Microbiological Studies on Shallow Marine Areas—VI

Thiosulfate-oxidizing bacteria isolated from shallow bay

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Abstract

Twenty two strains of thiosulfate-oxidizing bacteria were isolated from sea water and bottom sediments of Omura Bay, west coast of Kyushu. The majority (73%) of the isolates highly utilized thiosulfate in the sea water organic media, and they were identified as thiobacilli group 0 by classification of HUTCHINSON et al. The test strains belonging to this group oxidized thiosulfate by constitutive or inductive enzyme system, and in the former cases, the growth of bacteria was stimulated by addition of thiosulfate. The others (27%) utilized thiosulfate only in the sea water inorganic media, though they grew in sea water organic media.

In the previous paper[1], it was reported that every summer low concentration of hydrogen sulfide had been detected in the bottom layer of the bay indicating wide distribution of thiobacilli. The oxidation of hydrogen sulfide in sea water proceeds chemically to thiosulfate[2] and sulfate. The former is said to be oxidized further by the bacteria[3,4]. However, these marine bacteria have not been studied thoroughly.

Recently, studies on thiobacilli in the marine environment have been reported by TILTON et al[5]., and also by ADAIR & GUNDERSEN[6]. Since their studies were based mainly on the utilization of thiosulfate in conjunction with the decrease of pH value in the culture, they did not isolate the ecologically important bacteria that utilize thiosulfate with the increase of pH value though the procedure itself was useful. On the other hand, they isolated some bacterial strains that required acidic pH value which could not exist in the natural sea water habitat[7]. There are some problems which are yet to be solved.

This study was made in an attempt to isolate from the bay the bacteria which have the ability to oxidize thiosulfate, and also to verify their characteristics as well as to know the relation between the distribution and physiological activities in the bottom layer of the bay.

Materials and Methods

Isolatin in pure culture. Isolation was commenced by positive cultivation of thiobacilli enumeration[1]. After reinoculating bacteria three times on thiosulfate

agar plate (Medium A), the selected colonies were transplanted to the liquid medium A. The only criterion for identifying the isolated strains as *Thiobacillus* was the ability to grow on thiosulfate culture[8]. By using sulfate-free medium and detection test of sulfate ions, the ability of the isolates to oxidize thiosulfate was also tested. A total of 22 strains growing on the media was purely isolated. Of these, 16 strains were from sea water of more than 17.5 m of deep and 6 strains from bottom sediments. The samples were obtained from Omura Bay with the depth of about 20 m, west coast of Kyushu, in August 1968.

Culture media. The following culture media were employed :

A. Thiosulfate inorganic sea water medium : The composition of this medium was similar to TILTON's[5], but it was dissolved in 75% aged sea water, with pH value adjusted to 7.8–8.0.

B. Thiosulfate inorganic fresh water medium: $Na_2S_2O_3 \cdot 5H_2O$, 1.0%; NH_4Cl, 0.01%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.02%; $CaCl_2$, 0.01%; $FeCl_3 \cdot 6H_2O$, 0.0001 %; tap water, pH 7.5.

C. Thiosulfate organic sea water medium: Medium A added with polypeptone 0.1% and yeast extract 0.01%.

D. Thiosulfate organic fresh water medium: Medium B added with polypeptone 0.1% and yeast extract 0.01%.

E. Sulfate-free medium for identifying thiosulfate-oxidizing bacteria(NISHIJIMA's medium T for thiosulfate-oxidizing bacteria[9] : $Na_2S_2O_3 \cdot 5H_2O$, 0.4%; KNO_3, 0.2%; $NaHCO_3$, 0.1%; NH_4Cl, 0.05%; KH_2PO_4, 0.02%; K_2HPO_4, 0.02%; $MgCl_2 \cdot 6H_2O$, 0.01%; $CaCl_2 \cdot 2H_2O$, 0.01%; $MnCl_2 \cdot 4H_2O$, 0.002%; $FeCl_2 \cdot 4H_2O$, 0.001 %, Na-EDTA, 0.002%; artificial sea water. Composition of artificial sea water NaCl, 23.5 g; $MgCl_2 \cdot 6H_2O$, 5.0 g; KBr, 0.09 g; KCl, 0.6 g; $NaHCO_3$, 0.2g; $CaCl_2 \cdot 2H_2O$, 1.1g; tap water 1 liter.

F. Thiosulfate yeast extract sea water medium for growth test : Yeast extract, 0.2%; K_2HPO_4, 0.02%; $FeCl_3 \cdot 6H_2O$, 0.0001%; artificial sea water, pH 7.8. The above basal medium was added with $Na_2S_2O_3$ 1.0%, $Na_2S_2O_3$ 0.5% & Na_2SO_4 0.5% or Na_2SO_4 1.0%.

