopy and absence of growth in an anaerobic heterotrophic medium (NIH thioglycolate broth, Difco) amended with pyruvate (20 mM).

4.2.3 Morphology

For morphological analysis, strain OSK2A[T] was grown with acetate and amorphous Fe(III) hydroxide, followed by procedures described in chapter 3. Motility was observed with cultures grown on ethanol (10 mM) and amorphous Fe(III) hydroxide (ca. 100 mM) and examined by phase contrast microscopy under consideration of methods described by Childers *et al.*, (16). Reference strain PCA[T] (= DSM 12127[T]), was also checked for its motility under laboratory conditions with acetate (10 mM) replacing ethanol.

4.2.4 Physiology

Physiological tests on growth ranges for temperature, pH, and NaCl concentrations were performed with acetate (10 mM) and fumarate (20 mM), in a basal medium used previously to culture *Geobacter lutiola* OSK6[T], described in chapter 3. Tests for all growth ranges and optima for strain OSK2A[T] and the reference strain PCA[T] were, performed in duplicates with growth monitored at OD_{560} under our laboratory conditions.

4.2.4.1 Temperature optimum and range

Optimum temperatures for growth were tested at 4, 10, 20, 25, 30, 37, 40, 45, and 50 °C with 10 mM acetate and 10 mM nitrate in duplicates.

4.2.4.2 NaCl tolerance

Growth with NaCl was tested at 0, 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5% at near neutral pH with 10 mM acetate and 20 mM nitrate as electron donor and acceptor, respectively and incubated at 30 °C.

4.2.4.3 pH optimum and range

Likewise, optimal pH growth tests were carried out in vials containing basal medium with acetate and nitrate, but without sodium bicarbonate. The non-bicarbonate basal medium was prepared in the same way as that of all other phenotypic characterizations described above, but with N_2/CO_2 gas replaced by N_2 gas, and supplemented with buffers (MES-NaOH, 5.0–6.0; PIPES-NaOH, 6.0–7.0; HEPES-NaOH, 7.0–7.5; Tris-HCl, 8.0–11) at a final concentration of 10 mM to give the desired pH values. Incubations were done at 30 °C.

4.2.5 Substrates and Electron acceptor utilization

Fe(III) utilization and growth of the strain was observed at 30 °C with acetate as substrate and Fe(III)-NTA as an electron acceptor. Cell growth was determined by measuring protein concentrations and direct cell counting with SYBR Gold fluorescent dye.

The same composition of a bicarbonate-buffered basal medium described above was used for all phenotypic characterizations in which acetate and Fe(III) citrate were used as electron donor and acceptor respectively. Utilization of electron donors was examined at 30 °C in the presence of Fe(III) citrate (50 mM) with the following substrates (concentration in mM given in parenthesis, unless otherwise stated): formate (10), propionate (10), butyrate (10), pyruvate (10), lactate (10), fumarate (10), succinate (10), ethanol (10), butanol (10), glucose (10), phenol (1), benzoate (1), toluene (1), methanol (5), propanol (10), iso-propanol (10), H_2 (ca. 62 kPa), and CH_4 (ca. 62 kPa). Utilization of electron acceptors was done with nitrate (20), malate (20),

fumarate (40), Fe(III)-NTA (10), amorphous Fe(III) hydroxide (50), sulfate (20), elemental sulfur (3.0 g/L), sulfite (20) and thiosulfate (20) in the presence of acetate (10). Ethanol as the most distinguishing substrate that differentiated strain OSK2A^T from the closely related strain was further tested along with *G. sulfurreducens* PCA^T as the reference strain.

4.2.6 Isotopic methane incubation

Isotopic methane incubations were done in batch cultures cultivated in vials, according to procedures mentioned in chapter 2.

4.2.7 Chemo-taxonomic analysis

4.2.7.1 Respiratory Quinones

Cells for respiratory quinone analysis were extracted in chloroform/methanol and purified by TLC in hexane/benzene/chloroform mixture, then, determined by HPLC according to previously described procedures (20). *G. sulfurreducens* PCA^T was used as the reference strain for respiratory quinone analyses. Cells for both strains were cultured on acetate (20 mM) and Fe(III) citrate (100 mM) until early stationary phases for chemotaxonomic analyses.

4.2.7.2 Fatty acids

Analyses of cellular fatty acids were carried out by the Identification Service of the DSMZ, Braunschweig, Germany. *G. sulfurreducens* PCA^T was used as the reference strain for both respiratory quinones and fatty acids analyses. Cells for both strains were cultured on acetate (20 mM) and Fe(III) citrate (100 mM) until early stationary phases for chemotaxonomic analyses.

4.2.8 Molecular analysis

4.2.8.1 16S rRNA gene analysis

The 16S rRNA gene of strain OSK2A^T was amplified by PCR using primers EU10F and 1500R according to descriptions in chapter 2. Sequencing was performed according to previously described procedures (76).

4.2.8.2 Phylogenetic tree construction

For phylogenetic analyses, 16S rRNA gene sequences of the type strains of species of the genus *Geobacter* and other related taxa were aligned using the online alignment tool SINA (86) and manually edited in ARB (65). Aligned sequences were imported into the MEGA 5.0 and PHYML 3.0 (38, 118) to estimate phylogenetic trees using neighbor-joining (95) and maximum likelihood (31) methods, respectively, with Jukes and Cantor model (51). Reliabilities of the bootstrap values were inferred by both methods in accordance with Hall (39).

4.2.8.3 Rep-PCR fingerprinting

Genomic DNA for rep-PCR were obtained for strain OSK2A^T from cells grown with ethanol (10 mM) and Fe(III) citrate (50 mM) while, strain PCA^T cells were obtained from acetate (10 mM) and Fe(III) citrate (50 mM). Rep-PCR amplifications were performed with REP2-I and REP1R-I primers according to PCR conditions described by Versalovic *et al*. (126) and Sung *et al*. (114). The rep-PCR products were electrophoresed in 1.5% agarose gel with 1 × TAE buffer at 18V for 5 hours. Resulting band patterns were analyzed by visual comparison.

4.2.8.4 GC Content analysis

For determination of the G + C content (mol%), strain OSK2A^T and the reference strain PCA^T cells were grown with either ethanol (10 mM) or acetate (10 mM) and Fe(III) citrate (100 mM) and, cells were extracted according to the protocol described by Versalovic *et al*. (126).

The G + C content were determined by HPLC according to previously described procedures (117).

4.2.8.5 DNA-DNA hybridization

DNA-DNA Hybridization analyses (15, 26, 47) were performed by the Identification Service of the DSMZ, Braunschweig, Germany.

4.3 RESULTS and DISCUSSION

4.3.1 Morphology

Strain OSK2A^T was isolated from lotus field sediments in a roll tube method with agar as the solidifying agent with colonies appearing red and spherical in shape. No growth was observed when cultivated in the medium for heterotrophic anaerobes. Further microscopy observation of uniformity in cell morphology showed that strain OSK2A^T was eventually purified. The novel strain OSK2A^T is a rod shaped bacterium, 0.76-1.65 µm long and 0.28-0.45 µm wide (Fig. 4.2). Although there was no flagella observed under SEM observation, strain OSK2A^T is motile when observed with ethanol and Fe(III) hydroxide under light microscopy, unlike the closely related strain PCA^T which is not motile. Original description of strain PCA^T also found no motility (13).

4.3.2 Physiology

4.3.2.1 Temperature

Strain OSK2A^T grows at an optimum temperature of 30-37 °C with a growth range of 20-40 °C (Fig. 4.3) while the closest related strain PCA^T grows at similar optimum temperature but with a significant temperature range of 10-45°C.

