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Gene-based identification of bacterial colonies with an electric chip

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Abstract

A method for the identification of bacterial colonies based on their content of specific genes is presented. This method does not depend on DNA separation or DNA amplification. *Bacillus cereus* carrying one of the genes (*hblC*) coding for the enterotoxin hemolysin was identified with this method. It is based on target DNA hybridization to a capturing probe immobilized on magnetic beads, followed by enzymatic labeling and measurement of the enzyme product with a silicon-based chip. An *hblC*-positive colony containing 10^7 cells could be assayed in 30 min after ultrasonication and centrifugation. The importance of optimizing the ultrasonication is illustrated by analysis of cell disruption kinetics and DNA fragmentation. An early endpoint PCR analysis was used to characterize the DNA fragmentation as a function of ultrasonication time. The first minutes of sonication increased the signal due to both increased DNA release and increased DNA fragmentation. The latter is assumed to increase the signal due to improved diffusion and faster hybridization of the target DNA. Too long sonication decreased the signal, presumably due to loss of hybridization sites on the targets caused by extensive DNA fragmentation. The results form a basis for rational design of an ultrasound cell disruption system integrated with analysis on chip that will move nucleic acid-based detection through real-time analysis closer to reality.

Keywords: *Bacillus cereus*; Enterotoxin; Hemolysin; DNA fragmentation; Ultrasonication

Increasing concerns regarding food contamination by microorganisms have made more critical the importance of developing fast, reliable, and sensitive analytical methods for use in the monitoring of pathogens [1–5]. Traditional methods to detect food-borne bacteria rely on time-consuming growth in culture media followed by isolation, biochemical identification, and sometimes serological determination [6,7]. In many cases, it is not enough to identify a contaminating bacterium only at the species level. For instance, only approximately 50% of isolated *Bacillus cereus* was classified as pathogenic [8], and *Escherichia coli* is mostly not pathogenic, but some strains are harboring genes for toxins (e.g., shiga toxin [9]) that may cause fatal diseases. Furthermore, the antibiotics resistance of bacteria can mostly be genetically defined, and it is strain dependent rather than species dependent. These problems increase the demand for genetically based diagnostic assays.

The developments in bioinformatics have widened the basis for organism identification to also include nucleic acid analysis. Thus, new analytical instruments, monitoring devices, and rapid test kits have been created to detect and quantify bacteria [10–13]. Among them, DNA-sensing systems have become a powerful tool for the detection of various pathogenic microorganisms [2,14].

We recently reported on rapid detection of specific nucleic acid sequences by means of electric chips [15]. This method permits DNA analysis of microorganisms without prior nucleic acid purification or amplification

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Table 1
Characteristics of oligonucleotide primers and probes used in this study

Name	5′ position	Function	Nucleotide sequence[a] (5′–3′)
HblC U-ABCDEF	270	PCR upper primer ABCDE for *hblC* gene	TAATGTTTTAATGAACAACATAACT
HblC L-A	1180	PCR lower primer A for *hblC* gene	GATAGAGTTCCGATGACCATTCCTT
HblC L-B	1016	PCR lower primer B for *hblC* gene	ATATCCATGCCTTCCTGTTGAGTTT
HblC L-C	751	PCR lower primer C for *hblC* gene	TACTTACCTCTCACTTCGATACTCT
HblC L-D	624	PCR lower primer D for *hblC* gene	ACAGAACCGCGAGAATCAATAAACC
HblC L-E	456	PCR lower primer E for *hblC* gene	CACTTTTGTTATGCAGASAACTTAGA
HblC L-F	343	PCR lower primer F for *hblC* gene	CACTATAATTCCTATTAGCGTAACC
HblC C	270	Capture probe for *hblC* gene	xTCAGTAATGTTTTAATGAACAACATAACT
HblC D	296	Detection probe for *hblC* gene	GTATGACCAGACAGAAAGGATAAGGACTAy

[a] x and y are for amino group and biotin in that order.

