Specific electrochemical phage sensing for *Bacillus cereus* and *Mycobacterium smegmatis*

Miri Yemini [a], Yaron Levi [a], Ezra Yagil [b], Judith Rishpon [a,*]

[a] *Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*
[b] *Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

Abstract

The rapid and reliable detection of pathogenic microorganisms is an important issue for the safety and security of our society. Here we describe the use of a sensitive, inexpensive, amperometric, phage-based biosensor for the detection of extremely low concentrations of *Bacillus cereus* and *Mycobacterium smegmatis* as models for *Bacillus anthracis* (the causative agent of anthrax) and for *Mycobacterium tuberculosis* (the causative agent of tuberculosis), respectively. The detection procedure developed here enabled the determination of bacteria at a low concentration of 10 viable cells/mL within 8 h. This experimental setup allows the simultaneous analysis of up to eight independent samples, using disposable screen-printed electrodes.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Anthrax; Tuberculosis; Electrochemical; Bacteriophage; Detection

1. Introduction

The fields of medicine and public health require the rapid detection of bacterial pathogens that cause severe infectious diseases and poisonings. Although culturing methodology remains the gold standard for detection of food-borne pathogens, the technique is time-consuming (24–48 h of enrichment before analysis) [1,2]. Moreover, the identification of certain types of pathogens by culturing often results in false negatives due to a high background level of competing microorganisms. Another conventional method for detecting a bacterial pathogen involves the use of antibodies against the microorganism [3,4]. This method has some drawbacks as well: the sensitivity is inadequate and the bacteria must be purified, a process requiring many hours [5,6]. The polymerase chain reaction (PCR) has been used extensively in the last few years to detect bacteria and viruses. Although the sensitivity is improved, the technique is laborious and expensive [7,8]. Furthermore, PCR cannot differentiate between viable and non-viable bacteria. Therefore a more rapid, convenient and sensitive method would be extremely helpful.

Bacteriophages are extremely specific for their host because in the first step of phage infection the phage attaches to a specific receptor on the bacterial cell surface [9]. Bacteriophages have been used extensively in areas such as epidemiological phage typing [10,11] and the specific detection of bacteria [12,13]. The principle of phage-based detection is that the phage infection results in the bacterial lysis that results in the formation of visible plaques on solid medium plates. Alternatively, the lysis of phage-infected bacteria in a liquid growth medium, which occurs within a few hours, results in the release of cell contents into the surrounding medium. Thus, upon lysis in liquid medium, specific intrinsic cell compounds like enzymes can be measured and quantified rapidly by calorimetric or electrochemical methods. This provides a powerful means for the highly specific detection of a given bacterial strain. We have previously shown that the presence of low amounts of *Escherichia coli* cells can be specifically detected in an electrochemical assay of β-galactosidase released by cells lysed after being infected by bacteriophage lambda [14].

The detection of *Bacillus anthracis*, the causative agent of anthrax, is a very important issue because of its potential use in biological warfare. Once exposed to internal tissues of the host, anthrax bacteria can germinate and grow rapidly, which in many

---

* Corresponding author. Tel.: +972 3 6409366; fax: +972 3 6409407.
  E-mail address: Rishpon@post.tau.ac.il (J. Rishpon).

cases will result in the death of the human or animal host within several days [13]. Therefore, it is of particular importance that methods for a rapid analysis and detection in the environment be developed. Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the most neglected global health problems today and remains endemic in most parts of the world [5]. Fast and reliable detection of tuberculosis is critical from the point of view of effective medical treatment and epidemic prevention.

Among the abundant detection techniques [3,8,10] for the pathogen, none is available that integrates the following necessary features: speed, specificity, simplicity and economy. Here we show that the electrochemical method developed for the identification of low concentrations of *E. coli* can be applied to *Bacillus cereus* as a model for *B. anthracis* and to *Mycobacterium smegmatis* as a model for *M. tuberculosis*. This biosensor, developed here, is based on the phage-specific release of an intrinsic cell-marker enzyme that can be measured electrochemically, even when starting with a low amount of bacteria. The detection procedure takes less than 8 h and the results are shown on-line throughout the process. The sensor is inexpensive, easy to operate under field conditions, does not require early preparation of the specimen, and is not affected by the turbidity of the solution or the presence of other microorganisms. The electrochemical procedure allows the simultaneous examination of up to eight different samples.

