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Effect of Vitamins on the Growth of the Harmful Red Tide Dinoflagellate *Heterocapsa circularisquama* Horiguchi

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Abstract Effects of vitamin B_{12}, thiamine and biotin on the growth of the noxious red tide dinoflagellate *Heterocapsa circularisquama* were examined using a chemically defined medium. This species was found to require vitamin B_{12} and thiamine for its optimal growth. However, it showed considerable growth in a thiamine-lacking medium whereas little growth occurred in a vitamin B_{12}-lacking medium. Thiamine may be a growth promoting factor rather than an absolute requirement. The growth responses of *H. circularisquama* in natural seawater enriched with a full compliment of nutrients except for vitamin B_{12} were examined for seawater collected on September 1999 at Hiroshima Bay, an area where red tides by this species have frequently occurred. As a result, the growth of this species reached 10,000～40,000 cells ml^{-1} for each enriched seawater collected at 2 depths of 3 stations. Since the cell densities above 10,000 cells ml^{-1} are high enough to be recognized as a red tide in nature, it is considered that vitamin B_{12} may not have acted as the limiting factor on the growth of this species in Hiroshima Bay on September 1999.

Key words: harmful dinoflagellate, *Heterocapsa circularisquama*, thiamine, vitamin B_{12}, vitamin requirement

Heterocapsa circularisquama Horiguchi is a novel red tide species occurring in embayments of west Japan and causes mass mortality of bivalves (Matsuyama *et al.* 1995, 1997). In order to clarify the mechanism of red tide occurrence by this species, it is considered quite important at first to understand the physiological requirements of this species which has only been described recently (Horiguchi 1995). Yamaguchi *et al.* (1997) reported the growth response of this species under varying temperature and salinity conditions, and he further examined its nitrogen and phosphorus nutrient requirements (Yamaguchi unpublished). It is well known that many phytoplankton species require vitamins such as vitamin B_{12}, thiamine and biotin (Provasoli and Carlucci 1974). As for red tide occurrences, it is considered that the nutritional level of seawater is required to be at high levels to support the abnormal growth of the causative phytoplankton species. Iwasaki (1973) examined the vitamin requirements for 8 species of red tide flagellate and found that all species required vitamin B_{12}. Therefore, vitamins, especially B_{12} may be important factors supporting the occurrence of red tides. In the present study we tested the vitamin requirements of *H. circularisquama* using artificial seawater, and found that vitamin B_{12} is an indispensable vitamin for the growth of this species. Furthermore, the growth response of B_{12} starved cells to natural seawater was examined for the estimates of available vitamin B_{12} for this species in seawater of Hiroshima Bay where red tides of this species have frequently occurred since 1995 (Matsuyama *et al.* 1997, Tamai 1999).

Materials and Methods

The strain of *Heterocapsa circularisquama* used in the present experiments was HA92-1 (Uchida *et al.* 1995). This species has been reported to have endosymbiotic bacteria (Horiguchi 1995), and the strain HA92-1 used in the present study has also been shown to possess bacteria within the cell (Imai *et al.* 1999). However, no extracellular bacterial growth was found in the culture of *H. circularisquama* HA92-1 strain isolated from Ago Bay in 1992. The bacterial growth

