Review (special issue)

Polyphosphate-ATP Amplification Technology:
Principle and its Application to Detection of Specific Bacteria

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1. Bioluminescence assay is useful but not sensitive enough to detect a single bacterial cell

Adenosine triphosphate (ATP) plays a central role in all aspects of metabolism, and, therefore, the development of methods to detect very low concentrations of ATP is very important in many areas of pure and applied biochemistry[3]. The firefly luciferase-based (bioluminescence) assay for detecting ATP is a well-established technique and has been used as a way to rapidly monitor microorganisms in the environments or the hygiene of food and non-food contact surfaces[1]. Furthermore, the bioluminescence assay may also be used for warning and detection of attacks by biological warfare agents[4]. This assay technique, however, has a detection limit of approximately 10,000 colony-forming units (CFUs) of *Escherichia coli* per assay, which is not sensitive enough for detecting dangerous pathogenic bacteria[5].

2. Polyphosphate-ATP amplification allows detection of a single bacterial cell

To increase the sensitivity of the bioluminescence assay, we have developed the polyphosphate-ATP amplification (ATP amplification) reaction[5]. This amplification reaction takes advantage of (i) adenylate kinase (ADK), which converts adenosine monophosphate (AMP) and ATP to two molecules of adenosine diphosphate (ADP); and (ii) polyphosphate (polyP) kinase (PPK), which converts two molecules of ADP back to two molecules of ATP (Fig. 1). Using these reactions, ATP is amplified exponentially, resulting in high levels of bioluminescence in the firefly luciferase reaction[5]. The amplification enzyme was prepared from *E. coli* harboring a plasmid for expressing a PPK-ADK-His tag fusion. The highly purified PPK-ADK fusion enzyme successfully amplified ATP depending on its initial concentration (Fig. 2). ATP amplification for 60 min prior to the bio-

In the absence of ATP

$$AMP + polyP_n \rightarrow \quad X \qquad \text{(Adenylate kinase)}$$
$$\text{(Polyphosphate kinase)}$$

In the presence of ATP

1st
$$AMP + ATP \rightarrow 2ADP \qquad \text{(Adenylate kinase)}$$
$$2ADP + polyP_n \rightarrow 2ATP + polyP_{n-2} \qquad \text{(Polyphosphate kinase)}$$

2nd
$$2AMP + 2ATP \rightarrow 4ADP$$
$$4ADP + polyP_{n-2} \rightarrow 4ATP + polyP_{n-6}$$
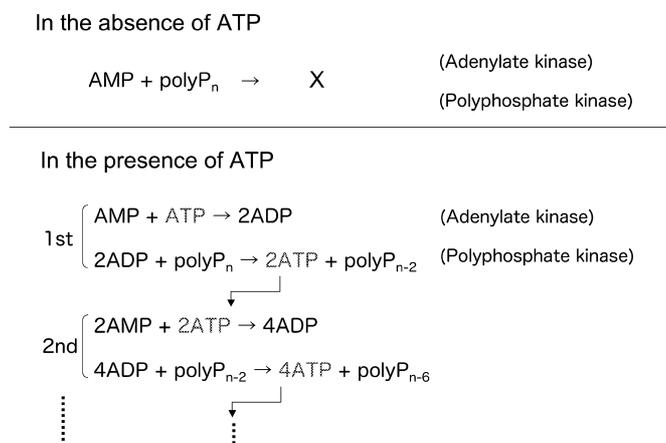
Fig. 1. Principle of ATP amplification.
The reaction mixture for the ATP amplification contains AMP, excess polyP, and the amplification enzyme (PPK-ADK). In the absence of ATP, ATP amplification is not initiated; ATP amplification starts only when exogenous ATP is added to the reaction mixture. n, number of phosphate residues in polyP.

