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Exponential ATP amplification through simultaneous regeneration from AMP and pyrophosphate for luminescence detection of bacteria

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ABSTRACT

Bacteria monitoring is essential for many industrial manufacturing processes, particularly those involving in food, biopharmaceuticals, and semiconductor production. Firefly luciferase ATP luminescence assay is a rapid and simple bacteria detection method. However, the detection limit of this assay for *Escherichia coli* is approximately 10^4 colony-forming units (CFU), which is insufficient for many applications. This study aims to improve the assay sensitivity by simultaneous conversion of PP_i and AMP, two products of the luciferase reaction, back to ATP to form two chain-reaction loops. Because each consumed ATP continuously produces two new ATP molecules, this approach can achieve exponential amplification of ATP. Two consecutive enzyme reactions were employed to regenerate AMP into ATP: adenylate kinase converting AMP into ADP using UTP as the energy source, and acetate kinase catalyzing acetyl phosphate and ADP into ATP. The PP_i-recycling loop was completed using ATP sulfurylase and adenosine 5′ phosphosulfate. The modification maintains good quantification linearity in the ATP luminescence assay and greatly increases its bacteria detection sensitivity. This improved method can detect bacteria concentrations of fewer than 10 CFU. This exponential ATP amplification assay will benefit bacteria monitoring in public health and manufacturing processes that require high-quality water.

Bacteria monitoring is essential for many industrial manufacturing processes, and particularly those involving food, semiconductors, and biopharmaceuticals. The presence of bacteria reduces production yield and may cause serious health problems in humans. Researchers have developed several rapid assays for detecting bacteria in water. These methods include polymerase chain reactions, fluorescence *in situ* hybridization [1], β-D-glucuronidase activity measurement [2], and firefly luciferase-based ATP luminescence detection [3].

The ATP luminescence assay is a rapid, sensitive, and easy-to-perform method based on the detection of ATP, a molecule ubiquitously present in all living cells. The enzyme luciferase catalyzes the oxidation of the substrate luciferin while transforming the energy derived from ATP into light, which can be quantified by a luminometer. This assay has been widely used in bacteria monitoring for food hygiene [4] and surface cleanliness [5]. Previous studies demonstrate the successful detection of bacteria, bacterial endospores, yeasts, and fungal spores using this method [6–8]. This assay can also be combined with immunomagnetic separation technology to detect specific pathogenic bacteria [9–11].

The current detection limit of the ATP luminescence method for *Escherichia coli* is approximately 10^4 colony-forming units (CFU)[1] [12,13], which is not sensitive enough for many industrial and medical applications. Several approaches have been adopted to improve the assay sensitivity. The first strategy involves the identification of chemical extractants that can effectively disrupt bacterial cells while not interfering with the luminescence assay. Both dimethyl sulfoxide (DMSO) [7,8] and benzalkonium chloride [11] showed a good activity in releasing ATP from living cells, and can achieve a 10-fold increase in luminescence emission. The second strategy is to improve the efficiency of the firefly luciferase used in the assay. One study used site-directed mutagenesis of *Photinus pyralis* luciferase to produce 10-fold higher luminescence intensity than the wild-type enzyme [14]. Alternative luciferases, including one from firefly *Luciola mingrelica*, have also been tested, although they typically require 6 h of assay time to detect bacteria below 10 CFU [15].

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[1] *Abbreviations used:* ADK, adenylate kinase; AGPPase, ADP-Glc pyrophosphorylase; APS, adenosine 5′ phosphosulfate; CFU, colony-forming units; DMSO, dimethyl sulfoxide; EAA, exponential ATP amplification.

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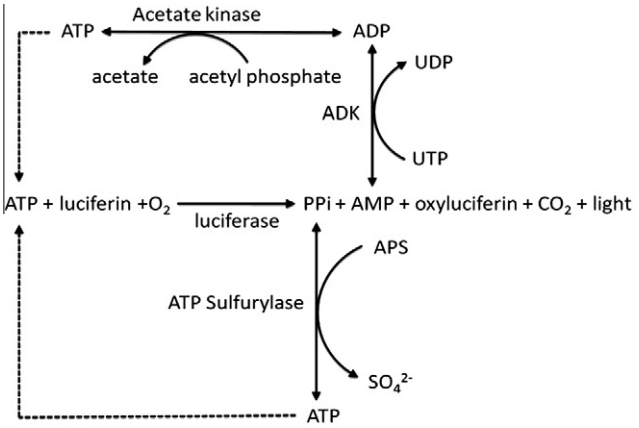

Fig.1. Schematic presentation of the biochemical pathways involved in the exponential ATP amplification system.

