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**Article**

# Isolation and Characterization of Sulfur-oxidizing Denitrifying Bacteria Utilizing Thiosulfate as an Electron Donor

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**Sulfur-oxidizing bacteria were enumerated and isolated from a steady-state anaerobic master culture reactor (MCR) operated for over six months under a semi-continuous mode and nitrate- limiting conditions using thiosulfate as an electron donor.Most are Gram-negative bacteria, which have sizes up to 12 m. Strains AD1 and AD2 were isolated from the plate count agar (PCA), and strains BD1 and BD2 from the solid thiosulfate/nitrate medium. Based on the morphological, physiological, FAME and 16S rDNA sequence analyses, the two dominant strains, AD1 and AD2, were identified as *Paracoccus denitrificans* and *Paracoccus versutus* (formerly *Thiobacillus versutus*), respectively. From the physiological results, glucose was assimilated by both strains AD1 and AD2. Heterotrophic growth of strains AD1 and AD2 could be a more efficient way of obtaining a greater amount of biomass for use as an inoculum. Even though facultative autotrophic bacteria grow under heterotrophic conditions, autotrophic denitrification would not be reduced.**

**Key words: Autotrophic denitrification, Sulfur-oxidizing bacteria, Denitrification, Thiobacillus**

## Introduction

Denitrification is carried out by either using heterotrophic or autotrophic bacteria. Many heterotrophic bacteria can reduce nitrate by utilizing organic substrates for the conversion of nitrate to free nitrogen gas under anoxic conditions while autotrophic bacteria use $H_2$ or a variety of reduced S compounds ($S^{2-}$, $S^{o}$, $S_2O_3^{2-}$, $S_4O_6^{2-}$-, $SO_3^{2-}$) instead of organic compounds as an electron donor. Heterotrophic denitrification is very efficient in terms of nitrate removal, provided adequate amounts of organic carbon are available (Flere and Zhang, 1999; Zhang and Lampe, 1999).

However, when organic carbon in the wastewater is insufficient compared to the nitrogen content, expensive chemicals, like methanol or similar organic compounds, must be added these kinds of wastewaterswould be landfill leachate, animal wastewater, and several industrial wastewaters. The rising cost and increasing scarcity of methanol and similar organic compounds make them increasingly undesirable as chemical additives. Furthermore,when heterotrophic bacteria are used, denitrification can produce excessive biomass and soluble microbial products that require subsequent treatment.For this reason, sulfur-based autotrophic denitrification has been receiving more attention recently. However, autotrophic denitrification increases sulfate concentration and consumes alkalinity in the wastewater. When wastewaters containing very high nitrate concentrations are treated, significant sulfate production might become a problem due to sulfide production if anaerobic conditions develop.

Colorless sulfur bacteria are able to oxidize reduced sulfur compounds, such as sulfides, sulfur, thiosulfate, polythionates and thiocyanate. These bacteriaare physiologically classified into four types: obligately chemolithoautotrophs, facultative chemolithoautotrophs, chemolithoheterotrophs and heterotrophs. Among them, many bacteria, such as *Thiobacillus denitrificans, Thiomicrospira denitrificans, Thiobacillus versutus, Thiobacillus thyasiris, Thiosphaera pantotropha,* and *Paracoccus denitrificans,* can reduce nitrate.

Facultative chemolithoautotrophs are apparently adaptable to different environments since they are capable of both autotrophic and heterotrophic growth (Matin, 1978). In engineering aspects, many researchers have used sulfur particles or thiosulfate as an electron donor to remove nitrate and have employed a mixed culture as an inoculum (Koenig et al., 1996; Batchelor et al., 1978;

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Flere and Zhang, 1999; Claus and Kutzner, 1985; Koenig and Liu, 1996; Zhang and Lampe, 1999). However, little research has been done on biological studies, such as the isolation, characterization, and identification of bacteria dominant in sulfur denitrification processes.

In a previous study, the kinetics and physiology of the mixed culture of autotrophic denitrifying sulfur bacteria were conducted by using a steady-state anaerobic master culture reactor (MCR) operated semi-continuously for over six months under nitrate-limiting conditions using thiosulfate as an electron donor (Oh et al., 2000). In this study, we isolated, enumerated, and identified dominant strains from the steady-state anaerobic master culture reactor.

