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# Study On Marine Pigmented Bacteria—I

## Distribution and Characteristics of Pigmented Bacteria

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**Abstract**

The pigmented bacteria distributed in the Kinko bay were *Pseudomonas, Corynebacterium, Flavobacterium, Micrococcus* and *Alteromonas* spp. Among them, *Pseudomonas* and *Corynebacterium* spp. were predominant. Purple bacteria belonged to the genus *Alteromonas*. As the mouth of the bay, *Pseudomonas* was predominant, but at the innermost station *Corynebactrium* predominated.

The pigment in these bacteria, except for strains of *Alteromonas* was found to be a carotenoid by means of spectrophotometric assessment of absorbance and Carr-Price reaction. One of the pigmented *Corynebacterium*, C–2 strain produced carotenoid pigment upon supplementation of a dialyzable constituent of yeast extract to a basal medium.

The pigment of *Alteromonas* strains was violacein. *Alteromonas* B, C, and D strains produced their purple pigment in a histidine or aspartic acid medium prepared as a sole source of nitrogen. Supplementation of tryptophan inhibited their growth and pigment production.

Many marine bacteria, both gram positive and gram negative, produce pigments on usual culture media. The characteristics of these bacteria resemble those of members of the genera *Pseudomonas*, *Flavobacterium*, *Corynebacterium* and *Alteromonas*.

The purpose of this study is not only to know the ecological importance of pigmented bacteria in sea water, but also to examine the characteristics of marine pigmented bacteria.

## Materials and Methods

**Bacterial strains.** The strains used in this study were isolated from the Kinko (Kagoshima) bay. Sampling stations are shown in Fig. 1. Sampling was carried out at four stations at three depths: surface, 50 m, 100 m. One exception, station C was from the surface of bottom due to shallowness. The isolation media used were modified ZoBELL 2216 E agar which was prepared with Herbst's artificial sea water, and another agar medium composed of glucose (0.5%), polypeptone (0.5%) and yeast extract (0.1%) (GPY). Viable counts of bacteria in sample water were determined on the two agar media described above. Three purple bacteria used in

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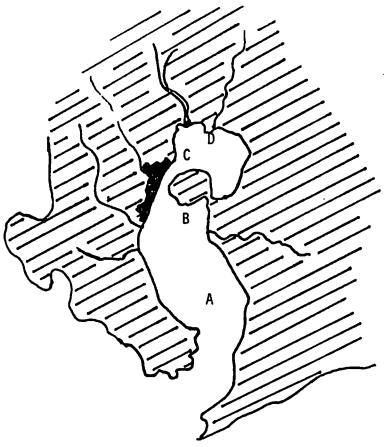


Fig. 1. Sampling stations in the Kinko bay.
A is off shore 300 m from Ibusuki B is near ferry boat harbour of Sakurazima
C is at Ryugamizu near the cage of yellow tail farm D is the innermost bay

this study were taken up randomly from three stations B, C, and D and named as strain B, C, and D respectively.

**Pigment extraction and identification.** Orange, greenish-yellow, yellow, brown and red pigmented bacteria were kept in acetone for 30 min, and successively centrifuged at 8,000 r.p.m. for 20 min, the supernatant was transferred into petroleum ether. The pigment which was dissolved in the solvent was examined to determine the absorption spectrum by means of Shimadzu double beam spectrophotometer UV 200. In order to confirm the carotenoid having $\beta$-ionone, the Carr-Price reaction was applied.

Purple bacteria were kept in acetone at 4°C for 30 min, and successively centrifuged at 8,000 r.p.m. for 20 min. Three volumes of distilled water were added to the supernatant and the resulting crude crystal was collected on the sintered glass filter, and washed 3 times with water and $CHCl_3$. The crystal was resuspended in acetone and dried over silica gel. The absorption spectrum of the dissolved pigment was measured on the spectrophotometer.

