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Boolean Logic-based Assays for the Analysis of Multiple *Bacillus cereus* Toxins

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To my father and my mother, for their support, encouragement, and love!

谨此献给我的父亲和母亲！

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ABBREVIATION

Ab	antibody
ALO	anthrolysin
α-PFT	alpha-pore-forming toxin
β-PFT	beta-pore-forming toxin
CDC	cholesterol-dependent cytolysin
CDT	cytolethal distending toxin
Cer	cereulide
ces	cereulide synthetase gene
CFU	colony forming unit
CLO	cereolysin O
ClyA	Cytolysin A
Cry	crystal toxin
CTB	cholera toxin B subunit
CwpFM	cell wall peptidase
CytK	Cytotoxin K
DNA	deoxyribonucleic acid
DSMZ	German Collections of Microorganisms and Cell Cultures
EF	edema factor
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EntFM	enterotoxin FM
Fur	ferric uptake regulator
Hbl	haemolysin BL
HlyI (II, III, IV, E)	haemolysin I (II, III, IV, E)
HlyIIR	HlyII regulator

HPLC	high-performance liquid chromatography
HPLC-MS	HPLC tandem mass spectrometry
HRP	horseradish peroxidase
HT_3	histamine type 3
IAC	immunoaffinity chromatography
InhA1	immune inhibitor A1
LDH	lactate dehydrogenase
LED	light-emitting diode
LF	lethal factor
LLO	Listeriolysin O
LOD	limit of detection
LPS	lipopolysaccharide
mAb	monoclonal antibody
MALDI-TOF-MS	matrix assisted laser desorption ionization time-of-flight mass spectrometry
MAPK	mitogen-activated protein kinase
mART	mono-ADP-ribosyltransferase toxin
MEM	minimum essential medium
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
Nhe	non-hemolytic enterotoxin
NprA	neutral protease A
NprB	neutral protease B
NRPS	non-ribosomal peptide synthetase
PA	protective antigen
PC	phospholipase C
PCR	polymerase chain reaction

PFO	perfringolysin O
PI	propidium iodide
PlcR	pleiotropic regulator
POCT	point-of-care testing
Ptx	pertussis toxin
RNA	ribonucleic acid
QDs	quantum dots
rNheC	recombinant NheC
RNS	reactive nitrogen species
ROS	reactive oxygen species
SLC	sequential logic circuits
Sec	general secretory pathway
SMase	sphingomyelinase
TLO	thuringiolysin O
TMB	tetramethylbenzidine
VIPs	vegetative insecticidal proteins
WCR	western corn rootworm
WST	water-soluble tetrazolium salt

I. INTRODUCTION

Food spoilage and contamination caused by unfavorable microorganisms, occurs frequently in food industrial systems (Shiby and Mishra, 2012; Doulgeraki et al., 2012). The presence or absence of pathogenic bacteria or their toxins determines the safety and quality of foods in a dose dependent manner regarding either the number of pathogens or the amount of toxins (Ceuppens et al., 2011; Farrokh et al., 2013). Along with the development of society, animal derived foods comprise a large proportion of human diet nowadays. Unfortunately, these foods such as dairy products are good growth media for many microorganisms including lactic acid bacteria, coliforms, psychrotrophic microorganisms, spore-forming bacteria, molds and yeasts (Quigley et al., 2013; Shiby and Mishra, 2012; Van Doren et al., 2013). During growth of these microorganisms many potential toxic substances may be produced, which can be catalogued into cytotoxic, enterotoxic, neurotoxic or immuno toxic compounds according to their functional effects (Antignani and Fitzgerald, 2013; Lemichez and Barbieri, 2013; Ribet and Cossart, 2010; Schiavo and van der Goot, 2001). In order to improve food safety, this thesis will be focused on multiple *Bacillus cereus* toxins.

Bacillus cereus is a ubiquitous Gram-positive, aerobic or facultative anaerobic, spore-forming rod-shaped bacterium, which completes its saprophytic life cycle mainly in soil. Based on the 16S rRNA gene sequences, *B. cereus* bears a closely genetic relationship to several other members in the *B. cereus* group, especially *Bacillus anthracis* and *Bacillus thuringiensis* (Baillie and Read, 2001; Ivanova et al., 2003; Vilas-Boas et al., 2002). They are, however, different in their phenotypic characteristics and in the symptoms of disease. *B. cereus* can not only easily spread to food of plant origin such as rice and vegetables, but it is also frequently found in meat and dairy products, especially the psychrotolerant strains (Ceuppens et al., 2013; Mols and Abee, 2011). Different isolates of *B. cereus* cover a broad range of habitats, ranging from symbiotic (commensalism) to pathogenic lifestyles, and include also probiotic strains, Figure 1. *B. cereus* strains have not only been reported for the positive interaction with certain plants to stimulate their growth and to protect them from some plant diseases (Yu et al., 2011), but have also been isolated from the gut of healthy individuals (Ghosh, 1978). Meanwhile, *B. cereus* spores from probiotic strains are sold as feed additive for poultry, rabbits, cattle and swine to improve performance and feed conversion rate (EFSA, 2012). This product named Toyocerin®, has been authorized in the European Union, although this strain harbors all of the genes coding for two main tripartite

enterotoxin complexes as shown by complete genome sequencing. In contrast, *B. cereus* is responsible for an array of symptoms, ranging from mild and transient diarrheal disease to life-threating diseases. In addition to gastrointestinal syndromes, *B. cereus* can cause numerous local and systemic infections, such as in the central nervous system, fulminant bacteremia, pneumonia, endophthalmitis and gas gangrene-like cutaneous infections (Bottone, 2010). Also, *B. cereus* is a known cause of diseases in animals such as bovine and caprine mastitis (Logan, 1988). Although the pathogenic mechanisms of most diseases are still not clear, many different toxins secreted by *B. cereus* have been reported as potential virulence factors in the past decades. A brief summary of the extracellular toxins produced by *B. cereus* and the related analytical assays is presented here.

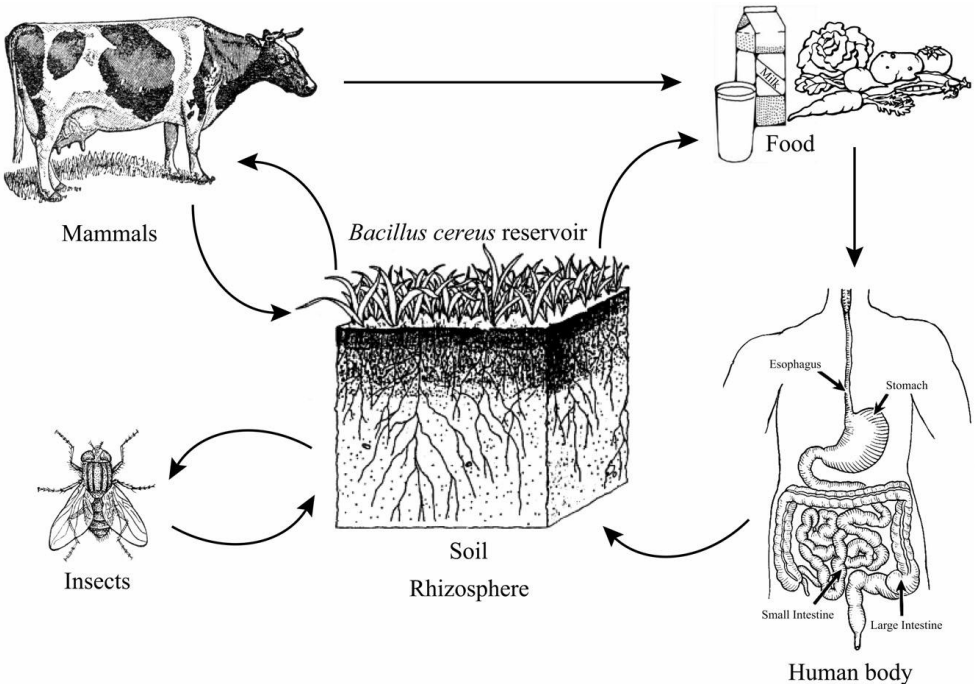


Figure 1 Ecological niches and transmission routes of *B. cereus*. Reprinted with permission from reference (Mols and Abee, 2011). Copyright 2011, Society for Applied Microbiology and Blackwell Publishing Ltd.

1. Toxins of *B. cereus*

1.1. Cereulide

The role of cereulide as the cause of emetic food poisoning, particularly after the consumption of contaminated cereal or dairy ingredients, is well documented (Shaheen et al., 2006). Cereulide, a 36-membered cyclic dodecadepsipeptide of 1.2 kDa consists of three repeats of four amino acid residues [-D-*O*-Leu-D-Ala-L-*O*-Val-L-Val-]$_3$ (Makarasen et

al., 2009), and is produced by a non-ribosomal peptide synthetase (NRPS), encoded by the plasmid mediated cereulide synthetase gene cluster (*ces*) (Ehling-Schulz et al., 2005). Although different in structure, cereulide biosynthesis is similar to that of surfactins, which represent a family of cyclic lipopeptides (EFSA, 2011). 5-6% of the *Bacillus* strains are able to produce various heat-stable surfactins with haemolytic activity, which can form pores on epithelial cells and are toxic to sperm cells. Owing to its structural similarity to valinomycin, a potassium ionophore, cereulide also acts as a cation ionophore to disrupt the electrochemical potential gradient on lipid membranes (Makarasen et al., 2009). The main mechanism of cereulide (Figure 2A) has been elucidated as: 1) binding to the 5-HT_3 receptor in the stomach; 2) inhibition of mitochondrial activity through the fatty acid oxidation pathway; 3) stimulating the afferent vagus nerve and causing vomiting (Mikkola et al., 1999; Stenfors Arnesen et al., 2008). In addition, cereulide caused fulminant liver failure and encephalopathy in children after consuming *B. cereus* contaminated foods (Dierick et al., 2005; Mahler H., 1997; Shiota et al., 2010).

There are several types of assays to detect cereulide in different food matrixes. Firstly, the biological effects of cereulide can be measured by sperm, or liver cells based on the inhibition of mitochondrial activity and cellular vacuolization (Stenfors Arnesen et al., 2008). Secondly, cereulide can be detected by instrumental methods, such as high-performance liquid chromatography (HPLC), HPLC tandem mass spectrometry (HPLC-MS) and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) MS (Stark et al., 2013; Tsilia et al., 2012). Thirdly, a monoclonal antibody (mAb 3C6) based immunoassay has been developed for analyzing a protein marker (NRPS) associated with cereulide production by *B. cereus* strains (Zhu et al., 2013c). An immunochromatographic test for this toxin marker is commercially available. Lastly, different primer systems for PCR amplification are routinely used to detect the *ces* gene.

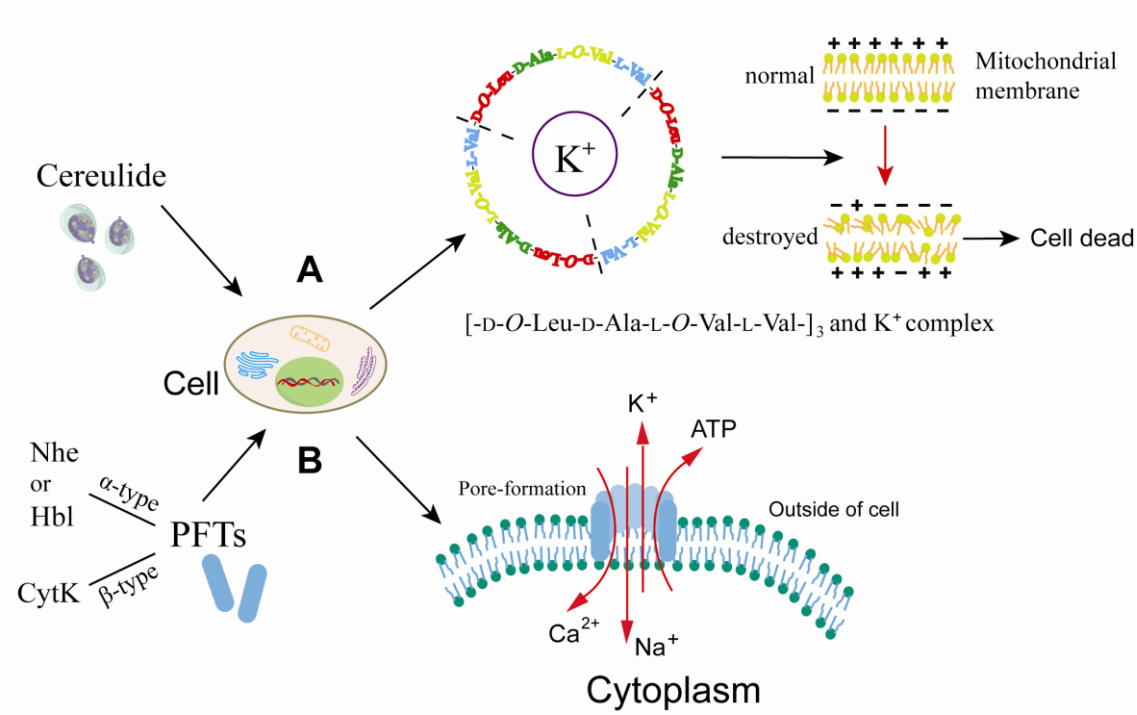


Figure 2 Proposed mechanisms of cereulide, Nhe, Hbl and CytK on cells. Cereulide acts as a potassium specific ionophore to disrupt the electrochemical potential gradient on lipid membranes (A). Both Nhe, Hbl (α-PFTs) and CytK (β-PFT) belong to the family of PFTs, which enable pore-formation on cell membranes (B).

1.2. Non-haemolytic enterotoxin (Nhe) and haemolysin BL (Hbl)

Both Nhe and Hbl are tripartite enterotoxins responsible for diarrhea caused by *B. cereus*. Nhe consists of three proteins NheA, B and C, molecular weights around 38 kDa, encoded by the *nheABC* operon (Lindbäck et al., 2004). Nhe has been identified as the major virulence factor and marker toxin among the various enterotoxins secreted by *B. cereus* (Moravek et al., 2006; Zhu et al., 2013c). Although the mode of action of Nhe is still elusive, the current model, which provides a framework for further work, is shown in Figure 2B. 1) The crystal structure of NheA and the predicted structures of NheB and C indicate that Nhe belongs to the family of alpha-pore forming toxins (α-PFTs) (Ganash et al., 2013); 2) the three components of the Nhe complex are produced at a ratio, close to 10:10:1 for NheA:B:C (Lindbäck et al., 2004); 3) NheC binds to NheB in solution to form different NheB-C oligomers of high molecular weight (Heilkenbrinker et al., 2013); 4) a sequential binding order is necessary to form pores on cell membrane. The cellular events are initiated by the binding of the NheB-C complex to the cell membrane, followed by other NheB molecules resulting in the formation of prepores. Finally, NheA is associated and penetrates the lipid bilayer, which leads to cell death (Lindbäck et al., 2010; Zhu et al.,

2014). There is a significant homology of the amino acid sequences between NheA, B, C and the Hbl components L2, L1, B. Combined with the fact that the crystal structure of Hbl B shows remarkable similarity to the well-known α-PFT ClyA or HlyE (Madegowda et al., 2008), Hbl, like Nhe, is probably also a member of the α-PFT family. In addition, given the similarities between the six *nhe* and *hbl* genes, it has been hypothesized that they originate from a common gene (Stenfors Arnesen et al., 2008), however, no cooperation between these two toxin complexes has been demonstrated and the single components cannot complement each other (Sastalla et al., 2013). Additionally, two types of the *hbl* operon, encoding for two homologous sets of the Hbl complex, sharing a high degree of heterogeneity and distinct physical properties (Beecher and Wong, 2000b), have been described.

