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Control of *Aliivibrio fischeri* Luminescence and Decrease in Bioluminescence by Fungicides

HITOMI KUWAHARA¹, JUNKO NINOMIYA^{1,2}, AND HIROSHI MORITA^{3*}

¹Graduate School of Environment Engineering, University of Kitakyushu, 1-1 Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0135, Japan

²National Institute of Technology, Oita College, 1666 maki, Oita city, Oita 870-0152, Japan

³Faculty of Environment Engineering, University of Kitakyushu, 1-1 Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0135, Japan

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Studies have reported that cell density, ultraviolet (UV) irradiation, and redox reactions, can induce bioluminescence in bacteria. Conversely, the relationship between seawater components and luminescence is not well understood. The efficacy of marine luminous bacteria as biosensors, and their reactivity to fungicides (for example postharvest pesticides) are also unknown. Therefore, we studied the relationship between the luminescence of *Aliivibrio fischeri* and the composition of artificial seawater media and analyzed the toxicity of fungicides using *A. fischeri* grown only with the elements essential to induce luminescence. Luminescence was activated in the presence of KCl, NaHCO₃, and MgSO₄. In addition, we cultivated *A. fischeri* with other compounds, including K⁺, HCO₃⁻, and SO₄²⁻ ions. These results suggested that *A. fischeri* requires K⁺, HCO₃⁻, and SO₄²⁻ ions to activate cell density-independent luminescence. Additionally, *A. fischeri* cultured in 2.81% NaCl solutions containing KCl, NaHCO₃, and MgSO₄ exhibited a decrease in luminescence in the presence of sodium ortho-phenylphenol at >10 ppm. This result suggests that *A. fischeri* can be used as a biosensor to detect the presence of sodium ortho-phenylphenol.

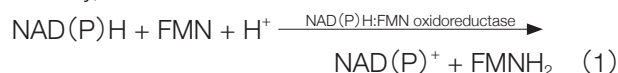
Key words : *Aliivibrio fischeri* / Luminescence / Artificial sea water / Fungicides.

INTRODUCTION

It is known that many species of bacteria, fungi, and animals exhibit bioluminescence (Węgrzyn and Czyż, 2002). Bioluminescent bacteria mainly live in marine environments (Węgrzyn and Czyż, 2002). They live freely in sea water, in symbiosis with certain squids and fishes, and are found either in the light organs of squids, on the surface of marine animals, or in the gut of fishes (Baumann et. al, 1973; Fitzgerald 1977; Meighen, 1994; Nealson, 1978; Ruby and Morin, 1979; Ruby, 1996; Węgrzyn and Czyż, 2002). In fact, marine luminous bacteria *Vibrio fischeri* are found in the special light organ of the Hawaiian sepiolid squid *Euprymna scolopes*; the light organ of a single adult contains a *V. fischeri* popu-

lation of between 10⁷-10⁹ cells (Fitzgerald, 1977; Ruby and Asato, 1993; Ruby, 1996). Thus, it is believed that marine luminous bacteria maintain high cell densities in light organs of marine animals.

Marine luminous bacteria emit blue-green light with a maximum wavelength of approximately 490 nm (Arakawa et. al, 2007; Yamada, 2014), and this light emission is caused by the catalytic action of a luciferase (Inouye, 1994; Miyashiro and Ruby, 2012). The bacterial bioluminescence reaction proceeds as follows (Inouye, 1994; Jablonski and DeLuca, 1978; Miyashiro and Ruby, 2012):



First, flavin mononucleotide (FMN) is reduced to

*Corresponding author. Tel: 81-93-695-3289, Fax: 81-93-695-3381, E-mail : morita(a)kitakyu-u.ac.jp

FMNH₂ by the catalytic action of NAD(P)H:FMN oxidoreductase in the presence of reduced NAD(P)H and H⁺ (Inouye, 1994; Ma et al., 2014) (Eq. 1). Bacterial luciferases oxidize long-chain aldehydes (RCHO) and Flavin mononucleotide (FMNH₂) in the presence of O₂, and bacterial luminescence takes place as an additional product of this reaction (Inouye, 1994; Jablonski and DeLuca, 1978; Ma et al., 2014; Miyashiro and Ruby, 2012) (Eq. 2). It is well known that the luminescence of marine luminous bacteria is dependent on cell density; when cell growth increases, luminescence also increases (Nealson, 1977). In other words, marine luminous bacteria do not exhibit luminescence at low cell density, but emit light when they reach a high cell density. Additionally, some studies have reported other factors such as ultraviolet (UV) irradiation (Czyz et al., 2002), redox state (Bose et al., 2007), cyclic AMP (Dunlap and Greenberg, 1988; Dunlap, 1989), heat shock proteins (Adar et al., 1992; Dolan and Greenberg, 1992), and osmolarity (Stabb et al., 2004) that induce luminescence. However, little is known about the relationship between sea water components and luminescence.

Recently, the marine luminous bacterium *V. fischeri* has been used as a biosensor. These biosensors are able to conduct qualitative analysis of toxic substances in the environment in an easy, quick, and cost-effective manner (Arakawa et al., 2007; Yamada, 2014). Marine luminous bacteria emit blue-green light; however, when they are exposed to toxic substances (e.g., heavy metals), the luminescence decreases rapidly (Arakawa et al., 2007; Yamada, 2014; Ma et al., 2014). Thus, biosensors using marine luminous bacteria analyze toxicity by luminescence inhibition rate (Parvez et al., 2006; Arakawa et al., 2007; Yamada, 2014; Ma et al., 2014).

In our previous study, we had investigated the relationship between cell growth and luminescence of four marine luminous bacteria (*Photobacterium leiognathi* ATCC 33469, *Vibrio harveyi* ATCC 14126, *V. fischeri* ATCC 33715, and *Aliivibrio fischeri* ATCC 7744) under nutrient-rich and nutrient-starvation conditions (Kuwahara et al., 2017). Under nutrient-rich conditions (i.e., under high cell density conditions), the luminescence of *P. leiognathi*, *V. harveyi*, and *V. fischeri* increased with the increase in cell density. In addition, their luminescence decreased or remained stable under nutrient-starvation conditions (i.e., under low cell density conditions) (Kuwahara et al., 2017). Therefore, it was assumed that these marine luminous bacteria regulate luminescence as a function of cell density (Kuwahara et al., 2017). Similarly, under nutrient-rich conditions, *A. fischeri* regulated luminescence in a cell density-dependent manner as did *P. leiognathi*, *V. harveyi*, and *V. fischeri*; however, under nutrient-starvation conditions, *A. fischeri* luminescence increased after 12 h despite

