Sensitive Detection of Bacteria and Spores Using a Portable Bioluminescence ATP Measurement Assay System Distinguishing from White Powder Materials

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The CheckLite™ 250 Plus portable bioluminescence assay system for measuring bacterial adenosine 5'-triphosphate (ATP) was investigated for its performance with respect to the field detection of bacteria in bioterrorism incidents. Vegetative bacteria, *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus*, and *Yersinia pseudotuberculosis* gave considerably high luminescence in a dose–dependent manner with responses of 10^4–10^5 relative light units (RLU) per 10^7 cells, whereas spore forms of *B. subtilis* showed considerably low luminescence with 214 RLU per 10^9 spores. Typical white powder materials such as wheat flour, sugar, and bovine serum albumin at concentrations of 0.1 and 0.5% (w/v) gave only negligible luminescence (lower than 400 RLU), and did not change the luminescence of *E. coli* cells significantly (42–102% of the control). The luminescence of *B. subtilis* spores increased considerably to over 10^4 RLU per 10^7 spores by pretreatment consisting of 37°C incubation for 30 min in nutrient broth medium containing 4 mM L-alanine. The increased luminescence by this pretreatment was not changed considerably (42–145% of the control) in the presence of the above tested white powder materials (0.1 and 0.5%).

Key words —— bioluminescence, adenosine 5'-triphosphate, bacteria, spore, bioterrorism, on-site detection

INTRODUCTION

The anthrax attack via postal letters that occurred in the United States in the autumn of 2001[1)] exposed us to the great threat of bioterrorism. Anthrax, Brucellosis, Plague, Q fever, Tularemia, Smallpox, Viral encephalitides, Viral hemorrhagic fevers, Botulinum toxin, and Staphylococcal enterotoxin B are typical biological warfare agents,[2)] and do not manifest diseases immediately after dispersion. Governments are dealing with bioterrorism incidents using surveillance to prevent infectious diseases. In contrast, on-site detection of causative biological agents can contribute to the early diagnosis and medical treatment before the outbreak of an infectious disease. After the 2001 anthrax attack, many incidents and disturbances relating to "white powder-like material" occurred.[3)] In such cases, first responders (police mobile teams or fire department) rush to the on-sites, and suspicious materials such as white powders are sampled to send to local public health research institutes for laboratory tests to identify *Bacillus anthracis* and other biological warfare agents. It is necessary to differentiate bioterrorism-probable samples from unrelated samples by on-site screening procedures. First, is the on-site sample organic matter (mainly proteinous compounds)? Secondly, does the sample contain living matter (metabolically active bacteria)? And finally, does the sample contain biohazardous bacteria? Several types of biological warfare agent detection equipment for field usage have been developed,[4)] and some are now commercially available.[5)]

In order to detect bacteria in samples, various kinds of technologies have been designed and adopted.[6)] Flow cytometry is used to detect and characterize bacteria,[7)] and our laboratory has developed

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on-site detection methods using a portable apparatus named "MICROCYTE."[8] A bioluminescence technique using adenosine 5'-triphosphate (ATP) assay with a luciferin-luciferase system is one sensitive method, and is widely used in food hygiene tests to check bacterial contamination of food and the sanitary condition of cooking materials.[9–11] One commercially available bioluminescence kit that is simple, reliable, and sensitive is the ATP measurement kit CheckLite™,[12,13] which uses a thermostable luciferase.[14] The kit is portable and its procedure is easy and simple. The reagent solutions are serially added to a sample solution in a test tube and the luminescence is measured by inserting the tube into the portable luminometer. It takes about 1 min for the bioluminescence assay and about 30 min for the specialized assay that uses pretreatment of eliminating extracellular ATP using CheckLite™ ATP Eliminating kit. According to the manufacturer, the 5 min incubation for the pretreatment using the ATP eliminating kit is sufficient to eliminate most extracellular ATP, and so within 10 min it is possible to obtain quantitative data for bacterial ATP. In this paper, we have investigated the performance of the CheckLite™ 250 Plus kit (Kikkoman Corp., Tokyo, Japan) for sensitively detecting bacteria by distinguishing from white powder-like materials, and also developed a pretreatment method for detecting spore forms of bacteria.