## Materials and Methods

**Media** The medium used for the enrichment and cultivation of autotrophic sulfur-oxidizing bacteria under denitrifying conditions was the *Thiobaccillus denitrificans* medium. It contained (per liter) 5 g $Na_2S_2O_3 \cdot 5H_2O$; 2 g $KNO_3$ 1 g $NH_4Cl$; 2 g $KH_2PO_4$ 0.8 g $MgSO_4 \cdot 7H_2O$; 2 g $NaHCO_3$ and 1 ml trace metal solution (Atlas, 1993). After being mixing thoroughly, this medium was sterilized by being autoclaved for 15 min at 15 psi pressure-121℃ and adjusted to pH 7. For the isolation of a single colony, the enrichment culture was spread on a solid medium containing 2 % agar and cultured in an anaerobic jar (Difco. Inc.) in the dark for a week. The medium for the heterotrophic and facultative autotrophic bacteria was plate count agar (PCA) (Difco. Laboratories, MI, U.S.A.), and the pH was 6.8-7.0.

**Enumeration and isolation of microorganisms** To numerate quantitatively the number of viable cells in the MCR, a serial dilution-agar planting technique was used. After diluting the MCR culture with 1% phosphate-buffered saline (PBS pH 7.2-7.4), the diluted sample was distributed uniformly on the *Thiobacillus denitrificans* medium and PCA plates with a sterile spreader. Plates were incubated in the dark in an anaerobic chamber at 33-35℃, and some ofthe main bacteria were isolated on the solidified media.

**Microscopy** Approximately 50 ㎕ of diluted pure culture isolated from MCR sludge was Gram stained, and pictures of the strains were taken with a Nikon Microphoto FXA microscope. In addition, bright-field and phase-contrast photomicrographs of the main microorganisms in the MCR were taken. Motility of each pure culture was determined using a microscopy.

**Denitrifying ability** The ability of the strains to denitrify was determined under autotrophic and heterotrophic conditions using a medium supplemented with sodium thiosulfate (5 g $Na_2S_2O_3 \cdot 5H_2O$/l) and nitrate (2 g $KNO_3$/l), or nitrate broth. The nitrate broth medium consisted of 5.0 g/l peptone, 3.0 g/l beef extract, and 5 g/l $KNO_3$, and the pH was 7.2. Each sterile Durham tube was filled with sterile nitrate broth and *Thiobaccillus denitrificans* liquid medium and was incubated at 35℃ under anaerobic conditions for 6 days. To characterize whether the strains were anaerobic or facultative, a shake-tube having media containing resazurin was inoculated and incubated for 6 days. The distributions of the cells were then determined and compared to the color distribution in the tube, an indicator of the presence of oxygen.

**Identification of sulfur-oxidizing bacteria** Some sulfur-oxidizing bacteria were isolated from the MCR. Two dominant strains, designated as strains AD1 and AD2, were selected and maintained on the solid medium for further study.

The isolates were identified on the basis of morphological, physiological, FAME and phylogenetic analyses. The physiological test was carried out using the commercially available API 20 NE rapid test kit (BioMerieux S. A., France). FAME profiles were analyzed according to the MIDI system (Microbial ID, Inc., Newark, Del.). The 16S rDNA sequencing of the isolates was performed as described previously (Yoon et al., 1998).Multiple alignments of sequences were analyzed by the ClustalX program (Thompson et al., 1997). The phylogenetic tree was drawn by the Treeview programusing the distance matrix and neighbor-joining method in the Phylogeny Inference Package (Page, 1996; Flesenstein 1993).