**Characteristics of pigmented bacteria.** The purple bacteria were classified according to Bergey's manual 8th ed. (1), SIVENDRA et al. (2), and GAUTHIER (3), other pigmented bacteria were classified according to Bergey's manual 8th ed. and the report of SHEWAN et al. (4).

**The effect of culture media on growth and pigment production.** *Corynebacterium* C–2 strain was used for the study of carotenoid production and growth on the various media. *Alteromonas* B and C strains were used as the representative strain of purple bacteria.

The experiments with *Corynebacterium* C–2 were carried out at 25°C and 37°C with 48 hrs incubation. Growth and pigment production were observed and judged with

the naked eye by several persons. The results observed and judged were compared with the results obtained in 0.5% polypeptone-ASW medium.

The experiments with purple strains B and C were based on the effect of amino acids and of tryptophan supplements. The incubation was at 25°C for amino acids experiments and at both 25°C and 37°C for tryptophan. All experimental results were obtained after 24 hr incubation.

## Results

As shown in Table 1, the total bacterial count was higher in the surface water than at the other two depths, while the distribution of pigmented bacteria did not show the same relationship. Among the bacteria, orange, yellow, greenish yellow, and pinkish red bacteria were abundant at all stations. No marked difference regarding the isolation of pigmented bacteria was observed between the two media (the modified ZOBELL 2216 E and GPY).

In Table 2, the characteristics of several representative strains isolated from each station (except for purple bacteria) are shown. The predominant pigmented bacteria in the Kinko bay were members of *Pseudomonas*, *Corynebacteriusm*, a few of *Flavobacterium*, *Micrococcus*, and purple bacteria. Considering the results shown in Tables 1 and 2, *Pseudomonas* and *Flavobacterium* were predominant at the mouth of the bay, while *Corynebacterium* was predominant at the innermost bay. The purple bacteria were distributed widely in the sea water of the bay.

Table 1. Bacterial count and pigmented bacterial count at each station and depth.

| Station | Bacterial count in 1 m*l* | | | Pigmented bacterial count in 1 m*l* | |
|---|---|---|---|---|---|
| | Depth | ZoBell | GPY | ZoBell | GPY |
| A | Surface | $1.3\times10^3$ | $5.0\times10^2$ | ND | ND |
| | 50 m | $4.2\times10^2$ | $1.2\times10^2$ | OR 10 | OR 10, Y 30 |
| | 100 | $7.6\times10^1$ | $6.6\times10^1$ | OR 20, Y 10 | ND |
| B | Surface | $2.4\times10^3$ | ND | P 15, OR 10 | OR 20, R 10, P 10 |
| | 50 m | $2.2\times10^2$ | $9.8\times10^1$ | OR 50, P 70 | OR 13, Y 20 |
| | 100 | $1.9\times10^2$ | $9.6\times10^1$ | — | — |
| C | Surface | $6.8\times10^2$ | $7.2\times10^1$ | P 20, OR 30 | OR 15, P 6, Y 5 |
| | 50 m | $1.2\times10^2$ | $5.0\times10^1$ | Y 30 | Y 10 |
| | Bottom | $6.4\times10^1$ | $5.2\times10^1$ | ND | OR 30 |
| D | Surface | $8.2\times10^2$ | $4.8\times10^2$ | Y 10 | Y 50, B 10 |
| | 50 m | $2.6\times10^2$ | $1.8\times10^2$ | OR 20 | OR 10, P 10 |
| | 100 | $7.0\times10^1$ | $3.8\times10^1$ | Y 10 | Y 10 |

ND not determine Y Yellow OR Orange
P Purple R Red B Brown

Table 2. Characteristics of selected pigmented bacteria.