low cell density (Kuwahara et al., 2017). Nealson demonstrated that *A. fischeri* ATCC 7744 exhibits cell density-dependent luminescence, and that an increase in luminescence activity occurred at optical densities of approximately >0.1 (Nealson, 1977). Nonetheless, the *A. fischeri* used in our previous study increased its luminescence activity when its optical density was approximately 0.004; therefore, the results suggested that *A. fischeri* may regulate luminescence independently of cell density under nutrient-starvation conditions. Therefore, we hypothesized that seawater components might induce cell density-independent luminescence in *A. fischeri* under nutrient-starvation conditions (i.e., under low cell density conditions). Here, it is considered that this “cell density-independent” indicate about luminescence induction system; the luminescence intensity in nutrient-starvation conditions is lower than that under nutrient-rich conditions. Tabei et al. showed that *V. fischeri* ATCC 49387 induced luminescence independent of cell density in artificial sea water (ASW) (Tabei et al., 2012). In the previous study, we selected *A. fischeri* as marine luminous bacteria and cultured it under nutrient-rich or nutrient-starvation conditions. The luminescence intensity displayed approximately 10⁸ orders at 12 h after inoculation under nutrient rich conditions (i.e. normal growth), however it displayed approximately 10⁶ orders in nutrient-starvation conditions (ASW) (Kuwahara et al., 2017). It is considered that the luminescence intensity is dependent on cell density. However, cell density increased between 0-12 h (OD₆₀₀=0.003 (0 h) to OD₆₀₀=0.667 (12 h)) under nutrient-rich conditions, but no such increase was observed in cell density (i.e. OD₆₀₀=0.001 (0 h) to OD₆₀₀=0.004 (12 h)) under nutrient-starvation conditions. These results suggest that *A. fischeri* shows cell density-dependent luminescence under nutrient rich conditions, however, they show cell density-independent luminescence, and this luminescence increased significantly (p<0.05) at 12 h under nutrient-starvation conditions. So, we called about “cell density-independent luminescence”.

In addition, as luminescence decreases after exposure to toxic substances, marine luminous bacteria are used as biosensors; *V. fischeri* is widely used biosensor, due to its susceptibility to various toxic substances. However, little is known about the reactivity of other marine luminous bacteria to toxic substances or their efficacy as biosensors. If a particular organism were able to analyze toxicity even at low cell densities (i.e., marine luminous bacteria grown with only the elements from sea water components essential to induce luminescence), we would be able to assess toxicity more easily and cost-efficiently.

Therefore, we investigated the relationship between cell density-independent *A. fischeri* luminescence and

sea water components, and we tested a new biosensor using *A. fischeri*, grown under only essential elements required to induce luminescence, for toxicity assays for fungicides, sodium ortho-phenylphenol and imazalil.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The marine luminous bacterium, *A. fischeri* (ATCC 7744), was purchased from American Type Culture Collection and used throughout this study. *A. fischeri* was grown at 26°C for 16 h in 300 ml Erlenmeyer flasks containing 100 ml of Photobacterium broth (pH 7.1) (Sigma-Aldrich Co.) for routine culturing. The culture flasks were agitated on a shaker at 70 rpm. Bacterial growth was quantified by measuring the optical density of the culture suspension at a wavelength of 600 nm using an Ultrospec 3100 pro spectrometer (Amersham Bioscience, Piscataway, NJ).

Luminescence assays

A. fischeri cells (ATCC 7744) were grown for 16 h in Photobacterium broth until OD₆₀₀ reached ~2.0. Then, 700 µl of 30% glycerin solution and 700 µl of preculture solution were mixed, and this mixture was used as glycerol stock. For luminescence measurements, 100 µl of glycerol stocked bacteria were inoculated into artificial sea water (ASW), or a combination of 1-3 elements of ASW medium components, and then cultured at 26°C. The ASW (Fulladosa et. al, 2007) composition was as follows: 28.1 g/l NaCl, 0.77 g/l KCl, 1.21 g/l CaCl₂, 10.2 g/l MgCl₂ · 6H₂O, 0.11 g/l NaHCO₃, and 7.15 g/l MgSO₄ · 7H₂O.

To study the effect of the K⁺, HCO₃⁻, and SO₄²⁻ on *A. fischeri* luminescence at low cell density, *A. fischeri* was grown in ASW and in ASW in which KCl, NaHCO₃ and MgSO₄ · 7H₂O were substituted with various potassium-, hydrogen carbonate-, and sulfate ion-containing compounds, each of which was diluted to 3 final concentrations. We used KBr (0.0123, 0.123, or 1.23 g/l), KHCO₃ (0.0103, 0.103, or 1.03 g/l), and KNO₃ (0.0105, 0.105, or 1.05 g/l) as sources of K⁺, and NH₄HCO₃ (0.001, 0.01, or 0.1 g/l) or KHCO₃ (0.0013, 0.013, or 0.13 g/l) provided the hydrogen carbonate ion (HCO₃⁻). In addition, we used CaSO₄ (0.02, 0.2, or 2 g/l), MnSO₄ (0.022, 0.22, or 2.2 g/l), FeSO₄ (0.0038, 0.038, or 0.38 g/l), CuSO₄ (0.0023, 0.023, or 0.23 g/l), Na₂SO₄ (0.21, 2.1 or 21 g/l), K₂SO₄ (0.25, 2.5 or 25 g/l), Na₂SO₃ (0.002, 0.02 or 0.2 g/l), K₂SO₃ (0.0024, 0.024, or 0.24 g/l), and Na₂S₂O₃ (0.18, 1.8, 18 g/l) as sources of the sulfate ion (SO₄²⁻). These concentrations were set to the approximately same molar concentration of KCl, NaHCO₃, or MgSO₄ in ASW. Moreover, we added 10- and 100-fold changes of these compounds

to investigate the effect of concentration. Additionally, when we investigated the effect of sulfur, we substituted MgSO₄ with various sulfur-containing amino acids, such as cysteine, cystine, methionine, serine, and taurine (all amino acid concentrations were 0.001, 0.01, or 0.1 g/l). At 12 h after inoculation, 1 ml samples were taken for luminescence and OD measurements. Luminescence of *A. fischeri* was measured on a luminescence PSN luminometer (AB-2200, ATTO, Tokyo, Japan), and luminescence intensity was determined in relative light units integrated over a 5-min period. Moreover, 100 µl glycerol stocked *A. fischeri* were inoculated to only 2.81% NaCl solution as a control, and then cultured at 26°C for 12 h. Analysis was conducted using the t-test between various culturing conditions (all samples) and the control.

Assay for susceptibility to the fungicides sodium ortho-phenylphenol and imazalil in KCl, NaHCO₃, and MgSO₄

The fungicides sodium ortho-phenylphenol (C₁₂H₉NaO) and imazalil (C₁₄H₁₄N₂O) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *A. fischeri* cells were cultured for 12 h in KCl, NaHCO₃, and MgSO₄ (essential elements to induce low cell density luminescence) containing 2.81% NaCl. Next, 900 µl of the culture suspension was mixed with 100 µl of sodium ortho-phenylphenol or imazalil stock solutions (see below) and vortexed. The samples were exposed to the fungicides for 5 min or 15 min. Sodium ortho-phenylphenol was dissolved in 3% NaCl solution, and the final concentrations of fungicides in the mixtures with bacteria were 0.1, 1, 10, 100, and 1000 ppm. Imazalil was dissolved in 10% Tween 80 solution containing 3% NaCl, and the final concentrations of fungicides were the same as those in the sodium ortho-phenylphenol solutions. The samples vortexed with fungicides were then analyzed for luminescence intensity using a luminescence PSN luminometer (AB-2200, ATTO, Tokyo, Japan); the data were used to calculate the rate of luminescence.