The third strategy of enhancing the luminescence assay signal is to divert the luciferase reaction products, either AMP or inorganic pyrophosphate (PP_i), to ATP regeneration. The conversion of AMP into ATP can be achieved through several approaches, including polyphosphate:AMP phosphotransferase and adenylate kinase in the presence of polyphosphates (Fig. 1) [16–18]. Some of these approaches are capable of detecting bacteria at a few CFU levels and reducing the required assay time to approximately 30 min. Recycling PP_i to generate ATP is commonly performed using ATP sulfurylase and its substrate adenosine 5′ phosphosulfate (APS) (Fig. 1) [19]. Our laboratory has shown recently that ADP-Glc pyrophosphorylase (Glc-1-P adenylyltransferase, AGPPase), which catalyzes the formation of ATP and Glc-1-P from ADP-Glc and PP_i, can increase the luminescence signals of luciferase-based assays and showed lower backgrounds than the ATP sulfurylase/APS system [20]. The AGPPase/ADP-Glc system can enhance the bacteria detection sensitivity about one order of magnitude.

The goal of this study is to enhance the sensitivity of the ATP bioluminescence assay and decrease the assay time to less than 1 min for detecting 10 CFU bacteria or below using a common laboratory luminometer. A novel strategy, called exponential ATP amplification (EAA), that significantly improves the sensitivity of the firefly luciferase-based ATP luminescence assay was developed to achieve the goal. This improved assay combines both the AMP- and the PP_i-recycling pathways in a one-tube reaction to amplify ATP exponentially. The proposed assay was applied to detect bacteria, and reliably detected fewer than 10 bacterial cells. The EAA method will find many applications in situations requiring highly sensitive bacteria monitoring.

Materials and methods

Enzymes and chemicals

All the chemicals and enzymes used in this study, including luciferin, nucleotide triphosphates, APS, sodium pyrophosphate, $MgCl_2$, acetyl phosphate, ATP sulfurylase, firefly luciferase, and acetate kinase, were either reagent grade or the highest purity available from Sigma–Aldrich Chemicals (St. Louis, MO). Recombinant ADP-Glc pyrophosphorylase (AGPPase) was overexpressed and purified from *E. coli* as described previously [20].

ATP luciferase assays

The basic ATP luciferase reaction mixture contained 50 mM Tris–HCl (pH 7.6), 3.0 mM $MgCl_2$, 200 μM luciferin, and 5–25 ng/μl firefly luciferase [14] as indicated. To ensure the regeneration of PP_i into ATP, 0.1 μM APS and 5×10^{-5} U ATP sulfurylase were added to the basic luciferase assay mixture. In addition to the basic luciferase and PP_i regeneration reaction components, the standard EAA assay includes 10 μM acetyl phosphate, 1.0 U adenylate kinase, 1.0 U acetate kinase, and 20 μM UTP. The light produced in these reactions was measured using either a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) or a Wallac 1420 VICTOR2 Multilabel Counter (Perkin Elmer, Waltham, MA) as indicated. No ATP was added to the reaction mixture when measuring background luminescence. All luciferase experiments were repeated three times, and data were expressed as mean ± standard deviation.

Detection of ATP from bacteria

The bacterial strains used in this study including *E. coli* BL21, *Pseudomonas aeruginosa* PAO1, and *Bacillus cereus* ATCC 14579 were obtained from the Bioresource Collection and Research Center, Hsin Chu, Taiwan. The bacteria were propagated in Luria–Bertani (LB) broth. Overnight cultures were harvested by centrifugation and serially diluted with M9 minimal medium to 10–10^5 CFU/ml to determine the limit of detection of the assays. M9 minimal medium contains 94.4 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 2.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose. Bacteria cells were disrupted by boiling at 95 °C for 10 min prior to adding the luminescence assay reagents. Unless otherwise indicated, the experiments in this study used a 100-μl bacterial suspension in each reaction. Alternatively, DMSO was added to the bacterial suspension to a final concentration of 10% with or without accompanying 10 min heat treatment in certain cases. The number of bacteria contained in a duplicated fraction was determined by plate counting CFU on LB agar.

Results

Rationale

Recycling an ATP hydrolysis product, either AMP or PP_i, back to ATP can replenish the original ATP pool, and therefore enhance the sensitivity of luminescence detection. If both AMP and PP_i are recycled simultaneously, two molecules of ATP can be generated from every ATP consumed in the luciferase reaction (Fig. 1), thus resulting in an exponential amplification of ATP (EAA) that would greatly improve the detection sensitivity.

