A DOCTORAL DISSERTATION

Diversity and Novel Strains of Sulfur Compounds Degrading Bacteria in the Swinery Sludge

GRADUATE SCHOOL
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Department of Life Science

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Diversity and Novel Strains of Sulfur Compounds Degrading Bacteria in the Swinery Sludge

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This dissertation has been examined and approved by

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Department of Life Science
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

돈사오니에서 유황화합물의 분해 세균의 다양성과 신규 균주의 특성

지도교수 오 덕 철

김 지 영

이 논문을 이학 박사학위 논문으로 제출함

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Chapter 1. Diversity of Sulfur Compounds Degrading Bacteria

Chapter 2. Polyphasic study and Characterization of Novel Sulfur-Oxidizing Bacteria from Swinery sludge

ABBREVIATION

BH	Bushnell Hass medium
DNA	Deoxyribonucleic acid
DPG	Diphosphatidylglycerol
GC	Gas chromatography
HPLC	High performance liquid chromatography
JCM	Japan collection of microorganisms
KCTC	Korean collection for type cultures
ORF	Open reading frame
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
Q-8	Uubiquinone-8
rRNA	Ribosomal RNA
SEM	Scanning Electron Microscope
Sox	Sulfur oxidizing
SOB	Sulfur-oxidizing bacteria
TLC	Thin layer chromatography
TSA	Tryptic soy agar
TSB	Tryptic soy broth

ABSTRACT

A study on the diversity and novel strains of sulfur compounds degrading bacteria was performed as the ultimate aim for the removal of harmful sulfur compounds from a swinery in Jeju. Sulfur-oxidizing bacteria were isolated using sulfur-oxidizing bacteria (SOB) medium from swinery sludge, and bacterial distribution was studied by phylogenetic analysis of the partial 16S rRNA gene. The s*oxB* genes essential for sulfur-oxidizing were successfully amplified from some of the isolates and characterized. The characteristics of thiosulfate oxidation were investigated.

As results, the distribution of bacterial populations in the sludge collected from 5 swineries was analyzed by 16S rRNA gene sequence analysis. A total of 351 strains of sulfur-oxidizing bacteria were isolated through the enrichment culture from swinery sludge and classified into 6 groups of phyla/class *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria*, *Bacteriodetes* and *Firmicute*. They were tentatively placed into 16 orders or suborders, 23 families and 48 genera by 16S rRNA sequence analysis. The sulfur oxidizers of the *Proteobacteria* cluster, *Comamonas*, *Paracoccus* and *Pseudomonas* in the swinery sludge were the evolutionary cousins of widespread swinery sludge bacteria of the same group. The *soxB* genes were found in 13 strains, which reveal the presence of sulfur-oxidizing bacteria in the swinery sludge. Among these were 16S rRNA sequences, 7 genera, including *Acinetobacter, Alicycliphilus, Comamonas, Hydrogenophaga, Paracoccus, Pseudomonas* and *Rhodobacter*, represented the new sulfur oxidizers in swinery sludge. Phylogeny based on the amino acids sequences of *soxB* gene was created using 9 strains of *Beta-proteobacteria* and 4 strains of *Alpha-proteobacteia*. The BLAST search showed the thiosulfate-oxidizing bacteria isolated from swine sludge fell into four different

genera, *Comamonas*, *Methylibium*, *Paracoccus* and *Thiobacillus* with 80-81 % sequence similarities. The sulfate assay using a modified standard turbidimetric method revealed that strains BB11 and BB12 produced the highest amount of sulfate accumulation 175.5 and 128.2 μg/mL, respectively.

Among the isolated strains, 5 strains were considered as candidates for novel genera or novel species according to combined genotypic and phenotypic characterization. Based on the differential phenotypic and chemotaxonomic properties, together with their phylogenetic and genetic distinctiveness, strain KBB12^T was considered to be a novel genus and species of the family *Comamonadaceae*, for which the name *Thiobacterium jejuense* gen. nov., sp. nov. is proposed, and strain BA15^T was also considered a novel genus and species of the family *Rhodobacteraceae*, for which the name *Caenirhodobacter jejuensis* gen. nov. was proposed. Strains KBB4^T and KBB8^T should be considered novel species in the genus *Comamonas*, for which the name *Comamonas jejuensis* sp. nov. and *Comamonas caeni* were proposed, and strain KBB11^T should be considered novel species in the genus *Hydrogenophaga*, for which the name *Hydrogenophaga thiooxydans* sp. nov. was proposed.

The results of this study could be used as the basic data for current issues in the livestock industry, especially in excrement treatment in Jeju. Since SOB reduce pollutants in livestock manure, they could be used in swineries to clean and improve the environment quality in Jeju. Future studies should focus on the isolation of such powerful SOB from swinery sludge or other sources. The study aimed to characterize the metabolism of sulfur by SOB to sulfide or other inorganic reduced forms of sulfur and determine the role of these bacteria on the degradation of sulfur compounds for the improving environmental conditions.

BACKGROUND

Recently, the agricultural community and large-scale livestock production have changed significantly. These trends include an overall reduction in the number of farms, but an increase in intensive livestock production facilities, which are major sources of unpleasant odor in rural communities. Odor nuisance and pollutant gas emissions continue to be a major issue for the livestock and poultry industries because of their potential environmental and health effects on animals, workers, and people who live near such confined animal feeding operations (Sun *et al.*, 2010).

Swinery odors are produced primarily via incomplete fermentation of livestock manure by bacteria. Some principal odorous compounds are ammonia, amines, sulfur-containing compounds, volatile fatty acids, indoles, skatole, phenols, alcohols, and carbonyls (Mackie, 1998; Laor *et al.*, 2007). However, the odor composition can vary with the type of animal raised, the season, the stage of animal growth, the type of feed, and the sampling location. Offensive odors are a problem that can lead to public opposition of establishing new livestock facilities or expanding existing facilities. In rural areas, odor emissions from livestock operations constitute a major issue. Pollutants, such as NH_3, H_2S, and others (particulate matter, odor, and pathogens) emitted by animal production units represent risks to the health and well-being of animals, workers, neighbors, and the global environment (Elenbaas *et al.*, 2005). As a result, animal producers are facing challenges from regulatory agencies and nearby communities to reduce offensive odors and pollutant gas emissions. Various treatment technologies have been developed to reduce malodor from animal feeding facilities (Powers *et al.*, 1999; Lacey *et al.*, 2004; Ullman *et al.*, 2004), which are primarily based on one of the following principle strategies: pre-excretion management, such as

inhibition of malodor formation by animals (i.e., dietary manipulation), and post-excretion control, including the treatment of volatilized compounds (i.e., biofiltration) and the suppression of malodor emissions at the source (i.e., manure amendments, impermeable/permeable covers). Post-excretion odor management targets the emitted odorous compounds to be mitigated through their adsorption or transformation mechanisms, such as oxidation, precipitation, chemical degradation, biodegradation, etc (Pahl *et al.*, 2002; Sliwinski *et al.*, 2002).

The industrial structure of the Jeju area is mostly taken up by primary and tertiary industries; in primary industry, the livestock industry makes up a large portion of the Jeju area economy, accounting for approximately 22.2 % of the gross income of primary industry. Moreover, in 2011, due to foot-and-mouth disease, which became widespread throughout the country, the livestock industry in the Jeju area became more popular than in other areas. However, various problems have arisen because of this large share of the economy that is taken up by the livestock industry in the Jeju area. These problems, mainly caused by livestock excretions generated from the livestock industry, have led to issues with greenhouse gas emissions on the global level, damage to the clean image of the country on a national level, complaints by residents, and damage to the tourism industry because of the foul smell, and pollution of underground water in the Jeju area.

Environmental contamination by bovine manure is not considered a huge concern on the island of Jeju since the majority of cattle are allowed to graze freely and only dairy cows are housed. However, swine that are raised under enclosed farming conditions pose a major contamination threat to the environment. Therefore, innovative manure treatment technologies that are economical and environmentally friendly have recently emerged as topic of concern. Disposing of animal waste without proper handling can increase the potential for air, soil, and water pollution. In particular, swine manure

contains a high concentration of pollutants that can seriously affect not only the soil near farming grounds but also the crops growing in the vicinity. Residents of such areas have also filed an increasing number of complaints regarding the offensive odor. The potential of animal waste as renewable raw material for biomass or natural fertilizer should not be disregarded. At present, 1,500 tons of manure are sprayed as fertilizer on soil and grass, but it has been regarded to have a negative impact on the pristine Jeju environment because of the offensive odor. Currently, the main types of swine manure management include the slats manure management system and the scraper method, the former being the most widely used. In the slats treatment, all wastes are transported to a processing facility, without performing liquid-solid separation. The advantages of this method are convenience and odor reduction. Alternatively, the scraper method involves the initial separation of liquids, which are then transferred to a storage facility for distinct processing. The solids are then dried or fermented for a variety of other uses. The scraper method is becoming increasingly unpopular because it is labor-intensive and odorous.

Both natural and anthropogenic sources contribute to the total emission of hydrogen sulfide. Hydrogen sulfide occurs naturally in the gases from volcanoes, sulfur springs, undersea vents, swamps, and stagnant bodies of water in crude petroleum and natural gas and as a product of the biological degradation of organic matter (Lomans *et al.*, 2002). Considerable amounts of hydrogen sulfide are also emitted from industrial activities such as petroleum refining, pulp and paper manufacturing, wastewater treatment, food processing, livestock farming, and natural gas processing.

Major problems associated with the anaerobic treatment of sulfate and sulfite containing wastewater from treated water and biogas are corrosion and a strong unpleasant smell of the treatment. Hydrogen sulfide is a toxic, colorless, flammable gas that has a characteristic odor of rotten eggs, and its

odor threshold is about 0.00047 ppm. Physicochemical methods for it's removal from gas emissions in use today have relatively high energy requirements or high chemical and disposal cost. Biological treatment using biofilter have been proposed as a convenient alternative for treating gaseous emissions containing hydrogen sulfide and reduced sulfur compounds (Cho *et al.*, 1991; Leson and Winer, 1991; Mackie *et al.*, 1998). The concentrations of hydrogen sulfide in gas emissions are usually very dilute and traditional physical - chemical technologies such as incineration, adsorption or chemical scrubbing tend to be costly and are associated with their own pollution problems. As a result, based on the cost of the equipment and operation, biological treatment is believed to be the most economical option for the removal of hydrogen sulfide. Microbial reactions in soils have been occurring naturally for many centuries, but only since the 1950's have such techniques been used to treat waste gases. Extensive biofilter research has been conducted only in the past 60 years, thereby limiting the quanity of information.

Biofiltration is an effective technology to reduce odor, hydrogen sulfide, and ammonia emissions from livestock facilities (Nicolai and Janni, 1998; Noren, 1985). Scholtens and Demmers (1990) reported that even though biofilters are known to reduce odor, hydrogen sulfide, and ammonia, they are hardly used in intensive livestock farming in the Netherlands. The cost of treating large quantities of exhaust air demonstrated that biofilters can be cost effective if they are produced with low construction costs and an efficient design is used (Nicolai *et al.,* 1998). For a biofilter to be both effective in removing odors and low cost, the biofilter size must be optimized. Two important parameters in the optimization of biofilter designs are the airflow rate and the residence time of the air being treated. The maximum livestock building ventilation rate establishes the biofilter airflow rate. The residence time is defined as the time the air is in contact with the biofilter media. It is a function of the media

depth, cross-sectional area, and airflow rate. An indicator of the residence time is called the "empty bed contact time" (EBCT). The EBCT is determined by dividing the volume of the biofilter media bed by the airflow rate. Biofiltration is a process that utilizes microorganisms growing or immobilized on an organic porous support; the organic medium acts as a physical support for active biomass and, in some cases, provides nutrients for growth. The contaminated gaseous stream passes through the filter bed, the bed material absorbs biodegradable volatile compounds, and the microorganisms degrade it into less harmful compounds (Groenestijn and Hesselink, 1993). In the case of a hydrogen sulfide removal system, the oxidation supplies energy to the cell and produces odorless compounds. Since the actions of the microorganisms in the biofilter causes the breakdown of the odorous compounds, it is important to understand the transformations and interactions of these microorganisms. Our knowledge of microorganisms' ecosystem is fragmentary and poor (Devinny, 1999) Biofilters may be self-inoculating, inoculated with activated sludge or compost, or induced with bacteria species. Most biofilters utilized in agricultural settings use compost as the source of microorganisms.

A biofilter is a biological waste gas treatment system that provides high porosity, high nutrient availability, high moisture retention capacity and high buffering capacity to sustain microbial growth on a suitable support matrix (Rene *et al.*, 2005; Syed *et al.*, 2006). The efficiency of any biofiltration process depends on the temperature, moisture content, pH level, flow rate, surface loading rate and the physical structure of the biofilter (Hong and Park, 2005). In biofilters the most commonly used carriers are compost and peat, although some researchers have added other materials such as perlite and/or wood chips in an effort to avoid compaction of the bed (Wani *et al.*, 1999). Activated carbons have also been used to remove H_2S and these provide high performance (Chung *et al.*, 2005; Ma *et al.*, 2006). The active

carbon allows a combination of adsorption and biological degradation. The effectiveness of a biofilter relies on the activity of the microbial population and the type of enrichment performed during the inoculation step. Several bacterial strains have shown the ability to remove malodor because of H_2S. They obtain energy for growth by oxidizing sulfide components. Several bacterial species have been evaluated for their sulfide oxidation properties (Syed et al., 2006). *Thiobacillus thioparus* was used for hydrogen sulfide treatment of industrial wastewater (Kanagawa and Mikami, 1989; Cho *et al.*, 1991; Chung *et al.*, 1996; Chung *et al.*, 1996; Vlasceanu *et al.*, 1997; Qiu *et al.*, 2006). This method is reported to be inexpensive and does not produce additional pollution. *T. thioparus* can oxidize sulfide into elementary sulfur under suitable physical, chemical and operational conditions (Qiu *et al.*, 2006). This kind of bacterium is also capable of degrading other sulfur containing compounds such as methanethiol, dimethylsulfide, and dimethyldisulfide. Some other species evaluated for sulfur reduced compound removal include *T. denitrificans, T. ferrooxidans* and *T. novellas* (Cha *et al.*, 1999; Ma *et al.*, 2006). Although it is possible to establish a microbial population that has the ability to oxidize H_2S, i.e. inoculating active sludge and allowing the operation conditions of the biofilter to select microorganisms with higher degradation activity (Hirai *et al.*, 1990; Cho *et al.*, 1991), its effectiveness is limited and generally the elimination capacities that such systems exhibit are not constant (Wani *et al.*, 1997).

CHAPTER 1

Diversity of Sulfur Compounds Degrading Bacteria

1.1. Introduction

Sulfur-oxidizing bacteria (SOB) play an important role in mineral cycling in the environment. Sulfide is toxic and poses a great threat to public and environmental health (Roth *et al*., 1995). Biological sulfide oxidation is currently the most widely used process for the treatment of sulfide wastewater (Liao *et al*., 2008 Sorokin *et al*., 2008). The sulfur compounds can be used as electron acceptors or electron donors in processes known as sulfate/sulfur reduction and sulfur oxidation, respectively (Fig. 1.1). The electrons derived from sulfur oxidation are used by aerobic chemotrophic archaea and bacteria for energy transformation of the respiratory chain and for autotrophic carbon dioxide reduction (Friedrich *et al*., 2005). Anaerobic phototrophic bacteria use light energy to transfer electrons from sulfur or other sources for autotrophic carbon dioxide reduction (Frigaard and Dahl, 2009).

Aerobic sulfur oxidizing bacteria are distributed in genera such as *Acidianus* (Friedrich, 1998), *Acidithiobacillus* (Kelly and Wood, 2000), *Aquaspirillum* (Friedrich and Mitrenga, 1981), *Aquifer* (Huber and Stetter, 1999), *Bacillus* (Aragno, M., 1991), *Beggiatoa* (Strohl, 1989), *Comamonas* (Pandey *et al*., 2009), *Methylobacterium* (Kelly and Smith, 1990; Zwart *et al*., 1996), *Paracoccus*, *Pseudomonas* (Friedrich and Mitrenga, 1981), *Starkeya* (Kelly *et al*., 2000), *Sulfolobus*, *Thermithiobacillus* (Kelly and Wood, 2000), *Thiobacillus* and *Xanthobacter* (Friedrich and Mitrenga, 1981). Phototrophic anaerobic sulfur oxidizing bacteria are distributed in genera such as *Allochromatium*, *Chromatium* (Imhoff *et al*., 1998), *Chlorobium*, *Chlorobaculum* (Rodriguez *et al*., 2011), *Rhodobacter* (Shibata and Kobayashi, 2001), *Rhodopseudomonas*, *Rhodovulum*, *Roseovarius* and *Thiocapsa* (Brune, 1989).

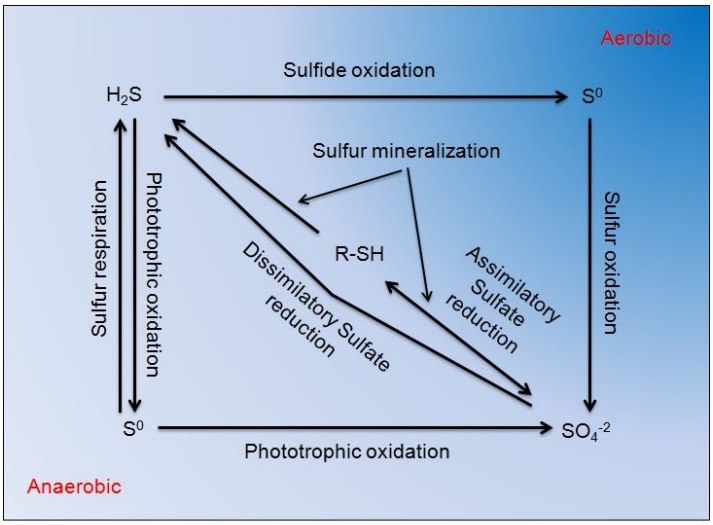

Fig. 1.1. The sulfur cycle (Raina *et al.*, 2009).

The wide taxonomic and ecological distribution of sulfur-chemolithotrophy also demonstrates the ability of various organisms to utilize different reduced sulfur compounds as chemolithotrophic substrates (Kelly *et al.*, 1997, 2000; Friedrich, 1998; Deb *et al.*, 2004; Ghosh *et al.*, 2005; Ghosh and Roy, 2007). The species-specific physiological distinctions are also related to the unequal efficiencies of energy conservation by different organisms from the same substrate, different electron transport mechanisms, and distinct substrate oxidation pathways and enzymes involved in dissimilar metabolic for sulfur (Lu and Kelly, 1988; Pronk *et al.*, 1990; Kelly *et al.*, 1997; Ghosh *et al.*, 2005; Ryu *et al.*, 2009; Masuda *et al.*, 2010). The thiosulfate is a common substrate oxidized by most sulfur-chemolithotrophs, and species distributed over the *Alpha-*, *Beta-* and *Gamma-proteobacteria* additionally utilize many other sulfur compounds including tetrathionate (Kelly *et al.*, 1997; Graff and Stubner, 2003; Ghosh *et al.*, 2005; Rohwerder and Sand, 2009; Vidyalakshmi *et al.*, 2009).

In many of these organisms, the sulfur-oxidizing (Sox) multienzyme complex is used in the oxidation of thiosulfate to sulfate (Friedrich *et al.*,

2001). Two different enzyme pathways for thiosulfate oxidation are found within these systems and different enzymes mediate the conversion of various reduced sulfur compounds (Fig. 1.2)(Kelly *et al.*, 1997; Friedrich, 1998). In the first pathway, which is found in bacteria such as the lithoautotrophic proteobacterium *Acidiphilium* and *Thiobacillus*, thiosulfate is first oxidized by a periplasmic thiosulfate dehydrogenase to tetrathionate either as the final or intermediate product (Meulenberg *et al.*, 1993; Kelly *et al.*, 1997; Sakurai *et al.*, 2010). In the second pathway, which is found in bacteria such as the facultative lithotrophic *Paracoccus pantotrophus* and the phototropic *Chlorobaculum*, thiosulfate oxidation is catalyzed by the collaboration of several periplasmic proteins, referred to as the sulfur-oxidizing system (Friedrich, 2001). The model Sox enzyme system comprises the 4 periplasmic complexes SoxXA, SoxYZ, SoxB and SoxCD that catalyze thiosulfate oxidation according to the following mechanism (Friedrich *et al.*, 2001; Hensen *et al.*, 2006; Meyer *et al.*, 2007; Weltle *et al.*, 2009).

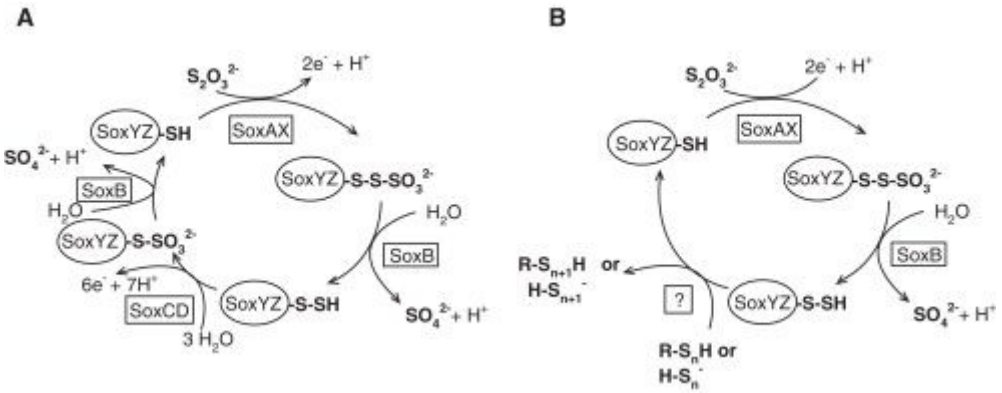

Fig. 1.2. Proposed pathway of thiosulfate oxidation catalyzed by Sox systems. (A) organisms that do not form sulfur globules en route to sulfate and (B) organisms that form sulfur globules as intermediates. All reactions take place in the periplasm (Figaard and Dahl, 2009).

This enzyme complex appears to be importance in many thiosulfate-oxidizing bacteria. In the Sox enzyme system, the functions of the monomeric, dimanganese containing proteins have been characterized (Cammack *et al.*, 1989; Epel *et al.*, 2005). First, SoxXA is a heterodimeric protein, complex oxidatively couples the sulfane sulfur of thiosulfate to a SoxY-cysteine-sulfhydryl group of the SoxYZ complex from which the terminal sulfone group is subsequently released by the activity of the SoxB component (Quentmeier and Friedrich, 2001; Ogawa *et al.*, 2008). Subsequently, the sulfur of the residual SoxY-cysteine persulfide is further oxidized to cysteine-S-sulfate by the SoxCD sulfur dehydrogenase complex from which the sulfonate moiety is again hydrolyzed off by SoxB, thereby restoring SoxYZ each of the previous proteins alone is catalytically inactive (Friedrich *et al.*, 2001; 2005) (Fig. 1.2). The soxB, an essential component of the bacterial Sox sulfur oxidation pathway (Schneider and Friedrich, 1994; Sauveet *et al.*, 2009), the thiosulfate-oxidizing multi-enzyme complex, that contains a prosthetic manganese cluster, and a dimer manganese(II) site in the reaction center, and is proposed to catalyze the release of sulfate from a protein-bound cysteine-S-thiosulfonate (Cammack *et al.*, 1989; Quentmeier and Friedrich, 2001). The previously published studies used polymerase chain reaction (PCR) (Petri *et al.*, 2001) to investigate the *soxB* distribution among different photo- and chemotrophically sulfur-oxidizing bacteria (SOB) strains considering thiosulfate-oxidizing, sulfur-storing species. The *soxB* gene is functional in all known thiosulfate-oxidizing and phototeophic SOB species, and likely in species that have not yet been reported to use sulfur compounds as electron (Mayer *et al.*, 2007).

Accordingly, this study investigated the bacterial diversity of thiosulfate-oxidizing bacteria strains from swinery sludge, and the possible modes of thiosulfate oxidation by these organisms and the phylogenetic distribution of the sulfur-oxidizing B gene (*soxB*).

1.2. Material and Methods

1.2.1. Sampling site

The swineries investigated in this study were selected in terms of the ventilation type. The 2 ventilation modes of the swined in Korea are 1) mechanical ventilation by wall exhaust fans and 2) natural ventilation by the operation of a winch-curtain. Generally, confinement style buildings for swine were mechanically ventilated and open style buildings for swine are naturally ventilated. Samples were collected from the swinery of natural ventilation type in A and B site, and mechanical ventilation type in C, D and E site (Table1.1, Fig. 1.3).

Table 1.1. The swinery structure of the sampling site

Site	Ventilation type	Excretion processing	Sampling date
A	Natural ventilation	Slats	July 10, 2010
B			July 27, 2012
C	Mechanical ventilation		June 30, 2011
D			August 10, 2011
E			August 10, 2010

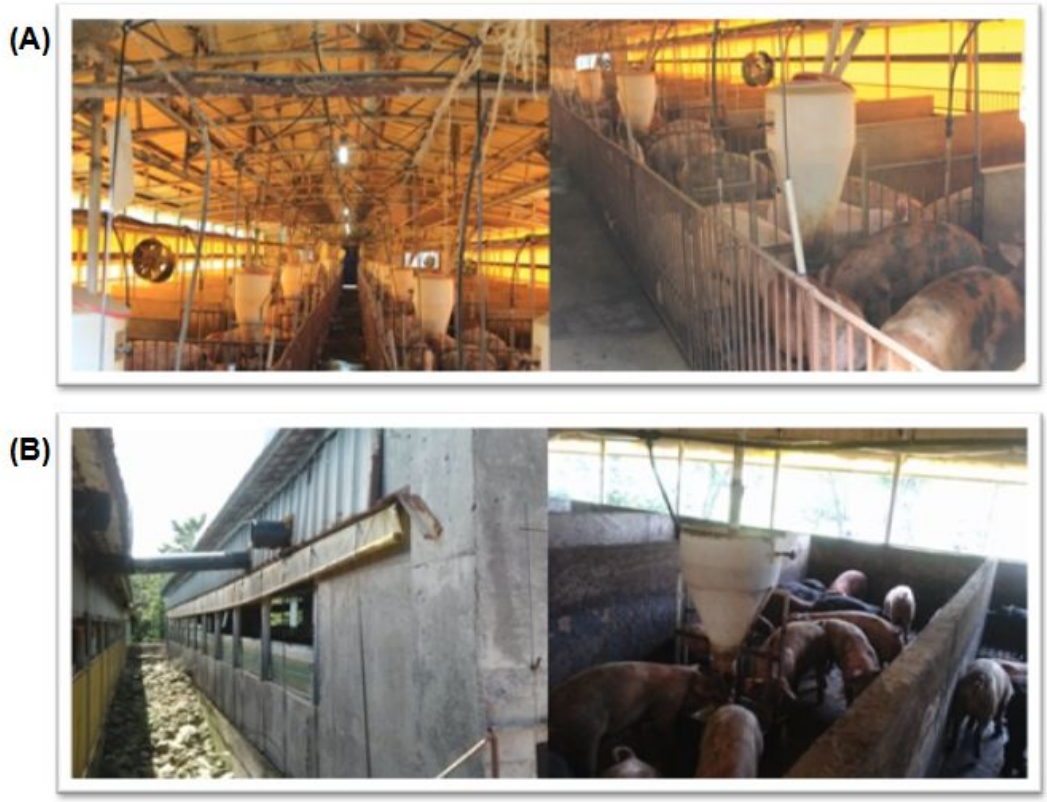

Fig. 1.3. The structural view of swinery sludge sampling sites in Jeju. (A) Mechanical ventilation and (B) Natural ventilation type.