## Results and Discussion

The pictures of the Gram-stained cells obtained from the MCR under phase-contrast and bright-field microscopy are shown in Fig. 1. We can conclude that there are only bacteria having a maximum size of. $1 \times 2$ ㎛ and that most of them are Gram-negative bacteria (red

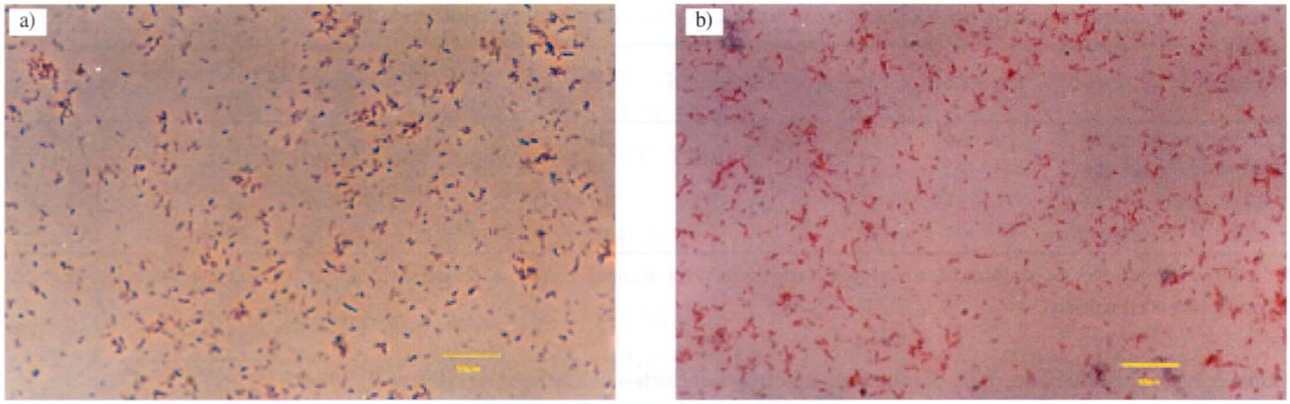


**Fig. 1. Gram stained cells obtained from MCR a) phase-contrast b) bright-field microscopy (bar = 10 m).**

color).

Two organisms mentioned in Table 1 were isolated on PCA plates, which contain high biodegradable organic matter, and designated strains AD1 and AD2. Strains BD1 and BD2 were isolated on a solid thiosulfate/nitrate medium without organic matterand chosen according to their growth patterns on each solid medium followed by microscopic confirmation. Enumeration results show that AD1 and AD2 constituted 93.5% and 6.5% of the CFUs on the PCA medium, respectively, while BD1 and BD2 were 95.7% and 4.3% on the solid thiosulfate plus nitrate medium, respectively. Therefore, the main strains in the MCR were AD1 and BD1. From the FAME and phylogenetic analyses results, AD1 and BD1 were found to be the same strain. AD1 can grow heterotrophically, use thiosulfate, and is a motile, Gram-negative short rod (0.5 by 1 ㎛).

Generally, the Genus *Thiobacillus* is Gram-negative, rod-shaped cells (~0.5 × 1.0-4.0 ㎛) with some species motile by means of polar flagella. Energy is derived from the oxidation of one or more reduced sulfur compounds, and sulfate is the end product of sulfur compound oxidation. Especially among the autotrophic sulfur bacteria (Genus *Thiobacillus*), when denitrification reactions are considered, the following five strains are typically thought of: *Thiobacillus denitrificans, Thiomicrospira denitrificans, Thiobacillus versutus, Thiobacillus thyasiris, Thiosphaera pantotropha, and Paracoccus denitrificans.* They are all mesophilic, denitrifying bacteria (see Tables 2 and 3). The strains of *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* are obligate autotrophic chemolithotrophic bacteria and *Thiobacillus versutus, Thiobacillus thyasiris, Thiosphaera pantotropha,* and *Paracoccus denitrificans* are facultative autotrophic chemolithotrophic bacteria. Within this group, the obligately chemolithotrophic Thiobacilli are virtually restricted to an autotrophic mode of growth since they cannot obtain energy from the oxidation of organic compounds and can utilize organic compounds only to a limited extent (Kuenen, 1979).In contrast, the facultatively chemolithotrophic (also called mixotrophic) Thiobacilli comprise a group of organisms