by PCR [16]. One application is determination of the presence of pathogenic strains of *B. cereus*. This organism is widely distributed in nature and commonly occurs in a variety of foods where it may produce different toxins [8,17]. The detection of this bacterium by classical methods often requires selective enrichments of up to 48 h followed by selective plating for 24–48 h. Thus, the rapidity and simplicity of *B. cereus* DNA analysis using electrochemical detection on a chip is a promising alternative. The method detects the selected pathogenicity-encoding nucleic acid sequence of *B. cereus* when it simultaneously hybridizes with a single-stranded DNA capture molecule immobilized on a solid surface of magnetic microbeads and a DNA detection probe molecule from a solution labeled with an enzyme. A miniaturized amperometric biosensor device enables evaluation of biomolecular interactions by measuring the redox recycling of enzymatic reaction products [18]. When applied to analysis of bacterial colonies, the main sample preparation includes only suspension of the colony in a buffer, ultrasonication, and centrifugation [16].

We report here on the optimization of the ultrasonication with the purpose of fragmenting the DNA and thereby improving the hybridization rate. We also explore an early endpoint semiquantitative PCR as a simple and inexpensive method for evaluating the DNA fragmentation without requiring expensive equipment or sophisticated probe preparation.

Materials and methods

Reagents

ExtrAvidin alkaline phosphatase conjugates (Ext-ALP),[1] bovine serum albumin (BSA), 100 mg/ml carbodiimide (EDC), 0.1 M ethanolamine/deoxynucleotide mix (each dNTP 10 mM), and *Taq* DNA polymerase (5 U/μl) and PCR buffer were purchased from Sigma (Steinheim, Germany). *p*-Aminophenyl phosphate (pAPP) was purchased from ICN Biomedicals (Aurora, OH, USA). Paramagnetic beads (Dynabeads M-270 carboxylic acid) were obtained from Dynal (Oslo, Norway). 2-[*N*-morfolino]ethonesulfonic acid (MES, 0.4 M) was adjusted to pH 5.0. Tris-buffered saline (TBS) was prepared by dissolving 30 mM tris(hydroxymethyl)aminomethane and 100 mM sodium chloride in water and adjusting to pH 8.0 by adding hydrochloric acid. Phosphate-buffered saline (PBS, pH 7.4) contained 2 mM sodium dihydrogen phosphate monohydrate, 8 mM disodium hydrogen phosphate dihydrate, and 150 mM sodium chloride. Dulbecco's buffered saline (DBS, pH 7.3) was prepared by dissolving 160 mM sodium chloride, 3 mM potassium chloride, 8 mM disodium hydrogen phosphate dihydrate, and 1 mM potassium hydrogen phosphate dihydrate.

Oligonucleotides

Purified oligodeoxynucleotides (with 5′ amino group or 3′ biotin modification) were purchased from Thermo Hybaid (Ulm, Germany). The designed primer pairs for PCR and probes for chip analyses are listed in Table 1. The oligonucleotide design is based on sequence complementarity to the selected toxin gene. The amino groups or biotin were linked to the probes with a spacer sequence of a few bases in length, each of which was selected noncomplementary to the target strand. HblC U-ABCDEF (upper primer) and HblC L-A, HblC L-B, HblC L-C, HblC L-D, HblC L-E, and HblC L-F (lower primer-A, -B, -C, -D, -E, and -F, respectively) were primer pairs designed from *hblC* sequence by computer analysis using the Oligo primer analysis software (MedProbe, Oslo, Norway). With the exception of the linker, the capture probe (C) was identical in sequence to the upper PCR primer HblC U-ABCDEF. The detection probe (D) was chosen to hybridize with only a 1-bp space directly next to the capturing probe [19]. In this way, the probe names HblC C and HblC D are abbreviated from *hblC* capture and *hblC* detection, respectively (Fig. 1).

[1] *Abbreviations used:* Ext-ALP, ExtrAvidin alkaline phosphatase; BSA, bovine serum albumin; EDC, carbodiimide; pAPP, *p*-aminophenyl phosphate; MES, 2-[*N*-morfolino]ethonesulfonic acid; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; DBS, Dulbecco's buffered saline; pAP, *p*-aminophenol.