2. Materials and methods

2.1. Bacterial strains and bacteriophages

B. cereus strain 7064 and its strain-specific bacteriophage B1-7064 were purchased from the American Type Culture Collection (ATCC) were obtained from Dr. McNerney and Dr. Hatfull. *M. smegmatis* strain mc^{2}155 and its phage-specific strain D29 [15,16].

2.2. Cultures and growth conditions

B. cereus was grown on nutrient broth medium (Difco) with shaking at 37 °C and *M. smegmatis* was grown in Middlebrook 7H9 at 30 °C [16].

2.3. Determination of bacterial concentration

The bacterial concentration was determined by spreading serial dilutions on 2% nutrient agar plates followed by incubation overnight at 37 °C. The concentration of viable cells in the original culture was expressed as colony forming units per milliliter (cfu/mL). The phage concentration was determined by mixing 100 µl of serial dilutions of the phage stock with $\sim 10^8$ cells followed by the addition 5% melted soft agar, and rapid plating on nutrient agar. Following an overnight incubation at 37 °C, the number of plaques formed on the lawn of bacteria was counted, and the concentration of live phage in the undiluted sample was expressed as plaque forming units per milliliter (pfu/mL).

2.4. Chemicals

para-Aminophenol (*p*-AP) and *para*-amino-phenyl-β-D-glucopyranoside (*p*-AP-β-GLU) were purchased from Sigma Chemicals Company, USA. *para*-Amino-phenyl-α-D-glucopyranoside (*p*-AP-α-GLU) was synthesized by Prof. Shmuel Carmeli from Tel-Aviv University.

2.5. Amperometric measurement of enzymatic activity

Enzymatic activity was determined electrochemically using as substrate *p*-AP-α-GLU for *B. cereus* and *p*-AP-β-GLU for *M. smegmatis*. The product of the reaction, *p*-aminophenol (*p*-AP) is oxidized at the carbon anode at 220 mV vs. (Ag/AgCl) reference electrode. Aliquots (300 µl) from the cultures were analyzed. Screen-printed electrodes consisted of carbon working and counter electrodes and a silver/silver chloride reference electrode, all printed on a ceramic support as described in [14]. The electrochemical cells consisted of polystyrene tubes (0.3-mL volume) attached to disposable screen-print electrodes. The electrochemical measuring system consisted of an eight-channeled, highly sensitive potentiostat (detection limit of 1 nA), constructed by Prof. Yarnitzky, Technion, Israel Institute of Technology. This multi-potentiostat allows the simultaneous measurement of eight electrochemical cells. The potentiostat was interfaced to a PC via an A/D converter employing visual basic software, so that the results of enzyme activity could be simultaneously visualized in real-time on a computer screen. We used a specific homemade apparatus for the electrical contacts of the screen print electrodes combined with suction-expulsion-based efficient stirring.

When we used different initial concentrations of cells (Figs. 3 and 5), 2 l of solution containing an initial concentration of 1000, 100 or 10 cfu/mL was filtered. The filter was re-suspended in 10 mL of medium and incubated for 3.5 h at 37 °C with continuous stirring. Then bacteria were infected by mixing 100 µl of bacteria with 10 µl of bacteriophage at MOI of 0.1 in a 15-mL test tube. Two milliliters of medium was added and the mixtures were incubated for 30 min without shaking. At the end of the absorption period, fresh medium was added to a volume of 10 mL. The mixture was incubated for an additional 4 h with vigorous shaking at which time lysis of infected cultures was complete. The resulting solution was filtered through a 0.22-µm filter and electrochemical measurement was performed on the filtrate.

2.6. Safety considerations

B. cereus and *M. smegmatis* bacteria were handled according to rules appropriate for biosafety level 2 microorganisms (no special hazard). Biosafety levels are defined by the Centers for Disease Control (CDC, USA).

