Research Paper

Interactions between bicarbonate, potassium, and magnesium, and sulfur-dependent induction of luminescence in *Vibrio fischeri*

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In spite of its central importance in research efforts, the relationship between seawater compounds and bacterial luminescence has not previously been investigated in detail. Thus, in this study, we investigated the effect of cations (Na^+, K^+, NH_4^+, Mg^{2+}, and Ca^{2+}) and anions (Cl^-, HCO_3^-, CO_3^{2-}, and NO_3^-) on the induction of both inorganic (sulfate, sulfite, and thiosulfate) and organic (L-cysteine and L-cystine) sulfur-dependent luminescence in *Vibrio fischeri*. We found that HCO_3^- (bicarbonate) and CO_3^{2-} (carbonate), in the form of various compounds, had a stimulatory effect on sulfur-dependent luminescence. The luminescence induced by bicarbonate was further promoted by the addition of magnesium. Potassium also increased sulfur-dependent luminescence when sulfate or thiosulfate was supplied as the sole sulfur source, but not when sulfite, L-cysteine, or L-cystine was supplied. The positive effect of potassium was accelerated by the addition of magnesium and/or calcium. Furthermore, the additional supply of magnesium improved the induction of sulfite- or L-cysteine-dependent luminescence, but not the L-cystine-dependent type. These results suggest that sulfur-dependent luminescence of *V. fischeri* under nutrient-starved conditions is mainly controlled by bicarbonate, carbonate, and potassium. In addition, our results indicate that an additional supply of magnesium is effective for increasing *V. fischeri* luminescence.

Keywords: *Vibrio fischeri* / Luminescence / Sulfur / Bicarbonate / Magnesium

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Introduction

Vibrio fischeri is a well-studied luminescent bacterium that can be found both free-living in seawater and in symbiotic association with the Hawaiian squid species *Euprymna scolopes* [1]. For many decades, *V. fischeri* has been a model organism for studying luminescence and its regulation. At a low cell density, *V. fischeri* produces very little luciferase and luminescence, whereas at a higher cell density, these levels are increased. This cell density-dependent induction of luciferase synthesis and resultant luminescence is controlled by diffusible luminescence inducing factors called autoinducers, which accumulate during bacterial growth and trigger induction of luciferase when it attains a particular threshold concentration [2–5]. In *V. fischeri*, these autoinducers are *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL), *N*-octanoyl homoserine lactone (C8-HSL), and presumably a furanosyl borate diester, which are produced by LuxI, AinS, and LuxS, respectively, and collectively stimulate the transcription of a set of luminescent genes, *luxICDABEG* [2, 6–10].

In contrast to cell density-dependent luminescence, some studies have shown that cell density-independent luminescence can be regulated by various factors, such as heat shock proteins [11, 12], UV irradiation [13], osmolarity [14], and redox state [15]. In addition, we recently discovered cell density-independent luminescence induced under nutrient-starved conditions [16]. Moreover, we demonstrated that this type of luminescence required the addition of sulfur compounds [16,

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17]. In particular, this luminescence type was induced both by inorganic sulfur sources, such as sulfate, sulfite, and thiosulfate, and by organic sulfur sources, including L-cysteine and L-cystine, but not by the addition of autoinducers, such as 3-oxo-C6-HSL or C8-HSL [17].

Generally, marine bacteria, such as *V. fischeri*, have special requirements for inorganic ions, partly to supply the needs of the organisms for growth and metabolism, partly to maintain the integrity of the cells [18]. These ions can be divided into cations and anions. Major cations are sodium, potassium, calcium, and magnesium, while anions include principally bicarbonate, chloride, biphosphate, and sulfate ions. It is usually explained that sodium (Na^+) is necessary for membrane stability, enzyme activity, and active transport [19]. In *V. fischeri*, Na^+ and K^+ are important for turgor, for the stability of the intracellular pH, and for membrane potential [20, 21]. In addition, it has been reported that Mg^{2+} has a stabilizing effect on the outer membrane in *V. fischeri* [22]. Furthermore, it has been reported that optimal migration of this organism required the addition of divalent cations such as Mg^{2+} and Ca^{2+} [23]. However, it remains unclear whether the composition of ions included in seawater affects the induction of sulfur-dependent luminescence in *V. fischeri*. Thus, the purpose of the present study was to investigate the effect of cations (Na^+, K^+, NH_4^+, Mg^{2+}, and Ca^{2+}) and anions (Cl^-, HCO_3^-, CO_3^{2-}, and NO_3^-) on both inorganic and organic sulfur-dependent luminescence in *V. fischeri*, as well as to analyze the relationships between cations/anions and the induction of the luminescence.

Materials and methods

Bacterial strain and culture conditions

All media reagents were obtained from Becton, Dickinson (Sparks, MD, USA); Kanto Chemical Co., Inc (Tokyo, Japan); Nacalai Tesque (Kyoto, Japan); and Wako Pure Chemical Industry (Osaka, Japan). *V. fischeri* (ATCC 49387) was obtained from the American Type Culture Collection and grown in 100 ml of nutrient broth (NB) (Becton, Dickinson) supplemented with 3% NaCl in 300 ml flasks, which were agitated on a shaker at 70 rpm at 22 °C. *V. fischeri* was also cultured at 22 °C in artificial seawater (ASW) [16, 17], which was composed of 28.1 g/l of NaCl, 0.77 g/l of KCl, 1.21 g/l of $CaCl_2$, 4.8 g/l of $MgCl_2 \cdot 6\ H_2O$, 0.11 g/l of $NaHCO_3$, and 3.5 g/l of $MgSO_4 \cdot 7\ H_2O$. Bacterial proliferation was monitored by measuring the increase in optical density of the culture suspension at 600 nm (OD_{600}).