MATERIALS AND METHODS

Chemicals —— The CheckLite™ 250 Plus kit and CheckLite™ ATP Eliminating kit were provided by Kikkoman Corp. Crystallized adenosine ATP disodium salt trihydrate and lysozyme from chicken egg white (biochemical reagent grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Albumin from bovine serum (fraction V powder, BSA) was obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MI, U.S.A.). Commercially available Nisshin flour Hakuriki (Nisshin Food Co., Tokyo, Japan) and Johakutou (Mitsui Seito Co., Tokyo, Japan) were used as wheat flour and sugar, respectively. The other chemicals used were analytical reagent grade.

Bacterial Growth and Spore Preparation —— The *Escherichia coli* (*E. coli*) KB759 (HB101), *Staphylococcus aureus* KB371 (ATCC 10832), and *Yersinia pseudotuberculosis* KB686 (serotype 4b) were kindly provided by Dr. Kazuyoshi Kawahara of The Kitasato Institute (Tokyo, Japan). The *Bacillus subtilis* (*B. subtilis*) 168 strain was kindly provided by Prof. Naoki Ogasawara of the Nara Institute of Science and Technology (Nara, Japan). The bacteria were grown in liquid medium of nutrient broth (8 g/l, Eiken Co., Tokyo, Japan) shaken overnight at 30°C and harvested by centrifugation. The pellets were dissolved in sterilized distilled water used for the bioluminescence assay.

The spores of *B. subtilis* were prepared according to the method of Zhou *et al*.[15] Briefly, the *B. subtilis* colony was grown on a agar plate [Nutrient Agar Nissui (Nissui Seiyaku Co., Tokyo, Japan), 35 g/l], suspended in Scheffer's sporulation medium, spread onto Scheffer's sporulation medium agar plates, and incubated at 37°C overnight. Spores were harvested using phosphate buffered saline (PBS), and washed with PBS, and treated with lysozyme [10 mg/ml 50 mM Tris•HCl pH 8.0, at 20°C for 0.5–1 hr with shaking (100 rpm)]. The cells were washed with sterilized water and then the pellets were vacuum dried and stored in closed glass containers in a cool, dark room.

The cell number of the bacterial solution was measured by the plate count method, and is represented as colony forming units (cfu).

Measurement of Luminescence of Samples —— The amount of ATP was measured using a CheckLite™ 250 Plus kit according to the manufacturer's manual. Briefly, 0.1 ml of sample diluted in sterilized distilled water was mixed with 0.1 ml of "ATP releasing reagent" solution. Twenty sec later, 0.1 ml of "Luciferin-Luciferase reagent" solution was added to the mixture, and the luminescence was immediately measured with a Lumitester C-100 [Kikkoman Corp., 18.5 (W) × 11 (D) × 7.5 (H) cm, 700 g, with built-in rechargeable battery].

As the ATP eliminating pretreatment, 1 ml of the sample solution was mixed with 0.1 ml of the CheckLite™ ATP Eliminating kit solution to remove the extracellular ATP. Thirty minutes later, 0.1 ml of the aliquot was subjected to the above bioluminescence assay using a CheckLite™ 250 Plus kit.

As the spore germination pretreatment, 0.1 ml of the spore solution was mixed with 0.9 ml of the germination medium (8 g/l nutrient broth medium and or 4 mM L-alanine) and incubated for the appropriate time. Then, 0.1 ml of the aliquot was subjected to the above bioluminescence assay using a CheckLite™ 250 Plus kit.

RESULTS AND DISCUSSION

Response of Bacterial Samples toward the Bioluminescence Assay System

Standard ATP solution showed concentration-dependent luminescence, where the calibration curve was linear for concentrations ranging from 0.1 to 100 nM ($\gamma = 0.9998$). One nM ATP gave 2893 ± 223 relative light unit (RLU) (average ± S.D., $n = 3$), a level which is compatible with the manufacturer's report.[13] The background level (sterilized distilled water) was under 100 RLU.