Immunoassays for the direct detection of each Nhe and Hbl component have been developed using specific mAbs (Dietrich et al., 1999; Dietrich et al., 2005; Heilkenbrinker et al., 2013). In addition, these mAbs served for the purification of NheB, and the NheB-C complex and for the neutralization of cytotoxicity of the Nhe complex (Didier et al., 2012; Heilkenbrinker et al., 2013; Lindbäck et al., 2010). There are three antibody-based commercial products for the detection of one or two components from the Nhe and Hbl complexes, the BDE VIA™ kit from 3-Tecra for NheA (ELISA), the BCET-RPLA kit from Oxoid for Hbl L2 (reversed passive latex agglutination) and the Duopath® kit from Merck for both NheB and L2 (lateral flow test) (Ceuppens et al., 2012a; Ceuppens et al., 2012b). A recently reported MALDI-TOF/MS for simultaneous detection of the Nhe complex and CytK1 from pathogenic strains of the *B. cereus* group, performed well and showed typical peptides of NheA and CytK1 with calculated masses at 1192.5 Da and 2310.2 Da (Tsilia et al., 2012). Also, indirect methods for assaying the presence of the Nhe complex and/or Hbl complex have been proposed. For example, multiplex PCRs for the differentiation of Nhe genes (*nheA, nheB* and *nheC*) and Hbl genes (*hblC, hblD* and *hblA*) have been applied to screen potentially pathogenic strains (Melnick et al., 2012; Wehrle et al., 2009). In addition, cytotoxicity assays are widely used to directly monitor pore formation on cell membranes (Wehrle et al., 2009; Zhu et al., 2013b). Due to the fact that some *B. cereus* strains harbor silent *nhe* genes (PCR positive results) without protein expression (antibody detection negative) (Zhu et al., 2014), detection of the Nhe complex or related products should be mainly based on cytotoxicity tests or immunoassays.

1.3. Cytotoxin K (CytK) and haemolysin II (HlyII)

CytK as a cytotoxic protein was originally described as haemolysin IV (HlyIV), due to its toxicity to retinal tissue *in vitro* and hemolytic activity (Beecher and Wong, 2000a). Subsequently, it was originally isolated and cloned from the *B. cereus* strain NVH391/98, after a severe foodborne poisoning outbreak in France (Lund et al., 2000). It has been proposed that CytK was solely responsible for the severe symptoms observed, however, a variant gene encoding for the intact Nhe complex has been identified later (Fagerlund et al., 2007). Therefore, the role of Nhe contributing to the pathogenicity of this strain cannot be ignored. Interestingly, a variant of CytK, namely CytK2, has also been isolated from the *B. cereus* strain NVH1230/88, with 98% similarity of the amino acid sequence to the original CytK (CytK1) (Fagerlund et al., 2004). Both CytK1 and CytK2 are β-PFTs (Figure 2B), like the α-toxin from *Staphylococcus aureus* and β-toxin from *Clostridium perfringens* (Stenfors Arnesen et al., 2008). Nevertheless, CytK1 shows five folds higher cytotoxicity to Vero and Caco-2 cells than CytK2 (Fagerlund et al., 2004). Because *B. cereus* strains carrying *cytK-1* genes are rare (only five strains, NVH391/98, NVH 883/00, INRA AF2, AFSSA 08CEB44 BAC and CVUAS2833 have been described), the new species *Bacillus cytotoxicus* sp. nov. , has been proposed (Guinebretiere et al., 2013).

Similar to CytK, Haemolysin II (HlyII) is another β-PFT that has been found in *B. cereus* (Andreeva et al., 2006). Unlike HlyI, HlyII and CytK are not dependent on cholesterol for cell binding (Ramarao and Sanchis, 2013). Both HlyII and CytK can form anion-selective channels or pores in planar lipid bilayers, by penetrating the membrane via their glycine-rich segment (Andreeva et al., 2007; Andreeva et al., 2006). A heptameric pore model has been proposed for HlyII using the crystal structure of *S. aureus* α toxin as a template (Miles et al., 2002). HlyII has only been found in potentially pathogenic strains, neither in food poisoning nor nonpathogenic strains (Cadot et al., 2010). Accordingly, HlyII has never been implicated as a virulence factor responsible for gastrointestinal diseases caused by *B. cereus* (Ramarao and Sanchis, 2013; Stenfors Arnesen et al., 2008). Also, trypsin in the small intestine can digest the β-loop in the transmembrane domain of HlyII to an inactive form (Granum, 1990; Lund et al., 2000). The intrinsic structure of HlyII differs from other β-PFTs, as there are 94 additional C-terminal amino acid residues, which are not required for pore formation or haemolytic activity (Miles et al., 2002).

Moreover, HlyII is one of the few toxins secreted by *B. cereus* that is independent on the regulation of pleiotropic regulator (PlcR). PlcR is the central transcriptional regulator for the genes of *nhe*, *hbl*, *cytk*, *hlyI* and many other virulence factors (Ceuppens et al., 2011).

In lieu of PlcR, the global ferric uptake regulator (Fur) and the specific transcriptional HlyII regulator (HlyIIR) have been demonstrated (Budarina et al., 2004; Harvie et al., 2005), to be dependent on iron and glucose concentrations, respectively. Both HlyIIR and Fur are negative regulators of *hlyII* expression in *B. cereus* in nutrient rich niches (Ramarao and Sanchis, 2013). Glucose passing the bacterial membrane as glucose-6-phosphate (glucose 6P), actives HlyIIR and inhibits the expression of HlyII in glucose rich environment (Guillemet et al., 2013). Analogously, iron binds to Fur, leading Fur to prevent RNA polymerase binding to the HlyII promoters (Sineva et al., 2012). If glucose and iron are limited and sensed by *B. cereus*, the repression of HlyIIR and Fur will be relieved, as shown in Figure 3. Regarding the cytotoxic effects of HlyII, it has been shown that susceptibility is strongly dependent on cell types. In contrast to the enterotoxins Nhe and Hbl, which are highly toxic to epithelial cells and not to monocytes (U937 cells) (Jeßberger et al., 2014), HlyII can induce the apoptosis of different monocytes and macrophages *in vivo*, by pore formation and activating the caspase 3 and 8 dependent pathways (Tran et al., 2011). But the relationship between apoptosis and pore formation is still unknown. With the help of HlyII together with other degradative enzymes and anti-ROS/RNS responses, *B. cereus* (spores) can survive and escape from macrophages (Tran and Ramarao, 2013). This unique property can endow *B. cereus* to withstand the host immune system. This capability could account for the persistence and dissemination of different nongastrointestinal diseases caused by *B. cereus*. Interestingly, HlyII has been demonstrated to enable penetration of plant cell walls and to reach the plasma membrane to form Ca^{2+}-permeable pores in the alga *Chara corallina* (Kataev et al., 2012).

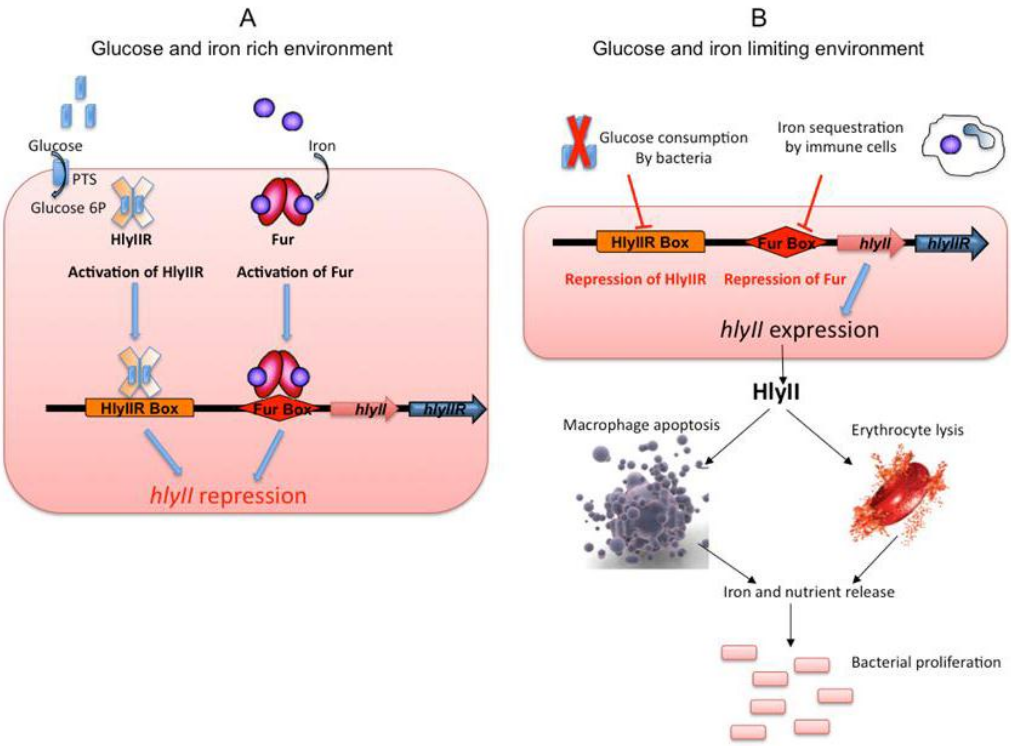


Figure 3 Alternative pathways involved in regulating the expression of HlyII from *B. cereus* in different environmental niches (from Ramarao and Sanchis 2013, open access article).

1.3. Haemolysin I (HlyI)

HlyI also known as cereolysin O (CLO) in *B. cereus*, is haemolytic and lethal for mice by intravenous injection (Bernheimer and Grushoff, 1967). As many other enterotoxins, the expression of HlyI is also controlled by the PlcR virulence regulon via a quorum sensing system in the stationary phase (Gohar et al., 2008). Interestingly, one small PlcR-regulated gene, encoding a 26-amino acid peptide, is co-expressed with HlyI (Brillard and Lereclus, 2007), yet the biological significance of this peptide hasn't been clarified. The release of lactate dehydrogenase in retinal toxicity assay induced by HlyI suggests that it is probably involved in the necrotic endophthalmitis, observed during eye infections by *B. cereus* (Beecher et al., 2000). The protein structure of HlyI shows high similarity (98% identity), with thuringiolysin O (TLO) of *B. thuringiensis* and anthrolysin O (ALO) of *B. anthracis* (Shannon et al., 2003). Meanwhile, these toxins still own close relationship with listeriolysin O (LLO) of *Listeria monocytogenes*, perfringolysin O (PFO) of *Clostridium perfringens* and streptolysin O of *Streptococcus pyogenes* (Shannon et al., 2003). HlyI is therefore a member of the cholesterol-dependent cytolysins (CDCs), and shares some common properties with other CDC toxins (Ramarao and Sanchis, 2013). For example,

HlyI contains a highly conserved tryptophan-rich undecapeptide in domain 4, which is able to form large pores on cell membranes and requires the presence of cholesterol as receptor. Even CD59 can serve as an alternative binding site for some CDCs (Hotze and Tweten, 2012). Although HlyI forms beta-barrel pores on cell membranes (β-PFT), it is obviously distinct from CytK and HlyII by the intrinsic cholesterol binding ability. However, its crystal structure is unknown and the function of HlyI during different infections is still elusive.

1.5. Certhrax and vegetative insecticidal proteins (VIPs)

Although the members of the *B. cereus* group show significant homology based on 16S rRNA analysis, they may contain various exchangeable plasmids (Modrie et al., 2010; Van der Auwera et al., 2007; Vilas-Boas et al., 2002; Yuan et al., 2007). For instance, the two plasmids pOX1 and pOX2, are unique for *B. anthracis*. The plasmid pOX1 contains the major virulence factors, protective antigen (PA), lethal factor (LF) and edema factor (EF) for causing anthrax, whereas plasmid pOX2 encodes the poly-γ-D-glutamic acid capsule for evading the host immune system (Sweeney et al., 2011). However, two similar plasmids pBCOX1 and pBC218 were found in the *B. cereus* G9241 strain, associated with severe pneumonia resembling inhalation anthrax (Hoffmaster et al., 2004). A new toxin Certhrax, encoded by the plasmid pBC218, has recently been purified from the same strain and the crystal structure reveals that it shares 31% sequence identity with anthrax LF (Visschedyk et al., 2012). Functional assays showed that, similar to LF and EF, Certhrax also needs PA to facilitate entry to host cells. However, Certhrax harnesses a mono-ADP-ribosyltransferase mechanism to trigger cytotoxicity, unlike LF, which uses a metalloprotease to hijack the MAPK kinase signal pathway (Sweeney et al., 2011). LF contains an active zinc metalloprotease domain and an inactive mono-ADP-ribosyltransferase toxin (mART) domain, whereas Certhrax differs by having gained a mART domain and having lost a metalloprotease domain (Visschedyk et al., 2012).

The family of bacterial mARTs can mimic the biological function of ADP-ribosylating enzymes to interfere with host cellular events. These toxins vary in the primary sequences, but conserve a protein fold in the active domain to bind NAD^+ and to catalyze it into ADP-ribose and nicotinamide (Deng and Barbieri, 2008). The covalent addition of ADP-ribose molecules to target proteins may result in inactivation of the targets, or may change cellular physiological effects leading to cell death or to signal upregulation. According to their target substrates, mARTs can be divided into actin- and Rho-targeting mARTs

(Visschedyk et al., 2012). Actin-targeting mARTs, like AB binary toxins, consist of two proteins, a cell binding component and a mART component which can inhibit actin ATPase activity, block polymerization or modify actin. This group includes the *Clostridium difficile* (*C. difficile*) ADP-ribosyltransferase, *C. botulinum* C2 toxin, *C. spiroforme* toxin, *C. perfringens* iota toxin, Photox from *Photorhabdus luminescens*, and the vegetative insecticidal proteins (VIPs) from *B. cereus* (Blöcker et al., 2000; Aktories, 2011; Haug et al., 2003). By contrast, Rho-targeting mARTs are single components, targeting host regulators of the actin cytoskeleton, namely the Rho GTPases. Examples are the C3-like toxins, C3bot1 and C3bot2 from *C. botulinum*, C3lim from *C. limosum*, C3cer from *B. cereus*, C3stau from *Staphylococcus aureus* and pertussis toxin (Ptx) from *Bordetella pertussis* (Aktories, 2011; Visschedyk et al., 2012). Certhrax integrates the properties of the Rho GTPase-ribosylating Cer3 and the actin-targeting VIP2 (Visschedyk et al., 2012), therefore it's hard to catalogue it to one subgroup. Certhrax contains a secondary catalytic Gln residue like in Rho-targeting mARTs. The conserved aromatic residue mid-loop can be found in both Rho- and actin-targeting mARTs. Compared to other C2 and C3-like mARTs, Certhrax has a shorter α3 helix for the specific binding of NAD^+, after binding the NAD^+ glycosidic bond is catalyzed and the ADP-ribose group is transfered to target proteins whereby nicotinamide is released.

VIPs are produced by bacteria in the vegetative state, unlike the crystal delta-endotoxins used as biocontrol agents, which are expressed during sporulation. VIPs produced by members of the *B. cereus* group have been considered as alternative agents against some agricultural insect pests, such as western corn rootworm (WCR), which is tolerant to pesticides or transgenic plants expressing insecticidal proteins (Tabashnik et al., 2013). The binary VIP1-VIP2 toxin is mainly derived from *B. cereus* and exhibits specific toxicity to coleopterans and homopterans, whereas VIP3 targeting lepidopterans is isolated from *B. thuringiensis* (Estruch et al., 1996; Yu et al., 2011). As mentioned above, the VIP1-VIP2 toxin belongs to the family of actin-targeting toxins, however, it's distinct from the classic binary toxins such as anthrax or cholera toxins that assemble into complexes exhibiting two functional sites (Han et al., 1999).

1.6. Other toxins or virulence factors

Enterotoxin FM (EntFM) was first isolated from the *B. cereus* FM1 strain, suspected to show vascular permeability activity in rabbits and to cause fluid accumulation in ligated mouse intestinal loops (Asano et al., 1997; Shinagawa et al., 1991). EntFM has now been

identified as a cell wall peptidase and was renamed to CwpFM (Tran et al., 2010). Although CwpFM shows no direct cytotoxicity to either epithelial cells or macrophages, it induces vacuolization of macrophages and contributes to *B. cereus* virulence in the insect model *Galleria mellonella*. Enterotoxin T, encoded by a single gene named *bceT*, was reported to be a cloning artifact (Hansen et al., 2003), and is on longer considered as a virulence factor. The gene of haemolysin III (HlyIII) has been cloned and expressed in *Escherichia coli* (Baida and Kuzmin, 1995), and the crude extracts can induce haemolytic activity via pore formation on erythrocytes (Baida and Kuzmin, 1996). So far, however, this toxin hasn't been purified and it remains unclear if it's secreted by *B. cereus*.

