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*Thiobacillus concretivorus*의 分離 및 同定에 關한 硏究

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Studies on Isolation and Identification of *Thiobacillus concretivorus*

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ABSTRACT

With respect to a bacterium isolated some of the similarities and differences previously indicated between *Thiobacillus thiooxidans* and *Thiobacillus concretivorus* were confirmed and the bacteria also provided considerable differences compared to the two species in some respects.

Severe precipitation of sulfur occurred in colonies and in liquid media of the organism isolated. The organism isolated utilized nitrate and asparagine as well as ammonium sulfate as a sole nitrogen source and grew well without being nearly inhibited by citrate and malate. This organism also showed the resistance to heats and external physical stimulations. Owing to some characters described above and the reports proposed, the organism isolated could be concluded to be *Thiobacillus concretivorus* and it was suggested that *Thiobacillus concretivorus* might be an apparently different species from *Thiobacillus thiooxidans*.

INTRODUCTION

The regular study on the genus *Thiobacillus* has been stimulated since Nathanson found the sulfur bacteria from sea in 1902 and Beijerinck reisolated it and described as *Thiobacillus thioparus* in 1904. Continuously *Thiobacillus thiooxidans* was described by Waksman and Joffe(1922) and then *Thiobacillus concretivorus* was also isolated by Parker (1945) in the concrete sewer corroded in the presence of H_2S. In the latest date London isolated a new species and described it as *Thiobacillus perometabolis* and so it is recorded at present that the genus *Thiobacillus* comprises 13 closely related species.

The genus *Thiobacillus* is a Gram-negative, non-sporulating, rod-shaped and motile or non-motile organism due to a single polar flagellum. All bacteria of this genus usually can utilize the reduced sulfur compounds as a energy source, carbon dioxide as a sole carbon source and ammonium nitrogen as a nitrogen source.

According to investigations by Vishniac *et al.* (1957) and Hutchinson *et al.* (1965; 1969). some species of this genus such as *Thiobacillus thioparus, Thiobacillus neapolitanus, Thiobacillus thioo-*

xidans, *Thiobacillus concretivorus* and *Thiobacillus ferrooxidans*, which are quite similar morphologically to one another, have shown difficulties to distinguish one another, especially *Thiobacillus concretivorus* and *Thiobacillus thiooxidans*. Parker and Prisk (1953) used the form of the nitrogen source as a diagnostic method in order to isolate *Thiobacillus thiooxidans* and *Thiobacillus concretivorus* and could classify them into two different species. The reports that *Thiobacillus thiooxidans* and *Thiobacillus concretivorus* must be assigned to same species have been continuously proposed by some investigators. For example, Jackson *et al.* (1968) noted that *Thiobacillus thiooxidans* and *Thiobacillus concretivorus* would be similar species since these two species showed a DNA base composition of 51—52 mole % G+C, and were therefore clearly separately from the rest of the genus. Hutchinson *et al.* (1965) stated there was not sufficient difference in "S" value between these two species and then these two species would occupy a same taxonomic position.

It must be also considered that the base sequence of DNA can be certainly different resulting in completely different phenotypic expression and physiological metabolism. The proposal by Hutchinson *et al.* will be ascribed to the fact that they could not obtain the authentic strain of Parker (1945) and their strains tested and any of the present isolates did not resemble *Thiobacillus concretivorus* in ability to utilize nitrate as a sole source of nitrogen.

It is necessary to reaffirm that *Thiobacillus concretivorus* is similar to *Thiobacillus thiooxidans* except that the former used nitrate as well as ammonium salt as a nitrogen source but the latter could not use nitrate and that the former oxidized thiosulfate to sulfric acid and free sulfur, *Thiobacillus thiooxidans* to sulfric acid only.

METHODS AND MATERIALS

Organism: The organism used was isolated originally from the sewer-soil obtained in Cheong Gyeo Cheon in Seoul, Korea, by enriching culture in the ONM medium for *Thiobacillus thiooxidans* described by Imai *et al.* (1964) and incubation on the agar medium.