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in the culture medium was tested using ST10^{-1} medium (Ishida *et al.* 1986). Cultures were conducted at 22±1°C on a 12 h light: 12 h dark cycle; illumination was provided by cool white fluorescent lamps at 90–120 μmol photon $m^{-2}s^{-1}$. Stock cultures of this species were maintained in modified SWM3 (Itoh and Imai 1987). Actively growing cells of this species in modified SWM3 medium were inoculated into modified ASP_{12} medium (Provasoli *et al.* 1957). Inoculum size was adjusted to 500–800 cells ml^{-1}. One liter of this medium (ASP_{12}HC) contains NaCl 28 g, $MgSO_47H_2O$ 7 g, $MgCl_26H_2O$ 4 g, KCl 700 mg, $CaCl_22H_2O$ 735 mg, $NaNO_3$ 100 mg, $NaH_2PO_42H_2O$ 10 mg, Fe-EDTA 1 μM, Tris hydroxymethyl aminomethane 500 mg, vitamin B_{12} 1 μg, thiamine-HCl 0.5 mg, biotin 1 μg, $MnCl_24H_2O$ 0.8 mg, $CoCl_26H_2O$ 2 μg, $ZnCl_2$ 8.4 μg, H_2SeO_3 7.4 μg, and pH was adjusted to 7.8–8.0. Then, 0.1 m*l* of this culture in the logarithmic growth phase was inoculated into 4.5 m*l* of ASP_{12}HC without either vitamin B_{12}, thiamine, or biotin. In the experiments, vitamins solutions were sterilized by passing them through a membrane filter (Kurabou Steradisc 25, pore size 0.2 μm), then were added to the basal medium without vitamins. The basal medium was sterilized in advance by autoclaving (120°C, 20 min). The growth of the organism on each of the three kinds of vitamin-lacking medium was monitored. Three successive cultures were carried out on each medium in order to identify the vitamin requirements of this species. If the growth in the vitamin-lacking medium was depressed compared to the control (ASP_{12} HC), *H. circularisquama* was regarded to require the vitamin, and then subjected to a growth test at various vitamin concentrations. Growth was measured by in vivo chlorophyll a fluorescence using a Turner Designs Model 10–100R fluorometer (Brand *et al.* 1981) at 2–4 day intervals. When necessary, growth was measured by counting cells in 0.01–0.1 m*l* culture samples using a Sedgewick-Rafter slide. When cell densities were greater than 20,000 cells ml^{-1}, the samples were diluted 10–20 times with autoclaved seawater.

Vitamin B_{12} starved cells growing on B_{12}-lacking ASP_{12} HC medium were inoculated into natural seawater medium enriched with the same concentrations of nutrients in ASP_{12} HC except B_{12} in order to evaluate the amount of available vitamin B_{12} in the seawater for the growth of this species. The inoculum size of B_{12} starved cells was from 500 to 800 cells ml^{-1}. The seawater samples used in this experiments were collected at 2 and 5 m depths at three stations in Hiroshima Bay on 5 September 1999 (Fig. 1) using a Niskin bottle sampler. The samples were brought to the laboratory, and passed through a membrane filter (Millipore, 0.45 μm), then stored below −30°C until use. In the experiments, each seawater sample was enriched with nutrients of ASP_{12} HC except for vitamin B_{12}. As a control, complete enrichment was carried out for each seawater sample. The thus prepared enriched seawaters were sterilized by passing through a membrane filter (Kurabou Steradisc 25, pore size 0.2 μm). Growth was monitored by measurements of *in vivo* chlorophyll *a* fluorescence or cell counts as described