RESULTS

Effects of ASW medium components on *A. fischeri* luminescence

First, we investigated the effects of ASW medium components (KCl, CaCl₂, MgCl₂, NaHCO₃, and MgSO₄) on cell density-independent luminescence (Fig.1). NaCl is necessary for the growth of *A. fischeri*; thus, 2.81% NaCl was always included in the medium in these experiments. *A. fischeri* luminescence increased significantly at 12 h after inoculation in ASW medium (p<0.05) (Fig.1(a)). However, the cell density (OD₆₀₀) was

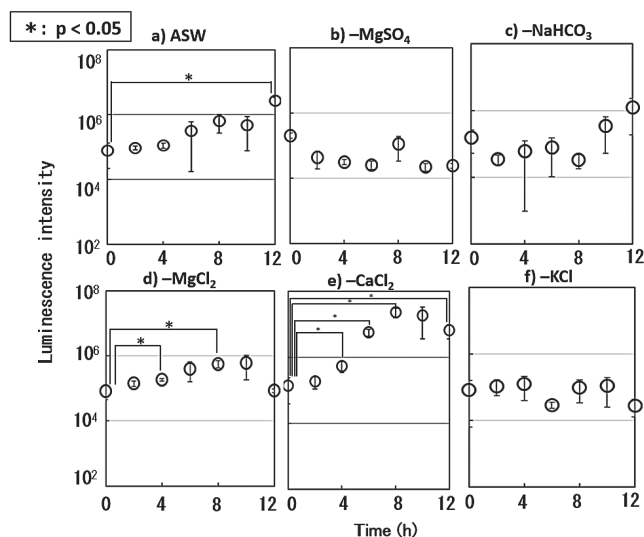


FIG. 1. Luminescence intensity of *A. fischeri* in a variety of modified Artificial Sea Water Media (ASW); (a) ASW, (b) MgSO_4 -starved conditions, (c) NaHCO_3 -starved conditions, (d) MgCl_2 -starved conditions, (e) CaCl_2 -starved conditions, (f) KCl -starved conditions.

Experiments were performed at least in triplicate and more, and the error bars represent standard deviations. Analysis was conducted using the t-test between each culturing time and 0 h.

approximately the same at both the time points, at 0.001 and 0.004 for 0 and 12 h, respectively. Under MgCl_2 -starvation conditions, the luminescence intensity of *A. fischeri* increased significantly at 4 and 8 h ($p < 0.05$) (Fig. 1(d)). Similarly, the luminescence intensity of *A. fischeri* increased significantly except at 2 and 10 h ($p < 0.05$) under CaCl_2 -starvation conditions (Fig. 1(e)). In addition, *A. fischeri* maintained an approximately steady OD_{600} value under these conditions (e.g. $\text{OD}_{600} = 0.000$ at 0 h and $\text{OD}_{600} = 0.005$ at 8 h in MgCl_2 -starvation conditions, and $\text{OD}_{600} = 0.001$ at 0 h and $\text{OD}_{600} = 0.003$ at 6 h in CaCl_2 -starvation conditions). These results suggest that MgCl_2 and CaCl_2 might not contribute in inducing luminescence. Conversely, luminescence decreased significantly under MgSO_4 -starvation conditions except at 8 h ($p < 0.05$) (Fig. 1(b)) and the OD_{600} was approximately same (e.g. 0.003 (0 h) and 0.012 (12 h)). Similarly, luminescence decreased significantly under NaHCO_3 -starvation conditions at 2 h and 8 h ($p < 0.05$) (Fig. 1(c)), and cell density was also maintained constant under these conditions (e.g. $\text{OD}_{600} = 0.001$ at 0 h and $\text{OD}_{600} = 0.002$ at 2 h). Furthermore, luminescence was not significantly different under KCl -starvation conditions for 0 to 12 h ($p > 0.05$) (Fig. 1(f)). These results suggest that cell density-independent luminescence of *A. fischeri* might require MgSO_4 , KCl , and NaHCO_3 under nutrient-starvation conditions.

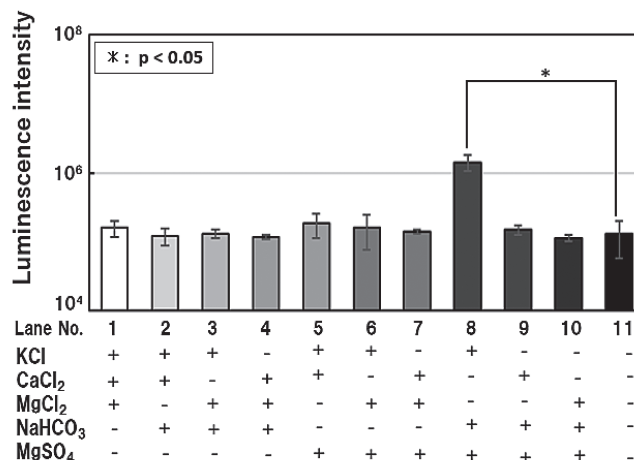


FIG. 2. Luminescence intensity of *A. fischeri* in a variety of modified ASW Media (3 elements).

Luminescence kinetics of *A. fischeri* in various modified ASW media. *A. fischeri* was cultured by combining three each of KCl , CaCl_2 , MgCl_2 , NaHCO_3 , or MgSO_4 with 2.81% NaCl . Experiments were performed at least in triplicate and more, and the error bars represent standard deviations. The presence and the absence of the components of the media are indicated by + and -, respectively. Analysis was conducted using the t-test between various culturing conditions and control (2.81% NaCl solutions).

We evaluated *A. fischeri* luminescence in the presence of only 1, 2 or 3 elements (Fig. 2) of the ASW medium components. *A. fischeri* luminescence increased significantly at 12 h in ASW (Fig. 1(a)). In the subsequent experiments, the luminescence intensity after 12 h of inoculation was measured. Luminescence of *A. fischeri* did not show a significant difference between control (2.81% NaCl) and only KCl , NaHCO_3 , or MgSO_4 ($p > 0.05$) (data not shown). In addition, *A. fischeri* did not activate luminescence with any combination of two components among KCl , MgSO_4 , and NaHCO_3 ; it did not show a significant difference between control and any combination of NaHCO_3 and MgSO_4 , KCl and MgSO_4 , or KCl and NaHCO_3 ($p > 0.05$) (data not shown). Nonetheless, *A. fischeri* luminescence increased in the presence of combinations of the three components KCl , MgSO_4 , and NaHCO_3 (Fig. 2, Lane No. 8), and there was a significant difference between these components and the control (Fig. 2, Lane No. 11) ($p < 0.05$). Additionally, cell density was $\text{OD}_{600} = 0.003$ at 0 h and $\text{OD}_{600} = 0.004$ at 12 h, therefore *A. fischeri* had minimal growth from 0 to 12 h. Moreover, it did not show a significant difference between other combinations of the three components (Fig. 2, Lane No. 1~7, 9~10) and control (Fig. 2, Lane No. 11). These results suggest that induction of *A. fischeri* luminescence under nutrient-starvation conditions (i.e., cell density-independent luminescence)