Several nonproteinaceous insecticidal exotoxins have been produced in six *B. cereus* strains, differing from beta-exotoxin I, a small and heat-stable insecticidal nucleotide analogue (Perchat et al., 2005). These partially characterized compounds are resistant to proteolysis and cannot be precipitated by ammonium sulphate, but show different heat stability, molecular weights and insecticidal spectra. Recently, delta endotoxin Cry4Aa was purified from a marine *B. cereus* isolate and identified by MALDI-TOF/MS (Poopathi et al., 2014). The characterization of the isolate as *B. cereus* was mainly based on the 16S rRNA gene sequence alignment, however, several authors reported that identification based on 16S rRNA sequences is not reliable to discriminate between *B. cereus* and other members in the *B. cereus* group (Baillie and Read, 2001; Ivanova et al., 2003).

Many degradative enzymes have been identified in the extracellular proteomes of *B. cereus* such as InhA1, phospholipase C and sphingomyelinase by 2-D electrophoresis (Gohar et al., 2005). InhA1 (immune inhibitor A1), a zinc metalloprotease, shares 96% indentify with that of *B. anthracis*, and can digest various substrates, e.g., antibacterial peptides and extracellular matrix proteins (Chitlaru et al., 2006). For nongastrointestinal infections, InhA1 enables the bacteria to counteract the host immune system, to protect the internalized spores and to escape from macrophages (Ramarao and Lereclus, 2005). Neutral protease A (NprA) also designated bacillolysin, neutral protease B (NprB) or Npr599 in *B. anthracis*, has displayed properties related to virulence factors (Chung et al., 2006). Cooperating with InhA, NprB can cleave a variety of host cell components and regulatory proteins, may allow the pathogenic strains to reach deeper tissues and contribute to their dissemination (Tran and Ramarao, 2013). Due to higher expression levels of *inhA1* and *nprA* in pathogenic than in nonpathogenic strains, InhA1, NprA and HlyII have been chosen as marker candidates for pathogenic *B. cereus* strains (Cadot et al., 2010).

Phospholipase C (PC) and sphingomyelinase (SMase) have been proposed to comprise a

cytolytic unit named cereolysin AB, according to their synergy during lysis of erythrocytes (Gilmore et al., 1989). Genetic analysis showed that cereolysin AB is encoded by two tandem genes, *cerA* for PC and *cerB* for SMase. Three single-base differences, two in *cerA* (nucleotides 866 and 875) and one in *cerB* (nucleotide 1287) are specific for *B. cereus*, and can be used to distinguish *B. cereus* from *B. thuringiensis* (Kim et al., 2000). SMase can hydrolyze sphingomyelin in cell membranes by binding to the sialic acid of the GM3 receptor through the beta-hairpin region, to phosphocholine and ceramide in a metal ion-dependent manner (Oda et al., 2013). This result is consistent with a previous report showing that SMase can inhibit neurite outgrowth in PC12 cells by inducing cell membrane damage (Tamura et al., 1994). This might be the reason why some *B. cereus* strains can cause diseases of the central nervous system. The structure of SMase from *B. cereus* is similar to the beta-toxin of *Staphylococcus aureus*, alpha-toxin of *C. perfringens* and the SMase of *Listeria ivanovii* (Gilmore et al., 1989; Huseby et al., 2007). Purified or recombinant SMase was essential for *B. cereus* induced mortality in mice and various insects (Doll et al., 2013; Oda et al., 2012; Usui et al., 2009). Like the synergistic interaction between SMase and the Hbl complex (Beecher and Wong, 2000a), a complementary effect of SMase and the Nhe complex has recently been demonstrated (Doll et al., 2013). Combined with Nhe, SMase is a strong inducer of polarized colon epithelial cell death. A possible mechanism might be that the pores formed on cell membranes by Nhe facilitate disruption of the inner membrane by SMase. On the other hand, the enzymatic breakdown of ceramide or other components may enhance Nhe binding to cell membrane or the pore formation. All these results indicate that SMase is an important virulence factor contributing to the pathogenesis of *B. cereus*.

2. Boolean logic analysis

Synthetic biology as a new emerging field, rooting in system biology, biochemistry, engineering, control theory and other disciplines, has bloomed in the past decade (Benenson, 2012; Purnick and Weiss, 2009). The prominent characteristics of synthetic biology are to redesign natural cellular functions for practical purposes, or to create artificial biological systems that differ from other existing engineering fields (Serrano, 2007). The goal of synthetic biology is both to advance our understanding of complex regulations in living systems as well as to foster an engineering discipline for various applications. For example, numerous biomedical areas have benefited from the achievements in synthetic biology such as diagnosis and therapeutics of disease, tissue engineering, hazardous chemical monitoring and antimicrobial drug production (Folcher

and Fussenegger, 2012; Riccione et al., 2012; Zakeri and Lu, 2012). Using the hierarchy of computer engineering as a model, the implemented procedures and the goal of synthetic biology may be illustrated from bottom to top (Andrianantoandro et al., 2006). At the bottom of the pyramid there are various biological building blocks, e.g., DNA, RNA, proteins and other components, analogous to transistors and resistors in computer engineering (physical level). Different biochemical reactions performed in cells or in vivo are similar to logic gates in computing (device level). Next, diverse cellular pathways resemble the integrated circuits in computers (module level). These complex pathways work together in a cell to perform sophisticated tasks, like a single computer (computer level). At last, many cells build different tissues or even organisms to meet more challenging tasks, equivalent to the internet connecting computers (network level).

Similar to the logic gate based man-made silicon computer, many Boolean logic procedures have been built up utilizing different systems from chemicals to biological components such as nucleic acids and proteins, to whole cells (bacterial or mammalian cells). Based on the computation of chemicals, chemical computers were constructed for assaying light, temperature, ions and low molecular weight molecules (Elstner et al., 2012; Gotor et al., 2013; Schweighauser and Wegner, 2013), associated with advanced devices, for instance, memory devices, half-adder, encoder and keypad locks (de Ruiter et al., 2010; de Ruiter and van der Boom, 2012; Zou et al., 2012). Meanwhile, biomolecular computing systems have made huge progresses in biosensors for various analytes of interest (Benenson, 2012; Katz et al., 2012). As biological building blocks of biomolecular computers mostly DNA or enzymes are used (Seelig et al., 2006; Xianyu et al., 2013; Zhu et al., 2013a). For example, a series of 3D DNA nanotetrahedra based logic gates has been constructed to generate fluorescent output responses to different input signals, e.g., H^+, $Hg2^+$ and ATP (Pei et al., 2012). Based on these basic gates advanced devices such as half-adder are used to implement more complicated computations. In addition, these nano-structured DNA materials can pass through cell membranes and detect cytoplasmic chemicals by fluorescent imaging. Also, a biomolecular system has been trained to work as Pavlov's dog, using four metabolic enzymes *in vitro* (MacVittie et al., 2013). This bio-inspired system shows the characteristic of memory and offers new ideas for biosensors and biocomputing applications. Although sophisticated networks consisting of concatenated logic gates have been designed for performing complex logic operations, due to the intrinsic properties of the single molecules in each Boolean logic system, it is hard to harness them to perform more complicated computations (MacVittie et al., 2012; Niazov et

al., 2006). Hence, whole bacterial or mammalian cells have been chosen to engineer complex computational modules or circuits and to furnish them with different applications (Benenson, 2009; TerAvest et al., 2011). For instance, a simple genetic circuit with quorum sensing has been designed to understand the intricate cell-cell communication patterns in *E. coli* (Tamsir et al., 2011). Based on the transcription of a repressor and quorum molecules, this system can perform complex calculations. Additionally, all 16 two-input Boolean logic gates have been created in living *E. coli* cells (Siuti et al., 2013). Another essential function of circuits, memory, could be long-term maintained for at least 90 generations in the same work. Recently, a set of synthetic transcription-translation based devices has also been achieved in mammalian cellular biocomputers (Auslander et al., 2012). Individual mammalian cells can perform programmable half-adder and half-subtractor, when different circuits are integrated into a combinatorial arrangement. Although the development of biocomputers is still in its infancy, the time for using these devices is nearing, especially for analytical or diagnostic purposes.

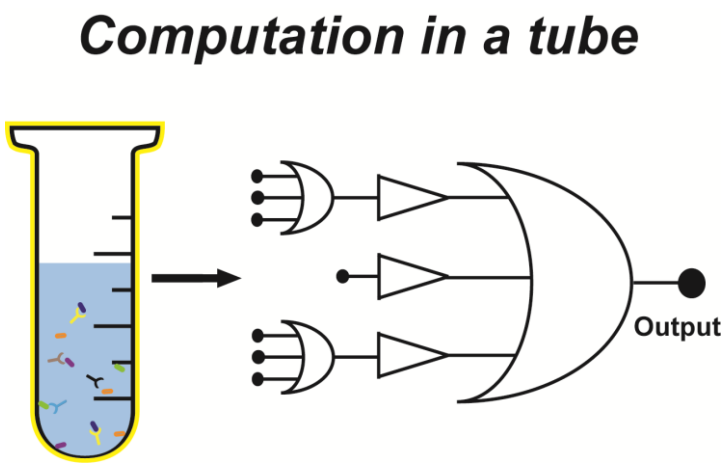
In the present work, we firstly performed an OR gate to sense seven components of bacterial toxins or toxin marker based on monoclonal antibodies. Furthermore, we constructed a cellular logic circuit to detect pore-forming toxins including both α-PFTs and β-PFT. At last combinatorial and sequential logic operations were used to decipher the information flow at the environment-cell interface, based on the tripartite Nhe complex triggered cytotoxicity.

Publication 1

Versatile antibody-sensing Boolean logic for the simultaneous detection of multiple bacterial toxins

Kui Zhu, Richard Dietrich, Andrea Didier, Gabriele Acar and Erwin Märtlbauer

Chem. Commun., 2013, 49, 9314-9316.



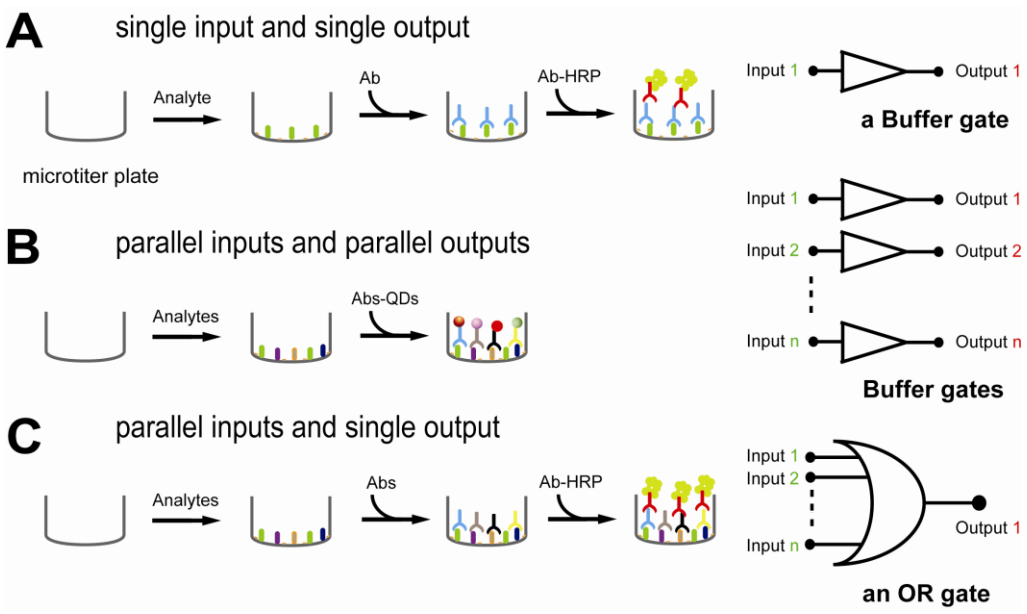
We present a monoclonal antibodies-based OR gate for the simultaneous detection of multiple bacterial toxins in a single tube. To further simplify the operating procedure, the Boolean rule of *simplification* was used to guide the selection of a marker toxin among the natural toxin profiles.

Abstract

We present an OR gate based on monoclonal antibodies for the simultaneous detection of multiple toxins in a single tube. To further simplify the operating procedure, the Boolean rule of *simplification* was used to guide the selection of a marker toxin among the natural toxin profiles.

Bacterial pathogens can cause various infectious diseases in humans and animals and may have a negative impact on environmental health as well.[1] To improve food safety, there is a strong demand to shift end-of-line product inspection to inline assessment during food production.[2] Therefore, a broad spectrum of methods has been developed to replace conventional culture methods, which usually require several days to provide results. The most promising alternative methods include genetic probes and immunochemical assays as well as instrumental analyses.[3] Particularly single-component immunoassays (Scheme 1A) are indispensable tools to detect the presence of bacterial toxins in food samples. A common way to achieve multi-component detection is the fabrication of microarray-based assays, in which the targets of interest are bound by antibodies, which are physically separated on the microarray.[4] Also, parallel quantification of four bacterial toxins has been achieved by measuring the fluorescence signals of antibodies labeled with different quantum dots (Scheme 1B).[5] However, relying on sophisticated biochemical reagents and/or equipment, is still a major limitation of these methods. On the other hand, for in-field controls simple assays covering a broad range of analytes and providing a simple 'YES/NO' answer would be sufficient. As a promising solution to this analytical problem, the application of a mixture of antibodies for the detection of multiple chemicals in a single well of a microtiter plate has been described.[6] These assays can be considered as a first step towards a simple and generic assay design, which can be easily adopted to serve for specific analytical purposes.

During the past years, another successful approach for simple qualitative analyses has been achieved by the development of Boolean logic-based biosensors, which hold out very promising perspectives for bioanalytical applications and enable the generation of a 'YES/NO' response.[7] The logic-based biosensors generally fall into two groups, the cellular logic systems and the abiotic logic systems.[8] The cellular logic systems are based on bacterial or mammalian cells and can perform sophisticated tasks, e.g., mammalian cells-based logic gates have been used to assay the interaction between bacterial toxins and cells.[9] On the other hand, many versatile abiotic logic biosensors have been developed based on various chemicals, biomolecules and nanoparticles for implementing challenging tasks.[10]



Scheme 1. Main types of assays for the detection of bacterial toxins in microtiter plates. (A) ELISA for a single analyte; (B) parallel detection of multiple analytes with multiple outputs; (C) single output for the detection of multiple analytes.

Herein, we integrate seven monoclonal antibodies (mAbs) to demonstrate a simultaneous multiplex assays for seven target proteins present in the same sample in a single well or tube with a binary 'YES/NO' answer (Scheme 1C). These proteins from *Bacillus cereus* (*B. cereus*) include a protein marker for the monomeric emetic toxin (cereulide, Cer) as well as the individual components of two tripartite enterotoxin complexes (the nonhemolytic enterotoxin, NheA, B and C, and hemolysin BL, Hbl L1, L2 and B).[11] To our best knowledge, this is the first example of a simple immunoassay for the simultaneous detection of multiple bacterial toxins in a single tube with colorimetric readout based on Boolean logic operations. In addition, the Boolean rule of *simplification* is used to guide the selection of a marker toxin among the natural toxin profiles and is confirmed by experimental results.