3. Results and discussion

The enzymes alpha-glucosidase and beta-glucosidase, which are constitutively expressed in *B. cereus* and in *M. smegmatis*, respectively (5, 14), catalyze the hydrolysis of *para*-amino-phenyl-α-glucopyranoside (*p*-AP-α-GLU) or *para*-amino-
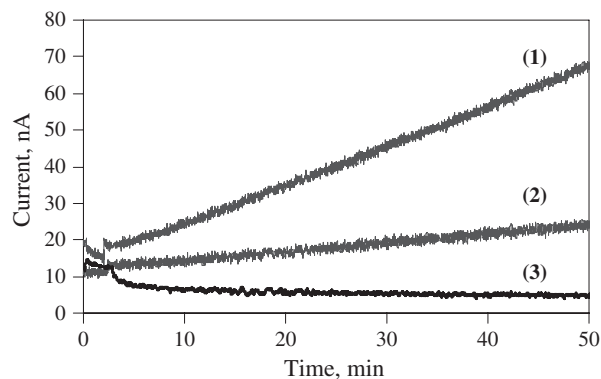
Fig. 1. Amperometric response of α-glucosidase in cultures of *B. cereus* infected with bacteriophage (1), of uninfected bacteria (2) and of the phage culture (3).

Fig. 3. Amperometric signals (presented as Current/Time (pA/s) of α-glucosidase in cultures of *B. cereus* at different initial concentrations, grown and infected at an MOI=0.1. The control was without bacteria.

phenyl-β-glucopyranoside (*p*-AP-β-GLU) to *para*-aminophenol (*p*-AP). After the oxidation of the reaction product (*p*-AP) at the carbon working electrode at 220 mV vs. an Ag/AgCl reference electrode, the current produced can be measured and analyzed. Thus, the specific cell markers, alpha- and beta-glucosidase, released into the medium upon phage-induced cell lysis, are quantified amperometrically. The measurement system is based on the on-line electrochemical monitoring of a mixture of culture medium and substrate placed in a disposable screen-printed electrochemical cell that is connected to an eight-channel based potentiostat as previously described [14].

Cells of *B. cereus* (strain 7064, obtained from the ATCC), grown overnight at 37 °C in nutrient broth, were infected at a concentration of 10^7 cfu/mL with phage particles (strain B1-7064, ATCC) at a multiplicity of infection (MOI) of 0.1 (one phage particle per 10 cells). The cell cultures were shaken in a water bath at 37 °C and following lysis, samples were placed into electrochemical cells for the amperometric monitoring of α-glucosidase (Fig. 1, line 1). In parallel, aliquots of non-infected bacteria (line 2) and of the phage culture alone (line 3) were used as controls. The results show that the phage-infected lysed culture (line 1) was over four-fold more active than was the uninfected culture (line 2), whereas the phage culture alone (line 3) was completely inactive. The residual activity of the uninfected culture is due to a partial penetration of the substrate into the uninfected cells (see below, Fig. 4B).
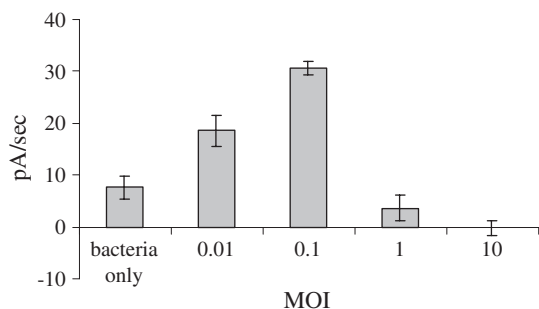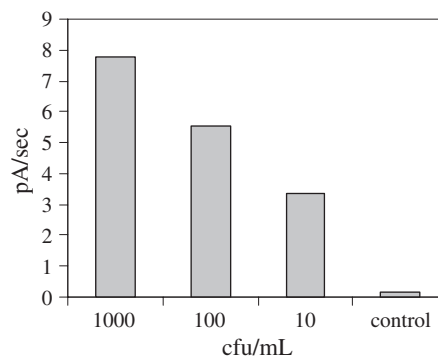
Next, we tested the effect of different MOIs on enzyme activity (Fig. 2). The best results were obtained at a MOI of 0.1. Apparently, at the lower MOI of 0.01, a significant fraction of the cells reached a growth phase that rendered them resistant to
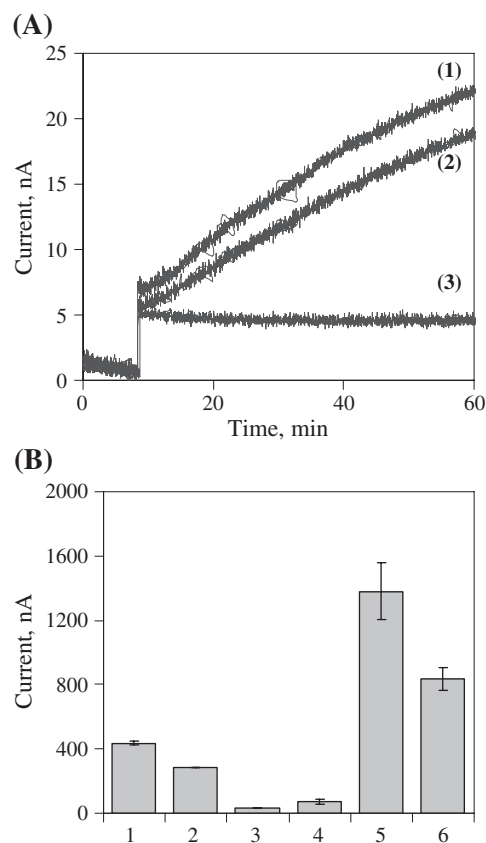
(A)