**Table 1. Sulfur utilizing autotrophic bacteria isolated from MCR.**

| Collection number | Size, color, and number($10^6$ CFU / mL) of isolated colonies | | Relation to oxygen | Motility | On nitrate broth | | Shape of the cell (size) |
|---|---|---|---|---|---|---|---|
| | Thiosulfate+nitrate | PCA | | | Grow | Gas production | |
| AD1 | Pinpoint Transparent | Medium light brown, 17.4 (93.5%) | Facultative | + | + | + | Short rod (0.5 by 1um) |
| AD2 | Pinpoint Transparent | Big yellow, 1.2(6.5%) | Facultative | + | + | + | Long rod (0.5 by 2um) |
| BD1 | Pinpoint Transparent, 18(95.7%) | Medium light brown | Facultative | + | + | + | Short rod (0.5 by 1um) |
| BD2 | Small Irregular, 0.8(4.3%) | Medium light brown | Facultative | rotation | + | ++ | Long rod (0.5 by 2um) |

Note : " -" means no gas production; "+", small amount; "++", large amount of gas production. The following terms were used to characterize the size of colony: *pin point, less than 1mm; small, 1 to 2 mm; moderate 2 to 5 mm; big, bigger than 5 mm

**Table 2. Basic characteristics of the obligately autotrophic colorless sulfur bacteria (Holt et al., 1994).**

| Species | %G+C | Motility | carboxysomes | pH | $NO_3^-$ reduction to: | | Opt. Temp. | Ubiquinone |
|---|---|---|---|---|---|---|---|---|
| | | | | | $NO_2^-$ | $N_2$ | | |
| *Thiobacillus thioparus* | 61-66 | + | + | 6-8 | + | - | 25-30 | Q-8 |
| *Thiobacillus tepidarius* | 66.6 | + | ND | 6-8 | + | - | 40-45 | Q-8 |
| *Thiobacillus denitrificans* | 63-68 | + | - | 6-8 | + | + | 25-30 | Q-8 |
| *Thiomicrospira denitrificans* | 36 | - | - | 7 | + | + | 20-25 | ND |

Carboxysomes indicates the posession of carboxysomes under some, if not all growth conditions; pH and temp. indicate the most favorable ranges for growth. ND, not determined.

**Table 3. Basic characteristics of the facultatively autotrophic colorless sulfur bacteria (Holt et al., 1994).**

| Species | %G+C | Motility | Carboxysomes | pH | $NO_3^-$ reduction to: | | Opt. Temp. |
|---|---|---|---|---|---|---|---|
| | | | | | $NO_2^-$ | $N_2$ | |
| *Thiobacillus versutus* | 65-68 | + | - | 6-8 | + | + | 30-35 |
| *Thiobacillus delicatus* | 66-67 | - | ND | 5-7 | + | - | 30-35 |
| *Thiobacillus aquaesulis* | 65-66 | + | ND | 7-9 | + | - | 40-45 |
| *Thiobacillus thyasiris* | 52 | - | + | 7-8 | + | + | 35-40 |
| *Thiosphaera pantotropha* | 66 | - | - | 7-9 | + | + | 30-40 |
| *Paracoccus denitrificans* | 64-67 | - | - | 7-9 | + | + | 25-35 |

Carbosysomes indicates the possession of carboxysomes under some, if not all, growh conditions; pH and temp. indicate the most favorable ranges for growth.

with a large metabolic flexibility that enables them to grow autotrophically, mixotrophically and heterotrophically on a variety of single, or a mixture of, inorganic and organic substrates(Kuenen, 1979). Therefore, the main strains in the MCR, AD1 and BD1, should be facultatively chemolithotrophic microorganism since they grow mixotrophically.

***Identification sulfur-oxidizing bacteria*** The physiological characteristics of the strains AD1 and AD2 are presented in Table 4. Both were able to assimilate glucose as a sole source of carbon and energy. Both AD1 and AD2 were similar to bacteria in the genus *Paracoccus*. The 16S rRNA sequences of the isolates were determined using PCR,and a phylogenic tree was drawn, as mentioned above, by comparing their sequences with other strains listed in the Blastin database (Fig. 2). Strains AD1 and AD2 had 99% and 98% similarities to *Paracoccus denitrifican* and *Paracoccus versutus* (old name: *Thiobacillus versutus*), respectively, and are located near the genus *Paracoccus* in alpha Proteobacteria.