G. S5, S6 and other test media for identification: These media were prepared as those of HUCHINSON et al.[8,10] With the exception of special test media, sodium chloride was supplemented to each medium in order to maintain the isotonicity with sea water.

In the preparation of all these media, the solutions of thiosulfate and phosphate were filter-sterilized separately before adding to the basal medium. Solid media were prepared by adding 1.5% agar. For stock culture, semi-solid medium A was used and maintained at 5°C.

Cultivation. Cultures of media G such as S5, S6 and other test media were incubated at 28°C and all others at 25°C. In the utilization test of thiosulfate, inoculum from medium A culture incubated for 3 days was transferred to 100 ml

of test medium contained in 200 ml flask. For growth test, medium F containing 1.0% sodium sulfate was used as preculture. After culturing for 48 hrs. in tube, 0.1 ml of it was inoculated to 7 ml of test medium. Agitating was made in the MONAD type water bath and growth was measured by turbidity at 660mμ. The same procedure was repeated three times.

Chemical test. Thiosulfate was determined iodometrically. First, 1 ml of culture from medium E was added with 0.2 ml of acidified salt solution (NaCl 240 g and conc. HCl 20ml/L) and then with a small amount of barium chloride powder. If white turbidity occurred, the solution was determined as positive of sulfate ions.

Results

The utilization of thiosulfate and change of pH in culture.

Each of the isolated 22 strains cultured in the thiosulfate organic or inorganic media prepared either with sea water or fresh water was incubated for 3 or 4 weeks. The utilization of thiosulfate and the change of pH value by growth in media A, C and D are shown in Fig.1. The utilization of thiosulfate exceeding 20% occurred only when organic media were used. In these cultures with the exception of 3 strains, oxidation of thiosulfate took place in conjunction with the increase of pH value in the medium. On the whole, the growth of isolated strains and their utilization of thiosulfate showed better results in the organic sea water media than in the inorganic sea water media. The results were poor in medium B. The stimulation of growth in inorganic medium by addition of growth factors was unsuccessful.

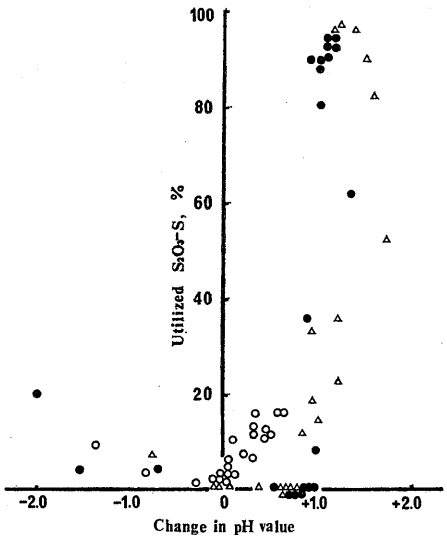

Fig. 1. The utilization of thiosulfate by thiobacilli isolated from seawater and bottom sediments. The results show 4 week-cultivation with medium A ○, and 3 week-cultivation with media C ● and D △ at 25°C.

Based on thiosulfate utilization and few other tests the 22 strains were divided into two groups. Group I with 16 strains including 12 strains from sea water and 4 strains from bottom sediments indicated better thiosulfate-utilizing ability in organic media than in inorganic media, but some fluctuation in the rate of utilization was observed. And group II with 6 strains grew in organic media, but thiosulfate oxidation occurred only in inorganic media.

Characteristics of the pure isolated strains.

As group I dominated group II and included strains of high thiosulfate-oxidizing potentiality, that group seemed to be more important ecologically. Therefore, group I was taken up for further study. The strains of this group were Gram-negative, nonsporeforming and motile rods, forming small colonies on thiosulfate culture plate, each cell measuring about 0.7 to 1.0 by 1.2 to 3.2 microns. Detection of sulfate ions from cultures of sulfate-free medium was positive in some strains but indistinct in several other.

Strains SW22, SW13, KA11 and S7 representing group I were further studied and identified by applying the diagnostic method of HUTCHINSON et al.[11] Their characteristics are shown in Table 1 and may be regarded as similar to group 0 of *Thiobacillus*. Detailed studies on group II were omitted on the assumption that it included the known species such as *T. novellus* or its related strains.

Table 1. Diagnostic tests for the thiobacilli isolated from shallow bay.