4.3.2.2 pH optimum and range

Strain OSK2A^T grows at pH range of 6.0-8.0 (Fig. 4.4). All the morphological and physiological results are summarized in (Table 4.1).

4.3.2.3 NaCl tolerance

Strain OSK2A^T strives in 0-1.0% NaCl (Fig. 4.5) as is the reference strain PCA^T which is in agreement to their closeness according to the 16S rRNA gene sequence.

4.3.3 Substrates and Electron acceptor utilization

Similar to other *Geobacter* species, strain OSK2A^T strives by coupling the reduction of ferric oxides (Fe(III)-NTA, Fe(III) citrate, and amorphous Fe(III) hydroxide) to the oxidation of acetate, coincided with increase in cell numbers and protein concentration (Fig. 4.6). Both the novel strain OSK2A^T and the reference strain PCA^T utilized the following electron donors with Fe(III) citrate; acetate, lactate, pyruvate and formate while, only the novel strain could utilize ethanol (Fig. 4.7). Original description of strain PCA^T (13) showed that strain PCA^T does not utilize carboxylic acids however, this study and others (59, 64, 109) found otherwise. Acetate or ethanol as the substrate the following electron acceptors were utilized by the novel strain OSK2A^T; amorphous Fe(III) hydroxide, Fe(III)-NTA, malate, fumarate and elemental sulfur (Table 1). Ethanol as the distinguishing substrate showed growth when measured by OD$_{560}$ unlike the reference strain with no growth after 10 days of cultivation (Fig. 4.7). Strain OSK2A^T has almost identical phenotypic characteristics with the phylogentically closest relative (*G. sulfurreducens* PCA^T) except for ethanol whereby the novel strain can utilize but which strain PCA^T does not. Other phenotypic characterizations between strain OSK2A^T and the closely related strain are given in Table 4.1.

Investigation on current production by the isolate, strain OSK2A[T] and reference strain PCA[T] in microbial fuel cells (MFC), in the presence of acetate as the substrate showed the former producing a significantly higher maximum current density than the latter (Fig. 4.9 and 4.10), which was, 6.5 times higher (7.1 and 1.1 A/m^2, respectively). A previous similar study conducted between an isolate named KN400 and wild-type strain of *G. sulfurreducens* also showed similar results to this study (137). In the previous study (137), strain KN400 produced higher current than the wild-type strain PCA[T] (Fig. 4.11). Furthermore, strain KN400 has 100% similarity to *G. sulfurreducens* PCA[T] based on the 16S rRNA gene, but a comparison of the complete genomes of KN400 and PCA[T] strains showed divergence of genes involved in current production (12). Similarly, the difference in current production between OSK2A[T] and PCA[T] strains may support strain OSK2A[T] as a separate strain from PCA[T], albeit belonging to the same species. On the other hand, although current production has not been officially recognized as a standard criterion for the taxonomic classification of *Geobacter* species, there are increasing studies on their promise for current production in MFC, and it is becoming an integral component of their physiological description (63, and references therein). Studies on the comparison of current production between the novel strain and reference strain, was conducted according to H. Yi et al (137).

4.3.4 Chemotaxonomic characterization

The novel strain has MK-8 as the major respiratory quinone, as is commonly reported for the genus *Geobacter* (41, 55, 64, 128). Unlike the respiratory quinones, fatty acids are specific for each strain. Strain OSK2A[T] contains the same but one less major fatty acids (≥ 1 mol %) compared to its closest relative; 16:1 ω7*c*, 16:0, 14:0, 15:0 iso, 16:1 ω5*c* and 18:1 ω7*c* (Table 4.2), in agreement with the findings of Hedrick *et al*. (41). Other fatty acids absent in the novel

strain but are present in the closely related strain PCA^T albeit in tiny portions (< 1 mol%) are 17:1 iso ω9c, 17:0 iso, 15:1 iso F and 13:0 iso. On the other hand, 18:1 ω7c 11-methyl (< 1 mol %) is found in the novel strain but absent in the reference strain PCA^T. Therefore, fatty acid composition do not clearly differentiates the novel strain from the most closely related strain PCA^T rather, it demonstrated their close relationship.

4.3.5 16S rRNA gene analysis/phylogenetic tree

Analysis of the near complete sequences of the 16S rRNA gene (1358) and eventual construction of the phylogentic tree as described above revealed that, strain OSK2A^T is positioned within the genus *Geobacter* (Fig. 4.8) and almost identical to *G. sulfurreducens* PCA^T with 99.6% similarity and, 95.6 % or lower similarity to other *Geobacter* species.

4.3.6 Rep-PCR

Although the novel strain is more than 99% similar to the most closely related strain based on the 16S rRNA gene, rep-PCR band patterns of the genomic DNA for both the novel strain and the reference strain PCA^T revealed separate fingerprints for both strains indicating that both strains are separate subspecies (Fig. 4.9).

4.3.7 GC Content/DNA-DNA hybridization

The G + C content of genomic DNA for strain OSK2A^T is 61.2 mol%, which is slightly lower but almost identical to the most closely related strain PCA^T (61.9 mol%), supporting their close phylogenetic relationship (Table 4.1). Previous values obtained for *G. sulfurreducens* by Coates *et al*. (19) showed a lower G + C content than values showed here, probably due to different cultivation conditions and protocols employed in this study (117) as opposed to the protocols of Mesbah *et al*. (70) as reported in Coates *et al*. (19). Furthermore, although DNA-DNA hybridization value of 60.7% ± 3.5% supports strain OSK2A^T as a novel species (110,

129), similar to strain TACP-2[T] and GS-15[T] which shared greater than 99% similarity according to their 16S rRNA gene sequence and yet, exhibit less than 70% DNA-DNA homology (19), phenotypic characterizations pointed otherwise to a novel subspecies.

4.3.8 Emended description of *Geobacter sulfurreducens* (12)

The description of *Geobacter sulfurreducens* is based on the data from (13), with the following modifications and additional features: utilizes formate, lactate, and pyruvate as substrates for growth with Fe(III) as electron acceptor. Grows at 10–45 °C and pH 5.6–8.0.

4.3.9 **Description of *Geobacter sulfurreducens* subsp. *ethanolicus*,subsp. nov.**

Geobacter sulfurreducens subsp. *ethanolicus* (etymologies: e.tha.no´li.cus. N.L. n. *ethanol* ethanol; L. suff. *-icus* suffix used with various meanings; N.L. masc. adj. *ethanolicus* belonging to ethanol, in reference to the ability of the species to utilize ethanol as a substrate for growth).

The cells are Gram-negative, motile, rod-shaped, strictly anaerobic, 0.76–1.65 μm long, and 0.28-0.45 μm wide. Growth occurs at 20–40°C with an optimum of 30–37°C, pH 6.0–8.1 (optimum pH 7.0) and can tolerate up to 1% NaCl. Electron donors utilized in the presence of Fe(III)-citrate include H_2, ethanol, acetate, lactate, pyruvate, and formate. Other electron donors tested but not utilized are propionate, butyrate, succinate, malate, fumarate, benzoate, butanol, methanol, propanol, iso-propanol, phenol, toluene, glucose, and yeast extract. Amorphous Fe(III) hydroxide, Fe(III)-citrate, Fe(III)-NTA, fumarate, malate, and elemental sulfur are utilized as electron acceptors with either acetate or ethanol as substrates, while nitrate, sulfate, sulfite, and thiosulfate are not utilized. The major respiratory quinone is MK-8. The major fatty acids are 16:1 ω7*c*,16:0, 14:0, 15:0 iso, 16:1 ω5*c*, and 18:1 ω7*c*. The G+C content of the genomic DNA is 61.2 mol%.