Since denitrifying bacteria using reduced sulfur compounds are likely to encounter both autotrophic and heterotrophic conditions in natural systems or in a denitrifying wastewater treatment process, it is of considerable interest whether their nitrate removal characteristics would be under autotrophic or heterotrophic conditions. Moreover, most research on sulfur utilizing autotrophic denitrification was aimed at finding key mechanisms and optimal conditions for

**Table 4. Some phenotypic characteristics of sulfur-oxidizing bacteria, strain AD1 and AD2.**

| Phenotypic Characteristics | AD1 | AD2 |
|---|---|---|
| Gram staining | - | - |
| $NO_3^-$ reduction | + | + |
| Indole production | - | - |
| Glucose acidification | - | - |
| Arginine dehydrolase | - | - |
| Urease | - | - |
| β-Glucosidase | - | - |
| Protease | - | - |
| β-Galactosidase | - | - |
| Oxidase | + | + |
| Catalase | + | + |
| **Assimilation of** | | |
| Glucose | + | + |
| Arabinose | - | + |
| Mannose | - | - |
| Mannitol | + | + |
| N-Acetylglucosamine | + | + |
| Maltose | + | + |
| Gluconate | + | + |
| Caprate | - | - |
| Adipate | - | -/+ |
| Malate | + | + |
| Citrate | - | - |
| Phenyl acetate | - | - |

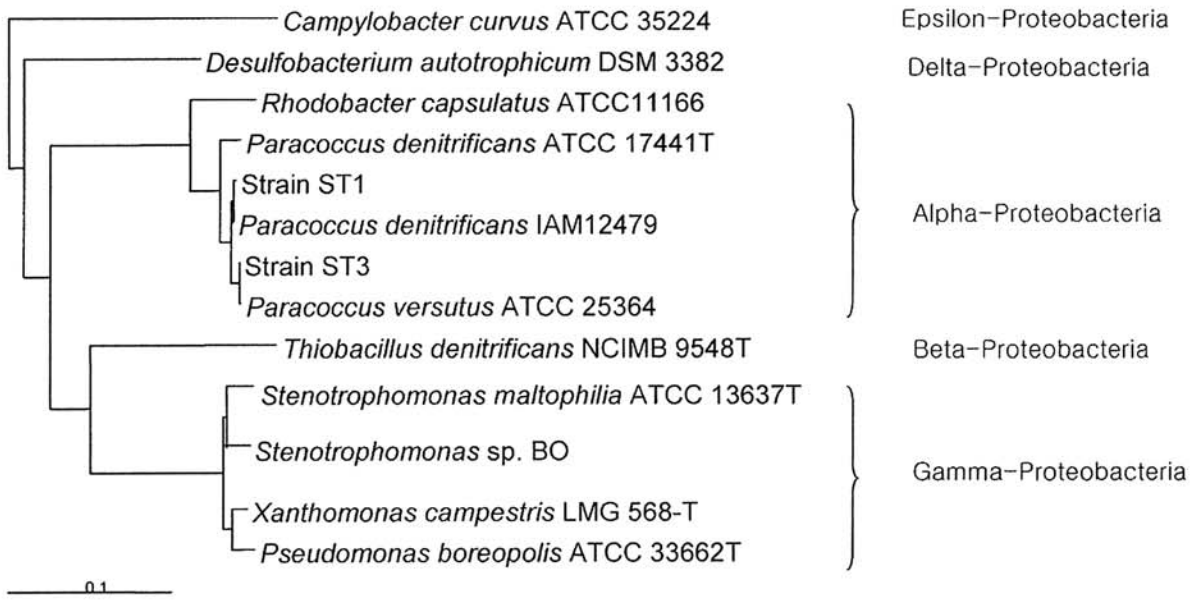


**Fig. 2. 16S rDNA sequence-based dendrogram shows the phylogenetic position of strain AD1 and AD2 in relation to members of the Proteobacteria. The scale bar indicates 10 % (0.1) estimated difference in nucleotide sequences.**