1.2.2. Enrichment culture and isolation of bacteria

Swinery sludge was used as the inoculums for enrichment cultures. Mineral media used for enrichment, isolation and cultivation were Sulfur-Oxidizing Bacteria medium (SOB; Na_2HPO_4 3.0 g/L, KH_2PO_4 1.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, NH_4Cl_2 0.4 g/L, $CaCl_2$ 0.2 g/L, $FeCl_3$ 0.01 g/L, $NaHCO_3$ 0.2 g/L and $Na_2S_2O_3$ 4.0 g/L) and Bushnell Hass (BH) medium (0.409 g/L $MgSO_4 \cdot 7H_2O$, 0.0265 g/L $CaCl_2 \cdot 2H_2O$, 1 g/L KH_2PO_4, 1 g/L NH_4NO_3, 6 g/L $Na_2HPO_4 \cdot 12H_2O$, and 0.0833 g/L $FeCl_3 \cdot 6H_2O$) and with 50 $\mu\ell$ trace elements (17 g/L $FeCl_3$, 0.6 g/L $CaCl_2$, 0.2 g/L $ZnSO_4$, 0.2 g/L $CuSO_4 \cdot 7H2O$, 0.2 g/L $MnSO_4$, 0.8 g/L $CoCl_2$, 0.1 g/L H_3BO_3, and 0.3 g/L $Na_2MoO_4 \cdot 2H_2O$) (Bushnell and, Haas, 1941). Enrichment of SOB and BH media was conducted using a liquid medium on a rotary shaker at 200 rpm at 30 °C. After 2 weeks, for the isolation and purification, solid medium was prepared and 0.2 mL aliquots of the enrichment cultures were transferred onto the solid medium. The plates were incubated in at 30 °C. The colonies formed on the agar plates of SOB and BH media were transferred at least three times to obtain pure strains.

1.2.3. DNA extraction

The bacterial DNA was extracted using the following protocol (Murray and Thompson, 1980). Each bacterium was inoculated with 10 mL of the above liquid medium. Cell cultures were transferred into microtube, centrifuged at 13,000 xg for 5 min and the supernatant liquid was discarded. The pellet was resuspended in 50 mM Tris-HCl/50 mM EDTA (TE) buffer, then 100 mg/mL lysozyme was added and incubated for 5 min. Next, 10 % SDS and 100 μg/mL proteinase K were added and the mixture was incubated for 1 hr at 37 °C. The solution of 5 M NaCl and CTAB/NaCl were added and incubated for

10 min at 65 ℃. DNA was extracted with equal volumes of Phenol/Chloroform/Isoamyl-alcohol (25:24:1) and centrifuged at 13,000 xg for 10 min. The aqueous phase was extracted with chloroform/isoamyl alcohol (24:1) and centrifuged at 13,000 xg for 10 min. The supernatant was removed to a fresh tube, 0.6 volumes of isopropanol was added to precipitate the nucleic acids and centrifuged at 13,000 xg for 5 min. The pellet was washed with 70 % ethanol, recovered by centrifuge at 13,000 xg for 5 min. Then the precipitated DNA was then dried and dissolved in TE buffer. The suspension was used as the DNA template for subsequent PCR amplification.

1.2.4. PCR amplification and sequencing of the 16S rRNA and *soxB* gene

The 16S rRNA gene was amplified by PCR with universal eubacterial primers 27F and 1522R (Table 1.2) in Thermal Cycler PTC 1000 (BIO-RAD, USA). The temperature program was as follows: denaturation at 94 ℃ for 5 min, annealing at 50 ℃ (Actinobacteria) and 55 ℃ (Bacteria) for 1 min, and extension at 72 ℃ for 1 min. The final cycle included an extension of 72 ℃ for 10 min to ensure full extension of the products. The PCR products were assayed by electrophoresis on a 1 % (w/v) agarose gel, stained with RedSafeTM (iNtRON, Korea), and visualized by a UV transilluminator. The PCR products were purified with Fragment DNA Purification (iNtRON, USA), according to the manufacturer's instructions.

Aamplification of the *soxB* gene fragment was performed using the primer set and PCR protocols desdcribed by Petri *et al.* (2001) (Table 1.2). The amplification program was as follows: one cycle comprising 94 ℃ for 2 min, followed by 10 cycles consisting of denaturation (94 ℃ for 30 s), annealing (55 ℃ for 40 s) and extension (72 ℃ for 30 s). Thereafter, 25 additional

cycles were performed at an annealing temperature of 47 ℃ and a final extension step consisting of 72 ℃ for 6 min.

The purified PCR products were ligated into a TOPO vector (Invitrogen, USA). The ligated products were transformed into TOP10-competent *Escherichia coli* JM109 cells (Invitrogen, USA). The recombinant plasmids DNA were extracted from the cloned cells and purified with a Wizard Plus Minipreps DNA purification system (Promega, USA). The 16S rRNA gene plasmid DNA were used as a template for sequencing. ABIPRISM Dye Terminator Cycle sequencing kits (Applied Biosystems, USA) were used, according to the manufacturer's instructions. The sequences were determined by an automated DNA sequencer (ABI Prism model 3730, Applied Biosystems, USA).

Table 1.2. Oligonucleotide sequences of primer used in this study

Primer	Sequence	Reference
27F	5'-AGA GTT TGA TCC TGG CTC AG-3'	Lane, 1991
1522R	5'-AAG GAG GTG ATC CA(AG) CCG CA-3'	Lane, 1991
soxB693FK39	5`-ATC GGN CAG GCC TTY CCN TA-3`	Petri *et al.*, 2001
soxB1446BK42	5`-CAT GTC SCC DCC BTG YTG-3`	Petri *et al.*, 2001
T7	5`-TAA TAC GAC TCA CTA TAG GG-3`	Invitrogen
M13R	5`-AGG AAA CAG CTA TGA CCA T-3`	Invitrogen

1.2.5. Phylogenetic analysis

The partial sequences of the 16S rRNA gene were aligned with the closest relative strains available in the GenBank database by using the BLAST program (nucleotide blast; http://www.ncbi.nlm.nih.gov/blast/). Sequence similarity values were computed using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007).

The soxB nucleotide sequences were translated into amino acid sequences, and deduced amino acid sequence data were compared to available data in the GenBank databases by using the BLAST program (tblastn; http://www.ncbi.nlm.nih.gov/blast).

The alignments were performed by CLUSTAL X 1.83 (Thompson *et al.*, 1997), and gaps were edited in BioEdit software (Hall, 1999). The phylogenetic trees were constructed based on the neighbor-joining method (Saitou and Nei, 1987) in MEGA 5 program (Kumar *et al.*, 2001). The resulting tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1,000 resampled data sets.

1.2.6. Thiosulfate oxidation

The thiosulfate oxidation was examined in a mineral salts medium that contained (g/L) $Na_2S_2O_3 \cdot 5H_2O$, 5.0; K_2HPO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 2.5; NH_4Cl, 1.0; bromocresol purple, 0.002; pH 7.5. The plates were incubated at 30 °C for 10 days and the experiments were replicated three times. The change in color from purple to yellow was indication of thiosulfate oxidation due to the production of sulfuric acid from thiosulfate. Additionally, control plates without thiosulfate were maintained (Anandham *et al*., 2009).

The thiosulfate determination in the culture supernatant was estimated turbidometrically (Kolmert *et al*., 2000). The 5 % $BaCl_2$ was added to the culture supernatant and the solution was mixed for 60 s at a constant speed. The absorbance of the resulting suspension at 420 nm was then determined. The standard sulfate solution were made by dissolving $Na2_2O_4$ in deionized water. The calibration curve obtained using standard solutions was fitted with a third degree polynomial curve over the range of 0-5 mM sulfate.

A batch culture experiment was performed to examine whether thiosulfate was oxidized directly into sulfate or accumulated as intermediate compounds. The bacterial strains were grown in 100 mL of SOB medium supplemented with a 0.1 % yeast extract to increase the cell yield in 250 mL Erlenmeyer flasks at 30 °C in a shaking incubator (120 rpm) for 3 days. Separate flasks were taken for analysis at the time intervals of 6, 12, 18, 24, 30, 36, 48, 60, 72, 84 and 96 h. The turbidity of the culture was measured with a UV spectrophotometer (Themer, USA) at an absorbance at 600 nm.

1.2.7. Thiosulfate oxidizing enzymes

The bacterial strains cultivated in SOB medium in a shaking incubator operating at 120 rpm for 7 days were harvested by centrifugation at 10,000 xg for 20 min. The harvested cells were washed and resuspended in 10 mM potassium phosphate buffer (pH 7.0) and disrupted by sonication using a sonopuls SM 2070 sonicator (Brandelin, Germary) at 20 kHz for 10 min at 0 °C. The cell debris was removed by centrifugation at 10,000 xg for 20 min at 4 °C. The clear supernatant were used as the cell-free extracts for the enzyme assay. The protein concentrations were determined by the standard method (Bradford, 1976).

The thiosulfate oxidase enzyme assay was based on that of Trudinger (1961) in which the ferricyanide reduction was measured by using the spectrophotometer at 420 nm. The reaction mixture was in a total volume of 1 mL in a 1 cm cuvette contained phosphate buffer pH 7.0 (100 mM), $Na_2S_2O_3$ (10 mM) and $K_3Fe(CN)_6$ (1 mM). The reaction was started by the addition of cell-free extract (500 ug/mL) and substrate. Decrease in absorbance at 420 nm was recorded and ferricyanide reduction was measured by using an extinction coefficient of 1.0 mM^{-1} cm^{-1}. The enzyme activity was expressed as nM ferricyanide reduced/min/mg protein.

The sulfite oxidase was assayed by the method described by Charles and Suzuki (1966), with slight modifications. The reaction was measured in a reaction mixture (1 mL) with slight modifications, containing 1 mL of Tris buffer (100 mM) and 25 mM EDTA, 0.1 mL of potassium ferricyanide (1 mM), Na_2SO_3 (1 mM) dissolved in 25 mM EDTA and cell-free extract (500 ug/mL). The reaction was initiated by adding sulfite, and decrease in absorbance at 420 nm was recorded by using buffer plus water as a blank. Ferricyanide reduction was measured by using an extinction coefficient of 1.0 mM^{-1} cm^{-1}.

1.3. Results

1.3.1. Isolation and phylogenetic analysis of sulfur-oxidizing bacteria from swinery sludge of site A

A total of 102 strains were isolated from sulfur oxidizing enrichment culture of the swinery sludge of site A. They were partially identified as 24 different genera and 41 species by 16S rRNA sequence analysis and were classified into 5 groups (phyla/class of *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria* and *Firmicutes*). While a substantial portion of the isolates belonged to *Gamma-proteobacteria*, (38 %), *Beta-proteobacteria* (28 %), *Alpha-proteobacteria* (15 %) and *Actinobacteria* (13 %), a few of isolates were affiliated with *Fimicutes* (6 %) (Fig. 1.4).

The 16S rRNA gene sequence of the isolated bacteria showed close similarity with reference sequences of the EzTaxon server database (http://www.EzTaxon.org/; Chun *et al*., 2007) (Table 1.3)

Most strains belonged to the phylum *Proteobacteria*, mainly to class *Gamma-proteobacteria*, and they were classified into different families: *Aeromonadaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* and *Moraxellaceae*. Of the 38 isolates, 14 isolates showed 99-100 % sequences similarity to *Stenotrophomonas* sp., 13 isolates exhibited 99-100 % to *Pseudomonas* sp. and 9 isolates exhibited 99-100 % to *Acinetobacter* sp.. The isolate SS47 showed a 97.4 % sequence similarity to *Zobellella* sp. that the isolate should be assigned to a novel species(Table 1.3).

The 29 isolates belonging to the phylum *Beta-proteobacteria* fell into the 3 genera, *Alcaligenes*, *Pusillimonas* and *Thauera* (Table 1.3). Twenty-five isolates showed 99-100 % sequences similarity to *Thauera* sp. being the

dominant species, 2 isolates showed 99-100 % to *Alcaligenes* sp. and 1 isolate showed 99-100 % to *Pusillimonas* sp.

The 15 isolates belonged to the phylum *Alpha-proteobacteria* fell into the 4 genera: *Aquamicrobium*, *Brevundmonas*, *Paenochrobactrum* and *Paracoccus* (Table 1.3). The 10 isolates showed 99-100 % sequence similarity to *Brevundmonas* sp., 2 isolates showed 99-100 % to *Aquamicrobium* sp., 2isolates showed 99-100 % to *Paenochrobactrum* sp. and 1 isolate showed 99-100 % to *Paracoccus* sp.

The 13 isolates belonging to genera *Arthrobacter*, *Brevibacterium*, *Dietzia*, *Leucobacter*, *Microbacterium*, *Streptomyces* and *Rhodococcus* were members within the phylum *Actinobacteria*. The remainder of 6 isolate, *Firmicutes* sequences were isolated bacteria, into the genera *Bacillus*, *Chryseomicrobium*, *Brevibacillus* and *Lysinibacillus* (Table 1.3).

Consequently 102 strains belonging to 24 different genera formed 5 taxonomic groups according to phyla, of which 6 strains proved to be candidates for novel taxa.

Table 1.3. Closest bacterial species to the bacterial strains isolated from site A Compared by 16S rRNA gene sequence similarity.

Strain	Phylogenetic group	Closest species	Similarity (%)
SB2	*Actinobacteria*	*Arthrobacter arilaitensis* CIP 108037^T	99.7
SS46		*Brevibacterium epidermidis* NCDO 2286^T	99.5
SS48		*Brevibacterium epidermidis* NCDO 2286^T	99.6
SB24		*Dietzia cercidiphylli* YIM 65002^T	99.8
SB19		*Leucobacter aridicollis* CIP 108388^T	98.9
SB22		*Leucobacter aridicollis* CIP 108388^T	99.3
SS43		*Microbacterium oxydans* DSM 20578^T	99.7
SS19		*Microbacteriumparaoxydans* CF36^T	99.2
SS49		*Microbacterium phyllosphaerae* DSM 13468^T	99.8
SB3		*Streptomyces violaceochromogenes* NBRC 13100^T	99.0
SS6		*Rhodococcus equi* DSM 20307^T	99.6
SS4		*Rhodococcus gordoniae* W4937^T	99.6
SB23		*Rhodococcus qingshengii* djl-6^T	99.8
SB46	*Firmicutes*	*Bacillus infantis* SMC 4352-1^T	99.4
SB21		*Chryseomicrobium imtechense* MW 10^T	99.3
SS27		*Brevibacillus agri* NRRL NRS-1219^T	99.3
SS42		*Brevibacillus brevis* NBRC 15304^T	99.7
SS14		*Lysinibacillus xylanilyticus* XDB9^T	98.5
SS13		*Lysinibacillus xylanilyticus* XDB9^T	99.2
SS40	*α - proteobacteria*	*Aquamicrobium defluvii* DSM 11603^T	100
SS21		*Brevundimonas bullata* IAM 13153^T	100
SS24		*Brevundimonas bullata* IAM 13153^T	99.2
SS26		*Brevundimonas bullata* IAM 13153^T	99.3
SS22		*Brevundimonas bullata* IAM 13153^T	99.7
SS23		*Brevundimonas bullata* IAM 13153^T	99.7
SS25		*Brevundimonas bullata* IAM 13153^T	99.7
SS28		*Brevundimonas naejangsanensis* BIO-TAS2-2^T	100
SS29		*Brevundimonas naejangsanensis* BIO-TAS2-2^T	98.4
SB28		*Brevundimonas naejangsanensis* BIO-TAS2-2^T	98.8
SB30		*Brevundimonas naejangsanensis* BIO-TAS2-2^T	98.9
SB20		*Paenochrobactrum gallinarii* Sa25^T	100
SS20		*Paenochrobactrum glaciei* Pi26^T	99.8
SB32		*Paracoccus denitrificans* DSM 413^T	99.0
SS33		*Paracoccus denitrificans* DSM 413^T	99.0
SS30	*β - proteobacteria*	*Alcaligenes aquatilis* LMG 22996^T	99.5
SB31		*Alcaligenes aquatilis* LMG 22996^T	99.6
SS18		*Pusillimonas noertemannii* BN9^T	98.9
SB47		*Thauera butanivorans* Bu-B1211^T	100
SS34		*Thauera butanivorans* Bu-B1211^T	99.4

Table 1.3. Continued

Strain	Phylogenetic group	Closest species	Similarity (%)
SS50	*β - proteobacteria*	*Thauera butanivorans* Bu-B1211^T	99.4
SS32		*Thauera butanivorans* Bu-B1211^T	99.6
SS35		*Thauera butanivorans* Bu-B1211^T	99.7
SS36		*Thauera butanivorans* Bu-B1211^T	99.7
SS37		*Thauera butanivorans* Bu-B1211^T	99.7
SS38		*Thauera butanivorans* Bu-B1211^T	99.7
SS39		*Thauera butanivorans* Bu-B1211^T	99.7
SS41		*Thauera butanivorans* Bu-B1211^T	99.7
SB44		*Thauera butanivorans* Bu-B1211^T	99.7
SB45		*Thauera butanivorans* Bu-B1211^T	99.7
SB33		*Thauera butanivorans* Bu-B1211^T	99.8
SB34		*Thauera butanivorans* Bu-B1211^T	99.8
SB35		*Thauera butanivorans* Bu-B1211^T	99.8
SB36		*Thauera butanivorans* Bu-B1211^T	99.8
SB37		*Thauera butanivorans* Bu-B1211^T	99.8
SB38		*Thauera butanivorans* Bu-B1211^T	99.8
SB39		*Thauera butanivorans* Bu-B1211^T	99.8
SB40		*Thauera butanivorans* Bu-B1211^T	99.8
SB41		*Thauera butanivorans* Bu-B1211^T	99.8
SB42		*Thauera butanivorans* Bu-B1211^T	99.8
SB43		*Thauera butanivorans* Bu-B1211^T	99.8
SS45		*Thauera butanivorans* Bu-B1211^T	99.8
SB48		*Thauera butanivorans* Bu-B1211^T	99.8
SB49		*Thauera butanivorans* Bu-B1211^T	99.8
SB17	*γ - proteobacteria*	*Acinetobacter johnsonii* DSM 6963^T	99.1
SB16		*Acinetobacter lwoffii* DSM 2403^T	98.0
SS15		*Acinetobacter lwoffii* DSM 2403^T	98.1
SS9		*Acinetobacter lwoffii* DSM 2403^T	98.4
SB14		*Acinetobacter lwoffii* DSM 2403^T	98.8
SS31		*Acinetobacter towneri* AB1110^T	97.7
SB27		*Acinetobacter towneri* AB1110^T	98.2
SS51		*Acinetobacter venetianus* RAG-1^T	99.1
SS44		*Acinetobacter venetianus* RAG-1^T	99.4
SS53		*Pseudomonas alcaligenes* LMG 1224^T	97.5
SB25		*Pseudomonas caeni* HY-14^T	99.8
SB5		*Pseudomonas corrugata* ATCC 29736^T	99.1
SS52		*Pseudomonas gessardii* CIP 105469^T	98.7
SS8		*Pseudomonas kilonensis* 520-20^T	99.1
SB1		*Pseudomonas koreensis* Ps 9-14^T	100
SB8		*Pseudomonas koreensis* Ps 9-14^T	100
SS5		*Pseudomonas koreensis* Ps 9-14^T	99.8
SS1		*Pseudomonas mohnii* Ipa-2^T	99.2

Table 1.3. Continued

Strain	Phylogenetic group	Closest species	Similarity (%)
SB4	γ - *proteobacteria*	*Pseudomonas mohnii* Ipa-2^T	99.2
SS7		*Pseudomonas mohnii* Ipa-2^T	99.4
SB6		*Pseudomonas mohnii* Ipa-2^T	99.4
SB13		*Pseudomonas taiwanensis* BCRC 17751^T	98.4
SB9		*Psychrobacter maritimus* Pi2-20^T	99.7
SB11		*Psychrobacter maritimus* Pi2-20^T	99.7
SB18		*Stenotrophomonas rhizophila* e-p10^T	98.9
SB15		*Stenotrophomonas rhizophila* e-p10^T	99
SS16		*Stenotrophomonas rhizophila* e-p10^T	99.1
SB10		*Stenotrophomonas rhizophila* e-p10^T	99.1
SS2		*Stenotrophomonas rhizophila* e-p10^T	99.3
SS11		*Stenotrophomonas rhizophila* e-p10^T	99.3
SS17		*Stenotrophomonas rhizophila* e-p10^T	99.4
SS10		*Stenotrophomonas rhizophila* e-p10^T	99.6
SS3		*Stenotrophomonas rhizophila* e-p10^T	99.8
SB7		*Stenotrophomonas terrae* R-32768^T	100
SB12		*Stenotrophomonas terrae* R-32768^T	100
SB26		*Stenotrophomonas terrae* R-32768^T	99.3
SS12		*Stenotrophomonas terrae* R-32768^T	99.6
SB29		*Stenotrophomonas terrae* R-32768^T	99.6
SS47		*Zobellella taiwanensis* ZT1^T	97.4

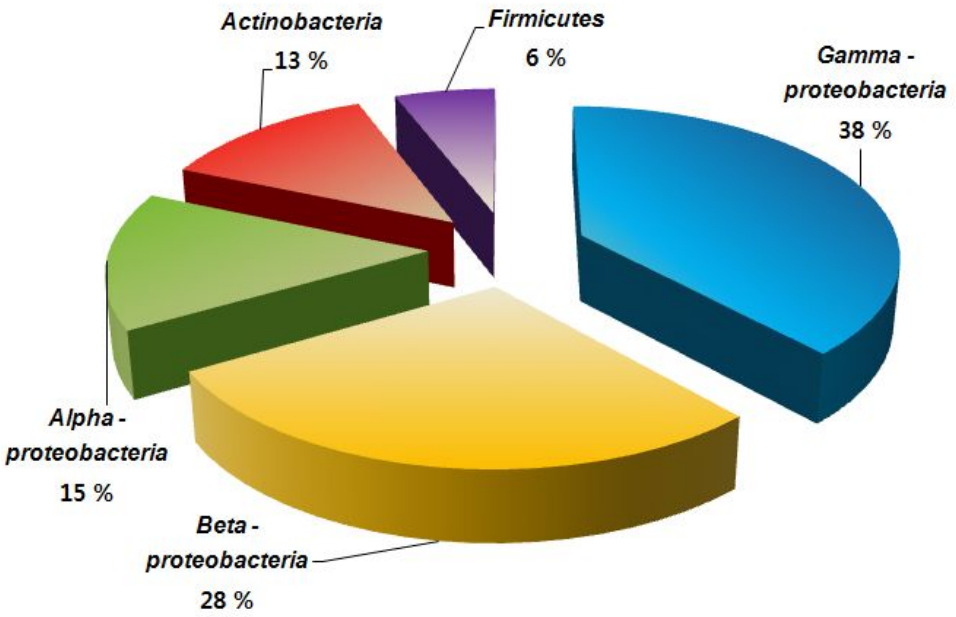

Fig. 1.4. Composition of bacterial community of swinery sludge site A in the level of the phylum/class.

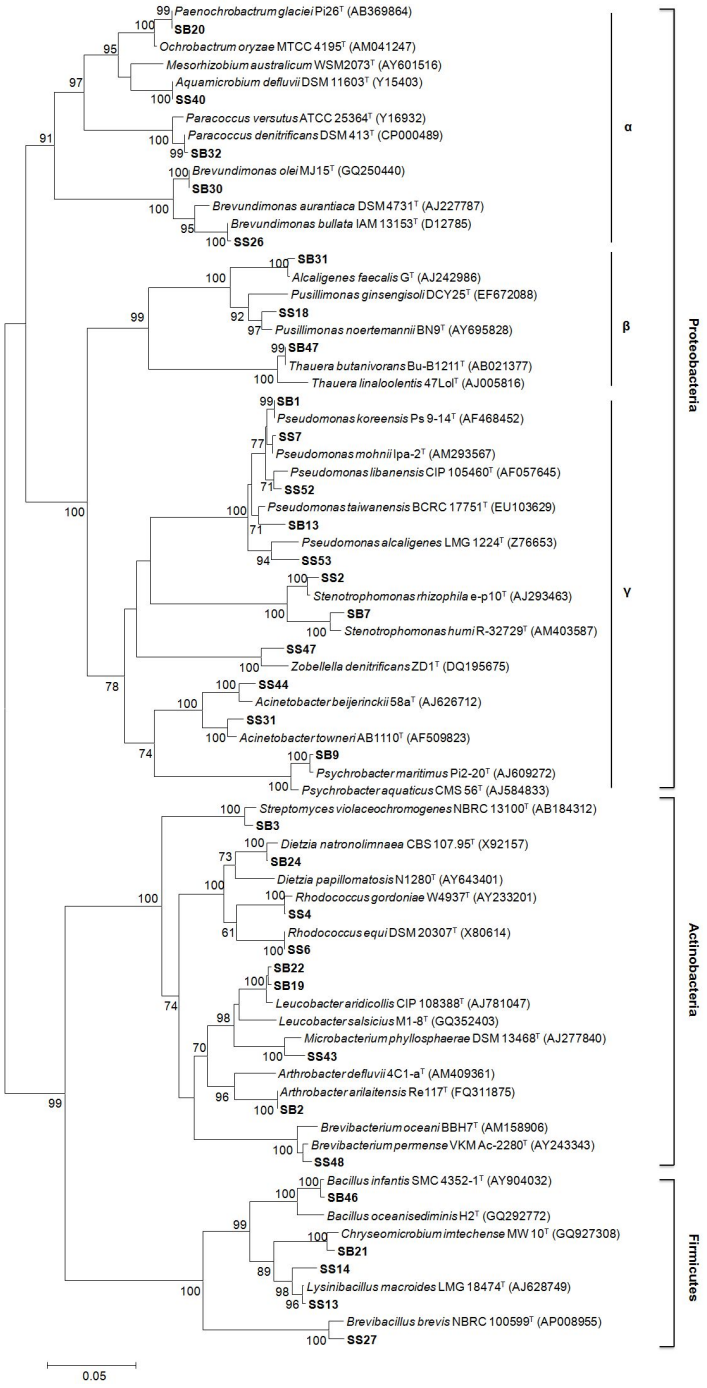

Fig. 1.5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria from site A. Bootstrap percentages (based on 1000 replicates) greater than 70 % are shown at branching points. Bar, 0.05 substitutions per nucleotide position.