Luminescence assay

To investigate the effects of cations and anions on sulfur-dependent luminescence in *V. fischeri*, the bacterium was grown in various media, including 2.81% NaCl and $MgSO_4 \cdot 7\ H_2O$ (3.5 g/l) or L-cysteine (10 mg/l), in addition to various compounds, each of which was dissolved at 3 different final concentrations, as follows: KCl (0.077, 0.77, and 7.7 g/l), NH_4Cl (0.056, 0.56, and 5.6 g/l), $NaHCO_3$ (0.011, 0.11, and 1.1 g/l), $KHCO_3$ (0.013, 0.13, and 1.3 g/l), NH_4HCO_3 (0.01, 0.1, and 1.0 g/l), Na_2CO_3 (1.3, 13, and 130 mg/l), K_2CO_3 (1.8, 18, and 180 mg/l), $(NH_4)_2CO_3$ (0.012, 0.12, and 1.2 g/l), $NaNO_3$ (0.011, 0.11, and 1.1 g/l), KNO_3 (0.1, 1.0, and 10 g/l), NH_4NO_3 (0.079, 0.79, and 7.9 g/l), KBr (0.12, 1.2, and 12 g/l), KI (0.17, 1.7, and 17 g/l), NaBr (0.1, 1.0, and 10 g/l), and NaI (0.16, 1.6, and 16 g/l).

To investigate the effects of various combinations of KCl, $MgCl_2$, $CaCl_2$, and $NaHCO_3$ on sulfur-dependent luminescence of *V. fischeri*, Na_2SO_4 (2.1 g/l), Na_2SO_3 (0.24 g/l), $Na_2S_2O_3$ (1.8 g/l), L-cysteine (10 mg/l), or L-cystine (10 mg/l) was used as a sole sulfur source.

V. fischeri was grown overnight in NB with 3% NaCl, and then subcultured in the various media at an initial concentration with an OD_{600} of 0.01 (approximately 10^6 colony forming units (CFU)/ml). At 14 and 24 h after inoculation, 1 ml samples were collected for examination of their luminescence measurements. A Luminescencer PSN luminometer (AB-2200; Atto Corp., Tokyo, Japan) was used to quantify the luminescent signal integrated during a 5 min period.

Statistical analysis

All assays in this study were carried out in triplicate. Statistical significance between groups was determined by 2-tailed Student's *t*-test (Excel; Microsoft). Differences were considered significant at $P < 0.05$.

Results

Effects of $MgCl_2$, $CaCl_2$, KCl, and $NaHCO_3$ on sulfur-dependent luminescence in ASW media

As an initial experiment, we investigated the effects of $MgCl_2$, $CaCl_2$, KCl, and $NaHCO_3$, which were included in the ASW medium, on the induction of sulfur-dependent luminescence in *V. fischeri* (Fig. 1). $MgSO_4$, an inorganic sulfur source, or L-cysteine, an organic sulfur source, was added into the media. We found that when *V. fischeri* was cultured in $MgSO_4$-supplemented conditions, its luminescence intensities were decreased in the presence of $MgCl_2$ or $CaCl_2$ between 14 and 24 h (Fig. 1a and b). In contrast, higher luminescence inten-

sity of the bacterium was observed upon the addition of KCl or $NaHCO_3$ between 14 and 24 h of culture (Fig. 1c and d). When *V. fischeri* was cultured in L-cysteine-supplemented conditions, its luminescence intensities were decreased in the presence of $MgCl_2$ or $CaCl_2$ between 14 and 24 h (Fig. 1e and f). In contrast to the case of the $MgSO_4$ + KCl-supplemented condition, the intensities of the time-course L-cysteine-dependent luminescence measurements were decreased in spite of the presence of KCl (Fig. 1g). On the other hand, higher luminescence intensities were observed after addition of $NaHCO_3$ between 14 and 24 h of culture (Fig. 1h). Since the cell density (OD_{600}) of *V. fischeri* in various media did not increase for 24 h, these phenomena appeared to be cell density-independent. These results suggest that $NaHCO_3$ is required for the induction of both inorganic and organic sulfur-dependent luminescence in *V. fischeri*, but KCl does not induce the latter type of luminescence.