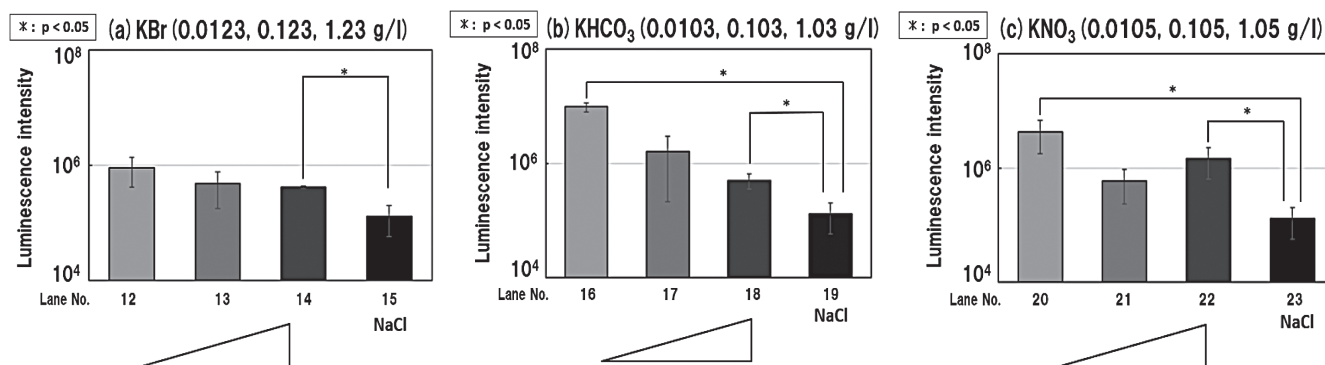


FIG. 3. Effects of various potassium compounds on induction of *A. fischeri* cell density-independent luminescence. KCl in the ASW medium was substituted with (a) KBr, (b) KHCO_3 , or (c) KNO_3 , and the intensity of luminescence in the cell was measured in each case. The right triangles below each graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compounds, while the black bars indicate the luminescence intensity in 2.81% NaCl solution. Experiments were performed at least in triplicate and more, and the error bars represent standard deviations. Analysis was conducted using the t-test between various culturing conditions and control (2.81% NaCl solutions).

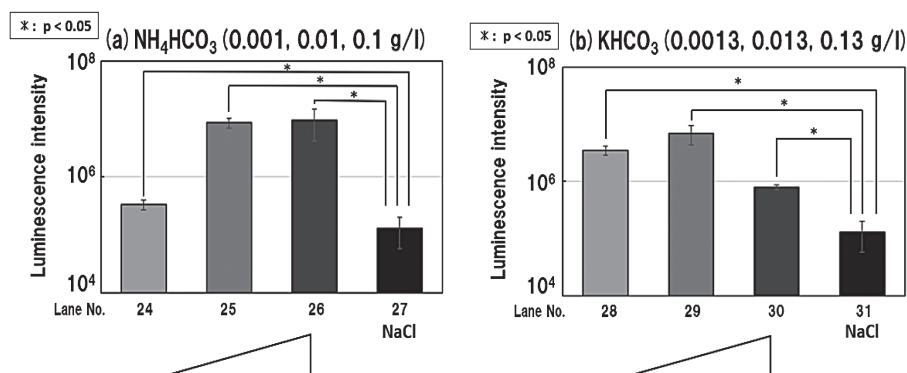


FIG. 4. Effects of various hydrogen carbonate compounds on induction of *A. fischeri* cell density-independent luminescence. NaHCO_3 in the ASW medium was substituted with (a) NH_4HCO_3 or (b) KHCO_3 , and we measured *A. fischeri* luminescence in each case. The right triangles below each graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compounds, while the black bars indicate the luminescence intensity in 2.81% NaCl solution. Experiments were performed at least in triplicate and more, and the error bars represent standard deviations. Analysis was conducted using the t-test between various culturing conditions and control (2.81% NaCl solutions).

might require the 3 elements of KCl, MgSO_4 , and NaHCO_3 . That is, KCl, MgSO_4 , and NaHCO_3 may affect the luminescence regulatory systems in *A. fischeri*.

Effects of K^+ on *A. fischeri* luminescence

A. fischeri required KCl, MgSO_4 , and NaHCO_3 to activate cell density-independent luminescence in the ASW medium; therefore, we assessed the effects of each ion (i.e., K^+ , HCO_3^- , and SO_4^{2-}) on *A. fischeri* luminescence.

First, we tested whether the K^+ stimulates the cell density-independent luminescence of *A. fischeri* (Fig.3). To investigate the effect of K^+ , we substituted KBr

(0.0123, 0.123, and 1.23 g/l), KHCO_3 (0.0103, 0.103, and 1.03 g/l), or KNO_3 (0.0105, 0.105, and 1.05 g/l) for KCl in the ASW medium. The luminescence of *A. fischeri* was significantly different between the substituted 1.23 g/l KBr (Fig.3(a), Lane No. 14) and control (Fig.3(a), Lane No. 15) ($p < 0.05$). Similarly, it increased significantly when replaced with 0.0103 g/l KHCO_3 (Fig.3 (b), Lane No. 16) ($p < 0.05$), or 1.03 g/l KHCO_3 (Fig.3 (b), Lane No. 18) compared to the control (Fig.3(b), Lane No. 19) ($p < 0.05$). Moreover, there was also a significant difference between the control (Fig.3(c), Lane No. 23) and 0.0105 or 1.05 g/l KNO_3 (Fig.3(c), Lane