A

B
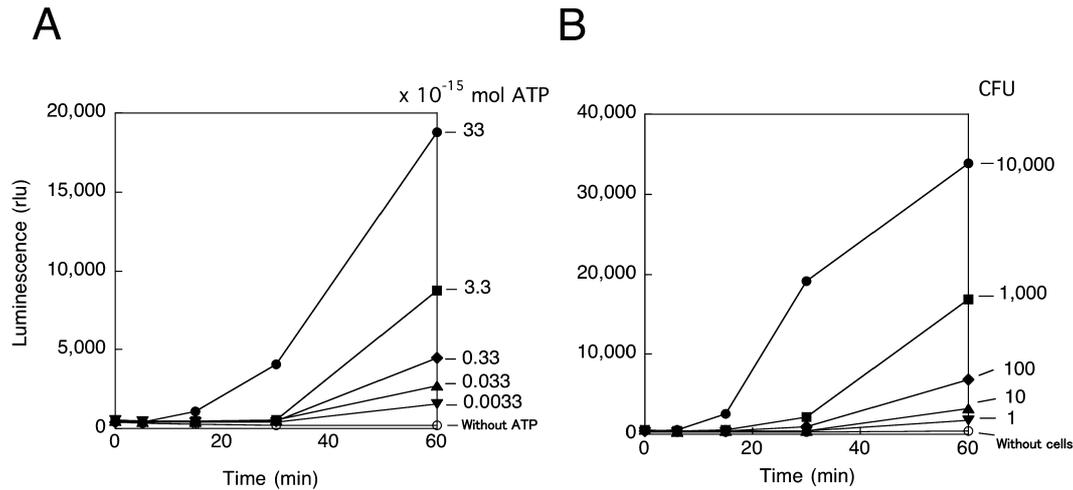
Fig. 2.   Time course of bioluminescence during ATP amplification.
(A) ATP amplification was started by adding a 2-µl ATP sample to 48 µl of a reaction mixture containing 0.16 µg of ADK-PPK, 10 µM AMP, 400 µM polyP, 8 mM MgCl$_2$, and 60 mM Tris-HCl (pH 7.4). The reaction mixture was incubated at 37°C. After appropriate periods of incubation, 5 µl of the reaction mixture was removed and mixed with 40 µl of ATP bioluminescence assay reagent (Roche). The amount of ATP that was initially present in the 5-µl reaction mixture is indicated. (B) *E. coli* cells (early stationary phase; 2.0×10⁹ CFU/ml) were appropriately diluted. The cell suspensions (500 µl) were mixed with 500 µl of lysis buffer (Roche) and incubated at 100°C for 2 min. Heated samples (2 µl) were subjected to ATP amplification prior to the bioluminescence assay. The CFU of *E. coli* present in the 5-µl reaction mixture is indicated.
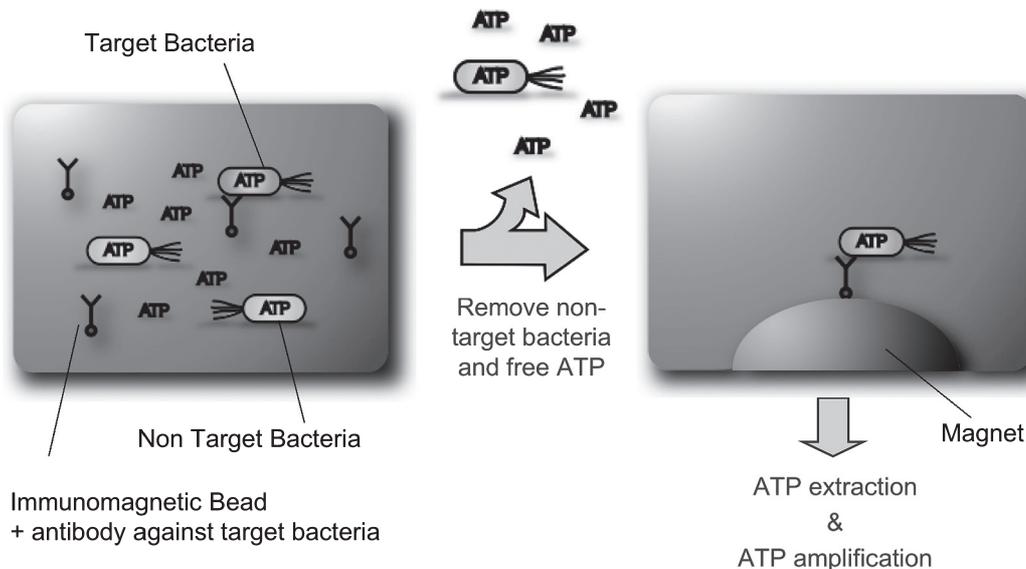
Fig. 3.   Schematic representation for detection of specific bacteria using immunomagnetic beads coupled with ATP amplification.