Requirement of bicarbonate, carbonate, or potassium for the induction of inorganic sulfur-dependent luminescence in *V. fischeri*

To verify the influence of cations and anions on inorganic sulfur-dependent luminescence of *V. fischeri*, we added the corresponding salts of the cations (Na^+, K^+, and NH_4^+) and anions (Cl^-, HCO_3^-, CO_3^{2-}, and NO_3^-) to the $MgSO_4$-supplemented media and subsequently measured the induction of luminescence at both 14 and 24 h (Fig. 2). All concentrations of KCl induced the inorganic sulfur-dependent luminescence of *V. fischeri* ($P < 0.05$) (Fig. 2a). Upon comparing the results generated in the presence of either KCl or NH_4Cl (Fig. 2a and b), we determined that the luminescence intensities induced by NH_4Cl were approximately 100–300-fold lower than those in the presence of KCl, which suggests that NH_4^+ and Cl^- were unnecessary for induction of inorganic sulfur-dependent luminescence. When moderate concentration of bicarbonates and carbonates of Na^+ (0.11 g/l and 13 mg/l, respectively) and NH_4^+ (0.1 and 0.12 g/l, respectively) were used, the inorganic sulfur-dependent luminescence intensities were higher than that in negative control ($P < 0.05$) (Fig. 2c, e, f, and h). On the other hand, bicarbonate and carbonate of K^+ had an inhibitory effect on the maintenance of luminescence during 24 h (Fig. 2d and g). In the presence of a low concentration of $KHCO_3$ (0.013 g/l) and a moderate to high concentration of K_2CO_3 (18 and 180 mg/l), the inorganic sulfur-dependent luminescence was similar to that in ASW media at 14 h, but the luminescence intensity was decreased at 24 h. Similarly, all concentrations of KNO_3 (0.1, 1.0, and 10 g/l) induced inorganic sulfur-dependent luminescence at 14 h, but this increase was not maintained at 24 h (Fig. 2j). Since the luminescence intensities induced by nitrates of Na^+ and NH_4^+ were approximately the same as that in negative control (Fig. 2i and k), it seems that NO_3^-, Na^+, and NH_4^+ had little effect on the induction of inorganic sulfur-dependent luminescence. These results suggest that bicarbonates and carbonates of Na^+ and NH_4^+ had a pronounced inducing effect on the inorganic sulfur-dependent luminescence of *V. fischeri*.

Similar to the inorganic sulfur-dependent luminescence intensities induced by various potassium compounds, KCl also induced inorganic sulfur-dependent luminescence, however, $KHCO_3$, K_2CO_3, and KNO_3 failed to maintain high luminescence intensities at 24 h (Fig. 2a, d, g, and j). Subsequently, to investigate

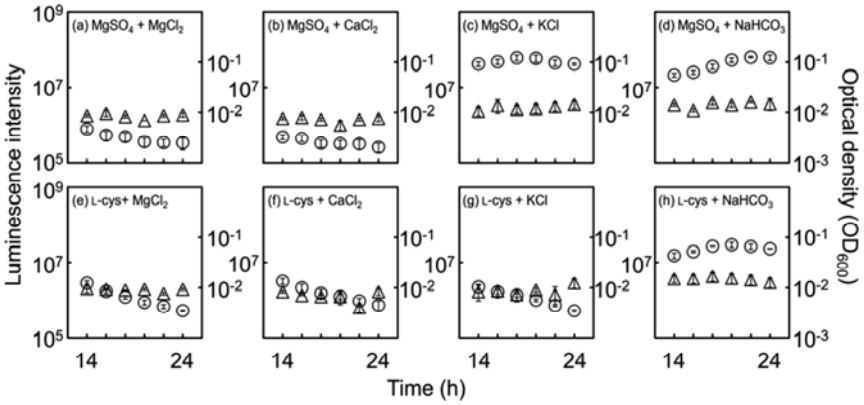

Figure 1. Luminescence kinetics (circles) and growth profiles (triangles) of *V. fischeri* in various media between 14 and 24 h. The media included (a, e) $MgCl_2$, (b, f) $CaCl_2$, (c, g) KCl, or (d, h) $NaHCO_3$ in addition to 2.81% NaCl and $MgSO_4$ (a–d), or L-cysteine (e–h). Data points represent the mean of 3 cultures of a representative experiment, and the error bars represent the standard deviation.

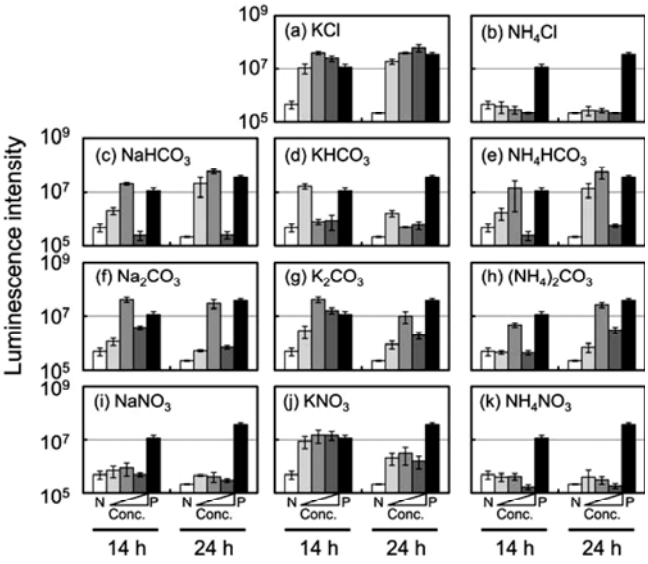

Figure 2. Luminescence intensity of *V. fischeri* in various media at 14 and 24 h. The media included (a) KCl, (b) NH_4Cl, (c) $NaHCO_3$, (d) $KHCO_3$, (e) NH_4HCO_3, (f) Na_2CO_3, (g) K_2CO_3, (h) $(NH_4)_2CO_3$, (i) $NaNO_3$, (j) KNO_3, or (k) NH_4NO_3, in addition to 2.81 g/l of NaCl and 3.5 g/l of $MgSO_4 \cdot 7\ H_2O$. The precise composition of the media is described in the Materials and Methods section. The right triangles below the graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compound. The black bars indicate the luminescence intensity in artificial seawater (ASW) medium (P: positive control), whereas the white bars indicate the luminescence intensity in 2.81 g/l of NaCl solution containing $MgSO_4$ (N: negative control). Experiments were performed in triplicate; the height of the bars indicates the mean and the error bars represent the standard deviation.