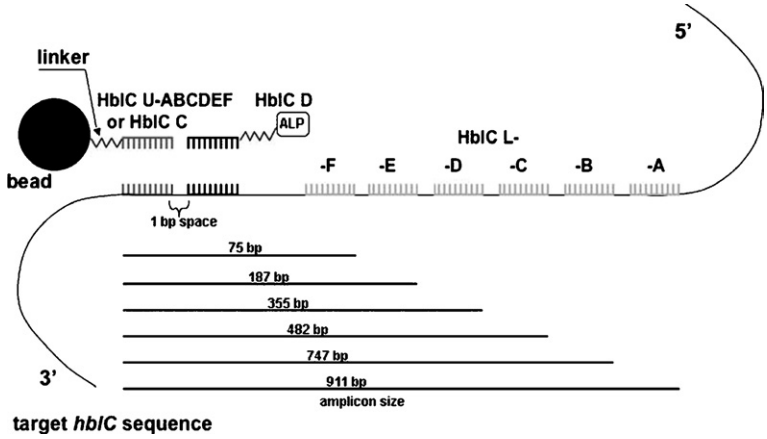

Fig. 1. Illustration of the bead-based sandwich hybridization and different alternative positions of the primers (HblC L-A, -B, -C, -D, -E, and -F) along the target *hblC* sequence. The positions and probe sequences are specified in Table 1. Different sizes of PCR products are indicated.

Cell sampling

One bacterial colony was transported from agar plate into an Eppendorf tube, suspended in 1 ml PBS buffer, and used for the analyses.

Preparation of crude cell lysates for hybridization assay

Cells were disrupted by ultrasonication to obtain lysates. The ultrasonic device was a Branson Ultrasonic Disruptor with a microtip 1 mm in diameter. The operating frequency was 30 kHz, and effective output power at the microtip was 100 W. During ultrasound cavitations, the samples were cooled in an ice water bath until completion of the procedure. After a heat treatment (95 °C, 10 min) and removal of the solid particles by centrifugation (5000*g*, 10 min), the lysates were subjected directly to the assay.

Electric signal generation

Fig. 1 illustrates the major steps of the electric chip assay. The detailed procedures of the conjugation of NH_2 single-stranded capture probe to M-270 carboxylic acid beads, as well as DNA sandwich hybridization and electrochemical detection, were described previously [15,16]. Also, details of the instrument and characteristics of the electrochemical detection were described previously [15,18]. In principle, the method is based on an electric chip combined with a bead-based sandwich hybridization that was directly employed on unpurified sample. The magnetic particles with carboxyl groups on the surface were used for covalent attachment of a capture probe containing amino group via a six-carbon atom linker. Target DNA in the cell lysate was exposed at the same time to capturing beads and biotin-labeled detection probes. Ext-ALP was then added to label the detection probe. For signal generation at the electric chip, we used an enzyme substrate, pAPP, that forms the product *p*-aminophenol (pAP) on reaction with ALP. pAP was redox cycled at the chip electrodes, thereby producing an electrical current in a nanoampere range that was related to the number of target DNA molecules present in the sample.

Flow cytometry

Flow cytometry was used to analyze the number of cells in colonies quantitatively isolated from agar plates. A PAS flow cytometer (Partec, Münster, Germany) with 488 nm excitation from an argon-ion laser at 20 mW was used. Interferences from system noise and nonmicrobial particles were minimized by appropriate instrument setup, careful calibration, and filtration (0.2 μm) of all solutions prior to use. The suspended colony was further diluted 10× with DBS buffer, resulting in 1 to 2×10^6 cells/ml, which is the recommended cell density for the flow cytometry measurements. The suspension was analyzed at a flow rate of 1500–2500 counts/s. Partec FloMax software (version 2.4b) and MATLAB were used for data analysis and for collecting histograms of forward scatter as a function of time. The forward scatter is considered to represent the size of cells and other measured particles [20,21].