luminescence assay enabled the detection of as little as 10⁻¹⁸ mol of ATP, whereas the conventional bioluminescence assay requires as much as 10⁻¹⁴ mol of ATP to obtain a signal[5]. The intracellular level of ATP in viable *E. coli* cells is known as approximately 2×10⁻¹⁸ mol of ATP per cell. This level of ATP is almost equal to that of the detection limit of our ultrasensitive bioluminescence assay. In fact, with this technology, we could detect a single CFU of *E. coli* per assay (Fig. 2). In the absence of *E. coli*, no signal was obtained even after a 60-min reaction (Fig. 2). The results show that this new biochemical reaction is useful for detecting contamination by as little as a single bacterial cell[5].

3.   Critical aspects for success of the ATP amplification technology

Using computer simulation, Chittock et al. also previously proposed an ATP amplification to enhance the sensitivity of ATP detection[2], but it has been very difficult to apply this idea because very small amounts of ATP contamination must be eliminated from the reaction mixture before examination. If not, ATP amplification starts even in the absence of exogenous ATP. Therefore, to eliminate possible contamination by ATP, all buffers and equipment were autoclaved for 120 min. In addition, commercial AMP used for the assay was purified by ion-exchange column chromatography.

The most difficult problem was that ADP bound to the amplification enzyme PPK-ADK even after purification[5]. Because this ADP could be converted to ATP by PPK in the presence of polyP, ATP amplification could be initiated without the addition of exogenous ATP. To eliminate ADP contamination, we treated PPK-ADK with polyP to release bound ADP from the enzyme. The resulting ATP was then degraded to AMP with apyrase, and the PPK-ADK was further purified with a chelating column[5]. The addition of 0.1 M pyrophosphate (almost saturated) to washing and elution buffers was effective at releasing any remaining ADP from the enzyme[5]. As a result of the combination of these precautions, ATP amplification in the absence of exogenous ATP was not observed for at least 60 min (Fig. 2).

4. Application of ATP amplification technology

With this technology, it is possible to amplify the very low levels of ATP extracted from a single bacterial cell to quantities that can be detected without a highly sensitive photometer. We therefore attempted to apply the ATP amplification technology to the monitoring of hygiene. The ATP amplification enhanced the sensitivity of swab monitoring by approximately 10,000-fold[5]. We further examined the ability of the ATP amplification to enhance the sensitivity of detecting bacterial contamination in drinking water. We found that the ATP amplification prior to bioluminescence assay allowed us to detect contamination by a single bacterium[5]. This sensitivity far exceeds that of conventional bioluminescence assays, and these results show that this new technology can be applied to a wide range of hygiene monitoring tasks.