The OR gate based assay showed good response to the toxins in the single tube assay as well as in the single well assay, with significant color changes compared to the negative control (Fig. 1A and Fig. S1). The corresponding absorbance values of each tube were quantified in Fig. 1B. The presence or absence of bacterial toxins is defined as input, 'True/False' or '1/0'; the relative absorbance value is defined as output, and the threshold value used was '0.1', as indicated by the blue dashed line in Fig. 1B. Due to the high specificity of the individual antibodies, there was no cross-reaction between the different target proteins and their corresponding mAbs. The assay is therefore suitable for a first

screening of the targets of interest in biological samples providing a rapid 'YES/NO' answer. In principle, this Boolean logic-based method could also be used for the detection of other multiple contaminants such as the residues of antibiotics in food samples or bacterial resistance genes. For example, the combination of YES and OR gates allowed the reduction of molecular beacon probes required for both the detection of *Mycobacterium tuberculosis* and the presence of rifampin resistance genes from five to two.[12]

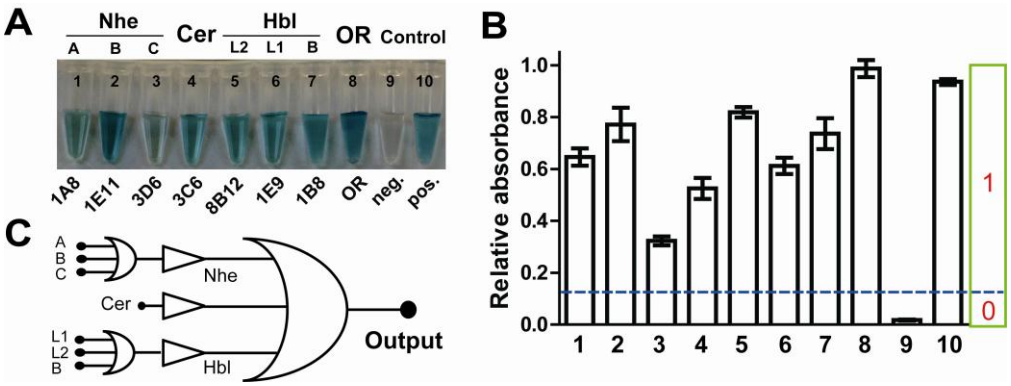


Fig. 1 Typical photograph (A) and corresponding relative absorbance (B) of the heterogeneous mAbs based OR gate for the detection of seven target proteins from *B. cereus* strain MHI 3016 (expressing all seven proteins). The mAbs used are listed in (A) and the colorless tetramethylbenzidine (TMB) is oxidized by horseradish peroxidase-antibody conjugate to blue colored TMB diimine; (C) the equivalent circuit of an OR gate based logic circuit.

Although qualitative and quantitative data about the toxin profile of a bacterial strain are essential to enable an unbiased assessment of the organism´s virulence, there is also an urgent need to select only one or a few markers for virulence in order to improve speed and efficiency of detection. Toward this end, we introduced the Boolean rule of *simplification* to reduce the selection of target toxins among the different possible combinations of Nhe, Hbl and Cer in food related strains. All natural occurring combinations of the three toxins are shown as logic circuit in Fig. 2A, namely Nhe alone (MHI 1491), Nhe plus Hbl (MHI 1505), Nhe plus Cer (MHI 165), and Nhe plus Hbl plus Cer (MHI 3016). To introduce Boolean algebra for the demonstration of *simplification*, we use three letters 'a', 'b' and 'c' to represent Nhe, Hbl and Cer, respectively. According to the logic circuit, we get the Boolean expression of all the combinations 'a + ab + ac + abc', and simplify it to 'a' (Fig. 2B). The corresponding proofs of *simplification* are in Fig. S2. The resulting term 'a' is much simpler than the original expression 'a + ab + ac + abc', yet possesses the same meaning, i.e. Nhe is the main toxin in all combinations of three toxins and it could serve as

the marker toxin for *B. cereus* strains.

This conclusion is supported by a previous report showing that the overall *B. cereus*-associated toxic activity is correlated with the Nhe expression level.[13] The former finding was based on the extensive characterization of 100 *B. cereus* strains originating from food poisoning cases as well as from randomly collected food samples. Selecting Nhe as the major virulence factor, based on Boolean logic as well as experimental data, will certainly simplify the current analytical procedures and accelerate the identification of virulent *B. cereus* isolates. Altogether, this is a convincing proof of principle of an experimental analogue circuit by using combinations of extracellular toxins of naturally occurring strains of *B. cereus*.

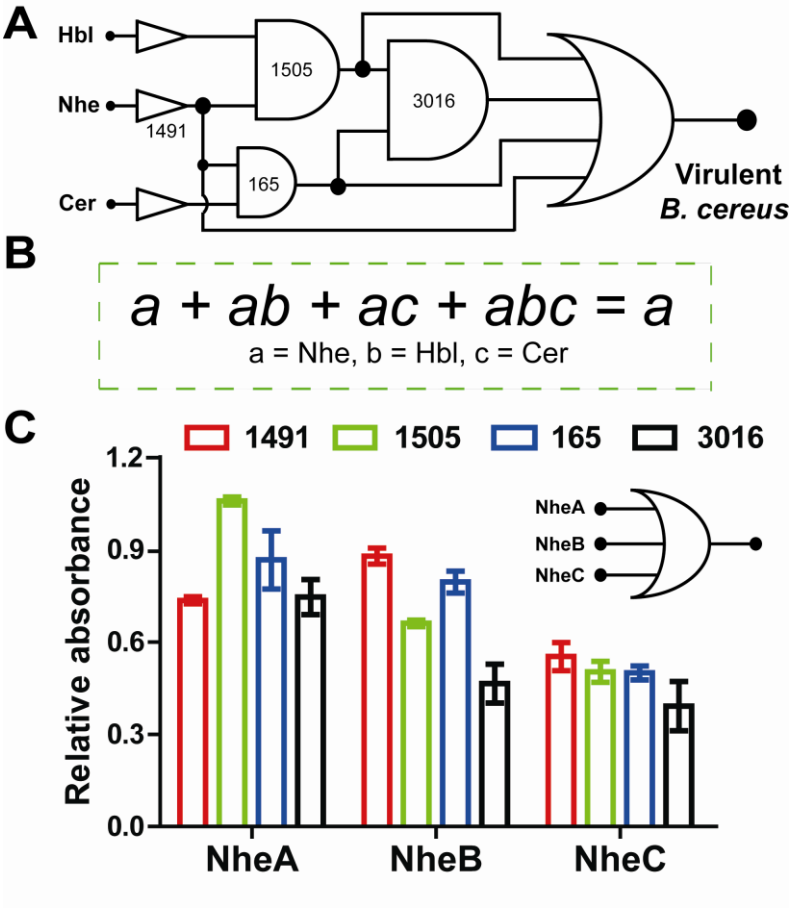
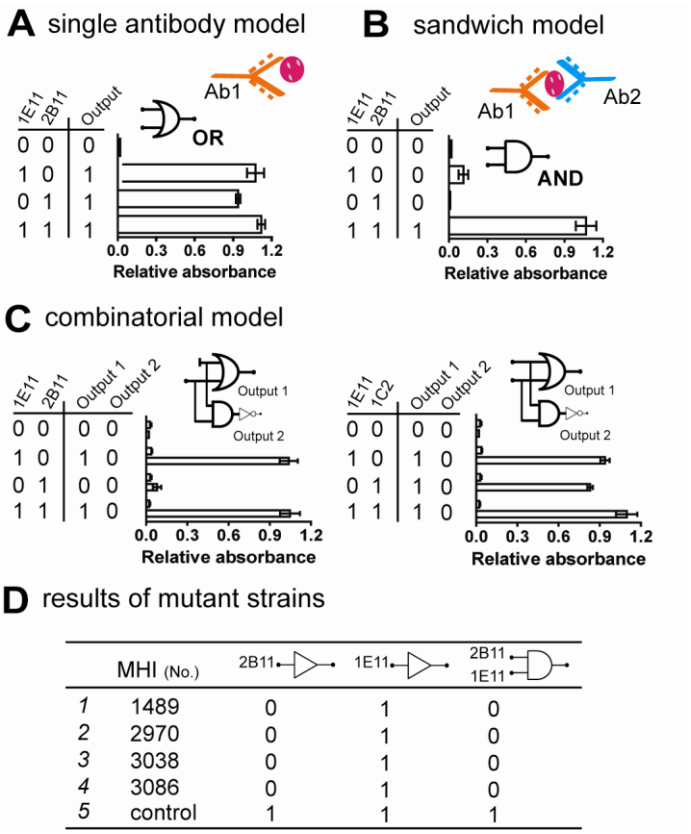


Fig. 2 Selection of the marker toxin from different *B. cereus* strains based on the Boolean rule of *simplification*. (A) All naturally occurring combinations of toxins presented as a logic circuit; (B) algebraic expression of the *simplification*; (C) OR gate of Nhe complex.

The fact that most *B. cereus* strains harbor a complete set of Nhe components provides a natural three-input OR gate for analytical purposes. In other words, measuring the expression levels of the individual Nhe components (NheA, B and C, Fig. 2C) indicates

that the analytical procedure could be even further simplified by reducing measurement to single component detection. For this purpose we chose NheB, which can be detected by different assay types. Using antibodies 1E11 and 2B11, which recognize different epitopes of NheB, in a single antibody assay, represents an OR gate (Fig. 3A). An AND gate using the same mAbs can be established by applying a double antibody (sandwich) assay (Fig. 3B). Considering that *B. cereus* strains may produce NheB owing mutations within the epitopes of the antibodies, but retaining the toxic activity, we analyzed several hundred strains by both assay types. Our results, which are exemplarily shown in Fig. 3C (left) and D, indicate that at least two different antibodies in the single antibody assay are necessary for reliable detection. The examples shown here, are mutant strains harboring amino acid exchanges in the range of amino acid residues 122-150 representing the epitope of mAb 2B11 and, therefore, drastically decrease the affinity of the antibody.[14] The output is 'TRUE' only for 1E11 in the single antibody model and 'FALSE', when combining mAbs 1E11 and 2B11 in the sandwich model. In a second example, a change in conformation upon adsorption of NheB to a surface (polystyrol or cell membrane) is demonstrated by using mAb 1C2. Although, Buffer and OR gates could be set up when replacing mAb 2B11 by mAb 1C2 (Fig. S3) in the single antibody model, no AND gate could be constructed in the sandwich model, Fig. 3C (right). This finding indicates that the epitope of NheB recognized by 1C2 is not accessible in solution, but only after adsorption of the protein to a surface, which has been shown to be a prerequisite for binding of NheA.[14] Altogether, these examples demonstrate that the antibody-based logic gates enable sophisticated tasks not only for accurate determination of bacterial toxins but also for the elucidation of structural properties of the individual toxin components.



	MHI (No.)	2B11	1E11	2B11 1E11
1	1489	0	1	0
2	2970	0	1	0
3	3038	0	1	0
4	3086	0	1	0
5	control	1	1	1

Fig. 3 Three antibody-based strategies for the detection of NheB in the Nhe complex with single antibody model (A), sandwich model (B) and combinatorial model (C). Table containing typical results for mutant strains (D).

In conclusion, this new antibody-based single tube assay generating a bioanalytical result with a qualitative 'YES/NO' answer demonstrates a robust approach for the detection of multiple bacterial toxins and could serve as a general model targets, but also holds the sophisticated power to simplify the selection of markers in complex biological systems. Therefore, the antibody-based logic system represents a versatile platform for the determination of multiple targets of interest and to perform sophisticated logic operations.

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Electronic supplementary information (ESI) available: Experimental details and supporting figures and tables. See DOI: 10.1039/c3cc45370g

Notes and references

1 (a) P. D. Cotter, C. Hill and R. P. Ross, Nat. Rev. Microbiol. 2005, 3, 777; (b) H. Pennington, Lancet. 2010, 376, 1428; (c) M. Kohanski, D. Dwyer, B. Hayete, C. Lawrence

and J. Collins, Cell. 2007, 130, 797; (d) M. F. Bavaro, Curr. Gastroenterol. Rep. 2012, 14, 317.

2 A. P. Craig, A. S. Franca and J. Irudayaraj, Annu. Rev. Food Sci. Technol. 2013, 4, 369.

3 (a) P. Capek, K. Kirkconnell and T. Dickerson, J. Am. Chem. Soc. 2010, 132, 13126; (b) M. Maizels and W. Budde, Anal. Chem. 2004, 76, 1342; (c) A. Løvseth, S. Loncarevic and K. Berdal, J. Clin. Microbiol. 2004, 42, 3869; (d) E. Wehrle, M. Moravek, R. Dietrich, C. Bürk, A. Didier and E. Märtlbauer, J. Microbiol. Methods. 2009, 78, 265.

4 J. B. Delehanty and F. S. Ligler, Anal. Chem. 2002, 74, 5681.

5 E. R. Goldman, A. R. Clapp, G. P. Anderson, H. T. Uyeda, J. M. Mauro, I. L. Medintz, H. Mattoussi, Anal. Chem. 2004, 76, 684.

6 (a) K. Zhu, J. Li, Z. Wang, H. Jiang, R. Beier, F. Xu, J. Shen and S. Ding, Biosens. Bioelectron. 2011, 26, 2716; (b) W. Jiang, Z. Wang, R. Beier, H. Jiang, Y. Wu and J. Shen, Anal. Chem. 2013, 85, 1995; (c) Y. Li, P. Li, X. Luo, Z. Hao, Z. Wang, J. Shen, X. Cao and S. Zhang, Anal. Bioanal. Chem. 2013, 405, 3307.

7 (a) Z. Xie, L.Wroblewska, L. Prochazka, R. Weiss and Y. Benenson, Science. 2011, 333, 1307; (b) P. Siuti, J. Yazbek and T. Lu. Nat. Biotechnol. 2013, 31, 448; (c) E. Katz and V. Privman, Chem. Soc. Rev. 2010, 39, 1835; (d) E. Katz, J. Wang, M. Privman and J. Halámek, Anal. Chem. 2012, 84, 5463; (e) T. Niazov, R. Baron, E. Katz, O. Lioubashevski and I. Willner, Proc. Natl. Acad. Sci. U.S.A. 2006, 103, 17160.

8 (a) M. A. TerAvest, Z. Li and L. T. Angenent, Energy Environ. Sci., 2011, 4, 4907; (b) Benenson, Y. Nat. Rev. Genet. 2012, 13, 455.

9 K. Zhu, U. Acaröz and E. Märtlbauer, Chem. Commun. 2013, 49, 5198.

10 (a) J. Chen, Z. Fang, P. Lie and L. Zeng. Anal. Chem. 2012, 84, 6321; (b) M. Elstner, K.Weisshart, K. Müllen and A. Schiller, J. Am. Chem. Soc. 2012, 134, 8098; (c) D. Liu, W. Chen, K. Sun, K. Deng, W. Zhang, Z. Wang and X. Jiang, Angew. Chem. 2011, 123, 4189; (d) Z. Zhou, Y. Liu and S. Dong, Chem. Commun. 2013, 49, 3107.

11 L. P. StenforsArnesen, A. Fagerlund and P. E. Granum, FEMS Microbiol. Rev. 2008, 32, 579.

12 E. M. Cornett, E. A. Campbell, G. Gulenay, E. Peterson, N. Bhaskar and D. M.

Kolpashchikov, Angew. Chem. Int. Ed. 2012, 51, 9075.

13 M. Moravek, R. Dietrich, C. Buerk, V. Broussolle, M. H. Guinebretière, P. E. Granum, C. Nguyen-the and E. Märtlbauer, FEMS Microbiol. Lett. 2006, 257, 293.

14 A. Didier, R. Dietrich, S. Gruber, S. Bock, M. Moravek, T. Nakamura, T. Lindbäck, P. E. Granum and E. Märtlbauer, Infect. Immun. 2012, 80, 832.

Supporting information --- ---

Experimental Section

B. cereus toxin production

The reference and mutant strains of *B. cereus* used in the current study are listed in Table S1. The standard medium for *B. cereus* growth was casein hydrolysat-yeast medium plus 1% glucose (CGY) according to the previous publication (1).

Monoclonal antibodies

Nine monoclonal antibodies (mAbs) specifically recognizing different components of the toxins or protein marker from *B. cereus* are involved in the present work. mAbs 1A8 for NheA, 1C2, 1E11 and 2B11 for NheB, and 3D6 for NheC; 8B12 for L2, 1E9 for L1 and 1E8 for B in the Hbl complex; 3C6 for a protein marker of cereulide producing by *B. cereus* strains (Table S2).

ELISA and single tube assay

Single antibody and sandwich type immunoassays in microtiter plates were performed as described earlier (1). For the single tube assay, 0.2 mL thin walled PCR tubes with flat cap were used. For the antibody-based OR gates, all primary mAbs were mixed together and applied simultaneously, the secondary antibody-horseradish peroxidase (HRP) conjugate was added subsequently after a washing step.