The type strain, OSK2A^T (= DSMZ 26126^T = JCM 18752^T), was isolated from lotus field sediments in Aichi prefecture, Japan.

Geobacter sulfurreducens subsp. *sulfurreducens* (13) was the name created based on the description of *Geobacter sulfurreducens* reported in (13).

4.4 Summary

A novel Fe(III)-reducing bacterium belonging to the genus *Geobacter* was isolated and characterized according to taxonomic criteria described by Tindall *et al*. (122). 16S rRNA gene analyses showed that the novel strain OSK2A^T is closely related to *Geobacter sulfurreducens* PCA^T. Phenotypic characteristics between strain OSK2A^T and the most closely related strain PCA^T showed almost identical phenotypic traits, agreeing with the closeness of their relationship based on the 16S rRNA gene sequence. However, ethanol utilization and the motility of strain OSK2A^T which are both absent for strain PCA^T, clearly distinguishes the novel strain. In addition, the limited temperature range observed for the novel strain compared to the most closely related strain PCA^T (Table 4.1) also delineate strain OSK2A^T from its closest relative. In support of the almost identical similarity between OSK2A^T and PCA^T according to the 16S rRNA gene sequences (99.6%), rep-PCR fingerprints, G + C content, DNA-DNA hybridrization values, cellular fatty acid composition and respiratory menaquinone analyses (Table 4.1 & 4.2), also corroborate classifying OSK2A^T as a novel subspecies belonging to *Geobacter sulfurreducens*. Strain OSK2A^T has been successfully published in the Journal of General and Applied Microbiology (JGAM) with the name *Geobacter sulfurreducens* subsp. *ethanolicus*, subsp. nov.

The type strain was deposited in DSMZ with identification number 26126^T and at Japan Collection of Microbes (JCM) with identification number 18752^T.
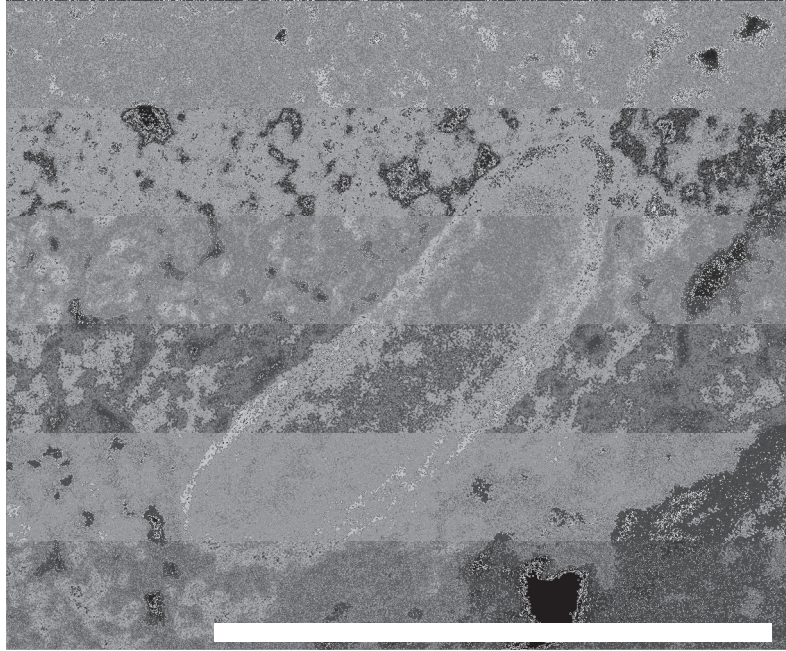
Fig. 4.1 Morphology of strain OSK2A^T **observed by scanning electron microscopy. Bar indicates 1 µm.**
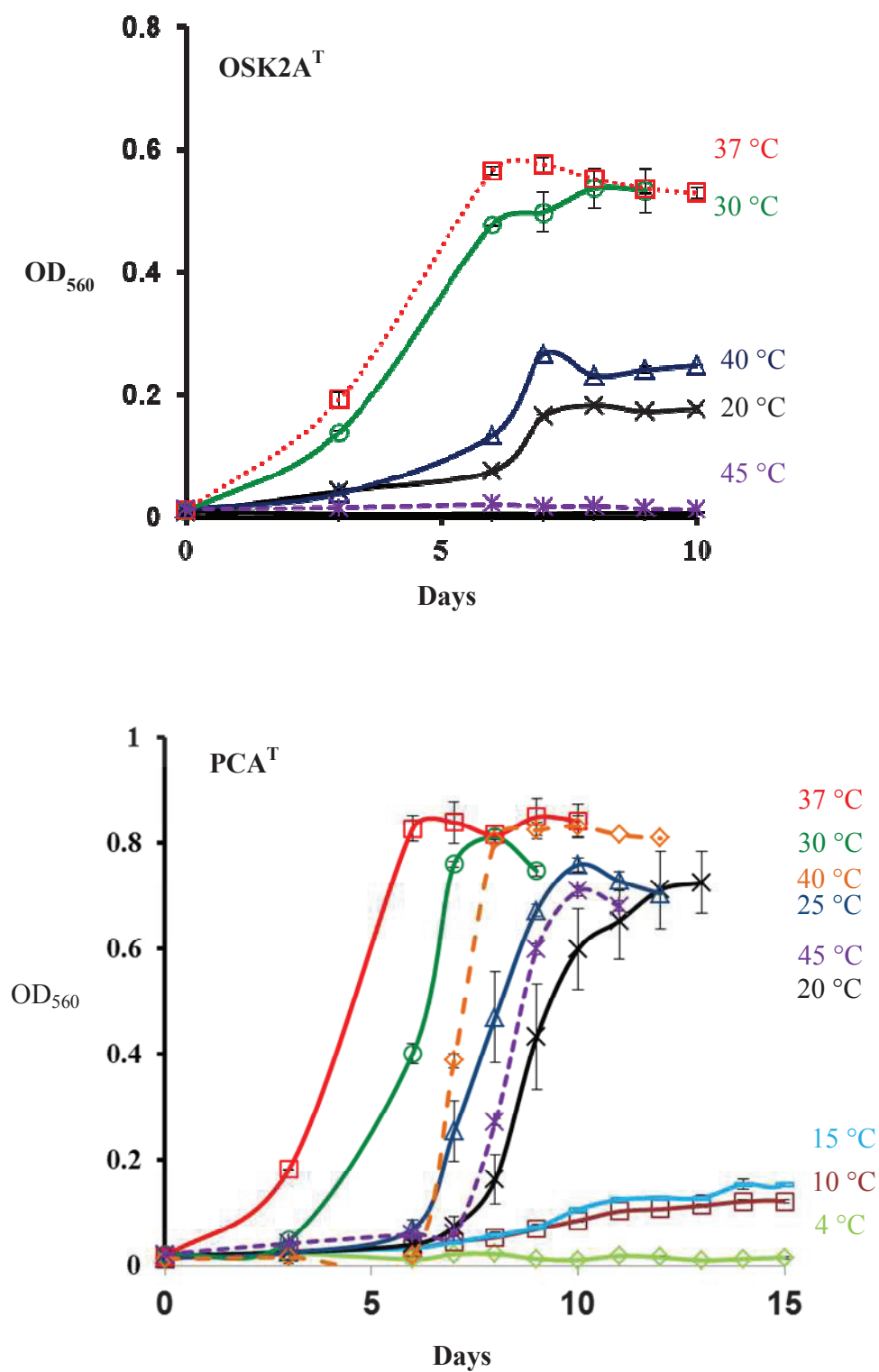
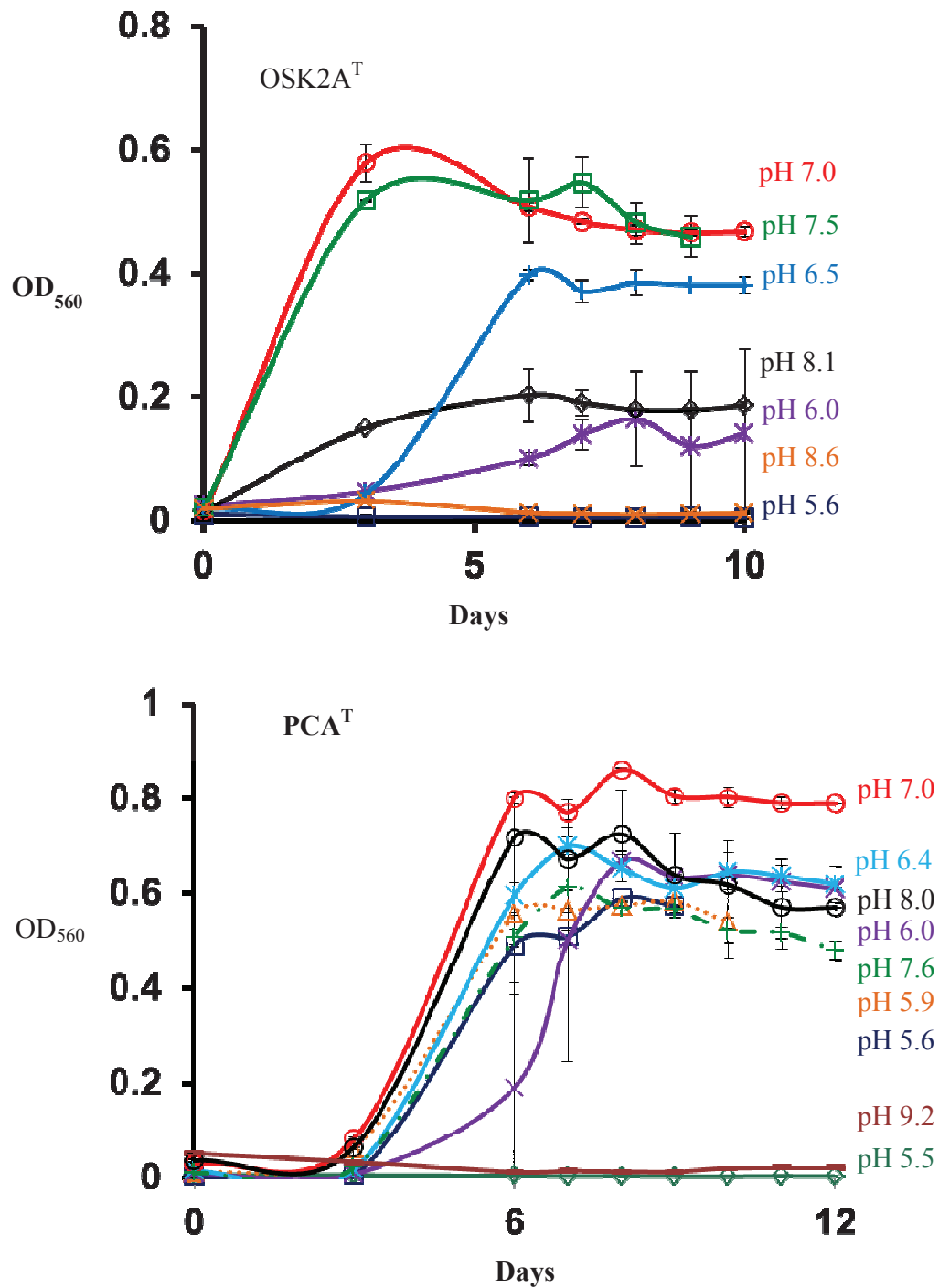**Fig. 4.2 Growth of strain OSK2A^T and the reference strain in various temperatures.**

