東京湾から分離されたHeterosigma akashiwo(Hada)Hadaの増殖特性
Growth Characteristics of a Strain of *Heterosigma akashiwo* (HADA) HADA Isolated from Tokyo Bay, Japan

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Abstract

The strain of *Heterosigma akashiwo* (HADA) HADA from Tokyo Bay, called *H. akashiwo* TKY825, was cultured axenically under laboratory conditions, and investigated its physiological characteristics. *H. akashiwo* TKY825 was extremely euryhaline and tolerable to very low salinities, i.e., growth was hardly affected by salinity change from 8 to 34%. This strain grew well at high pH above 8.5. *H. akashiwo* TKY825 was not able to use glycerophosphate (phosphorus source) and urea (nitrogen source). Among the strains of *H. akashiwo* for which urea utilization was examined, this strain was the only one incapable of utilizing urea effectively. The minimum cell quota of nitrogen and phosphorus were estimated to be 24 pg·cell⁻¹ and 1.82 pg·cell⁻¹, respectively. Vitamin B₁₂ and iron were essential for the growth of this strain. Any organic substrates such as Trypticase, yeast extract and glucose had no effects on the growth. From these results, it is concluded that *H. akashiwo* TKY825 is well adaptive to the extremely eutrophic waters with inorganic nutrients in the innermost part of Tokyo Bay.

Keywords: *Heterosigma akashiwo*, Tokyo Bay, red tide, growth characteristics, axenic culture

*Heterosigma akashiwo* (HADA) HADA, Raphidophyceae, is a well-known alga causing red tides in coastal waters of Japan. The occurrence and red-tide formation of *H. akashiwo* in Tokyo Bay have been recorded since 1977 by the Tokyo Metropolitan Bureau of Environmental Protection. *H. akashiwo* appears every warm season of the year and is recognized to be a predominant species next to *Skeletonema costatum* (GREVILLE) CLEVE, Bacillariophyceae, in Tokyo Bay.

In the past there were some taxonomical confusions in *H. akashiwo* and other related organisms. HARA et al. (1985) and HARA & CHIHARA (1987) demonstrated the morphological identity of *H. akashiwo* and *H. inlandica* HADA as well as the obvious distinction between *H. akashiwo* and *Olisthodiscs luteus* originally described by CARTER (1937). At present, the genus *Heterosigma* has a single species, *H. akashiwo*. On the other hand, some investigators (WATANABE et al. 1982, HARA

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& Chihara 1987) suggested that *H. akashiwo* was consisted of different strains because of some physiological differences between Fukuyama strain ("Entomosigma"), thereafter named *H. akashiwo*, Iwasaki et al. 1968) and Gokasho strain ("H. inlandica", Iwaki & Sasada 1969). Watanabe et al. (1982) concluded, on the basis of their comparative studies on physiological characteristics of Osaka strain and other previously reported strains including Narragansett strain ("O. luteus", Tomas 1978, 1979), that *H. akashiwo* was composed of at least three different strains, and each strain had the specific growth characteristics for its habitat.

As mentioned above, *H. akashiwo* is also distributed in Tokyo Bay, one of the most polluted areas in Japanese coastal waters, and water quality of the inner-most part of the Bay is in "hypertrophic" level. It is therefore expected that *H. akashiwo* in Tokyo Bay has some specific growth characteristics influenced by such water quality of the Bay.

In the present study, a Tokyo strain of *H. akashiwo* was cultured under laboratory conditions and compared with other known strains on its adaptive feature to the habitat.

**Materials and Methods**

A strain of *H. akashiwo*, hereafter called *H. akashiwo* TKY825, was isolated from a seawater sample collected at a station off Tokyo Port in May, 1982. An axenic clonal culture was obtained by micropipette washing method.

Basal culture media were SW II (Iwasaki 1961) with vitamin B$_{12}$ (2 µg·l$^{-1}$) and the synthetic medium ASP2M, a modification of ASP2 (Provasoli et al. 1957) from which sodium metasilicate and all other vitamins excluding vitamin B$_{12}$ (0.2 µg·l$^{-1}$), thiamine (100 µg·l$^{-1}$) and biotin (1 µg·l$^{-1}$) were omitted. SW II was used for the examination in salinity, and ASP2M for the others. The seawater for SW II was collected in winter from Sagami Bay, stored in the dark for a year, then treated with powdered activated charcoal, and filtered through a glass-fiber filter (GF/C).

Sterility tests in all experiments were done using ASP2M medium enriched with organic substances similar to STP (Provasoli et al. 1957) and the TYG medium (one liter of 80% seawater containing Trypticase (BBL) 0.5g, yeast extract (Difco) 0.25g and glucose 0.1g).