The bacteria could be readily purified by streaking on the solid media. Further pure cultures were attained by two successive subcultures of single colonies from the agar medium. Pure culture was maintained on slopes and plates of 1% thiosulfate medium, incubated at 30°C and subcultured every month. They were always incubated at 30°C until the growth occurred sufficiently and then stored at 5°C for the remaining time interval.

Media: The organism newly isolated was grown in the ONM medium containing: $(NH_4)_2SO_4$, 2g; $MgSO_4 \cdot 7H_2O$, 0.3g; $CaCl_2 \cdot 2H_2O$, 0.3g; $FeSO_4 \cdot 7H_2O$, 0.01g; KH_2PO_4, 4g; $Na_2S_2O_3 \cdot 5H_2O$, 10g; distilled water, 1000ml; *p*H 4.4 to 4.6. Elemental sulfur might be substituted for $Na_2S_2O_3 \cdot 5H_2O$ as a energy source at a same composition.

The 25% (w/v) thiosulfate was sterilized separately by filtration with the Seitz filter and then 1 ml of this solution was added to 24 ml ONM basal inorganic

Table1. Energy source substrates used in energy source tests.

Energy sources	Inorganic compounds	Organic compounds	Ferrous iron	Nutrient medium
Chemicals	H_2S*	Glucose		
	Na_2S	Xylose		
		Fructose		
	Na_2SO_3	K-glutamate	Medium 9K**	
		Na-acetate		Nutrient broth:8g
	$Na_2S_2O_3$	Na-citrate	and	
	Elemental sulfur	Na-malate	Leathen's	Agar:2g
		Na-pyruvate	medium***	
		Na-succinate		
Concentration	1%	0.5%		
*p*H	4.6	4.6-5.0	3.5 & 4.6	4.6
Remarks	Sterilized separately	Sterilized separately		added to 1000ml H_2O

* At a concentration of 200 ppm mixed with air.
** Described by Silverman and Lundgren (1959a).
*** Described by Leathen *et al.* (1951).

media which were sterilized by autoclave at 120°C for 15 min. Elemental sulfur was sterilized by intermittent steaming for 1 hour on 3 successive days and then added.

All of the solid media for use in following various tests were made with 2.5% agar (Difco).

Experimental tests: Studies on the utilization of energy sources were made by substituting various compounds containing glucose, xylose, hydrogen sulfide, sodium sulfite and etc. for sodium thiosulfate in the ONM medium, and also substituting some media for the ONM medium. Various compounds and the media substituted are shown in Table 1.

Organic compounds and inorganic compounds used as energy substrates were sterilized separately by autoclave or filtration. The sterilization of H_2S mixed with air was attained by passing through cotton.

$FeSO_4 \cdot 7H_2O$ of medium 9K and Leathen's medium for *Thiobacillus ferrooxidans* were also sterilized separately by filtration. Inoculum for the growth of organisms in the medium with organic substrates as a sole energy source was obtained from four successive subcultures so that the organism might be adapted to the true organic media before inoculation.

For the purpose of elucidating the nitrogen source, various nitrogen substances were treated as a nitrogen source instead of ammonium sulfate in the ONM medium. The inorganic compounds containing sodium nitrite, potassium nitrate and the organic compounds containing urea, peptone, glutamate+alanine and asparagine were used with a same composition of ammonium sulfate. They were all sterilized separately and

added.

Studies on the influence of organic matters upon the growth of the organism could be achieved, using same substances as used in detection of energy source described above.

The *p*H values of all media in these studies were adjusted 4.4 to 5.0 with 0.1N H_2SO_4 or 0.1N NaOH. The medium 9K and Leathen's medium were controlled at *p*H 3.5 and *p*H 4.6.

Cultures: A culture was attained with the test tubes, 3 cm in diameter, containing 25ml media and 500ml Erlenmeyer flasks containing 150ml media. The liquid medium was inoculated with inoculum of 7-day-old cultures of the organism isolated.