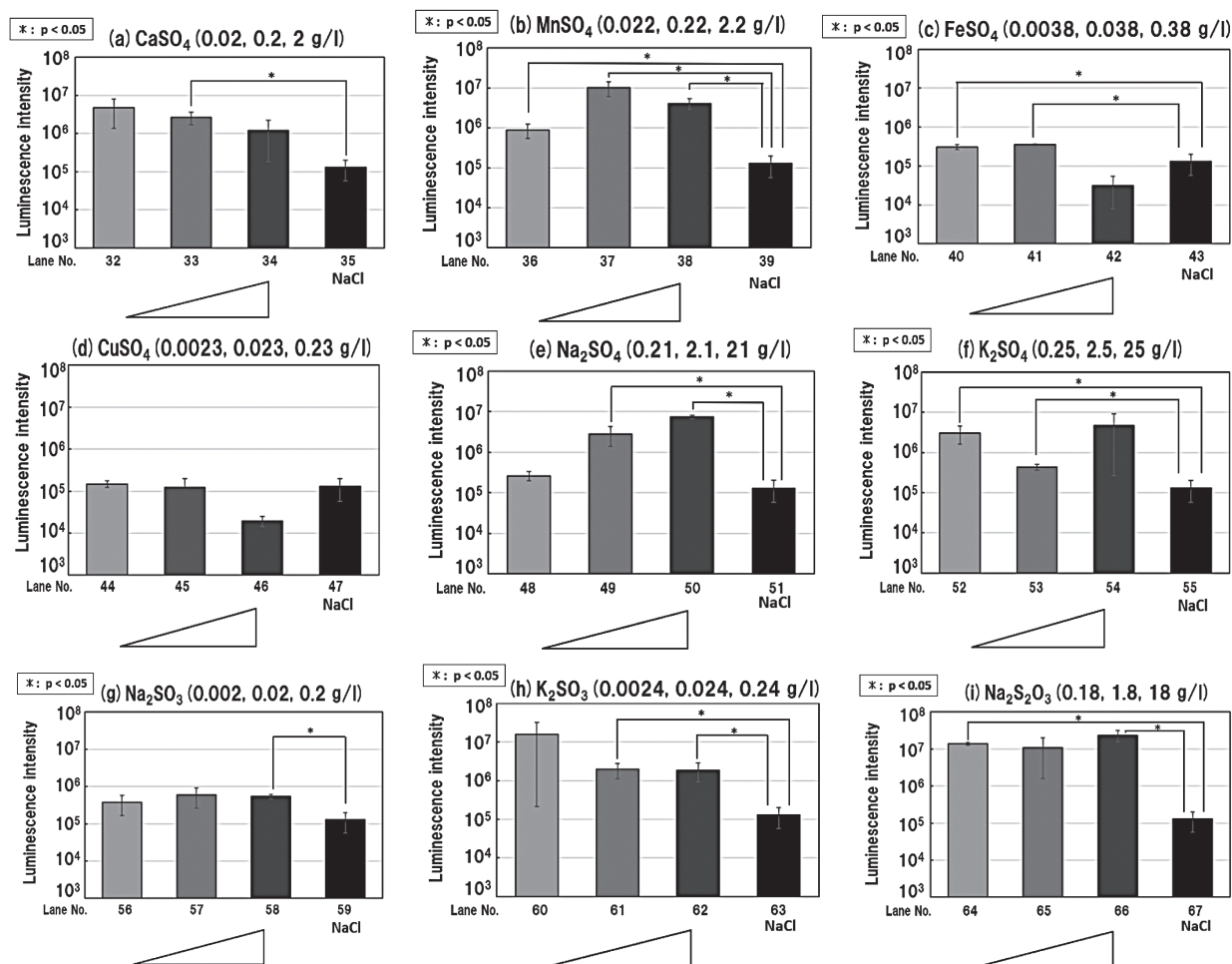


FIG. 5. Effects of various sulfate compounds on induction of *A. fischeri* cell density-independent luminescence. MgSO_4 in ASW medium was substituted with (a) CaSO_4 , (b) MnSO_4 , (c) FeSO_4 , (d) CuSO_4 , (e) Na_2SO_4 , (f) K_2SO_4 , (g) Na_2SO_3 , (h) K_2SO_3 , and (i) $\text{Na}_2\text{S}_2\text{O}_3$, and we measured *A. fischeri* luminescence in each case. The right triangles below each graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compounds, while the black bars indicate the luminescence intensity in 2.81% NaCl solution. Experiments were performed at least in triplicate and more, and the error bars represent standard deviations. Analysis was conducted using the t-test between various culturing conditions and control (2.81% NaCl solutions).

No. 20 or 22) ($p < 0.05$). Furthermore, cell density was nearly consistent under any conditions (e.g. $\text{OD}_{600} = 0.001$ at 0 h and $\text{OD}_{600} = 0.010$ at 12 h under replaced with 0.0103 g/l KHCO_3 conditions (Fig.3(b), Lane No. 16), and $\text{OD}_{600} = 0.002$ at 0 h and $\text{OD}_{600} = 0.005$ at 12 h under replaced with 0.0105 g/l KNO_3 conditions (Fig.3 (c), Lane No. 20)). These results suggest that inclusion of K^+ -containing compounds may induce cell density-independent luminescence in *A. fischeri*. That is, *A. fischeri* may require K^+ for activation of cell density-independent luminescence.

Effects of HCO_3^- on *A. fischeri* luminescence

Next, we explored the effects of the hydrogen carbonate ion (HCO_3^-) on *A. fischeri* cell density-inde-

pendent luminescence (Fig.4). Just as we substituted KCl, we cultivated *A. fischeri* by substituting NH_4HCO_3 (0.001, 0.01, or 0.1 g/l) or KHCO_3 (0.0013, 0.013, or 0.13 g/l) for NaHCO_3 in the ASW medium. When substituted with NH_4HCO_3 (Fig.4(a), Lane No. 24~26), luminescence intensity increased significantly ($p < 0.05$). Similarly, when substituted with KHCO_3 (Fig.4(b), Lane No. 28~30), the luminescence showed a significant difference to the control (Fig.4(b), Lane No. 31) ($p < 0.05$). Cell densities was also maintained constantly; when we replaced with 0.001 g/l NH_4HCO_3 (Fig.4(a), Lane No. 24), $\text{OD}_{600} = 0.001$ at 0 h and 12 h and replaced with 0.0013 g/l KHCO_3 (Fig.4(b), Lane No. 28), $\text{OD}_{600} = 0.003$ at 0 h and $\text{OD}_{600} = 0.011$ at 12 h. These results suggested HCO_3^- was required to induce

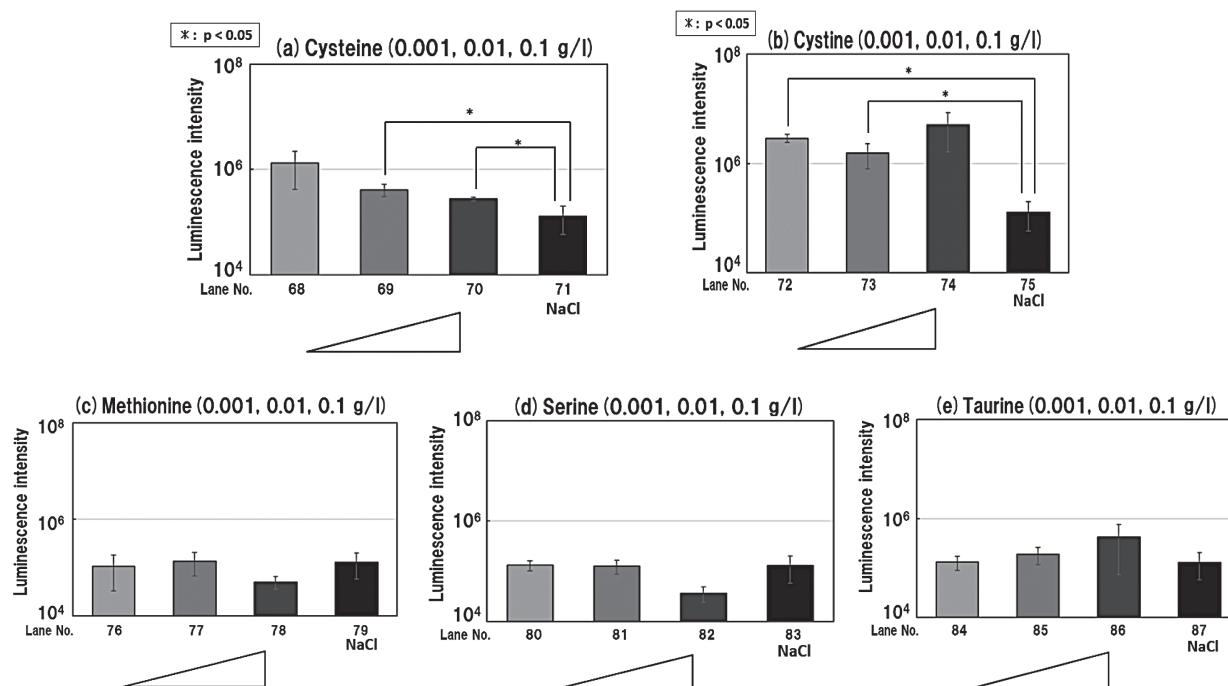


FIG. 6. Effects of various sulfur-containing amino acids on induction of *A. fischeri* cell density-independent luminescence. MgSO_4 in ASW medium was substituted with (a) cysteine, (b) cystine, (c) methionine, (d) serine, and (e) taurine, and we measured *A. fischeri* luminescence in each case. The right triangles below each graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compounds, while the black bars indicate the luminescence intensity in 2.81% NaCl solution. Experiments were performed at least in triplicate and more, and the error bars represent standard deviations. Analysis was conducted using the t-test between various culturing conditions and control (2.81% NaCl solutions).

the *A. fischeri* luminescence.

