Electric DNA chips for determination of pathogenic microorganisms

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Abstract

Silicon-based electric DNA chip arrays were utilized to fast identify pathogenic microorganisms with respect to the capacity to produce toxins involved in foodborne poisoning and infections. Bacteria of the *B. cereus* and the enterohemorrhagic *E. coli* (EHEC) groups contain different set-ups of various virulence factors that are encoded by the corresponding genes. The purpose of this work was to develop a fast and simple method for determination of the presence of these virulence genes in a colony from primary enrichment cultures. A target gene is detected through hybridization to a surface-immobilized specific capture probe and biotin-labeled detection probe. Following binding of an enzyme conjugate to this sandwich hybrid complex, a current signal is generated by electronic redox recycling of the enzymatic product p-aminophenol (pAP). Two versions of the assay were developed. In the first version the capture probes were immobilized on magnetic beads, which carried out all reactions until the pAP generation, while the final electric signal was created by transferring pAP to a single-electrode chip surface. In the second version a silicon chip array with 16 parallel sensing electrode positions each of them functionalized by capture probes, carried out all assay steps on the chip surface. This instrument can realize automatic and multiplexed gene detection. The kinetics of bacterial cell disruption and impact of DNA fragmentation by ultrasound were determined. The experimental data suggested that the increased signal after first minutes of ultrasonication were due to the accumulation of released DNA amount, while the further signal increase resulted from the improved hybridization with the shortened target DNA strands. Studies on probe localization on the 16-electrode chip assays indicated that the probe-targeting site, which was located at the 5’-end of strands, gave rise to the highest signal level due to the efficient target-probes hybridization and the following enzyme binding. When these functionalized chip arrays were exposed to the cell homogenates, the sensing electrodes were fouled by cellular proteins and therefore led to dramatically decreased redox-recycling current. To circumvent this, samples were treated by DNA extraction after the 1st sonication and then DNA fragmentation by a 2nd time sonication. The DNA extract removed most of
the interfering components from bacterial cell. This sample treatment was applied to characterize one “diarrheal” and one “emetic” strain of *B. cereus* with the chip arrays functionalized by eight DNA probes. The signal patterns of eight virulence genes from chip assays agreed well with PCR control analyses for both strains. By simply adding the SDS detergent to cell homogenates, chip surface blocking effect can be significantly reduced even without DNA extraction treatment. After optimization of some critical factors, the 16-electrode DNA chips with the improved sensing performance can directly detect multiple virulence genes from a single *E. coli* colony in 25 min after the introduction of supernatant of ultrasonicated cell lysate.

Keywords: electric DNA chip array, fast determination, virulence genes, multiplexed gene detection, bacterial colony, ultrasonication, DNA fragmentation, *Bacillus cereus*, enterohemorrhagic *E. coli*. 
To my parents
List of publications

This thesis is based on the following papers, which in the text are referred to by their Roman numerals, and some unpublished data:


Contribution to papers:

Paper I: Joined the experiment design, performed all the experiments and analyzed data.

Paper II: Joined the experiment design, performed all the experiments except the chip spotting, analyzed data and wrote the paper.

Paper III: Joined the experiment design, performed all the experiments except the chip spotting and selection of mismatch nucleotides and different linkers of probes, analyzed data and wrote the paper.

Paper IV: Joined the experiment design, performed all the experiments except the chip spotting and selection of the *E. coli* strains’ primers/probes, analyzed data and wrote the paper.
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1. Introduction

1.1 Foodborne microbial pathogens

Foodborne microbial pathogens cause diseases by intoxications and/or infections (Madigan, Martinko et al. 2000). Microbial intoxication results from the intake of toxins secreted by pathogens. *Staphylococcus aureus* and emetic *Bacillus cereus* are typical such pathogens, their secreted enterotoxins and the cereulide peptide toxin lead to severe nausea, vomiting and diarrhea symptoms. Microbial infection is due to the infection of the pathogens themselves and their further interactions with the host. *Salmonella* and pathogenic *Escherichia coli*, e.g. EHEC, are typical such pathogens, their proliferations in the human gut give rise to gastrointestinal diseases. Two main factors play a role in this microbial infection. One is the host colonization-associated genes carried by pathogens, another is the functional genes contributing to pathogenesis (Dobrindt 2005).

1.1.1 The *Bacillus cereus* group

*Bacillus cereus* are ubiquitous in the environment and also in food products. They are gram-positive, aerobic spore forming bacteria. The vegetative cells are normally large and rod-like (Fig. 1A). They can survive in different adverse conditions, e.g. during food processing, because of their ability to form resistant endospores (Fig. 1B).

![Vegetative cells and endospores of B. cereus ATCC14579](image)

Figure 1. A microscopic image of vegetative cells (A) and endospores (B) of *B. cereus* ATCC14579.
*B. cereus* has been reported to be an important pathogen responsible for food poisoning and food spoilage (Pruss et al. 1999; Ghelardi et al. 2002; Thaenthanee et al. 2005). Six different species have been classified and characterized in the *Bacillus cereus* group. *Bacillus anthracis*, a primary virulent pathogen for animals, possesses two specific virulence-encoding plasmids coding for a capsule and the anthrax toxin; *Bacillus thuringiensis*, a pathogen for some insects, produces parasporal crystal proteins encoded by its plasmid and has been commercialized for the use as an insecticide; the rhizoid colony forming *Bacillus mycoides* (Nakamura 1998) has been classified from other bacteria of *B. cereus* group due to their different fatty acid composition; *Bacillus pseudomycoides* (Nakamura 1998) recently was separated from *Bacillus mycoides* due to their distinct genotypes, but they are not distinguishable by their phenotypes; *Bacillus weihenstephanensis* (Lechner, Mayr et al. 1998) is classified by its psychrotolerant properties, and grows at 4 - 7 °C, not at 43 °C; *Bacillus cereus*, a common soil inhabitant and often identified from a variety of foods, including grains, dairy products and meat, is a foodborne pathogen and its toxins are encoded by both the chromosome and the plasmids. This organism grows in a broad temperature range of 8 - 55 °C, and optimally around 28 - 35 °C. *B. cereus* and *B. thuringiensis* are very similar according to their genomes (Priest, Barker et al. 2004). *B. thuringiensis* can only be distinguished from *B. cereus* by production of an intracellular crystal protein encoded by a plasmid.

### 1.1.2 *B. cereus* pathogenicity

*B. cereus* can secrete two main types of toxins, enterotoxins (Hansen and Hendriksen 2001) and emetic toxin (Paananen, Mikkola et al. 2002), which cause foodborne human illness (Fig. 2) (Lund and Granum 1996; Ghelardi, Celandroni et al. 2002; Schoeni and Wong 2005).

The enterotoxins consist of the haemolytic toxin BL (HBL), the nonhaemolytic toxin (NHE) and cytotoxin K (cytK). Both HBL and NHE are enterotoxin complexes composed of three proteins (Beecher and Macmillan 1991; Lund and Granum 1996): HBL is encoded cooperatively by the genes *hblA, hblD and hblC* in one operon; NHE is encoded cooperatively by the genes *nheA, nheB and nheC*. HBL and NHE are responsible for human diarrhoeal illness. This type of illness is normally mild and
usually lasts for less than 24 h. Cytotoxin K is a cytotoxic pore-forming protein encoded only by one gene (Fagerlund, Ween et al. 2004). It is necrotic, haemolytic and very toxic to human intestinal epithelial cells. A severe outbreak involved by cytotoxin K in 1998 led to the death of three people. Recently two different forms, encoded by cytK-1 and cytK-2 genes, have been described (Fagerlund, Ween et al. 2004). CytK-1 shows 89% protein sequence homology with that of cytK-2, but carries much higher toxicity. These toxins have been evaluated and studied well by different research groups. BceT and entFM, which before were believed to express enterotoxins, are now not considered to responsible for such diseases (Choma 2002; Hansen, Hoiby et al. 2003). Cereulide, a cyclic dodecadepsipeptide (Andersson 1998), can cause acute human emesis after 1 to 5 h of its ingestion. This peptide is a mitochondrion-toxin and synthesized by a non-ribosomal peptide synthetase (NRPS) from B. cereus (Horwood, Burgess et al. 2004). This small peptide is very thermo-stable and inert, can tolerate heat, protease, acid, alkaline, even autoclaving. So once it’s secreted by the food contaminating B. cereus, cereulide can’t be inactivated by the food processing and brings a risk to consumers.

Figure 2. Schematic display of different toxins secreted by B. cereus and their corresponding genes.

Around 60% of B. cereus carry HBL genes and most of them carry NHE genes, while cereulide-producing B. cereus are relatively rare. B. cereus bacteria are commonly present in raw and processed foods. They can survive different food treatments by forming spores, and pre-formed cereulide can’t be degraded during food processing. These facts pose a potential food safety problem. It’s normally believed that food
containing > 10³ B. cereus/g is not safe enough for consumption (Rosenquist, Smidt et al. 2005). Furthermore, we need to know accurately what types of toxins can be expressed by the food contaminator B. cereus bacteria. An additional food safety problem is the large similarities between the species in the B. cereus group. The B. cereus enterotoxins are also produced by some other Bacillus species (Hansen, Leser et al. 2001), so it is very important to identify the pathogenic potential of the bacteria of the B. cereus group when they are found in food, rather than the phenotypic properties of species determined by the traditional cultivation protocols.

