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Enhancing ATP-based bacteria and biofilm detection by enzymatic pyrophosphate regeneration

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ABSTRACT

The manufacturing processes of many electronic and medical products demand the use of high-quality water. Hence the water supply systems for these processes are required to be examined regularly for the presence of microorganisms and microbial biofilms. Among commonly used bacteria detection approaches, the ATP luminescence assay is a rapid, sensitive, and easy to perform method. The aim of this study is to investigate whether ATP regeneration from inorganic pyrophosphate, a product of the ATP luminescence assay, can stabilize the bioluminescence signals in ATP detection. ADPglc pyrophosphorylase (AGPPase), which catalyzes the synthesis of ATP from PP_i in the presence of ADPglc, was selected because the system yields much lower luminescence background than the commercially available ATP sulfurylase/adenosine 5′-phosphosulfate (APS) system which was broadly used in pyrosequencing technology. The AGPPase-based assay could be used to measure both PP_i and ATP quantitatively and shows 1.5- to 4.0-fold slight increases in a 10-min assay. The method could also be used to stabilize the luminescence signals in detection of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus cereus* in either broth or biofilm. These findings suggest that the AGPPase-based ATP regeneration system will find many practical applications such as detection of bacterial biofilm in water pipelines.

Many industrial manufacturing processes, including those for semiconductors and medicines, demand the use of high-quality water. Bacteria are one of the major pollutants in water. Many bacterial species are capable of colonization on water pipelines, filters, and containers to form biofilm, which is very difficult to eliminate. Hence the water supply systems for these processes should be monitored frequently for the presence of bacterial contamination and biofilm. Several rapid assays for detecting bacteria have been developed. These methods include PCR,[1] fluorescent *in situ* hybridization [1], β-D-glucuronidase activity measurement [2], and firefly luciferase-based ATP luminescence detection [3].

Among these bacteria detection approaches, the ATP luminescence assay is a rapid, sensitive, and easy to perform method. The assay is based on detection of ATP, a molecule that is ubiquitously present in all living cells, including bacteria. In the reaction, the enzyme luciferase catalyzes oxidation of the substrate luciferin while transforming the energy derived from ATP into light. This ATP detection assay is a well-established technique and has been used as a way to monitor the hygiene of food [4]. However, the detection limit of the method is approximately 10^4 colony-forming units (CFUs) of *Escherichia coli*, which is not sensitive enough for many industrial and medical applications [5,6].

One possible approach to enhance the bioluminescence signals of the assay is to divert the luciferase reaction products, AMP and inorganic pyrophosphate (PP_i), to ATP regeneration. AMP can be converted into ATP by several approaches, such as using polyphosphate:AMP phosphotransferase and adenylate kinase in the presence of polyphosphate (Fig. 1B) [7]. Utilization of PP_i to generate ATP could be achieved by using ATP sulfurylase and its substrate adenosine 5′-phosphosulfate (APS) (Fig. 1A) [8]. Despite that this approach has been commonly applied in detecting PP_i derived from nucleotide sequencing reactions, its usefulness in ATP detection has not been described.

In this study, we investigate whether ADPglc pyrophosphorylase (glc-1-p adenylyltransferase, AGPPase), which catalyzes the formation of ATP and glc-1-p from ADPglc and PP_i (Fig. 1A), could

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[1] *Abbreviations used:* ADK, adenylate kinase; ADPglc, ADP-glucose; AGPPase, ADP-glucose pyrophosphorylase; NDK, nucleoside diphosphate kinase; PCR, polymerase chain reaction; PK, polyphosphate kinase; PP_i, inorganic pyrophosphate; RLU, relative luminescence units; UGPPase, UDP-glucose pyrophosphorylase.