Cell numbers of 1ml inoculum employed were approximately 4×10^8/ml to 8×10^9/ml.

The flasks and test tubes plugged with cotton and then put on a reciprocal shaker at 112 rev/min and were shaken for a interval of required days at 30°C. The flasks containing sulfur as a energy source were allowed to stand for 3 days to permit contact between the organisms and sulfur and then placed on the shaker (Cook, T.M. 1964).

Examinations for motility were made in hanging drop preparations. The Gram reaction of the organism was determined on cells grown on thiosulfate agar. Smears were stained using the Hucker modification of the Gram stain with *E. coli* as controls.

The *p*H values for the test were recorded using a glass electrode *p*H meter.

Optical density for the measurement of turbidities was measured using Beckman model DU spectrophotomete r at 550 nm. Cell numbers were determined by direct microscopic counting and optical density at 550 nm. Cells were broken down with Biosonic III ultrasonic oscillator (24 kc/sec).

Analysis: Oxidation of thiosulfate was determined by volumetric iodine titration with 0.01N iodine solution.

RESULT AND DISCUSSION

1. Morphological and Cultural characters

a. Morphological characters

The genus *Thiobacillus* are all Gram-negative, small and rod-shaped, non-sporulating and motile or non-motile bacteria due to a single polar flagellum. The organism newly isolated has shown a vigorous motility and a rod-shaped type with round ends under the microscope. Cells measured 0.5 μ in width and 1 to 3μ in length. The single polar flagella were observed from cells, especially cells in 3-day-old cultures. The formation of spore was not observed.

Colonies were readily obtained on 2.5% agar plates. Small, circular and opaque colonies appeared within 3 days after inoculation with transparent whitish colour, developed into yellowish or canary yellowish colonies due to deposited sulfur in the center of colonies with round margin (see Fig. 3,4).

Their colour turned to brown or dark green in old cultures and the agar around the margin of colony was fogged by acid decrease. Colonies generally grew to 2 mm in diameter but some to 5 mm in 20-day-old cultures. These characters of colonies were quite similar to *Thiobacillus thioparus* in respect of

sulfur precipitation with yellowish colour (In Bergey's manual of determinative bacteriology, 1957).

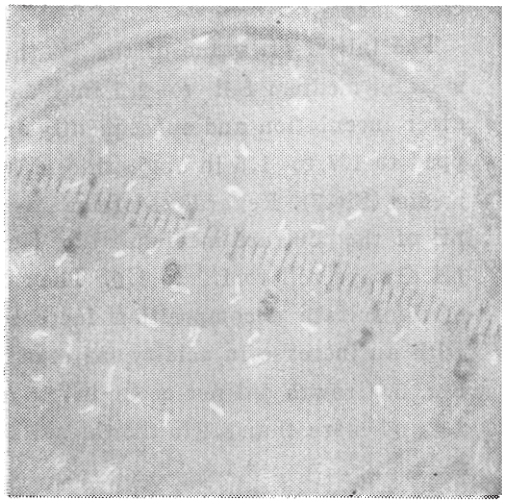

Fig. 1. Demonstration of the bacteria isolated by negative staining with nigrosine. Magnification, ×1,500.

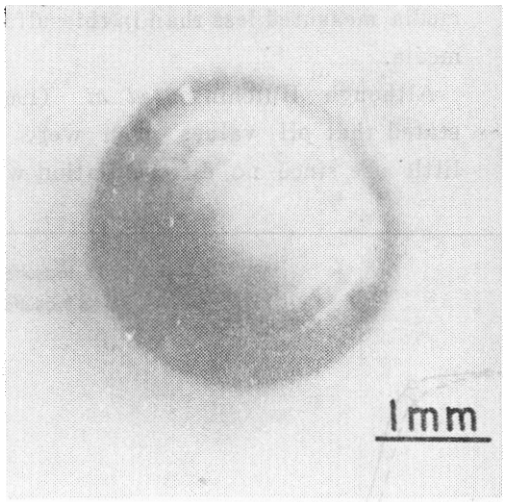

Fig. 3. Colonies with the deposited sulfur and surrounded with transparent sheath in 9 days after inculation.

b. Cultural characters

Liquid media became turbid and acid in 3 days after inoculation and severe turbidity appeared in 4 to 5 days with an associated fall in *p*H and increase in thiosulfate oxidized, showing a milky white colour (see Fig.5).