Toxins		Nhe complex			Cer	Hbl complex			References
		A	B	C		L2	L1	B	
B. cereus (MHI No.)	1491	+	+	+					*1, 2*
	165	+	+	+	+				*3*
	1505	+	+	+		+	+	+	*1*
	3016	+	+	+	+	+	+	+	*4*
	1489	+	+	+					*This study*
	2970	+	+	+					*This study*
	3038	+	+	+					*This study*
	3086	+	+	+					*This study*

Table S1 *B. cereus* strains used in the current study. The NheB components of MHI 1489, 2970, 3038 and 3086 contain point mutations in the range of amino acid residues 122 to 150.

		Nhe complex			Cer	Hbl complex			References
		A	B	C		L2	L1	B	
Antibodies	1A8	+							*5*
	1E11		+						*5*
	2B11		+						*5*
	1C2		+						*5*
	3D6			+					*6*
	3C6				+				*This study*
	8B12					+			*7*
	1E9						+		*8*
	1B8							+	*8*

Table S2 Monoclonal antibodies used for the detection of different components from *B. cereus* strains.

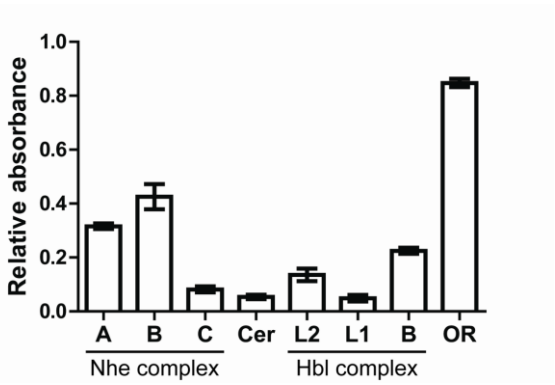

Figure S1 Sensitivity of the antibody-based OR gate in an ELISA microtiter plate.

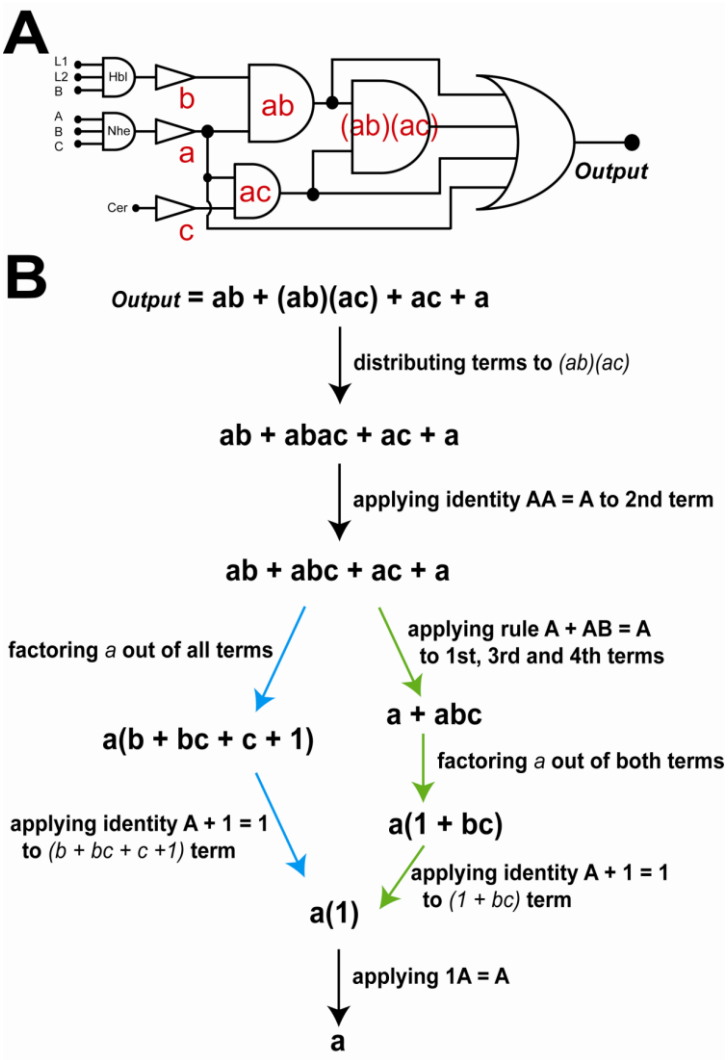

Figure S2 Proofs of the Boolean rule of *simplification*. (A) The Boolean expressions for the toxin profiles of the *B. cereus* strains; (B) The proofs of *simplification* using two methods.

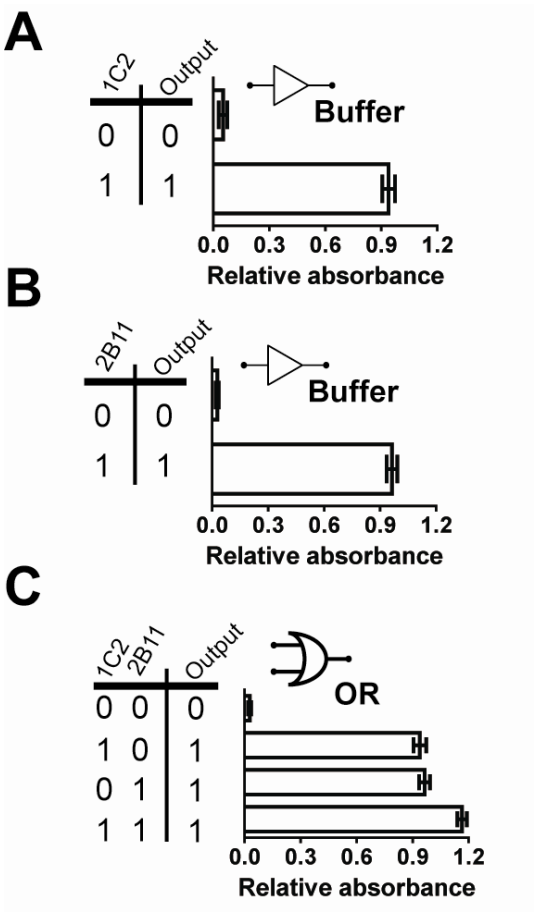

Figure S3 Single antibody model for constructing different logic gates. Two Buffer gates using mAbs 1C2 (A) and 2B11 (B), and an OR gate using mAbs 1C2 and 2B11 in the same assay(C).

References

1. K. Zhu, U. Acaröz, E. Märtlbauer. *Chem. Commun*. 2013, 49, 5198-5200.
2. T. Lindbäck, S. P. Hardy, R. Dietrich, M. Sødring, A. Didier, M. Moravek, A. Fagerlund, S. Bock, C. Nielsen, M. Casteel, P. E. Granum, E. Märtlbauer. *Infect. Immun.* 2010, 78, 3813-3821.
3. M. Ehling-Schulz, N. Vukov, A. Schulz, R. Shaheen, M. Andersson, E. Märtlbauer, S. Scherer. *Appl. Environ. Microbiol.* 2005, 71, 105-113.
4. J. W. Chon, J. H. Kim, S. J. Lee, J. Y. Hyeon, K. Y. Song, C. Park, K. H. Seo. *J. Appl. Microbiol*. 2012, 112, 1042-1049.
5. R. Dietrich, M. Moravek, C. Bürk, P. E. Granum, E. Märtlbauer. *Appl. Environ. Microbiol.* 2005, 71, 8214-8220.

6. U. Heilkenbrinker, R. Dietrich, A. Didier, K. Zhu, T. Lindbäck, P. E. Granum, E. Märtlbauer. *PLoS ONE*, 2013, 8, e63104.

7. R. Dietrich, C. Fella, S. Strich, E. Märtlbauer. *Appl. Environ. Microbiol.* 1999, 65, 4470-4474.

8. M. Moravek, R. Dietrich, C. Buerk, V. Broussolle, M. H. Guinebretière, P. E. Granum, C. Nguyen-the, E. Märtlbauer. *FEMS Microbiol. Lett*. 2006, 257, 293-298.

Publication 2

A cellular logic circuit for the detection of bacterial pore-forming toxins

Kui Zhu, Ulaş Acaröz and Erwin Märtlbauer

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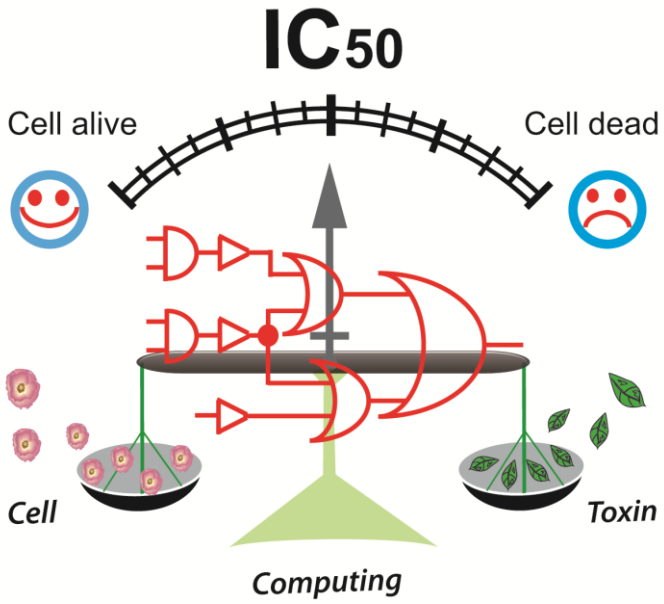


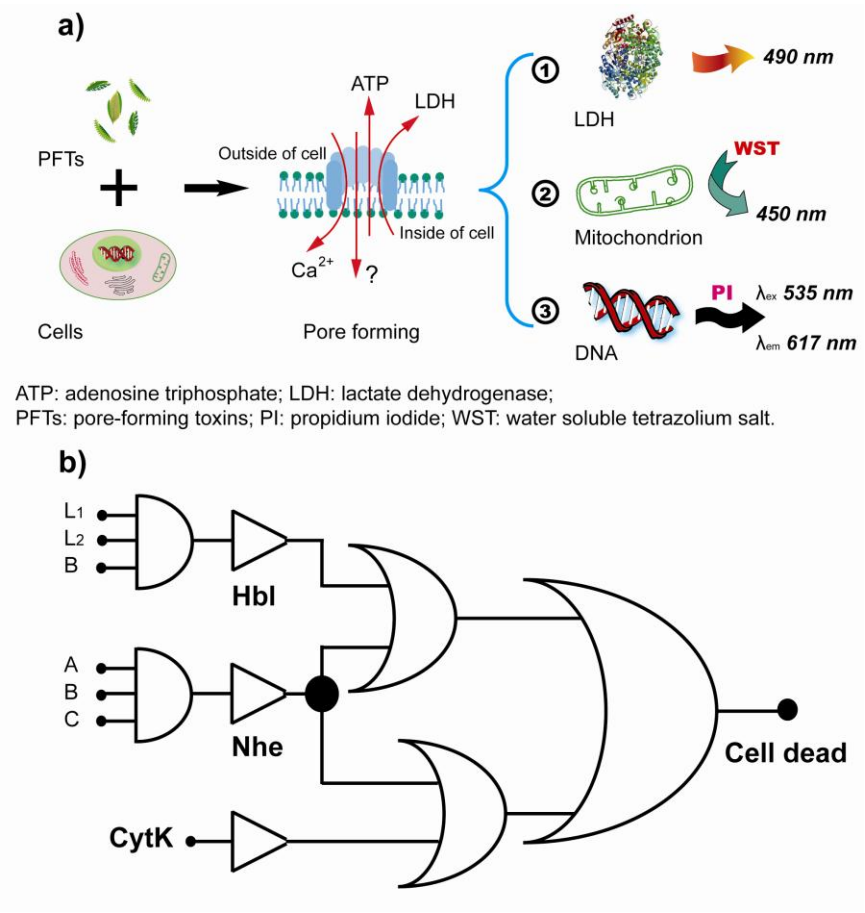
We have developed a cellular logic circuit for the detection of bacterial pore-forming toxins including AND, Buffer and OR gates.

Abstract

We present a cellular logic circuit for deciphering the profiles of toxin production in *B. cereus*, using multiple readout techniques based on the pore formation on the cell membrane. This new assay enables the simultaneous detection of seven biomarkers in pathogenic strains from various samples.

Bacterial toxins are produced by both Gram-positive and Gram-negative pathogens throughout their life cycles to adapt many different niches, which contribute to their virulence or sometimes are essential for their survival.[1] Some bacterial species are responsible for different illnesses due to the expression of diverse pathogenic factors, as exemplified by the *Escherichia coli* including the notorious O157:H7 strain.[2] Although there is a great variety of virulence factors participating into bacterial pathogenesis, pore-forming toxins (PFTs) comprise the largest group (>25%) of all bacterial protein toxins.[3] PFTs are generally produced by bacteria in single soluble monomeric forms, alpha-PFTs and beta-PFTs (depending on the mode of membrane binding, either by alpha-helical or beta-sheet units), and can perform highly ordered tasks by harnessing the versatile monomeric proteins to assemble into oligermeric structures.[4,5] The fact that the self-assembly of oligomers forms a highly ordered ring-like structure to penetrate the target cell membrane, which can be recognized as nanomachines, is particularly fascinating.[1,4a]

Bacillus cereus (*B. cereus*) is a Gram-positive, widely distributed opportunistic bacterium, responsible for two types of foodbore disease, the emetic and the diarrheal syndromes, which are caused by different toxins.[6] The emetic toxin is a heat-stable and ring-formed peptide causing vomiting, while the three protein enterotoxins are the main reasons for diarrhea. All the three toxins, nonhaemolytic enterotoxin (Nhe), haemolysin BL (Hbl) and cytotoxin K (CytK), belong to the group of PFTs.[6a,7] Both of Nhe, Hbl are tripartite toxins and alpha-PFTs, which consist of NheA, B and C, Hbl-L1, L2 and B, respectively. CytK, however, is a beta-PFT and consist employed as models for both alpha- and beta-PFTs to form pores on the cell membrane for constructing a cellular logic system. And the subsequent cell events can be monitored by determining the release of lactate dehydrogenase (LDH assay), activity of mitochondrion (WST assay) and propidium iodide binding to DNA (PI assay), as shown in Schme 1a.



Scheme 1 Cellular logic circuit for the detection of pore-forming toxins (PFTs) from *B. cereus*. (a) Schematic representation of 3 assays for evaluating the viability of the cells treated with PFTs. (b) Equivalent circuit of Nhe, Hbl and CytK based cellular circuit.

Biocomputing systems with sophisticated information processing possess various applications.[8] Novel biosensors based on Boolean logic operations with a simply binary Yes/No answer, have been achieved in abiotic biocomputing systems based on proteins or nucleotides, holding a glorious prospect for bioanalytical applications.[9] Bacterial cells based logic gates have also been developped for different aims.[10] However, the use of mammalian cells to perform biocomputing operations has been rarely reported.[11] Comparing with individual logic gates, logic circuits composed of the concatenated logic gates performing complex operations are urgently needed for the challenaging analysis of multiple inputs with limited selectivity. For example, this new approach can provide an alternative for simultaneous analysis of the presence of multiple biomarkers at different concentration levels, e.g., when a single biomarker analysis is not enough to draw a convinced conclusion.[12] Herein, we propose a sophisticated logic circuit included several types of concatenated cellular logic gates to perform Boolean logic operations processing multiple input signals for simultaneously computing the presence of PFTs in *B. cereus*, as

shown in Scheme 1b. In this work, the presence of all seven proteinic biomarkers of three PFTs from *B. cereus* is reported by African green monkey kidney epithelial (Vero) cells, to generate a qualitative binary result in the Yes/No form.