Serially-diluted bacterial solutions were examined by the CheckLite™ 250 Plus assay system. As shown in Fig. 1, the *E. coli* KB759 showed dose–dependent luminescence, and the detection limit was estimated to be about 10^4 cfu/ml. In the manufacturer's protocol, pretreatment using a CheckLite™ ATP Eliminating kit is performed to remove extracellular free ATP in various food samples, which enables the measurement of actual ATP content in bacteria.[12] Pretreatment using a CheckLite™ ATP Eliminating kit decreased the luminescence intensity of the *E. coli* KB759 cells (10^7 cfu/ml) to about the 60% level, suggesting that even freshly harvested bacterial culture samples contain high levels of free ATP, which is partly derived from dead and dying vegetative bacteria. The intra-day repeatability was 6.6% and 3.8% ($n = 3$, 10^7 cfu) without and with pretreatment using a CheckLite™ ATP Eliminating kit, respectively. The inter-day repeatability was 28.3% ($n = 3$, 10^7 cfu) without pretreatment using a CheckLite™ ATP Eliminating kit.

The *B. subtilis* 168, which was immediately subjected to the bioluminescence assay just after harvesting the culture on agar plates, also showed dose–dependent luminescence, which was stronger than that of *E. coli* KB759, and the slightly higher ATP content of *Bacillus* strain compared to that of *E. coli* is in agreement with the data of Honma.[13] In contrast, the spore forms of *B. subtilis* 168 showed considerably weak luminescence that was over one thousand-fold weaker than that of the vegetative forms. In addition, vegetative *B. subtilis* culture, which was left in sterilized water for a few hours after harvest, showed weak luminescence that was 10 to 20-fold lower than that of the freshly harvested bacteria. It is probable that the vegetative cells of *B. subtilis* instantly raise sporulation or die after the exponential proliferation,[16] and in such a physiological state cellular metabolic activity is depressed and the intracellular ATP level is reduced.

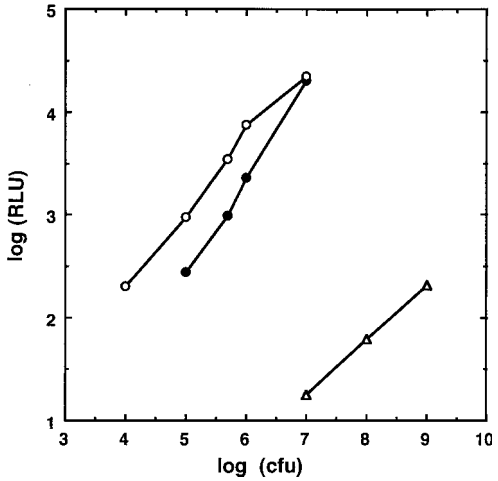

Fig. 1. The Luminescence Intensity of Bacteria Using the CheckLite™ 250 Plus

E. coli: closed circle; *B. subtilis* vegetative forms: open circle; *B. subtilis* spore forms: open triangle.

The *Staphylococcus aureus* KB371 and *Yersinia pseudotuberculosis* KB686 exhibited about 2.3-fold higher and 2.7-fold lower luminescence per 10^7 cells, respectively, than *E. coli* KB759. The detection limit of the CheckLite™ 250 Plus assay system for vegetative bacterial cells is so estimated to be around 10^4 cfu/ml, and this sensitivity is sufficiently high among bacterial detection techniques with a reasonably rapid analysis time (within one hour), except for fluorimetric immunoassay.[6]

Effects of White Powder-Like Materials on Luminescence Intensity

Typical white powder-like materials were examined with respect to their effects on the response of the bioluminescence assay system and their interference with the bacterial response. Wheat flour, which is composed of mainly starch and some proteins, gave 335 RLU in 0.5% solution, and 154 RLU after pretreatment using the CheckLite™ ATP Eliminating kit. Sugar (edible sucrose) gave 277 RLU in 0.5% solution. BSA (purified serum protein) gave 378 RLU in 0.5% solution. These rather moderate levels of luminescence intensity obtained by the white power samples may correspond to the practical background level of the field samples as the detection limit.