5. Detection of specific bacteria

Although ATP amplification is an interesting technology for detecting a single bacterial cell, but it can never inform us which bacteria contaminate a reaction mixture. Therefore, to adapt this technology to the detection of specific bacteria, we have combined it with the use of immunomagnetic beads technology[6] to trap target bacteria (Fig. 3). After washing out non-target bacteria and exogenous ATP, the ATP is extracted from the target bacteria and amplified (Fig. 3). Here we prepared *S. aureus* and *E. coli* suspension at concentrations of 10^4 cells/ml. Immunomagnetic separation was performed with an antibody against *S. aureus*. The recovery rates of cells were calculated on the basis of colony forming units of cell suspensions before and after immunomagnetic separation. The recovery rate of *S. aureus* cells by immunomagnetic separation with an antibody against *S. aureus* was about 30%, while *E. coli* cells were not recovered with an antibody against *S. aureus* (less than 0.1%). The cell suspensions after immunomagnetic separation were subjected to ATP amplification and bioluminescence reaction. Amplified bioluminescence signals were obtained from *S. aureus* suspension, while not from *E. coli* suspensions.
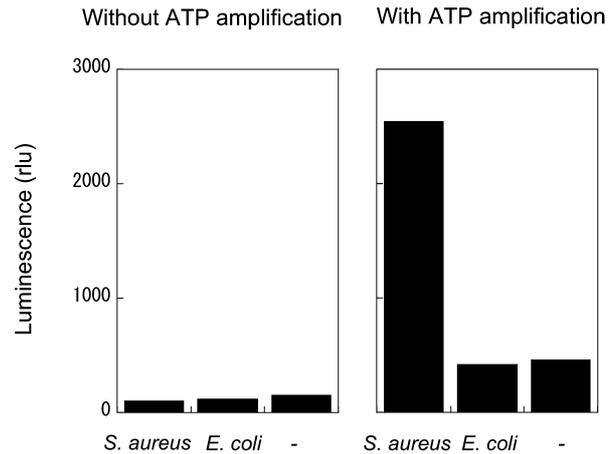
Fig. 4. Detection of *S. aureus* by using immunomagnetic beads coupled with ATP amplification.

The magnetic beads coated with anti-mouse IgG antibody (Fishers, IN, USA) were mixed with mouse monoclonal antibodies against *S. aureus,* (Funakoshi, Tokyo, JAPAN) in a PBS buffer (6 mM potassium phosphate, pH 7.2, 140 mM NaCl). The amount of antibodies and magnetic beads to achieve surface saturation was calculated following the manufacturer's instructions. The suspension was mixed for 30 min at 60 rpm (Dynal Sample Mixer, Dynal, Lake Success, NY, USA) at 4°C. The beads were then removed from the solution with a magnet and resuspended in the PBS buffer containing 1% BSA. These steps were repeated three times and resuspended in the PBS buffer containing 1% BSA and 0.02% NaN_3. Microbial cells that grew on nutrient medium were diluted with the PBS buffer. The cell suspension was mixed with the prepared immunomagnetic beads for 60 min at 60 rpm at 4°C. Using a magnetic separator, these complexes were concentrated to the magnet side of the tube wall. The supernatant was discarded by pipetting while the tube was on the magnet. The bead–bacteria complexes were resuspended in the PBS buffer (1 mL). The separation and washing steps were repeated twice. At the final washing step, the entire pellet was resuspended in 100 μl lysis buffer where ATP was extracted from the cells into the liquid portion. The entire liquid was then transferred to a microbial tube. ATP amplification was started by adding a 2-μl ATP sample to 48 μl of a reaction mixture containing 0.16 μg of ADK-PPK, 10 μM of AMP, 400 μM of polyP, 8 mM $MgCl_2$, and 60 mM Tris-HCl (pH 7.4). The reaction mixture was incubated at 37°C. After 60 min-incubation, a 5-μl reaction mixture was sampled and mixed with 40 μl of the ATP bioluminescence assay reagent (Roche, Basel, Switzerland). Bioluminescence was measured by using a multiplate luminometer (Wallac, Massachusetts, U.S.A).

These signals could not be obtained without using ATP amplification. The *S. aureus* suspensions after immunomagnetic separation subjected to ATP amplification contained ten CFUs of *S. aureus*.

PCR technology is now being used to detect contamination by low levels of specific bacteria within several hours. The ATP amplification can currently be completed in 10 to 20 min, and theoretically it could be much fast. Thus, we believe that this improved ATP amplification technology could be used for the detection of specific bacteria.

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