1.1.3 Diarrheagenic Escherichia coli
Pathogenic E. coli can cause different human infections, e.g. enteric diseases, urinary tract infection and meningitis symptoms. The group of diarrheagenic E. coli normally causes enteric infections. Based on their different virulence features, six categories have been identified (Nataro and Kaper 1998). They are enterotoxigenic E. coli (ETEC) causing the traveler’s diarrhea disease, enteroaggregative E. coli (EAEC), enteroinvasive E. coli (EIEC), diffusely adherent E. coli (DAEC), enteropathogenic E. coli (EPEC) and enterohemorrhagic E. coli (EHEC).

EHEC is associated with the gastrointestinal diseases featured by the syndromes of severe crampy abdominal pain and bloody diarrhea, and occasionally causes the hemolytic uremic syndrome. Studies showed that various virulence factors contribute to these symptoms (Kaper, Nataro et al. 2004). One major virulence factor is Shiga toxins, which are cytotoxic and may interact with epithelial cells by binding to glycolipid receptors. The Shiga toxin (Stx), Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2) all belong to the Shiga toxin family. These virulence factors are encoded by the chromosomal genes stx, stx1 and stx2, respectively. The enterohemolysin toxin Hly is associated with the distinct hemolytic property of EHEC strains, and is encoded by a gene located in a 60-MDa plasmid. The toxin complex intimin EAE, Tir and EspA/EspB/EspD is responsible for the formation of attaching and effacing lesions at epithelial cell surfaces, and the three factors are encoded by the genes eae, tir and the esp genes, respectively. Comparative genomics studies revealed that horizontal transfer, loss or rearrangement of the mobile genetic elements lead to diversified distribution
patterns of various virulence genes in different EHEC strains (Dobrindt 2005). Each distinct set-up of virulence factors in EHEC bacteria elucidates their unique pathogenicity features.

1.2 Toxin detection methods

To date different methods for the detection of microbial pathogens have been developed. Normally they can be divided into three categories: microbiological biochemistry method, immunological method and genetic method (Madigan, Martinko et al. 2000). Microbiological biochemistry method is based on the metabolic phenotype level. The toxin detection is normally carried out on agar or liquid cultures with selective media. However, toxin-encoding genes are not always expressed. Immunological method is based on the detection of presence of target proteins or enzymes expressed by microbes. Currently different methods, e.g. agglutination reactions, immunofluorescence, radioimmunoassay, immunoblot, ELISA and various protein-based biosensors, can be carried out. Genetic method is based on the direct detection of presence of target genes, which is not influenced by the metabolic control of toxin expressions. From different organisms, species down to genes, all can be determined by detecting their individual unique DNA sequences. PCR is a typical such method.

For *B. cereus* pathogenicity, some methods have been developed to detect different secreted toxins. For diarrhoeal toxin detection, a gel diffusion assay is to test a distinct serological entity, due to the discontinuous ring-shaped haemolysis pattern formed by HBL in the blood agar (Rhodehamel, Harmon et al. 2001); other microbiological methods include vascular permeability reaction assay, different cell lines cytotoxicity assay (Beecher and Wong 2000; Rowan, Deans et al. 2001). Based on immunological reactions, two diarrhoeal enterotoxin immunoassay kits were commercialized, OXOID Reverse Passive Latex Agglutination (BCET-RPLA) for the L2 component (encoded by the *hblC* gene) detection of the HBL complex, and TECRA Visual Immunoassay for the 41kDa protein component (encoded by the *nheA* gene) detection of the NHE complex. For the emetic toxin detection based on microbiological methods, the test of motility loss of boar spermatozoa (Andersson 1998), Hep-2 cell culture-based MTT conversion
assay (Finlay, Logan et al. 1999), a rat liver mitochondrial respiratory uncoupling activity assay (Kawamura-Sato, Hirama et al. 2005), and also a chemical method for ceruleide based on HPLC-MS analysis (Haggblom, Apetroaie et al. 2002), have been developed. These methods have some limitations: the time-consuming and laborious procedure, detection not specific for each individual function proteins. Recently, specific antibodies for each of the three components of the B. cereus HBL and NHE complexes (Dietrich 1999; Dietrich, Moravek et al. 2005) have been produced and characterized, so the immunological detection for each distinct virulence factor can be realized.

For the pathogenic E. coli, like the enterohemorrhagic E. coli (EHEC) bacteria, different cell line-culture cytotoxicity assays have been developed for the detection of their secreted Shiga toxins (Ball, Finlay et al. 1996), e.g. the cytotoxicity test of Vero cells. Immunoassays, e.g. ELISA, are also available for the analyses of Shiga toxins, i.e. Stx1, Stx2 and other types, and the attaching and effacing lesions-forming toxin complex intimin (EAE), EspA and others (Pulz, Matussek et al. 2003; Kuhne, Hawes et al. 2004).

As the outcome of the Genome Era, different genome-wide sequencing techniques have been dramatically developed, resulting a variety of sequenced both prokaryotic and eukaryotic organisms’ genomes and genes. This enables various nucleic acid-based methods to be applied for the detection of microbial pathogens and their toxins.

1.3 Nucleic acid-based methods for virulence gene detection
The genes (hblA, hblC, hblD) responsible for HBL toxin, the genes (nheA, nheB, nheC) responsible for NHE toxin, the genes cytK-1 and cytK-2, the gene ces responsible for synthesis of the emetic toxin ceruleide, and three EHEC diarrheal virulence factor genes (stx1, stx2, eae), have all been fully sequenced and can be downloaded from several major databases. The genetic sequence-based methods have impelled the identification of pathogenic microorganisms and types of secreted toxins from the phenotypic level to the genotypic level. Southern and northern blottings are among the earliest methods to detect genetic sequences.
PCR-based detection methods for eight *B. cereus* toxin-related genes, i.e. *hblA, hblC, hblD, nheA, nheB, nheC, cytK, cerNRPS*, and for different EHEC virulence genes, have been intensively explored (Mantynen and Lindstrom 1998; Hansen and Hendriksen 2001; Stenfors, Mayr et al. 2002; Ehling-Schulz, Fricker et al. 2004; Ehling-Schulz, Guinebretiere et al. 2006; Guinebretiere, Fagerlund et al. 2006). These detections are highly sensitive and can differentiate particular genes coding for each type of toxin specifically, even small differences between genes representing different forms or variants of the same type, e.g. *cytK1* and *cytK2* (Guinebretiere, Fagerlund et al. 2006), which can’t be fulfilled by any other conventional methods. With the introduction of real-time PCR, not only the presence, but also the number of initial target genes can be acquired quantitatively (Fykse, Olsen et al. 2003). The present development is towards a small-volume, high-speed, and microchip device-integrated PCR, which can realize the fast, efficient and automatic detection with the reduced cost of reagents and few amount of analytes needed (Roper, Easley et al. 2005).

More recently, along the development in the field of biosensors, different DNA chips or sensors have been derived and applied in many fields, e.g. food safety, medical diagnostics, environmental monitoring, biodefense and forensics (Passamano and Pighini 2006; Pejcic, De Marco et al. 2006; Rodriguez-Mozaz, Lopez de Alda et al. 2006). Different DNA sensor-based methodologies, which are designed for the potentially fast, simple, multiplexed and automatic foodborne or clinical pathogen detection, have been explored by worldwide research groups (Liu, Yang et al. 2004; Gabig-Ciminska, Liu et al. 2005; Elsholz, Worl et al. 2006). Since these DNA-based methods detect the toxin-related genes carried by the food contaminator, the presence of these genes does not indicate the expression and production of toxins by the bacteria (Ivanova, Sorokin et al. 2003; Slamti, Perchat et al. 2004). Gene detection shows that the cells have the capacity to produce the toxins and therefore are potential food-poisoning pathogens. Moreover, it is reported that the enterotoxins, except emetic toxin, produced by *B. cereus* are subject to inactivation in the human gut, so the gastrointestinal diseases caused are not directly correlated to the presence and ingestion of these enterotoxins in food; but rather to toxin production by the proliferation of vegetative cells germinated from *B. cereus* spores in the small intestine (Jaaskelainen,
Haggblom et al. 2004). On the other hand, enterotoxins and the emetic toxin are not produced by *B. cereus* present in food at all conditions. *B. cereus* toxin expression is regulated by its intracellular PlcR (Agaisse, Gominet et al. 1999; Slamti, Perchat et al. 2004) and stimulated at certain conditions (Fermanian, Lapeyre et al. 1997). For example, the production of the emetic toxin cereulide is induced at the end of the exponential growth phase and enhanced by agitation (Haggblom, Apetroaie et al. 2002), enough oxygen (Jaaskelainen, Haggblom et al. 2004) and mild temperatures (15 – 30°C). Its production is low or undetectable at temperatures below 8°C or above 37°C. Also the production of HBL and NHE toxins is strongly influenced by the bacterial growth rate and carbohydrate (Ouassila, Thierry et al. 2006). This limits the application of the phenotype-based detection methods for risk assessment, but do not influence that of the genetic sequence-based detection. Thereby the DNA chip-based detection method has become a promising tool to detect all existing disease-causing toxin encoding genes and their carriers, and assess the risk level of implicated food statistically. When combined with other assays, it can be used for the differential toxigenicity study among all such gene carrying bacteria.

### 1.3.1 The general background of DNA chips

DNA chips are developed from the integration of multidisciplinary fields, such as molecular biology, bioinformatics, surface chemistry, microelectronics, microfabrication and automation technologies, and other fields from the early 90s (Fodor, Read et al. 1991; Homs 2002). A DNA chip is generally produced by the immobilization of single-stranded DNA (ss-DNA) probes or analogs on a solid-phase with the communication of a transducer. Its detection scheme is: attached sensing probes can recognize and hybridize specifically with the target DNA or RNA in a sample solution according to the nucleotide base pairing, thereafter this binding event can be detected and finally converted to a signal, which is based on alteration of some parameters of the associated transducer induced by target bindings.