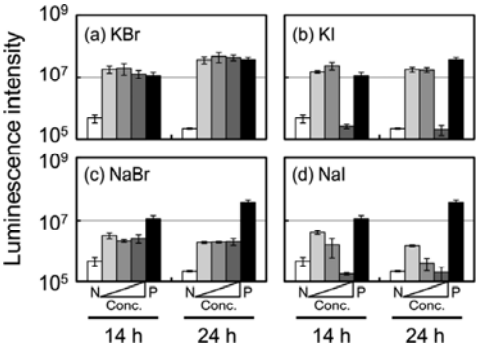

Figure 3. Luminescence intensity of *V. fischeri* in various media at 14 and 24 h. The media included (a) KBr, (b) KI, (c) NaBr, or (d) NaI, in addition to 2.81 g/l of NaCl and 3.5 g/l of $MgSO_4 \cdot 7\ H_2O$. The precise composition of the media is described in the Materials and Methods section. The right triangles below the graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compound. The black bars indicate the luminescence intensity in ASW medium (P: positive control), whereas the white bars indicate the luminescence intensity in 2.81 g/l of NaCl solution containing $MgSO_4$ (N: negative control). Experiments were performed in triplicate; the height of the bars indicates the mean and the error bars represent the standard deviation.

whether potassium compounds could stimulate inorganic sulfur-dependent luminescence, we focused on anion (Cl^-) in the form of KCl, in addition to using halogenated potassium compounds, including KBr and KI, for studying the induction of inorganic sulfur-dependent luminescence (Fig. 3). All concentrations of KBr (0.12, 1.2, and 12 g/l) and low to moderate concentrations of KI (0.17 and 1.7 g/l) induced inorganic sulfur-dependent luminescence at 14 h, which was also maintained at 24 h ($P < 0.05$) (Fig. 3a and b). In contrast, none of the concentrations of NaBr or NaI increased the induction of luminescence at 24 h (Fig. 3c and d), indicating that neither bromide nor iodide was necessary for induction of inorganic sulfur-dependent luminescence. These results suggest that potassium, at least in the forms of halogenated potassium compounds KBr and KI, has an inducing effect on inorganic sulfur-dependent luminescence in *V. fischeri*.

Requirement of bicarbonate or carbonate for the induction of organic sulfur-dependent luminescence in *V. fischeri*

To investigate the influence of cations (Na^+, K^+, and NH_4^+) and anions (Cl^-, HCO_3^-, CO_3^{2-}, and NO_3^-) on organic sulfur-dependent luminescence, we added various compounds to L-cysteine-supplemented media and measured the induction of luminescence after 14 and 24 h (Fig. 4). In line with our finding that all concentrations of KCl and NH_4Cl had little effect on the induction of organic sulfur-dependent luminescence, we found that K^+, NH_4^+, and Cl^- failed to stimulate induction of luminescence under these conditions (Fig. 4a and b). When moderate concentrations of $NaHCO_3$ (0.11 g/l) or NH_4HCO_3 (0.1 g/l) were used, the organic sulfur-dependent luminescence was induced at 14 h and increased at 24 h ($P < 0.05$) (Fig. 4c and e). Similarly, moderate concentrations of Na_2CO_3 (13 mg/l) and $(NH_4)_2CO_3$ (0.12 g/l) induced luminescence at 24 h ($P < 0.05$) (Fig. 4f and h). On the other hand, neither $KHCO_3$ nor K_2CO_3 induced high luminescence intensity, likely because potassium has an inhibitory effect under these conditions (Fig. 4d and g). In addition, sulfur-dependent luminescence was not induced by the addition of halogenated potassium compounds KBr or KI (data not shown). Since the luminescence intensities induced by nitrates of Na^+, K^+, and NH_4^+ were approximately 100-fold lower than that in ASW media (Fig. 4i, j, and k), it seems that NO_3^-, Na^+, and NH_4^+ had little effect on the induction of organic sulfur-dependent luminescence. These results suggest that bicarbonates and carbonates of Na^+ and NH_4^+ had a pronounced inducing effect on organic sulfur-dependent luminescence in *V. fischeri*.