(B)

Fig. 4. (A) Amperometric responses of α-glucosidase in phage-infected cultures of *B. cereus* (line 1) of a 1:1 mixture of *B. cereus* and *E. coli* (line 2) and of *E. coli* alone (line 3). (B) Amperometric signals of α-glucosidase (after 60 min of measurement) of the following cultures: *B. cereus* (column 1), *B. subtilis* (column 2), a filtered culture of *B. cereus* (column 3), a filtered culture of *B. subtilis* (column 4), a filtered culture of a phage-infected *B. cereus* (column 5), and a filtered culture of a phage infected 1:1 mixture of *B. cereus* and *B. subtilis* (column 6). All cultures in (A) and (B) started at an initial concentration of 10 cfu/mL.
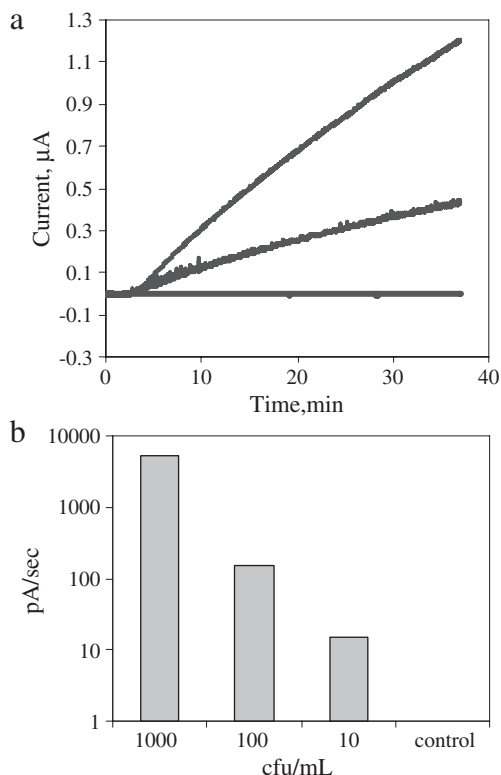

Fig. 2. Amperometric signals (presented as Current/Time (pA/s) of α-glucosidase in cultures of *B. cereus* infected at different MOIs.

