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Colorimetric dimethyl sulfide sensor using *Rhodovulum sulfidophilum* cells based on intrinsic pigment conversion by CrtA

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Abstract A colorimetric whole-cell sensor for dimethyl sulfide (DMS) was constructed based on the in vivo conversion of intrinsic pigments in response to the analyte. In a marine bacterium, *Rhodovulum sulfidophilum*, carotenoids are synthesized via the spheroidene pathway. In this pathway, demethylspheroidene, a yellow carotenoid, is converted to spheroidene under catalysis of *O*-methyltransferase. Spheroidene monooxygenase (CrtA) catalyzes the terminal step of the pathway and converts spheroidene to spheroidenone, a red carotenoid. Here, the CrtA gene in *R. sulfidophilum* was removed and then reintroduced downstream of the DMS dehydrogenase gene promoter. Using this whole-cell sensor, 3 μM DMS or dimethyl sulfoxide can be detected without adding any color-forming reagent. The ratio of the red spheroidenone to total carotenoids increased, as the DMS concentration was raised to 0.3 mM. Comparison of the signal to the background color indicated a shift in the color coordinate from a yellow to a red hue. An intense signal was obtained with 1-day incubation at a high cell density when sensor cells at the exponential growth phase were used. These results show that the genetically engineered *R. sulfidophilum* cells can be used to monitor the quality of marine aquacultural environments by the naked eye.

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Introduction

Carotenoid pigments accumulate in anoxygenic photosynthetic bacteria and have functions for the light harvesting and the protection of cells from photooxidative damage. Among the purple photosynthetic bacteria, five genera, *Rhodobacter*, *Rhodovulum*, *Rhodobaca*, *Rubrivivax*, and *Rhodoferax* synthesize carotenoids through the spheroidene pathway (Takaichi 1999; Takaichi et al. 2001). All the carotenogenesis genes in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* form a *crt* gene cluster, and the characteristics of the carotenogenesis enzymes have been investigated (Armstrong et al. 1989, 1990; Lang et al. 1995; Takaichi 1999). In *Rhodobacter sphaeroides*, spheroidene is formed by *O*-methylation of the C-1 hydroxy group of demethylspheroidene, catalyzed by *O*-methyltransferase (CrtF). Spheroidene monooxygenase (CrtA) introduces a 2-keto function at the C-2 of spheroidene and demethylspheroidene to form spheroidenone and demethylspheroidenone, respectively. CrtA is responsible for turning the color of carotenoids from a yellow hue derived from spheroidene to a red hue derived from spheroidenone (Yeliseev et al. 1996). Therefore, if expression of *crtA* in such photosynthetic bacteria can be modified to be under regulation of different transcriptional switches, colorimetry based on the catalytic conversion of carotenoids by CrtA might be established using the bacterial whole cells to detect analytes by the naked eye.

Dimethyl sulfide (DMS) is a volatile organo-sulfur compound, much of which in the atmosphere is formed from the enzymatic cleavage by marine algae and bacteria of dimethylsulfoniopropionate accumulated in the marine algae and plant halophytes (Hanlon et al. 1994; Vogt et al. 1997; Jonkers et al. 2000; Yoch 2002). DMS is also the essential source of the smell of the sea, which determines the quality of lavers as an Asian food. An easy method to detect DMS in the nursery of lavers will enable aquaculturists to control the flavor of the products.

As members of the genus *Rhodovulum* are halophilic bacteria found in tidal and seawater pools, application of the bacteria from a marine biotechnological standpoint can

be considered (Maeda et al. 1998). In *Rhodovulum sulfidophilum*, it was shown that DMS acceptor oxidoreductase activity was induced by DMS or dimethyl sulfoxide (DMSO) (Hanlon et al. 1994). If the DMS acceptor oxidoreductase activity might be regulated by transcriptional regulator protein(s), genetic manipulation techniques would enable the *crtA* gene expression under regulation of the DMS-responsible transcriptional switch in *R. sulfidophilum*.

The purpose of this study is the construction of a simple and cost-effective sensor independent from specific reagents and instruments for monitoring qualities of marine aquacultural fields. A sensor strain consisting of a *crtA*-deleted host strain of *R. sulfidophilum*, in which the CrtA gene was reintroduced downstream of the promoter for the DMS dehydrogenase (Ddh) gene cluster (McDevitt et al. 2002), was constructed to detect DMS in the nursery of lavers based on the conversion of the carotenoid colors.