Reaction background optimization

A potential problem with the EAA approach is that a large number of reagents must be included, which may create high assay backgrounds. The experiments in this study tested which of the two possible PP_i-recycling reactions, AGPPase/ADP-Glc or ATP sulfurylase/APS, would yield a lower background when combined with the standard AMP-recycling reaction. Combining an AGPPase/ADP system with the AMP-recycling reaction produced a higher background luminescence (Fig. 2). Further testing various combinations of enzyme and substrate indicates that the high background was due to nonspecific reaction of ADP-Glc and ADK (Fig. S1), presumably due to a nucleotide diphosphate contamination in the commercial ADP-Glc preparation. Hence, the subsequent EAA experiments combined the ATP sulfurylase/APS system with the AMP-recycling reaction. In addition, whether the concentration of the enzyme components in the EAA system is optimal was evaluated. By varying the enzyme concentration in the EAA system, it became clear that firefly luciferase (Fig. S2)

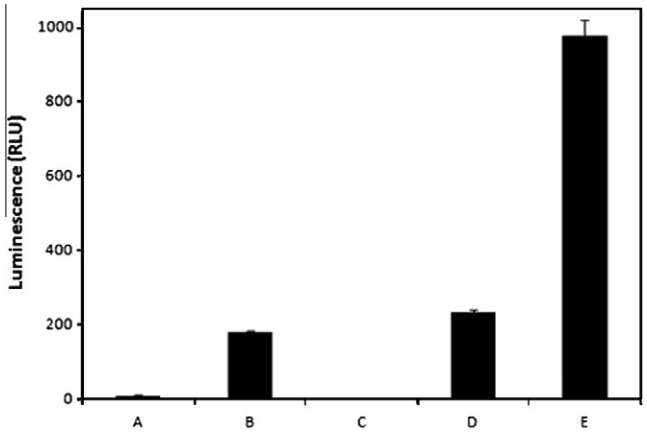

Fig.2. Comparison of background luminescence of AMP and PP_i-recycling reactions, and their combinations in the absence of exogenously added ATP and PP_i. A, Basic AMP-recycling assay; B, ATP sulfurylase/APS PP_i rescycling assay; C, AGPPase/ADPglc PP_i-recycling assay; D, ATP sulfurylase/APS reaction combined with the AMP-recycling assay; E, AGPPase/ADPglc reaction combined with the AMP-recycling assay. The luciferase concentration used in this study was 5 ng/μl. The experiments were repeated three times; data were expressed as mean ± standard deviation. Each of the reactions was recorded for 10 s using a Turner Designs TD-20/20 luminometer.

was the main rate-limiting step. Therefore, the luciferase concentration used in all subsequent experiments was increased to 25 ng/μl, which is the highest quantity the reaction mixture can accommodate.

Comparison of single-pathway ATP regeneration and EAA

This study compares the ATP regeneration efficiency of the EAA approach with that of single-product recycling reactions (Fig. 3). When applied in 1.0 pM ATP measurement, both the AMP and the PP_i-recycling pathways enhanced the luminescence signal approximately 2.5- to 4-fold in a 3-s reaction. On the other hand, when the two reactions were combined and carried out simultaneously in one tube, the signal could be enhanced more than 250-fold, reflecting the effectiveness of the improvement.

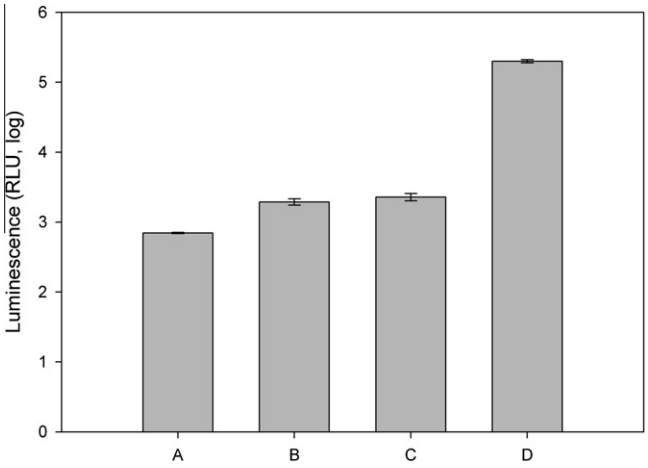

Fig.3. Comparison of EAA with the single-pathway ATP regeneration systems. A, basic luciferase ATP-luminescence reaction; B, ATP regeneration with PP_i recycling using the ATP sulfurylase/APS pathway; C, ATP regeneration using the AMP-recycling pathway; D, combination of both PP_i and AMP-recycling pathways. The ATP concentration used in this experiment was 1.0 pM. The luciferase concentration used in this study was 25 ng/μl. The experiments were repeated three times; data were expressed as mean ± standard deviation. Each of the reactions was measured for 3 s using a Wallac 1420 VICTOR2 Multilabel Counter.