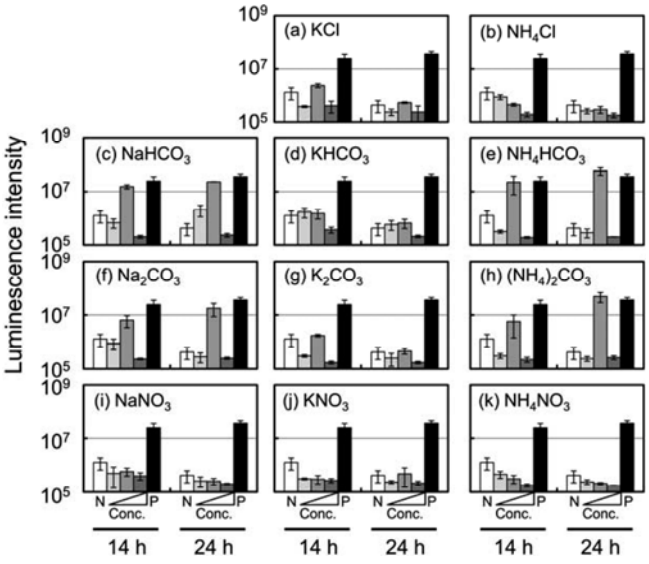

Figure 4. Luminescence intensity of *V. fischeri* in various media at 14 and 24 h. The media included (a) KCl, (b) NH_4Cl, (c) $NaHCO_3$, (d) $KHCO_3$, (e) NH_4HCO_3, (f) Na_2CO_3, (g) K_2CO_3, (h) $(NH_4)_2CO_3$, (i) $NaNO_3$, (j) KNO_3, or (k) NH_4NO_3, in addition to 2.81 g/l of NaCl and 10 mg/l of L-cysteine. The precise composition of the media is described in the Materials and Methods section. The right triangles below the graph and the bars with darker shades of gray correspond to increasing concentrations of the indicated compound. The black bars indicate the luminescence intensity in ASW medium in which $MgSO_4 \cdot 7\,H_2O$ was substituted with L-cysteine (P: positive control), whereas the white bars indicate the luminescence intensity in 2.81 g/l of NaCl solution containing L-cysteine (N: negative control). Experiments were performed in triplicate; the height of the bars indicates the mean and the error bars represent the standard deviation.

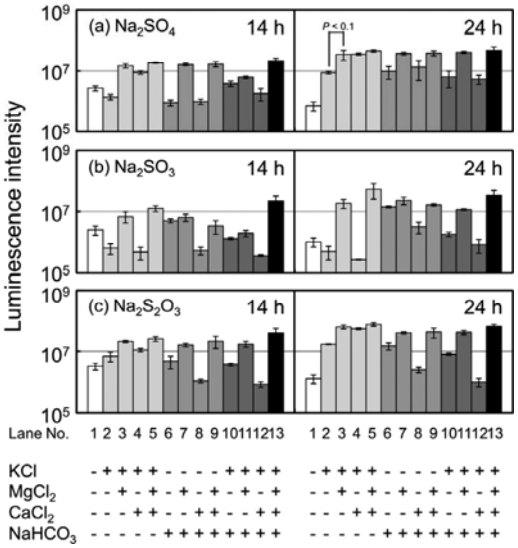

Figure 5. Luminescence intensity of *V. fischeri* in various media with different combinations of components at 14 and 24 h. (a) Na_2SO_4, (b) Na_2SO_3, or (c) $Na_2S_2O_3$ was added into the media as sulfur sources. The precise composition of the media is described in the Materials and Methods section. The white bars indicate the luminescence intensity in 2.81% NaCl media, whereas the black bars indicate the same but in ASW medium in which $MgSO_4 \cdot 7\,H_2O$ was substituted with Na_2SO_4, Na_2SO_3, or $Na_2S_2O_3$. The light gray, gray, and dark gray bars indicate the presence of KCl, $NaHCO_3$, and KCl/$NaHCO_3$, respectively. The presence and absence of the components in the medium are indicated as "+" and "–," respectively. Experiments were performed in triplicate; the height of the bars indicates the mean and the error bars represent the standard deviation.

Effects of potassium, bicarbonate, magnesium, and calcium on the induction of inorganic sulfur-dependent luminescence in *V. fischeri*

The ASW medium used in this study contained NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, and $MgSO_4$ as components (see Materials and methods). To elucidate the relationships between potassium, bicarbonate, magnesium, and calcium in the induction of inorganic sulfur-dependent luminescence, we measured the luminescence intensities of these components in various combinations after both 14 and 24 h (Fig. 5). In the presence of sulfate (here Na_2SO_4), sulfite (here Na_2SO_3), or thiosulfate (here $Na_2S_2O_3$) alone, the induction of high luminescence intensity did not occur (Fig. 5a, b, and c; lane 1). On the other hand, high luminescence intensity was induced in each sulfate, sulfite, and thiosulfate supplemented with KCl, $MgCl_2$, $CaCl_2$, and $NaHCO_3$ (Fig. 5a, b, and c; lane 13).

When sulfate (in the form of Na_2SO_4) was used as a sole sulfur source, the addition of KCl or $NaHCO_3$ did not induce luminescence at 14 h, but induced at 24 h ($P < 0.05$) (Fig. 5a; lanes 2 and 6). Moreover, the inducing effect of KCl was promoted by the addition of $MgCl_2$ and/or $CaCl_2$ at both 14 and 24 h (Fig. 5a; lanes 3, 4, and 5). Furthermore, the inducing effect of $NaHCO_3$ was similarly activated by the addition of $MgCl_2$, but not by $CaCl_2$ ($P < 0.05$) (Fig. 5a; lanes 6, 7, 8, and 9). In the presence of both KCl and $NaHCO_3$, the inducing effect of $MgCl_2$ was not clearly observed at 14 h, but appeared at 24 h ($P < 0.05$) (Fig. 5a; lanes 10 and 11). In contrast, the inducing effect of KCl and $NaHCO_3$ was not promoted by the addition of $CaCl_2$ (Fig. 5a; lanes 10 and 12), which indicates that the sulfate-dependent luminescence induced by potassium is repressed by the addition of $NaHCO_3$, and is mainly activated by a bicarbonate-involving pathway.