In a series of experiment including pH above 8.5, the culture medium was sterilized by membrane filtration with Millipore GS (pore size 0.22 µm) to avoid precipitation during autoclaving. Also in experiments in utilization of nitrogen and phosphorus, the filter-sterilization technique was applied to avoid decomposition of urea and Na$_2$glycerophosphate during autoclaving. The media used in all the other cases were sterilized by autoclaving (121°C, 15 min).

Screw-capped test tubes (18×160 mm) containing 10 ml of medium were used as culture vessels. All cultures were incubated in a growth chamber at 20±1°C and 4,000 lx, illuminated with white fluorescent lamps from a side of the tube for 12 h in a day.

Growth yield of *H. akashiwo* TKY825 was measured mainly on a spectrophotometer because the regression having a good linear correlation, the equation, $Y = (5.435 \times 10^{-7})X + 0.004$
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was observed between the number of cells per milliliter (X) and the extinction values (Y) at a wavelength of 430 nm in a microcell having 10-mm light path, and expressed as the relative values; percentage to the maximum value of each measurement. When necessary, number of cells were counted microscopically on a hemocytometer. The measurements were ordinarily made 12 days after inoculation, unless the organism was precultured for starvation.

In experiments concerning nutrition, starvation cultures were performed before inoculation for appropriate periods of days as indicated in each result.

The minimum cell quota ($q_m$) of nitrogen ($q_m$N) and phosphorus ($q_m$P) were estimated by the following equation:

$$q_m = \frac{(S_2 - S_1)}{(C_2 - C_1)}$$

where $q_m$ is minimum cell quota, and $C_1$ and $C_2$ are cell densities at two addition levels of limiting nutrient of nitrogen or phosphorus, $S_1$ and $S_2$, respectively in the linear portion of the maximum growth response. Complete disappearance of the limiting nutrient from the medium was confirmed by the preliminary examination when the growth reached its maximum.

**Results**

*Effect of Salinity*

*H. akashiwo* TKY825 showed almost equal growth over 8%, but the remarkable suppression was observed below 3% (Figure 1). No growth occurred at 1.7%.

*Effect of pH*

Effects of pH after sterilization are shown in Figure 2. *H. akashiwo* TKY825 grew well in the range of pH 8.5 - 9.0. Growth decreased with pH below this range. Fluctuation of pH between the start and termination of culture was 0.0 - 0.1 unit.

*Utilization of Nitrogen Sources*

Starvation culture was performed for 14 days in ASP2M without nitrogen sources. The availability of NaNO$_3$, NH$_4$Cl and urea as nitrogen sources was
TABLE 1. GROWTH OF *HETEROSIGMA AKASHIWO* TKY 825 WITH DIFFERENT NITROGEN SOURCES. (AFTER 35 DAYS)

<table>
<thead>
<tr>
<th>Concentration (N, mg·l⁻¹)</th>
<th>Growth (Number of cells × 10⁴·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added</td>
<td>1.79 1.79 1.79</td>
</tr>
<tr>
<td>1</td>
<td>6.64 24.23 1.99</td>
</tr>
<tr>
<td>10</td>
<td>36.53 0.00 3.13</td>
</tr>
</tbody>
</table>

tested (Table 1). Ammonium was effective at 1 mg N·l⁻¹, while nitrate gave good growth at 10 mg N·l⁻¹ without toxic effect. *H. akashiwo* TKY825, however, could not utilize urea effectively; it only doubled the growth of control culture (where no nitrogen source was added) even at 10 mg N·l⁻¹ of urea. Then, the relationship between added nitrogen concentration and the maximum growth of *H. akashiwo* TKY825 was examined with NaNO₃ and NH₄Cl. The maximum growth of *H. akashiwo* TKY825 showed a good linear response depending on nitrogen concentrations in the range tested (Figure 3). From this result, q_mN was estimated to be 24 pg·cell⁻¹.

Utilization of Phosphorus Sources

Cells of *H. akashiwo* TKY825 were precultured in ASP2M lacking phosphorus nutrient for 10 days. *H. akashiwo* TKY825 grew only in the medium with KH₂PO₄ (Figure 4). A good linear response of the maximum growth of *H. akashiwo* TKY 825 was also observed in the range of 0.03·0.3 mg P·l⁻¹. Estimated q_mP was 1.82 pg·cell⁻¹.

Effect of Iron and Other Metals
Fig. 3. Maximum growth yield of *Heterosigma akashiwo* TKY 825 in the presence of NaNO₃ (●) and NH₄Cl (▲).