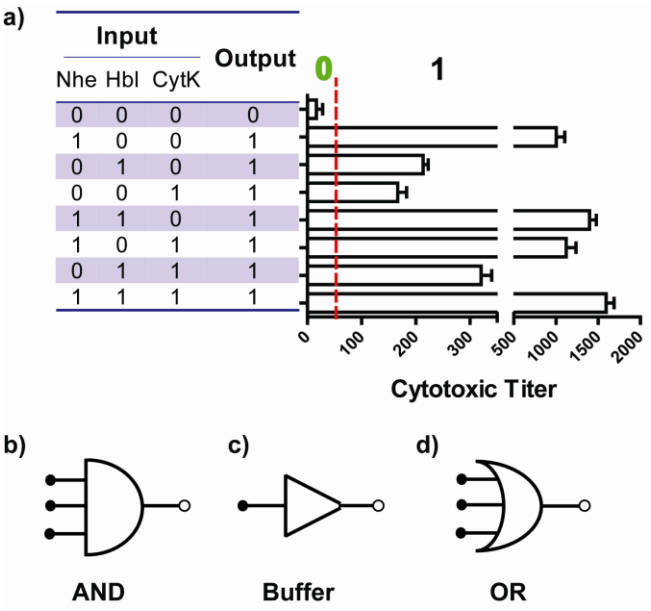


Fig. 1 Nhe, Hbl and CytK based logic gates. (a) Truth table of the cellular OR gate and the associated cytotoxic titers. The threshold value of cytotoxic titer (50) is labled by red dashed line. Equivalent circuit of a AND gate (b), a Buffer gate (c) and a OR gate (d).

As shown in Scheme 1b, the logic circuit is composed of several gates based on the three PFTs, which covers different toxin-types of *B. cereus*. Some strains can produce individual CytK or Nhe toxins, and the expression of Hbl proteins is always coupled with Nhe production, No natural strain can solely express Hbl without Nhe. The combination of CytK, Nhe and Hbl sometimes happens in a single strain, however, no strain harboring only CytK and Hbl has been identified yet. Anyone of the complete toxins can trigger cytotoxicty to Vero cells, e.g., the flat cells could be released from the adherent surface and suspend in the cell culture medium with round morphology when they were treated with the tripartite Nhe complex (Fig. S1). Since none of the three components from Nhe is enough for trigering full cytotoxicty to Vero cells, only simultaneous existence of all three components would provide potential toxicity. So, NheA, B and C are utilized as multiple inputs, the presence and absence are defined as ‘True’ input or ‘1’, and ‘False’ input or ‘0’, respectively. The viability of cells is employed output. To further quantify the information of output signals, the concept of cytotoxic titer is introduced in this work. Cytotoxic titer represents the most dilution fold of toxins that results in 50% loss of cell viability in a dose-dependent manner. A cytotoxic titer above ‘50’ is defined as ‘True’ output or ‘1’ (cell

dead), and a titer below '50' as 'False' input or '0' (cell alive). And that comes to be the same towards Hbl and CytK, as shown in Fig. 1a. Only in the absence of any input (0, 0, 0), the logic circuit is in the dissociated form with 'False' input (0), and all cells survive. In the presence of any input, it will report as a 'True' output, cells dead. Fig. 1b, c and d show the basic logic gates involved in the above cellular logic circuit. Both the three components of Nhe and those of Hbl construct the three-input 'AND' gates (Fig. 1b). The single input of CytK consists of a 'Buffer' gate (Fig. 1c). Meanwhile, the tripartite Nhe or Hbl can also be regarded as one whole input to build another two 'Buffer' gates, since most *B. cereus* strains produce the completed sets of Nhe or Hbl. Hence, all Nhe, Hbl and CytK are utilized as three inputs to construct an 'OR' gate (Fig. 1d).

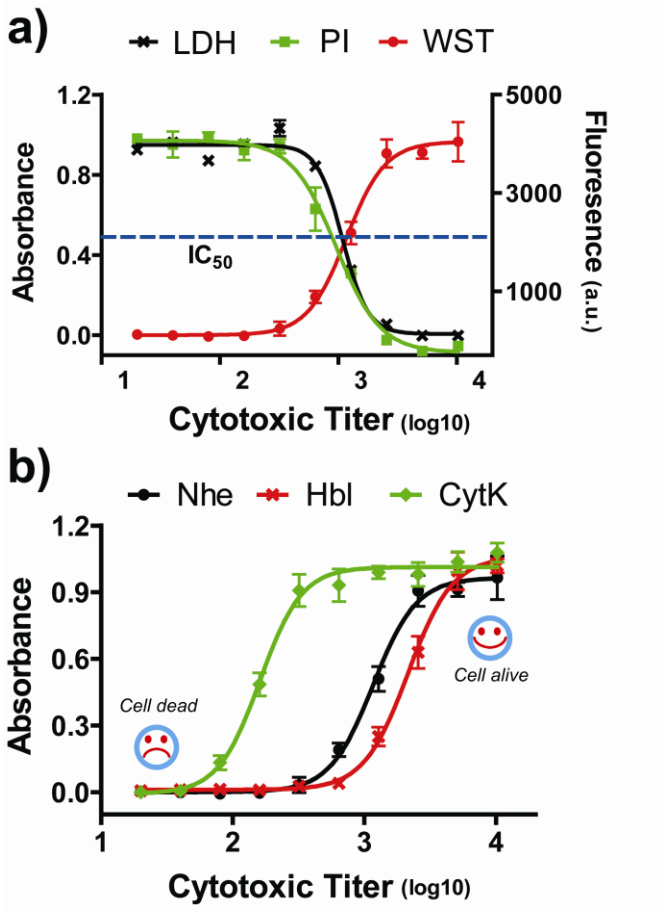


Fig. 2 (a) Comparison of Nhe treated Vero cells using LDH, PI and WST assays. The IC_{50} of Nhe in three assays is labeled by blue dashed line. (b) Standard curves of the reference strains with Nhe, Hbl and CytK productions for the cytotoxicity of Vero cells based on WST assays.

To optimize the cellular logic circuit for the detection of toxins from *B. cereus*, we firstly investigated the output signals of Vero cells with three different techniques (Scheme 1a). The cytotoxic titers of Vero cells with the Nhe treatment were detected by LDH, PI and

WST assays, respectively, as shown in Fig. 2a. All three methods show similar half maximal inhibitory concentrations (IC_{50}) with cytotoxic titers of approximately 1, 000, which means the lowest concentration of Nhe causing 50% Vero cells dead. In comparison among the procedures of three assays (schemes of each assay are shown in Fig. S2), WST assay is the most simplest and easy-to-use colorimetric method. Because: 1) LDH assay needs not only one more step for centrifugation to get LDH supernatant, but also an extra 96-well microplate to transfer the supernatant; 2) PI assay uses a fluorescent probe which is easily quenched by lights, for binding to cellular DNA and equipped with a specifical 96-well black microplate. Therefore, WST assay is used as a standard method for the following study in this work. Fig. 2b shows the standard curves of Nhe, Hbl and CytK (from reference strains, details are shown in supporting information) treated Vero cells in WST assays, the Hbl positive strain shows the highest toxicity to Vero cells and the CytK strain is the lowest one. And similar results were also obtained when using LDH and PI assays for the same Nhe, Hbl and CytK toxins (Fig. S3).

To further confirm the presence of Nhe, Hbl and CytK expressed by the reference strains, specific primers for each genes were used for PCR detection and ELISAs for the individual proteinic components were performed based on six different monoclonal antibodies (mAbs). All three strains harbor *nhe* genes, MHI 1505 and MHI 1307 contain additional *hbl* and *cytk* genes, respectively, comparing with the sole *nhe* gene in MHI 1491, in Fig. S4. The expressed six protein components of *nhe* and *hbl* genes were detected by ELISAs. The presence of NheA, B and C was found both in MHI 1491 and MHI 1505 and Hbl-L1, L2 and B were also positive in MHI 1505 (Fig. S5). Due to the lack of proper antibody against CytK and the fact that despite some strains harbor *cytk* gene without the expression of toxin, this developed cellular logic circuit provide an alternative for this situation.

Robust methods for the analysis of toxin production will not only facilitate the understanding of pathogensis but also provide useful information for diagnosis and food safety.[13] Given the huge diversity of PFTs, many approches have been developed.[14] These methods can be grouped into two general catalogues: those based on the direct detection of PFT genes or the proteinic components, such as PCR and ELISA; and those based on the indirect determination of activity tests, e.g., the released LDH from the cytosol of PFTs treated cells can cause a significant color change of the indicator, as shown in Scheme 1a. For proof-of-concept experiments, the cellular logic circuit was challenged with 20 *B. cereus* strains derived from food related samples, and the toxin profiles were deciphered by PCR and ELISA as well (Table S1). All the strains possess *nhe* genes, but only partial

components or none are expressed in some strains such as MHI 1647, MHI 1761 and MHI 1676, which were also confirmed by specific mAbs. Moreover, although the sandwich ELISA for NheB presents a negative result for MHI 2968, it is proved to be a false negative result based on the sophisticated cellular logic circuit which thus shows a higher accuracy than that of ELISA. The reason may be that the mutation in the epitope of NheB recognized by mAbs significantly decreases the affinity for the antibody binding. Both gene and protein based methodologies suffering from certain drawbacks, but the new developed cellular logic circuit has the particular advantages. For example, all the three components of Nhe are needed to trigger full cytotoxixity rather than the presece of nhe genes (PCR positive result) or incomplete expression of one or two components (ELISA positive result).

In conclusion, we have described a logic circuit based on mammalian cells for the rapid detection of toxin production in *B. cereus*, with simple binary Yes/No answers. The distinctive advantage of the new proposed assay will certainly accelerate the screening and identification of new pathogenic strains and probiotics comparing with the traditional assays that based on the presence of genes or single protein components, such as PCR and ELISA. Among numerous abiotic logic gates reported to date, few applications have been achieved to solve challenging practical problems. We have demonstrated herein that the mammalian cellular logic circuit can be used to perform a sophisticated analytical task efficiently and simply with multiple outputs. This work provides a versatile platform for studying the interaction between bacteria and cells integrating Boolean logic operations and biology.

This paper is dedicated to my dear Prof. Dr. Dr. h. c. Erwin Märtlbauer for his coming birthday.

Electronic supplementary information (ESI) available: Experimental details and supporting figures and tables. See DOI: 10.1039/c3cc41932k

Notes and references

1 J. Pizarro-Cerdá and P. Cossart, Cell, 2006, 124, 715.

2 M. Bielaszewska, A. Mellmann, W. L. Zhang, R. Köck, A. Fruth, A. Bauwens, G. Peters and H. Karch. Lancet Infect. Dis., 2011, 11, 671.

3 (a) R. Aroian and F. G. van der Goot, Curr. Opin. Microbiol., 2007, 10, 57; (b) R. J. Gilbert, Cell Mol. Life Sci., 2002, 59, 832.

4 (a) M. Bischofberger, I. Iacovache and F. G. van der Goot, Cell Host Microbe, 2012, 12, 266; (b) M. Mueller, U. Grauschopf, T. Maier, R. Glockshuber and N. Ban, Nature, 2009, 459, 726.

5 (a) N. Groulx, H. McGuire, R. Laprade, J. L.Schwartz and R. Blunck, J. Biol. Chem., 2011, 286, 42274; (b) M. Bischofberger, M. R. Gonzalez and F. G. van der Goot, Curr. Opin. Cell Biol., 2009, 21, 589.

6 (a) L. S. Arnesen, A. Fagerlund and P. E. Granum, FEMS Microbiol Rev., 2008, 32, 579; b) J.L. Schoeni and A. C. Wong, J. Food Prot., 2005, 68, 636.

7 D. J. Beecher and A. C. Wong, J. Biol. Chem., 1997, 272, 233.

8 (a) Z. Ezziane, Nanotechnology, 2006, 17, R27; (b) I. Willner and E. Katz, Ed. Bioelectronics: From Theory to Applications, Wiley-VCH, Weinheim, 2005; (c) E. Katz and V. Privman, Chem. Soc. Rev., 2010, 39, 1835.

9 (a) E. Katz, J. Wang, M. Privman and J. Halámek, Anal. Chem., 2012, 84, 5463; (b) E. E. May, P. L. Dolan, P. S. Crozier, S. Brozik and M. Manginell, IEEE Sens. J., 2008, 8, 1011; (c) von G. Maltzahn, T. J. Harris, J. H. Park, D. H. Min, A. J. Schmidt, M. J. Sailor and S. N. Bhatia, J. Am. Chem. Soc., 2007, 129, 6064.

10 (a) M. A. TerAvest, Z. Li and L. T. Angenent, Energy Environ. Sci., 2011, 4, 4907; (b) Z. Li, M. A. Rosenbaum, A. Venkataraman, T. K. Tam, E. Katz and L. T. Angenent, Chem. Commun., 2011, 47, 3060; (c) M. A. Arugula, N. Shroff, E. Katz and Z. He, Chem. Commun., 2012, 48, 10174; (d) J. M. Callura, D. J. Dwyer, F. J. Isaacs, C. R. Cantor and J. J. Collins, Proc. Natl. Acad. Sci. U S A, 2010, 107, 15898; (e) T. K. Lu, A. S. Khalil and J. J. Collins, Nat. Biotechnol., 2009, 27, 1139.

11 (a) S. Ausländer, D. Ausländer, M. Müller, M. Wieland and M. Fussenegger, Nature, 2012, 487, 123; (b) Z. Xie, L. Wroblewska, L. Prochazka, R. Weiss and Y. Benenson, Science, 2011, 333, 1307; (c) K. Rinaudo, L. Bleris, R. Maddamsetti, S. Subramanian, R. Weiss and Y. Benenson, Nat. Biotechnol., 2007, 25, 795.

12 (a) L. Halámková, J. Halámek, V. Bocharova, S. Wolf, K. E. Mulier, G. Beilman, J. Wang and E. Katz, Analyst, 2012, 137, 1768; (b) J. Zhou, J. Halámek, V. Bocharova, J. Wang and E. Katz, Talanta, 2011, 83, 955; (c) J. Halámek, V. Bocharova, S. Chinnapareddy, J. R. Windmiller, G. Strack, M. C. Chuang, J. Zhou, P. Santhosh, G. V. Ramirez, M. A. Arugula, J. Wang and E. Katz, Mol. Biosyst., 2010, 6, 2554.

13 (a) O. Lazcka, F. J. D. Campo and F. X. Munoz, Biosens. Bioelectron., 2007, 22, 1205; (b) Y. Shlyapnikov, E. Shlyapnikova, M. Simonova, A. Shepelyakovskaya, F. Brovko, R. Komaleva, E. Grishin and V. Morozov, Anal. Chem., 2012, 84, 5596.

14 V. Havlicek, K. Lemr and K. A. Schug, Anal. Chem., 2013, 85, 790.

Supporting information --- ---

Experimental Section

B. cereus toxin production

The wildtype *B. cereus* reference strains for Nhe, Hbl and CytK productions were designated MHI 1491, MHI 1505 and MHI 1307 in this work (1, 2). MHI means *B. cereus* strain collection of the Chair of Hygiene and Technology of Milk, LMU Munich. Caseinhydrolysat-Glucose-Yeast (CGY) medium plus 1% glucose was used for the toxin production according to the previous publication (3). Briefly, a single colony of *B. cereus* on the overnight cultured blood agar was inoculated into 20 mL CGY medium containing 1% glucose, then cultured in water bath for 17 h at 32 °C, subsequently transferred 0.1 mL of the culture liquid into a new 20 mL CGY medium containing 1% glucose for another 6 h incubation at 32 °C. 200 µL 0.1 M EDTA was added to the final concentration 1 mM before the harvest of toxin supernatants. The mixture was collected by centrifugation at 3 000 g for 20 min, at 4 °C, then passed through 0.22 µm Millipore filter to get bacteria-free supernatants for the further use.

Vero cells and culture condition

Vero cell line was purchased from the European Collection of Cell Cultures (ECACC). The culture condition Vero cells were recommended by the suppliers and incubated at 37 °C with 7% CO_2 (4). Minimum Essential Medium (MEM) Eagle was used for Vero cells, supplemented additional nutritional factors such as 1% fetal bovine serum (FBS), 1% sodium pyruvate and penicillin-streptomycin for the inhibition of bacterial growth.