Materials and methods

Bacterial strains and growth conditions

Rhodovulum sulfidophilum W-1S (FERM P-15320, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) (Maeda et al. 1998) as a wild-type (WT) strain and its *crtA*-deleted mutant (CDM) were used.

Modified Okamoto medium (Maeda et al. 2003) was used throughout this study. Tetracycline, when necessary, was added to cultures at the concentration of 2.5 μg ml^{-1}. Anaerobic light conditions were achieved in cultivation vessels by sealing those with a rubber cap fitted with a double stopper after ventilation with 99.99% argon through a set of needles; 0.2% (w/v) sodium bicarbonate was added to the medium. The light source was an incandescent lamp, and the photon flux intensity at the position of cultivation vessels was 40–45 μmol s^{-1} m^{-2}. Semiaerobic light conditions were achieved in cultivation vessels by covering those with a screw-on cap fitted with an inner gasket or the rubber cap without agitation. Light source and intensity were the same as those under anaerobic light conditions. Medium at one third volume of cultivation vessels was added, so that a ratio of culture to gas phase (v/v) was 0.5. Headspace was filled with air. Cultures at the exponential growth and stationary phases under semiaerobic light conditions exhibited dissolved oxygen (DO) of 0.65 and 0.71 ppm, respectively. Aerobic dark conditions were achieved in an Erlenmeyer flask by covering it with a silicone cap and agitating it strongly in the dark. Cultures at the exponential growth and stationary phases under aerobic dark conditions exhibited DO of 0.25 and 6.13 ppm, respectively. Before inoculation, DO of medium was 5.5–5.7 ppm.

Sensing conditions

Sensor cells were precultured under semiaerobic light conditions until the stationary phase. In 16-ml test tubes, 4.5 ml modified Okamoto medium, containing DMS or DMSO, was added, and then 0.5 ml of the preculture was inoculated. The test tubes were covered with the screw-on cap. Supposing a use of the sensor strain as immobilized beads under daylight, cells were incubated under semiaerobic light conditions for 3 days for sensing, and the culture finally reached the stationary phase.

For optimization of the sensing system, sensor cells at the exponential growth or stationary phase, precultured under anaerobic light, semiaerobic light, or aerobic dark conditions, were resuspended in modified Okamoto medium that does not contain ammonium chloride for sensing. Through resuspension, the cells were concentrated to one-, three-, and tenfold of the density in the precultures at the exponential growth phase and to one fifth-, one-, and fivefold of that in the precultures at the stationary phase. In 16-ml test tubes, 5 ml of the resuspended cells was added, and the test tubes were covered with the screw-on cap. Incubation for sensing was carried out under semiaerobic light conditions for a period indicated beside each panel figure.

Genetic manipulation

DNA and RNA isolation, enzymatic treatments for the construction of plasmids, polymerase chain reaction (PCR), and *Escherichia coli* transformation were carried out according to standard protocols (Sambrook et al. 1989) or the manufacturers' instructions. PCR was performed using LA *Taq* with guanine and cytosine (GC) buffer (Takara, Shiga, Japan). Oligonucleotides TTCCTCTAGA CGGTTTAGGAGAGTCGCAAA and AAGGTACCAGA AGGGAAAGGGTTGAGTC were used as a primer set for amplification of the DNA fragment that includes the Shine–Dalgarno (SD) sequence and the *crtA* open reading frame (ORF) (Fig. 1). Total RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo, Japan) supplemented with lysozyme for cell lysis. To remove contaminated DNA, an RNase-free DNase set (Qiagen) was used. Reverse tran-

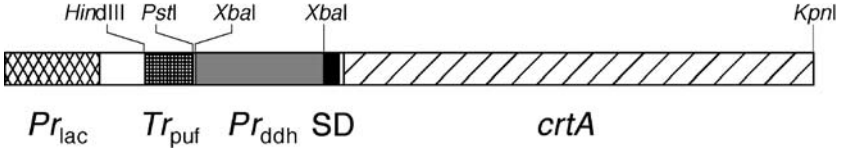

Fig. 1 DNA construct used for the colorimetric DMS sensor. The construct in pRK415 was introduced into CDM2 cells, resulting in the sensor strain. Pr_{lac} indicates lactose promoter; Tr_{puf}, *pufC* terminator; Pr_{ddh}, the upstream region of *ddhA*; and *SD*, Shine–Dalgarno sequence

scription was performed using an RNA PCR kit (AMV) ver. 2.1 (Takara) from 1 μg of the total RNA at 45°C for 28 min. For amplification of the DNA, 4 μl of the reaction mixture after reverse transcription was used as a template for PCR.