Turbidities maintained for log phase and stationary phase of cells and seemed whitish yellow, presumbly due to precipitated sulfur. The turbidity of the 1% thiosulfate culture medium became to disappear little by little over 6 days and then maintained at optical density 0.7 (550 nm) as showed in Fig. 5. No pellicle occurred in liquid thiosulfate media.

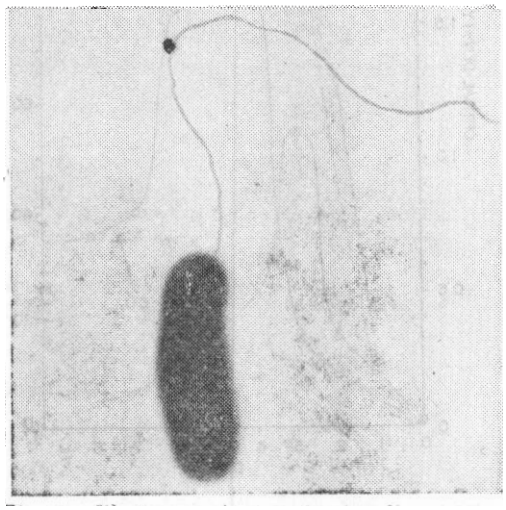

Fig. 2. Electron micrograph of cell with a single polar flagellum in 3-day-old culture media. Magnification, × 20,000.

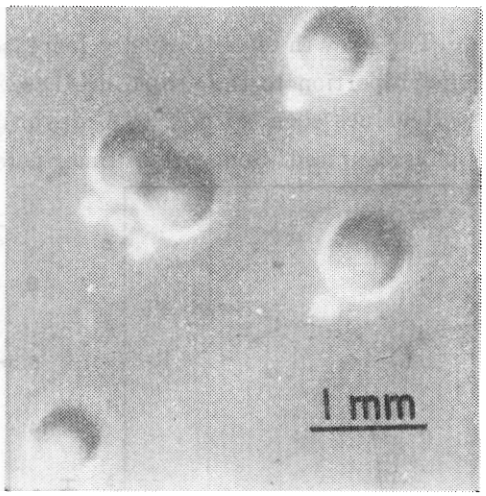

Fig. 4. The colony developed into dark green in 20 days.

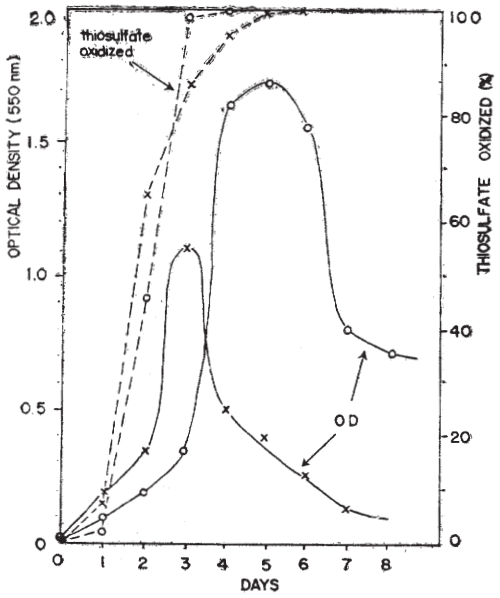

Fig. 5. Optical density and thiosulfate oxidized (%) by the organism isolated in the culture media. The concentration of $Na_2S_2O_3$ were:1%(0); 0.4%(x)

The growth of cells rapidly increased in proportion to the rapid decrease of *p*H values soon after 2 to 3 days lag phase expired. Generation time, determined from direct microscopic cell counting, varied between 7 and 8 hours, with an average of 7.2 hr in 1% thiosulfate ONM media.