Effects of sulfate ion SO_4^{2-} on *A. fischeri* luminescence

Finally, we tested the effect of SO_4^{2-} on cell density-independent *A. fischeri* luminescence (Fig.5). Just as we substituted KCl or NaHCO_3 , various SO_4^{2-} containing compounds were used instead of MgSO_4 (as shown in the MATERIAL AND METHODS). *A. fischeri* luminescence significantly increased in all of these conditions, except in CuSO_4 substitution (Fig.5(a)~(f)). When we replaced with 0.2 g/l CaSO_4 (Fig.5(a), Lane No. 33), there was a significant difference ($p < 0.05$) and cell density did not increase in this condition. Similarly, in MnSO_4 (Fig.5(b), Lane No. 36~38), 0.0038 or 0.038 g/l FeSO_4 (Fig.5(c), Lane No. 40 or 41), 2.1 or 21 g/l Na_2SO_4 (Fig.5(e), Lane No. 49 or 50), and 0.25 or 2.5 g/l K_2SO_4 (Fig.5(f), Lane No. 52 or 53) replaced conditions, the luminescence showed a significant difference from the control ($p < 0.05$). Cell density was also maintained constant under these conditions (e.g. $\text{OD}_{600} = 0.000$ at 0 h and $\text{OD}_{600} = 0.002$ at 12 h under 0.2 g/l CaSO_4 (Fig.5(a), Lane No. 33), and $\text{OD}_{600} = 0.003$ at 0 h and $\text{OD}_{600} = 0.007$ at 12 h under 0.022 g/l MnSO_4 (Fig.5(b), Lane No.36) replaced conditions, and OD_{600}

$= 0.005$ at 0 h and $\text{OD}_{600} = 0.005$ at 12 h under 0.038 g/l FeSO_4 (Fig.5(c), Lane No. 41), and $\text{OD}_{600} = 0.002$ at 0 h and $\text{OD}_{600} = 0.005$ at 12 h under 2.1 g/l Na_2SO_4 (Fig.5(e), Lane No. 49), or $\text{OD}_{600} = 0.005$ at 0 h and $\text{OD}_{600} = 0.009$ at 12 h under 2.5 g/l K_2SO_4 (Fig.5(f), Lane No. 53) replaced conditions).

Since the luminescence was increased when sulfide compounds were replaced, it seems that the sulfide compounds may be necessary to induce the luminescence of *A. fischeri*. *A. fischeri* luminescence was induced by all sulfide compounds tested except for CuSO_4 , therefore, we also evaluated the effects of SO_3^{2-} or $\text{S}_2\text{O}_3^{2-}$ ions (as other sulfur sources) on *A. fischeri* cell density-independent luminescence. We substituted Na_2SO_3 (0.002, 0.02, or 0.2 g/l), K_2SO_3 (0.0024, 0.024, or 0.24 g/l), or $\text{Na}_2\text{S}_2\text{O}_3$ (0.18, 1.8, or 18 g/l) for MgSO_4 in the ASW medium. When we replaced with 0.2 g/l Na_2SO_3 (Fig.5(g), Lane No. 58), the luminescence showed a significant difference ($p < 0.05$) and cell density did not increase. Furthermore, when we cultured *A. fischeri* under replacement with 0.024 or 0.24 g/l K_2SO_3 (Fig.5(h), Lane No. 61 or 62), and 0.18 or 18 g/l $\text{Na}_2\text{S}_2\text{O}_3$ (Fig.5(i), Lane No. 64 or 66), the luminescence showed a significant difference from the control

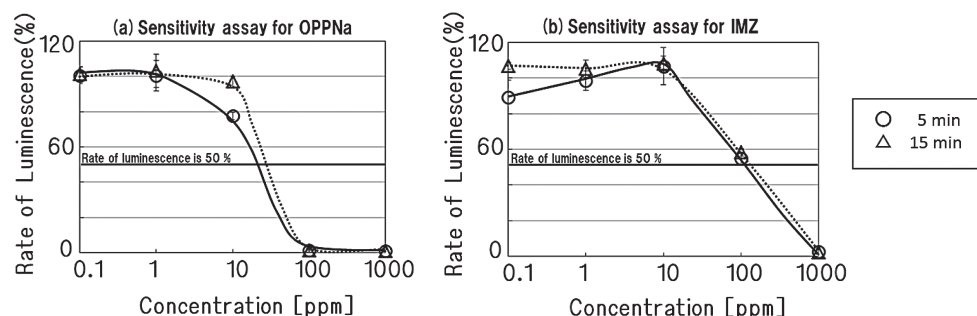


FIG. 7. Sensitivity of *A. fischeri* to the fungicides sodium ortho-phenylphenol and imazalil in KCl, NaHCO₃, and MgSO₄ containing 2.81% NaCl. *A. fischeri* was cultured in KCl, NaHCO₃, and MgSO₄ with 2.81% NaCl for 12 h, then mixed with sodium ortho-phenylphenol (a) or imazalil (b) solution and exposed for 5 min (circles and solid line) or 15 min (triangles and dotted line). Experiments were performed at least in triplicate and more, and the error bars represent standard deviations.

($p < 0.05$), and its cell density almost never increased in any condition. These results suggest that *A. fischeri* may require inorganic sulfur to induce luminescence cell density-independently. When we replaced with SO₄²⁻, SO₃²⁻, or S₂O₃²⁻ ion-containing compounds, except for CuSO₄, the luminescence increased significantly ($p < 0.05$). These results therefore suggest that *A. fischeri* activated cell density-independent luminescence in the presence of other SO₄²⁻ compounds and SO₃²⁻ or S₂O₃²⁻ ion-containing compounds, suggesting that *A. fischeri* may require sulfur for the induction of luminescence at low cell density. Finally, we investigated the effects of sulfur-containing amino acids as sulfur sources (Fig. 6). We added cysteine, cystine, methionine, serine, or taurine as sulfur-containing amino acids, and all amino acid concentrations were 0.001, 0.01, or 0.1 g/l. When we substituted 0.01 or 0.1 g/l cysteine (Fig. 6(a), Lane No. 69 or 70) and 0.001 or 0.01 g/l cystine (Fig. 6(b), Lane No. 72 or 73) for MgSO₄, the luminescence showed a significant difference from the control ($p < 0.05$); *A. fischeri* displayed luminescence at 12 h. Moreover, cell density was maintained under all conditions. On the other hand, other sulfur-containing amino acids did not induce luminescence; there was no significant difference between control and replacement with methionine (Fig. 6(c), Lane No. 76-78), serine (Fig. 6(d), Lane No. 80-82), or taurine (Fig. 6(e), Lane No. 84-86) ($p > 0.05$). Cysteine is known to be an essential component for bacteria, so these results suggest that the luminescence of *A. fischeri* and its metabolism might affect one another.