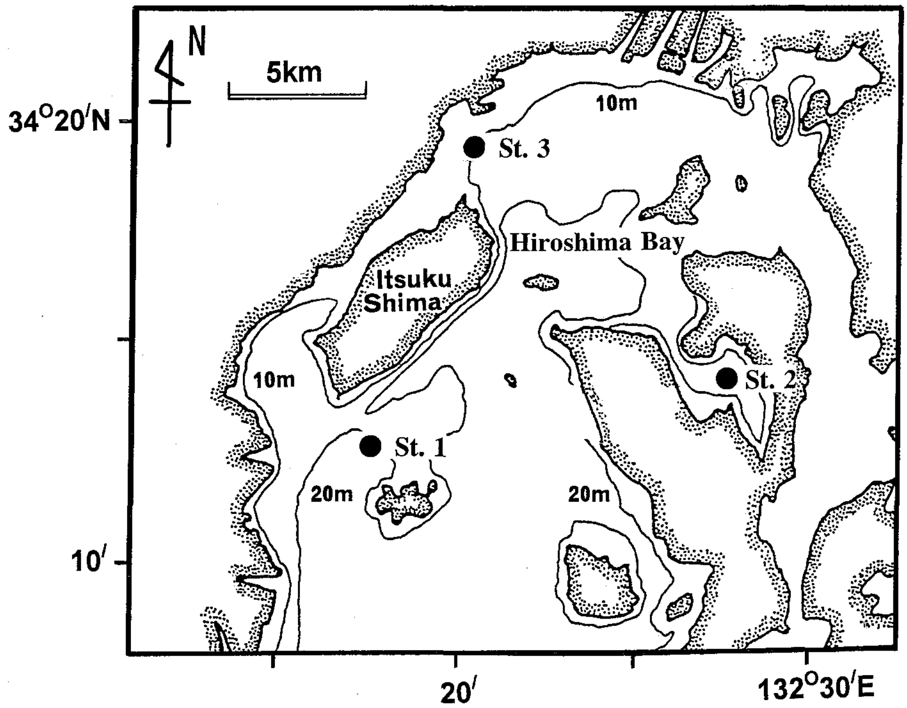

Fig. 1. The sites of seawater sampling.

above. The amount of available vitamin B_{12} in seawater was expressed as a maximum cell density of *H. circularisquama* grown in each seawater medium.

Results and Discussion

The growth of *Heterocapsa circularisquama* in the vitamin B_{12}-lacking medium was remarkably suppressed compared to the control ($ASP_{12}HC$). The growth of the B_{12} starved cells recovered when they were inoculated into complete ASP_{12} HC medium. As shown in Fig. 2, the maximum growth of this species was obtained at vitamin B_{12} concentrations higher than 8×10^{-3} $\mu g \cdot l^{-1}$. This value is almost the same level compared to other red tide flagellates. Iwasaki (1973) reported that the optimal concentrations of vitamin B_{12} varied from 10 to 20 $\times 10^{-3}$ $\mu g \cdot l^{-1}$ among three red tide dinoflagellates including *Exuviaella* sp., *Peridinium hangoei* Schiller and *Gymnodinium nelsoni* Martin (= *Gymnodinium sanguineum* Hirasaka). According to Nishijima (1985) three red tide flagellates (*Heterosigma akashiwo* (Hada) Hada, *Chattonella antiqua* (Hada) Ono and *Eutreptiella* sp.) required 10 to 20 $\times 10^{-3}$ $\mu g \cdot l^{-1}$ of vitamin B_{12} for their optimal growth. In this study, the maximum growth of *Heterocapsa circularisquama* at 1.6×10^{-3} $\mu g \cdot l^{-1}$ of vitamin B_{12} was 0.102 relative units of fluorescence which is equal to 13,000 cells m*l*. In this case, the growth of this spe-