| | A–1 | A–2 | A–3 | B–2 | B–5 | C–1 | C–2 | C–3 | D–1 | D–2 |
|---|---|---|---|---|---|---|---|---|---|---|
| Form* | R | R | R | R | R | R | R | R | C | R |
| Motility | + | + | − | + | + | + | − | + | − | − |
| Flagellation** | M | M | − | M | M | M | − | M | − | − |
| Gram stain | − | − | − | − | − | + | + | + | + | + |
| Hugh & A*** | − | − | − | − | − | − | − | − | − | − |
| Leifson An*** | − | − | − | − | − | − | − | − | − | − |
| Kovac oxiduse | − | − | + | + | + | − | − | − | − | − |
| Catelase | − | + | + | + | + | + | + | + | + | + |
| Arginine A*** | − | + | + | + | − | − | − | − | − | − |
| An*** | − | − | − | − | − | − | − | − | − | − |
| Indol | − | − | − | − | − | − | − | − | − | − |
| MR | − | − | − | − | − | − | − | − | − | − |
| VP | − | − | − | − | − | − | − | − | − | − |
| $H_2S$ | − | + | − | − | + | − | − | − | + | − |
| Caseinolysis | + | + | + | + | + | + | + | + | + | − |
| Gelantinolysis | + | + | + | + | + | + | + | + | + | − |
| Amylolysis | − | + | − | + | − | − | − | − | − | − |
| Opt. growth Temp. | 25–30 | 25–30 | 25–30 | 15–20 | 25–30 | 25–30 | 25–30 | 25–30 | 30 | 20–25 |
| Colony color**** | GY | LY | IOR | GY | IOR | OR | OR | GY | Y | PR |
| Genus | Pseud. | Pseud. | Flav. | Pseud. | Pseud. | Coryne. | Coryne. | Coryne. | Coccus | Coryne. |

A–1, A–2, and A–3 strains were isolated from A station
B–2 and B–5 from B station
C–1, C–2 and C–3 from C station
D–1 and D–2 from D station

* R: Rod, C: coccus
** M: Monotrichous
*** A: Aerobic An: Anaerobic
**** GY: greenish yellow, LY: Light yellow, IOR: Ivory orange, Y: Yellow, PR: Pinkish red, OR: Orange.

Table 3 shows the characteristics of purple bacteria. All of the isolates of purple bacteria were identified as *Alteromonas* based on these characteristics. The three strains of purple bacteria B, C and D were similar except for two observations. They differed in starch hydrolysis and sea water requirement as shown in Table 4.

Table 5 shows the results of the spectrophotometric measurement of isolated pigments, and the results of Carr-Price reactions. Except for A–2 and C–3 strains, the isolated pigments appear to be carotenoid in nature (6).

The growth and pigment production of *Corynebacterium* C–2 strain were shown in Fig. 2. It was ascertainable that both the growth and the pigment production mainly depended upon the addition of polypeptone and yeast extract. Pigment

Table 3. Selected characteristics of purple bacteria.

| | B | C | D |
|---|---|---|---|
| Form | Rod | Rod | Rod |
| Flagellation | single, polar to subpolar | | unsheathed |
| Motility | + | + | + |
| G C ratio | 44.9 | 43.4 | 43.9 |
| Gram stain | − | − | − |
| Methyl red | − | − | ND |
| Voges Proskauer | − | − | ND |
| Indol | − | − | − |
| Nitrate–Nitite | + | + | ND |
| Citrate | − | − | ND |
| Oxidase | − | − | − |
| Catalase | + | + | + |
| Urease | − | − | − |
| Casein hydrolysis | + | + | + |
| Starch hydrolysis | + | − | + |
| Haemolysis | + | + | ND |
| Lecithinase | + | + | + |
| Arginine dihydrolase | + | + | + |
| DNase | + | + | + |
| Hugh-Leifson | Oxi | Oxi | Oxi |

Table 4. Comparison of reported characteristics for violet pigmented-Gram negative rods.