The white powder like materials were added to the bacterial solution and the bioluminescence assay was conducted. As shown in Table 1, the addition of wheat flour (0.1% and 0.5%) decreased the luminescence intensity of the *E. coli* KB759 cells

Table 1. Effect of White Powder-Like Materials on the Luminescence Intensity of *E. coli* Vegetative Cells

Additive	Wheat flour	Sugar	Sodium chloride	Bovine serum albumin
0.1%	87.0%	90.2%	68.0%	90.6%
0.5%	83.9%	102.3%	42.0%	68.3%
2.0%	—*a)*	144.2%	—	25.1%

The vegetative cells of *E. coli* (10^7 cfu/ml) dissolved in sterilized water containing additive (final concentration: 0, 0.1, 0.5, 2.0%) were assayed for bioluminescence using the CheckLite™ 250 Plus kit. The values are the percent bioluminescence in the presence of additive compared to the control (no additive), and represent an average of two determinations. *a*) not determined.

(10^7 cfu/ml) slightly (87% and 84% of the control value). The addition of sugar did not change the *E. coli* luminescence intensity although a high level of sugar (2%) increased the intensity to 144%. The addition of sodium chloride (NaCl) and BSA decreased the *E. coli* luminescence intensity in a dose–dependent manner. However, the extent of the decreased level should never prevent the detection of bacteria in real samples. It is not clear whether this interference of the bioluminescence by additives is attributable to decreased luminescence of ATP itself or decreased ATP extraction from bacteria. Suppressed absorbance of the luminescence light should be also considered because our experimental results showed that the addition of wheat flour (0.1, 0.5, 2.0%) turned the test solution turbid and decreased the luminescence of standard ATP solution (20 nM) in this assay kit to 40%, 11%, and 7.5%, respectively. The other cause of the decreased bioluminescence can be ascribed to the inhibition of luciferase activity. According to the technical specifications for the CheckLite™ kit (1999), high levels of other inorganic salts can suppress the luminescence of ATP depending on the species of the additives. Our preliminary experiment also showed that the luminescence of the standard ATP solution (20 nM) in this assay kit was decreased to 65% and 23% in the presence of 0.5% and 2.0% sodium chloride, respectively. On the other hand, the luminescence of standard ATP solution (20 nM) increased to 120–150% in the presence of 0.2–2.0% sugar or BSA, indicating slight activation of ATP luminescence (or luciferase activity) by sugar or BSA. The considerable decrease in *E. coli* luminescence intensity (Table 1) in the presence of BSA may be attributed to the suppression of ATP release from bacteria with ATP releasing reagent by BSA. Therefore, possible suppression of the bioluminescence by high levels of matrix components should be considered when using the assay to test real world samples, and it is recommended that turbid or viscous samples be diluted before assaying.

Improvement of Luminescence of *B. subtilis* Spores

As shown in Fig. 1, it is impossible to detect the spore forms of *B. subtilis* by the bioluminescence ATP assay system, even at high spore concentrations. The reason why the spore forms gave negligible levels of luminescence compared to the vegetative forms can be attributed to the following two factors. The spore forms possess a low quantity of intracellular ATP because of their dormant nature, and ATP can not be sufficiently extracted from the spores during the assay procedure because of their mechanical resistance properties. It is known that spores germinate rapidly in response to changes in their external environment, such as the presence of trigger molecules and growing conditions.[16] It is also believed that spores produce amounts of ATP that correspond to the elevated intracellular metabolic activity during germination. We incubated spores in nutrient broth medium containing L-alanine as the germination trigger.[17] The spores were incubated in the germination medium and after an appropriate time interval the samples were assayed. As shown in Fig. 2, by 37°C incubation in water or L-alanine solution, the spores never increased the bioluminescence. In contrast, the luminescence intensity increased dramatically by incubation in nutrient broth with an increased incubation time. After 10 min, the luminescence surpassed the background level. Incubation at 37°C exhibited higher luminescence intensity than that at 25°C. Shaking during incubation did not increase the luminescence intensity, indicating aeration had little effect. The addition of L-alanine slightly increased the intensity. After 37°C incubation for 30 min and 25°C incubation for 60 min, the luminescence intensity almost reached the value obtained by the vegetative cells (~10^4 RLU). Under the pretreatment conditions (37°C, shaking, nutrient broth, 4 mM L-alanine), the luminescence in-