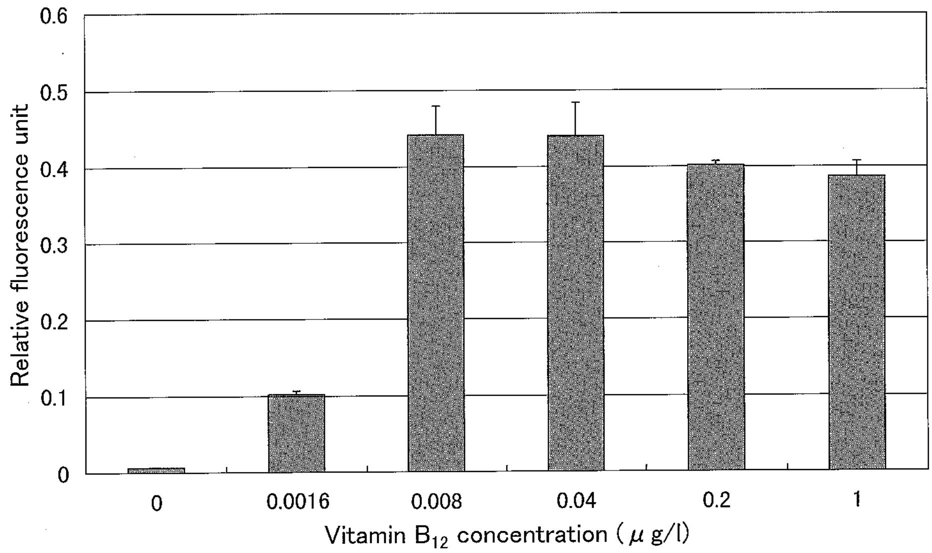

Fig. 2. Growth of *Heterocapsa circularisquama* in various concentrations of vitamin B_{12}. Vertical lines show the standard deviation (n=3).

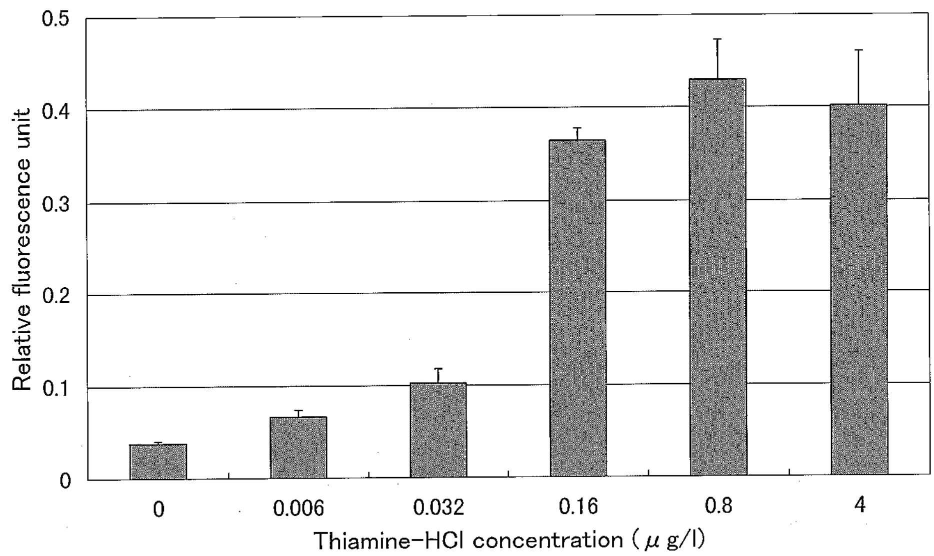

Fig. 3. Growth of *Heterocapsa circularisquama* in various concentrations of thiamine. Vertical lines show the standard deviation (n=3).

cies was limited to less than 1/4 of the maximum value at the optimal concentrations of B_{12}. Therefore, it is estimated that one cell of this species requires 12.3×10^{-3} pg of vitamin B_{12}.

The growth of this species was suppressed in thiamine-lacking medium compared to the control (ASP_{12} HC). When thiamine-starved cells were inoculated into ASP_{12} HC medium, the growth of *H. circularisquama* increased almost in proportion with thiamine concentrations up to 8×10^{-1} $\mu g \cdot l^{-1}$ (Fig. 3). However, slight growth (5,000 cells ml^{-1} on average) occurred in thiamine-lacking medium even after two successive cultures. Therefore, thiamine may be a growth promoting factor rather than an indispensable substance for growth of *H. circularisquama*. Different from vitamin B_{12} and thiamine, biotin was not required by *H. circularisquama*. The growth of this species was not suppressed even through three successive cultures in biotin lacking ASP_{12} HC medium. Thus, *H. circularisquama* was found to require vitamin B_{12} and thiamine for its optimal growth. The strain used here has endosymbiotic bacteria within the cell (Imai *et al.* 1999), however, the endosymbiotic bacteria perhaps do not supply vitamins in large enough amounts to sup-