Fig. 4.3 Growth of strain OSK2A^T and the reference strain in various pH. Strain OSK2A^T has an optimum pH7.6-7.9 for growth.
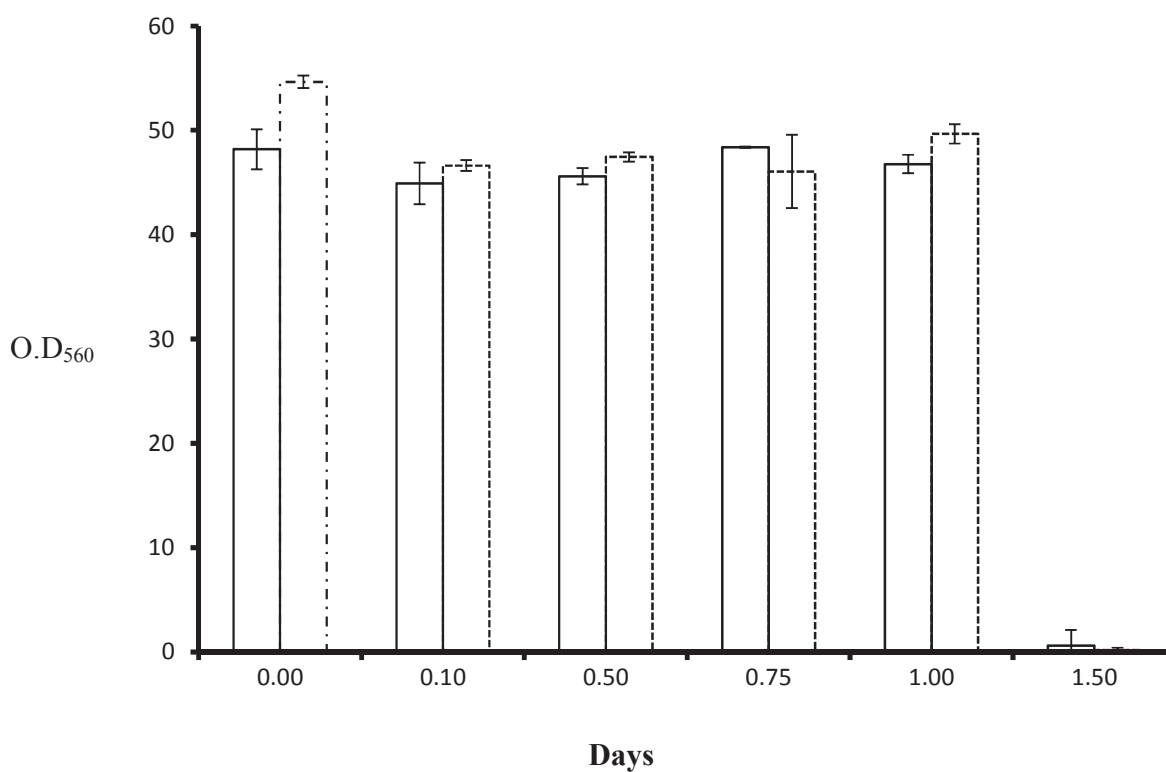
Fig. 4.4 Growth of strain OSK2A[T] and the reference strain in varying concentrations of NaCl. Strain OSK2A[T] can tolerate up to 1.0% (1.0 g/l) NaCl. Filled lines, strain OSK2A[T]; Dashed lines, reference strain.
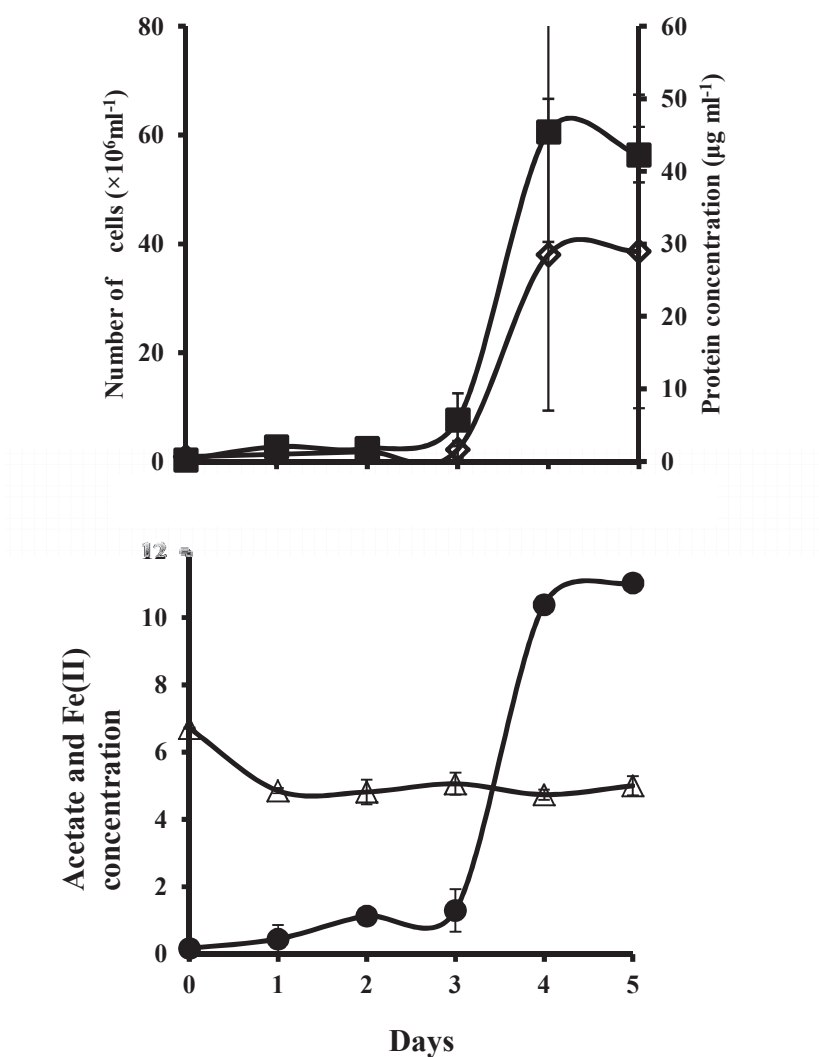
Fig. 4.5 Growth of strain OSK2A[T] with acetate and Fe(III)-NTA as electron donor and acceptor respectively. (a) Open diamond denote cell numbers and filled square represent protein concentration. (b) Acetate was utilized in concomitant reduction with Fe(III)-NTA over time, open triangle, acetate concentration; filled circle, Fe(II) concentration. Data presented are means of triplicate incubations with standard deviations as error bars.
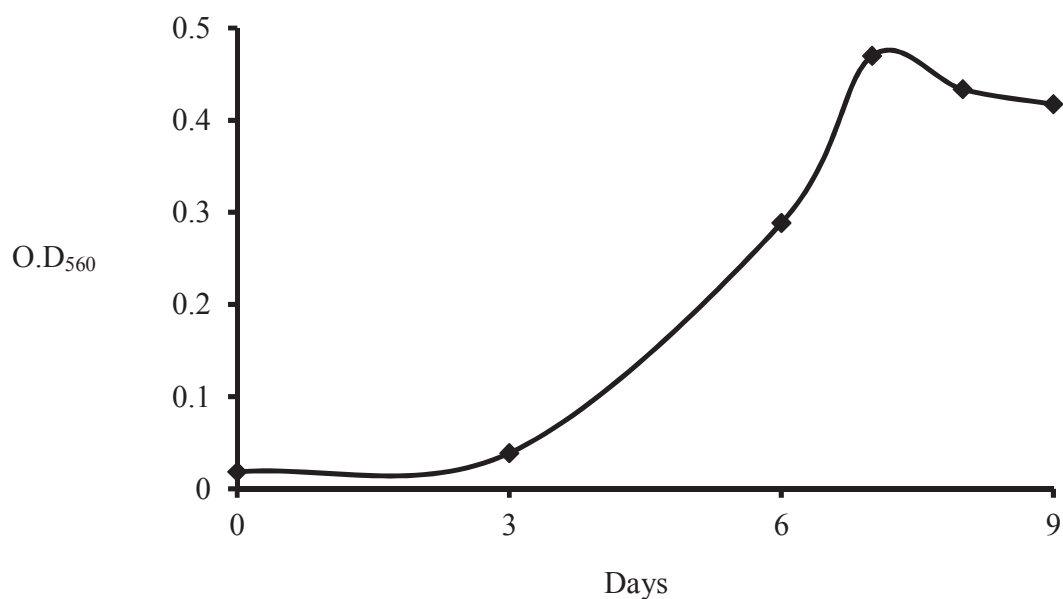