Fig. 4. Maximum growth yield of *Heterosigma akashiwo* TKY 825 in the presence of KH₂PO₄ (●) and Na₂-glycerophosphate (▲).
TABLE 2. EFFECT OF IRON AND PII METALS ON THE GROWTH OF *HETEROSIGMA AKASHIWO* TKY825. (AFTER 24 DAYS)

<table>
<thead>
<tr>
<th>Metals</th>
<th>Growth (Number of cells × 10⁴·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added</td>
<td>0.02</td>
</tr>
<tr>
<td>Fe* (5 µg·l⁻¹)</td>
<td>2.15</td>
</tr>
<tr>
<td>(50 µg·l⁻¹)</td>
<td>21.88</td>
</tr>
<tr>
<td>(500 µg·l⁻¹)</td>
<td>24.72</td>
</tr>
<tr>
<td>Fe (200 µg·l⁻¹)</td>
<td>20.22</td>
</tr>
<tr>
<td>+ PII metals (30 ml·l⁻¹)**</td>
<td></td>
</tr>
</tbody>
</table>

* Chelated with EDTA (1:1 molar ratio).
** Total metal concentration: Fe 500 µg·l⁻¹, Mn 1200 µg·l⁻¹, Zn 150 µg·l⁻¹, Co 30 µg·l⁻¹, B 6 mg·l⁻¹.

TABLE 3. EFFECT OF VITAMINS ON THE GROWTH OF *HETEROSIGMA AKASHIWO* TKY825. (AFTER 24 DAYS)

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Growth (Number of cells × 10⁴·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added</td>
<td>0.00</td>
</tr>
<tr>
<td>Vitamin B₁₂ (1 µg·l⁻¹)</td>
<td>29.28</td>
</tr>
<tr>
<td>Thiamine (100 µg·l⁻¹)</td>
<td>0.18</td>
</tr>
<tr>
<td>+ Biotin (1 µg·l⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂ (1 µg·l⁻¹)</td>
<td>29.64</td>
</tr>
<tr>
<td>+ Thiamine (100 µg·l⁻¹)</td>
<td></td>
</tr>
<tr>
<td>+ Biotin (1 µg·l⁻¹)</td>
<td></td>
</tr>
</tbody>
</table>

Starvation culture was performed for 13 days in ASP2M without addition of metals. No growth of *H. akashiwo* TKY825 occurred in the metal free medium, while good growth was observed in the medium containing only Fe at concentrations above 50 µg·l⁻¹ with EDTA (ethylenediaminetetraacetic acid) (Table 2). Both enhanced growth at 500 µg·l⁻¹ of Fe and synergistic effects due to the addition of other metals, such as Mn, Zn, Co and B in PII metals, were not observed.

*Vitamin Requirements*

Vitamin-starved cells were obtained by serial transfers in vitamin-free ASP2M for 36 days. Results shown in Table 3 indicated that *H. akashiwo* TKY825 required only vitamin B₁₂ essentially. Thiamine and biotin showed neither substitutional activity for vitamin B₁₂ nor growth stimulative effects under coexistence with
vitamin B_{12}.

**Effects of Organic Substances**

The presence of Trypticase, yeast extract and glucose in ASP2M in the range of 1-100 mg·l^{-1} gave no effects on the growth of *H. akashiwo* TKY825.

**Discussion**

*H. akashiwo* TKY825 was extremely euryhaline and tolerable to very low salinities. Although the growth responses of red-tide flagellates to salinity change were known to be influenced by temperature (Tomas 1978, Iwasaki 1979, Nozawa 1980), most of the previous results were comparable with this study because they were largely obtained at 20°C. Salinity of 3\% permitted half-maximum growth of this strain, while Fukuyama and Gokasho strains could not grow in this salinity. No data of Osaka strain in salinities below 10\% were presented. Tomas (1978) showed that Narragansett strain survived in 2\% of salinity at temperatures above 20°C, and the maximum growth rate at 3\% was 41 percent of that at 5\% and 20°C. Nomi strain showed similar responses to various salinities as the present strain (Nishijima & Hata 1984). According to other reports, Hakata strain (Honjo & Hanaoka 1973; named *Heterosigma* sp.), Shiogama strain (Matsudaira & Kawakami 1969; named *Olisthodiscus* sp.) and Tokuyama strain (Ikeda 1971; named *Olisthodiscus* sp.) showed little or no growth in 3\% of salinity. Hosaka (unpublished) also observed in axenic culture that Hakata strain collected in 1978 could not grow in 3\% of salinity. Therefore, *H. akashiwo* TKY825 is considered to be extremely adaptive to low salinity as Narragansett and Nomi strains. This character may explain the frequent red tide of *Heterosigma* in Tokyo Bay even in brackish canals.

Growth response of *H. akashiwo* TKY825 to pH was very similar to that of Gokasho strain for their preferences for high pH above 8, 0.