The solid interphase functions not only as the support of sensing probes, but also senses and transduces biorecognition events as the part of a transducer. It can be fabricated by glass, gold, graphite, silicon or metal oxide semiconductors, polymers-derived and other
materials. Sensing probes can be immobilized to the interface by covalent or non-covalent binding methods. Common covalent methods include: the NH$_2$-labeled probe binding via EDC-triggered esterization and amidation reactions, probe cross-linking in a polymerized matrix, the thiol-labeled probe self-assembled monolayer on gold surface. Non-covalent methods include: the random probe backbone multiple adsorption on a matrix, the biotinylated probe binding via the avidin-biotin affinity. Optimized by the surface mercaptohexanol (MCH) treatment, SAM has become one of the most employed methods that can generate a compact, ordered and specific adsorbed probe layer simply and efficiently (Levicky, Herne et al. 1998; Steel, Levicky et al. 2000). The other familiar immobilization strategy is the *in situ* synthesis of high-density oligonucleotides on a surface by applying photolithographic technology.

### 1.3.2 DNA chip classification and characteristics

DNA chips can be divided into high-density DNA microarrays and low-density DNA arrays/chips/sensors (DNA chips) approximately. High-density DNA microarrays are patterned by hundreds of thousand probe-spotting features on a glass slide-sized chip, so they are specialized for high-throughput screening analyses (Schena, Shalon et al. 1995; Chee, Yang et al. 1996; Panda, Sato et al. 2003). Ink-jetting and pin-deposition can print DNA oligonucleotides (oligos), PCR amplicons or other probes onto a surface to fabricate different kinds of high-density DNA microarrays. The maximal density DNA microarray of oligos can be achieved by photolithographic technologies, e.g. the commercial Affymetrix GeneChip Arrays, which can put the whole human genome on a single chip. These high-density DNA arrays are mainly applied in the genome-wide DNA sequencing, gene expression profiling, genome-wide DNA abnormalities and SNP surveys, cancer and other genetic disease related areas.

In contrast to high-density arrays, low-density DNA chips offer simpler, faster procedures and reusability for a limited number of analyses. Up to date, various types of DNA chips have been established (Fig. 3), which cater applications in different circumstances. Otherwise stated, DNA chips mentioned later refer to the low-density format. Detecting events can be measured by the change of optical, gravimetric, electric
and other detectable signals according to different transducers applied, before and after the application of an analyte to the sensing solid interface.

Figure 3. Schematic display of main transduction techniques applied with DNA chips.

Present optical chips are mainly based on fluorescence, chemiluminescence, colorimetry and surface plasmon resonance (SPR) detection techniques (Cheek, Steel et al. 2001; Broude 2002; Wirtz 2003; Zezza, Pascale et al. 2006). Optical chips, especially fluorescent chips are very sensitive (Hoang A. Ho 2005), and suitable for parallel DNA sequence analyses in the same sample, e.g. DNA microarrays (Cheung VG, Morley M et al. 1999). While target/probe-fluorophore labeling step is necessary for this method, also it normally need sophisticated and expensive instrumentations and is mainly used in a laboratory. On the other hand, DNA analytes need to be extracted purely from crude samples or non-nucleic acid contaminants before applied to an optical chip, so no obvious photo-induced background from samples can interfere in the final signal. Colorimetry, especially SPR-based methods detect the biomolecular binding events directly without any target labeling steps. SPR imaging, as a surface-sensitive optical technique, can monitor the kinetics of biorecognition process in real-time. Recent progress in SPR microscopy technology can provide the high-throughput and precise analyses simultaneously (Campbell and Kim. 2007).
Gravity-based DNA sensing instrument includes quartz crystal microbalance (QCM), surface acoustic wave (SAW) and microcantilever (Hansen, Ji et al. 2001; Minunni, Tombelli et al. 2005; Gronewold, Baumgartner et al. 2006). The detection mechanism of QCM and SAW is based on the shift of resonance frequency from quartz crystals or other surface acoustic resonators in response to the change of biomass attached, which finally are interrogated by a piezoelectric signal or a radio frequency signal. Microcantilever-based sensors implement the detection of binding biomass relying on the change of surface stress and followed by the bending of cantilevers, or the change of surface resonant frequency (Pinnaduwage, Ji et al. 2005). Gravity-based DNA sensors needn’t target labeling step for final signal generation and can be re-used multiple times. Meanwhile, they are sensitive and can carry out the real-time monitoring of target DNA binding reactions. Today’s microfabrication techniques can realize the compact cantilever arrays in nanoscale for parallel and sensitive target DNA detection in relative complex sample contents (Zhang, Lang et al. 2006). Their primary limitations are: delicate operation and expensive instruments are needed; mainly suitable for the research laboratories.

DNA chips, which finally generate the electrical readout by electrochemistry-based signaling, are called electrochemical chips. Hybridization of target DNA with surface-anchored probes induces a change of different electrochemical parameters (Fig. 3), e.g. capacitance, conductivity, current or potential, which are measured by the transducer. A well-studied electrochemical signaling module is based on the binding of target-probes hybrid complex to an enzyme tag, which triggers the production of a redox-active substance, and followed by a current signal generation due to redox-recycling between chip electrodes.

Various nanometer-sized particles (NPs) have been produced and introduced to the established DNA sensing systems (So-Jung Park 2002; Wang, Liu et al. 2003). Different material-based NPs, e.g. gold and other metal NPs, quantum dot NPs, magnet- and silicon-based NPs, present their unique and sensitive optical, electrical, catalytic and other properties based on their biomolecule-compatible sizes and very large surface area-to-volume ratios (Katz and Willner 2004; Fortina, Kricka et al. 2005). By integrating various DNA sensor devices with so called bio-bar-code NPs, each of whom
can carry and introduce hundreds of tag molecules upon binding to one target molecule, transduction of biorecognition events is greatly amplified and therefore a very sensitive and specific detection can be realized (Rosi and Mirkin 2005). Different nanowires and nanotubes have also emerged as new types of DNA sensing transistors (Bunimovich, Shin et al. 2006). They have small but very large contact area-to-volume-ratio surfaces that can be functionalized with sensing probes, which makes nano-sensors able to detect very few binding molecules due to their ultrasensitive surface electrical properties. All these developments are in order to amplify the signaling activity of DNA binding events and finally improve the detecting sensitivity and specificity.

### 1.3.3 Electrochemical DNA chips

Recently, increasing interest has been drawn to electrochemical signaling DNA chips (Fig. 4). Many related review articles have been written (Wang 2000; Albers, Grunwald et al. 2003; Drummond, Hill et al. 2003; Kagan Kerman 2004; Wang 2006).

![Diagram of electrochemical DNA chip](image)

**Figure 4.** The general working principle of an electrochemical chip-based DNA detection.

Also a number of electrochemistry-based signaling strategies and instruments have been rapidly developed with distinct characteristics. Overall, these DNA chips have been reported to exhibit the following promising features: low-cost platform and simple operation, no need for expensive and complicated instrumentation, e.g. silicon-based sensors (Gabig-Ciminska, Liu et al. 2005; Elsholz, Worl et al. 2006); simple analyte treatment from crude or multi-component samples, resistant to non-nucleic acid contaminants (Lee and Hsing 2002; Zwiglmaier, Ludwig et al. 2004; Liao, Mastali et
al. 2006; Lubin, Lai et al. 2006); high signal detection sensitivity and specificity (Zhang, Pothukuchy et al. 2004; Liao, Mastali et al. 2006); instrument integration and automation, the whole process from sample introduction to final signal generation can be realized automatically without manual aid (Liu, Yang et al. 2004; Elsholz, Worl et al. 2006); instrument miniaturization and portability, insensitivity to the surrounding environment are suitable for point-of-care analysis (Wang 2002; Liu, Yang et al. 2004; Wang 2006). All these offer the electrochemical DNA chip the potential candidate for realistic samples tests from environmental, clinical, food and other sources, and for genetic or infectious disease diagnosis, genetically modified organisms detection, foodborne pathogenicity detection, DNA–other molecules interaction detection and other utilities (Boon EM 2002; Drummond, Hill et al. 2003; Wang 2006). Label-free and label-based detections are two main categories in this field. For label-free detections, different strategies based on electrical properties of DNA backbones and reduction/oxidation of purine bases have been used to signal target DNA capturing (Boon EM 2002; Ozkan, Erdem et al. 2002). For label-based detections, many sensitive methods have been developed: ss- or ds-DNA nucleobases-specific intercalators binding and their redox-active electron detections (Boon, Ceres et al. 2000; Kara, Kerman et al. 2002), probe–target DNA hybridization and enzyme label-triggered signaling techniques (Umek, Lin et al. 2001; Gabig-Ciminska, Holmgren et al. 2004), different NP label-coupled detections (Peng, Soeller et al. 2006). Compared with label-free techniques, much higher detection sensitivity and sequence selectivity can be achieved by label-based techniques (Boon, Ceres et al. 2000; Zhang, Pothukuchy et al. 2004). Amperometric transducer is one of the most commonly used in combination with electrochemical sensing systems. In the present investigation, an amperometric DNA chip coupled with the enzyme-labeled sandwich hybrid detection method has been employed.

With the advance of present semiconductor manufacturing technologies, a silicon chip matrix array with high-density individual microelectrodes can be realized (Ghindilis, Smith et al. 2007). The Company CombiMatrix developed a silicon microarray featured by over ten thousand of individually addressable microelectrodes, which can detect thousands of biorecognition events by employing redox enzyme-conjugates and finally generate electrochemical signals simultaneously from all microelectrodes. This
indicates that high-throughput nucleic acid screening analysis or detection can be performed not only by fluorescence-based methodologies, but also by electrochemical transduction techniques.