Cytotoxicity detection assays

Cytotoxic effects of the different supernatants from *B. cereus* were performed on Vero cells using simultaneous incubation method with three different methods for detection as shown in Scheme 1a, WST assay (water-soluble tetrozolium salt, Roche Diagnostics), LDH assay (lactate dehydrogenase, Roche Diagnostics) and PI assay (propidium iodide, Fluka). The schemes of simultaneous incubation method for each assay are shown in Fig. S2. Briefly, the serially diluted toxin supernatants and Vero cells (1×10^4 cells/well) were seeded on 96-well microplates with the final culture medium volume 200 µL per well, and incubated at 37 °C with 7% CO_2 atmosphere for 18 h, then the morphology of Vero cells in the presence and absence of toxins were checked under inverted microscopy *in situ* (Biozero, Keyence), Fig. S1. Although the three mentioned above include the same

procedure for cytotoxicity assays using simultaneous method by mixing different toxins with Vero cells and cultured for 24 h, the quantification of cellular viability are based on different principles. LDH which is a stable cytoplasmic enzyme present in all cells, were rapidly released into the cell culture medium through the pore formation on cell membrane with PFTs treatment. After centrifugation, LDH in the cell-free supernatants was transferred to a new 96-well microplate and subsequently was used for catalyzing its specific substrate with a dramatic color change in solution detected at 490 nm, Fig. S2a. WST assay is based on the presence of WST reduced by the activity of mitochondria of viable cells and results in color changes (from red to yellow) for colorimetric detection as well at 450 nm Fig. S2b, (5). However, PI measurement is detected by fluorescence changes. Because PI can penetrate the damaged plasma membrane and binds to DNA in the nucleus with a large Stokes shift of the fluorescent probe, which can be measured at the excitation and emission wavelengths of 535 nm and 617 nm, respectively, Fig. S2c. The dose dependent manners were used to evaluate the 50% inhibitory concentrations (IC50) of the tested toxins by linear interpolation. The cytotoxic titers were defined as the highest serial dilution of the tested toxins that resulted in the 50% cells lost their viability, comparing with the parallel control.

PCR and ELISA

The *nhe, hbl* and *cytk* genes in each strain were detected according to the previous reports (2). The presence of each component of Nhe and Hbl complexes were deciphered by different monoclonal antibodies (mAb) in two types of ELISA (indirect and sandwich ELISAs), as shown in Fig. S5a. NheA (mAb 1A8), Hbl L1 (1E9) and Hbl B (1B8) were detected by indirect ELISAs, however, NheB (2B11 and 1E11), NheC (3D6 and 1E11) and Hbl L2 (1A12 and 8B12) were detected by sandwich ELISAs (6, 7). Subsequently, the horseradish peroxidase (HRP) conjugates were used to catalyze the TMB substrates (3,3',5,5'-tetramethylbenzidine) and the reaction was stopped by 1 M H_2SO_4 for colorimetric detection at 450 nm. Due to the lack of proper antibodies against CytK, so there was no available ELISA for this component in this work.

	Strain (MHI)	Gene			Nhe			Hbl			Cellular Circuit
		nhe	*hbl*	*cytk*	A	B	C	L1	L2	B	
	1491	+			+	+	+				1
Ref.	1505	+	+		+	+	+	+	+	+	1
	1307	+		+							1
1	1475	+	+	+	+	+	+	+	+	+	1
2	1489	+			+	+	+				1
3	1490	+	+	+	+	+	+	+	+	+	1
4	1501	+	+		+	+	+	+	+	+	1
5	1503	+			+	+	+				1
6	1513	+	+	+	+	+	+	+	+	+	1
7	1522	+		+	+	+	+				1
8	1556	+	+								0
9	1647	+			+						0
10	1669	+	+	+	+	+	+	+	+	+	1
11	1672	+			+	+					0
12	1676	+		+							1
13	1678	+	+		+	+	+	+	+	+	1
14	1700	+									0
15	1761	+				+	+				0
16	2964	+			+	+	+				1
17	2968	+		+		+	+				0
18	2969	+	+		+	+	+	+	+	+	1
19	2971	+		+	+	+	+				1
20	3016	+	+	+	+	+	+	+	+	+	1
	Total	**20**	**9**	**9**	**15**	**15**	**14**	**8**	**8**	**8**	**14**

Table S1 Comparison of the presence of PFT productions of 20 *B. cereus* strains isolated from food related samples at genetic, proteinic and cellular levels. *nhe, hbl and cytk* genes were detected by PCR; the proteins of NheA, B and C, Hbl L1, L2 and B were detected by different forms of ELISA with specific monoclonal antibodies against each components; results of cellular circuit were based on the cytotoxicty assay of Vero cells. **Note:** Ref., the reference strains of *B. cereus*; "+", positive detection.

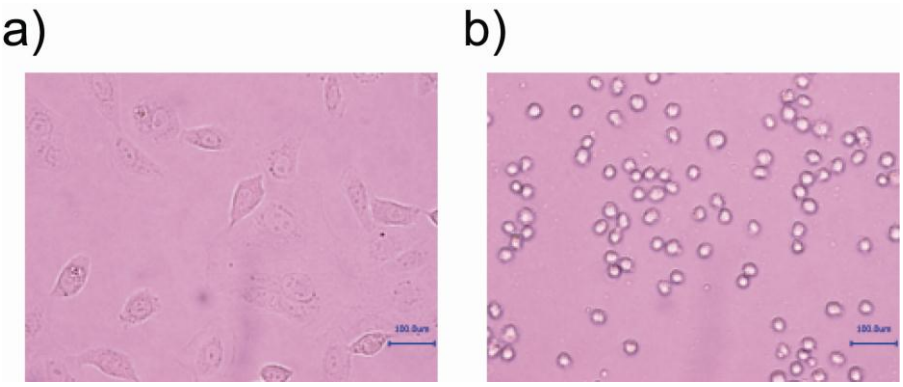


Figure S1 Morphologies of Vero cells in the absence (a) and presence (b) of the complete Nhe complex were shown as a model under an inverted microcopy *in situ*. The rose-colored background is due to the intrinsic color of MEM Eagle medium.

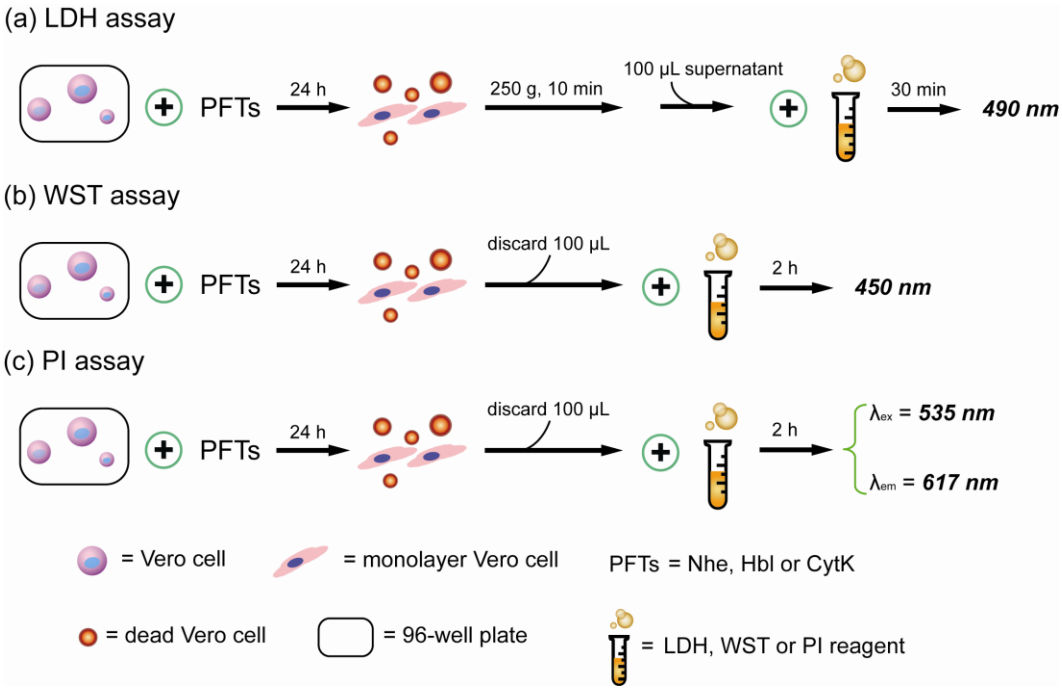


Figure S2 Schematic representation of the simultaneous methods used for cytotoxicity assays in the present work, (a) LDH assay, (b) WST assay and (c) PI assay.

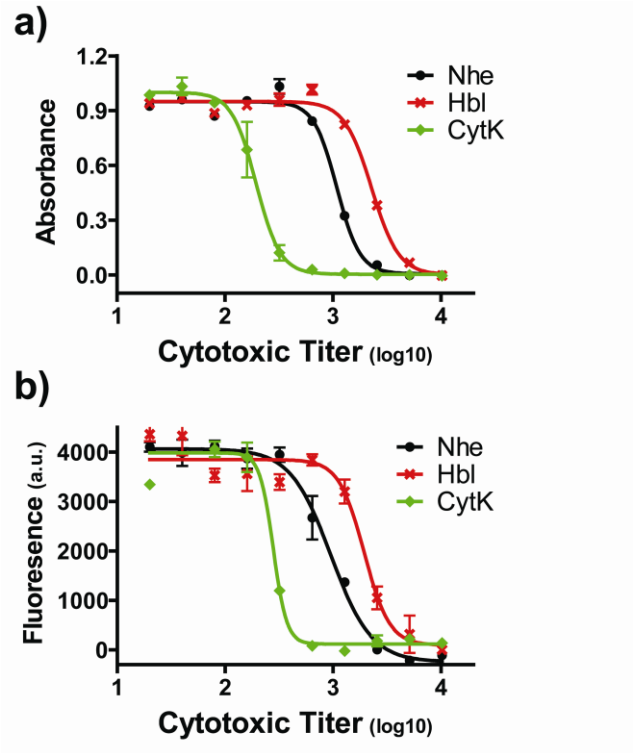


Figure S3 Standard curves of the reference strains with Nhe, Hbl and CytK productions for the cytotoxicity of Vero cells based on LDH assays (a) and PI assay (b).

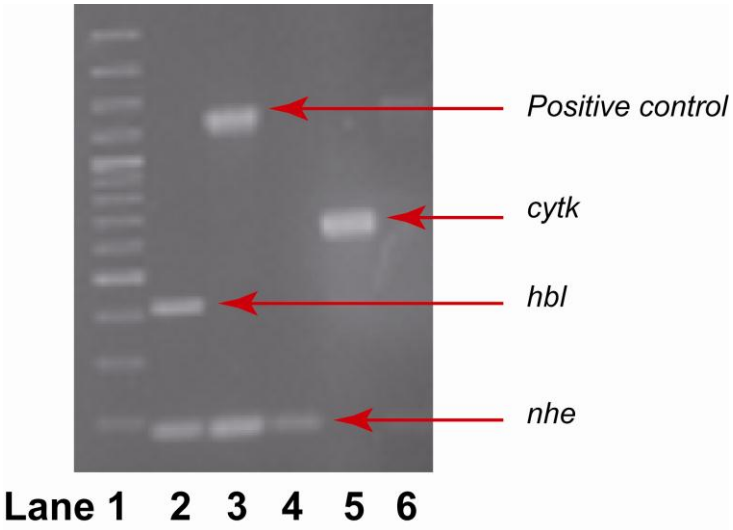
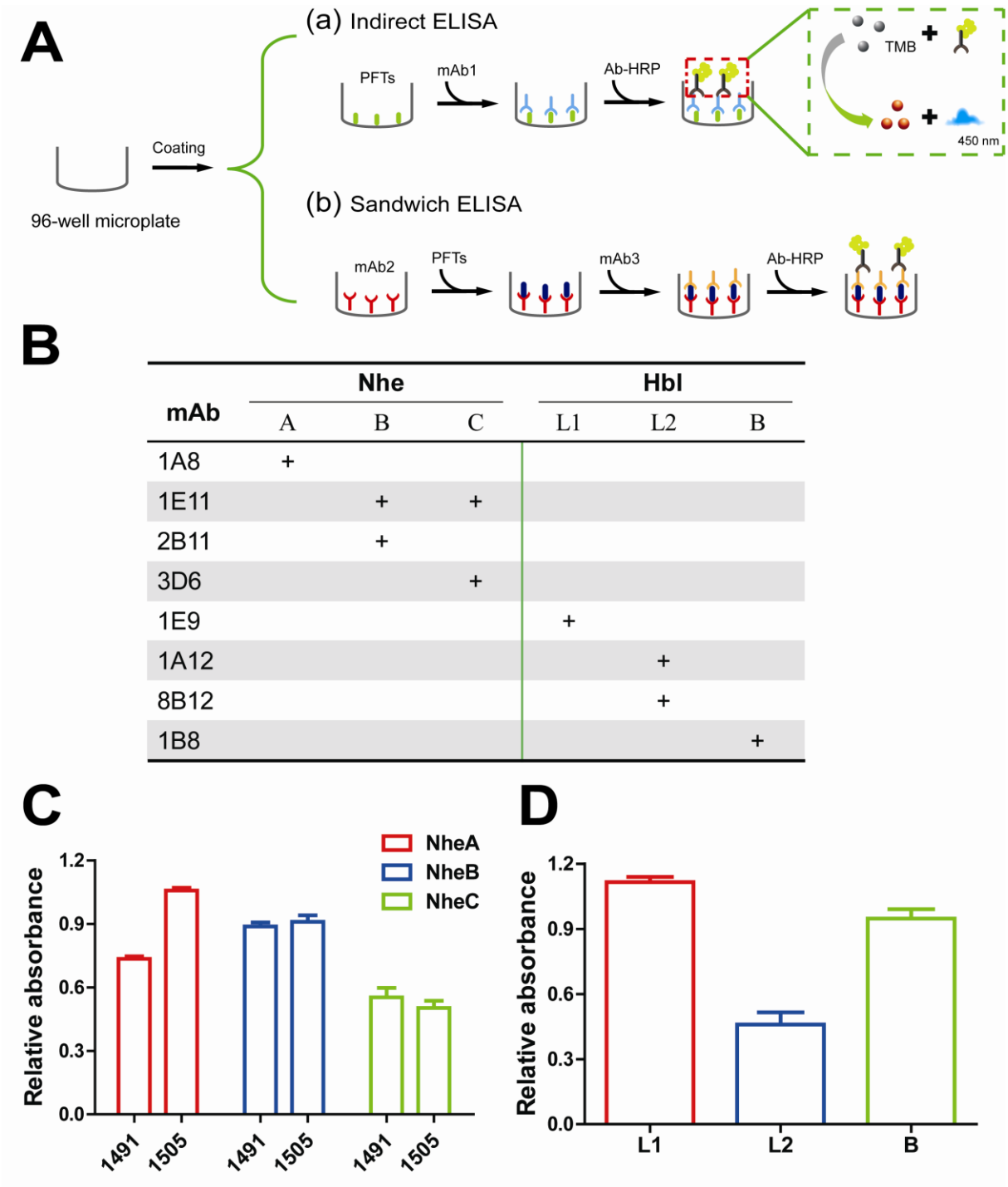


Figure S4 The presence of *nhe*, *hbl* and *cytk* genes were detected by PCR in *B. cereus* reference strains. Lane 1, DNA marker; Lane 2, MHI 1505 (*hbl* positive); Lane 4, MHI 1491 (*nhe* positive) and Lane 5, MHI 1307 (*cytk* positive); Lane 3 and Lane 6 were positive and negative controls, respectively. Due to the gene variant encoding Nhe in MHI 1307, *nhe* gene was not detected in the current PCR protocol for the generally designed primers.



mAb	Nhe			Hbl		
	A	B	C	L1	L2	B
1A8	+					
1E11		+	+			
2B11		+				
3D6			+			
1E9				+		
1A12					+	
8B12					+	
1B8						+

Figure S5 (A) schematic representation of two formats of ELISAs for the detection of different components in Nhe and Hbl complexes from *B. cereus*, (a) indirect ELISA and (b) sandwich ELISA; (B) diverse monoclonal antibodies (mAbs) involved for the specific binding to NheA (1A8), Hbl L1 (1E9) and Hbl B (1B8) with indirect ELISAs, NheB (2B11 and 1E11), NheC (3D6 and 1E11) and Hbl L2 (1A12 and 8B12) with sandwich ELISA; (C) the presence of NheA, B and C in the reference strains of MHI 1491 (for Nhe production) and MHI 1505 (for Hbl production); and (D) the presence of Hbl in MHI 1505.