Fig. 4.6 Growth of strain OSK2A^T, observed at 30 °C with ethanol and fumarate as electron donor and acceptor respectively. This figure shows that strain OSK2AT can utilize ethanol as a substrate for growth while, strain PCA^T (its closest relative) does not (data not shown). Data presented are means of triplicate incubations and error bars represent standard deviation values.
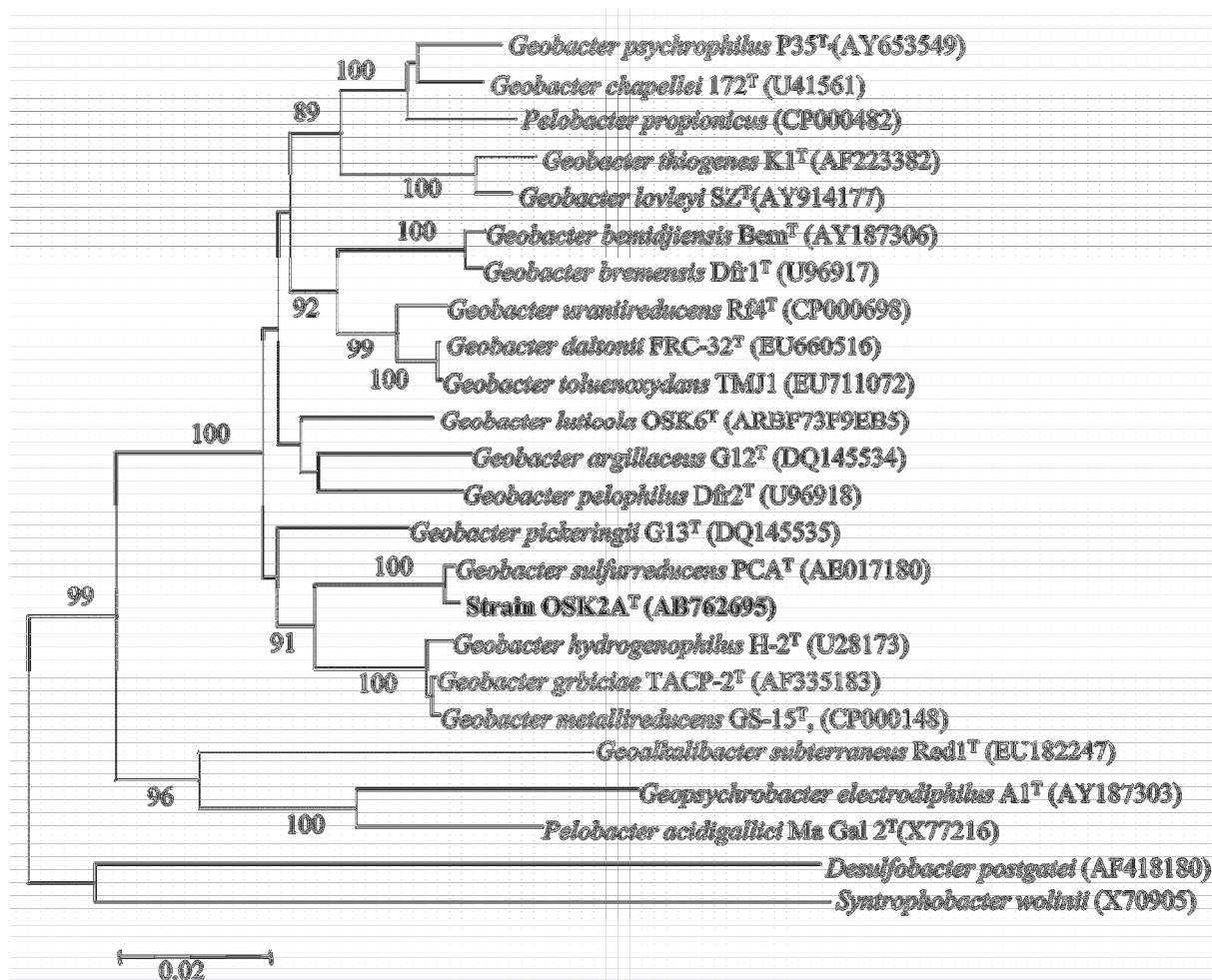
Fig. 4.7 Phylogenetic tree reconstructed from 16S rRNA gene sequences with Jukes–Cantor corrections, showing the placement of strain OSK2A[T] among closely related members of the family *Geobacteraceae*. The tree was constructed by MEGA5. Percentages at nodes show bootstrap values calculated by neighbor-joining (NJ) and maximum parsimony (MP) methods from 1000 replicates. NJ/MP values more than 70% reliability are shown while, values less than 70% are indicated as hyphen. Bars, 0.02 base substitutions per site.