Medium	Criterion	S7	SW13	SW22	KA11	NaCl,* %
S6	Final pH	6.8	6.5	6.5	6.8	2
S5	〃	6.8	5.5	7.0	6.0	2
S6	%, thiosulfate oxidation	75.6	38.8	65.6	26.4	2
S5	〃	54.7	4.2	68.4	26.3	2
S0+6% thiosulfate	〃	3.8	5.4	2.2	0	2
S6+4% phosphate	Inhibition	+	+	+	±	
S6+4% phosphate	〃	−	−	−	+	1
S5+4% phosphate	〃	+	+	+	+	
S5+4% phosphate	〃	+	+	−	+	1
S6+5% NaCl	〃	−	−	−	−	
S5+5% NaCl	〃	+	−	−	+	
S8	Growth	+	+	+	+	2
Anaerobic condition	Gas formation	−	−	−	−	
S7	Thiocyanate oxidation	−	−	−	−	2
Iron	Iron oxidation	−	−	−	−	2.5
Nutrient agar plate	Growth	+	+	+	+	2.5
Koser's citrate	〃	+	+	+	+	2.5
Thiosulfate agar	Sulfur deposition	−	−	−	−	2

These tests were carried out by the methods of HUCHINSON et al.* Sodium chloride was supplemented to the test media to maintain the isotonicity with sea water.

Effect of thiosulfate on the growth of test strains.

The comparison of growth was made on 4 differential strains by using medium F. The growth curve for these test strains indicated 2 different types as shown in Fig. 2. The growth of strains SW22 and KA11 was stimulated by thiosulfate at the initial growth phase, but at stationary growth phase they were of lower

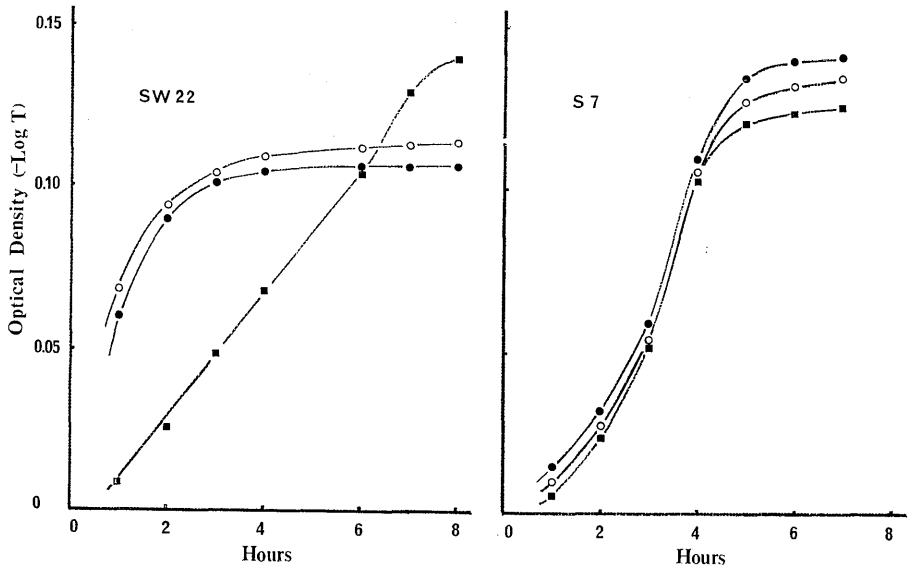

Fig. 2. The growth curves of strains SW22 and S7 in sodium thiosulfate yeast extract broth and sodium sulfate yeast extract broth.
○ 1% $Na_2S_2O_3$; ● 0.5% $Na_2S_2O_3$ plus 0.5% Na_2SO_4 and ■ 1% Na_2SO_4

population level than that of control culture. On the other hand, strains S7 and SW13 cultured with thiosulfate had little influence at the initial growth phase but following the late logarithmic growth phase to the stationary growth phase, the curves were somewhat higher. From these results, it is clear that thiosulfate played some role in the metabolism of these test strains.

In these experiments, yeast extract was used mainly as organic matter. However, both strains SW22 and S7 when cultivated with ammonium chloride as nitrogen source utilized glucose, lactate, malate and succinate. Acetate as carbone source was effective for strain SW22 but not for strain S7.

Influence of precultivation on biosynthesis of thiosulfate oxidation enzyme system.

