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# Requisite Morphologic Interaction for Attachment between *Ulva pertusa* (Chlorophyta) and Symbiotic Bacteria

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**Abstract:** In order to understand the morphogenesis-inducing mechanism of *Ulva pertusa* by symbiotic bacteria, we observed the requisite conditions of bacteria for attachment to *U. pertusa* for algal morphogenesis. Non-morphogenesis-inducing bacterial mutants derived by ultraviolet irradiation did not attach onto the surface of this alga. Scanning electron microscopic observation during the process of morphogenesis in *U. pertusa* revealed a network-like structure formed on the algal surface within 1 week after application of bacteria. The bacteria attached onto the alga after 2 weeks of incubation. After this attachment process the morphologic change was observed in *U. pertusa.*

**Key words:** *Ulva pertusa,* axenic culture, marine alga, morphogenesis, symbiotic bacteria

## Introduction

The marine foliaceous green macroalgae, such as Ulvalean plants, lose their typical morphology when cultured aseptically in defined synthetic media (Provasoli, 1958). However, the addition of adequate marine bacteria restores their typical morphology. The algal morphogenesis induced by bacteria has been reported for *Ulva lactuca* (Provasoli, 1958; Bonneau, 1977; Provasoli and Pintner, 1980), *Ulva pertusa* (Nakanishi et al., 1996), *Enteromorpha linza* (Fries, 1975), *Enteromorpha compressa* (Fries, 1975), and *Monostroma oxyspermum* (Provasoli and Pintner, 1964, 1980; Tatewaki et al., 1983). Tatewaki et al. (1983) reported that the addition of the culture filtrates of adequate bacteria, some brown or red algae could restore the typical morphology of axenic *Monostroma.* In a previous report we clarified that cocultivation of *U. pertusa* with certain symbiotic bacteria was thus far the only way to induce typical morphogenesis, which suggested that direct contact between *U. pertusa* and the bacterial strains is necessary for typical morphogenesis (Nakanishi et al., 1996). The morphogenesis-inducing mechanism for *U. pertusa,* therefore, is apparently different from that for *Monostroma.* In order to understand the morphogenesis-inducing mechanism of *U. pertusa* through bacterial attachment, in the present study we observed the requisite morphologic interaction for attachment between *U. pertusa* and bacteria that induce algal morphogenesis.