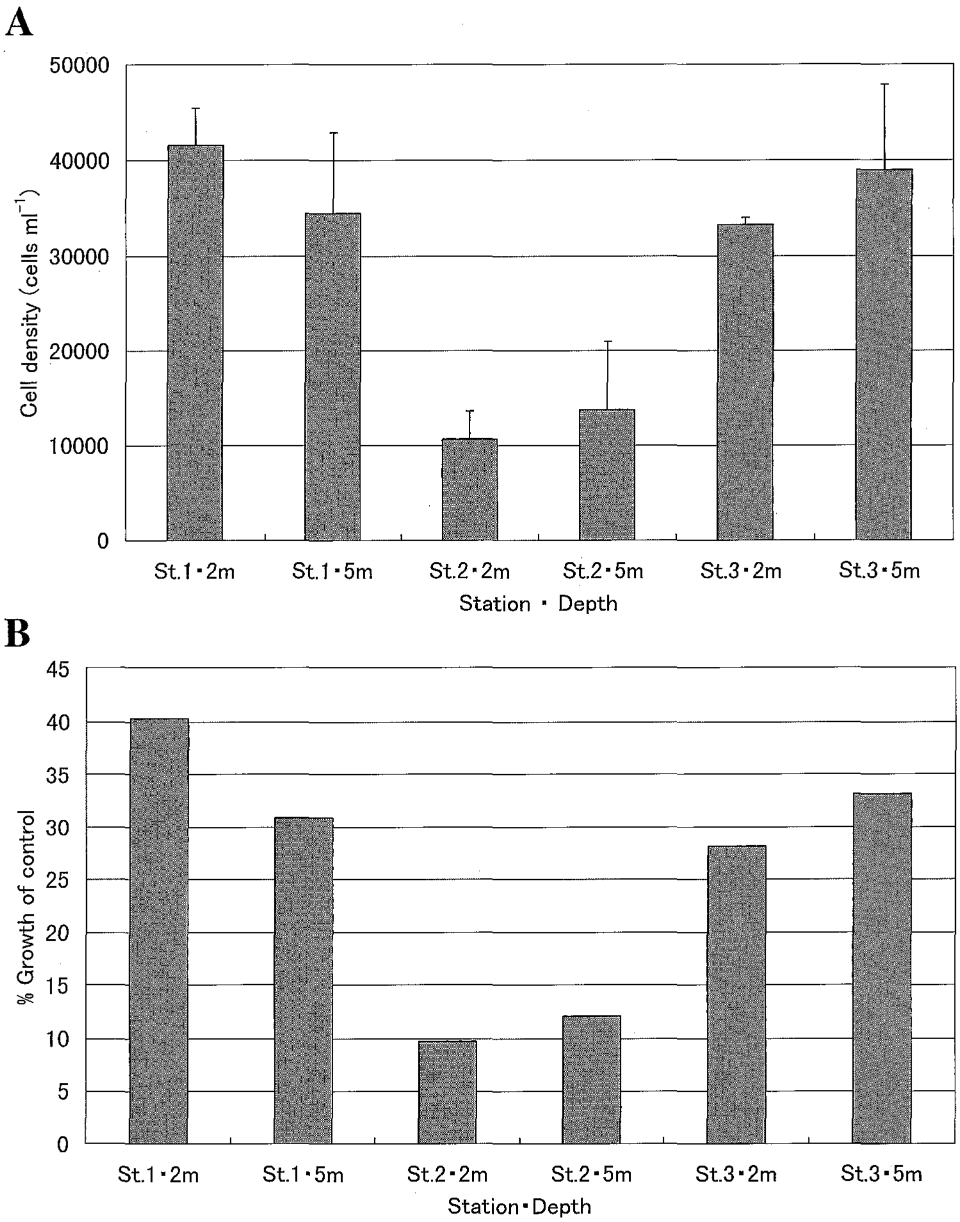

Fig. 4. Growth responses of *Heterocapsa circularisquama* in enriched natural seawater except for vitamin B_{12}. Maximum cell densities obtained in each seawater sample enriched with nutrients other than vitamin B_{12} at the same concentrations as modified SWM3 (Itoh and Imai 1987). A: cells ml^{-1}. Vertical lines show the standard deviation (n=3), B: % of control.