Another developing direction of electrochemical sensors, e.g. electrical nanowires, nanotubes and others, has been intensively explored along with the progressing of nanotechnology (Rosi and Mirkin 2005; Patolsky, Zheng et al. 2006). The detection strategy relies on the ultrasensitive field-effect characteristics of different nanoscale-structured transistors due to their sensing surface charge increase upon target hybridization (Chang, Yuan et al. 2007; Maki, Mishra et al. 2008). Researchers are making their efforts to realize a sensitive and multiplexed electrical DNA sensing in real-time and also label-free, based on the distinct field-effect properties of surface of nanoscaled-transistors.

1.4 Aim of the study

Most methods for determination of pathogenic microorganisms are based on the phenotype and species category. However, for improved risk assessment there is a trend to complement the phenotype with genotype detection. The aim of the present work was to develop a simple and fast method for determination of virulence genes in pathogens. For this purpose an electric DNA chip array-based detection method was developed. Firstly, an efficient DNA sample preparation from bacteria by ultrasound must be established. This treatment must release the genomic DNA from cells and also fragment the DNA strands to produce molecules with the proper presentation of targeting sequences for the efficient hybridization with sensing probes and following enzyme-triggered signal generation. Secondly, some critical parameters need to be explored in order to improve the detection capability of the DNA probe-functionalized chip arrays. Since several genes usually are involved in the pathogenicity of bacteria, a chip array with the capacity to determine multiple genes simultaneously should be developed. *Bacillus cereus* and the enterohemorrhagic *E. coli* (EHEC), which are known to produce various virulence factors, were chosen as model pathogens for the study. The regulatory demands on the protocols for analysis of many foodborne pathogens require detection of a single cell in 25 grams of food samples. This means that a primary enrichment
cultivation must be included before a characterization. In most analytical protocols this enrichment results in microbial colonies. For this reason the established DNA chip array-based method should be able to assay a single colony, which demands the detection sensitivity of the target genes in $10^7$ cells, after a simple sample preparation without the target sequences amplification and DNA extraction/purification steps.
2. Present investigation

2.1 Gene-based identification of bacterial colonies with an electric chip (paper I)

In order to develop a magnetic bead-based DNA detection method for the fast analysis of pathogenic bacteria from primary enriched culture samples, i.e. bacterial colonies or liquid cultures, without the requirement of additional target DNA amplification, different aspects should be considered: a simple and efficient crude sample treatment for the targeting sequence-site of DNA to be more accessible by sensing probes; a functionalized solid surface for immobilized sensing probes to hybridize to target DNA efficiently; a sensitive transduction for final signal amplification. In this work ultrasound was introduced to treat bacterial colony samples, and its kinetics to release and fragment target DNA strands was studied.

2.1.1 The experiment design

In this paper, a DNA probe-functionalized micro bead sensor provides high density of probes and a large contact surface for sensing target DNA. The gene hblC, encoding for the B. cereus enterotoxin haemolysin L2 component, was chosen as target for this analysis. Signal amplification was realized by the enzymatic generation of large amount of the redox-active product pAP, which finally gives rise to the redox-recycling current between the silicon chip electrode fingers. The whole detection procedure is illustrated in figure 5.

This work focused on bacterial colony sample treatment and its effect on electric signal generation level. An ultrasound technique can be applied for cell disruption, release and fragmentation of the DNA. Ultrasonic disruption efficiency is influenced by various parameters, such as output power and frequency, buffer ionic strength, ultrasound duration, amount and volume of bacterial samples, and also bacterial age (Fykse, Olsen et al. 2003; Mann and Krull 2004). In order to obtain target DNA samples with suitable characteristics, applied ultrasonic time should be adjusted according to specific operation parameters and sample situations. It is generally assumed that the longer an ultrasonic disruption time is applied to cells, the more DNA is released out and shorter sizes DNA strand is fragmented. Gel electrophoresis picture with the smeared staining DNA band was used to estimate overall resulting DNA amount and average lengths.
One emerging question is to define the correlation of overall DNA fragmentation to the fragmentation and hybridization behavior of particular DNA strands carrying the target sequences. Furthermore, the influence on disintegration of the target sequence sites and different factors’ contribution to generated signal level during ultrasonication need to be clarified.

Figure 5. The flowchart illustration of the BBSH-based gene detection procedure from bacterial colonies with an electric chip.

2.1.1.1 DNA oligonucleotide-functionalized beads
The paramagnetic beads with a diameter of 2.8 µm were activated by immobilization of the ss-\textit{hblC} capture probes via covalent binding of the amino group labeled at the 5’ end of the capture probes to the carboxylic acid groups on the bead surface. This functionalized magnetic bead provides a large sensing surface for the \textit{hblC} capture probes to recognize and hybridize to the \textit{hblC} gene present in cell homogenate samples.

2.1.1.2 BBSH and electric signal generation
Resulting cell homogenate from ultrasonicated \textit{B. cereus} was mixed well with the 3’ biotin-labeled \textit{hblC} detection probe and followed by denaturation of the ds-DNA analyte at 95 °C. Thereafter the denatured sample was immediately transferred to the capture probe-functionalized magnetic beads for bead-based DNA sandwich hybridization (BBSH) (Fig. 6A), which means simultaneous hybridization of target
DNA with the capture and detection probes. After washing, extravidin-ALP conjugate was applied to bind to the biotin of the detection probes. After another bead washing, the substrate pAPP was added and enzymatically hydrolyzed to the redox-active product pAP (Fig. 6B). By transferring this resulting pAP solution from reaction tube to an electric silicon chip, the pAP–QI redox-recycling (Fig. 6B) was triggered between electrode fingers under applied potentials to generate an electric signal (Fig. 6C).

Figure 6. (A) The schematic drawing of the BBSH-based target DNA detection principle. (B) The principle of the substrate pAPP enzymatic reaction and the pAP redox-recycling on the chip electrode surface. (C) Illustration of the single-electrode silicon chip and the electric signal.
2.1.2 Results

Ultrasonication was employed to disrupt *B. cereus* cells of a single colony, release and fragment cellular DNA for the detection of *hblC* gene by using BBSH and the electric chip. Different electric signals were generated when cell lysates treated with different ultrasonic time were applied (Fig. 7). The electric signal increased continuously during extended ultrasonication time up to 10 min. When even longer ultrasonication, 13 min, was applied, the electric signal started to decline. The maximal signal after 10 min sonication and the fact that the protein release reached a maximum after 5 min (Fig. 8) raised the hypothesis that part of the signal increase was due to improved hybridization of the target DNA resulting from continued sonication.

![Figure 7](image-url)

Figure 7. Effects of different ultrasonic time on the electric signal from analysis of the *hblC* gene of one *B. cereus* colony.

In order to study the effect of cell ultrasonication on the signal generation, we need to understand the fate of the targeting-site carrying DNA strands after different ultrasonic time. Three methods, i.e. protein release analysis (Fig. 8), agar gel electrophoresis (Fig. 9), semiquantitative early endpoint PCR analysis (Fig. 10), were used in this work. According to protein release analysis, the maximal cell disintegration was achieved within 5 min. The DNA electrophoresis pattern (Fig. 9) showed that the average size of

![Figure 8](image-url)

Figure 8. Analysis of the protein amount released from single *B. cereus* colony by the ultrasonic disruption as the function of time applied.
the released genomic DNA gradually declined with increasing sonication time. After 2.5 min sonication, little large-molecule DNA of more than 2000-bp can be observed, instead DNA fragmentation took place dominantly; more than 5 min treatment shortened most DNA to sizes in the range of 200 – 600 bp. The total size of the hybridization site for the two probes was 51 bp. This means that an increasing amount of target DNA probably becomes cleaved in the hybridization region at the longer sonication time.

![Figure 9. The fragment distribution image of B. cereus cellular DNA treated by different ultrasonic time in the agar gel.](image)

An alternative method to analyze the DNA fragment sizes is the semiquantitative early endpoint PCR analysis. The traditional PCR is completed at the plateau phase. Analysis taken at this saturated phase, i.e. endpoint analysis, will not reflect the initial amount of target DNA proportionally. Analysis taken at the earlier phase (exponential phase), i.e. early endpoint PCR analysis, can represent the initial target DNA number more accurately. In this experiment, different PCR primer pairs were designed and applied (Fig. 11).

![Figure 11. Illustration of the six primer pairs targeting-sites along the target hblC strands and the resulting sizes of amplicons used in the early endpoint PCR analysis.](image)
The targeting site of all upper primers was equivalent to that of the capture probe. Targeting sites of lower primers were arranged downstream in a distance from upper primers according to the different sizes of resulting PCR amplicons, which encompass the hybridization sites (51-bp) of the capture probe (25-bp) and the adjacent detection probe (25-bp). Any cut in the hybridization or surrounding PCR-amplified regions causes the reduced production of amplicons with different sizes. The kinetics of both cellular DNA release and DNA fragmentation regarding to the detecting sequence site during the ultrasonic processing can be characterized by using this early endpoint PCR method (Fig. 10).

![Figure 10. The relative amount and sizes distribution of targeting-site carrying DNA strands according to early endpoint PCR analysis after sample treatment with different ultrasonic time.](image)

Initially, the released larger target DNA strands (747 - 911 bp) increased only during the first minute. The target fragments with sizes in the range of 75 – 482 bp increased in number until 2.5 min ultrasonication. Then, this number declined with the extended treatment. However, the smaller the DNA size, the slower the less pronounced was the size reduction. The largest target DNA fragments were almost eliminated after 10 min, while more than 80% of the smaller target fragments remained. This indicates that more than 2.5 min ultrasound started to mainly cut released DNA strands, and even 10 min treatment did not lead to too much disruption at the hybridization sequence site.