tensity was increased to about 1.5-fold the background level (nutrient broth, 4 mM L-alanine) with 30 min incubation of the spores (10^5 cfu/ml) and to about 2.4 fold with 10 min incubation of the spores (10^6 cfu/ml).

As shown in Table 2, the inclusion of wheat flour (0.1, 0.5%) decreased the luminescence intensity of the spores slightly (65%, 53% of the control), which can be attributed to the suppression of ATP luminescence in turbid wheat flour solution. The inclusion of sugar increased the spore luminescence intensity in a dose–dependent manner. Sugar may promote spore germination or bacterial ATP production. The decreased luminescence intensity in the presence of NaCl may be due to the inhibition of luciferase activity by NaCl. Similar to the result in *E. coli* (Table 1), the inclusion of BSA decreased the luminescence intensity to about 60%. However, the extent of the altered fluorescence intensity level in the presence of these additives should not prevent the detection of the spores.

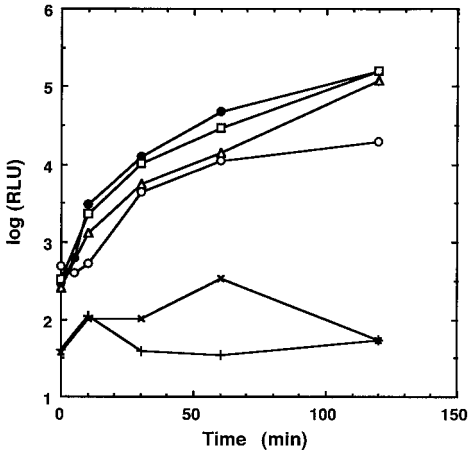

Fig. 2. Effect of Incubation on Luminescence Intensity of *B. subtilis* Spores

The spores of *B. subtilis* (10^7 cfu/ml) were incubated in nutrient broth medium (8 g/l) containing 4 mM L-alanine without shaking at 25°C (open circle) or 37°C (closed circle), for the appropriate time interval. They were also incubated with shaking at 37°C in water (cross), 4 mM L-alanine solution (saltire), nutrient broth (8 g/l, open triangle) or nutrient broth containing 4 mM L-alanine (open square). The samples were then assayed for bioluminescence using the CheckLite™ 250 Plus kit.

The effect of incubation in nutrient broth was also examined for the luminescence intensity of the vegetative *B. subtilis* cells which were left for a few hours in sterilized water after harvesting the culture and gave significantly decreased luminescence intensity compared to that of the freshly harvested cells. As shown in Fig. 3, the luminescence intensity did not increase by incubation in water or L-alanine solution. In contrast, by incubation in nutrient broth, the luminescence intensity increased from 2600 to 16100 RLU within the initial 10 min. The addition of L-alanine did not change the intensity. The luminescence level after 10 min incubation was almost equal to that of the freshly harvested cells. During the incubation for 60 min, the luminescence intensity did not so increase, which tendency was in contrast to that of the spores (Fig. 2).