Altogether, these results suggest that *A. fischeri* may require K⁺, HCO₃⁻, and SO₄²⁻ ions to induce cell density-independent luminescence under nutrient-starvation conditions in ASW.

Assay for susceptibility to the fungicides sodium ortho-phenylphenol and imazalil in KCl, NaHCO₃, and MgSO₄ containing 2.81% NaCl

When *A. fischeri* exhibited luminescence in low cell density conditions (i.e., as in sea water), it required K⁺, HCO₃⁻, and SO₄²⁻. We next considered whether these results could be used to create new biosensors, and we tested the susceptibility of *A. fischeri* to the fungicides sodium ortho-phenylphenol and imazalil. Sodium ortho-phenylphenol and imazalil are two fungicides designed to minimize postharvest decay and are used worldwide (Eckert and Brown, 1986; Ismail and Zhang, 2004; Smilanick et al, 2003; Smilanick et. al, 2005), but are known to be highly toxic substances (Kashiwakura, 1993). *A. fischeri* was grown in KCl, NaHCO₃, and MgSO₄ containing 2.81% NaCl for 12 h. Sodium ortho-phenylphenol and imazalil were then added, and the cultures were exposed to the fungicides for 5 min or 15 min. Final concentrations of sodium ortho-phenylphenol and imazalil were 0.1, 1, 10, 100 and 1000 ppm. We analyzed the *A. fischeri* luminescence and calculated the rate of luminescence.

When we exposed *A. fischeri* to sodium ortho-phenylphenol, the rate of *A. fischeri* luminescence remained >50% at 0.1 to 10 ppm, but it decreased to <50% at 100 and 1000 ppm (Fig. 7(a)). This result suggests that *A. fischeri* maintained luminescence under low sodium ortho-phenylphenol concentrations (0.1 ~ 1 ppm), and that the luminescence decreased under high concentrations (i.e. 100 or 1000 ppm). Sodium ortho-phenylphenol may be used in concentrations of <10 ppm in Japan, so this result suggests that *A. fischeri* might be useful to analyze sodium ortho-phenylphenol, as luminescence decreased at >10 ppm (Fig. 7(a)). Conversely, when we exposed *A. fischeri* to imazalil, the rate of luminescence remained at approximately 100% at 10

ppm, and remained >50% even at 100 ppm (Fig.7(b)). It then decreased to <50% at 1000 ppm (Fig.7(b)). As *A. fischeri* exposed to imazalil did not display any significant decrease in luminescence until very high concentrations (1000 ppm), and as imazalil is approved for use at concentrations under 2~5 ppm in Japan, we concluded that *A. fischeri* cultured under KCl, NaHCO₃, and MgSO₄ and containing 2.81% NaCl is not suited to be an efficient biosensor for imazalil. From the results of sodium ortho-phenylphenol and imazalil exposure, we concluded that a biosensor using *A. fischeri* might be useful in analyzing the toxicity of sodium ortho-phenylphenol. In addition, the results were obtained from *A. fischeri* cultured with only KCl, NaHCO₃ and MgSO₄ containing 2.81% NaCl, and the culture time was 12 h. Moreover, the toxicity test exposure time was 5 or 15 min. Thus, this method can be performed more quickly than existing methods.

DISCUSSION

In this study, we investigated the relationship between cell density-independent luminescence of *A. fischeri* and seawater components. *A. fischeri* was found to require K⁺, SO₄²⁻, and HCO₃⁻. In addition, its luminescence increased in a substituted medium containing other K⁺, SO₄²⁻, and HCO₃⁻ containing compounds. These results suggest that these ions (i.e., K⁺, SO₄²⁻, and HCO₃⁻) may help to induce cell density-independent luminescence. However, when we replaced them with CuSO₄ (Fig.5 (d)), the luminescence did not increase. It was believed that as copper is a heavy metal, luminescence was not induced under these conditions; heavy metals inhibit cellular metabolism (Ma et al., 2014; Parvez et al., 2006). In fact, Arakawa et al. showed *V. fischeri* NRRL B-11177 luminescence was inhibited when exposed to approximately <1.0 mg/l copper ion (Arakawa et al., 2007). In this study, we substituted with 2.3 mg/l CuSO₄ (Fig.5(d), Lane No. 44) as a minimum concentration and 230 mg/l CuSO₄ (Fig.5(d), Lane No. 46) as a maximum concentration for MgSO₄, so it was considered that the luminescence activity might be inhibited (Fig.5 (d)).

Commonly, marine luminous bacteria regulate their luminescence in a cell density-dependent way, a method called quorum sensing (Fuqua et al., 1994; Henares et al., 2012; Miyashiro and Ruby, 2012; Nealson et al., 1977). This luminescence regulatory system is triggered by an autoinducer, which is a species-specific signaling molecule, and which accumulates in the surrounding environment during cell growth (Miyashiro and Ruby, 2012; Nealson et al., 1977). One of the *V. fischeri* autoinducers, *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), binds to LuxR, a transcription factor. The LuxR/3-oxo-C6 complex activates the *lux* gene,

thereby increasing luminescence (Miyashiro and Ruby, 2012). The *lux* gene is a luminescence regulatory gene and encodes a transcription factor essential to the production of light (Miyashiro and Ruby, 2012). Luciferases are enzymes that catalyze bacterial bioluminescence and represent the core of light production (Miyashiro and Ruby, 2012; Węgrzyn and Czyż, 2002). As described above (Eq. 1 and 2), luciferases catalyze the oxidation of a long-chain aldehyde (RCHO) and Flavin mononucleotide (FMNH₂), to produce luminescence (Inouye, 1994; Jablonski and DeLuca, 1978; Ma et al., 2014; Miyashiro and Ruby, 2012). In fact, Nealson et al. reported that when decanal (i.e., the substrate of the luciferase reaction) is added to a culture of growing luminous bacteria, it stimulates luminescence (Nealson et al., 1970). In addition, some ions have been shown to affect this bioluminescence. Nealson et al. showed that arginine (incidentally, the HCO₃⁻ ion is involved in the biosynthesis of arginine (Caldara et al., 2008; Cunin et al., 1986)) stimulates bioluminescence in *Photobacterium fischeri*, and this phenomenon may affect luciferase synthesis (Nealson et al., 1970). In another study, Watanabe et al. found that intracellular K⁺ may affect the expression of a luminescence gene in *Photobacterium phosphoreum*. The concentration of the intracellular K⁺ ion is increased in *P. phosphoreum* concomitantly with the development of luminescence; therefore, it is believed that the increase in the concentration of the intracellular K⁺ ion may change the conformation of bacterial chromosomes, leading to transcription of the luminescence gene, and this results to the increase in luminescence of *P. phosphoreum* (Watanabe et al., 1991). Furthermore, Berglind et al. also found that addition of K⁺ has a positive effect on production of luminescence in *V. fischeri* (Berglind et al., 2010). K⁺ is also known to be an essential nutrient for the growth of marine bacteria. For example, it is required for formation of the Na⁺ pump (a nutrient transport system (Drapeau and Macleod, 1963; Wong et al., 1969)), for the uptake of oxygen, and for stability of intercellular pH (Tokuda et al., 1981; Unemoto, 2000). In this study, we did not evaluate the effects of K⁺ or HCO₃⁻ ions at the molecular level (i.e., their effects on luminescence-related genes), but it appears that these ions may influence the luminescence regulatory systems of *A. fischeri* at the metabolic level. It is also not known how sulfur affects the luminescence regulatory systems at the molecular level. Because *A. fischeri* required sulfur for activation of luminescence under nutrient-starvation conditions, sulfur is likely necessary for induction of luminescence in marine luminous bacteria, similar to K⁺ or HCO₃⁻ ions at the metabolic level.

