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Inhibitory effect of bacterial ubiquinones on the settling of barnacle, *Balanus amphitrite*

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Abstract. In an attempt to clarify the influence of marine bacteria on the settling of fouling invertebrate larvae, we screened for inhibitors, produced by marine bacteria, of settling by cyprids of the barnacle, *Balanus amphitrite*. We found that the culture broth of *Alteromonas* sp. strain number KK10304, which was associated with the marine sponge, *Halichondria okadai*, effectively inhibited settling of the cyprids. Bioassay-guided isolation indicated ubiquinone-8 (**1**) as an effective inhibitor of cyprid settling. As ubiquinones are widely distributed in bacteria, several related compounds were also tested.
Key words. Settlement inhibitor; barnacle; ubiquinone; sponge; *Halichondria okadai*; sponge associated bacteria; *Alteromonas* sp.

Marine fouling invertebrates, such as barnacles and blue mussels, cause serious problems on ship hulls, in cooling systems of power plants, and in aquaculture systems. Organotin compounds such as TBTO [bis-(*n*-tributyltin)-oxide] have been used as effective antifouling agents against those organisms. However, stern warnings have been issued regarding the toxic effects of such heavymetal compounds on marine environments and wildlife, including fish and shellfish. Therefore, antifouling substances with no or reduced toxicity must be found and developed. In our screening for antifouling substances, using laboratory-reared larvae of the barnacle, *Balanus amphitrite*, we have reported fatty acids[1], steroids[1], a sesquiterpene hydrocarbon[2], a betaine[3], a furanoterpenoic acid[4], a pukalide derivative[4], a gramine derivative[5] and others as settling inhibitors in marine invertebrates.

Crisp[7] reported that the larvae of fouling invertebrates were recruited to the settling surface in response to surface–associated stimuli. He also revealed[7] that the stimuli played an important role not only in attracting larvae but also in inducing their metamorphosis. Mitchell and Kirchman[8] proposed that the stimuli were produced by bacteria growing on the solid surface. Maki et al.[9] reported that films of bacteria on solid substrate could positively or negatively influence the settling of marine invertebrate larvae. They[9] tested the effects of culture media of 18 different strains of marine bacteria on the settling of cyprids of the barnacle, *B. amphitrite*. They found that 8 out of 18 culture media showed inhibition of the settling and that the culture medium of *Deleya* (*Pseudomonas*) *marina* showed the strongest activity. These papers have suggested the existence of settling inhibitors produced by marine bacteria, although many of these compounds have remained unknown. In the present paper, we report the isolation and identification of the settling inhibitors from bacteria associated with a marine sponge.

Materials and methods

Isolation of bacteria. A common marine sponge, *Halichondria okadai*, was collected from Numazu area in Suruga Bay of Shizuoka Prefecture, in 1990. This specimen was cut into small pieces (5 g), homogenized with 10 ml of sterilized seawater and diluted in a series of 10-fold dilutions. Each dilution sample was seeded onto agar medium: 750 ml sterilized seawater (pH 7.7), 250 ml distilled water, 5.0 g Bacto-peptone (Difco), 1.0 g Bacto-yeast extract (Difco), 15.0 g agar (Nacarai tesk) and 0.04 g $FePO_4$. After 4 days incubation at 20 °C, all colonies with a distinguishable appearance were isolated, resulting in the isolation of 32 bacterial strains.

Screening of settlement inhibitors of the barnacle cyprids. After culturing the isolated bacteria in the medium described above without agar and with supplement of glucose (2 g) at 20 °C for 4 days, supernatants and pellets were separated by centrifugation at 8,000 rpm for 15 min, and extracted with acetone. Each extract was concentrated under reduced pressure, and examined by the bioassay method developed by our group[6] for inhibitory activity against the settling of the reared cyprids of the barnacle, *B. amphitrite*.

Identification of the bacterium. Identification of the bacterium was performed according to Bergey's Manual of Systematic Bacteriology[10].

Isolation of a settling inhibitor from the bacteria. Ten liters of the 4 day-incubated culture broth of bacteria were centrifuged at 8,000 rpm for 15 min. The resulting pellet was extracted with acetone. The extract was concentrated in vacuo and separated into two layers by addition of ethyl acetate and distilled water. The ethyl acetate layer was chromatographed twice on silica gel (Silica gel 60, 230–400 mesh, Merck, Germany) with chloroform and dichloromethane, successively. Further purification of the active fraction was performed by

HPLC on silica gel (Cosmosil 5SL, Nacarai tesk) with a mixture of 5% acetone in *n*-hexane.