Stopa *et al.* validated the New Horizons Diagnostics Model 3550 luminometer system for bacterial detection in aerosol samples.[18] The kit adopted a special protocol that differentiated between bacterial and nonbacterial sources of ATP. The detection limit was about 10^5 cfu/ml, and incubation for 5 min in trypticase soy broth enabled the detection of 10^6 cfu/ml *Bacillus* spores. Compared to the result of Stopa *et al.*, the system examined in the present paper yielded more sensitive detection of vegetative cells (10^4 cfu/ml) and also compatible detection sensitivity for spores (10^5–10^6 cfu/ml) after 30 min incubation in nutrient broth including 4 mM L-alanine.

Field Utilization of the Bioluminescence System in Bioterrorism Incidents

Using the bioluminescence ATP measurement assay system it is possible to detect low level of bac-

Table 2. Effect of White Powder-Like Materials on the Luminescence Intensity of Germinating *B. subtilis* Spores

Additive	Wheat flour	Sugar	Sodium chloride	Bovine serum albumin
0.1%	65.2%	121.6%	63.1%	63.4%
0.5%	52.9%	144.6%	41.7%	64.4%
2.0%	—[a)]	232.9%	—	60.0%

The spores of *B. subtilis* (10^7 cfu/ml) were incubated in nutrient broth medium (8 g/l) containing 4 mM L-alanine and additive (final concentration: 0, 0.1, 0.5, 2.0%) with shaking at 37°C for 30 min, and then the incubation solutions were assayed for bioluminescence using the CheckLite™ 250 Plus kit. The values are the percent bioluminescence in the presence of additive compared to the control (no additive), and represent an average of two determinations. *a*) not determined.

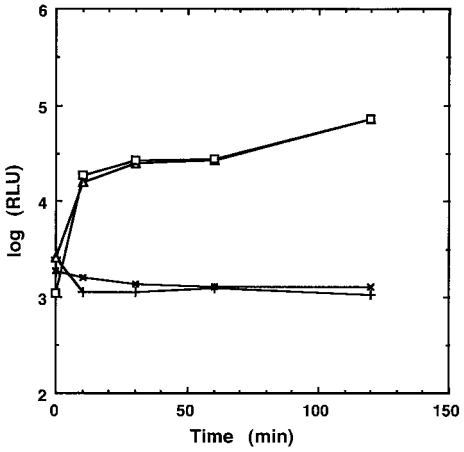

Fig. 3. Effect of Incubation on Luminescence Intensity of *B. subtilis* Vegetative Cells

The vegetative cells of *B. subtilis* were left for a few hours in sterilized water after harvesting and then incubated (10^7 cfu/ml) in water (cross), 4 mM L-alanine solution (saltire), nutrient broth media (8 g/l) without (open triangle) or with (open square) 4 mM L-alanine with shaking at 37°C for the appropriate time interval, and assayed for bioluminescence using the CheckLite™ 250 Plus kit.

teria (10^4 cfu/ml) even in crude matrix such as white powders, and *B. subtilis* spores (10^5 cfu/ml) can also be detected after pretreatment consisting of 30 min incubation in nutrient broth medium containing 4 mM L-alanine. Considering the cell number per wet volume (8–130 × 10^{10} cells/g)],[8] bacteria in white powder are assumed to be detected at least in the sample level of 0.1% (w/w), where about 10 mg on-site white power samples which is dissolved in 10 ml of sterilized water for testing. Bacterial spores can also be detected at a similar cell content level after the germination pretreatment. As the preliminary recommended field protocol for detecting bacteria, first, the target white powder is dissolved in sterilized water to about 0.1% and then assayed using the CheckLite™ 250 Plus kit. If the response is initially negative, incubation of the sample in nutrient broth containing 4 mM L-alanine at 37°C for 30 min is conducted, where a subsequent negative response means "no bacteria" and a positive response means "spore contamination." Second, if, the response of the initial test is positive, pretreatment using the CheckLite™ ATP Eliminating kit for 5 min is conducted, where a subsequent negative response means "no bacteria" and a positive response means "bacterial contamination." The construction of a detailed detection protocol is currently under consideration.

In addition, the bioluminescence ATP assay system can be used to ascertain the degree of sterilization of on-site equipment of first responders after the termination of on-site work in bioterrorism incidents.

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