The initial *p*H value of the 1% thiosulfate medium fell to 2.5 in 3 days after inoculation and subsequently dropped to 1.7 to 1.5 in 0.4% thiosulfate media (Fig.7). Regardless of the initial *p*H of the culture medium, the final *p*H always fell to 1.2 to 1.0. The rate of thiosulfate decomposition increased with an increase in acidity as seen in Fig. 5. Growth did not occur below *p*H 3.4 and more than 5.9 in the thiosulfate medium.

Optimum *p*H for the initiation of growth was measured to be 4.0 to 5.0. The optimum and final *p*H in sulfur media measured less than in thiosulfate media.

Although Hutchinson *et al.* (1965) stated that *p*H values given were of little use since no differentiation was

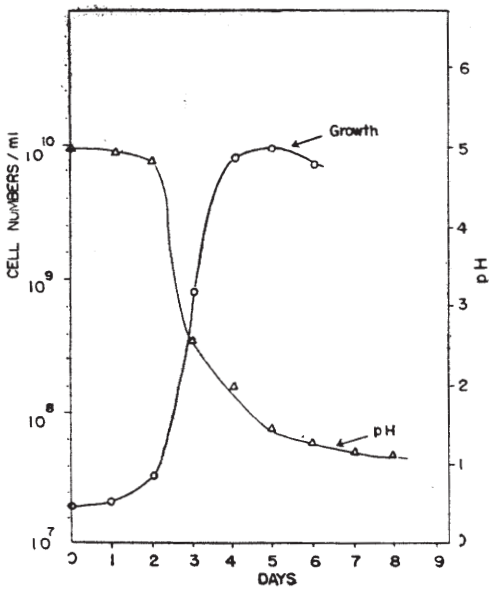

Fig. 6. Growth curve and *p*H changes during growth of the organisn in 1% thiosulfate media.

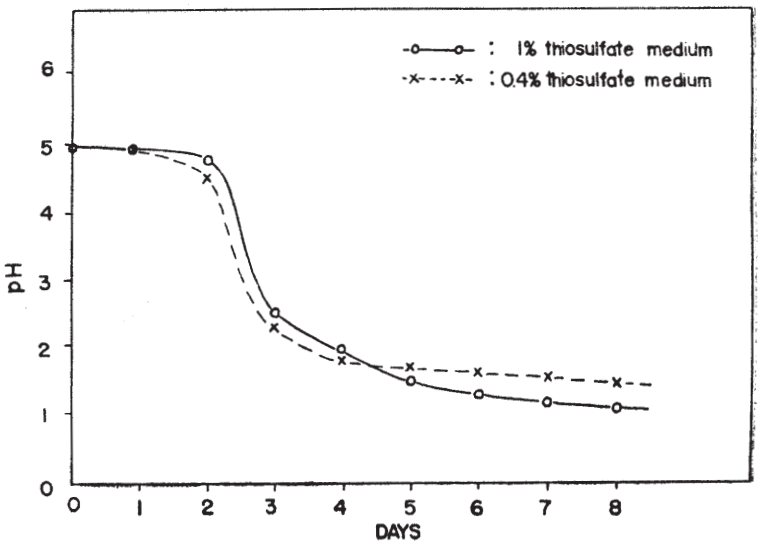

Fig. 7. *p*H changes during growth of the organism isolated in the thiosulfate media.

made between the limits for the initiation of growth and the final *p*H values attained by the culture, it must be emphasized that the genus *Thiobacillus* is divided into two or three groups on the basis of the final *p*H and optimum *p*H.