Thiosulfate oxidation system of thiobacilli are biosynthesized by constitutive enzyme or by inductive manner depending on the kind of species. The resting cells obtained from thiosulfate culture and thiosulfate-free culture were arbitrarily tested by manometric method on the oxidation of thiosulfate. Strain S7 growing in medium F with sodium sulfate did not oxidize thiosulfate immediately. But the resting cells of strain S7 obtained from medium F with thiosulfate oxidized thiosulfate inductively and showed similar mode as strain SW22 which oxidized thiosulfate by constitutive enzyme. These results are indicated in Fig. 3. The difference between the two types of growth curves is assumed to be due to the difference of biosynthesizing ability of the thiosulfate-oxidizing enzyme as observed in these experiments.

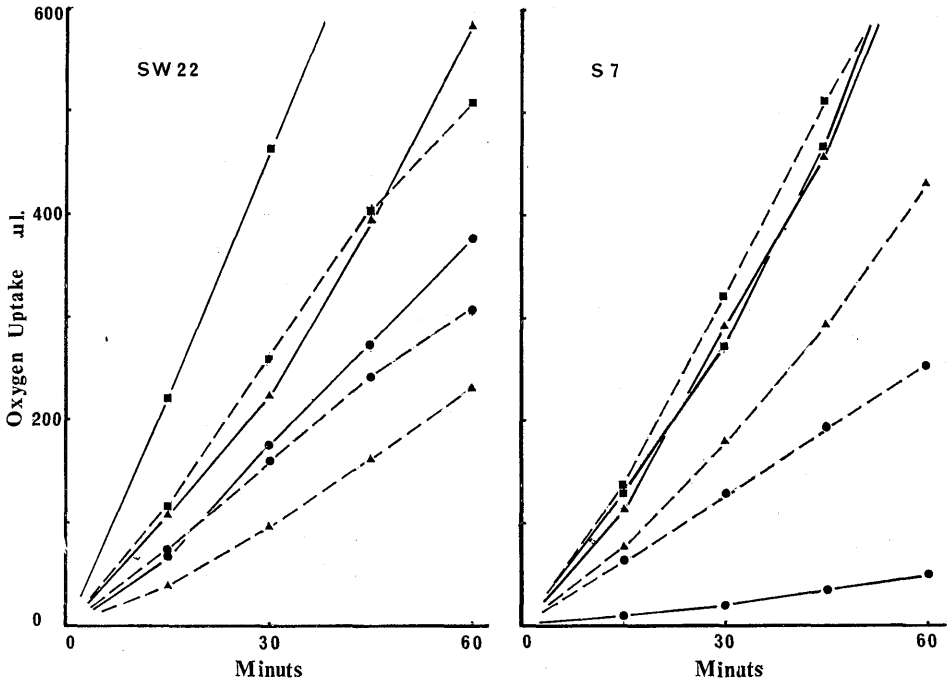

Fig. 3. The oxidation of thiosulfate and yeast extract by cell suspension of strains SW 22 and S7.
Reaction system : Gas phase, air. Temp., 25°C. 2 ml of cell suspension* with 0.05 M trisaminomethan buffer solution with pH 7.8. Total volume 2.2 ml. 0.2 ml of 20% KOH in center well.
▲ yeast extract 20 mg; ■ yeast extract 10 mg plus $Na_2S_2O_3$ 63μM and ● $Na_2S_2O_3$ 126μM. Solid line : cells from Na_2SO_4 yeast extract medium, 25°C, 2 days. Broken line : Cells from $Na_2S_2O_3$ yeast extract medium, 25°C, 2 days. Endogenous respiration which was measured in the reaction system with Na_2SO_4 140μM, was subtracted. Buffer solution was prepared by mixing NaCl 1.35%, $MgCl_2 \cdot 6H_2O$ 0.5%, $CaCl_2 \cdot 2H_2O$ 0.1% and KCl 0.05%.
* Optical density was adjusted to 0.600 at 660 mμ with 10 mm cuvette.

Discussion

In this study, obligate autotroph was not isolated. The pure isolated 16 strains having the ability of facultative autotroph — HUTCHINSON's group 0 predominating — may be classified as Pseudomonads. In the natural habitat, particularly in soil, the heterotroph is said to have a more important role in the sulfur cycle than the obligate autotroph[12,13]. *Thiobacillus* group I isolated by the authors, which utilizes organic matter and oxidizes thiosulfate, seems to be the dominant type in the bay.

In Omura Bay, organic matters are supplied by the growth and decomposition of the planktons, and they seem to support the growth and survival of these bacteria. KRISS[14] said that thiobacilli could be detected regularly in the planktonsphere. As have been previously reported of the thiobacilli's vertical

distribution[1)], the abundance in the lower layer and some distribution in the upper layer, seemingly the planktonsphere, would be explained by the fact that the dominant group of thiobacilli was of heterotroph to facultative autotroph.

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