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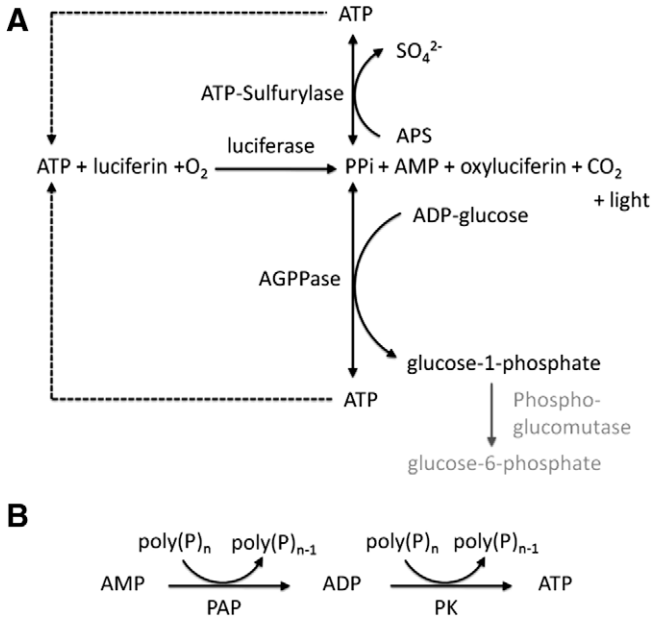

Fig. 1. (A) The proposed model of the AGPPase/ADPglc-based and ATP sulfurylase/APS-based ATP regeneration system. PP_i, the reaction product of luciferase, and ADPglc can be converted into ATP and glc-1-p by the catalysis of AGPPase. In the same mode, ATP sulfurylase can convert PP_i and APS to gain ATP. The regenerated ATP can be used by luciferase for light production. (B) The enzymatic pathway for conversion of AMP, using polyphosphate:AMP phosphotransferase, for ATP regeneration.

increase the luminescence signals of luciferase-based assays. Our result indicates that the AGPPase/ADPglc-based ATP regeneration system not only showed much lower backgrounds than the ATP sulfurylase/APS system but also stabilized the signals of the conventional luciferase-based ATP measurement assays. The results of the AGPPase/ADPglc-based assay is also more reliable than the conventional assays in bacteria and bacterial biofilm detection.

Materials and methods

Reagents

All chemicals used in this study, including ADPglc, UDPglc, luciferin, luciferase, ATP, sodium pyrophosphate, $MgCl_2$, 3-phosphoglycerate, NADH, UDPglc pyrophosphorylase (UGPPase), fructose-1,6-bisphosphate (FBP), phosphoglucomutase (PGM), glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase were reagent grade and were purchased from Sigma–Aldrich Chemicals (St. Louis, MO). The commercial APS was not contaminated with ATP as determined by the hexokinase/glucose-6-phosphate dehydrogenase-coupling assay [9,10] (Fig. S1) and by an HPLC qualitative analysis followed by the luciferase assay using the HPLC-purified APS fractions [11] (Fig. S2).

Cloning of AGPPase gene glgC into pET30a

A common laboratory bacteria strain *E. coli* W3110 genomic DNA was purified by the Wizard Genomic DNA Purification kit (Promega) and used as the template for PCR amplification of the AGPPase encoding gene *glgC*. The forward and reverse primers used in the PCR were 5′- ATCGCATATGGTTAGTTTAGAGAAGAA CG-3′, and 5′-TACGCTCGAGTCGCTCCTGTTTAGC-3′, respectively. The PCR-amplified *glgC* open-reading frame was purified from an agarose gel, digested with NdeI and XhoI restriction enzymes, and ligated directionally into the expression vector pET30a (Novagen Inc., WI). The correct clone with the insert was sent for sequencing to make sure the nucleotide sequences were correct and in frame.

Expression and purification of recombinant AGPPase

Escherichia coli BL21 (DE3) harboring the *glgC* overexpression plasmid was grown in 200 ml Luria–Bertani broth supplemented with 25 μg/ml kanamycin and 100 μM isopropyl thio-β-D-galactopyranoside at 30 °C. After 10 h of vigorous shaking, bacterial cells were collected by centrifugation at 5000 rpm for 15 min and washed with 50 mM HEPES buffer (pH 7.0). The pellets were resuspended in 50 mM HEPES buffer and cells were disrupted by sonication. Soluble proteins were separated from cell debris by centrifugation at 13,000 rpm for 15 min at 4 °C. The recombinant AGPPase protein was purified by nickel-charged affinity chromatography following the standard purification protocol (Novagen Inc.). The eluted protein was dialyzed against 50 mM HEPES buffer containing 10% glycerol to remove imidazole. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

AGPPase activity assay

The AGPPase activity was determined in a reaction mixture containing 0.1 mM ADPglc, 0.2 mM PP_i, 2 mM $MgCl_2$, 2 mM 3-phosphoglycerate, 0.25 mM NADH, 20 U glyceraldehyde-3-phosphate dehydrogenase, 10 U phosphoglycerate kinase, and the testing enzyme. The reaction catalyzed by AGPPase is reversible and may be inhibited by the accumulation of reaction product glc-1-p. Phosphoglucomutase was added to convert glc-1-p to glc-6-p to reduce the possibility of feedback inhibition. The decrease in NADH absorption at 340 nm was monitored by a UV spectrophotometer (Jasco V-530) [12].