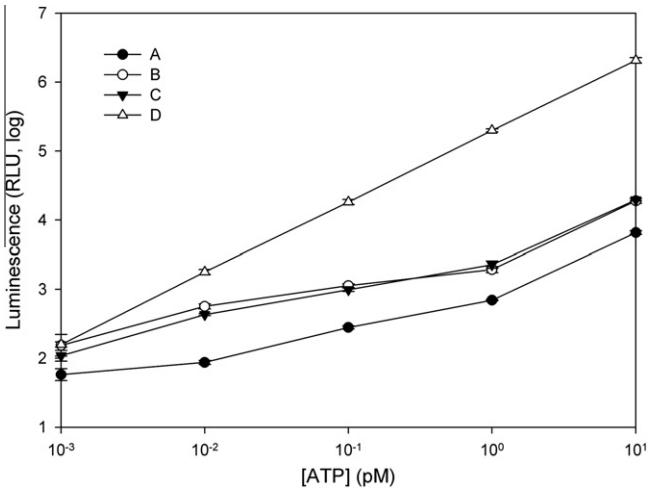

Fig.4. Detection limit of ATP using the exponential ATP amplification assay. Different ATP concentrations were tested at a luciferase concentration of 25 ng/μl, and the emitted luminescence was recorded for 3 s in a Wallac 1420 VICTOR2 Multilabel Counter. The experiments were repeated three times; data were expressed as mean ± standard deviation. A, basic luciferase ATP-luminescence reaction; B, ATP regeneration with PP_i recycling using the ATP sulfurylase/APS pathway; C, ATP regeneration using the AMP-recycling pathway; D, combination of both PP_i and AMP-recycling pathways.

Detection limit of ATP using the EAA assay

This study first tested the detection limit on ATP using the EAA method. While both of the single-product recycling methods could barely detect ATP at 1.0 pM under our standard assay conditions, their integration into the EAA assay allowed 10^{-2} pM ATP to be detected easily, yielding a 100-fold improvement (Fig. 4). In addition, the EAA reaction yielded a good linear dynamic measurement within the range of 10^{-3} pM to 10 pM ATP (Fig. 4)

Bacteria monitoring using the EAA method

This study also examines whether the EAA assay can detect bacteria in water. Two gram-negative bacteria, *Pseudomonas aeruginosa* and *E. coli*, and one Gram-positive bacterium, *Bacillus cereus*, were tested for this purpose. The bacteria were serially diluted and disrupted by boiling, and the ATP and PP_i released were measured by the ATP sulfurylase/APS and EAA assay individually. The EAA method was much more sensitive than sulfurylase/APS-based PP_i recycling alone (Fig. 5) that could reliably differentiate 10 CFU/ml from 100 CFU/ml with approximately 10-fold differences in RLU in all three tested bacterial species. The EAA method also achieved a good linearity in bacteria titers below 10^5 CFU/ml. On the other hand, only approximately 2-fold differences in RLU could be detected between 10 and 100 CFU/ml bacteria by the ATP sulfurylase/APS method.

Previous research shows that DMSO, particularly when combined with heat treatment, is an efficient way to extract ATP from microbial cells for conventional luminescence assay [7,8]. This notion was also tested using EAA as the detection method. No differences appeared between the heat treatment alone and heat combined with DMSO when EAA was used (Fig. S3).

Discussion

This study presents an improved firefly luciferase-based ATP luminescence assay, called EAA, that provides a highly sensitive method for bacteria detection. Unlike other published ATP regeneration systems, which replenish ATP to the original level, this