1.3.2. Isolation and phylogenetic analysis of sulfur-oxidizing bacteria from swinery sludge of site B

A total of 54 strains were isolated from sulfur oxidizing enrichment culture of the swinery sludge of site B. They were partially identified as 12 different genera and 26 species by 16S rRNA gene sequence analysis and were classified into 5 groups (*Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria* and *Firmicutes*). While a substantial portion of the isolates belonged to *Firmicutes* (43 %), *Actinobacteria* (18 %), *Beta-proteobacteria* (17 %) and *Gamma-proteobacteria* (15 %), a few isolates were members of the *Alpha-proteobacteria* (7 %) (Fig. 1.6).

The 16S rRNA gene sequence of the isolated bacteria showed close similarity with reference sequences of the EzTaxon server database (http://www.EzTaxon.org/; Chun *et al.*, 2007) (Table 1.4)

Most strains belonging to the phylum *Firmicutes* were distributed into the families *Bacillaceae* and *Paenibacillaceae* of the class *Bacilli*. Among 23 isolates, 9 isolates showed 99-100 % sequence similarity to *Paenibacillus* sp., 6 isolates exhibited 99-100 % to *Lysinibacillus* sp., 4 isolates exhibited 99-100 % to *Bacillus* sp., 2 isolates exhibited 99-100 % to *Brevibacillus* sp. and 2 isolates exhibited 99-100 % to *Cohnella* sp. (Table 1.4).

The 8 isolates belonging to the phylum *Actinobacteria* fell into the genus *Rhodococcus* showed 99-100 % similarity to the dominant species.

The 9 isolates belonging to the phylum *Beta-proteobacteria* fell into 2 genera, *Bordetella* and *Achromobacter* (Table 1.4). The 8 isolates showed 99-100 % sequence similarity to *Bordetella* sp. to being the dominant species and 1 isolates showed 99-100 % to *Achromobacter* sp..

The 8 isolates belonging to genera *Enterobacter*, *Escherichia* and *Shigella* are the members of the phylum *Gamma-proteobacteria*. The remaining 4

isolate, *Alpha-proteobacteria* sequences were isolated bacteria, 1 genera *Brevundimonas* showed 99-100 % similarity to the dominant species (Table 1.4).

Consequently, 54 strains belonging to 12 different genera formed 5 taxonomic groups according to the phyla and the phylogenetic tree based on 16S rRNA gene sequences showed *Proteobacteria* and other bacteria (Fig. 1.7).

Table 1.4. Closest bacterial species to the bacterial strains isolated from site B Compared by 16S rRNA gene sequence similarity.

Strain	Phylogenetic group	Closest species	Similarity (%)
HS4	*Actinobacteria*	*Rhodococcus gordoniae* W4937^T	100
HS5		*Rhodococcus gordoniae* W4937^T	100
HS6		*Rhodococcus gordoniae* W4937^T	100
HS8		*Rhodococcus rhodochrous* DSM 43241^T	100
HS10		*Rhodococcus equi* DSM 20307^T	100
HS14		*Rhodococcus equi* DSM 20307^T	100
HS17		*Rhodococcus kunmingensis* YIM 45607^T	100
HS20		*Rhodococcus kunmingensis* YIM 45607^T	100
HB20		*Rhodococcus zopfii* DSM 44108^T	100
HB24		*Rhodococcus zopfii* DSM 44108^T	100
HB21	*Firmicutes*	*Bacillus cereus* ATCC 14579^T	100
HB12		*Bacillus circulans* ATCC 4513^T	100
HS13		Bacillus *oceanisediminis* H2^T	96.7
HS16		Bacillus *oceanisediminis* H2^T	99.1
HB11		*Brevibacillus choshinensis* DSM 8552^T	99.8
HB10		*Brevibacillus choshinensis* DSM 8552^T	99.8
HB22		*Cohnella phaseoli* GSPC1^T	99.8
HS12		*Cohnella phaseoli* GSPC1^T	99.8
HB26		*Lysinibacillus macroides* LMG 18474^T	99.7
HS19		*Lysinibacillus macroides* LMG 18474^T	99.0
HS26		*Lysinibacillus macroides* LMG 18474^T	99.0
HB1		*Lysinibacillus sphaericus* C3-41^T	98.3
HB6		*Lysinibacillus sphaericus* C3-41^T	98.6
HB19		*Lysinibacillus xylanilyticus* XDB9^T	100.0
HB18		*Paenibacillus chibensis* JCM 9905^T	100.0
HS22		*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.7
HS25		*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.6
HB16		*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.5
HB9		*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.7
HS21		*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.7
HS24		*Paenibacillus pabuli* JCM 9074^T	99.5
HB8		*Paenibacillus wynnii* LMG 22176^T	98.0
HB17		*Paenibacillus wynnii* LMG 22176^T	98.5
HS23	*α - proteobacteria*	*Brevundimonas naejangsanensis* BIO-TAS2-2^T	99.8
HS1		*Brevundimonas naejangsanensis* BIO-TAS2-2^T	100
HB3		*Brevundimonas intermedia* ATCC 15262^T	100
HB7		*Brevundimonas aurantiaca* DSM 4731^T	100.0
HB4	*β - proteobacteria*	*Bordetella parapertussis* 12822^T	99.4
HS7		*Bordetella hinzii* LMG 13501^T	100
HS18		*Bordetella avium* ATCC 35086^T	100

Table 1.4. Continued

Strain	Phylogenetic group	Closest species	Similarity (%)
HS27	*β - proteobacteria*	*Bordetella parapertussis* 12822^T	98.3
HS28		*Bordetella parapertussis* 12822^T	100
HS29		*Bordetella parapertussis* 12822^T	100
HB14		*Bordetella parapertussis* 12822^T	100
HB25		*Bordetella parapertussis* 12822^T	100
HB2		*Achromobacter denitrificans* DSM 30026^T	100.0
HS2	*γ - proteobacteria*	*Escherichia fergusonii* ATCC 35469^T	99.8
HS3		*Enterobacter gergoviae* JCM 1234T^T	99.8
HS11		*Escherichia fergusonii* ATCC 35469^T	99.8
HS15		*Escherichia fergusonii* ATCC 35469^T	99.8
HB5		*Escherichia fergusonii* ATCC 35469^T	99.8
HB15		*Escherichia fergusonii* ATCC 35469^T	99.8
HB23		*Escherichia fergusonii* ATCC 35469^T	99.8
HB13		*Shigella sonnei* GTC 781^T	100.0

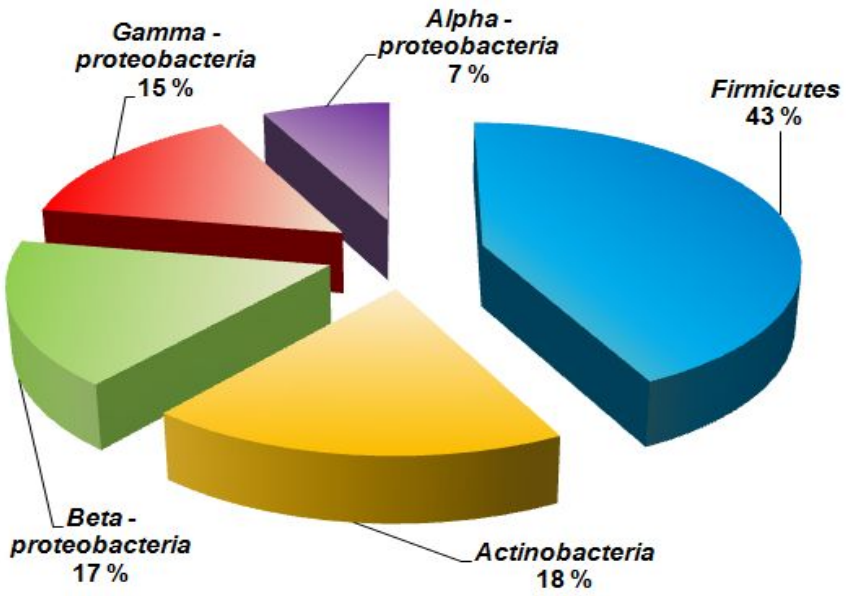

Fig. 1.6. Composition of bacterial community of swinery sludge site B in the level of the phylum/class.

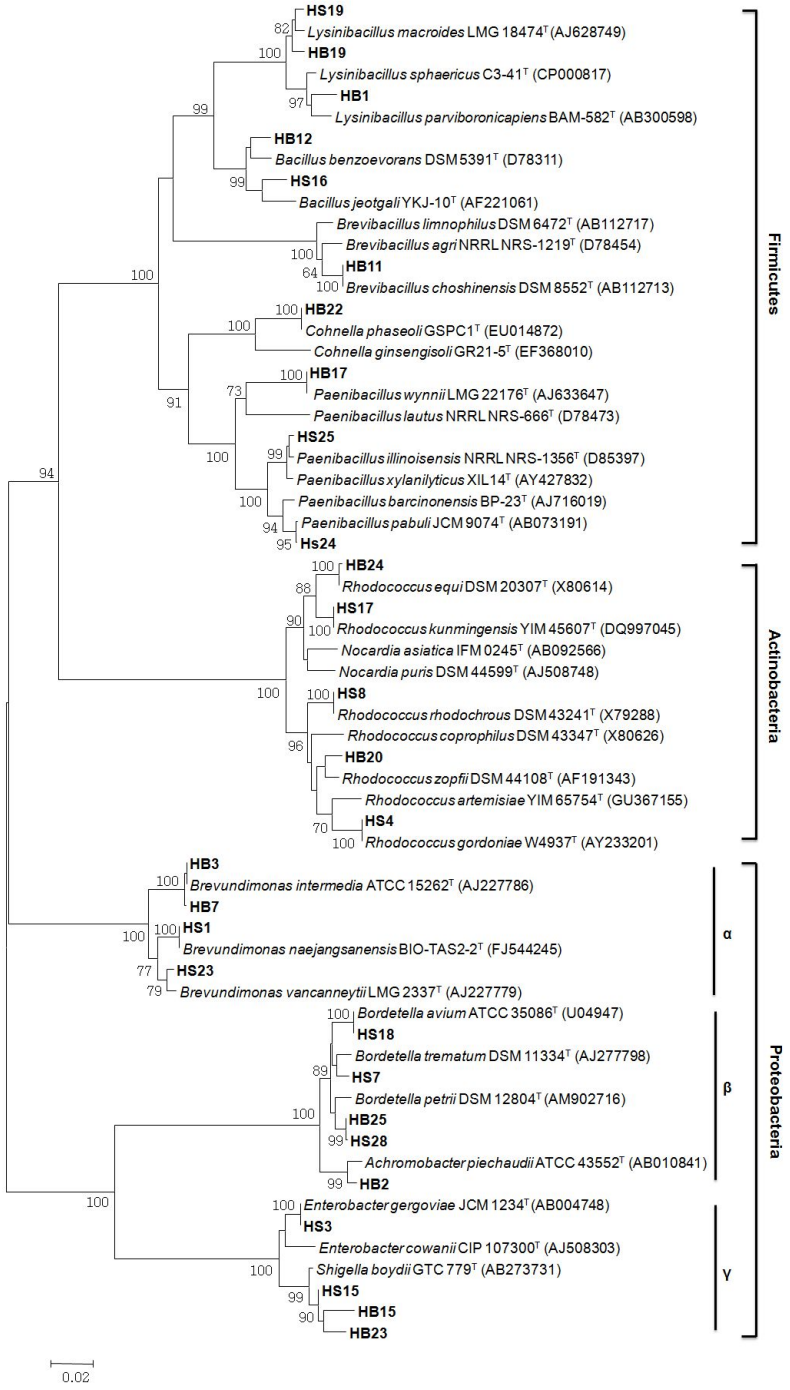

Fig. 1.7. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria from site B. Bootstrap percentages (based on 1000 replicates) greater than 70 % are shown at branching points. Bar, 0.02 substitutions per nucleotide position.

1.3.3. Isolation and phylogenetic analysis of sulfur-oxidizing bacteria from swinery sludge of site C

A total of 72 strains were isolated from sulfur oxidizing enrichment cultures derived from swinery sludge of site C. They were partially identified as 19 different genera and 29 species by 16S rRNA sequence analysis and were classified into 6 groups (*Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria, Bacteroidtes* and *Firmicutes*). While a substantial portion of the isolates belonged to *Actinobacteria* (35 %), *Beta-proteobacteria* (22 %), *Fimicutes* (15 %), *Gamma-proteobacteria*, (14 %) and *Alpha-proteobacteria* (10 %), a few isoletes were affiliated with *Bacteroidtes* (4 %) (Fig. 1.8).

The 16S rRNA sequence from the isolated bacteria showed close similarity with reference sequences of the EzTaxon server database (http://www.EzTaxon.org/; Chun *et al.*, 2007) (Table 1.5)

Most strains belonged to the phylum *Actinobacteria*, mainly to class fell into different 3 families: *Micrococcineae*, *Streptomycineae* and *Corynebacterineae*. The 25 isolates, 8 isolates showed 99-100 % sequence similarity to *Brevibacterium* sp. and 8 isolates exhibited 99-100 % to *Streptomyces* sp. Other into the genera *Arthrobacter*, *Corynebacterium*, *Kocuria*, *Leucobacter*, *Microbacterium* and *Micrococcus* (Table 1.5).

The 16 isolates belonging to the phylum *Beta-proteobacteria* fell into the 3 genera: *Alicycliphilus*, *Burkholderia* and *Castellaniella* (Table 1.5). The 14 isolates showed 99-100 % sequence similarity to *Castellaniella* sp. being the dominant species. The BA46^{T} isolate showed 97-98 % sequence similarity to *Burkholderia* sp., BA22^{T} showed a 97.5 % to *Alicycliphilus* sp. these isolates should be assigned to a novel species.

The 11 isolates belonging to the phylum *Fimicutes* fell into 2 genera *Bacillus* and *Staphylococcus* (Table 1.5). The 9 isolates showed 99-100 %

sequence similarity to *Bacillus* sp. and 2 isolates showed 99-100 % sequence similarity to *Staphylococcus* sp..

The 10 isolates belonging to the phylum *Gamma-proteobacteria* fell into the 2 genera *Pseudomonas*, *Raoultella* and *Stenotrophomonas*. The 5 isolates showed 99-100 % sequence similarity to *Pseudomona* sp., 4 isolates showed 99-100 % to *Stenotrophomonas* sp. and 1 isolate showed 99-100 % sequence similarity to *Raoultella* sp..

The 7 isolate, genera *Ochrobactrum* and *Rhodobacter* were members of the phylum *Alpha-proteobacteria*. The 4 isolates (BA15^{T}, BA24^{T}, BA31^{T} and BA36^{T}) showed a 96 - 97 % sequence similarity to *Rhodobacter* sp. that the isolate should be assigned to a novel species. The remaining 3 isolate, *Bacteroidetes* sequences were isolated bacteria, into the genera *Cloacibacterium* and *Sphingomonas* (Table 1.5).

Consequently 72 strains belonging to 19 different genera formed 6 representative taxonomic groups according to phyla, of which 6 strains proved to be candidates for novel taxa.

Table 1.5. Closest bacterial species to bacterial strains isolated from site C Compared by 16S rRNA gene sequence similarity.

Strain	Phylogenetic group	Closest species	Similarity (%)
BA 34	*Actinobacteria*	*Arthrobacter creatinolyticus* GIFU 12498^T	99.3
BA 30		*Arthrobacter protophormiae* DSM 20168^T	99.7
BA 39		*Brevibacterium epidermidis* NCDO 2286^T	98.8
BA 7		*Brevibacterium epidermidis* NCDO 2286^T	99.4
BA 8		*Brevibacterium epidermidis* NCDO 2286^T	99.5
BA 19		*Brevibacterium epidermidis* NCDO 2286^T	99.5
BA 35		*Brevibacterium epidermidis* NCDO 2286^T	99.6
BA 2		*Brevibacterium epidermidis* NCDO 2286^T	99.7
BA 5		*Brevibacterium epidermidis* NCDO 2286^T	99.7
BA 9		*Brevibacterium luteolum* CF87^T	99.5
BA 27		*Corynebacterium glutamicum* ATCC 13032^T	97.4
BA 29		*Corynebacterium glutamicum* ATCC 13032^T	99.3
BA 28		*Kocuria palustris* DSM 11925^T	99.8
BA 26		*Leucobacter iarius* 40^T	96.9
BA 18		*Microbacterium maritypicum* DSM 12512^T	99.5
BA 4		*Microbacterium hatanonis* JCM 14558^T	98.4
BA 16		*Micrococcus yunnanensis* YIM 65004^T	99.8
BA 38		*Streptomyces albolongus* NBRC 13465^T	100
BA 37		*Streptomyces albolongus* NBRC 13465^T	99
SA 13		*Streptomyces albolongus* NBRC 13465^T	99.8
SA 14		*Streptomyces albolongus* NBRC 13465^T	99.9
SA 4		*Streptomyces albolongus* NBRC 13465^T	99.4
SA 1		*Streptomyces albolongus* NBRC 13465^T	99.5
SA 2		*Streptomyces albolongus* NBRC 13465^T	99.6
SA 3		*Streptomyces albolongus* NBRC 13465^T	99.7
BA 20	*Bacteroidetes*	*Cloacibacterium normanense* CCUG 46293^T	98.4
BA 21		*Cloacibacterium normanense* CCUG 46293^T	98.9
BA 3		*Sphigomonas mizutaii* DSM 11724^T	99.1
BA 44	*Fimicutes*	*Bacillus anthracis* ATCC 14578^T	100
SA 5		*Bacillus anthracis* ATCC 14578^T	99.6
SA 6		*Bacillus anthracis* ATCC 14578^T	99.7
SA 15		*Bacillus aryabhattai* B8W22^T	99.6
SA 11		*Bacillus aryabhattai* B8W22^T	99.8
SA 12		*Bacillus aryabhattai* B8W22^T	99.9
SA 9		*Bacillus methylotrophicus* CBMB205^T	99.5
SA 10		*Bacillus methylotrophicus* CBMB205^T	99.6
SA 8		*Bacillus tequilensis* NRRL B-41771^T	99.8
SA 7		*Staphylococcus saccharolyticus* ATCC 14953^T	99.6
BA 17		*Staphylococcus xylosus* ATCC 29971^T	99.5

Table 1.5. Continued

Strain	Phylogenetic group	Closest species	Similarity (%)
BA 32	*α - aproteobacteria*	*Ochrobactrum tritici* SCII24^T	100
BA 42		*Ochrobactrum tritici* SCII24^T	99.6
BA 43		*Ochrobactrum tritici* SCII24^T	99.7
BA 15		*Rhodobacter blasticus* ATCC 33485^T	96.1
BA 24		*Rhodobacter blasticus* ATCC 33485^T	96.5
BA 31		*Rhodobacter blasticus* ATCC 33485^T	96.5
BA 36		*Rhodobacter blasticus* ATCC 33485^T	96.5
BA 22	*β - proteobacteria*	*Alicycliphilus denitrificans* K601^T	97.5
BA 46		*Burkholderia calva* 19620512^T	97.7
SA 22		*Castellaniella ginsengisoli* DCY36^T	98.2
SA 23		*Castellaniella ginsengisoli* DCY36^T	98.3
BA 25		*Castellaniella ginsengisoli* DCY36^T	98.4
SA 24		*Castellaniella ginsengisoli* DCY36^T	98.4
BA 6		*Castellaniella ginsengisoli* DCY36^T	98.5
BA 10		*Castellaniella ginsengisoli* DCY36^T	98.5
SA 25		*Castellaniella ginsengisoli* DCY36^T	98.5
SA 26		*Castellaniella ginsengisoli* DCY36^T	98.6
BA 23		*Castellaniella ginsengisoli* DCY36^T	98.8
BA 40		*Castellaniella ginsengisoli* DCY36^T	98.8
BA 41		*Castellaniella ginsengisoli* DCY36^T	98.8
BA 45		*Castellaniella ginsengisoli* DCY36^T	98.8
SA 20		*Castellaniella ginsengisoli* DCY36^T	99.2
SA 21		*Castellaniella ginsengisoli* DCY36^T	99.3
SA 16	*γ - proteobacteria*	*Pseudomonas brenneri* CFML 97-391^T	99.1
SA 17		*Pseudomonas brenneri* CFML 97-391^T	99.2
SA 18		*Pseudomonas brenneri* CFML 97-391^T	99.3
SA 19		*Pseudomonas brenneri* CFML 97-391^T	99.4
BA 14		*Pseudomonas xanthomarina* KMM 1447^T	98.4
BA 33		*Raoultella ornithinolytica* JCM 6096^T	100
BA 1		*Stenotrophomonas daejeonensis* MJ03^T	99.1
BA 11		*Stenotrophomonas daejeonensis* MJ03^T	99.1
BA 12		*Stenotrophomonas daejeonensis* MJ03^T	99.2
BA 13		*Stenotrophomonas daejeonensis* MJ03^T	99.2

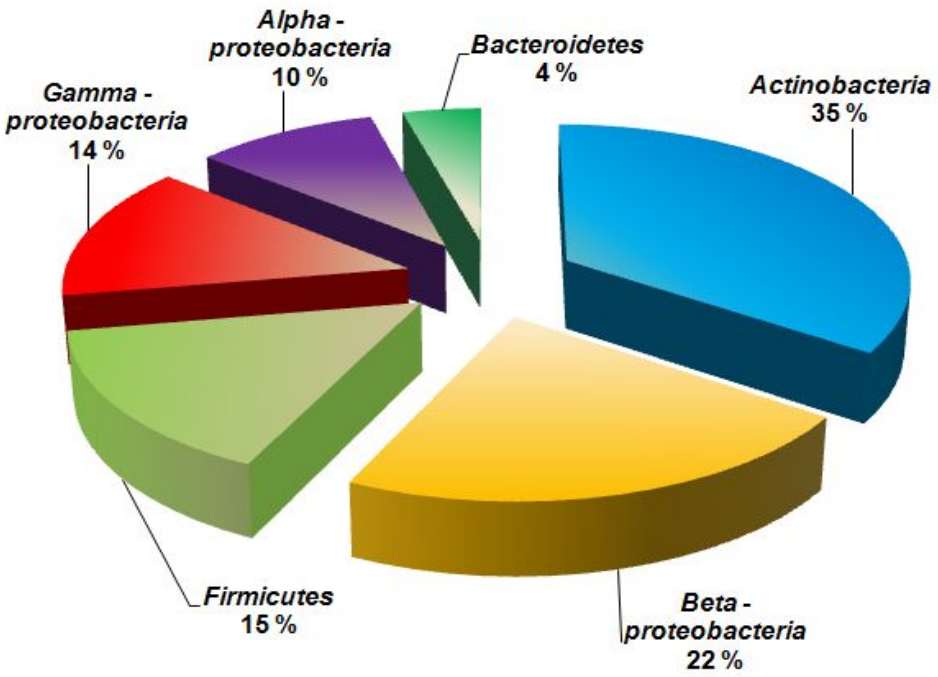

Fig. 1.8. Composition of bacterial community of swinery sludge site C in the level of the phylum/class.

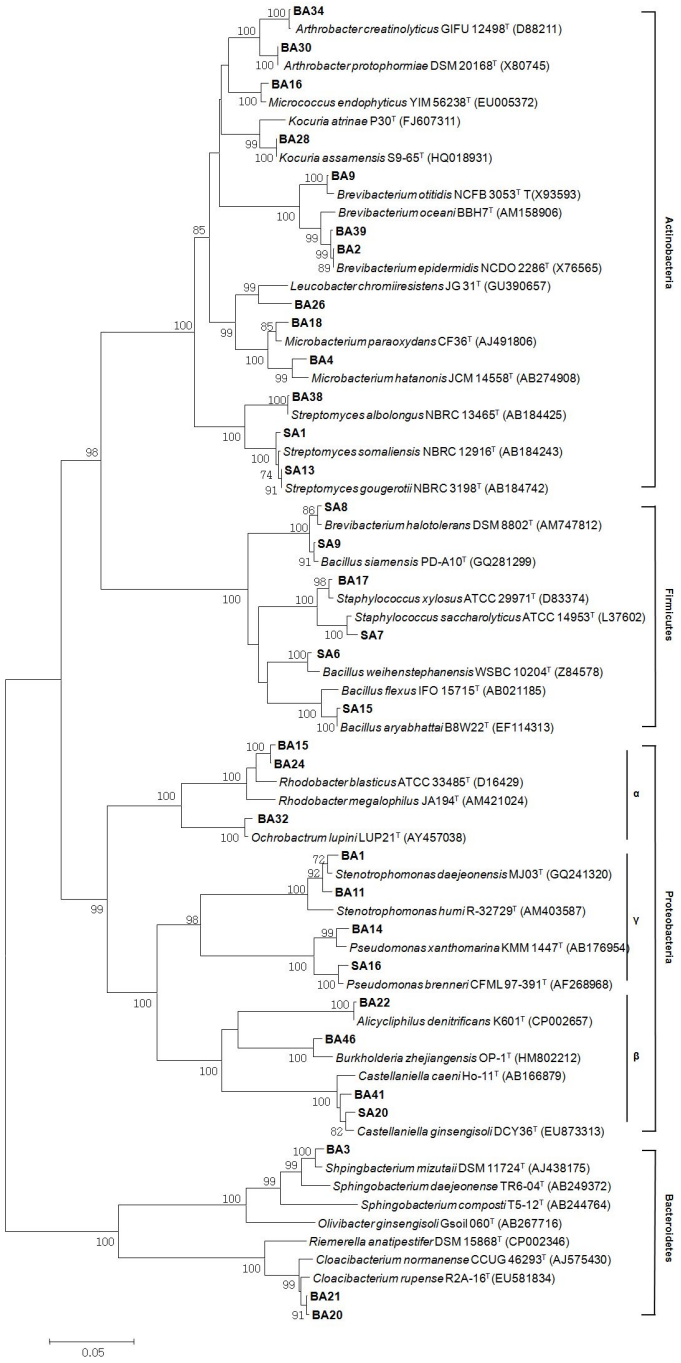

Fig. 1.9. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria from site C. Bootstrap percentages (based on 1000 replicates) greater than 70 % are shown at branching points. Bar, 0.05 substitutions per nucleotide position.