As a most remarkable feature in nutritional requirements, *H. akashiwo* TKY825 is unable to use urea as the nitrogen source and glycerophosphate as the phosphorus source. Fukuyama and Gokasho strains utilized glycerophosphate, while Osaka and Narragansett strains (Tomas 1979) were not. Osaka strain utilized urea, but less effectively than did Gokasho strain. Iwasaki et al. (1968) and Tomas (1979) did not describe urea utilization of their Fukuyama and Narragansett strains. However, Watanabe et al. (1982) noted the utilization of urea in Fukuyama strain. Therefore, *H. akashiwo* TKY825 is perhaps less heterotrophy than other strains, suggesting that its growth might be more dependent upon inorganic nutrients. According to the study of Ogawa & Ogura (1990), the following data were obtained: 0.56-2.30 mg·l^{-1} for DIN(NH_{4}-N+NO_{2}-N+NO_{3}-N) and 0.025-0.115 mg·l^{-1} for PO_{4}-P as averaged values during 1980-1988 at three stations in the innermost part of Tokyo Bay. These concentrations were twice as high as those of the innermost part of Osaka Bay (Joh & Yamochi 1986), and enough to cause a blooming of this strain.

The minimum cell quota of nitrogen (q_{mN}) and phosphorus (q_{mP}) of *H. akashiwo* TKY825 were estimated to be 24 pg·cell^{-1} and 1.82 pg·cell^{-1}, respectively. Tokyo strain had the same q_{mN} value as Narragansett or Osaka strain, but the smaller q_{mP} and larger N/P atomic ratio (=29.0) than Osaka strain. The small q_{mP} is considered to be an inherent nature of this strain because the growth...
of cells inoculated into the medium without phosphorus source from starvation culture was not observed.

WATANABE & NAKAMURA (1984) presumed the nitrogen and phosphorus limiting condition of seawater for the growth of Osaka strain by the following equation:

\[ Y = \frac{S}{q_m} \]

where \( Y \) = potential growth capacity (\( \times 10^3 \) cells\( \cdot ml^{-1} \); \( S \) = ambient concentrations of nitrogen and phosphorus (\( \mu g \cdot 1^{-1} \)); \( q_m \) = minimum cell quota of nitrogen and phosphorus (pg\( \cdot \)cell\(^{-1} \)). The element having smaller \( Y \) value is regarded as the more limiting one. \( Y \) values calculated from \( q_m \) of \textit{H. akashiwo} TKY825 and inorganic nutrient concentration of Tokyo Bay (OGAWA & OGURA 1990) are 23.3 -95.8 for nitrogen and 13.7 -63.2 for phosphorus. Thus, the nutritional condition of innermost part of Tokyo Bay for \textit{H. akashiwo} TKY825 is suspected to be more or less P-limiting.

Vitamin B\(_{12}\) was the sole essential vitamin for the growth of \textit{H. akashiwo} TKY825. This requirement is consistent with the results obtained previously (IWASAKI et al. 1968, IWASAKI & SASADA 1969, WATANABE et al. 1982, NISHIJIMA & HATA 1984, HOSAKA's unpublished data on Hakata strain).

Iron was also an essential element for the growth of \textit{H. akashiwo} TKY825. Osaka strain achieved only a half-maximum growth at a concentration of 50 \( \mu g \) Fe\( \cdot 1^{-1} \), whereas \textit{H. akashiwo} TKY825 showed an almost saturated response at the same concentration. Data are not available with other strains except Hakata strain (Honjo 1980) which showed the saturated growth at 10 \( \mu g \) Fe\( \cdot 1^{-1} \) in an enriched seawater medium. Requirements for the other metals added as PII metals were not demonstrated in this experiment, although they might be satisfied with metal contaminants from reagents (WATANABE et al. 1982).

A relatively high concentration of dissolved iron (averaged 78.6 \( \mu g \) Fe\( \cdot 1^{-1} \)) was detected in Tokyo Bay (TSUBOTA & KODAMA 1973). This value of dissolved iron seems enough to support the maximum growth of \textit{H. akashiwo} TKY825 (Table 2).

The innermost part of Tokyo Bay has been receiving a large amount of river water and discharge from many large sewage treatment plants and other urban wastewaters, resulting in an enormous dilution of seawater, and continued huge supply of organic and inorganic nutrients including metal pollutants. In addition, the elevation of pH (up to 8.6) all the year round with maximum in warm season resulted from high photosynthetic activity due to eutrophication of the Bay (HOSAKA 1990). From the results obtained in this study, it is concluded that \textit{H. akashiwo} TKY825 is quite adaptive to such an extremely inorganic nutrient-rich, eutrophic water quality of Tokyo Bay.

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