When sulfite (in the form of Na_2SO_3) was used as the sole sulfur source, the addition of $NaHCO_3$ induced luminescence, whereas the addition of KCl reduced it (Fig. 5b; lanes 2 and 6). This inhibitory effect of KCl was recovered by the addition of $MgCl_2$, but not by $CaCl_2$ alone at both 14 and 24 h ($P < 0.05$) (Fig. 5b; lanes 3, 4, and 5). The inducing effect of $NaHCO_3$ was not suppressed by the addition of $MgCl_2$, but was inhibited by $CaCl_2$ alone (Fig. 5b; lanes 6, 7, 8, and 9). The effect of

$NaHCO_3$ was also inhibited by the addition of KCl at 24 h ($P < 0.05$) (Fig. 5b; lanes 6 and 10). This inhibitory effect of KCl was overcome by $MgCl_2$, but not by $CaCl_2$ at 24 h ($P < 0.05$) (Fig. 5b; lanes 11 and 12). These results suggest that, in the presence of both KCl and $NaHCO_3$, sulfite-dependent luminescence was mostly activated by a bicarbonate-involving pathway at 14 h, and then activated by a potassium-involving pathway at 24 h.

When thiosulfate (in the form of $Na_2S_2O_3$) was used as the sole sulfur source, the addition of KCl or $NaHCO_3$ did not effect on luminescence at 14 h, but induced it at 24 h ($P < 0.05$) (Fig. 5c; lanes 2 and 6). The inducing effect of KCl was promoted by the addition of $MgCl_2$ at both 14 and 24 h ($P < 0.05$) (Fig. 5c; lanes 3 and 5). In contrast, the addition of $CaCl_2$ did not promote the effect of KCl at 14 h, but promoted at 24 h ($P < 0.05$) (Fig. 5c; lane 4). The inducing effect of $NaHCO_3$ was similarly activated by the addition of $MgCl_2$, but was inhibited by $CaCl_2$ ($P < 0.05$) (Fig. 5c; lanes 7, 8, and 9). In the presence of both KCl and $NaHCO_3$, the luminescence intensity was increased by the addition of $MgCl_2$, but was decreased by $CaCl_2$ at both 14 and 24 h ($P < 0.05$) (Fig. 5c; lanes 10, 11, and 12), indicating that the thiosulfate-dependent luminescence induced by potassium is repressed by $NaHCO_3$, and is mainly activated by a bicarbonate-involving pathway.

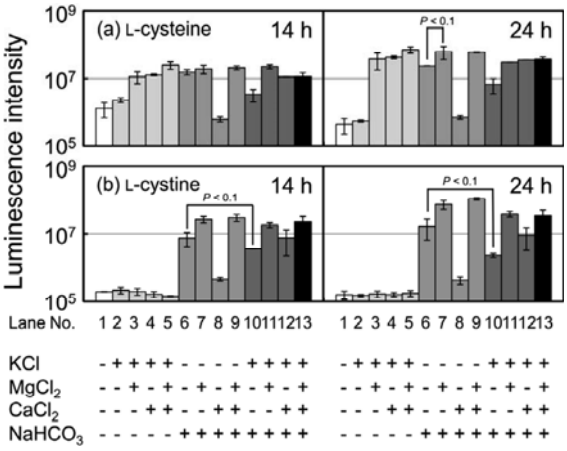

Figure 6. Luminescence intensity of *V. fischeri* in various media with different combinations of components at 14 and 24 h. (a) L-cysteine or (b) L-cystine was added into the media as a sulfur source. The precise composition of the media is described in the Materials and Methods section. The white bars indicate the luminescence intensity in 2.81% NaCl media, whereas the black bars indicate the same but in ASW medium in which $MgSO_4 \cdot 7\ H_2O$ was substituted with L-cysteine or L-cystine. The light gray, gray, and dark gray bars indicate the presence of KCl, $NaHCO_3$, and KCl/$NaHCO_3$, respectively. The presence and absence of the components in the medium are indicated as "+" and "–," respectively. Experiments were performed in triplicate; the height of the bars indicates the mean and the error bars represent the standard deviation.

Effects of potassium, bicarbonate, magnesium, and calcium on the induction of organic sulfur-dependent luminescence in *V. fischeri*

To elucidate the relationships between potassium, bicarbonate, magnesium, and calcium in the induction of organic sulfur-dependent luminescence, we measured the luminescence intensities of these components in various combinations after 14 and 24 h (Fig. 6). In the presence of L-cysteine or its oxidized form (L-cystine) alone, high luminescence intensity was not induced (Fig. 6a and b; lane 1). However, high luminescence intensity was observed in the presence of KCl, $MgCl_2$, $CaCl_2$, and $NaHCO_3$ (Fig. 6a and b; lane 13).