In order to describe the sensitivity of the assay, the amount of target DNA was measured as the number of carrying cells. For this purpose flow cytometry was used to count the number of cells in a single B. cereus agar colony that was used for the hblC gene detection. A typical 24 h B. cereus colony with the average diameter of 3.5±0.5 mm on the nutrient agar plate contained the average (5±1.4)×10⁷ cells. So the micro
bead-DNA sensor combined with the electric signaling chip can reach the detection sensitivity of $10^7$ target molecules.

2.1.3 Conclusions

When ultrasound at the experimental conditions is applied to prepare a cell lysate DNA sample from one *B. cereus* colony for the magnetic bead-based DNA detection, the first 2.5 min of treatment disrupts the bacterial cells and releases large cellular DNA. This is also reflected by an increasing chip signal due to the increasing number of target strands. However, continued ultrasonication mainly reduces the DNA fragment sizes and therefore contributes to the further increase of signal. This signal increase is probably due to the increased diffusion rate of the target DNA to the probes. The shortened DNA may also provide less steric hindrance to the access of probes for hybridization (Liu, Guo et al. 2007). The maximal signal was obtained from the target DNA treated with 10 min ultrasonication. The signal decrease after 13 min treatment, which was observed in all experiments, may be due to the damage or over-fragmentation of target DNA molecules by the extended ultrasonication. So for the DNA sensor-based gene detection, the optimized cell lysate DNA preparation should provide not only a maximal amount of released DNA, but also shortened sizes without too much detecting-site disintegration of the target DNA.

This work shows that it is possible for the micro bead DNA sensing system established here to analyze the presence of a gene in one single bacterial colony by only ultrasonication and centrifugation as sample treatment.
2.2 Confirmative electric DNA array-based test for food poisoning *Bacillus cereus* (paper II)

In paper I a single-electrode silicon chip was used to detect one of the eight known pathogenicity coding genes (*hblC*) of *B. cereus*. It was shown that the assay principle could be applied directly to disintegrated cell homogenates without DNA amplification. To apply the detection principle of the electrochemical DNA sensing system to a more advanced instrument, chip arrays with simultaneous and automatic analysis of multiple genes in a sample was developed and presented in paper 2. In this work, a 16-electrode silicon DNA chip array was used for simultaneous analyses of eight genes encoding four toxins of *B. cereus*.

2.2.1 The experiment design

2.2.1.1 DNA chip array and its functionalization by eight toxin-sensing probes

Each DNA chip array contained sixteen gold electrode positions (Fig. 12A). These electrode surfaces were activated by immobilizing capture probes furnished with a thiol group at the 5’ end for self-assembling on the gold. 14 of these electrode positions were functionalized by capture probes for eight toxin-related genes of *B. cereus* (*hblA, hblC, hblD, nheA, nheB, nheC, cytK-2, ces*) on one chip array by a random localization. One electrode position was functionalized by a negative control probe (NC), the sequence of which was neither biotinylated nor relevant to the genomic sequences of both *B. cereus* strains ATCC14579 and F4810/72. Another electrode was functionalized by a positive control probe (PC), the sequence of which was equivalent to that of the NC but biotinylated at the 3’ end.

2.2.1.2 The instrument and assay program

When a chip array was placed into the cartridge of the electrochemical array analyzer (Fig. 12B), a flow chamber with an internal reaction volume of about 7 µl was created on the chip surface. All assay steps, from introduction of DNA samples to final electric signal data readout (Fig. 12C), can be performed automatically on one chip array surface. The detection procedure was usually completed within 30 min and controlled by the software “MCDDE”. Signal readout was acquired differently from that of the single–electrode chip in paper 1, which reads the maximum amperometric signal (nA) after a certain time of enzymatic reaction. The signal (nA/min) from the DNA chip
array was read as the initial (2nd – 10th sec.) rate of signal increase under the stop-flow condition after addition of the substrate pAPP to the flow chamber (Fig. 12D).

Figure 12A. Photo of the DNA chip array with 16 electrode positions and a scheme of the spotting location of the eight sensing probes, which represent three HBL encoding genes: hblA, hblC, hblD, three NHE encoding genes: nheA, nheB, nheC, cytK-2 encoding gene cytK-2 and the emetic toxin-related gene ces, respectively. NC and PC are negative and positive controls, respectively.

Figure 12B. Photo of the DNA chip array analyzer ‘eMicroLISA’.

Figure 12C. Scheme of the target DNA analyzing procedure on one sensing electrode position of chip array.
2.2.2 Results

Two *B. cereus* strains ATCC14579 and F4810/72, reported to be diarrheal and emetic pathogens, respectively, were applied to PCR for analysis of presence of the *hblA, hblC, hblD, nheA, nheB, nheC, cytK-2* and *ces* toxin-related genes. Gel pictures of the amplicons confirmed the presence of *hblA, hblC, hblD, nheA, nheB, nheC, cytK-2*, and the absence of *ces*, in the diarrheal strain ATCC14579; while only *nheA, nheB, nheC* and *ces* were presented in the emetic strain F4810/72. This indicates that ATCC14579 contains all genes for the haemolysin BL (HBL), the nonhaemolytic enterotoxin (NHE) and the cytK-2 toxin, while F4810/72 contains genes only for the NHE toxin and the cereulide synthetase involved in the synthesis of the emetic toxin cereulide.

*Analysis of PCR amplicons.* Detection protocol and program (Table 1) were designed and optimized to achieve simultaneous detection of all target genes. The total assay was completed within 30 min without any manual operation. This included 15 min for simultaneous target DNA hybridization with the capture and detection probes. All PCR amplicons of the eight genes were applied for DNA chip array analysis individually.
Signals for all amplicons were generated only from their specific capture probe-spotting electrode positions. There were no obvious cross-reactions observed from non-specific amplicons or negative control positions.

Table 1. Assay program for the 16-electrode DNA chip array-based detection system.

<table>
<thead>
<tr>
<th>step No.</th>
<th>program step</th>
<th>temperature [°C]</th>
<th>time [sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffer flushing reaction chamber</td>
<td>RT</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Temperature adjust of reaction chamber</td>
<td>45</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Sample transfer to reaction chamber</td>
<td>45</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>DNA hybridization in reaction chamber</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>Sample renewal</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Recycling from step 4 to 5 for 12 times</td>
<td>45</td>
<td>65 × 12 cycles</td>
</tr>
<tr>
<td>7</td>
<td>Buffer washing and cooling of reaction chamber</td>
<td>38</td>
<td>130</td>
</tr>
<tr>
<td>8</td>
<td>Enzyme conjugate transfer to reaction chamber</td>
<td>38</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>Enzyme binding to target DNA-probes-hybrid</td>
<td>38</td>
<td>300</td>
</tr>
<tr>
<td>10</td>
<td>Buffer washing</td>
<td>38</td>
<td>130</td>
</tr>
<tr>
<td>11</td>
<td>Substrate pAPP transfer to reaction chamber</td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>Stop flow (pAPP), pAP generation, redox recycling and electric signal readout</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>13</td>
<td>Buffer washing</td>
<td>RT</td>
<td>60</td>
</tr>
</tbody>
</table>

Analysis of genomic DNA samples. For analysis of presence of the toxin-related genes in genomic DNA from agar colonies or other enrichment cultures, cells were disintegrated by ultrasonication according to the method developed in paper 1. When the DNA chip array was exposed to these samples, no or very weak signals were obtained. However, this problem was not observed when working with the single-electrode signaling chip in paper 1. A difference between these two modes of operation is that the sensing electrode surfaces of the DNA chip array are exposed to the whole cell lysate which was not the case in the previous work with the single-electrode chip, in which all reactions except the final step pAP redox-recycling took place on the magnetic beads in an eppendorf tube. To investigate if the eliminated signal from the array was caused by problems from DNA hybridization or following enzyme-triggered signaling steps, measurements were made only on the positive control (PC) position after exposure to the purified PCR amplicons in buffer and cell lysates treated with none, two or four times of DNA extraction step, respectively. Figure 13 shows different
blockage effects of these materials on the PC signals. The signal generated from the purified amplicons was believed not interfered by any non-DNA impurities and then used as the reference. The cell lysates treated without any DNA extraction blocked more than 70% of the reference signal; the cell lysates treated with two times of DNA extraction still blocked 40% of the reference signal but to a less degree; the cell lysates treated with four times of that recovered the reference signal completely and had no blockage effect at all. Since the PC signal originated from the assay steps of enzyme binding to sensing probes spotted at chip surface, problems from DNA hybridization were not the predominant reason for signal suppression. Probably the signal generation was interfered by other cellular components than DNA (Gong, Lee et al. 2006), e.g. proteins, lipo-polysaccharides and others, which were not or not thoroughly removed from the DNA fraction of cell lysate samples treated without or with less than four times DNA extraction.