The CDM2 strain (Maeda et al. 2005), used as a host strain in this study, was constructed using the homologous recombination technique. Briefly, a DNA fragment, consisting of the 600-bp upstream and 800-bp downstream regions of *crtA* ligated with a kanamycin resistance cassette, was used to make a deletion of the *crtA* ORF. The genotype at the insertion region of the CDM2 strain was confirmed by sequencing and Southern hybridization (Maeda et al. 2005).

A DNA fragment consisting of the *pufC* terminator (Masuda et al. 1999), the *ddhA* upstream region (Genbank-EMBL-DDBJ accession number AB201470), the SD sequence, and the *crtA* ORF was constructed (Fig. 1). As a negative control, the DNA fragment, from which the *ddhA* upstream region was excised, was also constructed. The DNA fragments with and without the *ddhA* upstream region were ligated with *Hin*dIII and *Kpn*I sites of a shuttle vector, pRK415 (Keen et al. 1988), resulting in pSDMS and pRV5Ω, respectively. pSDMS and pRV5Ω were introduced into *E. coli* S17-1 (Simon et al. 1983) cells by electroporation and then transferred to CDM2 cells by conjugation, respectively, as described previously (Yun et al. 1990). Transformants were selected by tetracycline resistance. A CDM2 strain transformed with pSDMS was designated as the sensor strain.

Quantification of carotenoids

The sensor strain was cultured under semiaerobic light conditions for 3 days. Cells were collected by centrifugation, stored at −80°C overnight, and then lyophilized. Pigments were extracted from the lyophilized cells with a mixture of acetone and methanol (7:2, v/v) by vortexing for 2 min, and cell debris was removed by filtration. The extract was analyzed by a high-performance liquid chromatography (HPLC) system equipped with a μBondapak C_{18} column (3.9×300 mm, 125-Å pore size, Nihon Waters, Tokyo, Japan) as described previously (Takaichi et al. 2001).

Colorimetry

The light spectrum transmitted through the culture was measured with a spectrophotometer (CM-3500d, Konica Minolta Sensing, Tokyo, Japan). The illuminant D65, which was the Commission Internationale de l'Éclairage (CIE)- and International Organization for Standardization (ISO)-standard light source, was used. The color of the cultures was defined by converting the spectrum to the L*a*b* coordinates (CIE, 1976) (Davis et al. 1992; Fortner and Meyer 1997). In the CIE LAB system, the L* coordinate is a measure of lightness, and the a* and b* coordinates determine chroma and hue (Davis et al. 1992). The chroma and hue of the colors were calculated as the square root of $(a^*)^2$ plus $(b^*)^2$ and $\tan^{-1}(b^*/a^*)$, respectively.

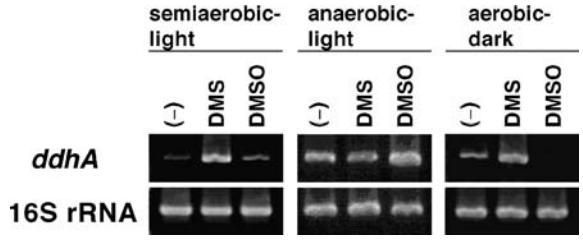

Fig. 2 Changes in the levels of the *ddhA* mRNA of WT cells grown under different conditions detected by reverse transcription-polymerase chain reaction. DMS or DMSO was added at 3×10^{-2} M, and cells were collected after 3 days' cultivation for total RNA extraction. A *minus sign* in parenthesis means the absence of DMS and DMSO. After 40 and 20 thermal cycles for *ddhA* and rDNA, the DNA was fractionated by electrophoresis and stained with ethidium bromide

Statistical analysis

Student's *t* test was performed for statistical comparisons.