When L-cysteine was used as the sole sulfur source, the addition of KCl did not affect the induction of luminescence (Fig. 6a; lane 2). On the other hand, the addition of $MgCl_2$ and/or $CaCl_2$ promoted the positive effect of KCl and induced organic sulfur-dependent luminescence at both 14 and 24 h ($P < 0.05$) (Fig. 6a; lanes 3, 4, and 5). The high luminescence intensity induced in the presence of $NaHCO_3$ was slightly increased by $MgCl_2$ at 24 h ($P < 0.1$), but was decreased by $CaCl_2$ at both 14 and 24 h ($P < 0.05$) (Fig. 6a; lanes 6, 7, 8, and 9). In the presence of both KCl and $NaHCO_3$, the inducing effect of $NaHCO_3$ was inhibited by the addition of KCl, but the inhibitory effect of KCl was dissolved by $MgCl_2$ or $CaCl_2$ ($P < 0.05$) (Fig. 6a; lanes 6, 10, 11, and 12), which indicates that L-cysteine-dependent luminescence was mostly activated by a potassium-involving pathway in the presence of both KCl and $NaHCO_3$.

When L-cystine was used as the sole sulfur source, the addition of KCl had no effect on the induction of luminescence (Fig. 6b; lane 2). The positive effect of KCl was not observed by the addition of $MgCl_2$ and/or $CaCl_2$ (Fig. 6b; lanes 3, 4, and 5). The L-cystine-dependent luminescence induced by $NaHCO_3$ was increased in the presence of $MgCl_2$, but was decreased in the presence of $CaCl_2$ ($P < 0.05$) (Fig. 6b; lanes 6, 7, 8, and 9). In the presence of both KCl and $NaHCO_3$, the luminescence induced by $NaHCO_3$ was slightly decreased by KCl ($P < 0.1$) (Fig. 6b; lanes 6 and 10), but the inhibitory effect of KCl was overcome by the addition of $MgCl_2$ (Fig. 6b; lanes 10 and 11). Moreover, the inhibitory effect of $CaCl_2$ was suppressed by KCl, and its luminescence was induced ($P < 0.05$) (Fig. 6b; lanes 8 and 12). These results indicate that L-cystine-dependent luminescence was mainly activated by a bicarbonate-involving pathway, but not by a potassium-involving pathway under nutrient-starved conditions.

Discussion

The present screening study showed that, under inorganic sulfur-supplemented conditions, the addition of various bicarbonates, carbonates, or halogenated potassium compounds were effective for inducting sulfur-dependent luminescence. Under organic sulfur-supplemented conditions, the addition of various bicarbonates or carbonates was effective for the induction of luminescence, whereas the addition of potassium was not. Furthermore, our results indicate that the effects of potassium, bicarbonate, magnesium, and calcium on the inorganic sulfur-dependent luminescence were appeared to be changed between 14 and 24 h (Fig. 7), but that on the organic sulfur-dependent luminescence were not changed between 14 and 24 h (Fig. 8).

For several luminescent bacteria, including *V. fischeri*, *V. harveyi*, and *Photobacterium phosphoreum*, an association between their luminescence and the respiratory system has been extensively documented [24, 25]. It has been reported that marine bacteria possess a Na^+-dependent respiratory chain, in addition to an H^+-dependent respiratory chain [26–29]. Previously, it was demonstrated that there is a close correlation between luminescence and Na^+-dependent respiratory activity in *V. harveyi* [30, 31]. In our study, in spite of the fact that enough sodium ions were contained in all test conditions (all media contained 2.81 g/l of NaCl), the sulfur-dependent luminescence was not induced by the addition of a sulfur source alone, but instead required the additional supply of bicarbonate compounds. These results suggest that bicarbonates have an auxiliary function for the activation of a Na^+-dependent respiratory reaction and for the induction of sulfur-dependent luminescence under nutrient-starved conditions.

Sequencing of many bacterial genomes has revealed that some species possess genes that encode the SulP family of proteins. One SulP subfamily includes transporters fused to homologs of carbonic anhydrase, which suggests that these chimeric proteins function in bicarbonate and/or carbonate transport. Some SulP proteins possess putative Na^+/H^+ antiporter or Na^+/bicarbonate symporter domains. It has been shown that one of the distant SulP homologs identified in marine cyanobacteria is a Na^+/bicarbonate symporter [32, 33]. Although these SulP family proteins have not yet been identified in *V. fischeri*, it has been speculated that a putative Na^+/bicarbonate symporter, like that of SulP, is

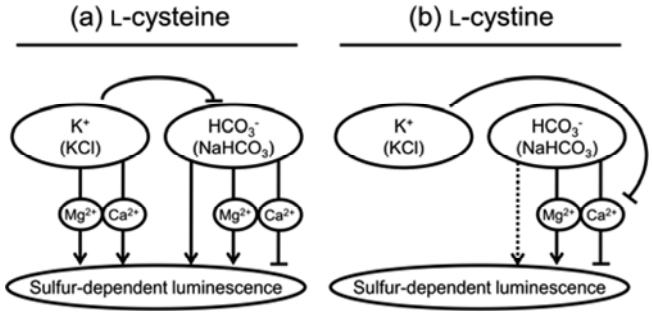

Figure 8. Conceptual model for the relationships between bicarbonate, potassium, magnesium, and calcium and organic sulfur-dependent induction of luminescence in *V. fischeri*. Arrows indicate positive inducing effects, and bars indicate negative inhibitory effects. Broken lines indicate weak inducing effects. See the Results and Discussion sections for further explanation.