port the growth of their host. Therefore, it is clear that these two vitamins, particularly vitamin B_{12} are the important nutrient factors for the growth of this species. It is necessary hereafter to clarify if there exist some differences in vitamin requirements among strains of *H. circularisquama*

Many species of dinoflagellates have been reported to require vitamins of the B group including vitamin B_{12}, thiamine and biotin (Provasoli and Carlucci 1974, Loeblich 1967). Thiamine in some cases appears to be a growth stimulator rather than an absolute requiment. Thiamine and vitamin B_{12} were required by *Glenodinium halli*, but the organism could grow without thiamine (Gold 1964). Similarly, *Glenodinium* sp. required vitamin B_{12} and thiamine for its optimal growth although thiamine was not an absolute requirement but a growth stimulator (Uchida 1975). It is noteworthy that these two *Glenodinium* species and *Heterocapsa circularisquama* show a similar pattern in vitamin requirements; B_{12} is an indispensable growth factor and thiamine is rather a growth promoting substance, since taxonomically both *G. halli* and *Glenodinium* sp. are closely related in the genus *Heterocapsa* (Loeblich *et al.* 1981).

The maximum growth of *H. circularisquama* in enriched natural seawater except for vitamin B_{12} is shown in Fig. 4. The growth in the seawater collected at St. 2 was considerably less than in water from St. 1 and St. 3. Even at St. 2, however, the cell density reached more than 10,000 cells ml^{-1}, a density which is high enough to be recognized as a red tide in nature comparing with previous field observations (Matsuyama *et al.* 1996, Tamai 1999). Since various analogues of vitamin B_{12} are considered to be present in seawater (Loeblich 1967), and a vitamin B_{12} binder, which inactivates B_{12} utilization by some phytoplankters, is excreted by other members of phytopkankton (Nishijima *et al.* 1995), the growth responses of *H. circularisquama* to natural seawater demonstrated here are considered to express the total available B_{12} to this species. The cell density of this species in St. 1 on the sampling day was 100 cells ml^{-1} at 2 m depth and 150 cell ml^{-1} at 5 m depth, respectively while it was below 20 cells ml^{-1} at the other two stations. However, the cells of this species did not grow to form red tides afterwards. Previous reports showed that red tides by *H. circularisquama* occur from August to November in Hiroshima Bay (Matsuyama *et al.* 1997, Tamai 1999). It is unclear why this species did not form red tides in autumn of this year (1999), but vitamin B_{12} may not have acted as the limiting factor on the growth of this species although the assay of seawater was conducted only on seawater samples collected on 5 September. It is necessary hereafter to demonstrate in detail the changes of available B_{12} concentration in relation to the growth of *H. circularisquama in situ.*

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有害赤潮渦鞭毛藻 *Heterocapsa circularisquama* の増殖に及ぼすビタミンの影響について

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新型赤潮原因種である *Heterocapsa circularisquama* の生理的特性を明らかにし，その赤潮発生機構解明に資するため，本種のビタミン要求をビタミンB_{12}, チアミン，ビオチンについて検討した。その結果，*H. circularisquama* はビタミンB_{12} 及びチアミンを要求することが明らかになった。ビタミン B_{12} 欠乏培地において本種は殆ど増殖しなかったが，チアミンを除いた培地ではある程度増殖することから，チアミンは絶対要求というよりも増殖促進的な効果を持つと考えられた。広島湾で９月に採取した海水にビタミン B_{12} 以外の栄養物質を添加し，本種の増殖を調べたところ，３地点・２層のいずれの試水においても最大増殖密度は 10,000～40,000 cells ml^{-1} であった。これらの値は天然において赤潮として十分に認識できる細胞密度であることから，広島湾において９月の時点ではビタミン B_{12} が *H. circularisquama* の増殖の制限要因になっている可能性は低いと判断された。