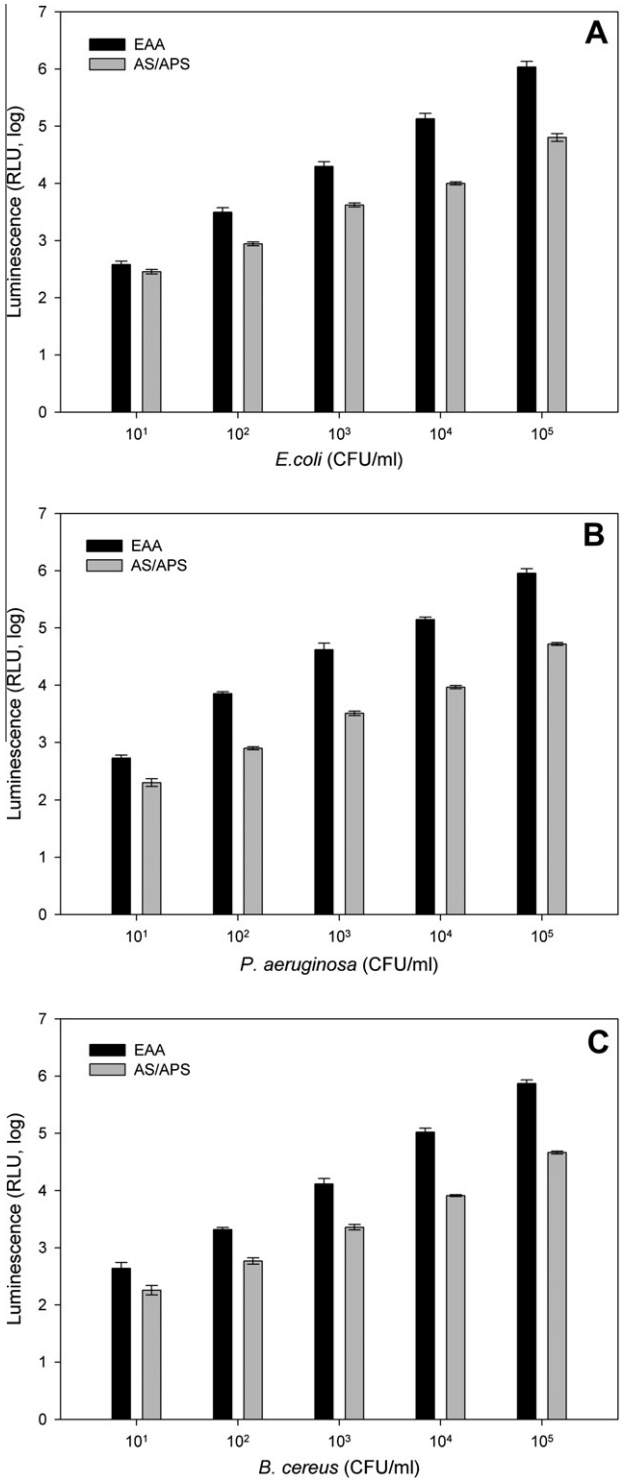

Fig.5. Comparison of the exponential ATP amplification assay with the single-pathway ATP sulfurylase/APS system in bacteria detection. The bacteria used (A) were *E. coli* BL21 (A), *P. aeruginosa* PAO1 (B), and *B. cereus* (C). The emitted luminescence from each of the reactions was recorded for 3 s in a Wallac 1420 VICTOR2 Multilabel Counter. The experiments were repeated three times; data were expressed as mean ± standard deviation.

improved assay recycles both AMP and PP_i. This method achieves exponential amplification of ATP and significantly enhances the luminescence signals. Besides for use in luciferase-based ATP detection, EAA may be applied in enzyme-catalyzed ATP-dependent phosphoryl transfer reactions in preparative organic synthesis [21]. Because EAA recycles both ATP hydrolysis products, it may reduce feedback inhibition of the biosynthetic enzyme system, leading to better yields.

The primary drawback of the EAA assay is that approximately 10 different components must be included in the reaction. This makes the optimization and quality control of the assay more complicated than the conventional luciferase assay. The concentration and activity of each of the components may not be optimal and some of the reagents may produce background luminescence, compete with one another, or inhibit the enzymes used in the reaction. These factors may explain why the EAA reaction does not show an ideal exponential reaction curve. Nevertheless, this study demonstrates that the use of ATP sulfurylase/APS in the EAA reaction could yield reasonably low assay backgrounds. Future research should optimize the EAA reaction by testing different concentrations of each reagent. Identifying the most active enzyme required in the assay will further improve the amplification efficiency of the EAA system.

Because both AMP and PP_i were recycled to regenerate ATP, the EAA assay may not be suitable for quantification of ATP if PP_i is present in the specimen. Nevertheless, the inclusion of PP_i detection in the assay improves bacteria detection, particularly of a pure water system, in which PP_i is primarily of microbial origin. Because of its smaller molecular weight and nonpolar nature, PP_i is more likely than ATP to leak out of intact cells, and therefore is a better target for bacteria and biofilm detection. The EAA assay can also be applied in various molecular diagnosis studies based on DNA or RNA polymerization reactions, which often produce large quantities of PP_i [22–25].

In summary, this study describes a highly sensitive ATP luminescence assay that recycles both luciferase reaction products back to ATP. This improved assay may be a method of choice for bacteria monitoring in water used in semiconductor and biopharmaceutical industries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.07.013.

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