Fig. 5. (a) Amperometric detections of β-glucosidase in cultures of *M. smegmatis* (a) cells (10^8 cfu/mL) infected with phage (strain D29) (line 1), uninfected cells (line 2) and the phage culture (line 3). (b) Assays of infected cells at a different initial concentration of cells. The control is without cells. Amperometric signals presented (presented as Current/Time (pA/s).

further phage infection. In contrast, at the high MOIs of 1 and 10, the cells did not have a chance to propagate and lysed when the concentration of cells in the culture was still low. Thus, in all other experiments, we used an MOI of 0.1.

In the following experiment, we tested the sensitivity of the amperometric response to the initial concentration of cells in the culture. Cultures of different initial cell densities were grown, infected with the phage and tested for α-glucosidase as described in Materials and methods. The results presented in Fig. 3 shows that even in a culture that started with only 10 cfu/mL a significant enzyme activity could be detected within 8 h.

To test the specificity of the phage-promoted amperometric test, we used a 1:1 mixed culture of *B. cereus* and *E. coli*, each at an initial low concentration of 10 cfu/mL. Fig. 4A, line 2 shows that the enzyme activity of the mixed culture infected with the *B. cereus*-specific phage was similar to the non-mixed phage-infected culture of *B. cereus* (line 1), whereas *E. coli* cells alone, in the presence of the *B. cereus*-specific phage, did not show any activity (line 3). Another *Bacillus* species, *B. subtilis*, also produces β-glucosidase constitutively but does not support the growth of the *B. cereus* phage B1-7604. Fig. 4B shows that uninfected cells of both *B. subtilis* (column 1) and *B. cereus* (column 2) display a low amount of background activity, due to substrate penetration into the cells. Columns 3 and 4 show the amperometric signal obtained when the cell cultures were

filtered through a 0.22-μm filter. The lower background activity in these cultures demonstrates the importance of sample filtration before the measurement. A filtered culture of a 1:1 mixture of a low concentration (10 cfu/mL) of phage-infected *B. subtilis* and *B. cereus* exhibited a significantly increased activity (column 6), although much lower than that of a filtered culture of phage-infected *B. cereus* alone (column 5). One reason for the difference (here as well as in the mixed culture with *E. coli* shown in Fig. 4A) might be that the competitive growth conditions between the two bacterial species yielded fewer *B. cereus* cells.

Finally, we repeated the same experiments using *M. smegmatis* grown at 30 °C, using its specific phage (strain D29). Fig. 5a illustrates that the activity of the phage-infected cells was more than four-fold stronger than that of uninfected cells. As shown in Fig. 5b using *M. smegmatis*, the assay can be done within 8 h beginning with a culture that starts with 10 viable cells/mL. Also it should be mentioned that pre-filtration of the culture before the measurement is essential for increasing the quality of the detection. The results presented here demonstrate the high efficiency of our model system for the specific identification of *B. cereus* and *M. smegmatis*, either alone or in a mixture. Enzymatic activity of a bacterial cell content at a lower detection limit of 10 cfu/mL was recorded in the extremely short time (after as fast as 10 min from the beginning of measurement). The ability to measure the enzymatic reaction rate directly in the electrochemical cell eliminates the need for additional treatments of the bacteria.

Our approach allows identification of pathogenic bacteria from variable sources (such as air, water, body-fluids, etc.) with minimum adaptation of the general procedure. Moreover, the biosensor described here has a generic structure and can easily be applied in the identification and quantification of other bacteria by measuring their specific enzymes on the electrodes.

4. Conclusion

In conclusion, a phage-sensed amperometric method that was originally developed to identify very low concentrations of a laboratory *E. coli* strain is applicable to close relatives of the pathogens *B. anthracis* and *M. tuberculosis*. It is therefore plausible that the same method can be used for an early identification of low concentrations of the actual pathogens. Even at a low concentration of bacteria, the entire detection process takes no longer than 8 h. The process is simple and can be applied under field conditions or in a physician's laboratory, thus facilitating early treatment of the relevant diseases. Moreover, due to the specificity of the phage, a mixture containing bacteria that are not attacked by the phage has only a small effect on the sensitivity of the detection.