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, 5×10^{-6} mg/μl luciferase, and the testing sample unless otherwise indicated [13]. The AGPPase-based luminescence assay system includes, besides the basic reaction mixture, 20 μM ADPglc, 5×10^{-3} mg/ml AGPPase and 1 μM FBP. Similarly, 0.1 μM APS and 5×10^{-5} U ATP sulfurylase were added to the basic luciferase assay mixture for PP_i regeneration into ATP. All luminescence assays were performed using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). All experiments have been done for three times, independently.

Luminescence detection of bacteria in both bacterial broth and biofilm

Overnight *E. coli* BL21, *Pseudomonas aeruginosa* PAO1 and *Bacillus cereus* cultures were serially diluted with M9 minimal medium to 10^2–10^5 CFU/ml for the determination of 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 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. Biofilm formation was performed as described earlier [14] with slight modification. Bacterial biofilm was generated by incubating 100 μl of the 10-fold diluted overnight bacterial cultures in a 96-well microtiter plate at 37 °C for 48 h. The nonadherent bacteria were then removed and the biofilm formed on the plate was washed twice with deionized water. Finally, M9 minimal medium was added to the 96-well plate at 100 μl/well and incubated at room temperature for 1 h. Subsequently, the

broth was transferred into a new microcentrifuge tube and the bacterial cells removed by centrifugation at 13,000 rpm for 10 min. The supernatant was used directly in the luciferase assay. All experiments were done for three times, independently.

Results

AGPPase yields lower backgrounds than ATP sulfurylase in the luciferase assays

Two PP_i-utilizing enzymes, AGPPase and ATP sulfurylase (Fig. 1A), were compared for their efficiency in regenerating PP_i into ATP. Under the conditions described under Materials and methods for ATP luciferase assay, when both enzymes produced comparable luminescence, ATP sulfurylase showed a much higher background even in the absence of PP_i (Fig. 2), presumably due to nonspecific reactivity of firefly luciferase with the energy donor APS [15,16] (Fig. S2B). The high background would prohibit reliable quantification of PP_i below 1.0 pM using the ATP sulfurylase. Because the AGPPase/ADPglc-based system showed very low background in the luciferase reaction, it was therefore chosen for the subsequent studies.

Optimization of the AGPPase-based assay

Two additional components were individually included in the luminescence assay system to test for their capability in enhancing PP_i regeneration efficiency by AGPPase. The components were FBP, an activator of AGPPase [17], and PGM, which could reduce accumulation of glc-1-p, the nonrecyclable reaction product of AGPPase to prevent product inhibition on the enzyme. While the addition of PGM increased the PP_i utilization activity of AGPPase slightly, FBP showed more significant enhancement. Since a combination of FBP and PGM did not further enhance the enzyme activity (Fig. 3), only FBP was included in the subsequent assays.

Comparison of the AGPPase-based and UGPPase-based ATP regeneration methods was also performed. Both enzymes increased the luminescence intensity about 2-fold in a 10-min assay (Table S1). Nevertheless, because the UGPPase-based system yielded lower RLU readings and required two extra reagents, ADP and nucleoside diphosphate kinase, that complicate the assays, the AGPPase-based ATP regeneration system was selected for subsequent studies.