Structural elucidation of the substance. The structure of the substance was elucidated mainly by spectroscopic analysis as follows. The UV absorption spectrum (UV) in hexane was recorded on a Shimadzu UV-2100S recording spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Unity 500 Spectrometer using $CDCl_3$ as solvent. Electron-impact mass spectra (EIMS) were obtained with a JEOL JMS-SX102 mass spectrometer.

Results and discussion

Identification of the bacterium which shows antifouling activity against cyprids of reared barnacle. Of the bacterial strains tested, we found that the pelleted organic extract of a bacterium with tentative strain number KK10304 most effectively inhibited settlement of the cyprids. The bacterial colony was circular and raised. The strain was an aerobic Gram negative straight-rod (1.2×2.0 μm), and moved using a polar flagellum (4.5 μm). Catalase, oxidase and gelatin tests were positive, whereas O/F and glucose tests were negative. The strain required seawater salts for growth. From these observations and the description of the genus *Alteromonas* in Bergey's Manual of Systematic Bacteriology[10], this strain was considered to be identifiable as *Alteromonas* sp.

Structural elucidation of the settlement inhibitor. At the final stage of purification by HPLC, 9 mg of pale yellowish oil was obtained. The spectral data of the pigment were as follow; UV (hexane) λ_{max} 269 nm; EIMS *m/z* 726 (48, M$^+$), 711 (4), 359 (9), 235 (100), 197 (22), 149 (26); ^{1}H-NMR ($CDCl_3$, 500 MHz, ppm) δ 1.56 (3 H, s), 1.58 (3 H, s), 1.59–1.61 (15 H, brs), 1.68 (3 H, d, J = 1.0), 1.74 (3 H, d, J = 1.0), 1.95–1.99 (14 H, m), 2.01 (3H, s), 2.02–2.10 (14 H, m), 3.18 (2 H, brd, J = 6.5), 3.98 (3 H, s), 4.00 (3 H, s), 4.93 (1 H, t, J = 7.0, 1.0), 5.04–5.13 (7 H, m); ^{13}C-NMR ($CDCl_3$, 125 MHz, ppm) δ 11.9 (q), 15.9–16.1 (6 C, each q), 16.4 (q), 17.7 (q), 25.3 (t), 25.7 (q), 26.4–26.8 (7 C, each t), 39.7–39.8 (7C, each t), 61.1 (q), 61.2 (q), 118.9 (d), 123.8–124.4 (7 C, each d), 131.2 (s), 134.8–135.2 (6 C, each s), 137.6 (s), 138.8 (s), 141.7 (s), 144.3 (s), 144.4 (s), 183.9 (s), 184.7 (s). These spectral data correspond to those of ubiquinone-8 (**1**) (fig. 1) described by Komagata and Suzuki[11], and those of ubiquinone-7 by Terao et al.[12]. Therefore the compound was identified as **1** which are widely distributed in bacteria[10,11].

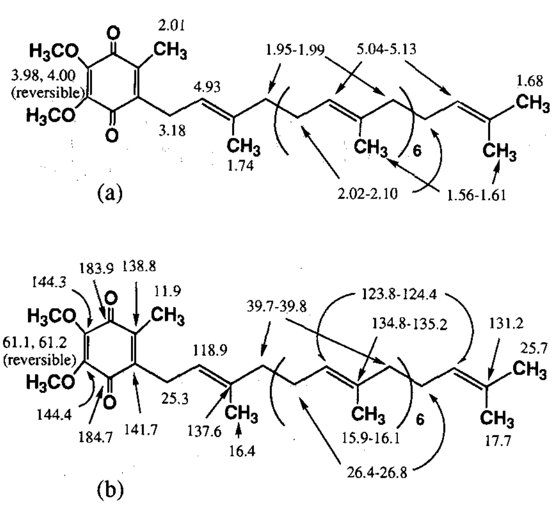

Figure 1. Chemical structure of ubiquinone-8 (**1**) with assignments of ^{1}H (a) and ^{13}C-NMR (b) spectra.