98
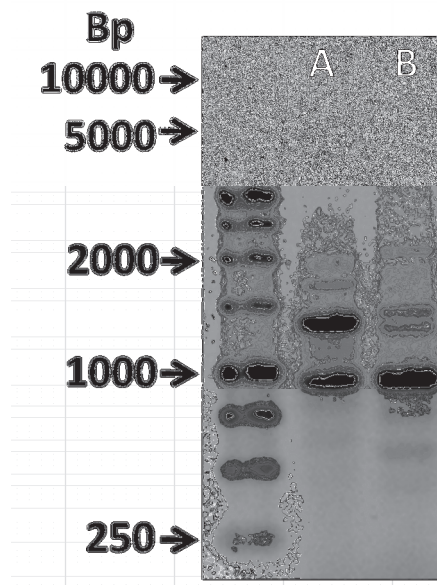
Fig. 4.8 Rep-PCR fingerprints of strain OSK2A^T (B) and strain PCA^T (A). The first lane shows the 1-kb size marker (SibEnzyme).
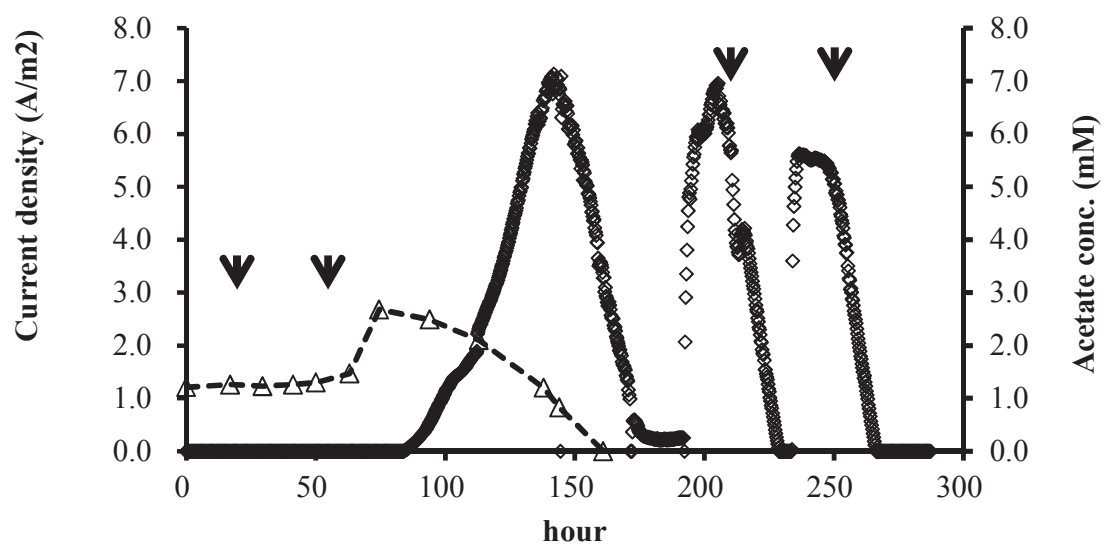
Fig 4.9 Current production by OSK2A^T. Δ, represent acetate concentration (mM) within the culture. ◊, represent current production in (A/m^2). ↓, showed time at which acetate was added to the culture.
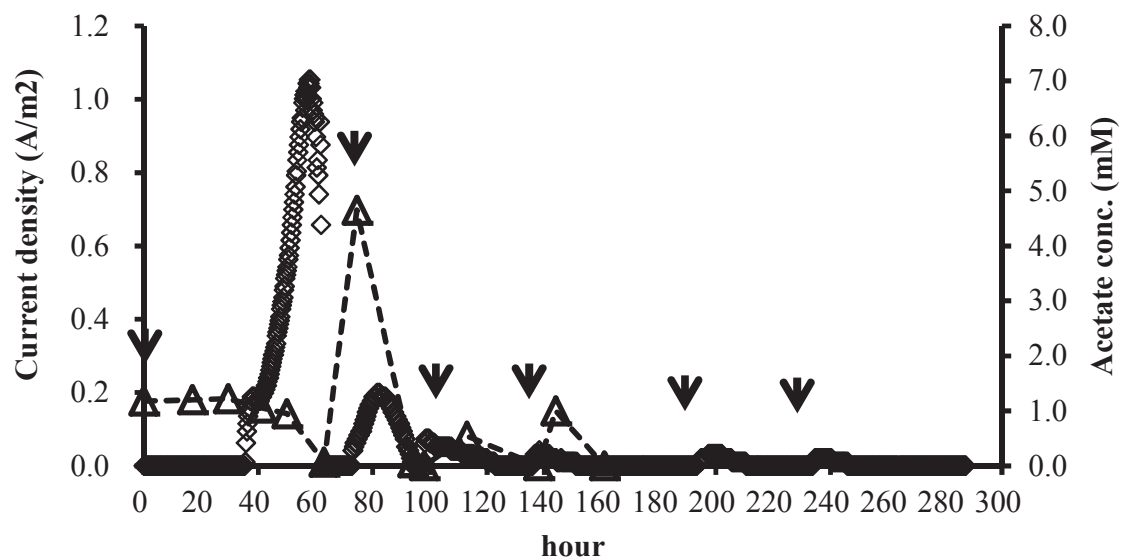
Fig 4.10 Current production by PCA^T. Δ, represent acetate concentration (mM) within the culture. ◊, represent current production in (A/m^2). ↓, showed time at which acetate was added to the culture. .
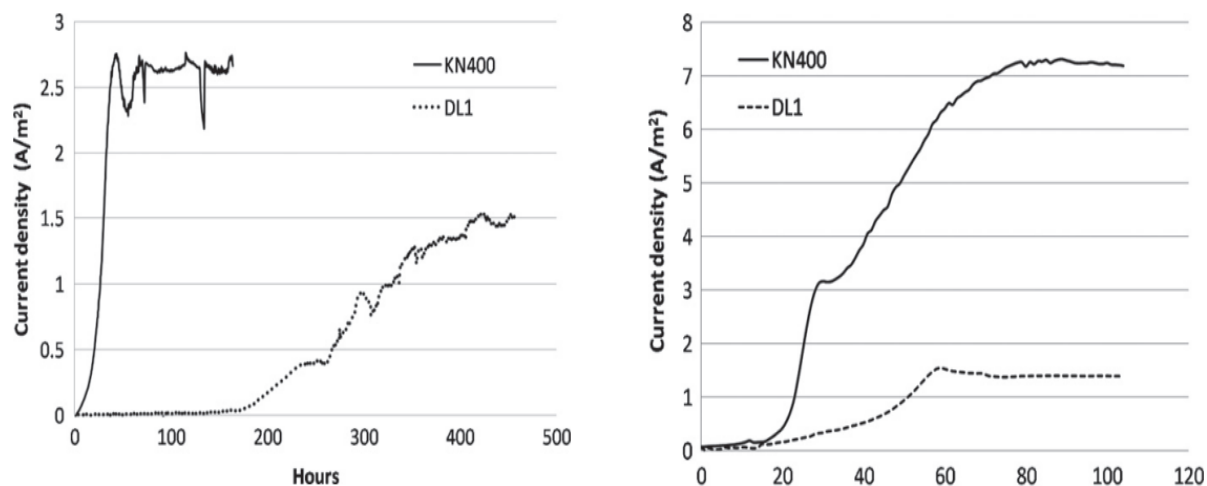
Fig 4.11 (A) Current produced by KN400 and DL1 strains in a system with anodes poised at -400 mV. (B) Current produced by KN400 and DL1 in fuel cell mode with a platinum wire anode. The external resistance was 560 . Adopted from H. Yi et al (2009).