The organism newly isolated was tolerable at the extremely low *p*H, below 2.0, for a long time and afterwards grew well in the ONM medium. It was therefore suggested that this organism would be an acidophillic cell, perhaps was one of some species including *Thiobacillus thioparus*, *Thiobacillus neapolitanus*, *Thiobacillus thiooxidans*, *Thiobacillus concretivorus* and *Thiobacillus intermedians*. *Thiobillus thioparus* and *Thiobacillus neapolitanus* grew most rapidly near the neutral *p*H and were inhibited severely by the low *p*H values, below 3.0 (Hutchinson *et al.*, 1966). Hutchinson *et al.* also reported that the final *p*H value in thiosulfate and sulfur media for *Thiobacillus thioparus* was always below 5.0 but never less than *p*H 3.5, and that the final *p*H of *Thiobacillus intermedians* was 2.0 to 2.8 but never less than 2.0. The final *p*H of *Thiobacillus neapolitanus* is also never less than *p*H 2.8.

All these investigations and the results attained indicated that newly isolates might be certainly one of three acidophillic bacteria, *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Thiobacillus concretivorus*, since these three species grow and reproduce readily at the lower extremes of the *p*H scale.

Table. 2. Growth of the organism newly isolated in the medium containing various energy substrates.

Chemical		$Na_2S_2O_3$	Na_2S	Na_2SO_3	H_2S	Ferrous iron medium	9K	Leathen's	Organic compounds		Nutrient agar	Elemental sulfur
*p*H	Initial	4.6	4.6	4.6	4.6	4.6	3.5	4.6	3.5	4.6	4.6	4.6
	Final	1.2			4.1							0.9
Growth		+++*	—	—	+	—	—	—	—	—	—	+++

* The growth of the organism isolated are indicated by +++ (good growth), + (poor growth), and — (no growth).

Table 3. Growth of the organism isolated and *Thibacillus thiooxidans* in the media with various nitrogen compounds.

Chemicals			K-nitrate	Na-nitrite	Urea	Asparagine	Peptone	K-glutamate Alanine	Ammonium sulfate	None
A*	Liquid	Growth	++++**	—	+	+++	++	+++	+++	—
	Agar	Growth	++	—	—	+++	+		+++	—
		Colony	RY***	—	—	RY & IRG	RY & IRG		RY	—
B	Liquid	Growth	—	—	+—	—	+—		+++	—

* A: The organism newly isolated, B: *Thiobacillus thiooxidans.*

** The relative growth of the organism are indicated by ++++ (excellent growth), +++ (good growth), ++ (growth), + (poor growth), +— (doubtful growth) and — (no growth).

*** Abbreviations: RY, yellowish round colonies; IRG, grey colonies with irregular margin.

2. Energy source test.

The growth of bacteria tested with various energy sources are shown in Table 2.

Ferrous iron in the medium 9K and Leathen's medium were never utilized as a energy source and the organism isolated could oxidize two reduced sulfur compounds such as sodium thiosulfate and hydrogen sulfide and elemental sulfur for the growth of cell.

In Bergey's manual of determinative bacteriology (1957) hydrogen sulfide can be utilized by *Thiobacillus concretivorus* and *Thiobacillus thioparus* but not by *Thiobacillus thiooxidans*, but recently it has been proposed that *Thiobacillus thiooxidans* also might oxidize H_2S as a energy source in the environment below *p*H 2.0 (Hutchinson *et al.*, 1969).

For this reason, it was clear that this organism would not be an iron-oxidizing bacterium, *Thiobacillus ferrooxidans* because of no growth in the medium 9K and Leathen's medium. The fact that no growth occurred in the nutrient medium and the medium with organic substrates as a sole energy source confirmed this organism was a strict autotrophic cell.