1.3.4. Isolation and phylogenetic analysis of sulfur-oxidizing bacteria from swinery sludge of site D

A total of 58 strains were isolated from sulfur oxidizing enrichment culture derived from swinery sludge of site D. They were partially identified as 14 different genera and 22 species by 16S rRNA sequence analysis and were classified 5 into groups (*Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Bacteroidetes* and *Actinobacteria*). A substantial portion of the isolates belonged to *Beta-proteobacteria*, (39 %), *Gamma-proteobacteria* (30 %), *Bacteroidetes* (14 %), *Actinobacteria* (9 %) and *Alpha-proteobacteria* (8 %) (Fig. 1.10).

The 16S rRNA sequence from the isolated bacteria showed close similarity with reference sequences of the EzTaxon server database (http://www.EzTaxon.org/; Chun *et al*., 2007) (Table 1.6)

Most strains belonged to the phylum *Proteobacteria*, mainly to class *Beta-proteobacteria* into the different families: *Alcaligenaceae* and *Comamonadaceae*. The 22 isolates, 14 isolates showed 99-100 % sequence similarity to *Alcaligenes* sp., and 3 isolates exhibited 99-100 % to *Stenotrophomonas* sp.. The 4 isolates ($BB4^T$, $BB8^T$, $BB9^T$ and $NB13^T$) showed a 97-98 % sequence similarity to *Comamonas* sp., 2 isolates ($BB5^T$, $BB11^T$) showed a 97-98 % to *Hydrogenophaga* sp. and 1 isolates ($BB12^T$) showed a 95-96 % to *Alicycliphilus* sp. that the isolate should be assigned to a novel species.

The 18 isolates belonging to the phylum *Gamma-proteobacteria* fell into 1 isolate exhibited 99-100 % sequence similarity to *Aeromonas* sp., one genus *Acinetobacter* showed 97-100 % to the dominant species and the isolate should be assigned to a novel species. The 8 isolates belonged to the phylum *Bacteroidetes* fell into the 1 genus *Sphingobacterium* showed 94-100 % sequence similarity to being the dominant species and the isolate should be

assigned to a novel species.

The 5 isolates belonging to the phylum *Alpha-proteobacteria* fell into the 4 genera, *Brevundimonas* and *Pseudochrobactrum* (Table 1.6). The 4 isolates showed 99-100 % sequence similarity to *Brevundmonas* sp. and 1 isolate showed 99-100 % to *Pseudochrobactrum* sp..

The remaining 5 isolate, *Actinobacteria* sequences were isolated bacteria, into the genera *Gordonia* , *Arthrobacter* and *Micrococcus* (Table 1.6).

Consequently 58 strains belonging to 14 different genera formed 5 representative taxonomic groups according to phyla, of which 7 strains proved to be candidates for novel taxa.

Table 1.6. Closest bacterial species to the bacterial strains isolated from site D Compared by 16S rRNA gene sequence similarity.

Strain	Phylogenetic group	Closest species	Similarity (%)
BS 2	*Actinobacteria*	*Gordonia malaquae* IMMIB WWCC-22^T	100
BS 3		*Gordonia malaquae* IMMIB WWCC-22^T	100
BS 4		*Gordonia malaquae* IMMIB WWCC-22^T	100
BB 3		*Arthrobacter mysorens* LMG 16219^T	99.8
BB 10		*Micrococcus luteus* NCTC 2665^T	99.5
BB 2	*Bacteroidetes*	*Sphingobacterium mizutaii* DSM 11724^T	95.0
BS 18		*Sphingobacterium mizutaii* DSM 11724^T	98.3
BS 16		*Sphingobacterium mizutaii* DSM 11724^T	99.6
BB 1		*Sphingobacterium mizutaii* DSM 11724^T	99.7
BS 17		*Sphingobacterium mizutaii* DSM 11724^T	99.7
BB 6		*Sphingobacterium composti* T5-12^T	97.8
BB 7		*Sphingobacterium composti* T5-12^T	97.9
NB 4		*Sphingobacterium shayense* HS39^T	94
NB 12	*α - proteobacteria*	*Brevundimonas diminuta* ATCC 11568^T	99.8
BS 5		*Brevundimonas terrae* KSL-145^T	99.1
BS 6		*Brevundimonas terrae* KSL-145^T	99.1
BS 7		*Brevundimonas terrae* KSL-145^T	99.1
BS 1		*Pseudochrobactrum saccharolyticum* CCUG 33852^T	99.8
NS 5	*β - proteobacteria*	*Alcaligenes aquatilis* LMG 22996^T	99.7
NS 13		*Alcaligenes aquatilis* LMG 22996^T	99.8
NS 9		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.3
NS 7		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.4
NS 10		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.4
NS 12		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.4
NS 1		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.7
NS 2		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.8
NS 3		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.8
NS 8		*Alcaligenes faecalis* subsp. *faecalis* IAM12369^T	99.8
NS 14		*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.5
NS 11		*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.6
NS 4		*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.7
NS 6		*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.7
BB 12		*Alicycliphilus denitrificans* K601^T	95.7
BS 10		*Castellaniella denitrificans* NKNTAU^T	99.7
BB 4		*Comamonas thiooxidans* S23^T	98.6
BB 8		*Comamonas thiooxidans* S23^T	98.6
NB 13		*Comamonas thiooxidans* S23^T	98.6
BB 9		*Comamonas thiooxidans* S23^T	98.7
BB 5		*Hydrogenophaga temperata* TR7-01^T	98.7
BB 11		*Hydrogenophaga temperata* TR7-01^T	98.7

Table 1.6. Continued

Strain	Phylogenetic group	Closest species	Similarity (%)
BS 12	*β - proteobacteria*	*Stenotrophomonas acidaminiphila* AMX19^T	99.4
BS 13		*Stenotrophomonas acidaminiphila* AMX19^T	99.5
NB 1		*Stenotrophomonas ginsengisoli* DCY01^T	98.8
BS 11	*γ - proteobacteria*	*Aeromonas punctata* subsp. *punctata* NCIMB 13016^T	99.7
NB 3		*Acinetobacter bouvetii* 4B02^T	97.2
NB 10		*Acinetobacter bouvetii* 4B02^T	97.2
BS 8		*Acinetobacter bouvetii* 4B02^T	97.2
NB 2		*Acinetobacter bouvetii* 4B02^T	97.5
NB 6		*Acinetobacter bouvetii* 4B02^T	97.5
NB 15		*Acinetobacter bouvetii* 4B02^T	97.5
NB 7		*Acinetobacter bouvetii* 4B02^T	99.6
NB 8		*Acinetobacter bouvetii* 4B02^T	99.7
NB 9		*Acinetobacter bouvetii* 4B02^T	99.8
BS 14		*Acinetobacter johnsonii* DSM 6963^T	99.8
NB 5		*Acinetobacter johnsonii* DSM 6963^T	96.9
BS 15		*Acinetobacter johnsonii* DSM 6963^T	96.9
NB 11		*Acinetobacter johnsonii* DSM 6963^T	97.5
BS 9		*Acinetobacter johnsonii* DSM 6963^T	98.2

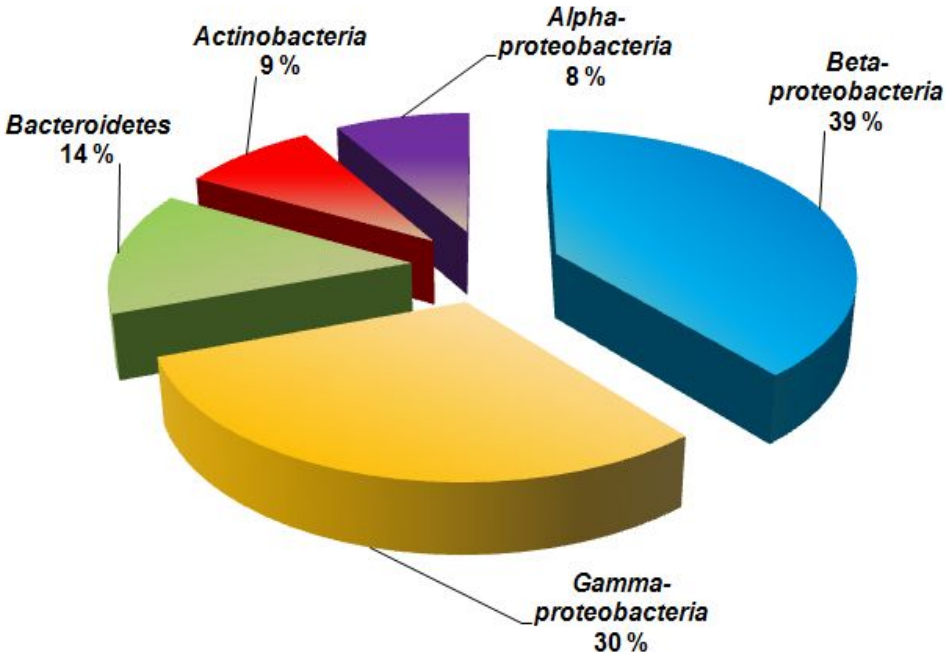

Fig. 1.10. Composition of bacterial community of swinery sludge site D in the level of the phylum/class.

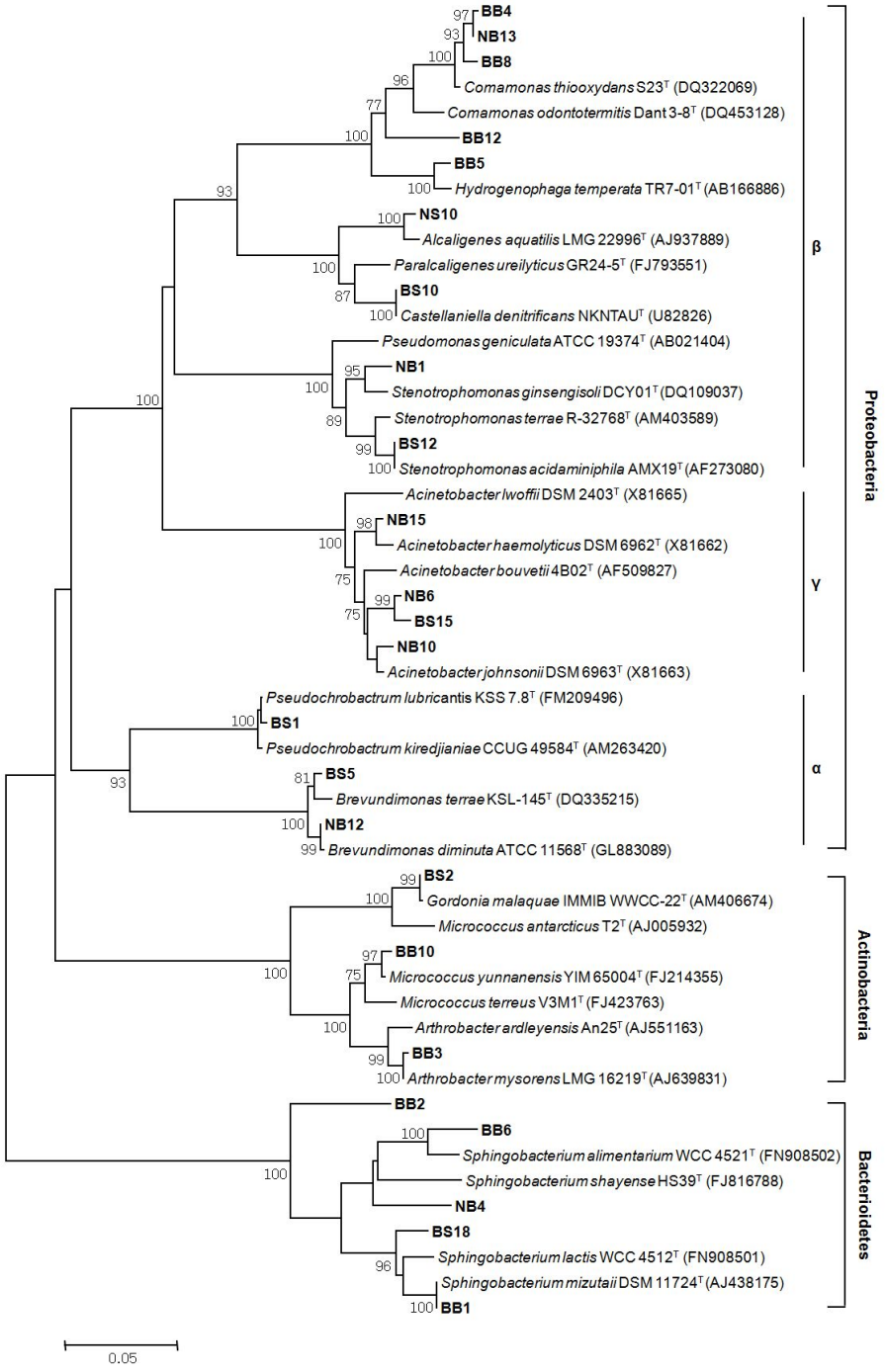

Fig. 1.11. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria from site D. Bootstrap percentages (based on 1000 replicates) greater than 70 % are shown at branching points. Bar, 0.05 substitutions per nucleotide position.

1.3.5. Isolation and phylogenetic analysis of sulfur-oxidizing bacteria from swinery sludge of site E

A total of 64 strains were isolated from sulfur oxidizing enrichment culture derived from swinery sludge of site E. They were partially identified as 11 different genera and 22 species by 16S rRNA sequence analysis and were classified 5 into groups (*Beta-*, *Gamma-proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*). While a substantial portion of the isolates belonged to *Gamma-proteobacteria*, (65 %), *Beta-proteobacteria* (12 %), *Actinobacteria* (9 %) and *Bacteroidetes* (8 %) and a few isolated were affiliated with *Fimicutes* (6 %) (Fig. 1.12).

The 16S rRNA sequence from the isolated bacteria showed close similarity with reference sequences of the EzTaxon server database (http://www.EzTaxon.org/; Chun *et al.*, 2007) (Table 1.7)

Most strains belonging to the phylum *Proteobacteria*, mainly to class *Gamma-proteobacteria* into different families: *Aeromonadaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* and *Moraxellaceae*. The 42 isolates, 23 isolates showed 79-100 % sequence similarity to *Pseudomonas* sp. being the dominant species. The 11 isolates exhibited 98-100 % sequence similarity to *Acinetobacter* sp., 4 isolates exhibited 99-100 % to *Aeromonas* sp. and 4 isolates exhibited 99-100 % to *Stenotrophomonas* sp..

The 8 isolates belonged to the phylum *Beta-proteobacteria* fell into the 3 genera, *Alcaligenes* and *Comamonas* (Table 1.7). The 5 isolates showed 99-100 % sequence similarity to *Comamonas* sp. and 3 isolates showed 99-100 % to *Alcaligenes* sp..

The 8 isolates belonged to the phylum *Actinobacteria* fell into the 4genera, *Microbacterium* and *Rhodococcus* (Table 1.7). The 5 isolates showed 99-100 % sequence similarity to *Rhodococcus* sp. and 1 isolate showed 99-100 % to

Microbacterium sp..

The 5 isolate, genera *Dyadobacter* and *Sphingobacterium* were members of the phylum *Bacteroidetes*. The remainder of four isolate, *Firmicutes* sequences were isolated bacteria, into the genera *Paenibacillus* (Table 1.7).

Consequently 64 strains belonging to 11 different genera formed 5 representative taxonomic groups according to phyla.

Table 1.7. Closest bacterial species to the bacterial strains isolated from site E Compared by 16S rRNA gene sequence similarity

Strain	Phylogenetic group	Closest species	Similarity (%)
KS 26	*Actinobacteria*	*Microbacterium keratanolyticum* IFO 13309^T	99.7
KS 19		*Rhodococcus gordoniae* W4937^T	99.8
KB 12		*Rhodococcus gordoniae* W4937^T	99.8
KB 19		*Rhodococcus gordoniae* W4937^T	99.8
KB 25		*Rhodococcus gordoniae* W4937^T	99.8
KB 28		*Rhodococcus gordoniae* W4937^T	99.8
KB 1	*Bacteroidetes*	*Dyadobacter fermentans* DSM18053^T	98.4
KB 17		*Sphingobacterium lactis* WCC 4512^T	99.8
KS 1		*Sphingobacterium multivorum* IAM14316^T	99.5
KS 24		*Sphingobacterium multivorum* IAM14316^T	99.5
KB 13		*Sphingobacterium multivorum* IAM14316^T	99.5
KB 14	*Firmicutes*	*Paenibacillus chibensis* JCM 9905^T	99.1
KS 5		*Paenibacillus chibensis* JCM 9905^T	99.2
KB 6		*Paenibacillus chibensis* JCM 9905^T	99.2
KS 23		*Paenibacillus chibensis* JCM 9905^T	99.5
KS 2	*β - proteobacteria*	*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.5
KS 3		*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.3
KS 20		*Alcaligenes faecalis* subsp. *parafaecalis* G^T	99.7
KB 20		*Comamona stestosteroni* ATCC 11996^T	100
KS 16		*Comamonas thiooxydans* S23^T	100
KS 27		*Comamonas thiooxydans* S23^T	99.2
KS 7		*Comamonas thiooxydans* S23^T	100
KB 10		*Comamonas thiooxydans* S23^T	100
KB 16	*γ - proteobacteria*	*Acinebacter lwoffii* DSM 2403^T	98.9
KB 22		*Acinebacter lwoffii* DSM 2403^T	98.8
KB 15		*Acinetobacter baumannii* ATCC 19606^T	97.8
KB 23		*Acinetobacter baumannii* ATCC 19606^T	98.1
KS 17		*Acinetobacter baylyi* B2^T	97.6
KB 21		*Acinetobacter bouvetii* 4B02^T	97.5
KS 15		*Acinetobacter johnsonii* DSM 6963^T	99.7
KS 14		*Acinetobacter johnsonii* DSM 6963^T	99.3
KS 13		*Acinetobacter junii* LMG 998^T	98.7
KB 18		*Acinetobacter lwoffi* iDSM 2403^T	99.1
KB 24		*Acinetobacter lwoffii* DSM 2403^T	98.8
KS 8		*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966^T	99.8
KS 12		*Aeromonas veronii* ATCC 35624^T	100
KS 21		*Aeromonas veronii* ATCC 35624^T	99.1
KS 22		*Aeromonas veronii* ATCC 35624^T	99.7
KS 9		*Pseudomonas aeruginosa* LMG 1242^T	99.7
KS 10		*Pseudomonas aeruginosa* LMG 1242^T	99.6

Table 1.7. Continued

Strain	Phylogenetic group	Closest species	Similarity (%)
KS 30	γ - *proteobacteria*	*Pseudomonas aeruginosa* LMG 1242^T	99.8
KS 31		*Pseudomonas aeruginosa* LMG 1242^T	99.6
KB 7		*Pseudomonas aeruginosa* LMG 1242^T	100
KB 8		*Pseudomonas aeruginosa* LMG 1242^T	99.8
KB 9		*Pseudomonas aeruginosa* LMG 1242^T	100
KB 26		*Pseudomonas aeruginosa* LMG 1242^T	99.8
KB 27		*Pseudomonas aeruginosa* LMG 1242^T	99.8
KB 29		*Pseudomonas aeruginosa* LMG 1242^T	99.7
KB 30		*Pseudomonas aeruginosa* LMG 1242^T	99.6
KB 31		*Pseudomonas aeruginosa* LMG 1242^T	99.7
KB 32		*Pseudomonas aeruginosa* LMG 1242^T	99.7
KB 33		*Pseudomonas aeruginosa* LMG 1242^T	99.8
KS 11		*Pseudomonas aeruginosa* LMG 1242^T	100
KS 28		*Pseudomonas aeruginosa* LMG 1242^T	100
KS 29		*Pseudomonas aeruginosa* LMG 1242^T	99.8
KS 32		*Pseudomonas aeruginosa* LMG 1242^T	100
KB 2		*Pseudomonas aeruginosa* LMG 1242^T	99.5
KB 3		*Pseudomonas aeruginosa* LMG 1242^T	99.2
KB 4		*Pseudomonas aeruginosa* LMG 1242^T	99.6
KB 5		*Pseudomonas aeruginosa* LMG 1242^T	99.8
KS 6		*Pseudomonas mendocina* LMG 1223^T	97.0
KS 18		*Stenotrophomonass ginsengisoli* DCY01^T	99.7
KS 25		*Stenotrophomonass ginsengisoli* DCY01^T	99.3
KS 4		*Stenotrophomonass ginsengisoli* DCY01^T	99.4
KB 11		*Stenotrophomonas terrae* R-32768^T	99.3

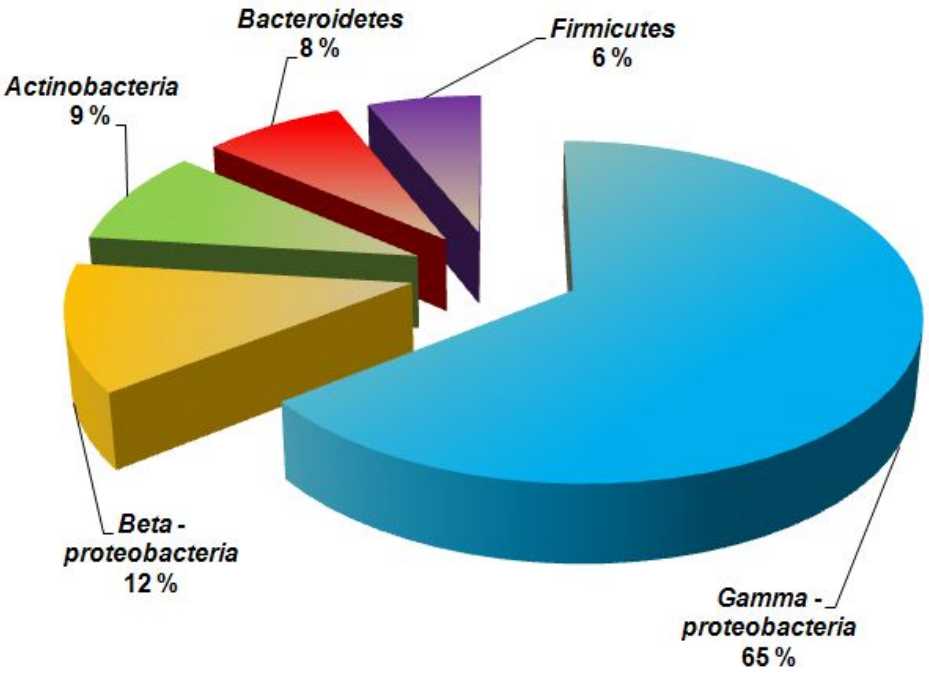

Fig. 1.12. Composition of bacterial community of swinery sludge site E in the level of the phylum/class.

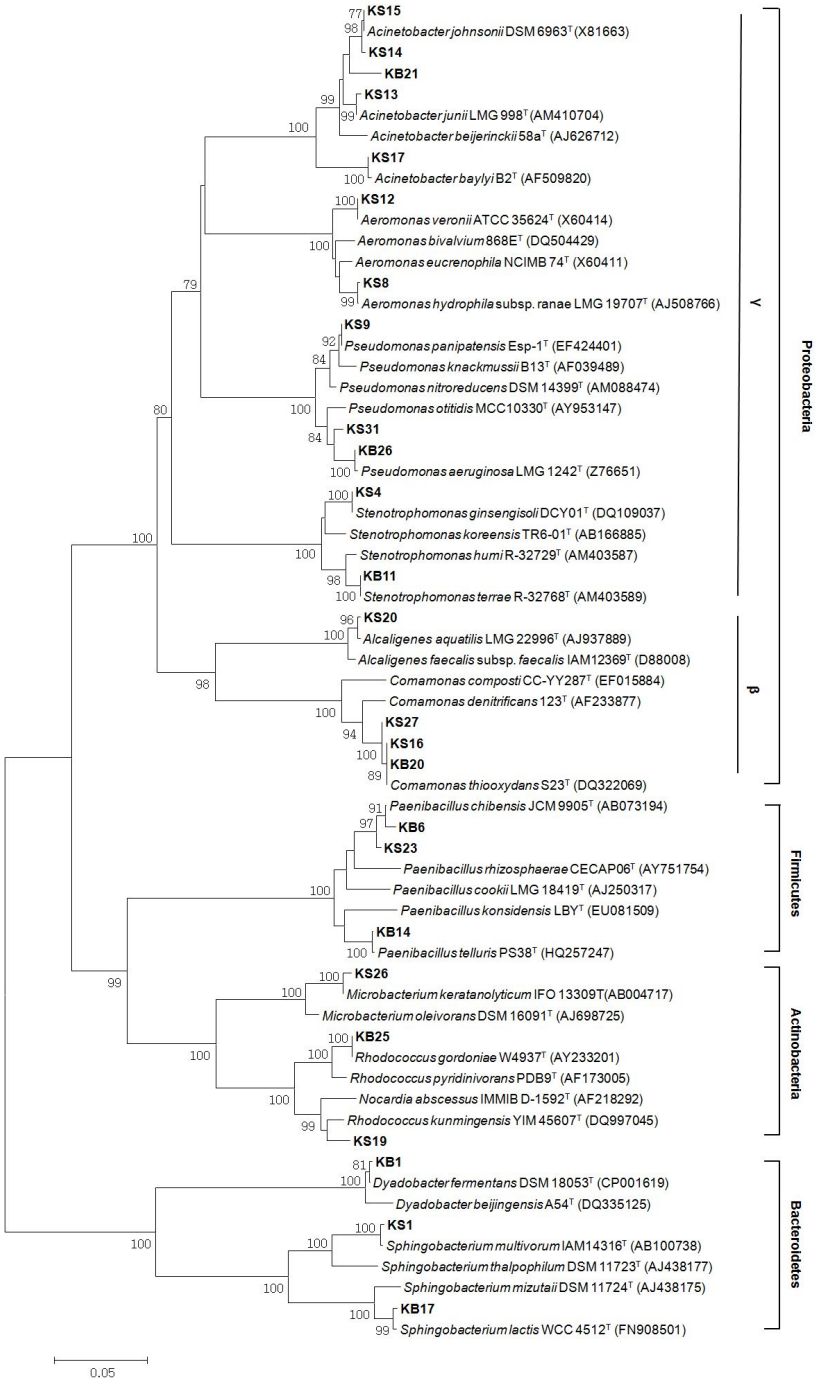

Fig. 1.13. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria from site E. Bootstrap percentages (based on 1000 replicates) greater than 70 % are shown at branching points. Bar, 0.05 substitutions per nucleotide position.