Table 4.1 Differentiating characteristics of strain OSK2A[T] from closely related members of the genus *Geobacter*. Taxa: 1, strain OSK2A[T] (data from this study); 2, *G. sulfurreducens* PCA[T] (Caccavo *et al.*, 1994, and this study); 3, *G. grbiciae* TACP-5[T] (Coates *et al.*, 2001); 4, *G. metallireducens* GS-15[T] (Lovley *et al.*, 1993). All strains utilize acetate as an electron donor. +, positive; −, negative; NR, not reported in the original reference.

| Characteristics	1	2	3	4
Cell width (μm)	0.28-0.45	0.5	0.6	0.5
Cell length (μm)	0.76-1.65	2-3	1.0-2.0	2-4
Motility	+	−*	−	+
Temperature range (°C)	20-40	10-45*	30[‡]	30-35[‡]
pH range	6.0-8.0	5.6-8.0	NR	6.7-7.0[‡]
Electron acceptor usage				
Fe (III)-citrate	+	+	−	+
amorphous Fe(III) hydroxide	+	+	+	+
Fe(III)-NTA	+	+	+	+
nitrate	−	−	NR	+
fumarate	+	+	NR	−
malate	+	+	NR	NR
Elemental sulfur	+	+	NR	−
Electron donor usage				
H_2	+	+	+	−
formate	+	+*	+	−
propionate	−	−	+	+
butyrate	−	−	+	+
pyruvate	+	+*	+	+
Lactate	+	+*	−	−
succinate	−	−	−	NR
benzoate	−	−	+	+
ethanol	+	−*	+	+
Propanol	−	−	NT	+
butanol	−	−	NR	+
phenol	−	−	−	+
toluene	−	−	+	+
DNA G + C content (mol %) [†]	61.2	61.9*	57.3[LC]	56.6™
16S rRNA gene (%)	100	99.6	95.6	95.6

[†], determined by HPLC (LC), melting temperature (TM); [‡], optimum; *, this study.

Table 4.2 Fatty acid compositions of strain OSK2A^T and the closest relative, strain PCA^T.

Total lipid fatty acids (mol %)	OSK2A^T	PCA^T
16:1 ω7*c*	47.63	49.31
16:0	33.55	29.53
14:0	7.11	4.89
15:0 iso	3.89	5.73
16:1 ω5*c*	2.48	2.52
18:1 ω7*c*	2.24	3.95
16:0 3OH	tr	1.11
16:1 iso I	tr	tr
18:0	tr	tr
13:1 at 12-13	tr	tr
15:0 anteiso	tr	tr
18:1 ω5*c*	tr	tr
17:1 anteiso ω9*c*	tr	tr
15:0 iso 3OH	tr	tr
18:1 ω7*c* 11-methyl	tr	–
17:1 iso ω9*c*	–	tr
17:0 iso	–	tr
15:1 iso F	–	tr
13:0 iso	–	tr

Both strains were grown in 20 mM acetate and 100 mM Fe(III) citrate until early stationary phase. Fatty acid analyses were carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. Values of one or higher mol % of fatty acids are shown. tr, less than 1 mol %; -, not detected.

CHAPTER 5 GENERAL CONCLUSION OF THESIS

Methane is an odor less and colorless gas produced through methanogenesis, the decomposition of organic matter by methanogens. Only a slight fraction of methane produced in both natural and anthropogenic sources are utilized by mankind while, 90% of methane produced in paddy fields such as rice and lotus fields escaped into the atmosphere. Since methane is a radiative gas twenty times more potent than carbon dioxide, it is classified as a significant greenhouse gas (GHG) and roughly contributes 20% to the overall global warming. Paddy fields therefore constitute as major anthropogenic sources for methane emission and such environment provides conducive conditions for vast microbial communities.

Microbes are able to utilize methane as a substrate for growth by oxidizing methane to carbon dioxide thus, acts as methane sinks by preventing the direct escape of methane into the atmosphere. Paddy fields are well known sources for methane sinks particularly for methane oxidizing bacteria or methanotrophs that could utilize oxygen as the terminal electron acceptor in the oxidation of methane. Additionally, such environments are potential sites for anaerobic oxidation of methane with Fe(III) oxides.

Although stride progress has been made in discovering the occurrence of anaerobic oxidation of methane, bio-chemical mechanisms and the pathways involved in AOM has so far eluded our countless discovery attempts. Thus, current proposed pathways in AOM are based on circumstantial evidences due to absence of any isolates mediating AOM. Three AOM processes have been reported so far, the first one is AOM coupling to sulfate reduction (the most widely reported of the three) and is a major methane sink particularly in marine sediments. The second AOM process is coupled to nitrate or nitrite reduction and has been steadily discovered in polluted streams across several continents. The final and least understood AOM process is

coupled to manganese or iron oxide reduction and so far, only a valid report had been published to date, occurring in a marine sediment site but no reports for terrestrial environment to date.

Fe is the fourth most abundant element on earth and ubiquitous in soils as Fe oxides. The high concentration of Fe oxides in soils and its recyclability as a terminal electron acceptor in the decomposition of organic matter as well as thermodynamic calculations predicts their favorability for coupling to AOM. Fe(III)-reducing bacteria are the most predominant microbes in Fe(III)-reducing environments with decomposition of organic matter. These microbes contribute largely to the global cycling of metals and carbon. Some of these microbes are also capable of utilizing hydrocarbon and aromatic contaminants. One such kind of microbe belongs to the genus *Geobacter.* Since their initial discovery and establishment as a separate genus within the deltaproteobacteria, they have been found to be very versatile and widespread in numerous environments. Their versatility has given rise to their use as bacteria of choice for model studies on bioremediation and electrical generation in microbial fuel cells. In my PhD thesis, Fe(III)-reducing microbes were enriched in an anaerobic reactor inoculated with mud from lotus field and induced with methane. Fe oxide in the form of amorphous Fe(III) hydroxide was regularly supplemented at above 20 mM and samples for observation in occurrence of microbial activities were monitored over time. The aim was to cultivate Fe(III)-reducing microbes that are capable of AOM and isolate these microbes for characterization for potential use as model studies in the future.