| | Bergeys[1] Ch. violaceum | Sivendra et al.[2] Ch. violaceum | Gauthier[3] Alteromonus | B, C, D Strains |
|---|---|---|---|---|
| Temp, Maximum | 37°C | 37°C | 30°C | 37°C |
| Catalase | + | + | − | − |
| Oxidase | + | + | + | + |
| Casein | + | + | + | + |
| Gelatin | + | + | ++ | + |
| Citrate | + | Variable | − | − |
| H & L/glucose | Ferment | Ferment | No react. or oxid. | Oxidative |
| Starch hydrolysis | − to weak | − | + | B & D + C − |
| Nitrate reduction | + | + | − | + |
| Motile | + | + | +(polar) | + |
| Lecithinase | + | + | + | + |
| Hemolysis | + | + | − | + |
| G/C | 63–68% | ND | 41% | |
| Indol | − | − | − | − |

Table 4. (Continued)

| | Bergeys[1] Ch. violaceum | Sivendra et al.[2] Ch. violaceum | Gauthier[3] Alteromonus | B, C, D Strains |
|---|---|---|---|---|
| Methyl red | ND | − | ND | − |
| Voges-Proskaur | − | − | ND | − |
| EMB | ND | + | ND | − |
| McConkey | ND | + | ND | − |
| Pigment: wave length | 579 nm | Pig & nonpig | 580±5 nm | 575 nm |
| $O_2$ | Strict or Fac. | Fac. | Strict | Strict |
| Single carbon nutrition | Many | ND | ND | Some |
| Sea water | No | No | Required | Required B & D |
| | | | Killed at 37°C 2 hrs | |

[1] Bergey's Manual 8th Edition

[2] Sivendra, R., H. S. Lo and K. L. Lim. 1975. Identification of Ch. violaceum: pigmented and non-pigmented strains. J. Gen. Microbiol. **90**: 21–31

[3] Gauthier, M. J. 1976. Morphological physiological and biochemical characteristics of some violet pigmented bacteria isolated from sea water. Can. J. Microbiol. **22**: 138–149

Table 5. Spectrophotometric measurement and Carr-Price reaction.

| λmax in | A–1 | B–2 | A–2 | A–3 | B–5 | D–1 | D–2 | C–1 | C–2 | C–3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Acetone | 402 | 371 | 395 | 406 | 406 | ND | 500 | 441 | 408 | 415 nm |
| | 422 | 415 | | 429 | 432 | | 468 | 458 | 445 | 432 |
| | | 442 | | 452 | 452 | | 530 | 491 | 465 | |
| Methanol | 420 | 440 | 395 | 403 | 406 | 425 | 465 | 463 | 463 | 427 |
| | | | | 425 | 429 | 447 | 495 | 485 | 485 | |
| | | | | | 450 | 473 | 528 | | | |
| Petroleum ether | 405 | ND | insoluble | 408 | 408 | 445 | 500 | 445 | 445 | insoluble |
| | | | | 427 | 427 | 465 | 535 | 465 | 465 | |
| | | | | 453 | 453 | 495 | | 495 | 495 | |
| Carr-Price reaction | + | ND | ND | + | + | ND | ND | + | + | ND |

\+ positive
ND not determined

production was especially dependent upon the dialysable constituent in yeast extract.

Purple bacteria were treated according to the method previously described, and the pigment crystal obtained was subjected to the spectrophotometric procedure to determine maxima absorbance of the pigment. The absorbance maxima obtained

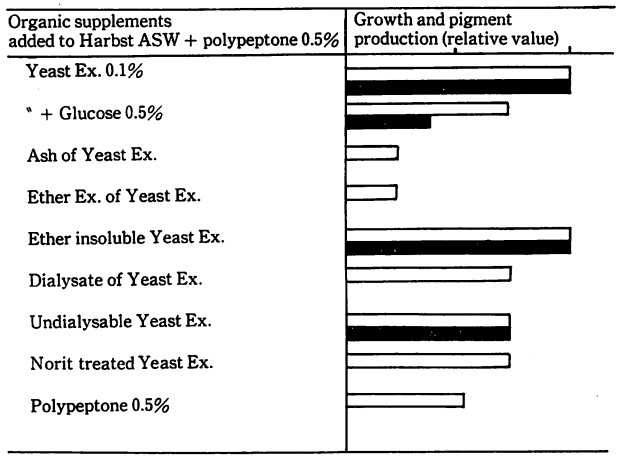


Fig. 2. The effects of organic supplements on growth and pigment production of Corynebacterium C–2 strain.