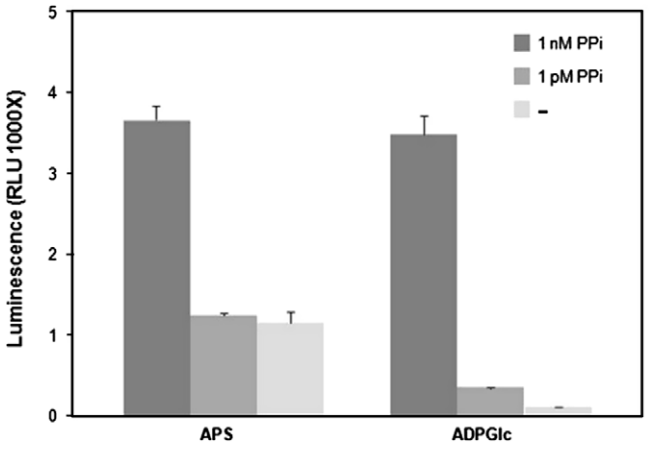

Fig. 2. The enhancement and background of AGPPase/ADPglc- and ATP sulfurylase/APS-based methods in luciferase assay. APS (0.1 μM) or ADPglc (20 μM) was incubated in the absence and presence of 1.0 pM and 1.0 nM of pyrophosphate (PP_i). Both methods increase the luminescence intensity. The enhancement is dependent on the PP_i concentrations.

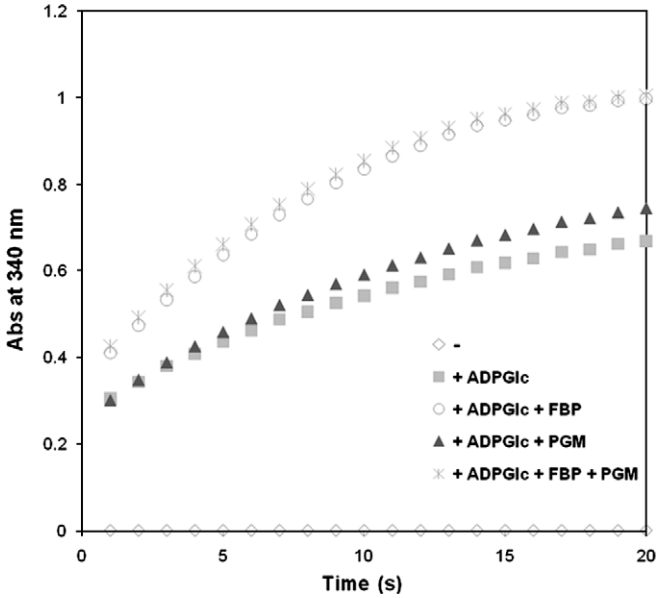

Fig. 3. The time-dependent curve of AGPPase activity assay. The NADH absorbance at 340 nm immediately decreases after 3-phosphoglycerate (3-PG) was added. Phosphoglucomutase (PGM) and the activator of AGPPase, fructose-1,6-bisphosphate (FBP), were tested in this coupling reactions. The labels display reaction without ADPglc (◇), with ADPglc (■), with ADPglc and FBP (○), with ADPglc and PGM (▲), with ADPglc, FBP, and PGM (∗), respectively.

The AGPPase-based ATP regeneration system is useful, for enhancing rate with respect to duration, for PP_i quantification

Because of its low background, the AGPPase-based method was first tested for detection limit of PP_i. Quantification in the range of 0.1–10^4 pM concentration of PP_i was performed. There was no luminescence detected below 0.1 pM PP_i. However, this method could reliably detect as low as 1.0 pM PP_i and exhibited good linearity above 10 pM PP_i (Fig. 4A). In addition, the reaction could continue steadily for at least 20 min (Fig. 4B), suggesting that it is possible to improve the detection rate, and thereby accuracy, simply by prolonging the reaction time without compromising the quantification activity through this modified luciferase assay.

AGPPase-based method is more stable for ATP detection than the conventional luminescence method

The ATP quantification activity of AGPPase-based method was compared with the conventional luminescence assay at 0.1–10^4 pM of ATP. As demonstrated in Fig. 5A, the luminescence intensity obtained by the AGPPase-based method was clearly superior to that of the conventional method at all ATP concentrations tested. Approximately 4.5-fold increases in luminescence could be achieved at 1.0 pM ATP in a 10-min reaction (Fig. 5A). The increased luminescence intensity was contributed primarily by ATP regenerated from PP_i, because the addition of ADPglc alone in the reaction yielded very low background (Fig. 2). The differences between the presence and the absence of AGPPase/ADPglc were more significant at lower ATP concentrations, indicating that the modified system might be particularly useful in quantifying ATP at subpicomolar ranges. However, the most significant observation was that unlike the conventional ATP luciferase assay system in which light production started declining after 5 min of reaction, the luminescence accumulation remained steadily at a constant rate in the presence of AGPPase and ADPglc (Fig. 5B). The finding provides direct evidence supporting that the ATP regeneration from PP_i by AGPPase and ADPglc is more stable and efficient than the ATP