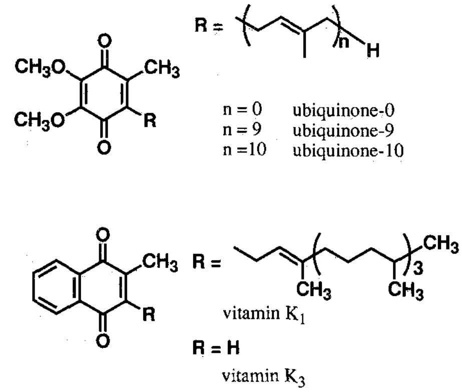

Figure 2. Chemical structures of ubiquinone-8 (**1**)-related compounds tested for inhibitory activities on larval settlement.

Inhibitory activities of 1 and related compounds. Compound **1** and related compounds, ubiquinone-0 (**2**), ubiquinone-9 (**3**), ubiquinone-10 (**4**), vitamin K_1 (**5**) and vitamin K_3 (**6**) (fig. 2), were examined for inhibitory activities and toxicities according to the method described by our group[6]. The degree of inhibition of the settling of reared cyprids by these compounds was expressed as minimum inhibitory concentration (MIC), and toxicities were expressed as 30% lethal concentration (LC_{30}) (table). Compound **2**, which had no polyprenyl group, showed higher inhibitory activity and higher toxicity than other polyprenylated ubiquinones, suggesting that the length of a polyprenyl side chain is an important factor influencing both inhibitory activity and toxicity of ubiquinones. This is true for **5** and **6**: the latter, with no

Table. MIC (minimum inhibitory concentration) against larval settlement and LC_{30}(30% lethal concentration) on reared cyprids.

Compound	MIC (ppm)	LC_{30} (ppm)
1	12.5–25.0	50.0 <
2	1.3–2.5	5.2
3	50.0–100.0	100.0 <
4	25.0–50.0	100.0 <
5	50.0–100.0	100.0 <
6	1.3–2.5	2.5

polyprenyl group, was more active and more toxic than the former, which has a polyprenyl group. A number of research groups[7-9] reported that films of bacteria on solid substrates played important roles as repellents or attractants for the settlement of marine invertebrate larvae. As ubiquinones and related compounds are commonly found in bacteria[10,11], they may retard the settling of invertebrate larvae. Thus the repellent activity of the *D. marina* culture reported by Maki et al.[9] may be due to its isoprenoid quinones. This is the first report to show that slime films of marine bacteria produce repellent substances against the settling of marine invertebrate larvae.

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1 Mizobuchi, S., Shimidzu, N., Katsuoka, M., Adachi, K., and Miki W., Nippon Suisan Gakkaishi *59* (1993) 1195.
2 Shimidzu, N., Katsuoka, M., Mizobuchi, S., Ina, K., and Miki, W., Nippon Suisan Gakkaishi *59* (1993) 1951.
3 Kawamata, M., Kon-ya, K., and Miki, W., Fisheries Sci. *60* (1994) 485.
4 Mizobuchi, S., Kon-ya, K., Adachi, K., Sakai, M., and Miki, W., Fisheries Sci. *60* (1994) 345.
5 Kon-ya, K., Shimidzu, N., Adachi, K., and Miki, W., Fisheries Sci. *60* (1994) 773.
6 Kon-ya, K., and Miki, W., J. mar. Biotechnol. *1* (1994) 193.
7 Crisp, D. J., in: Marine Biodeterioration: an Interdisciplinary Study, p. 103. Eds J. D. Costlow and R. C. Tipper, Naval Institute Press, Annapolis 1984.
8 Mitchell, R., and Kirchman, D., in: Marine Biodeterioration: an Interdisciplinary Study, p. 49. Eds J. D. Costlow and R. C. Tipper, Naval Institute Press, Annapolis 1984.
9 Maki, J. S., Rittschof, D., Costlow, J. D., and Mitchell, R., Mar. Biol. *97* (1988) 199.
10 Palleroni, N. J., in: Bergey's Manual of Systematic Bacteriology, p. 141. Eds N. R. Krieg and J. G. Holt, Williams & Wilkins, Baltimore 1984.
11 Komagata, K., and Suzuki, K., in: Methods in Microbiology, vol. 19: Current Methods for Classification and Identification of Microorganisms, p. 161. Eds R. R. Colwell and R. Grigorova, Academic Press, London 1987.
12 Terao, S., Kato, K., Shiraishi, M., and Morimoto, H., J. chem. Soc., Perkin Trans. *1* (1978) 1101.