Results

Regulation of gene expression by the *ddhA* promoter

The quantity of *ddhA* mRNA in WT cells grown in the presence and absence of DMS or DMSO was compared (Fig. 2). Incubation with DMS under semiaerobic light and aerobic dark conditions, and with DMSO under semiaerobic light conditions, resulted in an increase in *ddhA* mRNA. Under anaerobic light conditions, there was a high background of *ddhA* mRNA.

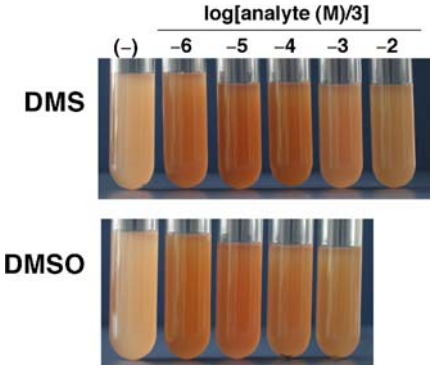

Fig. 3 Changes in the cultural color of the sensor strain in response to various concentrations of DMS or DMSO. A *minus sign* in parenthesis means the absence of DMS or DMSO

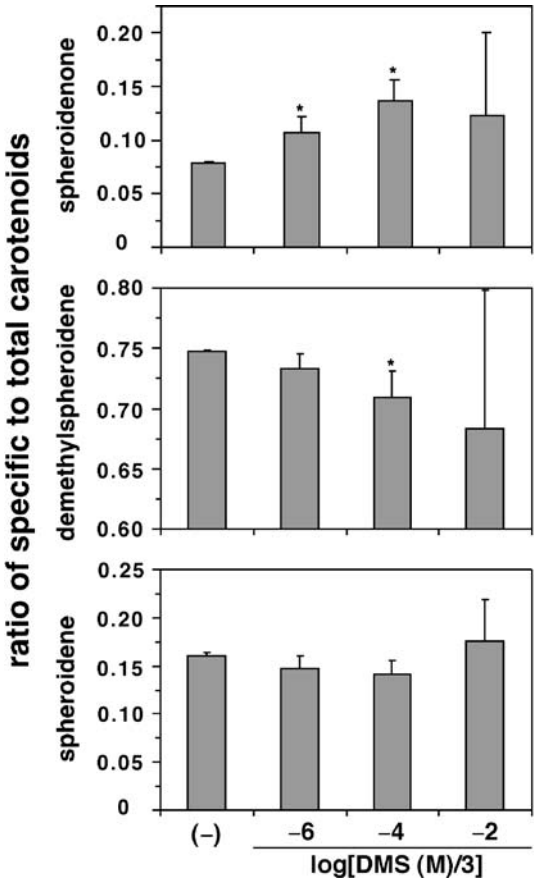

Fig. 4 Changes in the carotenoid composition in the sensor strain in response to various concentrations of DMS. The ratio of the specific to total carotenoids is the mean of data from three independent cultures and shown by a column with a *bar* showing the SD. *Asterisk* indicates $P<0.05$ in the statistical comparison of the data, with those in the absence of DMS indicated by (−)

Detection of DMS and DMSO by the colorimetric sensor

When the carotenoid composition was analyzed in a CDM2 strain transformed with pRV5Ω, the *pufC* terminator was found to prevent transcription from the lactose promoter of pRK415 (data not shown).

Sensor cells were inoculated at an optical density (OD) at 660 nm (OD_{660}) of 0.21 and grown in the presence and absence of either DMS or DMSO. After 1 day, at which an OD_{660} of the culture incubated in the absence of DMS and DMSO was 1.01, there was no difference in the color between cultures incubated in the presence and absence of DMS or DMSO. The signal color became distinguishable from the background one after 2 days, at which the OD_{660} was 2.03, and was redder after 3 days (OD_{660} 1.90) than after 2 days. The colors of the cultures after 3 days were compared (Fig. 3). At 3×10^{-6} to 3×10^{-3} M DMS and 3×10^{-6} to 3×10^{-4} M DMSO, the sensor cells became redder compared with the background color obtained when neither DMS nor DMSO was added in the medium. At 3×10^{-2} M DMS and 3×10^{-3} M DMSO, the cells became brownish.