Early endpoint multiple-priming PCR

DNA of *B. cereus* strain ATCC 14579 (bacterium purchased from the American Type Culture Collection, Manassas, VA, USA) was used as template. PCR assays were performed in a DNA Thermal Cycler (MJ Research, Waltham, MA, USA). Reaction volumes of 50 μ l contained 5 μl of genomic DNA (~5 ng of DNA), 2.5 U of *Taq* polymerase, deoxynucleoside triphosphates at a concentration of 200 μM each, and primers at 0.5 μM each in reaction buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3). The amplification of specific fragments was performed by PCR with

the following parameters: one cycle of DNA predenaturation (95 °C, 4 min); 16, 20, or 23 cycle repeats, each consisting of denaturation (95 °C, 45 s), primer annealing (55 °C, 1 min), and DNA elongation (72 °C, 2 min); and a final extension step (72 °C, 10 min). The GeneBank sequence for the *B. cereus* (Accession No. AJ237785) hemolysin gene, *hblC*, was used to design the primer pairs HblC U-ABCDE and HblC L-A, -B, -C, -D, -E, and -F (Fig. 1) for the amplification of 911-, 747-, 482-, 355-, 187-, and 75-bp fragments, respectively. The upper oligonucleotide from each primer pair was identical in sequence to the capturing probe with the exception of the linker used in the *hblC* assays (Fig. 1). All primers were considered to have a similar annealing temperature of 55 °C. Amplification products were detected by subsequent agarose gel electrophoresis, and the results were quantified by densitometric scanning.

Results

Determination of cell number in colony

The average diameter of the *B. cereus* colonies on agar plate was 3.5 ± 0.4 mm. The cell numbers in these colonies were counted by flow cytometry and evaluated against data of viable cell counting on agar plates (cfu). Both methods showed comparable values of 10^7 cells per colony (data not shown).

Assay for identification of hemolysin encoding B. cereus colonies

Electrochemical measurements for detection of DNA of the *B. cereus hblC* gene were performed with the electric silicon chip according to the protocol described in Materials and methods. Prior to the analysis, a single colony was ultrasonicated for 30 s, 1 min, 5 min, 10 min, or 13 min and was subjected directly to the assay after heat treatment and centrifugation. The negative control with nonultrasonicated cells did not yield any signal (Fig. 2). In general, the electric signal increased with ultrasonication time up to 10 min, but further sonication reduced the signal. This reduction presumably was a result of excessive DNA degradation that occurs after sonication disintegrates the cells.

In addition to the enzyme reaction, the hybridization step is a major signal-limiting reaction in the protocol [15,16]. To study this, the signal was documented under conditions where hybridization time was varied (10 min, 2 h, or 3 h) while the other protocol steps were constant. Samples sonicated for 10 min generated higher signals with longer hybridizations, presumably due to increased time for diffusion of DNA across the chip (data not shown). Thus, the increased signal with increased ultrasonication time (Fig. 2) might be due to improved hybridization efficiency caused by fragmentation of the large DNA molecules. However, when samples sonicated for 13 min were assayed, a decrease in signal was observed, possibly due to DNA overfragmentation.

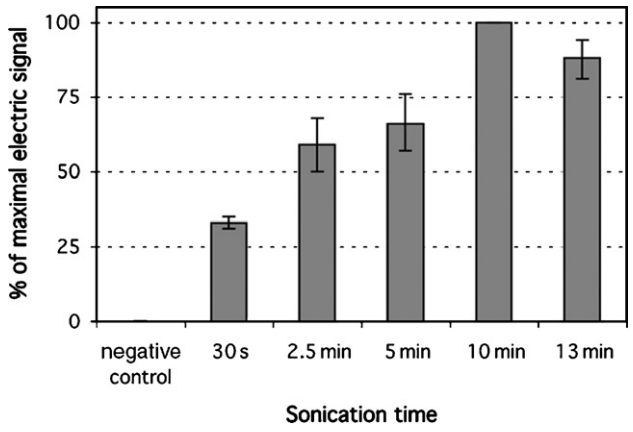

Fig. 2. Influence of ultrasonic disruption time on signal of *B. cereus* DNA in crude cell lysate. A suspension of vegetative cells was continuously disrupted with sampling after 30 s, 1 min, 5 min, 10 min, and 13 min. The ultrasonic power output was 100 W. Target cells (10^7) from the lysates were analyzed in an assay containing 2×10^7 capturing beads and 10 nM HblC D probe (2 h hybridization at 40 °C, 30 min enzyme binding at room temperature, and 30 min enzymatic reaction at 30 °C). Bars represent the standard errors on at least three independent determinations per sonication time (error bars at 10 min are not visible).