1.3.6. Analysis of the sulfur-oxidizing bacteria distributed in all swinery sites

In order to identify sequence types that constitute the impacted system, the 16S rRNA gene sequences of the isolated bacteria were searched in the GenBank database. As a result, most of the isolates in swinery sludge were assigned to previously reported cultured classes.

Totally 351 strains of sulfur-oxidizing bacteria were isolated through enrichment cultures from swinery sludge and classified as members of the 6 groups, *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria*, *Bacteriodetes* and *Firmicutes* (Table 1.8, Fig. 1.14). They were partially indentified as 16 ordesr or suborders, 23 families and 48 genera by 16S rRNA sequence analysis (Table 1.8).

Overall, many bacteria isolated from 5 sampling sites belonged to the phylum *Proteobacteria,* and isolates belonging to *Gamma-proteobacteria* include a various of class. It was reported that diversity of sulfur-oxidizing bacteria either natural ventilation or mechanical ventilation

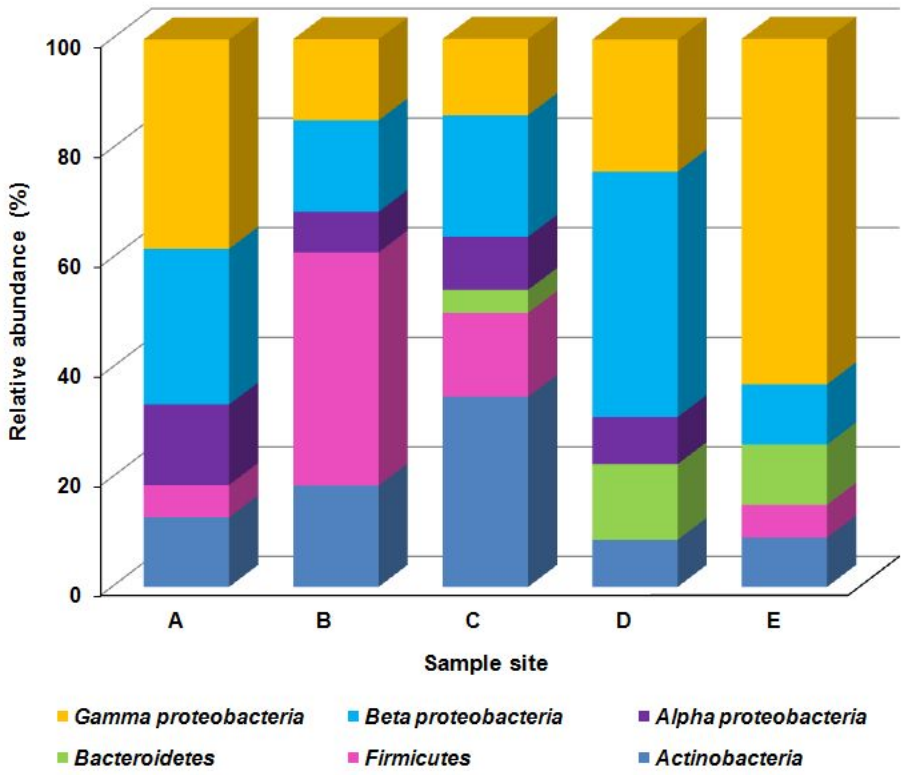

Fig. 1.14. Comparison of bacterial distribution of swinery sludge in the phylum/class level.

Table 1.8. list of sulfur-oxidizing bacteria from different sample site (A-E)

Phylum/Class	Order or Suborder	Family	Genus	A	B	C	D	E	Total
Actinobacteria	*Corynebacterineae*	*Dietziaceae*	*Dietzia*	1					1
			Rhodococcus	3	10			5	18
		Gordoniaceae	*Gordonia*				3		3
	Micrococcineae	*Micrococcineae*	*Arthrobacter*	1		2	1		4
			Brevibacterium	2		8			10
			Corynebacterium			2			2
			Kocuria			1			1
			Leucobacter	2		1			3
			Microbacterium	3		2		1	6
			Micrococcus			1	1		2
	Streptomycineae	*Streptomycetaceae*	*Streptomyces*	1		8			9
Bacteriodetes	*Cytophagales*	*Cytophagaceae*	*Dyadobacter*					1	1
	Flavobacteriales	*Flavobacteriaceae*	*Cloacibacterium*			2			2
	Sphingobacteriales	*Sphingobacteriaceae*	*Sphingobacterium*			1	8	4	13
Firmicutes	*Bacillales*	*Bacillaceae*	*Bacillus*	1	4	9			14
			Lysinibacillus	2	6				8
		Staphylococcaceae	*Staphylococcus*			2			2
		Paenibacillaceae	*Brevibacillus*	2	2				4
			Cohnella		2				2
			Paenibacillus		9			4	13
		Planococcaceae	*Chryseomicrobium*	1					1
α-proteobacteria	*Caulobacterales*	*Caulobacteraceae*	*Aquamicrobium*	1					1
			Brevundimonas	10	4		4		18
	Rhizobiales	*Brucellaceae*	*Ochrobactrum*			3			3
			Paenochrobactrum	2					2
			Pseudochrobactrum				1		1
	Rhodobacterales	*Rhodobacteraceae*	*Paracoccus*	2					2
			Rhodobacter			4			4
β-proteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Achromobacter*		1				1
			Alcaligenes	2			14	3	19
			Bordetella		8				8
			Castellaniella			14	1		15
			Pusillimonas	1					1
		Comamonadaceae	*Alicycliphilus*			1	1		2
			Comamonas				4	5	9
			Hydrogenophaga				2		2
		Burkholderiaceae	*Burkholderia*			1			1
	Rhodocyclales	*Rhodocyclaceae*	*Thauera*	26					26
γ-proteobacteria	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*				1	4	5
			Zobellella	1					1
	Enterobacteriales	*Enterobacteriaceae*	*Enterobacter*		1				1
			Escherichia		6				6
			Raoultella			1			1
			Shigella		1				1
	Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	9				11	20
			Psychrobacter	2					2
		Pseudomonadaceae	*Pseudomonas*	13		5	14	23	55
	Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas*	14		4	3	4	25
6	16	23	48	102	54	72	58	65	351

1.3.7. Amplification and phylogeny of the *soxB* gene

The s*oxB* genes from the different thiosulfate-oxidizing bacteria and from the natural samples were successfully amplified and yielded PCR products of the expected length in high quantities. The products could be purified and sequenced. The primer pairs soxB693F/soxB1446B, ioffered the most successful and reliable amplification results. All amplified PCR products were sequenced on both strands and translated into 250-260 amino acid sequences (Fig. 1.16). The phylogenetic tree was calculated from the aligned amino acid and nucleotide sequences to infer branching orders. The *soxB* amino acid and nucleotide alignments yielded nearly identical trees and the overall topologies of both trees were strongly substantiated by high bootstrap values (Fig. 1.15).

The *soxB* genes were detected in 13 isolated strains, including 9 strains of *Beta-proteobacteria* and 4 strains of *Alpha-proteobacteia*. The BLAST search results showed that the thiosulfate-oxidizing bacteria isolated from swinery sludge fell into four different genera, *Comamonas*, *Methylibium*, *Paracoccus* and *Thiobacillus* (Table 1.9). The partial alignment of predicted amino acid sequences is shown in Fig. 1.16. Residues conserved in *soxB* sequences are highlighted. The level of conservation of the *soxB* amino acid sequences ranged to 70-95 %.

The strains BB5 and BB11 revealed 80-81 % sequence similarity of the 16S rRNA gene to the type strain of *Hydogenophaga* sp, and 6 strains revealed 89 % sequence similarity of 16S rRNA gene to the type strain of *Comamonas* sp, which belongs to *Beta-proteobacteria*, its *soxB* gene sequences were associated with this branch of *Beta-proteobacteria*. The strains BA15, BA31 and SS33 revealed 91-95 % amino acid similarity to the *soxB* gene sequence of *Paracoccus denitrificans,* which belongs to *Alpha-proteobacteria*, its *soxB* gene sequences were associated to this branch of *Alpha-proteobacteria* (Table 1.9, Fig. 1.15). The branching order of the

phylogenetic trees calculated from the *soxB* sequences and the corresponding *16S rRNA* data are basically consistent regarding the separation of sulfur-oxidizing bacteria and the different representatives of the *Alpha-*, *Beta-*, and *Gamma-proteobacteria*.

Table 1.9. The *soxB* gene amino acid similarity of bacteria isolated from swinery sludge to closest species

Strain	Closest species	Similarity (%)	Accession NO. (*soxB* gene)	Amino acids[a]
SS33	*Paracoccus denitrificans*	95	JX867762	251
BA15	*Paracoccus denitrificans*	91	JX867763	251
BA31	*Paracoccus denitrificans*	91	JX867764	251
KS16	*Comamonas* sp.	89	KC295218	255
KS27	*Comamonas* sp.	89	KC295216	255
KB20	*Comamonas* sp.	89	KC295217	255
BB4	*Comamonas* sp.	89	KC295212	255
BB8	*Comamonas* sp.	90	KC295213	255
BB9	*Comamonas* sp.	89	KC295214	255
BB5	*Thiobacillus aquaesulis*	80	KC295219	255
BB11	*Thiobacillus aquaesulis*	81	KC295220	255
BB12	*Methylibium petroleiphilum*	79	KC295221	255
NB11	*Comamonas* sp.	89	KC295215	255

[a] *soxB* nucleotide sequences were then translated into amino acid sequences

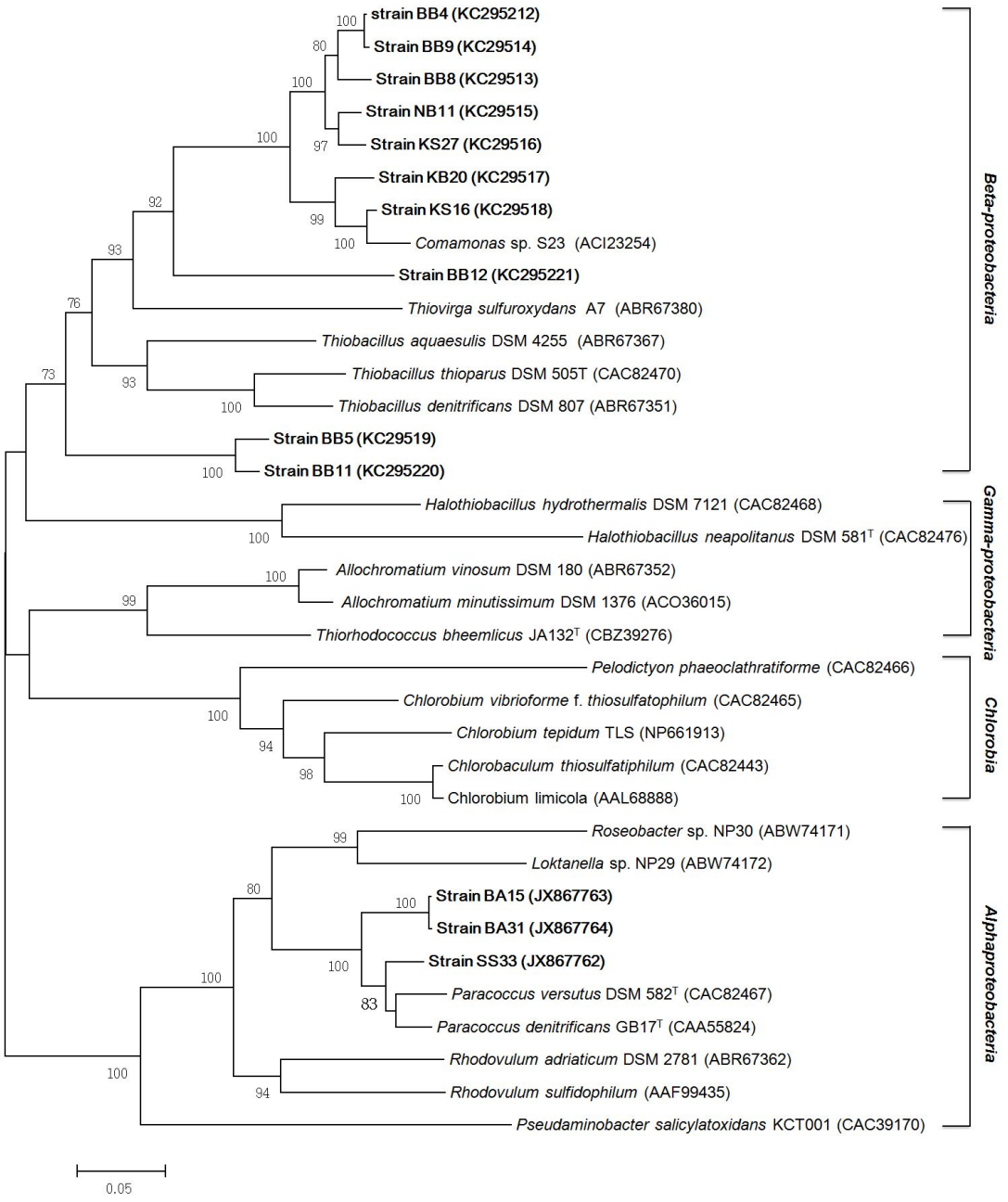

Fig. 1.15. Phylogenetic analysis of *soxB* amino acid sequences from thiosulfate-oxidizing strains isolated from swinery sludge. Bootstrap values above 70% calculated from 1000 replicates are shown at the nodes as percentages. The scale bar indicates 0.1 substitutions per site.

```
BB5     IGQAFPYTPIANPRYMVPDWTFGIQDEHMQTVVDQARGEGAQVVVVLSHNGMDVDIKMAS
BB11    IGQAFPYTPIANPRYMVPDWTFGIQDEHMQTVVDQARGEGAQVVVVLSHNGMDVDIKMAS
KB20    IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARAKGAKVVVVLSHNGMDVDLKMAS
KS16    IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARAKGAKVVVVLSHNGMDVDLKMAS
BB4     IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARGKGAKVVVVLSHNGMDVDLKMAS
BB8     IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARGKGAKVVVVLSHNGMDVDLKMAS
BB9     IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARGKGAKVVVVLSHNGMDVDLKMAS
KS27    IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARGKGAKVVVVLSHNGMDVDLKMAS
NB11    IGQAFPYTPIANPRYMVADWSFGIQDENMQKMVDEARGKGAKVVVVLSHNGMDVDLKMAS
BA15    IGQAFPYMPIANPKWMFPEYSFGIREERMQEMVDELRAEGVDLVVVLSHNGFDVDKKMGG
BA31    IGQAFPYMPIANPKWMFPEYSFGIREERMQEMVDELRAEGVDLVVVLSHNGFDVDKKMGG
SS33    IGQAFPYMPIANPKWMFPEYSFGIREERMQHVVDELRAEGVDLVVVLSHNGFDVDKKMGG
        ******* *****  *     ***  * **  **  *  *   ******** *** **

BB5     RVRGIDAILGGHTHDGMPAPTIVKNGGGQTLVTNAGSNSKFLGVLDFDVRGGKVQDFRYK
BB11    RVRGIDAILGGHTHDGMPAPTIVKNGGGQTLVTNAGANSKFLGVLDFDVRGGKVQDFRYK
KB20    RVRGIDAILGGHTHDGMPVPTLVQNAGGKTIVTNAGSNGKFLGVLDLDVRDGKVRGFQYR
KS16    RVRGIDAILGGHTHDGMPVPTLVQNAGGKTIVTNAGSNGKFLGVLDLDVRDGKVRGFQYR
BB4     RVRGIDAILGGHTHDGMPVPTIVQNAGGKTIVTNAGSNGKFLGVLDLDVKDGKVRDFQYR
BB8     RVRGIDAILGGHTHDGMPVPTIVQNAGGKTIVTNAGSNGKFLGVLDLDVKDGKVRDFQYR
BB9     RVRGIDAILGGHTHDGMPVPTIVQNAGGKTIVTNAGSNGKFLGVLDLDVKDGKVRDFQYR
KS27    RVRGIDAILGGHTHDGMPVPTIVQNAGGKTIVTNAGSNGKFLGVLDLDVKDGKVRDFQYR
NB11    RVRGIDAILGGHTHDGMPVPTLVQNAGGKTIVTNAGSNGKFLGVLDLDVKDGKVRDFQYR
BA15    RVKGIDVILSGHTHDAVPEPILIG----ETILIATGSNGKFVSRVDLDVRDGRMMGFRHK
BA31    RVKGIDVILSGHTHDAVPEPILIG----ETILIATGSNGKFVSRVDLDVRDGRMMGFRHK
SS33    RVKGIDVILSGHTHDAVPEPILIG----ETILIATGSNGKFVSRVDLDVRDGKMMGFRHK
        ** *** ** *****  * *         *    * * **    * **  *    *

BB5     LLPVFSNLLPADAGMQAYIDQVRAPYKNKLEEKLAVTEDLLYRRGNFNGSWDQLICDALM
BB11    LLPVFSNLLPADPGMQAYIDQVRAPYKNKLEEKLAVTEDLLYRRGNFNGSWDQLICDALM
KB20    LLPVFANILPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
KS16    LLPVFANILPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
BB4     LLPVFANLLPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
BB8     LLPVFANLLPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
BB9     LLPVFANLLPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
KS27    LLPVFANILPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
NB11    LLPVFANILPADAQMQALITKIRAPYEGRLNEVLARTDGTLYRRGNFNGTGDQLLLDAMM
BA15    LIPIFSDVIAPDADMAALIDAERAPFKAQLEERIGTTESLLYRRGNFNGTWDDLICDAVR
BA31    LIPIFSDVIAPDADMAALIDAERAPFKAQLEERIGTTESLLYRRGNFNGTWDDLICDAVR
SS33    LIPIFSDVIAPDADMAALIDAERAPFKAQLEERIGTTESLLYRRGNFNGSWDDLICDAVR
        * * *     *  * * * *   ***    * *    *   *********  * *  **
```

Fig. 1.16. A alignment of the predicted amino acids encoded by *soxB* of bacteria isolated from the swinery sludge.

```
BB5     EVKGADMAFSPGVRWGTSLLPGDTITYERMMDQMAMTYPATTLNEFTGEQIKGILEDVAD
BB11    EVKGADMAFSPGVRWGTSLLPGDTITYERMMDQMAMTYPATTLNEFTGEQIKGILEDVAD
KB20    EVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
KS16    EVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
BB4     AVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
BB8     AVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
BB9     AVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
KS27    AVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
NB11    AVQDAPIAFSPGFRWGTSLLAGQDITREWLMDMTATTYSYATVTEMTGATIKTVLEDVAD
BA15    SERDAQIALSPGVRWGTTLLPGEAITREDIHNVTSMTYGAVYRNEMTGEMLKTILEDVAD
BA31    SERDAQIALSPGVRWGTTLLPGEAITREDIHNVTSMTYGAVYRNEMTGEMLKTILEDVAD
SS33    SERDAQIALSPGVRWGTTLLPGDAITREDIHNVTSMTYGAVYRTEMTGEMLKTMLEDVAD
            *  * *** **** ** *  ** *        **      * **   *  ******

BB5     NIFNPDPYYQHGGDM
BB11    NIFNPDPYYQHGGDM
KB20    NLFNPDPYYQHGGDM
KS16    NLFNPDPYYQHGGDM
BB4     NLFNPDPYYQHGGDM
BB8     NLFNPDPYYQHGGDM
BB9     NLFNPDPYYQHGGDM
KS27    NLFNPDPYYQHGGDM
NB11    NLFNPDPYYQHGGDM
BA15    NIFNTDPYYQQGGDM
BA31    NIFNTDPYYQQGGDM
SS33    NIFNTDPYYQQGGDM
        * ** ***** ****
```

Fig. 1.16. Continued.

1.3.8. Thiosulfate oxidation

The thiosulfate-oxidizing ability of the isolated bacteria was examined in the solid mineral salts thiosulfate medium. A change in color of the plate from purple to yellow was observed in 6 strains (KS16, KB20, BB4, BB8, BB9 and BB11). Moreover, these strains were able to grow in the SOB medium without thiosulfate and no change in color was noted (Table 1. 11). The sulfate assay described here is a modification of a standard turbidimetric method based on precipitation of sulfate ions with barium chloride in such a manner as to form barium sulfate crystals. The thiosulfate (20 mM initial concentration) in the growth medium after the production of acid (SO_4^{-2}). The definite growth of *Paracoccus pantotrophus* as a control strain confirmed that the presently used medium was suitable for sulfur-oxidizing bacteria and lithotrophic growth. Among our isolates, BB11 (*Hydrogenophaga* sp.) and BB12 (*Alicycliphilus* sp.) consumed the highest amount of accumulated sulfate of 175.5 and 128.2 μg/mL respectively. The concomitant reduction in pH of the spent medium was noted (Table 1. 11). Hence, these two strains were selected for further studies. Other strains, KS16, KB20 and SS33 accumulated a slightly lower amounts of sulfate, 89.2 μg/mL, 72.7 μg/mL and 70.8 μg/mL respectively. The strain that was able to accumulate the highest amount of sulfate and achieve growth with oxidation of thiosulfate was investigated in mixotrophic medium containing 20 mM sodium thiosulfate over a total incubation period of 96 h (Fig. 1.16). A maximum increase in the optical density of the absorbance at > 3.0 was noted for KB20, KS16 and SS33, while the other 2 strains, BB11 and BB12, grew less well, with optical density of less than at < 1.0 in SOB medium (Fig. 1.16). During the time course of thiosulfate oxidation by chemotrophic bacteria in sulfur oxidizing medium, thiosulfate was oxidized directly to sulfate. The thiosulfate disappearance was always accompanied by an increase in the celluar yield of the bacteria (Fig. 1.16).

Table 1.10. The thiosulfate oxidation by isolated bacteria strains from swinery sludge

-, not detected.

Strain	Closest species	Similarity (%)	Accession NO. (16S rRNA)[a]	Sulfate formation (ug/mL)	Color change[b]
NB11	*Acinetobacter johnsonii*	97.5	KC295210	5.6	Light purple
BB12	*Alicycliphilus denitrificans*	95.6	JX997988	128.2	Light purple
KS16	*Comamona stestosteroni*	100	KC295206	89.2	yellow
KB20	*Comamona stestosteroni*	99.9	KC295208	72.7	yellow
BB4	*Comamonas testosteroni*	98.6	JX997984	60.7	yellow
BB8	*Comamonas testosteroni*	98.6	JX997985	41.0	yellow
BB9	*Comamonas testosteroni*	98.6	JX997986	39.8	yellow
BB5	*Hydrogenophaga bisanensis*	99.0	KC295209	9.7	Light purple
BB11	*Hydrogenophaga bisanensis*	97.6	JX997987	175.5	yellow
SS33	*Paracoccus denitrificans*	99.2	JF820843	70.8	Light purple
KB27	*Pseudomonas aeruginosa*	100	KC295207	6.6	Light purple
BA15	*Rhodobacter megalophilus*	95.5	JX029075	-	Light purple
BA31	*Rhodobacte rmegalophilus*	95.5	KC295211	-	purple
GB17^T	*Paracoccus pantotrophus*	-	-	23.1	purple

[a] Strains were accession number to GenBank (NCBI).

[b] Color change of the plate from purple to yellow.

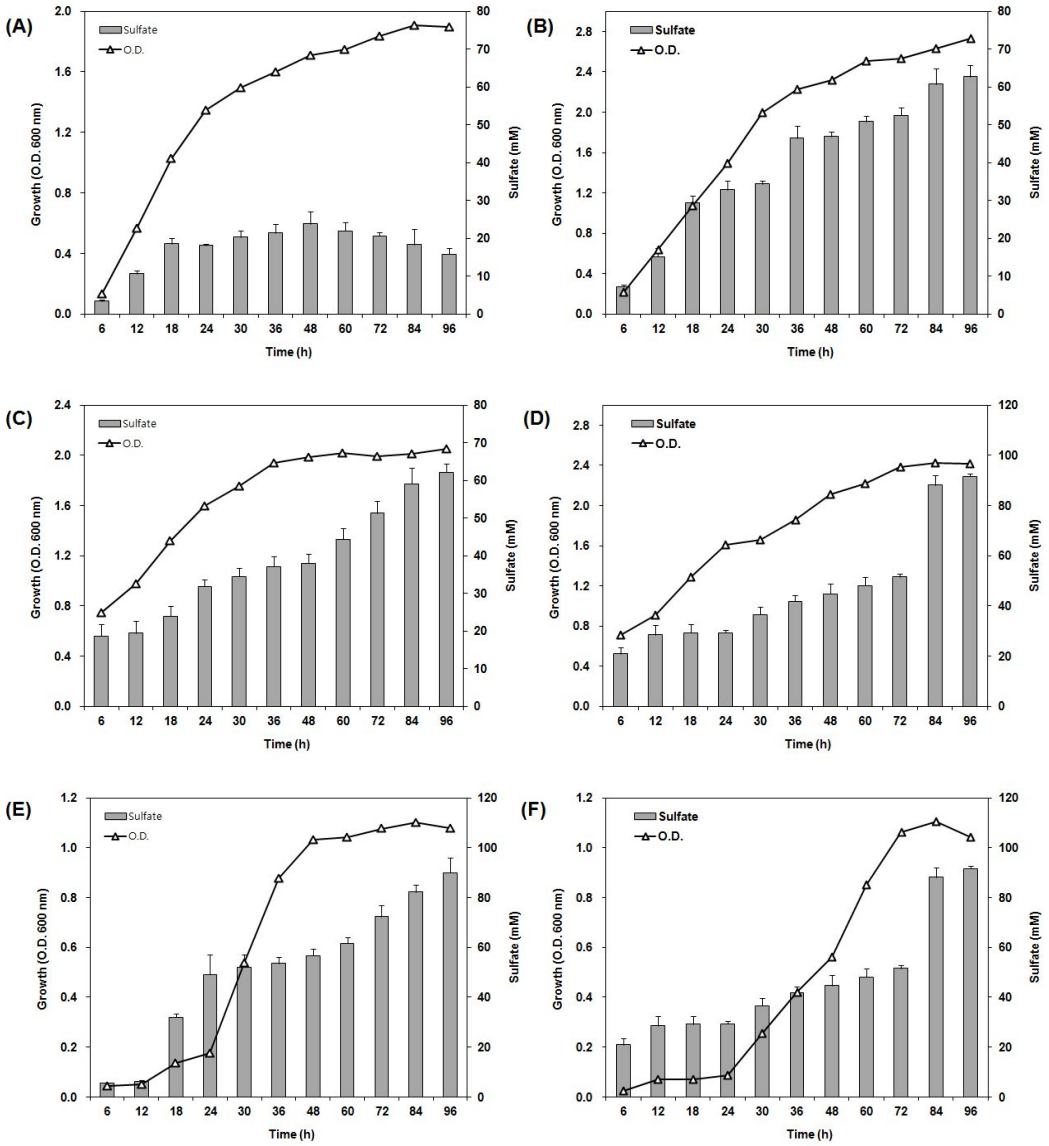

Fig. 1.17. Sulfate accumulation by bacterial strains isolated from swinery sludge during incubation time. (A) *Paracoccus pantotrophus* GB17, (B) SS33, (C) KB20, (D) KS16, (E) BB11 and (F) BB12 in SOB medium containing 20 mM thiosulfate. Values are the mean±SD of 3 determinations.