Quantification of signal in sensing DMS

The carotenoid composition was analyzed in the sensor strain after exposure to DMS for 3 days (Fig. 4). The addition of 3×10^{-6} and 3×10^{-4} M DMS significantly increased the ratio of spheroidenone to total carotenoids, and 3×10^{-4} M DMS decreased the ratio of demethylspheroidene. A decrease in the ratio of spheroidene at DMS concentrations of 3×10^{-6} and 3×10^{-4} M was not significant.

The color of the sensor strain was defined after exposure to DMS using the CIE LAB L*, a*, and b* coordinates (Table 1). With increased DMS concentration, the degree of hue decreased, mainly because of an increase in the a* coordinate, and the L* coordinate slightly decreased. The b* coordinate and the chroma decreased at DMS concentrations of 3×10^{-4} and 3×10^{-3} M.

Optimization of the sensing system

Sensor cells, precultured under the different conditions and resuspended at the different cell densities, were incubated in the presence of 3×10^{-3} M DMS. Under anaerobic light conditions, OD_{660} of the preculture reached a plateau around 1.1. The intense signals, compared with the colors of the cultures incubated in the absence of DMS, arose in the sensor strain at the exponential growth phase, precultured under semiaerobic light or aerobic dark conditions (Fig. 5). The signal of the sensor strain at the exponential growth phase, precultured under aerobic dark conditions, became distinguishable within 26 h of incubation. The OD_{660} value of 2.22 or more was needed to clearly recognize the signal.

Table 1 CIE LAB color coordinates for cultures of the sensor strain after incubation in the presence and absence of DMS

log[DMS (M)/3]	L*[a]	a*[a]	b*[a]	Chroma	Hue (°)
(−)[b]	93.1	1.53	9.30	9.43	80.7
−6	93.0	1.51	9.39	9.51	80.9
−5	92.5	1.77	9.40	9.57	79.3
−4	92.5	1.99	8.69	8.91	77.1
−3	91.5	2.30	8.68	8.98	75.2

[a]Values are the means of three independent measurements
[b]DMS was not added

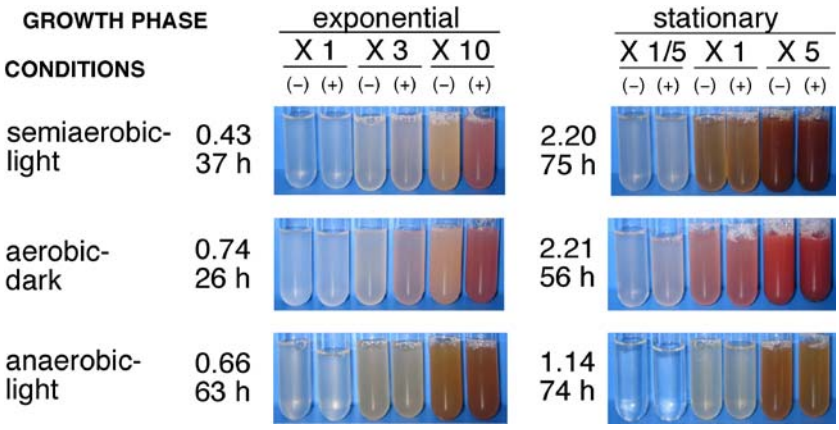

Fig. 5 Differences in the resolution of signal of the sensor strain precultured under different conditions and adjusted at different cell densities. The preculture conditions and the growth phase of cells, used for sensing, are indicated on the *left* and *upper* sides of the panels. An OD_{660}, at which preculture was completed, and an incubation period of sensing are indicated on the *left* of each panel. Cell densities for sensing are shown as the OD_{660} times the *value* indicated *above* the panels. *Minus* and *plus* signs in parentheses mean the absence and presence of DMS. DMS at 3×10^{-3} M was added

Discussion

To our knowledge, a colorimetric whole-cell sensor, in which the signal generates based on the conversion of intrinsic pigments of a host strain, has not been developed elsewhere. Compared to the DMSO-reductase-based amperometric DMSO sensor (Abo et al. 2003), the colorimetric signal derived from the intrinsic pigments confer unique properties on the sensor, including freedom from the need for adding specific reagents and from devices and instruments to discern the signal; it is easy even for untrained hands to assay. To test whether such a reporter event can generate a signal in response to analytes, the gene for CrtA, which converts a yellow carotenoid to a red one, was ligated to the 320-bp upstream region of the ddhA SD sequence.