Kinetics of cell disruption by ultrasonication

To evaluate the cell disruption during ultrasonication, single colonies containing 10^7 *B. cereus* cells were subjected to ultrasonic disintegration followed by flow cytometry analysis. Fig. 3 shows the forward scatter profiles obtained for each sample. Initially, one broad peak with a strong signal representing nondisrupted cells was observed. With increasing ultrasonication time, this signal gradually became weaker and most of the main peak

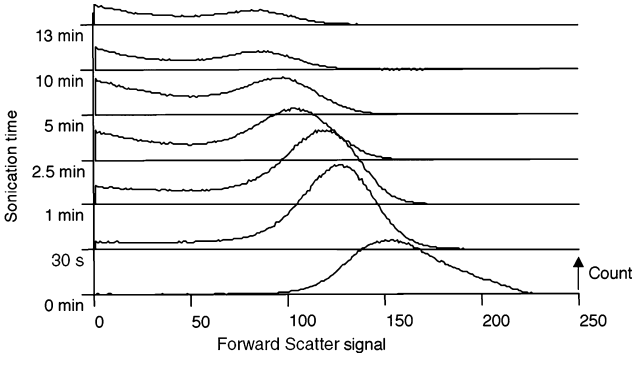

Fig. 3. Kinetics of cell disruption by ultrasonication as shown by a histogram of forward scatter values from *B. cereus* cells subjected to 0–13 min ultrasonication.

corresponding to the undisrupted cells disappeared after 13 min sonication.

DNA fragmentation pattern during ultrasonication

Agarose gel electrophoresis was used to determine the size distribution of DNA released from cells subjected to ultrasonic disruption (Fig. 4). Highly fragmented DNA is evident from the presence of a DNA smear rather than high-molecular weight bands that were eliminated from samples sonicated for 2.5 min or longer. Longer sonication gradually reduced fragment lengths to approximately 1.5–0.15 kb, and sonication for 13 min further degraded these fragments, as can be seen by the lower intensity of the smear as compared with the 10-min sample. Thus, the average DNA fragment size gradually declined with ultrasonication time.

Although this protocol is simple, it is only a rough method due to its limitations in sensitivity and accuracy. For this reason, a semiquantitative early endpoint PCR was also evaluated.

Semiquantitative early endpoint PCR analysis of DNA fragment size

This PCR-based method was used to measure the number of copies of a particular DNA fragment from colony samples that were sonicated for 30 s, 1 min, 2.5 min, 5 min, 10 min, and 13 min (Fig. 5). The most striking result is the different patterns for higher molecular weight PCR products (747 and 911 bp) as compared with lower molecular weight products (75, 187, 355, and 482 bp). For the long DNA fragments, a maximum number of amplicons was observed after 1 min ultrasonication, followed by a rapid decline with further increases in sonication time. When the shorter fragments were analyzed, the number of amplicons increased with sonication time up to approximately 5 min (75-, 187-, 355-, and 482-bp fragments). The number of amplicons for these fragment sizes also decreased after more than 5 min ultrasonication. The extent of amplicon decrease was related to fragment size, with the largest decreases for the longest fragments.

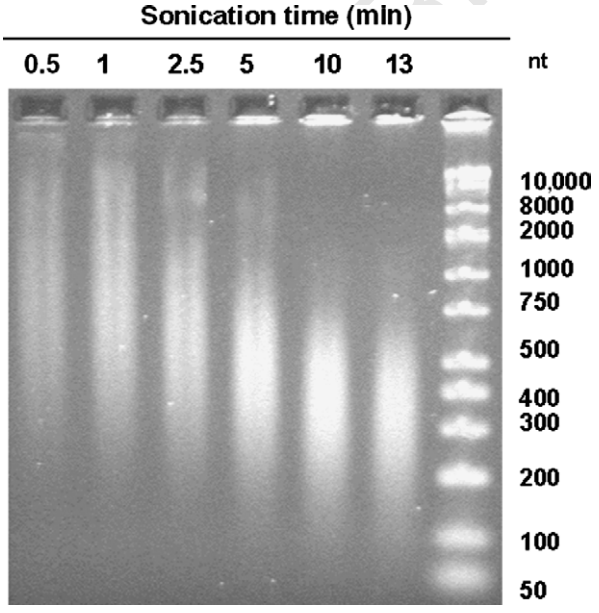

Fig. 4. Distribution of sonicated genomic *B. cereus* DNA in agarose gel.