Previous studies have shown that iron performs an essential function in cell growth (just as the K⁺ ion

does) in microorganisms, and it might affect luciferase synthesis in *V. fischeri* (Haygood and Nealson, 1985). In addition, Septer et al. reported about the relationship between Fe and the luminescence regulatory systems of *V. fischeri* ES 114 at the molecular level. Under iron-rich conditions, Fur (ferric uptake regulator), a regulator of iron uptake genes, was suppressed (Septer et al., 2013). The biosynthesis of siderophores in response to a change in cellular Fe levels represses the expression of LitR, which is a protein that activates the expression of *luxR*. Conversely, under low-Fe conditions, Fur activates *litR*, which activates expression of *luxR*; thus, *V. fischeri* emits brighter luminescence (Fidopiastis et al., 2002; Hantke, 2001; Miyashiro and Ruby, 2012; Septer et al., 2013). That is, *V. fischeri* shows regulation of LitR and LuxR by Fur; regulation of luminescence may depend on Fe, and this Fe-dependent luminescence control requires Fur (Septer et al., 2013).

Similarly, certain studies have suggested that inorganic salts (e.g., potassium, sodium hydrogen carbonate, and iron) that are essential for cellular growth of bacteria might affect luminescence in the process of their metabolism. Therefore, there is likely to be a relationship between metabolism and luminescence in marine luminous bacteria. In fact, Wada et al. found a relationship between the respiratory chain and luminescent system in *V. harveyi* (Wada et al., 1992). The *A. fischeri* used in this study required sulfur for activation of luminescence under nutrient-starvation conditions (i.e., under very low cell density conditions). Sulfur is an essential component for the growth for all organisms and makes up 0.5-1% of cell dry weight in bacteria (Kertesz, 1999). It is known that sulfur also plays an essential role in various enzyme cofactors and is crucial for many redox processes, both as a building block for the iron-sulfur cluster and as the redox-active component of disulfide bonds (Kertesz, 1999). In addition, sulfur is needed for production of the side chains of the amino acids cysteine and methionine in bacteria, whereas inorganic sulfate and sulfite (i.e., SO_4^{2-} and SO_3^{2-} ions), like those used in this study, are necessary components for the biosynthesis of cysteine. Cysteine biosynthesis by the sulfate assimilation pathway proceeds via the transport of inorganic sulfate into the cell (Kertesz, 1999). In fact, when the various substances containing SO_4^{2-} or SO_3^{2-} were added, *A. fischeri* luminescence increased (Fig.5), and when the MgSO_4 was substituted with cysteine or cystine, luminescence also increased (Fig.6(a)(b)). Moreover, Liu et al. showed that *V. harveyi* produced cysteine protease (Liu et al., 1997). In contrast, the sulfur cycle of marine luminous bacteria is poorly understood, and it is not clear how sulfur affects the luminescence regulatory system at the molecular level. One study showed that the K^+ or arginine (synthesized from HCO_3^-) may affect the

synthesis of luciferase in marine luminous bacteria; in fact, the *A. fischeri* used in this study required these ions for the induction of luminescence. In addition, these ions are required for the growth of marine bacteria. Therefore, we can hypothesize that sulfur has some effect (e.g., the biosynthesis of luciferase or upregulation of luminescence genes) that increases luminescence after its assimilation. It seems that *A. fischeri* has another luminescence regulatory system (i.e., sulfur-dependent luminescence) for the induction of luminescence. Nonetheless, the relationship between sulfur and the luminescence regulatory system is not known at the molecular level, and further investigation into how sulfur affects the induction of luminescence in marine bacteria is needed.

In this study, we also investigated luminescence behavior after exposure to sodium ortho-phenylphenol and imazalil (Fig.7). When *A. fischeri* was exposed to sodium ortho-phenylphenol, luminescence intensity decreased until < 50% with > 10 ppm added (Fig.7(a)), similarly the luminescence intensity decreased < 50% at 1000 ppm of imazalil (Fig.7(b)). Recently, marine luminous bacteria have been used as biosensors; toxicity is analyzed by luminescence inhibition rate (Arakawa et al., 2007; Yamada, 2014; Ma et al., 2014; Parvez et. al, 2006). This inhibition is closely linked to light emission and cellular metabolism. When marine luminous bacteria are exposed to toxic substances (e.g. heavy metals), luminescence decreases rapidly because luciferase activity is inhibited when marine luminous bacteria are exposed to toxic substances (Ma et al., 2014; Parvez et. al, 2006). Fungicides inhibit ergosterol biosynthesis or related products in microorganism (Schepers, 1983; Björnlund et al., 2000; Onyewu et al., 2003). Additionally, Jang et al. showed that ortho-phenylphenol down-regulated many amino acids biosynthesis pathways in *Staphylococcus aureus* at 20 min, and also up-regulated transcription ribosomal proteins at 20 min (Jang et al., 2008). In this way, fungicides affect the metabolism of microorganisms, therefore it was considered that when fungicides (ortho-phenylphenol or imzalil) were administered to *A. fischeri* in this study, they inhibited the metabolism and reduced luminescence (Fig.7).

In particular, when *A. fischeri* was exposed to sodium ortho-phenylphenol, luminescence decreased at > 10 ppm. As sodium ortho-phenylphenol may be used in concentrations of <10 ppm in Japan, we concluded that *A. fischeri* might be useful in analyzing the toxicity of sodium ortho-phenylphenol. However, *A. fischeri* displayed quenching when we added only sodium ortho-phenylphenol in this study. If *A. fischeri* is used as a biosensor for analysis of fungicides, we will have to consider the effect of other substances, pH, or selec-

tivity, for example. In fact, when they were exposed to 3 % NaCl solutions prepared at approximately pH 2.0 for 5-15 min, *A. fischeri* maintained the rate of luminescence approximately 90% (data not shown), so we considered that pH had minimal effect. In the future studies, we assume that the citrus peel will be washed and scuffed (e.g. abrasive such as silica or brushing), subsequently, the suspended solution will be produced and analyzed for toxicity using this biosensor. In this study, it was found that *A. fischeri* luminescence was decreased by exposure to fungicides, so it is considered that it could be used as a biosensors at low cell density, and therefore it may be useful for primary screening of remaining fungicides in citrus fruit. It is considered that as *A. fischeri* cultured in KCl, MgSO₄, and NaHCO₃ can be analyzed more quickly and at lower cost than existing methods, it may be useful. We need further research toward the practical use of *A. fischeri* cultured in the presence of KCl, NaHCO₃, and MgSO₄ as biosensors.

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