In chapter 2, the study revealed that over the course of the microbial enrichment cultivation, heterotrophic bacteria are the most dominant and presence of Fe(III)-reducing bacteria, sulfate reducers and oxidizers were detected. Active Fe(III) reduction was also detected reaching a high of 28 mM Fe(II) and an average difference of 1.8 mM methane was measured

between the inlet and outlet methane flow. The microbial activity seen in the active Fe(III) reduction suddenly declined steadily to an all-time low of 2 mM Fe(II) accompanied with a major shift in the microbial community profile of the enrichment culture as observed by the DGGE profile. This coincided with the replacement of methane gas bottles that were used to supply methane to the enrichment culture. An abundance of aerobic microbe belonging to the *Rhodococcus* genus was detected in the same duration as observed for decline in Fe(II) production, indicating an occurrence of oxygen contamination within the anaerobic reactor. Abiotic Fe(II) production may have occurred in parallel with Fe(III) reduction since Fe(III) easily reacts with oxygen to form Fe(II) and consequently low concentration of Fe(II) observed. Although methane measurements showed an average difference in concentrations of 3 mM methane between the inlet and outlet, isotopic methane incubations in batch cultures showed no production of isotopic carbon dioxide, indicating that there is no occurrence of AOM in the batch cultures inoculated with inoculums from the enrichment culture. However, further studies are needed to characterize such microbial ecosystem induced with methane and a properly designed anaerobic reactor which caters for isotopic methane studies in the near future.

In chapter 3, isolation of a novel Fe(III)-reducing bacteria from the microbial enrichment and published in the International Journal of Systematic Microbiology (IJSEM) as *Geobacter luticola* OSK6[T] is reported. Samples for isolation of strain OSK6[T] was collected from the microbial enrichment culture reported in chapter 2 on 495 days of incubation, the same period at which highest production of Fe(II) was observed. Strain OSK6[T] was isolated in deep gellan gum tubes and purified in the six well-plate method. It couples growth from the reduction of Fe and acetate oxidation. It is one of the very few *Geobacter* species that is capable of nitrate respiration and is red and spherical in shape when grown in solid medium. The new isolate is a strictly

anaerobic, Gram-negative, motile, straight rod-shaped bacterium, 0.6–1.9 µm long and 0.2–0.4 µm wide. The growth of the isolate occurred at 20–40 °C with optima of 30–37 °C and pH 6.5–7.5 in the presence of up to 0.5 g NaCl l^{-1}. The G + C content of the genomic DNA was determined by HPLC to be 59.7 mol %. The major respiratory quinone is MK-8. The major fatty acids are 16:1 ω7*c* and 16:0. Strain OSK6^T was able to grow with Fe(III)-NTA, ferric citrate, amorphous Fe(III) hydroxide, and nitrate, but not with fumarate, malate, and sulfate as electron acceptors. Among examined substrates grown with Fe(III)-NTA, the isolate grew on acetate, lactate, pyruvate, and succinate. Analysis of the near full-length 16S rRNA gene sequence revealed that strain OSK6^T is closely related to *G. daltonii* and *G. toluenoxydans* with 95.6% similarity.

In chapter 4, a novel strain belonging to *Geobacter sulfurreducens*, named as *Geobacter sulfurreducens subsp. ethanolicus* OSK2A^T, in reference to its ability to utilize ethanol as substrate for growth in comparison to its closest relative, is reported. This is the second strain of the genus *Geobacter* to be isolated in Japanese soils from the microbial enrichment reported in chapter 2. Similar to *Geobacter luticola* OSK6^T, the novel isolate was initially isolated in deep gellan gum tubes and further purified in roll tubes with agar. It is spherical in shape and red in color when grown in solid medium. Strain OSK2A^T is Gram-stained negative, motile, rod shaped bacterium, strictly anaerobic, 0.76-1.65 µm long and 0.28-0.45 µm wide. Growth of the isolate occurred at 20-40 °C, pH 6.0-8.1 and can tolerate up to 1 % NaCl. Phylogenetic analysis of the 16S rRNA gene sequence with comparisons of 1358 positions by ARB showed 99.6% similarity to *Geobacter sulfurreducens* strain PCA^T and 95.6 % or lower similarity to other *Geobacter* species. The G+C content of the genomic DNA is 61.2 mol% and a DNA-DNA hybridization value of 60.7%. The major respiratory quinone is MK-8. The major fatty acids are 16:1 ω7*c*,

16:0, 14:0, 15:0 iso, 16:1 $\omega 5c$ and 18:1 $\omega 7c$. Strain OSK2A^T is able to utilize H$_2$, ethanol, acetate, lactate, pyruvate and formate as substrates with Fe(III)-NTA as electron acceptor. Amorphous Fe(III) hydroxide, ferric citrate, fumarate, malate and elemental sulfur are utilized as electron acceptors with either acetate or ethanol as substrates. Based on all these physiological, phylogenetic, chemotaxonomic and genotypic differentiation of strain OSK2A^T from its closest relative, the isolate is assigned as a novel subspecies of *Geobacter sulfurreducens* with the name *Geobacter sulfurreducens* subsp. *ethanolicus*, subsp. nov. was proposed. The type strain is OSK2A^T (= DSMZ 26126^T = JCM) and has been successfully published in the Journal of General and Applied Microbiology (JGAM).

The successful establishment of a microbial enrichment culture and subsequent isolation and taxonomic characterization of two novel Fe(III)-reducing isolates from the lotus field demonstrated;

1) The pragmatism of the current research as a viable approach to future studies in microbial Fe(III) reduction in terrestrial environment. I propose that such microbial enrichment in an improved anaerobic reactor is applicable for cultivation, isolation and characterization of Fe(III)-reducing bacteria which could be used for model studies on terrestrial AOM.

2) That lotus field mud is a conducive habitat for both methanogensis and Fe(III)-reducing bacteria, hence add further evidence to the widespread occurrence of *Geobacter* species in terrestrial environments.

3) An improvement to the design of the currently used anaerobic reactor, to cater for carbon isotopic studies to observe growth of microbes in presence of isotopic methane as a substrate is paramount. Suggested improvement should be made to both gas inlet and

outlet pipes located at the bottom and top end of the reactor, respectively. This is to cater for precise quantitative measurements of induced methane concentration entering and exiting the anaerobic reactor.

4) That rather than vertically positioning the anaerobic reactor, it will be more effective for sampling purposes if the anaerobic reactor was horizontally positioned for ease in sample collection and avoidance of unnecessary oxygen contamination as well as to effectively prevent breakage of the anaerobic reactor, during handling.

5) The need for addition of a stirrer bar into the anaerobic reactor to keep the homogeneity of cultures during sample collections.

In conclusion, with further improvements suggested above are made to the anaerobic reactor, laboratory investigation on the roles of Fe(III)-reducing bacteria in terrestrial environment especially their involvement in AOM, will be more effective, hence, the need to conduct such model studies will still remain a top priority in science, towards identifying and understanding the bio-chemical mechanisms involved in AOM, and also to further investigate possible alternative microbial mechanisms in Fe(III)-reduction and subsequently effective bioremediation in commercial scales.

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To the people in parts of Japan affected by the earthquake and subsequent tsunami that followed suit in March 2011, I wish you recovery as you continue to rebuild your lives from scratches.

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