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Detection of somatic coliphages through a bioluminescence assay measuring phage mediated release of adenylate kinase and adenosine 5′-triphosphate

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The feasibility of detecting somatic coliphages by phage infection of *Escherichia coli* WG5 and measurement of phage propagation by the lysis mediated release of the bacterial host adenylate kinase (AK) and adenosine 5′-triphosphate (ATP) detected by a bioluminescent signal was evaluated. After 2 h of incubation, all cultures infected with reference bacteriophage ϕX174 showed a significant increase in the bioluminescent signal, even with number of phages as low as less of 10 plaque forming units (PFU). Naturally occurring somatic coliphages ensured a significant bioluminescent signal after 3 h of infection when >10 PFU were inoculated. These results indicate that an easy and reliable method to detect low numbers of coliphages in less than 3 h is feasible.

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1. Introduction

Somatic coliphages is a group of bacteriophages that has been promoted as indicator microorganisms in the assessment of faecal contamination of water (Grabow, 2001; IAWPRC, 1991; Jofre, 2007) and food (Hsu et al., 2002; Pillai, 2006) and have recently been included as a water quality parameter in the US EPA Ground Water Rule (USEPA, 2006). Different strains of *Escherichia coli* and different assay media give different phage counts and detect different somatic coliphages (Havelaar and Hogeboom, 1983; Muniesa et al., 1999; Rajala-Mustonen and Heinonen-Tanski, 1994). To avoid this source of potential variability in somatic coliphage determination, standardized methods for detecting and quantifying this group of phages had been established and are now available (APHA, 2001; ISO, 2000; USEPA, 2001a,b). These standardized methods recommend *E. coli* C as host strain, either wild strain ATCC13706 (APHA, 2001) or strains WG5 (Havelaar and Hogeboom, 1983; ISO, 2000) or CN13 (Payment and Franco, 1993; USEPA, 2001a,b), which were isolated as spontaneous nalidixic resistant mutants of wild strain *E. coli* C. All these methods include a quantitative version which provides numbers of plaque forming units (PFU) and a presence/absence one, based in phage enrichment and a posterior testing of the presence of phages in the supernatant of the enrichment culture on a host monolayer. Whereas the PFU method for somatic coliphages provides results in 6–8 h, the presence/absence is an overnight procedure (ISO, 2000).

The literature was surveyed for alternative tests allowing to detect the presence of bacteriophages in few hours in order to speed up obtaining presence/absence results. Different methods based in the propagation of phages in bacterial hosts had been explored in food microbiology for detecting the presence of bacteria (Jassim and Griffiths, 2007; Ress and Voorhees, 2005; Stewart et al., 1998; Wu et al., 2001). Amid them are those based in the measurement of phage mediated release of intracellular molecules such as adenosine 5′-triphosphate (ATP) and/or adenylate kinase (AK) (Blasco et al., 1998; Wu et al., 2001). These are measured by the following reactions: firstly, the adenylate kinase (AK) released converts exogenous adenosine 5′-diphosphate (ADP) to adenosine 5′-triphosphate (ATP); secondly, the released ATP and the ATP produced by the AK activity is determined by the ATP bioluminescent assay based in the activation of the firefly luciferase. All the reagents necessary for these reactions are available commercially.

The purpose of the research was to explore the feasibility of detecting somatic coliphages by phage infection of *E. coli* WG5 and measurement of phage propagation by the lysis mediated release of the bacterial host AK and ATP detected by a bioluminescent assay based in the activation of the firefly luciferase. The rational assumption of this approach is that one infectious bacteriophage when inoculated into a liquid culture of specific host cells should produce, in few rounds of replication, the lysis of a number of bacterial cells

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high enough to release amounts of AK and ATP detectable easily by the bioluminescence reactions described above.

2. Materials and methods

2.1. Microorganisms

2.1.1. Bacterial host

Escherichia coli WG5 (ATCC 700078), which is the strain recommended in the ISO 10705-2 (ISO, 2000) standard procedure for the detection of somatic coliphages, was used as the host strain.

2.1.2. Bacteriophages

Bacteriophage ϕX174 that is the reference phage recommended in the ISO 10705-2 standard (ISO, 2000), different suspensions containing naturally occurring coliphages and phage isolates CB501 and CB502 (isolated during this study), were used.

Naturally occurring somatic coliphages were phages from real world samples (raw sewage from two different municipalities, tertiary effluents from two water reclamation facilities and river water). Prior to testing for phages, sewage and river water samples were decontaminated by filtration through 0.22 μm pore size diameter Millex-GP membranes, which are low binding protein polyether-sulphone membranes (Millipore Corporation, Bedford, MA, USA). The bacteriophages from the tertiary effluents were concentrated according to Mendez et al. (2004) prior to testing them.

Stocks of ϕX174, CB501 and CB502 were obtained as indicated in the ISO 10705-2 standard (ISO, 2000).