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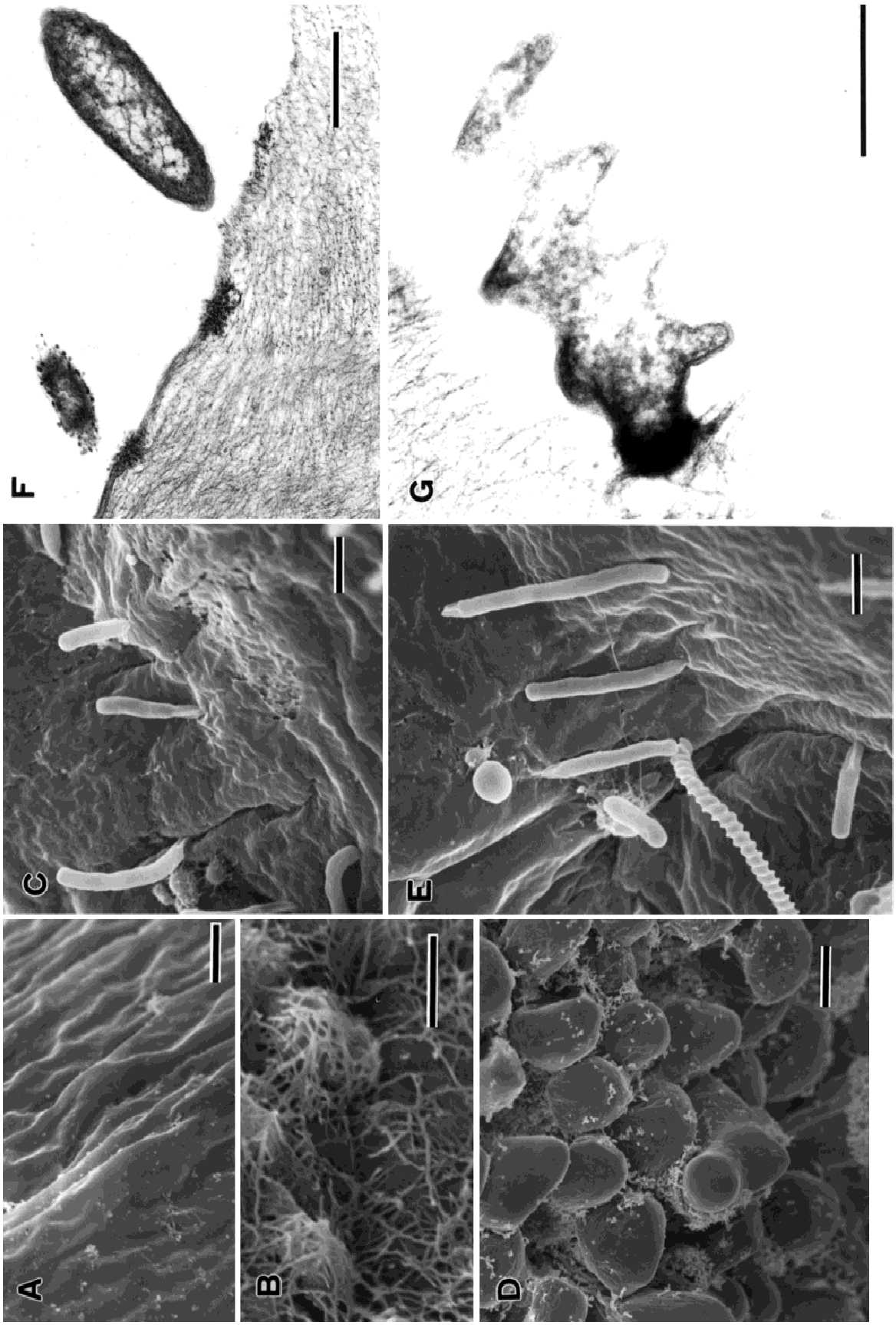
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**Figure 1.** The contact process of morphogenesis-inducing bacteria BUP-7 on the algal surface: **A,** initial algal surface of axenic culture (control), scale bar = 1 μm; **B,** 1-week-old algal surface (network-like structure spreaded on the algal surface), scale bar = 1 μm; **C,** 2-week-old algal surface, scale bar = 1 μm; **D,** 4-week-old algal surface with bacteria, scale bar = 20 μm; **E,** 4-week-old algal surface, scale bar = 1 μm; **F,** bacterial contact point on the algal surface (rod shape), scale bar = 1 μm (TEM); **G,** bacterial contact point on the algal surface (corkscrew shape), scale bar = 1 μm (TEM).

## Materials and Methods

### Plant Material for the Bioassay of Morphogenesis Inducing Activity

The axenic female strain of *Ulva pertusa* Kjellman (strain UP-203) was established according to the method reported previously (Saga and Sakai, 1982). Callus-like masses used in the present study were induced spontaneously under axenic culture conditions. The morphogenesis-inducing activity was assayed and estimated as described previously (Nakanishi et al., 1996). The following procedures were used to assay morphogenesis-inducing activity. Callus-like masses were homogenized with a glass homogenizer and transferred into 24-cell wells containing bacteria. Each cell well contained 50 to 150 *Ulva* cellular aggregates in 2 ml of $ASS_2$ medium. The colonies were cultured in a growth chamber maintained at 20°C under white fluorescent lamps (80 μmol $m^{-2}$ $s^{-1}$) with a daily regimen of 14 hours light and 10 hours dark for 4 weeks. The morphogenetic cellular aggregates were observed using a microscope. The cellular aggregates that formed primodia of foliaceous thalli from single cells or cellular masses and showed parenchymatous three-dimensional growth were defined as morphogenetic cellular aggregates. All other cellular aggregates that formed only formless and callus-like growth were defined as nonmorphogenetic.

### Morphogenesis-Inducing Bacterial Materials and Selection of Non-Morphogenesis-Inducing Mutants

The bacteria used were *Cytophaga* sp. BUP-7 and *Flavobacterium* sp. BUP-41, which were isolated from field-collected *Ulva pertusa* at the Omaezaki beach, Shizuoka Prefecture, Japan, in 1994. Both had morphogenesis-inducing activity. Non-morphogenesis-inducing mutants were induced by ultraviolet irradiation for 7 minutes from BUP-7 and BUP-41 treated to give approximately $1 \times 10^{-4}$ percent survival. Ultraviolet irradiation was supplied by a 10-W germicidal lamp (254 nm), which provided 2.4 Kerg $mm^{-2}$ $min^{-1}$ at an irradiation distance of 15 cm. The mutants were selected using the 24-cell well plate method. The bacterial strains were preserved under liquid nitrogen according to the method of Floodgate and Hayes (1961).