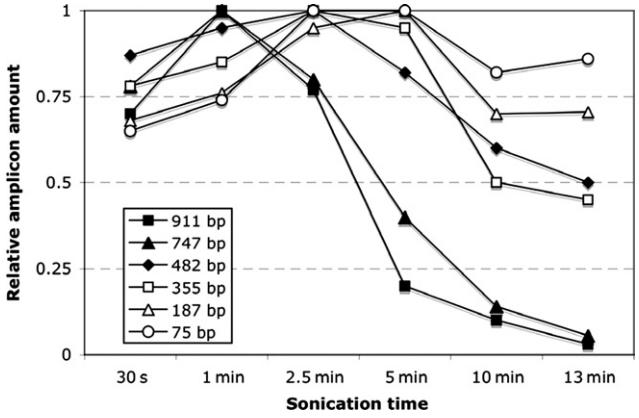

Fig. 5. PCR product analysis via semiquantitative early endpoint PCR for *B. cereus* DNA fragmentation assessment. Determination of the exponential range of amplification for *hblC* was carried out after 23 cycles for 747- and 911-bp fragments; 20 cycles for 187-, 355-, and 482-bp fragments; and 16 cycles for the 75-bp fragment. Reactions were performed in the same conditions but with different primer sets (Table 1), and samples were sonicated for times ranging from 30 s to 13 min.

Discussion

The goal of the hybridization step in the assay is to distribute target DNA sequences in the solution for annealing with complementary probes fixed to a solid surface. However, this requires that the target genomic DNA be released from bacterial cells so that it is available in the assay solution. Among the methods available for cell disruption at the laboratory scale, ultrasonication is one of the most commonly employed methods [22–24] because it requires neither sophisticated equipment nor extensive technical training. The amount of energy that must be put into the breakage of cells depends greatly on the type of organism and, to some extent, on the physiology of the cell. Some types of cells are broken readily (e.g., some gram-negative bacteria such as *E. coli*), whereas some gram-positive microorganisms are more resistant (e.g., *B. cereus*). Therefore, an implementation of a small-scale disruption procedure with the use of ultrasonication was presented here.

The study achieved a sensitivity of 10^7 molecules using an electric chip assay of 30 min (data not shown). There are three main parameters that determine the sen-

sitivity of this assay: the sonication efficiency (Fig. 2), the hybridization time (data not shown) [15], and the enzyme reaction time [15]. However, the shape of the plot of the chip signal against sonication time (Fig. 2) required further characterization of the cell disruption.

Flow cytometry was used to monitor the ultrasonication process because it allows quantitative measurements at rates of several thousand cells per second and can reveal some physical properties of the particles being measured. The disintegration of cells and the formation of small particles were observed by the gradual decrease of the forward scatter signal (Fig. 3), which is considered to reflect the particle size [20,21]. The 30-s sonication reduced the mean particle size considerably; after 5 min, no forward scatter signal was observed at the value representing the mean value of the untreated cells, and further sonication decreased the signal even more. Microscopic observation confirmed this trend (data not shown).

However, there was a strong increase in the electric signal from 5 to 10 min sonication (Fig. 2) even though additional cell disruption was very low at more than 5 min sonication (Fig. 3). This indicates that sonication effects on the DNA may be involved in the amplification of the signal at greater sonication times. The DNA first released from the cells has a very high molecular weight, creating a highly viscous sample. Under these conditions, the DNA diffusion to the capture probes is probably severely hindered, and this should result in a low signal.