Only slight signals from the gene sensing electrode positions were observed by four times extracted DNA analyte \((5 \times 10^8 \text{ cells})\), which indicated the insufficient DNA hybridization. Due to this low specific signal generated from extracted DNA analytes, the sample treatment needed to be improved to enhance the detection sensitivity. A possible explanation could be that mainly large-MW DNA was recovered by the DNA

![Figure 13. Blockage effect of differentially treated cell lysates on the signals from the chip PC positions. Column 1 represents the reference signal obtained when the purified PCR amplicons in buffer were applied. Column 2 stands for the PC signal generated after the subjection of the cell lysate without any DNA extraction. Column 3 shows the signal obtained from the assay of the DNA fraction of cell lysate treated with two times DNA extraction. Column 4 shows the signal after the introduction of the DNA fraction of cell lysate treated with four times DNA extraction. The data represent average values from at least three independent replicates.](image)
extracellular DNA from the sonicated cells. Further ultrasound treatment of the DNA extract was therefore studied (Fig. 14). Clear signals from all sensing probes were achieved from 4×DNA extracts treated with first 2.5 or 5 min ultrasonication, followed by another 10 min ultrasonication after the DNA extraction, but not from those treated with only once time of 2.5, 5, 10 or 15 min ultrasonication, nor with samples by first long time ultrasonication of 10 to 15 min and followed by another 10 min treatment after DNA extraction. According to the results in paper 1, these data implicated that: large cellular DNA molecules were obtained by first 2.5 or 5 min ultrasonication, which do not favor DNA hybridization and signal generation; after their extraction, shortened target DNAs were produced by another 10 min fragmentation. Smaller DNA molecules can be generated by direct 10-15 min ultrasonication of cell lysate, but due to their low extraction efficiency by organic solvents, neither large nor smaller target DNA molecules can be extracted sufficiently to generate clear signals by one time of 10-15 min sonication. The highest and most reproducible signals were achieved when B. cereus ATCC14579 (5×10⁸ cells) was treated with first 5 min ultrasonication, followed by four times DNA extraction and another 10 min ultrasonication.

Figure 14. Comparison of the hblD, nheB, cytK-2 gene signals generated from differentially ultrasound-treated DNA analytes applied for chip array assays.
To detect all eight toxin-related genes simultaneously from microbial samples, one chip array functionalized by eight corresponding probes on the 16 electrode positions (Fig. 12A) was prepared and applied for assay of both strains. Responses to all eight genes agreed well with results from the PCR. Signals of eight sensing probes (Fig. 15A-B) for both strains were clearly generated from $10^8$ cells. There was no proportionally increased signal level generated when increasing the cell number from $10^8$ to $5 \times 10^8$, indicating that the assay protocol was not optimized for quantitative analysis.

Figure 15. Analysis of presence of eight toxin-related genes from the diarrheal strain ATCC14579 (A) and the emetic strain F4810/72 (B). Signals were obtained from $1 \times 10^8$ (white columns) and $5 \times 10^8$ (grey columns) cells, respectively.

2.2.3 Conclusions
A fast and automated method for the multiplex detection of toxin-related genes can be realized by the application of the 16-electrode DNA chip array-based detection system and properly designed assay program. One DNA chip array can be functionalized by multiple gene-sensing probes and applied for the detection of these genes simultaneously in one sample. The whole instrumentation automates all detection steps
of the sample and handles final data in a 30 min assay procedure. Proper sample preparation from bacteria is very important. Ultrasound was introduced to disrupt cells, release and fragment cellular DNA. Target DNA fragmentation is important to increase the signal. Contrary to previous results in paper 1, where DNA hybridization and enzyme-triggered pAP generation took place on magnetic beads instead of chip surface, only ultrasonic disruption treatment was not enough to prepare suitable DNA analyte, because background components in cell lysate block the pAP redox-recycling pathway on chip electrodes. A DNA extraction step was therefore needed. Due to low extraction efficiency for small DNA molecules, large cellular DNA are mainly harvested in the extraction and therefore resulting extracted DNA must are fragmented by a second ultrasonication. The need of the DNA extraction increases the assay time by about 20 min and is not included in the automation program. Therefore it is important to further study the fouling mechanisms to circumvent DNA extraction and enable direct application of ultrasonicated cells to the chip assays.
2.3 Critical factors for the performance of chip array-based electrical detection of DNA for analysis of pathogenic bacteria (paper III)

A fast and automated gene detection method based on a 16-electrode position DNA chip array system was developed and presented in paper II. Extracted DNA samples were derived from the whole microbial genomic DNA material and applied without additional target sequences amplification. This sample format with high content of background DNAs demands excellent sensing performance of the chip array. In order to improve the detection sensitivity and specificity based on this electric DNA chip, various parameters were examined here. Activation of chip surface-functionalized probe layer, hybridization buffer ionic strength and probe linker length, which determine target DNA sensing capability of capture probes immobilized on chip surface, were investigated. To properly prepare the analyte from bacteria by ultrasound, the optimal presentation of targeting sequences in DNA strands for hybridization and following enzyme-triggered signal generation, i.e. different probe-binding locations, effect of ultrasonication on DNAs of varied lengths, was studied. To investigate the detection specificity, different single- and multiple-mismatched sequences were designed and different assay performances were tested to improve the nonspecific signal discrimination from perfect-matched signals. Chip reusability was also explored.

2.3.1 The experiment design

2.3.1.1 Chip surface functionalization via probe linkers of different lengths

Probe linker is used to anchor capture probe DNA to chip electrode surface via the reaction of its end-labeled thiol group to gold. Commonly used probe linker is a six-carbon chain. Here, longer linkers, i.e. 9-, 12- and 18-carbon chains, were also applied to attach capture probes to chip arrays. This experiment was to study the effect of steric hindrance of probes controlled by carbon linkers of varied lengths on detection sensitivity and specificity.

2.3.1.2 Different probe targeting locations along DNA strands

Different capture/detection probe-binding locations along DNA strands and their impacts on generated signal level were explored. Three alternative sequence fragments from both nheB and cytK-2 genes were amplified by PCR. The resulting 317-bp nheB(1), 332-bp nheB(2) and 321-bp nheB(3) amplicons comprise the same probe-
binding sequences and similar sizes, but different probe-binding locations (Fig. 16). Probe-binding sites were located at the 3’ end, 5’ end and middle positions of the targeted nheB(1), nheB(2) and nheB(3) strands, respectively. The same principle was applied to three cytK-2 PCR amplicons, i.e. 815-bp cytK-2(1), 811-bp cytK-2(2) and 847-bp cytK-2(3).

2.3.1.3 Electric chip array-based detection strategy
Prepared samples were first mixed with biotin-labeled detection probes (1 µM working conc. for each) in hybridization buffer. The mix was first treated by denature at 95 °C for 5 min and cooling on ice for 1 min, then immediately applied to DNA chips to form the sandwich hybrid complex, capture probe-target-detection probe, at chip surface. The resulting hybrid complex can capture the Streptavidin-β-Gal (Str-β-Gal) by the specific biotin-avidin bound after introduction of the enzyme conjugate. The redox-active product pAP was generated by the enzymatic hydrolysis of the flushing substrate pAPG. Conversion of the accumulating pAP and its oxidation form quinoneimine (QI) between interdigitated anodes and cathodes results in the increasing redox-recycling current curves from all 16 electrode-positions of one chip simultaneously after the stop flow of pAPG. The slope of 8-sec current increase from the 2nd sec of stop flow was employed as the signal (nA/min). All analyzing steps after sample introduction were run automatically in the instrument ‘eMicroLISA’ and controlled by the assay program.
2.3.2 Results

Before applying sample analysis, the sensing surface of dry-stored chip arrays was streamed by buffer to rehydrate immobilized capture probe layers. Capture probes rehydrated by PBS buffers of varied ionic strengths exhibited different target DNA hybridization (Fig. 17B) and following enzyme-binding efficiencies (Fig. 17A). The similar PC signal level (60 nA/min) was generated after rehydration with 1× or 2×PBS buffers and therefore showed the similar binding efficiency of biotinylated probes to Str-β-Gal. Rehydration by 4×PBS buffer resulted the highest PC signal level (176 nA/min), which indicated that the accessing and binding efficiency of immobilized probes to the enzyme conjugate was three times of that treated by PBS buffers with lower ionic strengths. Further rehydration by 5×PBS decreased the enzyme binding capacity apparently. It is assumed that rehydration by high buffer ionic strength causes immobilized probe layers stand more upright from sticky gold surface and then expose

![Graph A](image.png)

![Graph B](image.png)

Figure 17. Effect of chip sensing surface rehydration on signal generation. Before sample analysis, a chip surface was flushed by buffer to rehydrate the immobilized probe layer. Effects on generated signal level by different rehydration temperatures, incubation time and buffer ionic strengths were compared from chip PC positions (A) and the gene ces sensing probe positions by applying the ces amplicon target (B).
more explicitly to reactive compounds on chips (Herne and Tarlov 1997; Rant, Arinaga et al. 2004). Too high buffer ionic strength stimulates the formation of secondary structures intra- or inter-probe strands, which reduce the probe accessibility to enzyme conjugate. Further experiment (Fig. 17B) demonstrated that rehydration treated by different ionic strengths had the same impact on probe’s hybridization ability. The rehydration process was fast and not influenced by incubation time (5 – 15 min).

It is obvious that DNA hybridization kinetics is affected by the buffer ionic strength (Park, Germini et al. 2007). High hybridization ionic strength reduces the electrostatic repulsion between negatively charged DNA strands and triggers fast and efficient target DNA hybridization to probes, while too high buffer ionic strength decreases hybridization efficiency. To find out the optimal hybridization buffer for the enhanced probe sensing capability, buffer ionic strengths ranging from 1×PBS to 5×PBS were tested. It was concluded that 4×PBS buffer gave rise to the highest hybridization efficiency.