3. Nitrogen source test

Thiobacillus thiooxidans which has been stocked in this laboratory was used for this test in addition to the organism newly isolated. The results indicated that nitrate was apparently utilized as a nitrogen source better than ammonium salt by the organism isolated and peptone, asparagine also could be utilized as seen in Table 3. The data seen in Table 3 indicated that the organism isolated utilized more extensive nitrogen sources than *Thiobacillus thiooxidans*. The utilization of urea by the organism were doubtful in our test. No growth occurred in the medium contained nitrite-ammonium as a sole nitrogen source.

In 1923 it was already demonstrated that *Thiobacillus thiooxidans* required ammoniacal nitrogen and could not use nitrate which was toxic(Starkey). Waksman and Starkey also noted a concentration of 0.15 to 0.20 mole of the various nitrate was sufficient to repress completely the growth of *Thiobacillus thiooxidans* and these results indicated that nitrate was toxic to the organism and not merely that it was an unfavorable nitrogen source and then *Thiobacillus thiooxidans* required ammonium nitrogen. Urea, peptone and asparagine has been known not to be used as a nitrogen source by *Thiobacillus thiooxidans* (In the Bergey's manual of determinative bacteriology, 1957) but urea was suggested to be utilized by *Thiobacillus thiooxidans* (Brierley, 1968) and peptone was widely used by the genus *Thiobacillus* (Skerman, 1967).

For these reasons, whether ammonium salt and nitrate can be utilized by the organism or not, was already regarded to play a significant role to identify two species *Thiobacillus thiooxidans* and *Thiobacillus concretivorus* (Parker, 1953).

Subsequently it was suggested that this organism newly isolated would be *Thiobacillus concretivouss* on account of utilization of nitrate and asparagine as a nitrogen source and utilization of

Table 4. Influence of organic matters upon the growth of the organism isolated.

Organic compounds		Glucose	Fructose	Xylose	K-glutamate	Na-citrate	Na-succinate	Na-malate	Na-pyruvate	Na-acetate	None
Time for maximum growth (days)		5	5	5	6	9	—	7	—	—	5
Thiosulfate oxidized(%)		99	90	99	90		—				100
Growth		+++*	+++	+++	+++	++	+−	++	−	−	+++
*p*H	Initial	5.0	5.0	4.9	5.0	4.8	4.9	4.9	4.8	4.9	4.9
	Final	1.4	2.2	1.3	1.6	2.2	4.5	2.3	—	—	1.2

* The relative growth of the organism isolated are indicated by +++(good growth), ++ (growth), +(poor growth), +−(doubtful growth) and−(no growth).

more nitrogen source compounds than *Thiobacillus thiooxidans.*

4. Influence of organic matters on the growth of the organism

The growth of the organism isolated was not effected by organic substances such as glucose, fructose, xylose and glutamate at a concentration of 0.5% (w/v). The growth of this organism, as it were, was the same as the organic compounds were not added. As shown in Table 4, citrate and malate influenced a little on growth of the organism owing to prolonging a period of lag phase but grew well soon after the lag phase terminated. Pyruvate and acetate seemed to inhibit the growth of cells during cultures. Poor colonies appeared only in the agar medium with 0.5% succinate in 7 days cultures, but in liquid media the growth of cells did not occur.

Thiobacillus thiooxidans is quite tolerant to appreciable concentration of organic materials without being affected by them (Waksman and Starkey, 1923;1925) and so far it has been well acknowledged glucose caused little or no inhibition of substrates oxidation of *Thiobacillus thiooxidans.* Waksman and Starkey (1922) and Emoto (1933) found that the rate of sulfur oxidation by *Thiobacillus thiooxidans* increased slightly in the presence of glucose and glucose present during sulfur oxidation slowly disappeared and the amount of glucose consumed was proportional to the growth of the organism.

It could be, therefore, said that *Thiobacillus concretivorus* and *Thiobacillus thiooxidans* were not effected by glucose and glucose rather penetrated the cell wall, incorporated into cell organisms. Borichewski (1966) reported that when 2×10^{-5} to 4×10^{-5}M pyruvate added with the inoculum, growth compeletely was inhibited and citric, isocitric, succinic, malic and α-ketoglutaric acid have occurred comparable in hibition of 2×10^{-4}M but the cause of the toxicity was not clear.