Growth pigment

Table 6. Minimal nutrition of Alteromonas B and C strains.

| Amino Acid[1] | Marine strain B | | | | Non-Marine strain C | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose[2] | | Glycerol[2] | | Glucose | | Glycerol | |
| | 37°C | 25°C | 37°C | 25°C | 37°C | 25°C | 37°C | 25°C |
| Histidine | +p[3] | +p | + | + | + | +p | + | + |
| Aspartic acid | + | +p | − | + | − | − | − | − |
| Proline | − | − | − | + | − | − | − | + |
| Arginine | − | − | − | + | − | − | − | + |
| Alanine | − | − | − | + | − | − | − | + |
| Glutamic acid | − | − | − | − | − | − | − | − |
| Cysteine | − | − | − | − | − | − | − | − |
| Methionine | − | − | − | − | − | − | − | − |
| Valine | − | − | − | − | − | − | − | − |
| Leucine | − | − | − | − | − | − | − | − |
| Tryptophan | − | − | − | − | − | − | − | − |
| Phenylalanine | − | − | − | − | − | − | − | − |
| Tyrosine | − | − | − | − | − | − | − | − |
| Serine | − | − | − | − | − | − | − | − |

[1] Added at concentration of 50 mg percent

[2] Concentration 0.5 percent

[3] + growth; − no growth; p=pigment

were 370 nm and 560 nm. The pigment changed from purple to blue in pyridine, acetone and HCl, and to green with addition of 10% NaOH. The pigment was identified as violacein from these characteristics (5). Purple bacteria B and C strains grew and produced pigment in the minimum media as shown in Table 6, that is,

Table 7. Effect of tryptophan on growth of Alteromonas B and C strains.

| Medium constituents | Marine Strain B | | Non-Marine Strain C | |
|---|---|---|---|---|
| | 37°C | 25°C | 37°C | 25°C |
| Glucose+Histidine | +p[1] | +p | + | +p |
| +25 mg % tryptophan | ±* | ±* | − | − |
| +50 mg % tryptophan | ±* | ±* | − | − |
| Glucose+Aspartic acid | +p | +p | + | +p |
| +25 mg % tryptophan | +p | +p | ± | ± |
| +50 mg % tryptophan | − | − | − | ± |
| Glycerol+Histidine | + | + | − | − |
| +25 mg % tryptophan | − | − | − | − |
| +50 mg % tryptophan | − | − | − | − |
| Glycerol+Aspartic acid | + | − | − | − |
| +25 mg % tryptophan | − | − | − | − |
| +50 mg % tryptophan | − | − | − | − |

[1] + growth; − no growth; p=purple pigment
* Brown water soluble pigment

they could grow and produce pigment in pyruvate and histidine with ASW, pyruvate, $(NH_4)_2SO_4$, and phosphate with ASW, or glucose, histidine with ASW. In the case of the last medium, C strain could not produce pigment. Tryptophan inhibited not only the growth, but also pigment production (Table 7).

## Discussion

It is assumed that there are ecological complexities involved in the distribution of pigmented bacteria in the Kinko bay, because the bay is constantly affected by the Kuroshio Current and inflow of river water accompanied with pollution. The importance of pigmented bacteria in the bay are never negligible not only from an ecological view point but also from the standpoint of pollution.

The result of this study reveals that the bacteria which possess carotenoid as their pigment are predominant in the bay, and purple bacteria belonging to *Alteromonas* are few but widely distributed.

As the pigment production of bacteria is affected by nutrition supplements, the distribution of the pigmented bacteria in the bay may be dependent on environmental conditions such as pollution and temperature.

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