It has been reported that the flexibility and solubility of capture probes in reaction solutions from anchoring solid phase have impact on their target DNA interaction performance (Franke-Whittle, Klammer et al. 2005; Peplies, Glöckner et al. 2003). Probe linkers are used to connect sensing probes to the thiol-reacted gold surface and therefore support probes extending to solution phase. The most used probe linker is a six-carbon chain. Longer length of carbon linker gives more freedom to tethered probes, which increases the frequency of probe interaction to target DNA in solution. Probes anchored by 6-, 9- and 12-carbon linkers generated the similar signal levels, while 18-carbon linked probes produced the highest signal level and resulted a 1.6-fold increase of signal. However, longer linker lengths led to the higher unspecific signals, especially for 18-carbon linked probes. From another point of view, 18-carbon linker may reduce spatial hindrance of probes from solid interface, it also poses higher spatial impedance for pAP electron redox-recycling between interdigitated electrodes triggered by enzyme, which is the final electrical signal generation step. In the balance of assay sensitivity and specificity, 6-carbon chain plus 4 nucleotides-spacer of capture probe was suggested as the proper probe-anchoring linker for enzyme-triggered electric DNA chip detection.
In order to systematically study ultrasound fragmentation on DNA strands and the effect on generated signal level, PCR amplicons with varied sequences and sizes were applied (Fig. 18). Except the shortest strands (187-bp), ultrasonication up to 10 min did not take effect on fragmentation of short DNA strands ($\leq 400$ bp) nor resulted in obvious damage on them, and therefore produced the similar signal level as without treatment. However, for large DNA strands ($\geq 400$ bp) the signal increased transiently with the sonication time, up to 2-fold. This indicates that the overtime sonication might lead to the disruption of the entire targeting sequence region. By comparing different operation time, 10 min ultrasonication showed the highest signal from large DNA strands. Furthermore, a reference experiment, which showed influences of varied sizes of *hblC* amplicon targets on signal generation, was performed. With gradually shortened *hblC* strands, i.e. the longest 911-bp, 747-bp, 355-bp to the shortest 187-bp, resulting signals increased from 1.4, 2.4 to 3 times of the signal level generated by the longest one (911-bp), respectively (unpublished data). This illustrated that the efficient fragmentation for longer DNA targets can be achieved by simple ultrasonication.

![Figure 18. Amplicon targets ultrasound treatments by different time and their effects on signal generation.](image)

Target DNA strands with the same probe-targeting sequences and similar lengths, but differing in probe-binding sites, were designed. Effects of these different probe-targeting sites on generated signal level were investigated by employing two groups of
PCR amplicon targets (Fig. 19), i.e. *nheB* amplicons with the small sizes of around 320-bp and *cytK-2* amplicons with the large sizes of over 810-bp.

Data showed the same effects of targeting sequence-locations on signal for both short and large DNA targets. The highest signal level was always generated from DNA with probe-binding site at 5’ end of strands; the lowest signal was generated from DNA with binding sites in the middle region; DNA strands with binding site at 3’ end generated the intermediate signal level. Interior shift of probe-binding sites from 5’ end dramatically diminished signal level more than 10-fold for large DNA (*cytK-2* amplicons), while the same shift only reduced half of signal level for short DNA (*nheB* amplicons). It showed that probe-binding site variation influenced signal generation to a less degree for small-sized DNAs when comparing with larger ones. Binding site at 5’ end presents optimal accessibility for both probe hybridization and following enzyme conjugate binding, while 3’ end binding site only gives optimal access for probe interaction while it causes some steric impedance for enzyme binding. Interior binding site creates both conformational difficulty for probe interaction and also steric hindrance for enzyme binding (Lane, Evermann et al. 2004). The larger the size of DNA is, the more steric hindrance and signal suppression is expected from both 3’ end and the interior region of strands.

In order to investigate the chip sensitivity to minor mutations in the hybridization site of the target DNA, various single- and multiple-mismatched probe sequences were designed. Different parameters, i.e. hybridization temperature and buffer ionic strength,
washing stringency after hybridization, were studied. Only 55 °C stringent washing by PBS buffer effectively improved the signal differentiation of single-mismatched (M1MM) and three middle-mismatched (M3MM) sequences to perfect-matched one. This washing can remove background signals from three random-mismatches (R3MM) and five middle-mismatches (M5MM). Increasing hybridization temperature or decreasing buffer ionic strength much reduced absolute signal levels from both matched and mismatched sequences, but did not improve the detection specificity clearly. It was also observed that nonspecific bindings with discontinued nucleotide mismatches (R3MM) were much weaker than those with contiguous nucleotide mismatches (M3MM).

Chip reusability, as one characteristic of electric DNA chip detection system, was investigated (Fig. 20). For the DNA chip array instrument established here, at least two times of sample analysis can be implemented for one functionalized chip. High level of signal recovery and negligible background signal were obtained after the second use of chip. The third use resulted in the less effective level of signal regeneration and the relatively higher background.

![Figure 20. Chip reusability test. White, grey and black columns displayed the signal recovery level after the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} time analyses of different target sequences and sizes on one chip array.](image)

### 2.3.3 Conclusions

DNA sensors are sensitive to many assay variables. To improve the sensing performance of the amperometric DNA chip, two main aspects were investigated here. One was to study the effect of different presenting forms of probe-targeting sequences
in DNA strands on signal generation and therefore how to properly prepare sample analyte by ultrasound, the second was to optimize some critical parameters to improve sensing ability of probe-functionalized chip arrays.

Rehydration of chip surface-immobilized probe layer by proper buffer ionic strength activates probe’s sensing capability, so it is an important assay step before probe hybridization to target DNA. Hybridization efficiency of sensing probes and detection reproducibility can be improved by optimized hybridization buffer ionic strength. Generally shorter DNA strands generate higher signal level. Ultrasonication was demonstrated as a simple and efficient method to lyse whole bacterial cells and further fragment large genomic DNAs. However, too extensive sonication reduces the signal, probably by cleaving some DNA in the hybridization site. Not only target DNA sizes, but also probe-binding sites have much impact on probe hybridization, following enzyme binding and enzyme-triggered electrical signal generation. DNA 5’-end provided the best location for probe binding and following signaling reactions. Large DNA strands probably create higher intra-spatial hindrance if the probe-binding site is not at the ends, therefore resulting in higher signal suppression when comparing to short DNA strands, which have less influence of differed probe-binding sites on signal level. This further confirms why better sensitivity is achieved from smaller-sized targets. Stringent washing after hybridization is necessary for improving detection specificity. Each functionalized chip array can be applied for sample analysis at least two times, or even three times. Different factors studied here need to be considered in the optimization of sample preparation and DNA chip detection protocol. Optimal combination of all these parameters should result in improvement for this electric chip array-based detection methodology.
2.4 Rapid determination of virulence factors in EHEC colonies with an electric DNA chip array (paper IV)

In paper II, DNA extraction was needed before a sample being applied for the electric DNA chip assays. In paper III, the assay protocol was readily improved to enable analysis on the supernatant of the cell homogenates without DNA extraction. Agar colonies, as obtained in most primary enrichment cultures in common analytical protocols of pathogenic microorganisms, were chosen as samples. Three EHEC virulence gene-containing \textit{E. coli} strains, i.e. O157:H7 K'EDL933, O157:H7 BC8624Δstx2 and DH5αstx2, and one nonpathogenic \textit{E. coli} strain MG1655, were selected as model microbes. Distributions of three virulence genes (\textit{stx1}, \textit{stx2} and \textit{eae}) in these four strains were examined.

2.4.1 The experiment design

2.4.1.1 Single colony sample preparation

\textit{Sample preparation with DNA extraction}. Single colonies with the average diameter of 2±0.5 mm from each studied strain, were first collected from the cultured agar plate and suspended in H$_2$O. Then these cell suspensions were subjected to 95 $^\circ$C water-bath for 10 min, thereafter applied for 1 min sonication by an ultrasound disruptor to open cells and release genomic DNA. Cellular DNA was extracted by the organic solvent of phenol:chloroform:isoamyl alcohol from crude cell lysate, and followed by again 9 min sonication to fragment large DNA molecules. The resulting extracted DNA sample was ready for use.

\textit{Sample preparation without DNA extraction}. Single colony-cell suspensions from each \textit{E. coli} strain were first prepared. After this, the cells were subjected to 95 $^\circ$C water-bath for 10 min, direct 10 min sonication was applied to open cells, release and also fragment cellular DNA. Afterwards, crude cell lysate was centrifuged and the resulting supernatant cell homogenate was collected and ready for use.
### 2.4.1.2 DNA chip assay program

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Program step</th>
<th>Temperature [°C]</th>
<th>Time [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flushing buffer flow</td>
<td>RT</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Chip rehydration and temperature adjustment</td>
<td>50</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>Sample analyte transfer to reaction chamber</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>4*</td>
<td>Hybridization in reaction chamber</td>
<td>50</td>
<td>20° / 40°</td>
</tr>
<tr>
<td>5*</td>
<td>Sample renewal</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>Wash flow</td>
<td>50</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>Enzyme conjugate transfer to reaction chamber</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Incubation</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>9</td>
<td>Wash flow</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>10</td>
<td>Temperature adjustment</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>Substrate pAPG transfer to reaction chamber</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>Stop flow and electrical signal readout</td>
<td>55</td>
<td>35</td>
</tr>
<tr>
<td>13</td>
<td>Wash flow</td>
<td>RT</td>
<td>30</td>
</tr>
</tbody>
</table>

RT represents the room temperature of around 22 °C.
* Steps 4 and 5 are repeated in a sequence of 12 times.

20° means that the hybridization time (20 sec/sequence) was applied for the sample analytes treated with DNA extraction; 40° means that the hybridization time (40 sec/sequence) was applied for the sample analytes treated without DNA extraction.