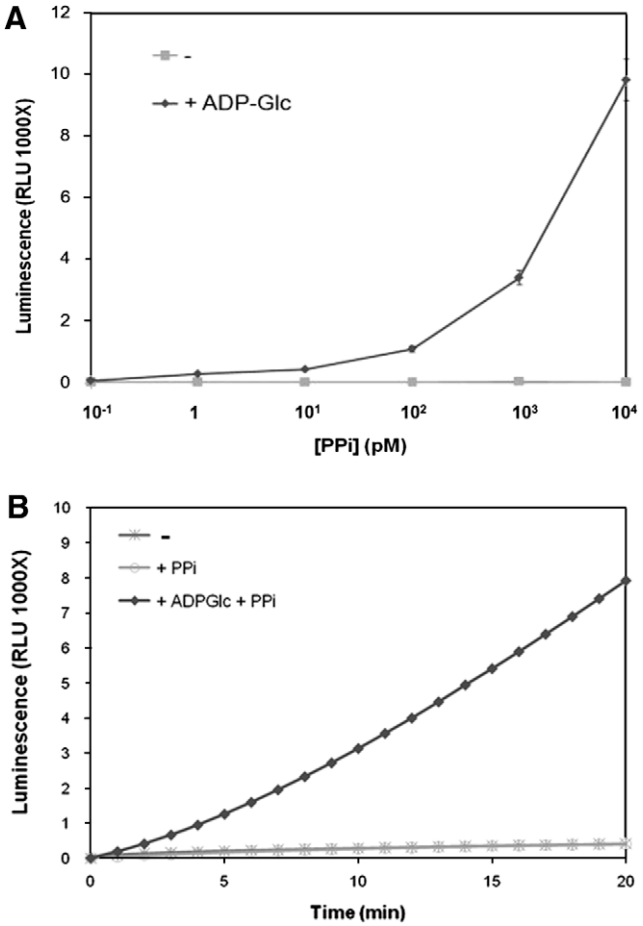

Fig. 4. The linear dynamic range of the conventional luminescence assay and AGPPase/ADPglc-based ATP regeneration systems under different concentrations of PP_i. (A) The AGPPase/ADPglc-based ATP regeneration system gradually increases the luminescence intensity when the concentration of PP_i is between 1 and 10^4 pM. (B) The time-dependent curve of the conventional luminescence assay and AGPPase/ADPglc-based ATP regeneration systems. The PP_i concentration is 1 nM.

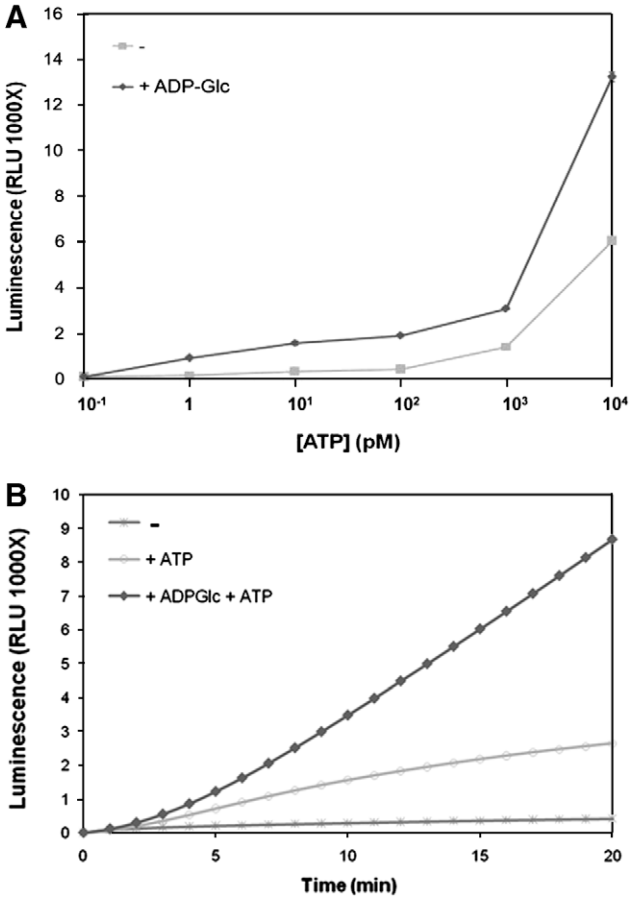

Fig. 5. The linear dynamic range of the conventional luminescence assay and AGPPase/ADPglc-based ATP regeneration systems under different concentrations of ATP. (A) The AGPPase/ADPglc-based ATP regeneration system increases about 2- to 4.5-fold of luminescence intensity when the concentration of ATP is below 10^4 pM. (B) The time-dependent curve of the conventional luminescence assay and AGPPase/ADPglc-based ATP regeneration systems. The ATP concentration is 1 nM.