**Table 5. The stoichiometric equations of autotrophic denitrification.**

| Electron donor | Equation | Reference |
|---|---|---|
| Thiosulfate | $8NO_3^- + 5S_2O_3^{2-} + H_2O \rightarrow 4N_2 + 10SO_4^{2-} + 2H^+$ | Claus and Kutzner(1985) |
| | $0.141NO_3^- + 0.125S_2O_3^{2-} + 0.0643\ CO_2 + 0.100H_2O$ | Matsui and Yamamoto |
| | $\rightarrow 0.064N_2 + 0.25SO_4^{2-} + 0.109H^+ + 0.0129C_5H_7O_2N$ (biomass) | (1986) |

obligate autotrophic denitrification using pure or mixed cultures (Kuenen, 1979; Koenig and Liu, 1996; Flere and Zhang, 1999). In this study, however, we found that the majority of bacteria in the MCR fed with nitrate and thiosulfate were facultatively autotrophic colorless sulfur bacteria, and they can grow heterotrophically. The bacteria produce sulfate and consume alkalinity autotrophically, while reducing nitrate (Table 5). When nitrate concentration is high, the sulfate that is produced will be very high. This high sulfate concentration would deteriorate rivers or wastewaters since sulfate-reducing bacteria (SRB) would produce hydrogen sulfide with biodegradable organics in sediments or water. To overcome the disadvantages of autotrophic denitrification, a system with reduced sulfur would be operated in a modified manner. If small amounts of external organics relative to heterotrophic stoichiometiric requirements are applied to the sulfur denitrification system,nitrate could be removed by means of simultaneous heterotrophic and autotrophic denitrification. In that case, high alkalinity consumption and high effluent sulfate concentrations, which are the disadvantages of autotrophic sulfur denitrification, could be decreased.

## Acknowledgements

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# 황(thiosulfate)을 이용하는 탈질 미생물의 분리 및 특성 파악

**오상은* · 주진호 · 양재의**

강원대학교

황산화 미생물인 *Genus Thiobacillus* 중 몇 종류의 탈질균은 여러 종류의 황 화합물($S^{2-}$, So, $S_2O_3^{2-}$, $S_4O_6^{2-}$, $SO_3^{2-}$)을 황산염이온으로 산화시키면서 동시에 질산성질소를 질소 가스 형태로 전환시킨다. 이는 독립영양 미생물이므로 외부 탄소원이 필요치 않으며, C/N비가 낮은 폐수에 메탄올 대신 값이 싼 황 입자의 투입으로 경제적이며 효과적인 탈질화를 유도할 수 있다. 후탈질시 인위적인 유기물의 투입대신 값 싼 황입자를 사용하여 질소를 제거하며, 처리효율이 안정적이고, 운전이 쉬워, 최근 이 공정은 세계적으로 많이 연구되고 있다. 그러나 탈질시 알칼리도가 파괴되어 특히 알칼리도가 낮은 폐수의 경우 고농도 탈질시 pH가 떨어져 탈질이 더 이상 진행되지 않으며, 고농도의 질산성질소를 처리할 경우 부산물로서 고농도의 황산염이온이 생성된다는 부수적인 단점을 안고 있다. 본 연구에서는 MCR로부터 독립영양 미생물을 분리 characterization 및 미생물 동정을 하였다. MCR내에는 주로Paracoccus denitrificans and Paracoccus versutus (formerly Thiobacillus versutus)가 주종을 이루었으며 이들 미생물들은 유기물도 에너지원으로 이용할 수 있는 facultative autotrophic denitrifier이었다. 이들 미생물을 탈질에 이용할 시 유기물이 있는 조건에서도 성장하기 때문에 유입 폐수 내 유기물 농도에 영향을 받지 않고 또는 인위적으로 소량의 유기물을 넣어 독립영양탈질과 종속영양탈질을 동시에 일어날 수 있도록 함으로서 독립영양탈질의 단점인 높은 황산염이온 생성 및 알칼리도 파괴를 막을 수 있을 것으로 사료된다.