References

[1] A.J. Baeumner, B. Leonard, J. McElwee, R.A. Montagna, A rapid biosensor for viable *B. anthracis* spores, Analytical and Bioanalytical Chemistry 380 (2004) 15–23.

[2] C.L. Turnbough, Discovery of phage display peptide ligands for species-specific detection of *Bacillus* spores, Journal of Microbiological Methods 53 (2003) 263–271.

[3] M. Diaz-Gonzalez, M.B. Gonzalez-Garcia, A. Costa-Garcia, Immunosensor for *Mycobacterium tuberculosis* on screen-printed carbon electrodes, Biosensors and Bioelectronics 20 (2005) 2035–2043.

[4] V.A. Petrenko, I.B. Sorokulova, Detection of biological threats. A challenge for directed molecular evolution, Journal of Microbiological Methods 58 (2004) 147–168.

[5] A.Y. Rubina, V.I. Dyukova, E.I. Dementieva, A.A. Stomakhin, V.A. Nesmeyanov, E.V. Grishin, A.S. Zasedatelev, Quantitative immunoassay of biotoxins on hydrogel-based protein microchips, Analytical Biochemistry 340 (2005) 317–329.

[6] B. Zhou, P. Wirsching, K.D. Janda, Human antibodies against *Bacillus*: a model study for detection of an protection against anthrax and the bioterrorist threat, Proceedings of the National Academy of Sciences of the United States of America 99 (2002) 5241–5246.

[7] S.S. Iqbal, M.W. Mayo, J.G. Bruno, B.V. Bronk, C.A. Batt, J.P. Chambers, A review of molecular recognition technologies for detection of biological threat agents, Biosensors and Bioelectronics 15 (2000) 549–578.

[8] D.G. de Viedma, Rapid detection of resistance in *Mycobacterium tuberculosis*: a review discussing molecular approaches, Clinical Microbiology and Infection 9 (2003) 349–359.

[9] T.D. Brock, M.T. Madigan, Biology of Microorganisms, 6th ed, 1991.

[10] C. Carriere, P.F. Riska, O. Zimhony, J. Kriakov, S. Bardarov, J. Burns, J. Chan, W.R. Jacobs, Conditionally replicating luciferase reporter phages: improved sensitivity for rapid detection and assessment of drug susceptibility of *Mycobacterium tuberculosis*, Journal of Clinical Microbiology 35 (1997) 3232–3239.

[11] A.S. Mittelmann, E.Z. Ron, J. Rishpon, Amperometric quantification of total coliforms and specific detection of *Escherichia coli*, Analytical Chemistry 74 (2002) 903–907.

[12] R. Capita, C. Alonso-Calleja, L. Mereghetti, B. Moreno, M.D. Garcia-Fernandez, Evaluation of the international phage typing set and some experimental phages for typing of *Listeria monocytogenes* from poultry in Spain, Journal of Applied Microbiology 92 (2002) 90–96.

[13] R. Schuch, D. Nelson, V.A. Fischetti, A bacteriolytic agent that detects and kills *Bacillus anthracis*, Nature 418 (2002) 884–889.

[14] T. Neufeld, A. Schwartz-Mittelmann, D. Biran, E.Z. Ron, J. Rishpon, Combined phage typing and amperometric detection of released enzymatic activity for the specific identification and quantification of bacteria, Analytical Chemistry 75 (2003) 580–585.

[15] R. McNerney, B.S. Kambashi, J. Kinkese, R. Tembwe, P. Godfrey-Faussett, Development of a bacteriophage phage replication assay for diagnosis of pulmonary tuberculosis, Journal of Clinical Microbiology 42 (2004) 2115–2120.

[16] A.I. Kim, P. Ghosh, M.A. Aaron, L.A. Bibb, S. Jain, G.F. Hatfull, Mycobacteriophage Bxb1 integrates into the *Mycobacterium smegmatis* groEL1 gene, Molecular Microbiology 50 (2003) 463–473.