1.3.9. Enzyme activity of thiosulfate oxidation

Activities of enzymes associated with thiosulfate metabolism, particularly, thiosulfate oxidase and sulfite oxidase, were observed in the cell-free extracts of all thiosulfate-oxidizing strains (Table 1.11). The highest activities of thiosulfate oxidase (16.9 nmol ferricyanide reduced/min/mg protein) and sulfite oxidase (14.5 nmol ferricyanide reduced/min/mg protein) were found in SS33 and BB12 respectively.

Table 1.11. Activities of enzymes for thiosulfate and sulfite oxidation of sulfur-oxidizing bacteria isolated from swinery sludge

ND, not detected.

Strain	Protein content (mg/mL)	Thiosulfate oxidase (nmol ferricyanide/ min/mg protein)	Sulfite oxidase (nmol ferricyanide/ min/mg protein)
NB11	9.53	10.4	0.4
BB12	12.54	11.3	14.5
KS16	9.39	ND	3.7
KB20	12.24	1.2	5.5
BB4	8.64	0.8	1.3
BB8	5.47	7.7	1.3
BB9	9.54	ND	1.6
BB5	6.41	0.9	ND
BB11	13.04	10.4	5.2
SS33	14.12	16.9	4.2
KB27	12.37	16.0	12.3
BA15	5.59	ND	1.9
BA31	5.41	ND	ND
GB17^{T}	9.31	5.12	3.26

1.4. Discussion

The aerobic sulfur-oxidizing bacteria have been isolated from sulfur-rich swinery sludge using enrichment culture. Therefore, high concentrations of thiosulfate (20 mM) were added to the medium for cultivation and biochemical and ecological analyses of these sulfur-oxidizing bacteria. In total 351 strains of sulfur-oxidizing bacteria were isolated and classified into 6 groups *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria*, *Bacteriodetes* and *Firmicutes* (Table 1.8, Fig. 1.14). They were partially indentified as 16 orders or suborders, 23 families and 48 genera by 16S rRNA sequence analysis (Table 1.8). The 16S rRNA-based identification of cultured sulfur-oxidizing bacteria allowed a sharper focus on this group, with evolutionary and ecological implications. The sludge isolates of the *Proteobacteria* cluster, the most frequently isolated strains in this study, showed an unexpected phylogenetic and physiological link with the microbial populations of the swinery sludge. The swinery sludge contained thiosulfate oxidizers of the *Proteobacteria* cluster, *Comamonas*, *Paracoccus*, and *Pseudomonas* are the evolutionary cousins of the widespread swinery sludge bacteria of the same group. The perspective of molecular genetics and the phylogeny of sulfur oxidation are extremely important, species distributed the *Alpha-, Beta-,* and *Gamma-proteobacteria* (Kelly *et al*., 1997; Ghosh *et al*., 2005).

The complex metabolic process of sulfur chemolithotrophy is unlikely to have originated as multiple parallel evolutionary events (Ghosh and Roy, 2007). All of sulfur-oxidizing microorganisms are thought to have originated from some ancient stock possessing lithoautotrophic potential governed by a primordial genetic system, while also possessing additional abilities to oxidize

tetrathionate or thiocyanate, possibly converting them into substrates that could be utilized by Sox-mediated pathways (Deb *et al*., 2004; Anandham *et al*., 2008; Masuda *et al*., 2010).

Mixotrophic growth (i.e., concurrent utilization of organic and inorganic substrates) may be metabolically advantageous for these bacteria. Since low concentrations of S compounds can limit growth, the use of organic carbon for biomass synthesis or even co-oxidation of S compounds together with organic substrates may ensure better survival and growth of S-oxidizing bacteria in the rhizosphere (Graff and Stubner, 2003).

The aim of this study was to develop a PCR-based assay and probe sulfur-oxidizing bacteria based on a functional gene essential for sulfur oxidation and to screen its distribution among recognized sulfur-oxidizing bacteria as well as, new isolates from swinery sludge. The possible modes of thiosulfate oxidation were identified in the bacteria isolated in the present study. Thus, based on data related to thiosulfate oxidation products, thiosulfate metabolizing enzymes and the absence of the *soxB* gene, it is postulated that *Dyella*, *Lysinibacillus*, *Alcaligenes* and *Microbacteium* (Kelly *et al*., 1997; Suzuki, 1998). The highly degenerated primers used in this study were complementary to target sites of *Chlorobiaceae*, *Beta-proteobacteria* and most *Gamma-* and *Alpha-proteobacteria soxB* sequences (Meyer *et al*., 2007). Thiosulfate oxidation pathways operating in sulfur-oxidizing bacteria have been tentatively grouped into three categories (Meyer *et al*., 2007). The frist pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which are common in extremophilic sulfur oxidizers (Kelly *et al*., 1997). The second a pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by a multienzyme complex (Sox) system, active in photo- and chemotrophic members of *Alpha-proteobacteria* (Friedrich *et al*., 2001; Mukhopadhyaya *et al*., 2000). The last branched thiosulfate oxidation pathway involving formation

of sulfur globules operating in sulfur-storing bacteria (Hensen *et al.*, 2006). The Sox enzyme system is present in diverse thiosulfate-oxidizing bacteria. Phylogenetic trees constructed with diverse *soxB* gene homologs available in public databases revealed that *Beta-proteobacteria* and *Alpha-proteobacteria* were not monophyletic and formed at least 4 and 2 clusters respectively, while *Alpha-proteobacteria* were shown to be monophyletic (Anandham *et al.*, 2008; Meyer *et al.*, 2007; Petri *et al.*, 2001). With the introduction of two novel soxB gene homologs of *Rhodobacter* sp. (strains, BA15 and BA31) in the database, the s*oxB* phylogenetic tree presented in this report has established for the first time that *Alpha-proteobacteria* were not monophyletic but formed at least 2 distinct groups. The level of conservation of the *soxB* amino acid sequences ranged from 80 to 100 % and was comparable to other functional genes, such as ammonia monooxygenase (amoA) and nitrous oxide reductase (nosZ) (Rotthauwe *et al.*, 1997; Scala and Kerhof, 1999). The amino acid sequences of different clusters revealed low similarity values of 80-89 %, whereas closely related sequences within clusters shared much high similarities of 90-91 %, thereby reflecting a strong separation between the different lines of descent of the *soxB* gene. This observation may indicate that the *soxB* gene has a long evolutionary history (Petri *et al.*, 2001). According to the *soxB* phylogeny, 3 and 2 distinct *soxB* groups were noted in the *Gamma-* and *Alpha-proteobacteria*, respectively, indicating lateral gene transfer (Meyer *et al.*, 2007; Petri *et al.*, 2001).

The accumulation of intermediate products of thiosulfate oxidation such as trithionate, sulfite, sulfur and thiosulfate metabolizing enzymes and the presence of the *soxB* gene were noted in *Alicycliphlus* sp., *Comamonas* sp., *Hydrogenophaga* sp. and *Paracoccus* sp. (Table 1.10). Hence, it is postulated that these bacteria could possess the S4 intermediate pathway, in addition to the sulfate oxidizing multi-enzyme mediated sox system which is essential for thiosulfate oxidation in the *Paracoccus* sulfur oxidation (PSO) pathway

(Friedrich *et al*., 2001; Hensen *et al*., 2006; Kelly *et al*., 1997).

In many sulfate oxidizing bacteria, the genes encoding sulfate oxidizing multi-enzyme system proteins occur in etheir a single cluster or multiple clusters (Friedrich *et al*., 2000). The genesencoding soxAX, soxB and soxYZ are invariably present in these bacteria, and the encoded proteins share relatively high amino acid sequence identities with each other irrespective of the presence or absence of soxCD (Welte *et al*., 2009; Zander *et al*., 2011). In *Paracoccus versutus* (formerly *Thiobacillus versutus*), a thiosulfate-oxidizing periplasmic multienzyme system comprising soxA, soxB, and multiheme cytochromes was characterized (Lu, 1986; Lu and Kelly, 1988; Kappler *et al*., 2000). The proposed mechanism is designated the PSO pathway (Kelly *et al*., , 1997). The function of soxA and soxB enzymes were not demonstrated. The sequence analysis revealed a partial open reading frame (ORF), soxA, and 5 additional ORFs (soxBCDEF) downstream of soxA (Wodara *et al*., 1994; Wodara *et al*., 1997).

The recent whole-genome sequence analysis revealed a homologous gene cluster similar to the Sox locus in a large number of diverse bacterial species. Future studies should consider isolating SOB from this sulfide-rich wastewater, characterizing the metabolism of sulfur in these bacteria to sulfide or other inorganic reduced sulfur, and determining the role of these bacteria in sulfide degradation.

In addition, efficient and environmentally friendly manure treatment technologies can reduce odors through the utilization of microorganisms and also produce organic fertilizer, which can then be linked to sustainable agricultural production. The current manure treatment issues in Jeju can be addressed using SOB to reduce the pollutants in manure; this will lower environmental risks, thereby restoring the image of Jeju as a clean region.

Chapter Ⅱ

Polyphasic study and Characterization of Novel Sulfur-Oxidizing Bacteria

2.1. Introduction

Taxonomy comprises classification, nomenclature, and identification. The modern bacterial taxonomy uses the polyphasic approach, which is based on the several molecular techniques, each one retrieving the information at different cellular levels (quinones, fatty acids, phospholipids, and DNA G+C content). The obtained results are combined and analyzed to reach the 'consensus taxonomy' of the microorganism. In principle, all genotypic, phenotypic, and phylogenetic information may be incorporated in polyphasic taxonomy. The genotypic information is derived from the nucleic acids (DNA and RNA) in the cell, whereas phenotypic information is derived from proteins. Several methods described briefly below such as determination of G+C mol% and DNA-DNA hybridization studies, are classical approached and have been applied in taxonomic analysis (Vandamme *et al*., 1996).

The family *Comamonadaceae* (Wen *et al*., 1999), belongs to the class *Beta proteobacteria*, currently consists of more than 29 genera with validly published names. Most isolates of this family were obtained from soil, freshwater, wastewater, activated sludge, and pond water and indicating that this evolutionary cluster has a wide spectrum of habitats and various metabolism pathways. Some isolates are capable of degrading hydrocarbons, accumulating phosphorous, oxidizing ammonia and performing denitrification.

The genus *Comamonas* was first described by De Vos *et al*. (1985). Subsequently, a number of novel species have been added into this genus. The genus *Comamonas* is group of Gram-negative, non-spore-forming, and rod-shaped. These bacteria are catalase- and oxidase positive and a DNA G+C content of the genomic DNA of this genus ranges from 60 to 66 mol%. Members of the genus *Comamonas* have been isolated from various

environments such as wetlands, termite gut, soil and waste.

The genus *Hydrogenophaga* was created by the reclassification of the genus *Pseudomonas* (Willems *et a*l., 1989). The organisms that belong to these genera are chemo-organotrophic or chemolithoautotrophic, using the oxidation of H_2 as an energy source and CO_2 as a carbon source, this is one of the major differentiating characteristics for distinguishing them from other genera of the family *Comamonadaceae* (Wen *et al*., 1999; Kämpfer *et al*., 2005).

The family *Rhodobacteraceae* (Garrity *et al*., 2005), which belongs to the class *Alpha-proteobacteria*, contains approximately 90 recognized genera (type genus, *Rhodobacter*) at the time of writing. Members of the family *Rhodobacteraceae* was phenotypically, metabolically, and ecologically diverse. Includes phoroheterophically that can also grow photoautotrophically or chemotrophically under approproate environmental conditions, chemoorganotrophs with either strictly aerobic or facultatively anaerobic respiratory metabolism, facultatively fermentative organism, and facultative methylotrophs. The family Rhodobacteraceae have been isolated from seawater, saline, sediments, microbial mats, seaweeds, fresh water and animal tissues in various habitats

Based on partial 16S rRNA sequences, novel strains were selected for polyphasic taxonomic analysis. These novel strains were further characterized by genotypic and phenotypic methods and were compared to the profiles of the SOB isolated from swinery sludge.

2.2. Material and Methods

2.2.1. Isolation of the bacterial strains and culture condition

The sulfur-oxidizing bacteria were isolated from swinery sludge in Jeju, Republic of Korea. The sludge samples were subjected to enrichment culture, 100 mL of on BH medium and incubated at 30 °C for 2 weeks. The culture slurries were serially diluted and appropriate dilutions were spread onto BH medium and incubated at 30 °C for 7 days. The agar plates were incubated for 7 days and colonies exhibiting different morphologies were transferred to new media. Pure culture were obtained through a series of re-plating to check for purity. It was subcultivated on tryptic soy agar (TSA; Difco, USA) at 30 °C. The strains were preserved in a glycerol solution (20 %, w/v) at -70 °C.

2.2.2. Cell morphology

Gram-staining was performed using the Gram-stain kit (BD) according to the manufacturer's instructions. A motility test was performed by motility test agar (0.5 % agar). Bacterial motility was observed macroscopically by a diffusion zone of growth spreading from the line of inoculation. For scanning electron microscopy (SEC, Korea), cells attached to the filter were fixed for 2 h with 2.5 % glutaraldehyde and washed three times for 5 min in 0.1 M sodium phosphate buffer, pH 7.2. The dehydration of the bacterial specimen was carried out sequentially using 50 %, 70 %, 80 %, 90 %, 95 %, and 100 % ethanol. Bacterial specimen was treated with isoamyl acetate and kept at room temperature for 1 hr. The bacterial specimens were then frozen at -70 °C. and then lyophilized. After plantinum coating (20 mA, 90 sec).

2.2.3. Analysis of phenotypic characteristics

Growth at 4 to 50 °C was measured on TSA. The pH range for growth was investigated on TSB adjusted to pH 3.0–11.0 in increments of 0.5 pH units using 1 M HCl or 1 M NaOH. Salt tolerance was tested by incubation for 7 days on TSA supplemented with 1–10 % (w/v in increments of 1 % unit) NaCl. Catalase activity was observed by bubble production in a 3 % solution (v/v) of hydrogen peroxide, and oxidase activity was determined by the oxidation of 1 % (w/v) tetramethyl *p*-phenylenediamine (Merck, USA). Hydrolysis of casein, starch, and Tween 20, 40, 60, and 80 was performed on TSA using the substrate concentrations described previously (Cowan and Steek, 1965). DNase activity was examined using DNase test agar (Difco, USA) with methyl green. Growth under anaerobic condition was determine after incubation for 4 weeks in an AnaeroPack (Oxoid, UK) on TSA and supplemented with potassium nitrate (0.1 %, w/v). Other biochemical tests were carried out using the API20E, API20NE, and APIZYM kits (bioMerieux, UK) according to the mamufacturer's instructions. Utilization of different carbon sources was assessed using GN2 microplate (Biolog, USA) according to the mamufacturer's instructions. Susceptibility to antibiotics was tested on TSA plates using antibiotic discs (BBL, USA) containing the following: ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), erythromycin (15 μg), kanamycin (30 μg), lincomycin (2 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), oleandomycin (15 μg), penicillin G (10 IU), polymyxin B (300 IU), rifampicin (5 μg), streptomycin (10 μg) and tetracycline (30 μg).

2.2.4. Phylogenetic analysis

Genomic DNA was extracted and purified by using a commercial genomic DNA extraction kit (Promega, USA), and the nearly complete 16S rRNA gene sequence was amplified using bacterial universal primers (Weisburg *et al.,* 1991).Sequencing of the 16S rRNA gene of strains was carried out as described previously (Lane, 1991). Full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR, USA) and compared with available 16S rRNA gene sequences on the GenBank database by using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). Sequences of related taxa and sequence similarity value were obtained from EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Multiple alignments were performed using the CLUSTAL X version 1.83 program (Thompson *et al.*, 1997) and gaps were excluded by using the BioEdit program (Hall, 1999). A phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981), and maximum parsimony (Kluge and Farris, 1969) algorithms contained in MEGA version 5.0 (Tamura *et al.*, 2011). Bootstrap analysis based on 1,000 resamplings was used to evaluate the reliability of tree topology (Felsenstein, 1985).

2.2.5. Analysis of chemotaxomic characteristics

The respiratory quinones were extracted and purified as described by Komagata and Suzuki (1987), and separated by using reversed-phase HPLC with a Spherisorb 5 μm ODS2 column (250 x 4.6 mm; Waters) in an elution of methanol and isoprophyl ether (4:1) as described previously (Tamaoka, 1986).

For fatty acid methyl ester analysis, cells were allowed to grow on TSA. The fatty acid methyl esters were saponified with saponifying reagent (NaOH

in aqueous methanol) by boiling at 100 °C, for 30 min and methylated with methylating reagent (6 N HCl in aqueous methanol) by reacting at 80 °C for 10 min. Fatty acids were then extracted with extraction solvent (hexane/Methyl-tert butyl ether; 1:1, v/v). Next, the base was washed by saturated NaCl. The Sherlock Microbial Identification System (MIDI; version 6.1) (Sasser, 1990), and analyzed by gas chromatography (GC 7890A, Agilent, USA) with the TSBA6 library.

The DNA G+C content of strains was determined using the thermal denaturation method (Marmur & Doty, 1962) using an UV-visiable spectrophotometer evolution 300 (Thermo scientific, USA). DNA from *Escherichia coli* K-12 was used as a control.

Polar lipids were extracted and examined by two-dimensional TLC (Minnikin *et al.*, 1984). The lipid extracts were dissolved in 50 uL of chloroform: methanol (2:1, v/v) and 10 uL of samples was spotted on the thin layer chromatography plates (Merck, USA). Chromatography was carried out using chloroform: methanol: water (65:25:4, v/v/v) in the first direction, followed by chloroform: acetic acid: methanol: water (80:15:12:4, v/v/v/v) in the second one. Extracted lipids were separated by TLC and identified by spraying with appropriate detection reagent (Minnikin *et al.*, 1984; Komagata and Suzuki, 1987). Ninhydrin reagent (ninhydrin reagent 0.2 % solution, Sigma, USA) was used to detect free amino groups containing lipids, zinzadze reagent (molybdenum blue spray reagent, 1.3 %, Merck, USA) for phosphorus containing lipids, α-naphtol reagent (α-naphtol reagent 15 % solution, Sigma, USA) for glycolipids containing lipids and molybdophoshpolic acid (phosphomolybdic acid reagent, 10 % solution in ethanol, Sigma, USA) for total lipids. Finally, the lipids on the TLC plate were identified by comparing their mobility with those of authentic lipids (Sigma, USA)

2.3. Results and Discussion

2.3.1. Strain KBB12^T

The family *Comamonadaceae* belongs to the class *Beta-proteobacteria* and contains many genera including *Acidovorax*, *Comamonas*, *Variovorax*, *Xylophilus*, *Hydrogenophaga*, *Aquaspirillum*, *Rhodoferax*, *Polaromonas*, *Alicycliphilus* and *Ramlibacter* have been added to the family (Wen *et al.*, 1999; Hiraishi *et al.*, 1991; Irgens *et al.*, 1996; Heulin *et al.*, 2003; Mechichi *et al.*, 2003).

A 16S rRNA gene sequence analysis revealed that strain KBB12^T was placed into the family *Comamonadaceae* of the class *Beta-proteobacteria*. A sequence similarity calculation using the EzTaxon server (http://www.EzTaxon.org/; Chun *et al.*, 2007) indicated that the closest relatives of strain KBB12^T were *Alicycliphilus denitrificans* K601^T (95.8 %) and *Diaphorobacter nitroreducens* NA10B^T (94.8 %). Lower sequence similarities (less than 95 %) were found with members of all other genera such as (*Alicycliphilus*, *Diaphorobacter* and *Acidovorax*) shown in Fig. 2.1. neighbour-joining phylogenetic tree confirmed the separate position of strain KBB12^T. The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms also supported the notion that there is no genus group that shows a clear phylogenetic relationship with strain KBB12^T in the family *Comamonadaceae*.

Strain KBB12^T was facultative aerobic, Gram staining negative and rod-shape. The colonies grown on TSA for 3 days were pale yellow, circles, measuring 0.5-1 mm in diameter. The optimal condition for growth was 37 °C and pH 7.0. The physiological characteristics of strain KBB12^T were

summarized in the species description and a comparison of selected characteristics with related strains is shown in Table 2.1.

The fatty acids of strain KBB12^T showed high amounts of $C_{16:1}$ ω7*c* (40.5 %), $C_{16:0}$ (27.7 %) and $C_{18:1}$ ω7*c* (16.5 %), as reported for strains of the genera *Acidovorax*, *Alicycliphilus*, *Giesbergeria*, *Simplicispira* and *Diaphorobacter*, but also showed relatively high amounts (>8 %) of $C_{17:0}$ cyclo (Table 2.2). The profile of polar lipids included phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and unknown lipid (L) (Fig. 2.2).

Strain KBB12^T belonged to the family *Comamonadaceae* and formed a distinct phyletic line with the clades of the related genera. Moreover, strain KBB12^T was differentiated from members of the genus *Alicycliphilus* and *Diaphorobacter* by several phenotypic characteristics including fatty acid composition, carbon utilization (Table 2.1. and 2.2) and polar lipid compositions (Fig. 2.2). Based on polyphasic data strain KBB12^T represents a novel genus and species of the family *Comamonadaceae*, for which the name *Thiobacterium jejuense* gen. nov., sp. nov. is proposed.

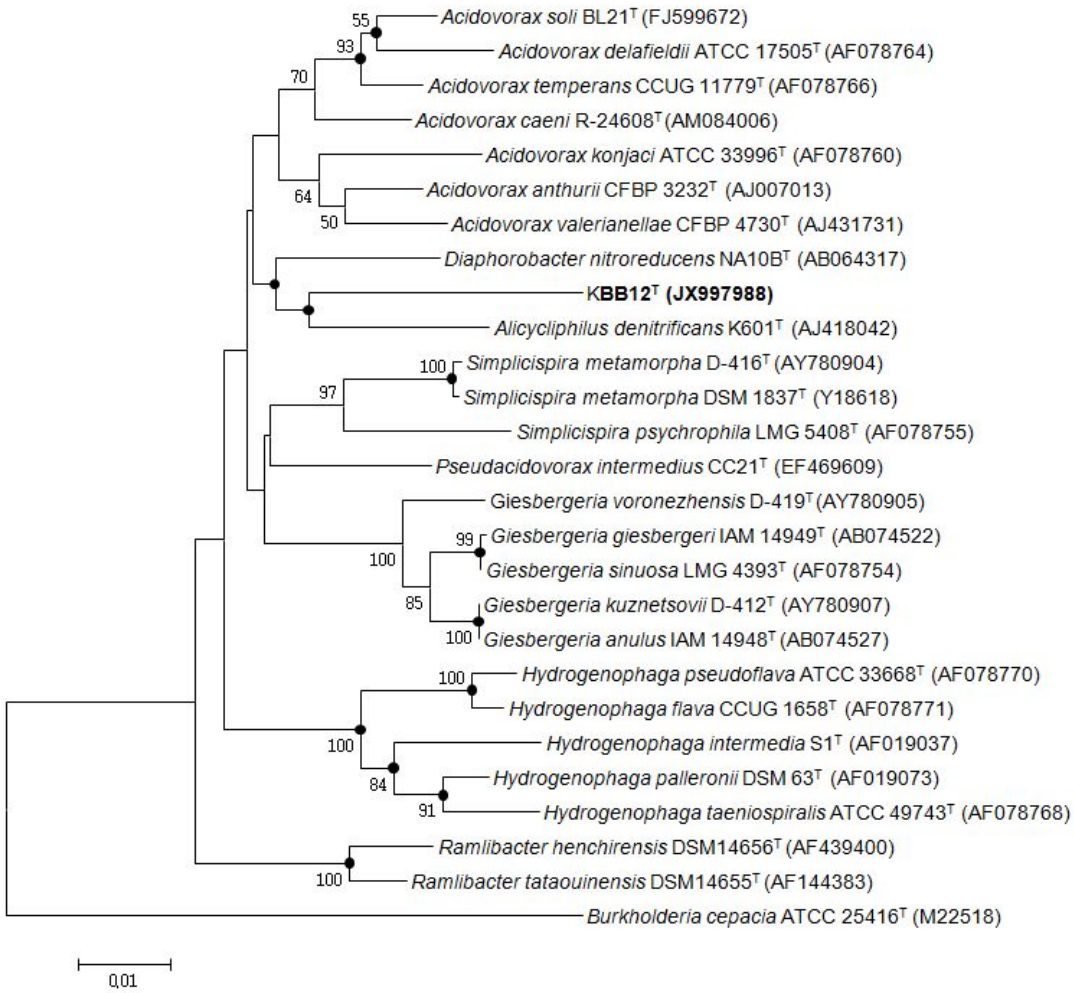

Fig. 2.1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain KBB12^T and members of the family *Comamonadaceae*. Bootstrap value (>50 %) based on 1,000 replications are shown. *Burkholderia cepacia* ATCC 25416^T was used as an out-group. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Genbank accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

Table 2.1. Differential characteristics of strain KBB12^T and related genera within the family *Comamonadaceae*

Strains: 1. KBB12^T; 2, *Alicycliphilus*; 3, *Diaphorobacter*; 4, *Acidovorax*. Data from Khan and Hiraishi (2002), Mechich *et al.* (2003) and Choi *et al.*, (2010). +, Positive; -, negative; v, variable; NA, not reported.