It was confirmed by reverse transcription–PCR analyses that the mRNA level of *ddhA* increased in the presence of DMS or DMSO under semiaerobic light conditions. Consistent with this result, the colorimetric DMS sensor, consisting of the *crtA*-deleted host strain and *crtA* under regulation of the upstream region of *ddhA*, showed a responsiveness to DMS or DMSO at concentrations above 3×10^{-6} M under the same conditions. Therefore, it is likely that the upstream region of *ddhA* used in this study contains the responsive element to DMS and DMSO and *crtA* functions as a reporter gene of the colorimetric sensor. *R. sulfidophilum* has been shown to contain the DMSO reductase activity, which catalyzes the backward reaction of Ddh (Hanlon et al. 1994). Therefore, the reductase activity may confer the responsiveness to DMSO on expression of *ddhA* and interfere in precise sensing to DMS. It might positively impact on the responsibility and specificity of the sensing system to DMS if the control of the DMSO reductase activity would become possible.

The carotenoids in *R. sulfidophilum* W-1S (WT) were identified as demethylspheroidenone, demethylspheroidene, spheroidenone, and spheroidene (Maeda et al. 2005). The CDM2 strain, where *crtA* had been deleted, exhibited the phenotype that demethylspheroidene is predominant and none of demethylspheroidenone and spheroidenone accumulates (Maeda et al. 2005). In WT cells cultivated in the presence of oxygen, where the CrtA activity is supposed to be high, the demethylspheroidene content was less than 10%, and the spheroidenone content was more than 80% of the total carotenoids (Maeda et al. 2005). Evaluating these knowledge together with the carotenoid composition in the sensor strain, which is derived from the CDM2 strain, it is conceivable that in the sensor strain, the CrtA activity expresses even in the absence of DMS, enhances under the regulation of the *ddhA* promoter by the addition of DMS, and then facilitates the conversion of demethylspheroidene to spheroidenone via spheroidene synthesis, which is catalyzed by CrtF (Takaichi 1999). It has been shown in the WT strain that the content of 2-keto carotenoids (spheroidenone and demethylspheroidenone) is more than 90% under semiaerobic light conditions and about 50% even under anaerobic light conditions (Maeda et al. 2005). On the other hand, the spheroidenone content in the sensor strain did not exceed 20% even in the presence of DMS. Therefore, it seems likely that a shortage of oxygen source (s), including molecular oxygen to the 2-keto group, might not quantitatively limit the synthesis of spheroidenone in the sensor strain under semiaerobic light conditions, the sensing conditions to DMS.

In the color coordinates of the sensor defined by the CIE LAB system, the L* coordinate is a measure of lightness, and the a* and b* coordinates determine chroma and hue (Davis et al. 1992). Changes in hue occur circumferentially around the L* axis, and changes in chroma increase radially from the L* axis (Davis et al. 1992). The +a* and +b* axes are set along red and yellow hues, respectively (Davis et al. 1992). The difference between the signal and background colors, discernible by the naked eye, can be explained as differences in these color coordinates. It is conceivable that the signal generated by *crtA* expression is

mediated through a shift of the color coordinate from the yellow toward the red hue as well as through decreases in the lightness and chroma. An intense signal can be obtained with 1-day incubation at an OD_{660} of 4.3 or more under semiaerobic light conditions using sensor cells at the exponential growth phase, precultured under aerobic dark conditions.

Once spheroidenone was formed, it was rather stable for several days at room temperature (data not shown). Although oceanic dissolved DMS is found at nanomolar levels (Anderson et al. 2001; Toole and Siegel 2004), it has been shown that DMS concentration in the headspace of test tubes containing the marine intertidal sediment reaches more than 400 μM (Jonkers et al. 2000). By putting the immobilized sensor cells within cultures, it will therefore become possible for aquaculturists to monitor DMS levels in the nursery of lavers by the naked eye for controlling the intenseness of the flavor of the products. Apart from DMS, sensor tools to monitor the various qualities of marine aquacultural fields can be developed by combining *crtA* with the kind of transcriptional switches in the marine photosynthetic bacterium.

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