Sonication fragmented large DNA molecules [25] once the whole cell was disintegrated. Given that the size of the DNA will influence the hybridization rate, it is important to know the size of DNA fragments generated for the assay. Agarose gel electrophoresis studies demonstrated that 10 min ultrasonication converted high-molecular weight DNA of *B. cereus* to fragments ranging from approximately 100 to 600 bp (Fig. 4). Semiquantitative early endpoint PCR was used to assess the DNA fragmentation more accurately. In general, amplification of DNA fragments can be detected either with endpoint analysis (when cycling is complete) or with real-time analysis (while the reaction is occurring). For endpoint analysis, PCRs may be run on any thermal cycler and analyzed with gel electrophoresis; there is no requirement for an instrument capable of real-time quantitative PCR. However, endpoint analysis can provide only semiquantitative results due to differences between samples in the concentrations of reaction components that may be limiting as the reaction progresses. Thus, in this study, we performed an early endpoint PCR analysis. In general, early endpoint PCR includes only the initial and logarithmic phases and the plateau stage is omitted. Thus, the amount of amplified product in each sample is determined by the initial copy number of template for that sample. By attempting to amplify different length products, we can gauge the degree of DNA fragmentation in the samples.

Using the early endpoint PCR, the relative amount of fragmented nucleic acid targets was analyzed from the lysates processed with different ultrasonic times. The results showed that the ultrasonic treatment for lysis of bacterial cells first leads to disruption of cellular structures and release of DNA. Thus, an increased number of potential target DNA molecules was obtained during the first minutes of sonication. As the extent of exposure to ultrasound increased, the proportion of fragmented DNA molecules increased (Fig. 5). As a result, an improved diffusion-driven target movement is assumed to increase the efficiency of the hybridization. These data are consistent with our biochip assay results. Using a longer time than 10 min of the disruption caused a decrease in the signal, possibly due to a loss of potential DNA target molecules caused by cleavage of DNA fragments containing hybridization sites for both the capture probe and the detection probe (Figs. 2 and 5).

These results emphasize the importance of controlling not only the cell disruption efficiency but also the DNA fragmentation in the preparation of samples for DNA hybridization on solid surfaces. Initially, increased fragmentation improves the signal strength by making it easier for the DNA to diffuse and hybridize. However, if sonication continues for too long, the target DNA fragments become so small that they lose at least one of their hybridization sites.

The method demonstrated here should be applicable to a wide variety of microbial analyses for several reasons. First, in food and clinical analytical microbiology, the initial step in the analysis is often a precultivation that results in colonies that are then subjected to confirmative analyses. Second, the cell disintegration by ultrasonication is a common technique with large applicability, and the DNA fragmentation by ultrasonication can be expected to be organism independent. Finally, the hybridization reaction used to detect the specific gene of interest is a common principle in gene analysis.

Acknowledgments

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References

[1] N. Cook, The use of NASBA for the detection of microbial pathogens in food and environmental samples, J. Microb. Methods 53 (2003) 165–174.