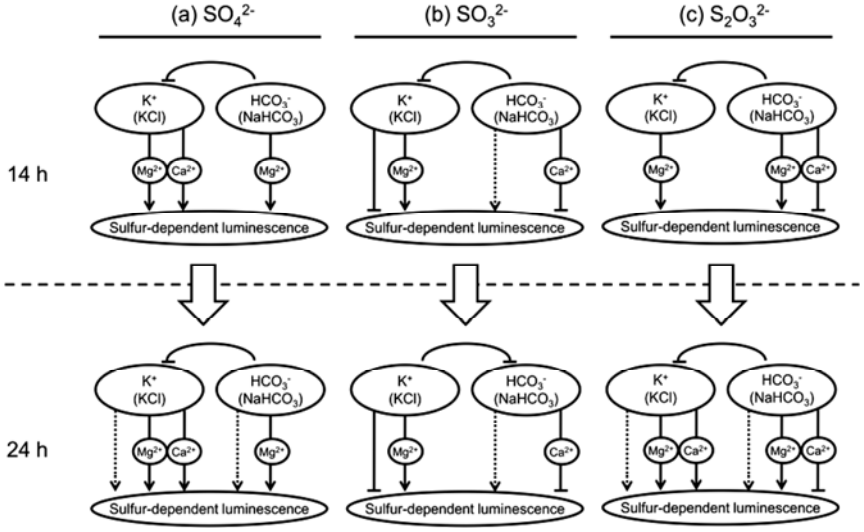

Figure 7. Conceptual model for the relationships between bicarbonate, potassium, magnesium, and calcium and inorganic sulfur-dependent induction of luminescence in *V. fischeri* at 14 and 24 h. Arrows indicate positive inducing effects, and bars indicate negative inhibitory effects. Broken lines indicate weak inducing effects. See the Results and Discussion sections for further explanation.

activated under bicarbonate-supplemented conditions, and that Na^+ is transported along with bicarbonate into the cell. Subsequently, incorporated Na^+ would activate the Na^+-dependent respiratory chain and its activation might subsequently lead to the induction of sulfur-dependent luminescence in *V. fischeri*.

Potassium also had a positive effect on the induction of sulfur-dependent luminescence when sulfate or thiosulfate was supplied as a sulfur source (Figs. 3, 5, and 7). However, this positive effect was not observed in organic sulfur-supplemented conditions (Figs. 6 and 8). This stimulatory effect of potassium is in accordance with the idea that the osmolarity of a medium has a positive effect on luminescence (mediated by accumulation of intracellular K^+), which plays a key role in osmoregulation and simultaneously increases the content of long-chain aliphatic aldehydes in cells [34]. A high K^+ concentration is important for the transcription of luminescence genes in *P. phosphoreum*, and can be achieved by a high concentration of Na^+ passing through the Na^+/K^+-pump [34, 35]. In this study, although we did not investigate the content of intracellular long-chain aliphatic aldehydes or the transcription of luminescence genes, we speculate that the intracellular long-chain aldehydes and luminescent genes required for the induction of sulfur-dependent luminescence may be able to reach optimal values upon the addition of potassium.

Under nutrient-starved conditions, the properties of the outer membrane of gram-negative bacteria are modified because of the changes in the extracellular proteins and the synthesis of polysaccharides [36, 37]. Several studies have indicated that magnesium is a vital biological divalent cation (Mg^{2+}) functioning both as a cofactor and regulator of numerous proteins and as a regulatory and stabilizing factor for membranes and other cellular structures [38, 39]. Indeed, it has been reported that Mg^{2+} has a stabilizing effect on the outer membrane of gram-negative bacteria, including *V. fischeri* [22]. In this study, magnesium had a positive effect on the sulfur-dependent luminescence that was induced by the addition of bicarbonate or potassium under nutrient-starved conditions (Figs. 5–8). Although the detailed mechanisms responsible for this finding are unclear, our results suggest that sulfur-dependent luminescence under nutrient-starved conditions requires outer membrane stabilization, and that Mg^{2+} might play a key role in *V. fischeri*.

Both theoretical calculations and experimental measurements suggest that perhaps 10% of the energy expended by bright cells is devoted to luminescence [40]. This energetic cost suggests that activity of luminescence system plays an important role in the physiology of luminous bacteria. One possibility is that the luminescence systems arose evolutionarily as a detoxification [41]. A related possibility is that bacterial luminescence may stimulate DNA repair [13, 42, 43]. Since the induction of high luminescence intensity in *V. fischeri* requires low pH value [44], these evolutionary advantageous functions might not be significant for *V. fischeri* living at high pH conditions. However, in this study, we demonstrated that sulfur-dependent luminescence can be induced by KCl and $NaHCO_3$. Since the pH values of KCl- and $NaHCO_3$-supplemented media were approximately 5.5 and 8.5 respectively, the sulfur-dependent luminescence was occurred at wide pH range conditions. As a result, the evolutionary advantages of inducible luminescence in bacteria may apply to *V. fischeri* living in various environments.

The detailed molecular mechanisms of the interactions between sulfur-dependent luminescence and bicarbonate/carbonate, potassium, or magnesium in *V. fischeri* remain unclear. Further studies are necessary to elucidate the molecular mechanism(s) of this phenomenon, which will provide further insight into the control of bacterial luminescence and, potentially, an increased understanding of the apparently atypical induction of luminescence in this species. Moreover, these results should be useful in guiding future research efforts that involve bioluminescence and related marine organisms.

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