Characteristic	1	2	3	4
Motility	-	+	+	v
Temp.	30-37	30	28-35	28-30
Conditions for growth				
Oxygen	Facultative aanerobic	Facultative anaerobic	Aerobic	Aerobic
Utilization of :				
D-Fructose	-	-	+	+
D-Glucose	-	-	+	v
Glycerol	-	NA	+	+
β-Alanine	-	+	+	v
Malonate	+	+	-	v
Fumarate	-	+	+	v
Major fatty acid	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$ ω7*c*	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$ ω7*c*	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$ ω7*c*	$C_{16:0}$, $C_{18:1}$ ω7*c*
DNA G+C mol%	62.7	66	64-65	62-66
Source	swinery sludge	Waste water	Activated sludge	Soil, freshwater, clinical isolate

Table 2.2. Cellular fatty acid composition of strain KBB12^T and closely related genera

Strains: 1. KBB12^T; 2, *Alicycliphilus*; 3, *Diaphorobacter*; 4, *Acidovorax*. Data from Khan and Hiraishi (2002), Mechich *et al.,* (2003) and Choi *et al.*, (2010). Values are percentages of total fatty acids; -, not detected.

Fatty acid	1	2	3	4
$C_{10:0}$ 3-OH	4.8	4	4.6	3.1-8.5
$C_{12:0}$	3.5	4	2.7	3.2-7.8
$C_{14:0}$	1.5	2	2.2	1.5-7.4
$C_{16:0}$	27.7	24	29.2	23.5-32.1
$C_{17:0}$ cyclo	2.5	2	1.4	1.6-3.2
$C_{18:0}$	1.0	-	0.1	-
$C_{18:1}$ ω7*c*	16.5	21	18.1	11.7-22.6
summed feature 3*	40.5	37	40	31-43.1
summed feature 7*	1.4	-	8.7	-

* Summed feature 3 contained iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7*c* and summed feature 7 contained $C_{19:1}$ cyclo ω10*c* and/or $C_{19:1}$ ω6*c*.

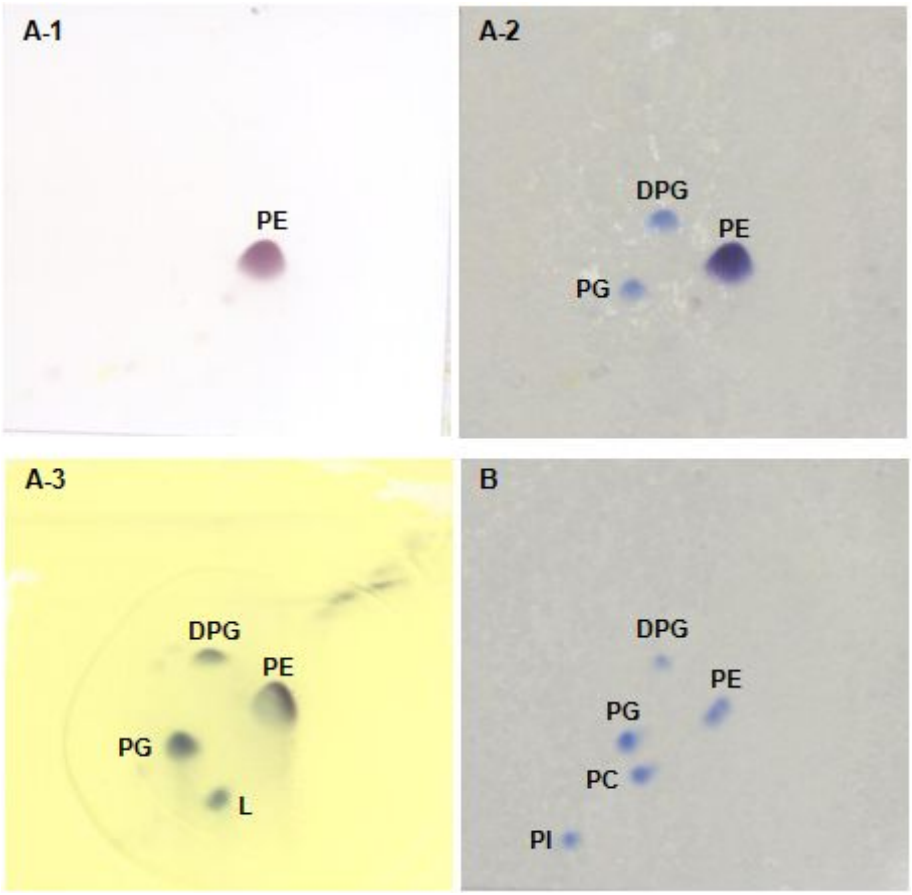

Fig. 2.2. Polar lipid profiles of strain KBB12^T (A) and the authentic standards used (B) after two dimensional thin-layer chromatography (TLC). PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; L, unknown lipid. Stained with ninhydrin reagent for detection of aminolipids (A-1), Zinzadze reagent for phospholipids (A-2) and 10% ethanolic molybdophosphoric acid for detection of the total polar lipids (A-3).

Description of *Thiobacterium* gen. nov., sp. nov.

Thiobacterium (Thi.o.bac.te.ri.um. Gr. n. thion sulfur; N.L. n. bacterium from Gr. n. bakterion rod; N.L. neut. n. *Thiobacterium* a rod-shaped bacterium oxidizing sulfur).

Cells are Gram-negative, facultative aerobic, non-motile rods, at 37 °C on TSA. Catalase- and oxidase-positive. Nitrate is reduced to nitrite and thiosulfate is oxidized to sulfate. Contain phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unknown lipid as polar lipids. The major isoprenoid quinone is ubiquinone-8 (Q-8). The DNA G+C content of the type strain of the type species is 62.7 mol% (HPLC). Phylogenetically, the genus belongs to the family *Comamonadaceae*. The type species is *Thiobacterium jejuense*.

Description of *Thiobacterium jejuense* sp. nov.

Thiobacterium jejuense (je.ju.en'se. N.L. neut. adj. *jejuense* pertaining to Jeju, Republic of Korea, from where the type strain was isolated).

Cell are oxidase and catalase positive, denitrifying, with overall dimensions of 0.5-0.7 ㎛ (width) by 2.0-3.5 ㎛ (length), as assessed in 3 days cultures grown at 37 °C on TSA. Optimum growths is observed at 30-37 °C, pH 7-8 and 0-1 % NaCl. Hydrolyses gelatin and Tween 20, 40, and 80 but does not hydrolyze casein, urea, Tween 60, tyrosine, aesculin and starch. Utilizes N-acetyl galatosamine, N-acetyl glucosamine, N-acetyl mannosamine, D-arabitol, adipate, glucuronamide, gluconate, *myo*-inositol, lactulose, malate, maltose, D-mannose, palatinose, raffinose, D-sorbitol, sucrose, bromosuccinic

acid, β-hydroxybutyric acid, malic acid, succinamic acid. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine -, valine -, cystine arylamidase and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, a-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α -fucosidase, arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Susceptible to ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), penicillinG (10 IU), polymyxinB (300 IU), and tetracycline (30 μg).

The major fatty acids found in strain KBB12^{T} were $C_{16:1}$ ω7c (40.5 %), $C_{16:0}$ (27.7 %) and $C_{18:1}$ ω7c (16.5 %). The polar lipids are phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and unknown lipid (L).

The type strain, KBB12^{T} (= KCTC 32230^{T}), was isolated from swinery sludge in Jeju, the Republic of Korea.

2.3.2. Strain BA15^T

The family *Rhodobacteraceae* (Garrity *et al*., 2005), which belongs to the class *Alpha-proteobacteria*, contains approximately 70 recognized genera (type genus, *Rhodobacter*). The family *Rhodobacteraceae* typically comprises phototrophic purple non-sulfur bacteria characterized by the presence of photosynthetic pigments. However, a few non-pigmented, non-phototrophic strains have been reported that phylogenetically branch within the radius of new genera *Pseudorhodobacter* (Uchino *et al*., 2002) and *Haematobacter* (Helsel *et al*., 2007).

A 16S rRNA gene sequence analysis revealed that strain BA15^T was classified in the family *Rhodobacteraceae* of the class *Alpha-proteobacteria*. A sequence similarity calculation using the EzTaxon server (http://www.EzTaxon.org/; Chun *et al*., 2007) indicated that the closest relatives of strain BA15^T were *Rhodobacter megalophilus* JA194^T (95.2 %), followed by members of the genera *Haematobacter* (95 %) and *Pseudorhodobacter* (94 %). Lower sequence similarities (95 %) were found with members of all other genera shown in Fig. 2.3 neighbour-joining phylogenetic tree confirmed the separate position of strain BA15^T. The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms also supported the notion that there was no genus group that showed a clear phylogenetic relationship with strain BA15^T in the family *Comamonadaceae*.

Strain BA15^T was aerobic, Gram-staining negative and rod-shaped. The colonies were grown on TSA agar plate for 3 days. The optimal condition for growth was 30 °C and pH 7.0. The physiological characteristics of strain BA15^T was summarized in the species description and a comparison of selected characteristics with related strains is shown in Table 2.3.

The major fatty acids of strain BA15^T were $C_{18:1}$ ω7*c* and $C_{10:0}$ 3-OH

consistent with members of the genera *Rhodobacter*, *Haematobacter* and *Pseudorhodobacter* (Table 2.5). The polar lipids profile of strain BA15^T contained phosphatidylcholine (PC), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), unknown aminophophoslipids (APL), unknown aminolipids (AL) and unknown lipids (L1-L3) (Fig. 2.4). The polar lipid content of strain BA15^T was similar to genus *Rhodobacter*, but was distinguishable from those of reference strains by the absence of the aminophophoslipids (APL), unknown aminolipids (AL) and unknown lipids (L1-L3).

Strain BA15^T belonged to the family *Rhodobacteraceae* and formed a distinct phyletic line with the clades of the ralated genera. Moreover, strain BA15^T was differentiated from members of the genus *Rhodobacter*, *Haematobacter* and *Pseudorhodobacter* by several phenotypic characteristics, including fatty composition, carbon utilization (Table 2.3 and 2.4) and polar lipid compositions (Fig. 2.4) Based on polyphasic data presented in this study, strain BA15^T represents a novel genus and species of the family *Rhodobacteraceae*, for which the name *Caenirhodobacter jejuensis* gen. nov., sp. nov. is proposed.

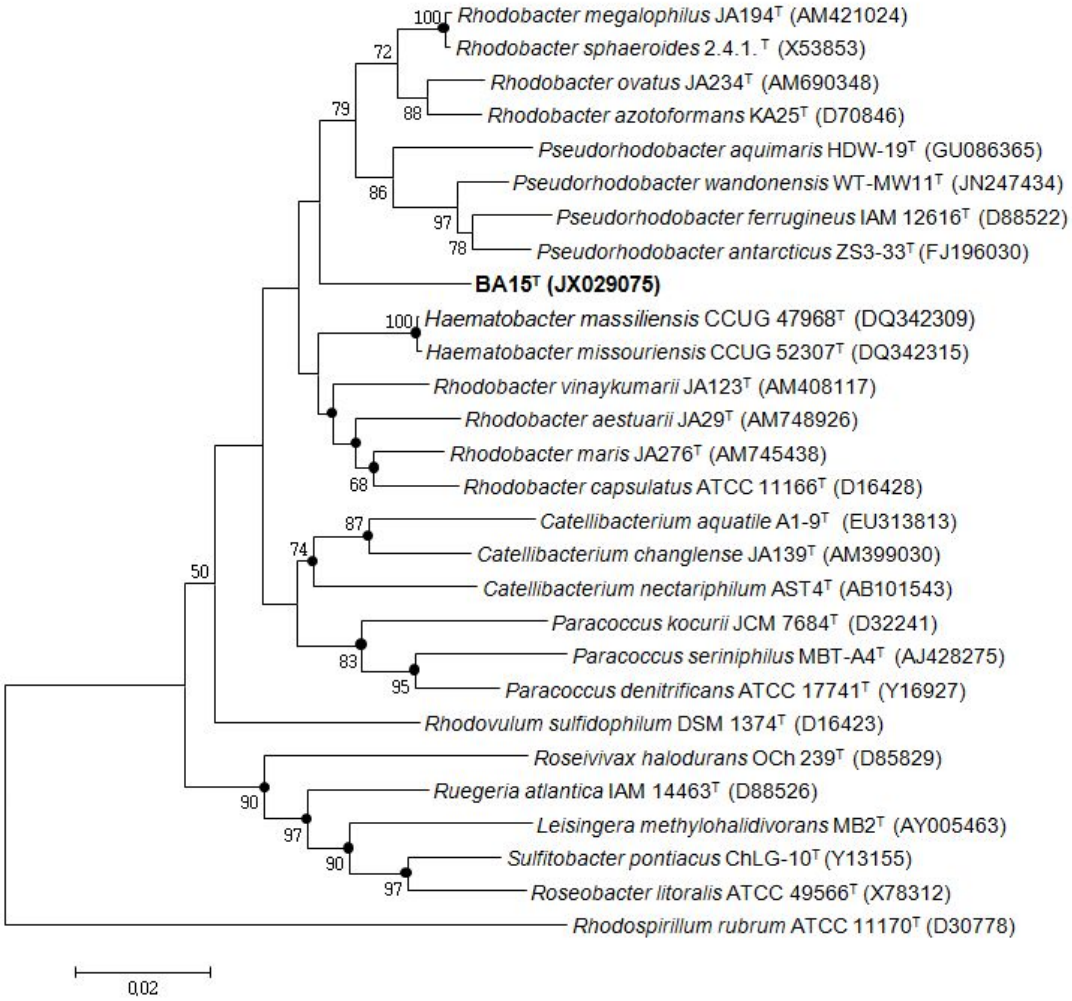

Fig. 2.3. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of strain BA15^T and members of the family *Rhodobacteraceae*. Bootstrap value (>50 %) based on 1,000 replications are shown. Filled circles indicate nodes also recovered reproducibly with maximum-likelihood. *Rhodospirillum rubrum* ATCC 11170^T was used as an out-group. Genbank accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position.

Table 2.3. Differential characteristics of strain BA15^T and related genera within the family *Rhodobacteraceae*

Strains: 1, BA15^T; 2, *Rhodobacter*; 3. *Pseudorhodobacter*; 4, *Haematobacter*. Data for reference strains were taken from Jung *et al*., (2002), Helsel *et al*., (2007) and Venkata *et al*., (2009). +, Positive; -, negative; v, variable.

Characteristic	1	2	3	4
Motility	-	v	-	-
NaCl requirement	-	-	+	-
Anaerobic photosysthetic growth	-	+	-	-
Utilization of :				
Maltose	-	v	-	-
Sucrose	+	v	-	-
D-Glucose	+	v	-	-
D-mannitol	+	v	v	-
D-xylose	-	v	-	-
DNA G+C mol%	51.6	62.8-73	56.6-58.0	65
Source	Swinery sludge	Fresh water, marine	Marine	Blood

Table 2.4. Cellular fatty acid composition of strain BA15^T with closely related genera

Strains: 1, BA15^T; 2, *Rhodobacter*; 3. *Pseudorhodobacter*; 4. *Haematobacter*. Data for reference strains were taken from Jung *et al*., (2002), Helsel *et al*., (2007) and Venkata *et al*., (2009). Values are percentages of total fatty acids; -, not detected.

Fatty acid	1	2	3	4
$C_{10:0}$ 3OH	12.6	2-6	2-4	2-11
$C_{12:0}$	0.9	-	-	-
$C_{16:0}$	2.2	2-7	1-2	2-15
$C_{17:0}$	0.9	-	0-1	11.00
$C_{18:0}$	5.0	1-7	3.2-4.5	1-2
$C_{18:0}$ 3OH	4.3	-	-	-
$C_{18:1}$ ω7*c*	69.5	60-80	81-86	56-90
$C_{18:1}$ ω9*c*	-	1-2	0-1.5	1.00
$C_{18:1}$ ω7*c* 11-methyl	2.9	-	-	-
$C_{19:0}$ cyc ω8*c*	-	4	0-1.6	3-20
$C_{19:0}$ 10-methyl	0.6	1-3	-	-
Summed feature 3*	1.2	1-9	0.8 - 3	1-11

*Summed feature 3 contained $C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2-OH.

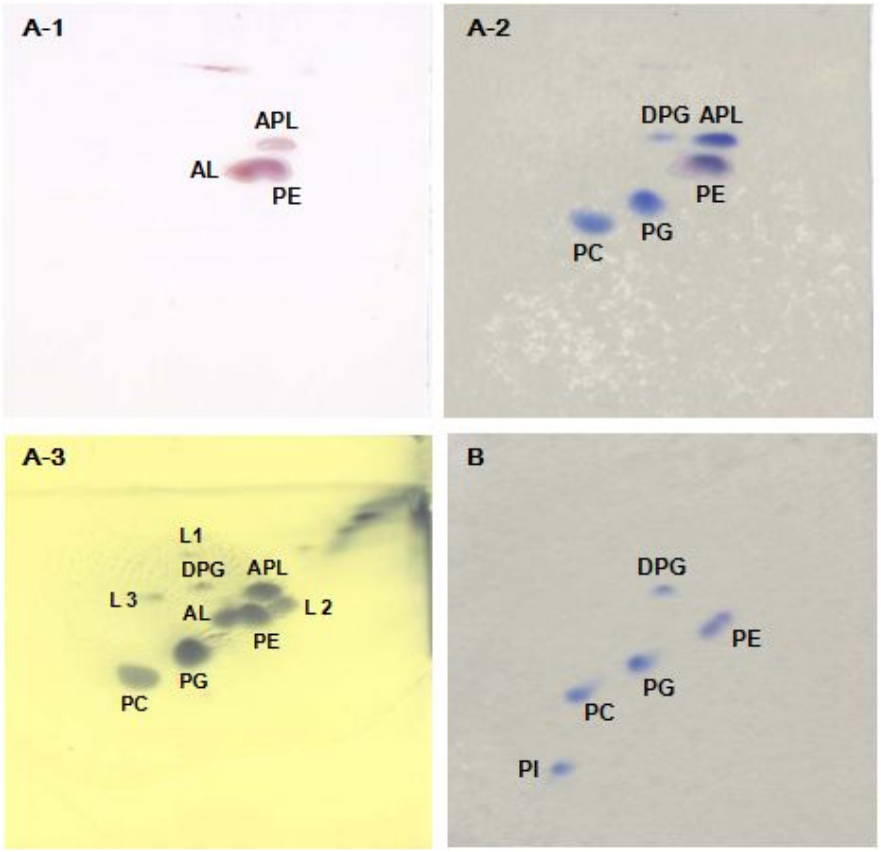

Fig. 2.4. Polar lipid profiles of strain BA15^T (A) and the authentic standards used (B) after two dimensional thin-layer chromatography (TLC). PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; APL, unknown aminophophoslipids; AL, unknown aminolipids; L1 - L3, unknown lipids. Stained with ninhydrin reagent for detection of aminolipids (A-1), Zinzadze reagent for phospholipids (A-2) and 10% ethanolic molybdophosphoric acid for detection of the total polar lipids (A-3).

Description of *Caenirhodobacter* gen. nov.

Caenirhodobacter (Cae'ni.rho.do.bac'ter. L. n. caenum mud, sludge; N.L. masc. n. *Rhodobacter*, a bacterial generic name; N. L. fem. n. *Caenirhodobacter* monad isolated from sludge).

Cells are Gram-negative, aerobic, non-motile rods, at 30 ℃ on TSA. Catalase- and oxidase-positive. Nitrate is reduced to nitrite and thiosulfate is oxidized to sulfate. Contain are diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) as polar lipids. The major isoprenoid quinone is ubiquinone-8 (Q-8). The DNA G+C content was 51.6 mol%. Phylogenetically, the genus belongs to the family *Rhodobacteraceae*. The type species is *Caenirhodobacter jejuensis*.

Description of *Caenirhodobacter jejuensis* sp. nov.

Caenirhodobacter jejuensis (je.ju.en'sis. N.L. fem. adj. *jejuensis* referring to Jeju Island in the Republic of Korea, where the type strain was isolated).

Cell are oxidase and catalase positive, denitrifying, with overall dimensions of 0.2-0.4 ㎛ (width) by 1.4-2.6 ㎛ (length), as assessed in 3 days cultures grown at 30 ℃ on TSA. Optimum growths is observed 25-30 ℃, pH 7-8 and on 0-1 % NaCl. Hydrolyses aesculin and DNase but does not hydrolyse casein, gelatin, Tween 20, 40, 60, 80 urea, starch or tyrosine. Utilizes arabinose, cellobiose, β-cyclodextrin, erythritol, glucose, glucuronamide, glycerol, *myo*-inositol, maltitol, maltotriose, melibiose, asicose, salicin, D-sorbitol, sorbose, stachyose, sucrose, tagatose, turanose, ɣ-aminobutyric acid, bromosuccinic acid, β-hydroxybutyric acid, ɣ-hydroxybutyric acid,

malate, saccharate, succinamate, succinate, alaninamide, aspartate, threonine, adenosine. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine-, valine-, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase but not lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, α-glucosidase, N-acetyl-b-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Susceptible to ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), naldic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), oleanomycin (15 μg), penicillin G (10 IU), polymyxinB (300 IU), rifamysin (5 μg), streptomycin (30 μg) and tetracycline (30 μg).

The major fatty acids found in strain BA15^{T} were $C_{18:1}$ ω7*c* (69.5 %) and $C_{10:0}$ 3OH (12.6 %). The polar lipids are diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG).

The type strain, BA15^{T} (= KCTC 32231^{T}= JCM 18654^{T}), was isolated from swinery sludge of in Jeju, the Republic of Korea.

2.3.3. Strains KBB4^T, and KBB8^T

The nearly complete sequences of the 16S rRNA genes KBB4^T, and KBB8^T, which were 1453 and 1443 nucleotides, respectively. Strains KBB4^T, and KBB8^T was classified in the family *Comamonadaceae* of the class *Beta-proteobacteria*. A sequence similarity calculation using the EzTaxon server (http://www.EzTaxon.org/; Chun *et al*., 2007) indicated tha the closest relatives of strains KBB4^T, and KBB8^T were *Comamonas testosteroni* ATCC 11996^T (98.6 %), *Comamonas odontotermitis* Dant 3-8^T (97.4 %) and *Comamonas composti* CC-YY287^T (96.4 %). The phylogenetic consensus tree clearly showed the relationship of strains KBB4^T, and KBB8^T to the entire type specied of the genus *Comamonas* (Fig. 2.5). In the phylogenetic trees reconstructed using both maximum-likelihood and maximum-parsimony algorithms, strains KBB4^T, and KBB8^T fell under the clade encompassing the genus *Comamonas*.

The physiological characteristics of strains KBB4^T, and KBB8^T were summarized in the species description and a comparison of selected characteristics with related strains shown in Table 2.5.

The major cellular fatty acids of strains were summed feature 3 ($C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2-OH) and $C_{16:0}$. Total fatty acid profile was similar to those of the type strains of *Comamonas* species, although there were differences in relative amounts of some fatty acids (Table 2.6). The predominant ubiquinone was Q-8. The major polar lipids found of strains KBB4^T, and KBB8^T were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE) (Fig. 2.6).

Based on the results of phenotypic and phylogenetic analyses, strains KBB4^T, and KBB8^T is considered to represent a new species of the genus *Comamonas*, the names *Comamonas jejuensis* sp. nov. and *Comamonas caeni* sp. nov. is proposed.

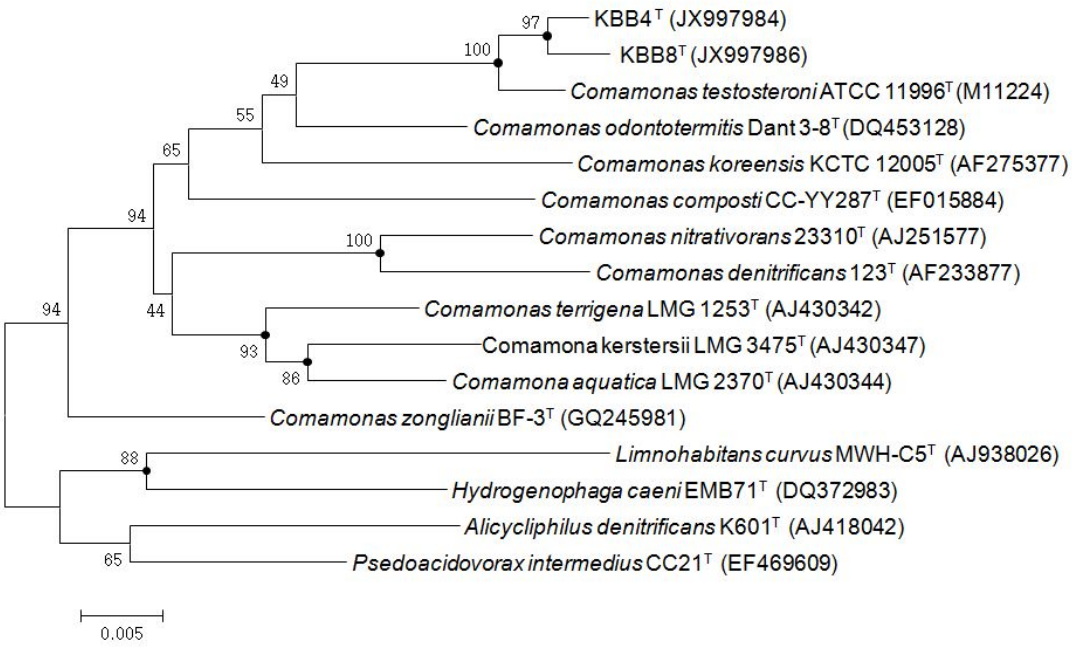

Fig. 2.5. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of strains KBB4^T, and KBB8^T, *Comamonas* species and representatives of some other related taxa. Bootstrap value (>50 %) based on 1,000 replications are shown. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Paracoccus versutus* ATCC 25364^T was used as an out-group. Genbank accession numbers are given in parentheses. Bar, 0.005 substitutions per nucleotide position.

Table 2.5. Differential characteristics among strains KBB4^T, and KBB8^T closely related type strains of the genus *Comamonas*

Strains: 1, KBB4^T; 2, KBB8^T; 3, *C. testosteroni* ATCC 11996^T; 4, *C. odontotermitis* LMG 23579^T; 5, *C. composti* LMG 24008^T; 6, *C. koreensis* KCTC 12005^T. Data from Chang *et al.*, (2002), Chou *et al.*, (2007) and Young *et al.*, (2008). +, positive; −, negative.