Butler and Umbreit(1965) also noted that succinic acid was somewhat toxic to their strains of *Thiobacillus thiooxidans.*

Contrary to these reports,succinate stimulates the respiration of *Thiobacillus thiooxidans* and *Thiobacillus thioparus,*

this compound must be able to enter the cell (Vishniac and Santer, 1957). Vogler *et al.* (1942) noted that the C_4-dicarboxylic acids enhanced the rate of respiration of *Thiobacillus thiooxidans.*

Some substances such as acetate, pyruvate and glucose could be incorpcrated into cell substances of *Thiobacillus thiooxidans* and metabolized to CO_2(Still and Wang, 1965). The uptakes of substrate are not due to mere absorption to the cell but represent definite incorporation during growth.

The reports described and the results obtained shown in Table 4 indicated that keto acids including citrate, succinate, acetate, malate and pyruvate would be signigicant materials to isolate and identify *Thiobacillus concretivorus.* Citrate and malate were considered to be utilized by the organism isolated better than *Thiobacillus thiooxidans.*

All bacteria of the genus *Thiobacillus* are generally dead at 55°C except for *Thiobacillus thermophilica,* which grows rapidly at higher temperature, 57°C. The bacteria isolated in this laboratory did not die at 55°C despite of treatment for 30 min, moreover some of these bacteria never died even in treatment with boiling at 100°C for 30 min.

Thiobacillus thioparus, Thiobacillus novellus and *Thiobacillus thiooxidans* cells were readily broken down in treatment with sonicating in 10 kc/sec oscillator for 20 minutes. The organism isolated, however, was not broken down in those treatments and cell extracts could be obtained for the first time, when suspension of the organism isolated was given ultrasonic treatment for 30 min. at 5°C with Biosonic model III disintegrator (24kc/sec). Cell walls were not lysed even by treatment of lysozyme for 6 hours. These results was corresponding to the report of Jackson *et al.*(1968). Jackson *et al.* stated that the gram-negative bacteria, *Thiobacilli* were easily lysed by detergent described by Marmur. There were, however, two exceptions, *Thiobacillus concretivorus* and *Thiobacillus ferrooxidans.* These two species did not lyse with sodium lauryl sulfate to prepare a DNA sample from two species.

These properties, the resistances to heats and extracellular physical stimulations, of the organism newly isolated which was understood to be *Thiobacillus concretivorus,* seem to produce a character distinguishable from *Thiobacillus thiooxidans.* The property and structure of the cell membrane of *Thiobacillus concretivorous* may differ from that of *Thiobacillus thiooxidans* with an association of the resistance to heat and stimulation.

摘 要

今番 새로이 分離한 菌에 對한 硏究의 結果 *Thiobacillus thiooxidans*와 *Thiobacillus concretivorus* 간의 前부터 알려진 몇몇 相異點과 類似點을 確認함과 아울러 몇가지 주목할만한 特徵을 밝힐 수 있었다. 即 새로이 分離한 本 菌은 培養 過程中 液體 및 固體 培地상에서 상당한 量의 硫黃을 沈出하였고 單一 窒素源으로서 ammonium sulfate뿐 아니라 nitrate 및 asparagine도 잘 利用하였다.

Citrate와 malate같은 keto acid에 依하여 本 菌의 成長은 거의 沮害받지 않았으며 熱 및 外部의 物理的 刺戟에 대하여서도 커다란 抵抗性을 나타내었다.

數種의 實驗結果와 지금까지 報告된 論文을 根據로 하여 本菌을 *Thiobacillus concretivorus*라고 同定할 수 있었으며 더욱이 *Thiobacillus concretivorus*는 *Thiobacillus thiooxidans*와는 分明히 相異한 種일 것이라는 結論을 얻게 되었다.

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