luciferase assay. It also indicates that the modified reaction is more linear and is therefore more reliable in quantitative analysis of ATP.

Bacteria and bacterial biofilm detection by the improved method

It has been shown in a previous report that the limit of the conventional ATP luciferase method in bacterial detection was approximately 10^4 CFU [6]. To assess whether the AGPPase-based method can have advantages over conventional methods for bacterial detection, serial dilutions of two gram (−) bacteria, *E. coli* BL21 and *P. aeruginosa* PAO1, and one gram (+) bacterium, *B. cereus,* were prepared and tested. In either of the cases, the luminescence intensity of the groups measured by the AGPPase/ADPglc method was slightly higher (1.4- to 3.0-fold) than that of the conventional method (Fig. 6). The higher luminescence readings in the modified assay ensured the detection of 10^3 CFU more reliably than that without modification. Similarly, when the AGPPase/ADPglc assay was applied to detect biofilm, the luminescence values were also higher than the control groups for 1.8- to 3.7-fold (Fig. 7).

Discussion

Because of the advantages including high sensitivity, large linear dynamic range, and ease to perform, the ATP luminescence assay has been the method of choice in detecting bacterial contamination in water. Until recently, researchers are still attempting many ways to improve the detection limit of the method. One research direction involves the use of genetic modification on the firefly luciferase per se to lower its K_M and improve the substrate turnover efficiency [18]. The other kind of approaches concerns use of different coupling enzymes, such as adenylate kinase and polyphosphate kinase, to recycle AMP produced by the luciferase [5,19,20]. Such an approach generally shows good enhancement in detection sensitivity and is highly specific to ATP.

On the other hand, there has not been any study employing ATP regeneration from PP_i, the other firefly luciferase reaction product, in ATP and bacteria detection and quantification assays. One possible reason is that ATP sulfurylase and its substrate APS, the most well-known PP_i-utilizing enzyme system, show relatively high backgrounds which prohibits the quantification of low concentrations of ATP. This problem, however, can be overcome by using the AGPPase/ADPglc system described in this study.

The other possible reason for little description of PP_i-mediated ATP regeneration is that PP_i detection may be complicated by the presence of preexisting PP_i and therefore may not reflect the original quantity of ATP. However, this problem can be overcome simply by boiling the testing sample to hydrolyze PP_i prior to the reaction. Even in a condition where sample boiling is not allowed, the PP_i-recycle method is still useful for bacteria detection, particularly in a pure water system in which PP_i is mostly of microbial origin. Furthermore, because of its smaller molecular weight and