### 2.4.1.3 Chip sensing electrode fouling study

To find out which type of interfering component in cell homogenates and possible methods to eliminate this fouling effect from the chip electrodes, the cell homogenates after treatments with 0.1% Tween 20, 0.15% SDS and proteinase K, respectively, were applied to the chip surface to test the redox-recycling blocking effect and the signal recovery.

*pAP-QI redox-recycling experiment.* The chip surface was first rehydrated before test, followed by flushing with the redox-active substance 4-aminophenol (pAP) solution to generate an electronic redox-recycling current (nA) between the interdigitated electrode fingers, before and after the sample exposure to the chip surface.

### 2.4.2 Results

For four *E. coli* strains studied, data from PCR and gel electrophoresis (Fig. 21) revealed that: all three virulence genes, i.e. *stx1*, *stx2* and *eae*, exist in strain O157:H7 K:EDL933; only the *eae* gene is found in strain O157:H7 BC8624Δstx2; the *stx2* gene
is found in strain DH5αstx2, and the nonpathogenic strain MG1655 contains none of these genes.

The DNA chip array instrument and the optimized assay procedure were employed for determination of virulence genes in studied strains derived from single agar colonies. Two preparations were used to treat the single colony sample (about 5×10^7 cells). One was to first apply sonication to release the cellular DNA, followed by DNA extraction and again sonication to fragment the extracted DNA, as presented in paper II. The second was a more simplified preparation, which excluded the DNA extraction and only applied one sonication to release and fragment cellular DNA. The whole sample preparation was ready in 30 min, which was 10 min shorter comparing with that of the preparation applying DNA extraction. When the assay program with shorter DNA hybridization time (7 min) was applied, clear signals were only generated from the single colony samples treated by DNA extraction (Fig. 22). When the hybridization time was prolonged from 7 to 12 min, single colonies treated without DNA extraction gave rise to the clear signals (Fig. 23). The generated signal patterns of the three virulence genes from single colony samples treated by both protocols, were completely consistent with the PCR control data for all tested strains.
The arising problem after applying cell homogenate samples for the chip assay, which has been discussed in paper II, was the dramatic suppression of the redox-recycling signals (about 2/3 signal reduction) (Fig. 24). To study signal recovery from the fouled electrode, direct washing of the chip surface with Tween 20 was first applied after exposed to cell homogenate. While the washing of the fouled chip surface for up to 37 min only led to the slightly recovered signal (unpublished data). Secondly, chip surfaces were applied by the cell homogenate samples treated by two detergents (Tween 20 and SDS) and a proteinase K, respectively, before flushing pAP to generate the redox-
recycling signal. Results showed that the cell homogenate treatments by Tween 20 or proteinase K for 20 min did not reduce the fouling effect (data not shown). On the contrary, the SDS treatment of the cell homogenate effectively prevented the interfering components from sticking to the chip surface and regenerated most of the original current signal level (Fig. 24). This result indicates that cellular proteins can adsorb to the chip electrodes and mainly contribute to this signal blocking effect. The removal of cellular lipids by Tween 20 did not take effect on the signal recovery. Nor hydrolysis of the cellular proteins by proteinase K helped removing adsorbed interferers. SDS has distinct functions, by denaturing proteins and binding to the resulting peptides, loading them with strong negative charges. This function may lead to the electrostatic repulsion of cellular proteins from the surface-immobilized negative-charged DNA probe layers and thereby reducing protein adsorption to the chip surface.

Figure 24. Chip sensing surface fouling study. pAP-QI redox-recycling current signals were generated from the chip electrodes after exposed to the buffer or differentially treated samples. Chip surfaces were treated by 1: only buffer flushing; 2: extracted genomic DNA (gDNA) incubation; 3: cell homogenate incubation; 4: incubation with the cell homogenate treated by 0.15% SDS, individually.

### 2.4.3 Conclusions

Four *E. coli* strains studied here have different set-ups of the three EHEC virulence genes *stx1*, *stx2* and *eae*. After improving the sensing ability of the DNA chips, and establishing simpler sample preparation method, a protocol for fast gene identification from a single microbial colony without additional target DNA amplification was
developed. The signal pattern of the three virulence genes in studied strains agreed well to those of the PCR analysis, which demonstrated sufficient detection specificity for this DNA chip array method. Analysis of presence of multiple virulence genes can be realized in 25 min automatically after introduction of supernatant of a sonicated colony. The detection sensitivity was sufficient to directly analyze a single colony of a primary enrichment culture.

Analyses of samples in complex matrix resulted in chip sensing-surface fouling, which reduced the detection sensitivity probably by this decreased signal transduction capability of the DNA chip. The chip fouling study indicates that cellular proteins in cell homogenate samples are the main interferers, which can adsorb to the redox-recycling pathway between the chip electrodes and reduce the electric signal. By simply adding SDS to cell homogenates, this signal blocking effect was significantly reduced. This sample treatment should be applicable to different surface-based DNA sensings for analysis of complex samples.
3. Concluding remarks

Current methods for determination of pathogenic bacteria are mainly based on determination of phenotypic properties. However, for food safety assessment organisms like *E. coli* and the *B. cereus* group are better characterized by their potential for production of toxins, many of which are encoded by well-known genes. Thus, simple and fast determination of a quite limited number of such genes should be a useful tool.

Silicon-based electric DNA chip arrays can here be very useful. For the electric DNA chip-based gene detection, proper presenting form of target sequences in DNA strands is essential. Short target strands and the close to 5’-end localization of the target sequences exhibit the best conformation for probe sensing and following enzyme-triggered signal generation. Ultrasound, as a simple and fast cell disruption method, performs different functions on cellular DNAs. Dominant function of the first minutes treatment is to open bacterial cells and release DNA; continued sonication functions as DNA fragmentation, large DNA molecules are gradually shortened. The signal therefore first increases with sonication time due to increase in the free DNA amount in the sample, then due to increased rate of hybridization of the smaller DNA fragments. Finally, the signal may decline due to breakage in the hybridization site of the target strands.

These mechanisms, unfortunately, make it difficult to use the DNA chips, with the current protocol, for quantitative analysis, since it would require a control of cell lysis yield and DNA size distribution. On the other hand, the demand on many food analyses is to detect one cell, i.e. mostly one DNA molecule, in 10-25 g of sample. This is far from realistic for assays which do not include DNA amplification. Most current analytical protocols for pathogenic bacteria therefore include a primary enrichment culture in selective media, and colonies from such culture contain sufficient DNA for determination with the current DNA chip assay and protocol.

To make a realistic application of the electric DNA chip array for such confirmative analysis of colonies, simple, fast and highly automatic procedures need be developed. The instrument reaction used in this study is completely automatic from application of the DNA analyte to readout of the signals from the assays, which typically requires 25
min. This time can be shortened by reducing the hybridization time but on the expense of sensitivity. The sample preparation used in this work was ultrasonication, centrifugation and addition of SDS, which requires about 30 min. If the removal of cell debris by centrifugation could be eliminated or replaced by filtration, these operations can be included in the automated procedure making it possible to assess the genetic sequence of pathogenicity of many microorganisms by subjecting a colony directly to the instrumental analysis.
### 4. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>BBSH</td>
<td>bead-based sandwich hybridization</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cerNRPS</td>
<td>gene of non-ribosomal peptide cereulide synthetase</td>
</tr>
<tr>
<td>ds-</td>
<td>double stranded-</td>
</tr>
<tr>
<td>eae</td>
<td>gene encoding the toxin intimin</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EHEC</td>
<td>enterohemorrhagic <em>E. coli</em></td>
</tr>
<tr>
<td>Ext-ALP</td>
<td>extravidin–alkaline phosphatase conjugate</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>HBL</td>
<td>haemolytic toxin B and L complex</td>
</tr>
<tr>
<td>IDA</td>
<td>interdigitated anode-cathode array</td>
</tr>
<tr>
<td>M1MM</td>
<td>single middle-mismatched sequence</td>
</tr>
<tr>
<td>M3MM</td>
<td>triple middle-mismatched sequence</td>
</tr>
<tr>
<td>M5MM</td>
<td>quintuple middle-mismatched sequence</td>
</tr>
<tr>
<td>MCH</td>
<td>mercaptohexanol</td>
</tr>
<tr>
<td>NC</td>
<td>negative control</td>
</tr>
<tr>
<td>NHE</td>
<td>nonhaemolytic toxin complex</td>
</tr>
<tr>
<td>NP</td>
<td>nanometer-scaled particles (diameter ≤ 100 nm)</td>
</tr>
<tr>
<td>pAP</td>
<td>p–aminophenol</td>
</tr>
<tr>
<td>pAPG</td>
<td>4-aminophenyl β-D-galactopyranoside</td>
</tr>
<tr>
<td>pAPP</td>
<td>p–aminophenyl phosphate monosodium</td>
</tr>
<tr>
<td>PC</td>
<td>positive control</td>
</tr>
<tr>
<td>PlcR</td>
<td>pleiotropic regulator</td>
</tr>
<tr>
<td>QCM</td>
<td>quartz crystal microbalance</td>
</tr>
<tr>
<td>QI</td>
<td>quinoneimine</td>
</tr>
<tr>
<td>R3MM</td>
<td>triple random-mismatched sequence</td>
</tr>
<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
</tr>
<tr>
<td>SAW</td>
<td>surface acoustic wave</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>ss-</td>
<td>single stranded-</td>
</tr>
<tr>
<td>Str-β-Gal</td>
<td>streptavidin-β-Galactosidase conjugate</td>
</tr>
<tr>
<td>stx1</td>
<td>gene encoding the Shiga-like toxin 1</td>
</tr>
<tr>
<td>stx2</td>
<td>gene encoding the Shiga-like toxin 2</td>
</tr>
</tbody>
</table>
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References