[2] A.K. Deisingh, M. Thompson, Biosensors for the detection of bacteria, Can. J. Microbiol. 50 (2004) 69–77.
[3] R.H. Liu, J. Yang, R. Lenigk, J. Bonanno, P. Grodzinski, Self-contained, fully integrated biochip for sample preparation, polymerase chain reaction amplification, and DNA microarray detection, Anal. Chem. 76 (2004) 1824–1831.
[4] T.A. McMeekin, Detecting Pathogens in Food, Woodhead Publishing, Cambridge, UK, 2003.
[5] N. Sergeev, M. Distler, S. Courtney, S.F. Al-Khaldi, D. Volokhov, V. Chizhikov, A. Rasooly, Multipathogen oligonucleotide microarray for environmental and biodefense applications, Biosens. Bioelectron. 20 (2004) 684–698.
[6] R.L. Buchanan, F.J. Schultz, Comparison of the Tecra VIA kit, Oxoid BCET-REPLA kit, and CHO cell culture assay for the detection of *Bacillus cereus* diarrhoeal enterotoxin, Lett. Appl. Microbiol. 19 (1994) 353–356.
[7] T.L. Day, S.R. Tatani, S. Notermans, R.W. Bennnett, A comparison of ELISA and RPLA for detection of *Bacillus cereus* diarrhoeal enterotoxin, J. Appl. Bacteriol. 77 (1994) 9–13.
[8] V. Mäntynen, K. Lindström, A rapid PCR-based DNA test for enterotoxic *Bacillus cereus*, Appl. Environ. Microbiol. 64 (1998) 1634–1639.
[9] J.C. Paton, A.W. Paton, Methods for detection of STEC in humans: an overview, Methods Mol. Med. 73 (2003) 9–26.
[10] A.E. Grow, L.L. Wood, J.L. Claycomb, P.A. Thompson, New biochip technology for label-free detection of pathogens and their toxins, J. Microbiol. Methods 53 (2003) 221–233.
[11] D. Ivnitski, I. Abdel-Hamid, P. Atanasov, E. Wilkins, S. Stricker, Application of electrochemical biosensors for detection of food pathogenic bacteria, Electroanalysis 12 (2000) 317–325.
[12] Y.-R. Kim, J. Czajka, C.A. Batt, Development of a fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in nonfat dry milk, Appl. Environ. Microbiol. 66 (2000) 1453–1459.
[13] V.A. Petrenko, V.J. Vodyanoy, Phage display for detection of biological threat agents, J. Microbiol. Methods 53 (2003) 253–262.
[14] F. Lucarelli, A. Kicela, I. Palchetti, G. Marrazza, M. Mascini, Electrochemical DNA biosensors for analysis of wastewater samples, Bioelectrochemistry 58 (2002) 113–118.
[15] M. Gabig-Ciminska, A. Holmgren, H. Andresen, K.B. Barken, M. Wümpelmann, J. Albers, R. Hintsche, A. Breitenstein, P. Neubauer, M. Los, A. Czyz, G. Wegrzyn, G. Silfversparre, B. Jürgen, T. Schweder, S.-O Enfors, Electric chips for rapid detection and quantification of nucleic acids, Biosens. Bioelectron. 19 (2004) 537–546.
[16] M. Gabig-Ciminska, H. Andresen, J. Albers, R. Hintsche, S.-O Enfors, Identification of pathogenic microbial cells and spores by electrochemical detection on a biochip, Microbiol. Cell Factories 3 (2004) 1–11.
[17] A.B. Gi, M. Skeie, T. Sorhaug, T. Langsrud, P.E. Granum, Growth and toxin profiles of *Bacillus cereus* isolated from different food sources, Intl. J. Food Microbiol. 28 (2001) 237–246.
[18] J. Albers, T. Grunwald, E. Nebling, G. Piechotta, R. Hintshe, Electrical biochip technology: a tool for microarrays and continuous monitoring, Anal. Bioanal. Chem. 377 (2003) 521–527.
[19] K.B. Barken, M. Gabig-Ciminska, A. Holmgren, S. Molin, Effect of unlabeled helper probes on detection of an RNA target by bead-based sandwich hybridization, BioTechniques 36 (2004) 124–132.
[20] G. Boeck, Current status of flow cytometry in cell and molecular biology, Intl. Rev. Cytol. 204 (2001) 239–298.
[21] H.M. Shapiro, Optical measurements in cytometry: light scattering, extinction, absorption, and fluorescence, Methods Cell Biol. 63 (2001) 107–129.
[22] K.A.J. Borthwick, W.T. Coakley, M.B. McDonnell, H. Nowotny, E. Benes, M. Groschl, Development of a novel compact sonicator for cell disruption, J. Microbiol. Methods 60 (2005) 207–216.
[23] J.X. Feliu, R. Cubarsi, A. Villaverde, Optimized release of recombinant proteins by ultrasonication of *E. coli* cells, Biotechnol. Bioeng. 58 (1998) 536–540.
[24] P. Belgrader, D. Hansford, G.T.A. Kovacs, K. Vanketeswaran, R. Mariell, F. Milanovich, S. Nasarabadi, M. Okuzumi, F. Pourahmadi, M.A. Northrup, A minisonicator to rapidly disrupt bacterial spores for DNA analysis, Anal. Chem. 71 (1999) 4232–4236.
[25] E.M. Fykse, J.S. Olsen, G. Skogan, Application of sonication to release DNA from *Bacillus cereus* for quantitative detection by real-time PCR, J. Microbiol. Methods 55 (2003) 1–10.