2.2. Reagents and equipment

DNAse and RNAse free distilled water (ultra PURE™, GIBCO™, New York, USA) was used to dilute reagents when necessary. Adenosine 5′-diphospate (ADP) purified to ≥95% by HPLC (Sigma–Aldrich, Steinheim, Germany) was used as the substrate for adenylate kinase. The BacTiter-Glo™ Assay (Promega, Madison, USA), which utilizes a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase, Promega, Madison, USA) that supports a stable luminescent signal, was used to measure ATP. The GLoMAX® 20/20 luminometer (Promega, Madison, USA) was used to determine light emission and hence ATP concentrations.

2.3. Bacteriophage enumeration

Somatic coliphages (ϕX174, CB501, CB502 and naturally occurring coliphages) in the suspensions added to the host strain cultures and those in the supernatants of the cultures after different post-infection times were enumerated as plaque forming units (PFU) by the double agar layer technique according to the ISO 10705-2 standard (ISO, 2000).

2.4. Assays performed to optimize the provisional method for the measurement of bioluminescence due to AK + ATP released by phage mediated cell lysis

Several small methodological variations were tested on the host culture and in the reaction for measuring free AK + ATP in order to minimize the background luminescence and to maximize the bioluminescent signal of the phage mediated release of AK and ATP.

2.4.1. Removal of non-ruptured cells

Since the rational of the method is to measure the AK and ATP released by cell lysis caused by bacteriophages, the full non-ruptured cells had to be removed. Filtration through low binding protein polyether-sulphone membranes (0.22 μm pore size diameter) and centrifugation at 4000, 8000 and 16,000 × *g* for 5 min at room temperature were evaluated.

2.4.2. Density and physiological condition of the host culture

Host cultures in the logarithmic growth phase were used because their relatively low extracellular adenylate kinase levels and because the proportion of extracellular kinase stays relatively constant throughout the logarithmic growth phase (Blasco et al., 1998). Moreover, according to the ISO 10705-2 standard for detection and enumeration of somatic coliphages (ISO, 2000), the optimal density and physiological condition of the cells for phage enumeration is when the host culture reaches approximately 10^8 cells per ml in the logarithmic growth phase. According to the ISO method, this optical density requirement is reached after 0.5–2.5 h after inoculation of a volume of a frozen culture (working culture), prepared according to ISO specifications, in Modified Scholtens' Broth (MSB) followed by incubation at 37 °C. The time elapsed between the inoculation of the working culture and infecting the culture with bacteriophages will be denominated pre-incubation period. To determine the best pre-incubation period, four time intervals were tested (0, 30, 90 and 150 min). Bacteriophage ϕX174 was used in this set of experiments.

2.4.3. Potential effect of culture medium in the background levels of AK + ATP

Modified Scholtens' Broth (MSB), the ISO recommended culture medium for the host cells, contains 3 g per litre of yeast extract that is a potential source of AK and ATP. Different concentrations (1:2, 1:5 and 1:10) of the MSB medium were assayed in comparison to MSB to assess whether the background values of bioluminescent signal were influenced by the culture media.

2.4.4. Application of an extractant reagent to the supernatant

An extractant reagent (Extractant B/S, BioThema AB, Handen, Sweden) recommended to extract AK and ATP from full bacterial cells was applied to the supernatants obtained after removal of the non-disrupted cells to liberate AK and ATP that could have remained attached to membrane residues after cell lysis caused by bacteriophages.

2.4.5. Concentrations of exogenous ADP

Different concentrations of exogenous ADP were assayed. Concentrations of ADP tested ranged from 100 to 0.1 mmol/l.

2.5. Determination of bioluminescent signal in the supernatant of bacteriophage infected and non-infected (control) cultures

After the results reported in Section 3.1 the protocol used in the experiments to determine the changes in bioluminescent signal, which measures the presence of AK + ATP in the supernatant of control and phage-infected cultures, produced by the addition of phages to the host culture was as follows. One hundred microlitre of a working culture, which was obtained as indicated in the ISO 10705-2 standard, warmed to room temperature was added to 10 ml of MSB prewarmed at 37 °C. Immediately, phages were added and the mixture was incubated under gentle shaking at 37 °C. After the incubation time tested, 1 ml of an infected culture was transferred into a 1.5 ml microcentrifuge tube (Eppendorf, Noviglio, Milano, Italy). After centrifuging at 4000 × *g* for 5 min at room temperature, 25 μl of the supernatants were mixed with 25 μl of ADP (solution containing 4.6 mmol/l) and after gentle agitation, they were incubated for 5 min at room temperature. Then, 25 μl of BacTiter-Glo ™ Reagent (Promega Corporation, Madison, USA) was added. The mixture was mixed gently and incubated again for 5 min at room temperature. The reaction was then read 3 times in the luminometer. All the biochemical reactions were performed in the dark.

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