Characteristic	1	2	3	4	5	6
Source	Swinery sludge	Swinery sludge	Soil	Termite gut	Compost	Wetland
Motility	+	+	+	+	+	−
Nitrite reduction to nitrogen	+	+	−	−	−	−
Assimilation of :						
D-Gluconate	−	−	+	+	+	+
Adipate	−	−	+	−	+	+
Caprate	−	+	+	−	−	−
Citrate	−	−	+	+	−	+
Oxidation of :						
ɣ-Hydroxyburate	−	+	−	+	+	+
L-Threonine	−	−	+	+	−	+
Glycyl L-asparate	−	+	+	+	−	−
N-Acetyl-D-glutamate	+	+	−	−	−	−
Tween 80	−	+	+	+	+	+
Susceptibility to:						
Ampicillin	−	−	−	−	+	−
Gentamicin	+	+	+	−	−	−
Rifampicin	−	−	+	−	+	+
Streptomycin	+	−	+	−	−	+
DNA G+C mol%	61.3	62	62.5	61.6	62.8	66

Table 2.6. Cellular fatty acid composition of strains KBB4^T, and KBB8^T with closely related species

Strains: 1, KBB4^T; 2, KBB8^T; 3, *C. testosteroni* ATCC 11996^T; 4, *C. odontotermitis* LMG 23579^T; 5, *C. composti* LMG 24008^T; 6, *C. koreensis* KCTC 12005^T. Data from Chang *et al.*, (2002), Chou *et al.*, (2007) and Young *et al.*, (2008). Values are percentages of total fatty acids; -, not detected.

Fatty acid	1	2	4	5	6	7
$C_{10:0}$ 3-OH	6.1	10.8	4.8	3.8	5.6	3.5
$C_{12:0}$	5.2	6.1	2.4	2.7	3.2	2.3
$C_{14:0}$	0.7	1.1	1.0	1.2	1.4	1.0
$C_{15:0}$	3.1	0.7	1.0	-	-	9.4
$C_{15:0}\omega6c$	1.3	0.2	-	-	-	-
$C_{16:0}$	34.1	23.2	30.4	33.6	33.3	29.9
$C_{16:0}$ 2-OH	4.3	4.4	2.0	2.5	-	2.2
$C_{16:1}$ 2-OH	-	2.3	0.8	-	-	-
$C_{17:0}$	1.0	-	0.8	-	1	2.6
$C_{17:0}$cyclo	10.5	8.7	3.8	5.9	1.4	12.3
Summed feature 3*	26.5	33.5	33.1	33.9	40.8	26.1
Summed feature 8*	6.7	8.1	17.9	16.2	12.9	8.7

*Summed feature 3 contains $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; Summed feature 8 contains $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$.

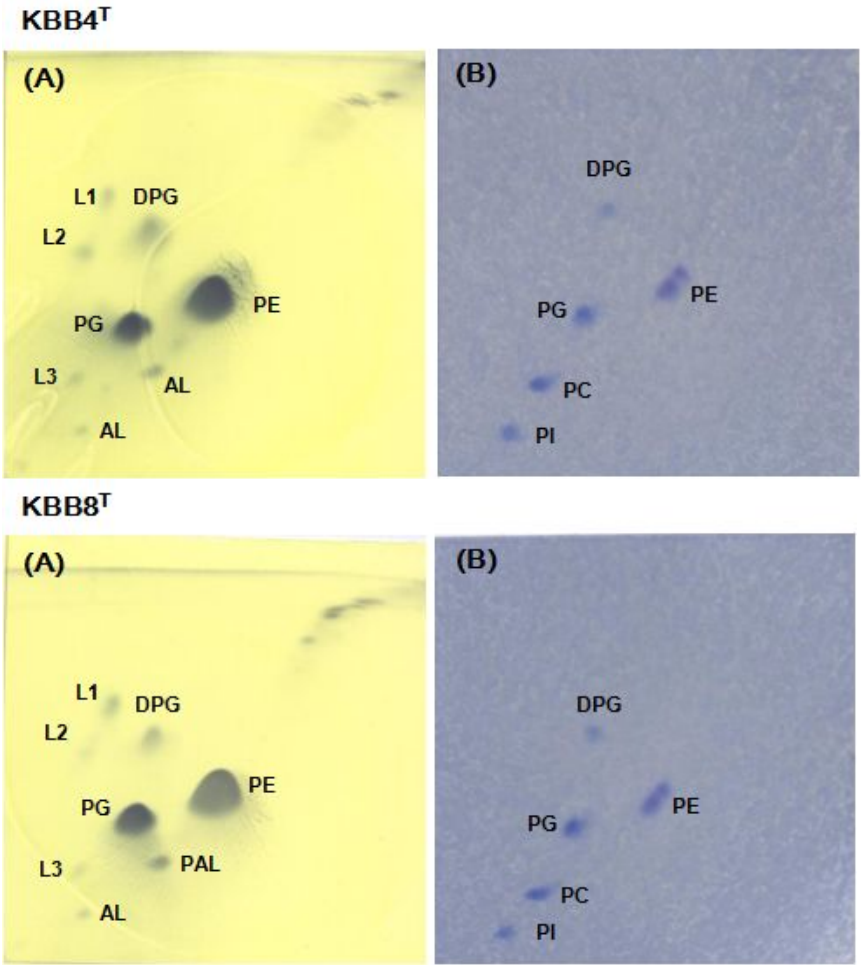

Fig. 2.6. Polar lipid profiles of strains KBB4^T, and KBB8^T (A) and the authentic standards used (B) after two dimensional thin-layer chromatography (TLC). PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; APL, unknown aminophophoslipids; AL, unknown aminolipids; L1 - L3, unknown lipids.

Description of *Comamonas jejuensis* sp. nov.

Comamonas jejuensis (je.ju.en′sis. N.L. fem. adj. jejuensis referring to Jeju Island in the Republic of Korea, where the type strain was isolated).

Cell are oxidase and catalase positive, denitrifying, with overall dimensions of 0.5-0.6 ㎛ (width) by 1.0-2.5 ㎛ (length), as assessed in 2 days cultures grown at 37 °C on TSA plate. Optimum growths is observed 30-37 °C, pH 7-8 and on 0-3 % NaCl. Hydrolyse acetoin, casein, DNase, gelatine and starch but does not hydrolyse urea, Tween 20, 40, 60, 80, aesculin or tyrosine. Nitrate is reduced. Anaerobic growth does not occur on TSA, but does occur on TSA supplemented with potassium nitrate. The following compounds are utilized as the sole carbon source: N-acetyl-D-glucosamine, D-arabitol, maltose, palatinose, D-psicose, D-raffinose, D-rhamnose, sorbose, sedoheptulos, stachyose, D-xylose, ɣ-aminobutyric acid, β-hydroxybutyric acid, lactate, malate, succinate and alaninamide. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine-, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Susceptible to cephalothin (30 ㎍), chloramphenicol (30 ㎍), erythoromycin (15 ㎍), gentamicin (10 ㎍), naldic acid (30 ㎍), neomycin (30 ㎍), novobiocin (30 ㎍), oleanomycin (15 ㎍), polymyxinB (300 IU), sterptomycin (10 ㎍) and tetracycline (30 ㎍). The major fatty acids (>10 % of the total) of strain were $C_{16:0}$ (34.1 %), Summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 26.5 %) and $C_{17:0}$ cyclo (10.5 %). The polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE), two aminolipids and three unkonown lipids. The DNA G+C content was 61.3 mol%.

The type strain, KBB4^T (= KCTC 32226^T), was isolated from swinery sludge of in Jeju, the Republic of Korea.

Description of *Comamonas caeni* sp. nov.

Comamonas caeni (ca.′ni. L. gen. n. caeni of mud, referring swinery sludge, from where the type strain was isolated).

Cell are oxidase and catalase positive, denitrifying, with overall dimensions of 0.3-0.5 ㎛ (width) by 1.0-1.5 ㎛ (length), as assessed in 2 days cultures grown at 30 °C on TSA. Optimum growths is observed 30-37 °C, pH 7-8 and on 0-3 % NaCl. Hydrolyses acetoin and tween 80 but does not hydrolyse casein, DNase, Tween 20, 40, 60 and starch. Nitrate is reduced. The following compounds are utilized as sole carbon source: N-acetyl-D-glucosamine, D-arabitol, N-acetyl-D-mannosamine, maltose, mannitol, mannose, melibios, D-psicose, D-raffinose, ribose, salicin, sorbitol, sorbose, sucrose, D-xylose, β-hydroxybutyric acid, lactate, malate, succinate, Itaconate, asparagine, aspartate, glutamate, putescine and adenosine. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine-, valine arylamidase, acid phospatase and naphthol-AS-BI- phosphohydrolase. Susceptible to cephalothin (30 ㎍), chloramphenicol (30 ㎍), erythoromycin (15 ㎍), gentamicin (10 ㎍), naldic acid (30 ㎍), neomycin (30 ㎍), novobiocin (30 ㎍), oleanomycin (15 ㎍), polymyxinB (300 IU), sterptomycin (10 ㎍) and tetracycline (30 ㎍).

The major fatty acids (>10 % of the total) of strain were Summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 33.5 %), $C_{16:0}$ (23.2 %) and $C_{10:0}$3-OH (10.8 %). The DNA G+C content was 62 mol%.

The type strain, KBB8^T (= KCTC 32227^T), was isolated from swinery sludge of in Jeju, the Republic of Korea.

2.3.4. Strain KBB11^T

The 16S rRNA gene sequence of strain KBB11^T comprosed 1487 nucleotides and revealed that strain KBB11^T was classified in the family *Comamonadaceae* of the class *Beta-proteobacteria*. A sequence similarity calculation using the EzTaxon server (http://www.EzTaxon.org/; Chun *et al*., 2007) indicated tha the closest relatives of strain KBB11^T were *Hydrogenophaga bisanensis* K102^T (97.6 %), *Hydrogenophaga flava* CCUG 1658^T (96.5 %) and *Hydrogenophaga pseudoflava* ATCC 33668^T (96.5 %). The phylogenetic consensus tree clearly showed the relationship of strain KBB11^T to the entire type specie of the genus *Hydogenophaga* (Fig. 2.7). In the phylogenetic trees reconstructed using both maximum-likelihood and maximum-parsimony algorithms, strain KBB11^T fell under the clade encompassing the genus *Hydrogenophaga*.

Strain KBB11^T was aerobic, Gram negative, rod-shape and the colonies were grown on TSA agar plate for 3 days were pale yellow, circles, 0.5-1 mm in diameter. The physiological characteristics of strain KBB11^T was summarized in the species description and a comparison of selected characteristics with related strains shown in Table 2.7.

The major cellular fatty acids (>10 % of the total) of strain were Summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 38.9 %) and $C_{16:0}$ (23.7 %). Total fatty acid profile was similar to those of the type strains of *Hydogenophaga* species, although there were differences in relative amounts of some fatty acids (Table 2.8). The polar lipids found of strain KBB11^T were phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), and aminophospholipids (PAL1) and unknown lipids(L1-L2) (Fig. 2.8).

Based on the results of phenotypic and phylogenetic analyses, strains KBB11^T is considered to represent a new species of the genus *Hydogenophaga*, the name *Hydogenophaga thiooxydans* sp.nov., is proposed.

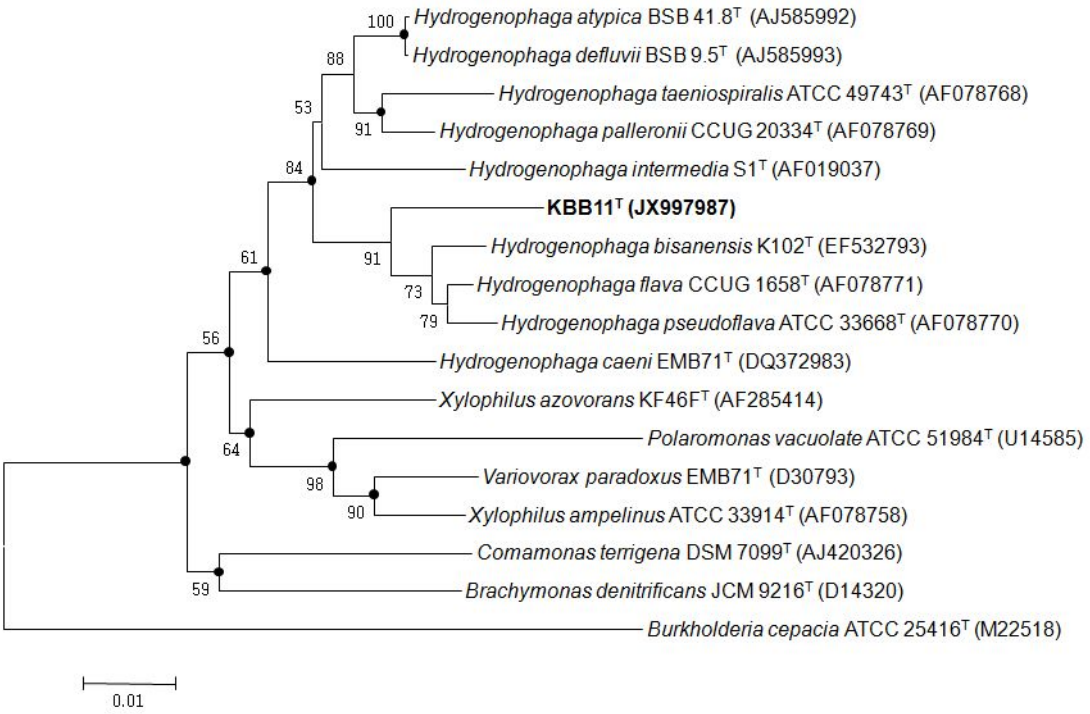

Fig. 2.7. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of strain KBB11^T, *Hydrogenophaga* species and representatives of some other related taxa. Bootstrap value (> 50 %) based on 1,000 replications are shown. Filled circles indicate nodes also recovered reproducibly with maximum-likelihood. *Burkholderia cepacia* ATCC 25416^T was used as an out-group. Genbank accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

Table 2.7. Differential characteristics of strain KBB11^T closely related type strains of the genus *Hydrogenophaga* species

Strains: 1, KBB11^T; 2, *H. bisanensis* K102^T; 3, *H. flava* DSM 619^T; 4, *H. pseudoflava* LMG 5945^T. Data for reference strains were taken from Yoon *et al.* (2008).

Characteristic	1	2	3	4
Cell size (um)	0.3-0.5	0.4-0.6	0.5	0.5
Cell size (um)	1.9-2.5	1.0-5.0	1.0-2.0	1.0-2.5
Temp.	30-37	30-37	30	35-38
Growth at 40 ℃	+	+	-	+
Denitrification	+	+	-	+
Utilization of :				
L-Arabionse	-	-	+	+
Adipate	-	+	-	+
cellobiose	-	-	+	+
D-Fructose	-	+	+	+
D-Galactose	-	-	+	+
D-Mannose	-	-	+	+
Mannitol	-	-	+	+
Maltose	+	-	+	+
D-xylose	-	-	-	+
DNA G+C mol%	63.2	64.8	67	66

Table 2.8. Cellular fatty acid composition of strain KBB11^T with closely related species

Strains: 1, KBB11^T; 2, *H. bisanensis* K102^T; 3, *H. flava* DSM 619^T; 4,, *H. pseudoflava* LMG 5945^T.

Fatty acids that represented <0.5 % in all strains were omitted. −, Not detected; tr, trace amount (<0.1 % of total fatty acids). Data for reference strains were taken from Yoon et al. (2008).

Fatty acid	1	2	3	4
Saturated				
$C_{12:0}$	5.2	-	-	-
$C_{14:0}$	5.3	tr	3.6	3.5
$C_{15:0}$	0.6	1.3	1.1	1.1
$C_{16:0}$	23.7	49.8	25.5	25.5
$C_{17:0}$	-	1.2	1.4	1.4
$C_{17:0}$ cyclo	7.7	tr	tr	2.3
Unsaturated				
$C_{16:1}$ ω5c	1.6	tr	-	tr
$C_{17:1}$ ω6c	-	tr	1.2	-
$C_{17:1}$ ω8c	-	0.5	-	1.2
$C_{18:1}$ ω7c	5.7	7.9	-	-
Hydroxy				
$C_{8:0}$ 3-OH	tr	0.8	1.2	tr
$C_{10:0}$ 3-OH	4.9	-	3.5	3.8
Summed feature 3*	38.9	35.1	44.7	46.2
Summed feature 6*	-	-	16.9	12.6
Summed feature 7*	5.7	1.6	-	-

*Summed feature 3 contained $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; Summed feature 6 contained $C_{18:1}\omega7c$, $C_{18:1}\omega9t$ and/or $C_{18:1}\omega12t$; Summed feature 7 contained unknown fatty acid (ECL) 18.846, $C_{19:1}\omega6c$ and/or cyclo $C_{19:0}\omega10c$.

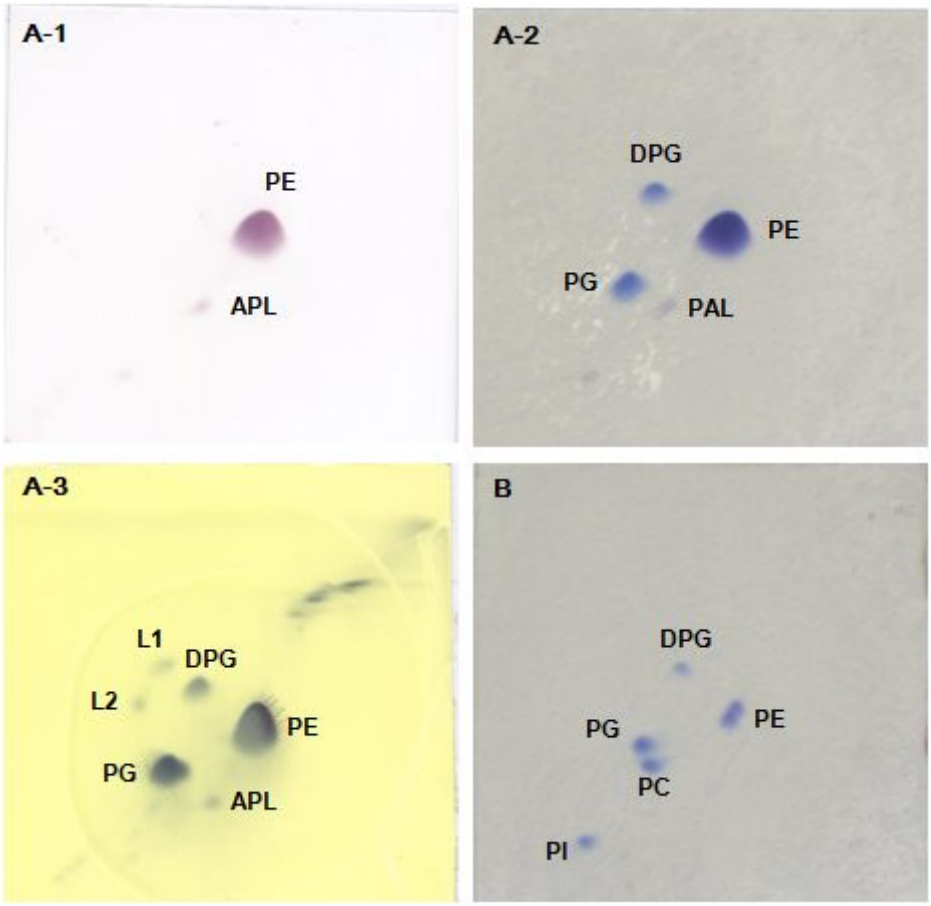

Fig. 2.8. Polar lipid profiles of strain KBB11^T (A) and the authentic standards used (B) after two dimensional thin-layer chromatography (TLC). PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; APL, unknown aminophophoslipids; L1 - L2, unknown lipids. Stained with ninhydrin reagent for detection of aminolipids (A-1), Zinzadze reagent for phospholipids (A-2) and 10 % ethanolic molybdophosphoric acid for detection of the total polar lipids (A-3).

Description of *Hydrogenophaga thiooxydans* sp. nov.

Hydrogenophaga thiooxydans (thi.o.ox'y.dans. Gr. n. *thion* sulfur; N.L. v. *oxydo* to make acid, to oxidize; N.L. part. adj. thiooxydans oxidizing sulfur).

Cell are oxidase and catalase positive, denitrifying, with overall dimensions of 0.3-0.5 ㎛ (width) by 1.9-2.5 ㎛ (length), as assessed in 2 days cultures grown at 30 ℃ on TSA plate. Optimum growths is observed 30-37 ℃, pH 7-8 and 0-2 % NaCl. Hydrolyse starch but does not hydrolyse casein, urea, Tween 20, 40, 60 and 80. Nitrate is reduced.

The following compounds are utilized as sole carbon source: D-arabitol, α-cyclodextrin, dextrin, 2-keto-D-gluconic acid, lactulose, maltose, palatinose, D-psicose, D-raffinose, sorbitol, sorbose, Stachyose, β-hydroxybutyric acid, ɣ-methyl-D-galactoside, α-ketoglutaric acid, lactate, malate, succinate, alanine, alaninamide, asparagine, ornithine and threonine. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but not trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, a-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Susceptible to cephalothin (30 ㎍), chloramphenicol (30 ㎍), erythoromycin (15 ㎍), gentamicin (10 ㎍), nalidixic acid (30 ㎍), neomycin (30 ㎍), novobiocin (30 ㎍), penicillinG (10 IU), polymyxinB (300 IU), streptomycin (10 ㎍) and tetracycline (30 ㎍). The major fatty acids (>10 % of the total) found in strain KBB12^{T} were Summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{16:0}$..

The type strain, KBB11^{T} (= KCTC 32229^{T}), was isolated from swinery sludge of in Jeju, the Republic of Korea.

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국문 초록

돈사오니에서 유황화합물의 분해 세균의 다양성과 신규 균주의 특성

본 연구는 양돈장에서 발생하는 황화합물의 악취를 효과적으로 제거 할 수 있는 세균을 탐색하기 위하여 돈분오니에서 유황화합물을 분해하는 세균을 분리하고 그 특성을 조사하였다. 황화합물 분해 세균은 thiosulfate가 함유된 배지를 이용하여 분리하였으며, 분리된 세균의 분포는 16S rRNA 유전자 서열로 계통학적 분석하였다. 유황화합물 분해능에 관여하는 *soxB* 유전자를 PCR 기법을 이용하여 황화합물 분해 균주를 선발하고 thiosulfate 산화능을 조사하였다.

제주 지역 5군데의 양돈장 분뇨 오니에서 총 351개의 균주가 분리하였고, 16S rRNA 유전자 서열을 비교 분석한 결과, *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria*, *Becteriodetes* 그리고 *Bacillia* 등 6 class와 16 order 및 suborder, 23 family, 48 genus으로 나타났다. 분리된 균주 중에 sulfur-oxidizing 세균은 *Comamona*s, *Paracoccus*, *Pseudomonas* 속 등으로 *Proteobacteria* 에 속하였다.

돈사에서 분리된 균에서 *soxB* gene을 갖는 균주는 총 13개로 16S rRNA 유전자 분석결과, *Acinetobacter*, *Alicycliphilus, Comamonas, Hydrogenophaga, Paracoccus, Pseudomonas* 그리고 *Rhodobacter* 속에 속하였다. *soxB* gene의 amino acid 서열로 계통 분석한 결과, *Alpha-proteobacteria*에 9균주, *Beta-proteobacteria* 4균주가 속하며, BLAST 검색 결과 *Comamonas*, *Methylibium*, *Paracoccus* 그리고 *Thiobacillus* 속에 75-95 %의 유사도를 나타내었다. Thiosulate 산화능의 측정에서는 BB11 균주가 175.5 μg/mL로 가장 높은 sulfate을 형성하였고 그 다음으로는 BB12 균주가 128.2 μg/mL 로 형성하였다.

돈사 오니에서 분리된 균주 중 5개의 균주가 genotypic 및 phenotypic 특성에

따라서 새로운 속이나 종으로 보인다. KBB12^{T} 균주는 *Comamonadaceae* 과의 새로운 속으로서 *Thiobacterium jejuense* 로 명명하였고, BA15^{T} 균주는 *Rhodobacteraceae*에 속하는 새로운 속으로서 *Caenirhodobacter jejuensis* 로 명명하였다. KBB4^{T} 와 KBB8^{T}는 *Comamonas* 속의 새로운 종으로 *Comamonas jejuensis*와 *Comamonas caeni*고, KBB11^{T} 균주는 *Hydrogenophaga* 속의 새로운 종으로 *Hydrogenophaga thiooxydans* 로 명명하였다.

본 연구의 결과를 통하여 현안문제인 제주지역 내 가축분뇨 처리를 위한 기초 데이터로 활용하며, 황산화 세균을 가축분뇨로부터 발생되는 오염물질을 저감하는데 이용함으로써 환경의 질을 향상시키고 더 나아가 제주도의 청청이미지의 더욱 부각 시킬 수 있을 것이라 사료된다. 또한 분리된 황화합물의 제거에 대한 황산화 세균의 대사과정과 역할에 대해 더 많은 연구가 이루어져야 할 것이다.

감사의 글

끝이 보이지 않던 시간동안 많은 깨달음과 가르침을 주신 모든 분들게 지면으로나마 감사의 말씀 드립니다.

우선 이 논문이 완성되기까지 부족한 저에게 많은 관심과 격려해주신 오덕철 교수님께 진심으로 감사드립니다. 그리고 바쁘신 와중에도 미흡한 논문을 세심하게 다듬어 주신 김세재 교수님, 허문수 교수님, 김명숙 교수님과 강형일 교수님께 깊은 감사 말씀 드립니다. 그리고 많은 가르침을 주시고 격려해주신 김문홍 교수님, 이화자 교수님, 고석찬 교수님과 이선령 교수님께 감사의 마음을 전합니다.

박사과정동안 관심과 조언으로 많은 도움을 주신 미생물학 실험실에 감사의 말을 전합니다. 부족한 저에게 많은 가르침을 주셨던 이동헌 선생님, 병준 오빠, 그리고 후돈, 까탈스러운 선배의 짜증도 다 받아주는 한수, 가영, 지현에게도 고맙고, 수고 했다는 말을 함께 전하고 싶습니다.

학위과정 내내 의지하며 도와준 오대주 박사, 강성일 박사, 유경, 후배 은영, 혜선 그 외의 생물학과 모든 대학원생... 그리고 사회생활 하면서 인연을 맺으며 이 논문을 위해서 열정적으로 도와주시고 격려해주신 언니 같은 김영주 박사님, 많은 이야기를 나눌 수 있었던 송관필 박사님, 같이 고생한 소현, 정민씨, 효선에게도 감사의 말을 전함과 동시에 앞으로도 더 좋은 연구 성과를 기대합니다.

마지막으로 힘든 시간 동안 포기하지 않도록 지켜봐주고 힘 복돋아 준 사랑하는 남편과 항상 사랑과 정성으로 보살펴 주신 부모님과 동생, 언니와 형부 그리고 늘 격려해주시는 시부모님께 말로 다 표현할 수 없는 고마움과 사랑의 마음을 전하며, 열심히 살아가는 모습으로 보답하겠습니다. 사랑합니다.

감사합니다.