### Observation of the Algal Surface Using the Electron Microscope

Samples of *U. pertusa* were gently washed in sterile seawater 3 to 4 times to remove any debris and bacteria and then fixed in 2.5% glutaraldehyde in sterile seawater overnight at 4°C. The next day they were washed in sterile seawater, postfixed in 2% $OsO_4$ in 50% seawater for 2 hours, washed in distilled water, and dehydrated in a graded ethanol series from 50% to 100%. For scanning electron microscopy (SEM), the samples were further dehydrated in amyl acetate before critical point drying with liquid $CO_2$ in a Hitachi HCP-2 drying apparatus. These specimens were coated with a platinum/palladium alloy in a Hitachi E102 ion sputter. Non-morphogenesis-inducing bacterial mutants on the algal surface and morphogenesis-inducing bacteria cells on the algal surface were viewed at various time points after inoculation using a Hitachi S-2500 scanning electron microscope. For transmission electron microscopy (TEM), after ethanol dehydration, *U. pertusa* samples were placed in the transitional solvent propylene oxide, infiltrated in Epon 812 resin (TAAB Co., U.S.A.), and polymerized in BEEM capsules. Thin sections were cut using a diamond knife and counterstained with saturated aqueous uranyl acetate and Reynolds' lead citrate. The morphogenesis-inducing bacterial cells that attached onto the algal surface were observed and photographed using a Hitachi H-7000 transmission electron microscope.

## Results and Discussion

The following are the sequential morphologic changes of axenic UP-203 with BUP-7. Two weeks after inoculation, primordia had not yet formed in the cellular aggregate with bacteria. Two to 4 weeks after bacterial inoculation, primordia differentiated from a single cell or small number of cells into the cellular aggregate. Primordium of foliaceous

thalli was observed in the cellular aggregates 4 weeks after inoculation. In contrast, no morphologic change was induced in the axenic colony.

The changing appearance of the algal surface and the subsequent attachment process of BUP-7 onto the algal surface are shown in Figure 1. One week after inoculation there was as yet no contact with the bacteria, but a network-like structure was seen on the algal surface (compare Figures 1A and 1B). After about 2 weeks the network mostly disappeared, and bacteria were often seen attached to the algal surface (Figure 1C). After 4 weeks highly populated bacteria could be seen on the algal surface (Figure 1D); some of these bacteria were corkscrew-shaped and others were regular and smooth in shape (Figure 1E). In both cases, we were able to observe the unique narrowed and pointed shape of the end of the bacteria attached onto the algal surface. This unique shape was distinctly lacking in non-morphogenesis-inducing bacterial mutants. It appears that these mutant bacteria only lay on the algal surface without developing any obvious attachment to the surface.

These results showed that *U. pertusa* requires a unique physical attachment of bacteria for complete algal morphogenesis. There are at least two steps in the process of morphogenesis. First, the network-like structure was formed on the algal surface, at the time when bacteria did not attach. The bacteria attached within 2 weeks, and a single cell or cellular aggregates of the callus-like structure differentiated into the primordia, then developed into leafy or tubular fronds. Our further observations showed that the network-like structure was induced on the algal surface by the addition of the filtrate of the bacterial growth medium (data not shown).

The attachment point of BUP-7 on the alga is shown in Figure 1. In both the rod-shaped and corkscrew-shaped bacteria, the narrowed and pointed ends were observed (data not shown). But in thin sections of the samples we were not able to see any part of the bacteria actually entering the algal tissue itself (Figures 1F and 1G).

Mateous et al. (1995) reported that *Rhizobium* attached to the white clover root epidermal surface as syntropism. Their SEM results showed firm attachment onto the root surface by *Rhizobium* similar to the attachment of morphogenesis-inducing bacteria on *U. pertusa.* The factors inducing morphologic change of the alga were neither plant growth regulators nor extracellular substances (Provasoli, 1965; Provasoli and Carlucci, 1974; Nakanishi et al., 1996). At this point we can only surmise that there is an interaction between the alga and the bacteria. We have not been able to get a clear-cut solution of network-like structure, but we speculate that the network-like structure is formed without the bacterial attachment, and this structure performs some function concerned with cell-to-cell interaction between the algal surface and the bacteria. In conclusion our observations show a firm relation between bacterial attachment onto *U. pertusa* and the induction of complete algal morphogenesis from callus-like masses.

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