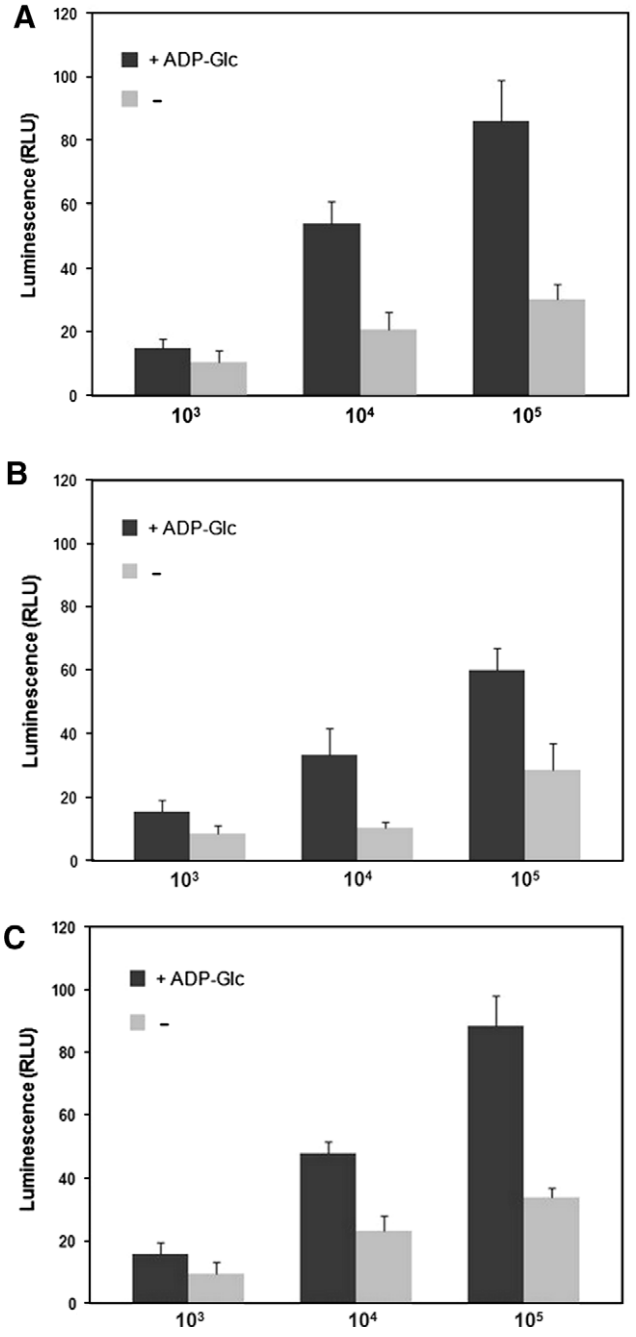

Fig. 6. AGPPase enhances bacteria detection by luciferase. (A) *E. coli* BL21; (B) *P. aeruginosa* PAO1; (C) *B. cereus*. The bars from left to right are 10^3 to 10^5 CFU of bacteria.

less polar nature, PP_i has a higher chance than ATP to leak out from intact cells and therefore is a better target for bacteria and biofilm detection. Finally, PP_i is known to inhibit firefly luciferase. Utilization of PP_i to synthesize ATP can reduce PP_i accumulation and therefore can potentially improve the sensitivity and linearity of the assay.

Our results clearly indicate that this novel method can increase the efficiency of ATP detection limits, and also it was found to be more reliable and superior for quantification of ATP by using luminescence assays where quantification was not hindered due to background as described in a previously reported method [14]. Although, quantification was performed at the subpicomolar level, there was only a slight increase (~3- to 4-fold) in sensitivity and

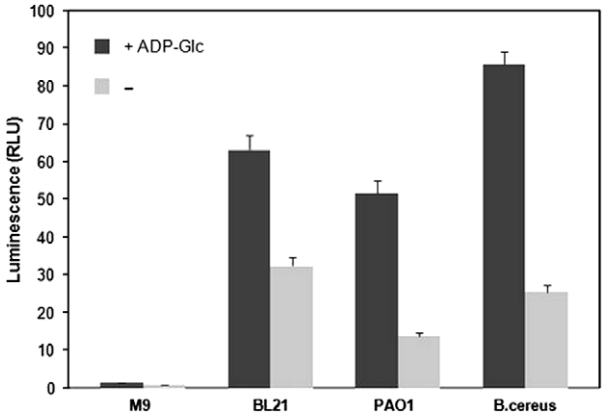

Fig. 7. AGPPase enhances bacterial biofilm detection by luciferase. The bars from left to right are M9 minimal medium, *E. coli* BL21, *P. aeruginosa* PAO1, and *B. cereus*.

also the detection time was longer, which does not make it a very quick diagnostic tool for detection or quantification studies. Irrespective of such a drawback, what was most alluring about this method was its ability to be more stable and progressively linear, providing accuracy as a result of which it can detect PP_i from lesser colony-forming units (~10^3 CFU) of bacteria or biofilm.

In summary, we believe that the AGPPase/ADPglc method described in this study can be applied for water quality control in high-tech industries to reduce the loss due to bacterial contamination in water. The method can also be applied in many molecular diagnostic studies concerning DNA/RNA polymerization, in which large quantities of PP_i production are accompanied [21–23]. The lower background of the AGPPase/ADPglc method will make it a preferred system than the currently used ATP sulfurylase/APS system.

Acknowledgments

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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.2009.12.032.

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