References

1. T. Lund, P. E. Granum. *FEMS Microbiol Lett.*, 1996, 141,151-156.
2. E. Wehrle, M. Moravek, R. Dietrich, C. Bürk, A. Didier, E. Märtlbauer. *J. Microbiol. Methods*. 2009, 78, 265-270.
3. D. J. Beecher, A. C. L. Wong. *Infect Immun.*, 1994, 62, 980-986.
4. A. Didier, R. Dietrich, S. Gruber, S. Bock, M. Moravek, T. Nakamura, T. Lindbäck, P. E. Granum, E. Märtlbauer. *Infect Immun.*, 2012, 80, 832-838.
5. T. Lindbäck, S. P. Hardy, R. Dietrich, M. Sødring, A. Didier, M. Moravek, A. Fagerlund, S. Bock, C. Nielsen, M. Casteel, P. E. Granum, E. Märtlbauer. *Infect Immun.*, 2010, 78, 3813-3821.
6. R. Dietrich, M. Moravek, C. Bürk, P. E. Granum, E. Märtlbauer. *Appl. Environ. Microbiol.*, 2005, 71, 8214-8220.
7. R. Dietrich, C. Fella, S. Strich, and E. Märtlbauer. *Appl. Environ. Microbiol.*, 1999, 65, 4470-4474.

Publication 3

Ordered self-assembly of proteins for computation in mammalian cells

Kui Zhu, Jianzhong Shen, Richard Dietrich, Andrea Didier, Xingyu Jiang and Erwin Märtlbauer

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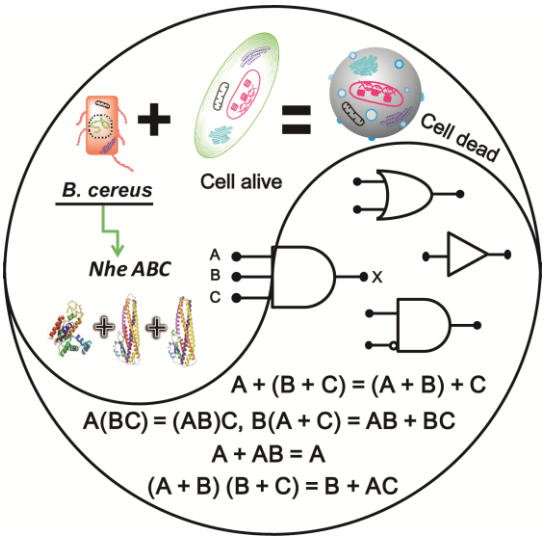


A cellular logic system capable of combinatorial and sequential logic operations based on bacterial protein-triggered cytotoxicity was constructed. Advanced devices such as a keypad lock, half-adder and several basic Boolean properties were demonstrated on the cells.

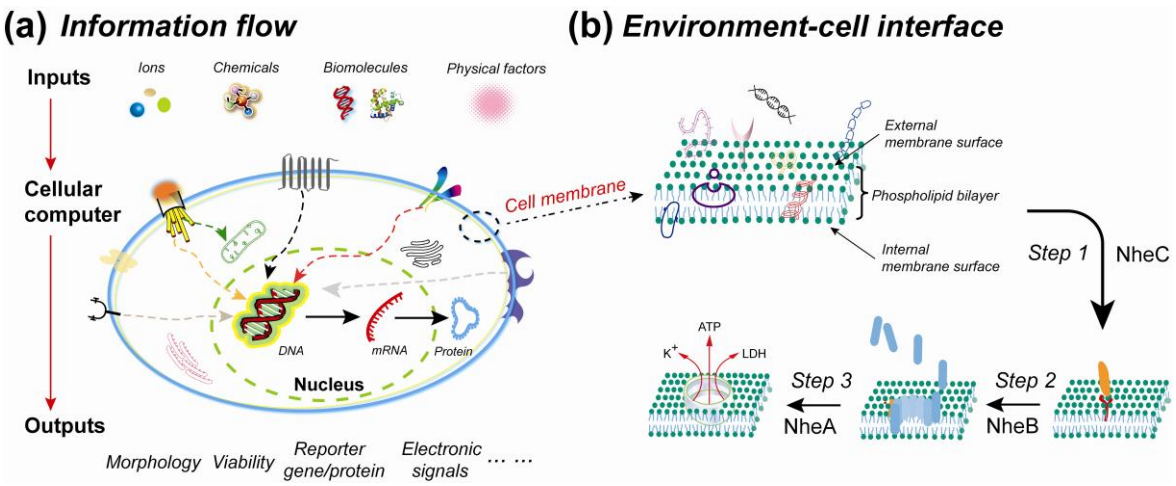
Abstract

A cellular logic system capable of combinatorial and sequential logic operations based on bacterial protein-triggered cytotoxicity was constructed. Advanced devices such as a keypad lock, half-adder and several basic Boolean properties were demonstrated on the cells.

Note: This article has been highlighted by *Chemistry World*, with the title ‘Steering cells towards biocomputers’, 25 Nov. 2013.

Recent advances in our understanding of biology are critical to the development of future biocomputers, with several distinct advantages, such as flexible design, efficient energy usage, memory functions and error checking.[1,2] These developed biocomputing systems can be generally grouped into two categories, the bottom-up approach uses biomolecules for sensing and performing computation; on the other hand, the top-down method utilizes whole cells including prokaryotic or mammalian cells to implement sophisticated tasks.[3-5] For example, bacterial virulence proteins have been used as valuable synthetic biology tools to engineer mammalian cells for therapeutic and analytical applications.[6]

Compared to the silicon-based computers using sole electrical signal for information processing, natural cellular biocomputing systems harness various inputs including chemicals, biomolecules even physical factors such as light and heat. After the extracellular inputs have been sensed by cells, they pass through the first barrier, the cell membrane, then activate downstream effectors in the cytoplasm, and eventually trigger different gene regulators in the nucleus leading to the expression of corresponding proteins, which will execute specific tasks.[7] All these events take part in the integral information flow from Input to Output, as shown in Scheme 1a. Whereas the information flow from DNA to RNA and to protein synthesis, according to the central dogma of molecular biology, is well known,[8] the crucial role of the cell membrane, the environment-cell interface, in sensing, filtering, amplification, and storage of external signals is still not fully understood. The environment-cell interface shows a phospholipid bilayer architecture with which various receptors and other recognition elements interacting with extracellular stimuli are anchored.[9] To elucidate the process of information transduction between Input and environment-cell interface will facilitate the development of biocomputers with useful human-machine interfaces. In this work, we utilize the self-assembly of a bacterial toxin complex on the cell membrane of immortalized mammalian cells as a 3-input framework to conduct different logic operations (Scheme 1b).



Scheme 1. a) Schematic representation of a mammalian cell-based biocomputer. b) The environment-cell interface mediated sequential logic circuit of the Nhe multicomponent toxin complex.

This extracellular protein complex, the nonhemolytic enterotoxin (Nhe), is expressed by most strains of *Bacillus cereus*, but can also be produced by non-*B. cereus* strains.[10] The Nhe complex consists of the cytolytic protein NheA and the binding components NheB and NheC. Nhe shows some unique properties: (i) the individual Nhe proteins are not toxic, (ii) toxicity can be evoked only by a specific ordered binding sequence, provided that (iii) the Nhe proteins are present in a specific molar ratio, and (iv) Nhe toxicity can be neutralized by antibodies interfering with the binding order.

These properties render the Nhe complex interesting for biocomputing and particularly for constructing Boolean logic gates, which could serve as the basis for sequential logic circuits (SLC). SLCs are commonly used for the construction of memory devices and storage elements.[11] Although many logic operations have been achieved in cell-free systems, only a few logic gates have been developed in bacterial and mammalian cells.[3-5] However, no sequential logic system based on cells has been reported. In this work, we propose a cellular SLC on the natural phospholipid bilayer of the mammalian cell membrane, engineered by the information encoded in the Nhe tripartite protein complex ("Inputs"). In addition, the self-assembly of the ring-shaped nanopore formed by the Nhe complex on the membrane enables multiple readouts for monitoring the dynamic process between the Nhe complex and the cell. We used the developed cellular logic circuit to generate the output signals in this protein-based cellular logic system.[6b] We first evaluated the response of several cell lines to the Nhe complex, including A549, Caco-2, HEp-2, IPEC-J2 and Vero cell lines. Vero cells were chosen as a model cell line to perform the following logic operations due to their high susceptibility (Fig. S1). The dynamic response

curves of the propidium idodide (PI) and water-soluble tetrazolium salt (WST-1) assays show that the Nhe complex triggers a rapid cellular response in a time-dependent manner (Fig. S2).

We employed the Nhe components as input, and defined the presence and absence of the individual components (NheA, B and C) as a “True” input, or “1” and a “False” input, or “0”, respectively. The cellular activity of viable cells was regarded as the output, for which we defined a cytotoxicity above 10% as “True” output, or “1” and a cytotoxicity below 10% as “False” output, or “0”. So, we initially constructed a combinatorial AND gate requiring the presence of all three components of the Nhe complex (NheA, B and C = 1/1/1), which caused high cytotoxicity to Vero cells (output = 1), as shown in Fig. 1b. Applying a mixture of all three components simultaneously represents a Buffer gate (Fig. 1c). If, however, the Nhe components are added individually to the cell membrane and unbound proteins are removed after each step, an intrinsic binding order was observed (Scheme 1b). The binding order requires that NheC binds to the cell membrane first (step 1). Together with NheB, which is added in the second step, a stable pre-pore is formed. During this step NheB undergoes a conformational change allowing association of NheA (step 3), which finally leads to cell lysis.[12] This self-assembly of the three Nhe proteins, triggered by the contact with the cell membrane, forms a natural model of sequential logic and allowed the set-up of an intelligent keypad lock (Fig. 1d and e). As a practical application, we characterized the toxin profiles of 30 *B. cereus* strains isolated from food samples by using the supernatants of the strains as a Buffer gate (Fig. 1c) together with partial Nhe components (Table S1). All the strains harbored the *nhe* genes, but some strains did not express the full tripartite Nhe complex at the protein level resulting in a “False (0)” output in the Buffer gate. By supplementing the Buffer negative strains with either NheA and B (input X2 = AB) or NheB and C (input X2 = BC), the toxin profile could be deciphered.

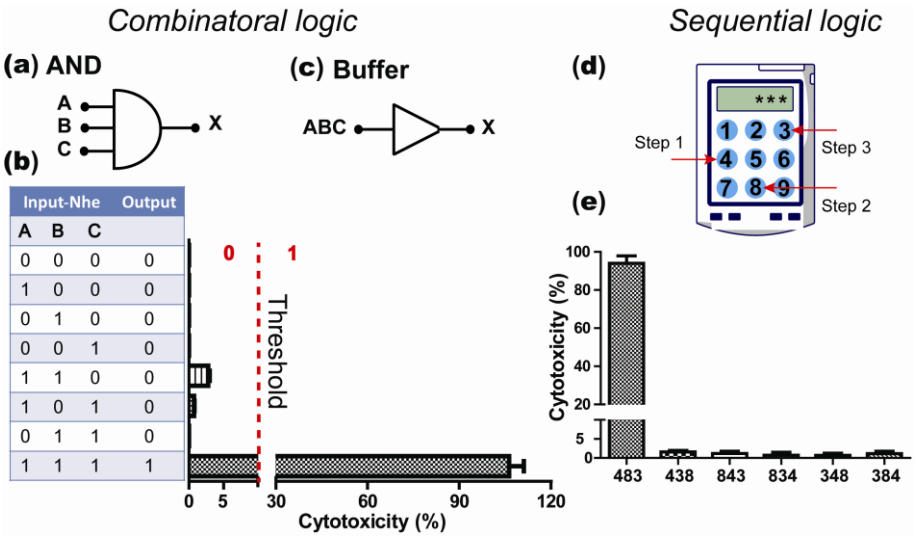


Fig. 1 Nhe-based cellular logic. Equivalent circuit (a) and truth table of the cellular AND gate and the associated cytotoxicity (b). The threshold value (10% of cytotoxicity) is indicated by the dashed red line. (c) Equivalent circuit of a Buffer gate. Scheme (d) and response of the WST-1 assay (e) of all permutations of a three-bit keypad lock.

Based on the recombinant proteins from *E. coli* (rNheA and rNheC) as well as the mutant strains MHI 1672 (NheA and B) and MHI 1761 (NheB and C), we constructed three "OR" gates for each component of the Nhe complex in the reference strains MHI 1491 (NheA, B and C), Table S2 and Fig. S3. To construct INHIBIT gates, physical and biochemical treatments were employed to neutralize the cytotoxic effects of the Nhe complex (Fig. 2a). The Nhe complex is heat-labile[13] and the cytotoxicity of Nhe is abolished in a temperature-dependent manner. Almost no cytotoxic effect was observed when Nhe was heated at 70 °C for 10 min. Both NheA and B showed a sharp decrease of cytotoxicity above 50 °C, whereas NheC was heat-stable even at 100 °C (Fig. S4). A second INHIBIT gate was constructed using a monoclonal antibody (mAb 1E11) against NheB. Addition of mAb 1E11 to the Nhe components nearly completely neutralized the cytotoxic effect caused by the toxin complex (Fig. S5). Disrupting the optimum ratio of the three Nhe components provides yet another means to construct an INHIBIT logic gate. With Nhe the maximum cytotoxicity is achieved when the ratio of the three components is kept at a proper level (NheA : B : C = 10 : 10 : 1).[14] Interestingly, as the concentrations of NheC increases, the cytotoxicity of the Nhe complex decreases (Fig. S6). Therefore, to obtain a third INHIBIT gate, we just had to add an excess concentration of rNheC to the Nhe complex.

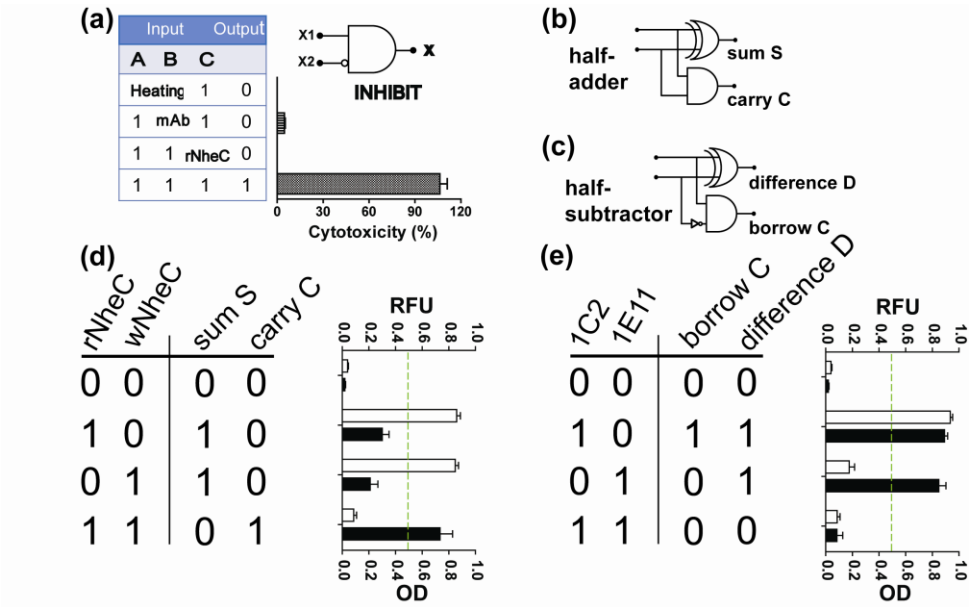


Fig. 2 Equivalent circuit and truth table of the cellular INHIBIT gates and the associated cytotoxicity (a) after applying heat treatment, antibody, or excess rNheC (X2) to the Nhe complex (X1). Symbolization, true tables and experimental results of half-adder (b, d) and half-subtractor (c, e). OD (absorbance, black bars) and RFU (fluorescence, white bars) represent the results of ELISA and cellular assay.

Towards a programmable cellular biocomputer, we constructed two commonly used combinational circuits, a half-adder and a half-subtractor, based on the described OR and INHIBIT gates. The half-adder unit performing the addition of two bits, combines a XOR gate with an AND gate (Fig. 2b and d). The XOR gate producing a sum S output, is based on the fact that excess wild-type NheC (wNheC) and rNheC together with NheB will form a stable NheB-C complex in solution, resulting in no toxicity to cells. It reports a ‘False’ or ‘0’ answer, if and only if the presence of inputs is the same (0 + 0 = 0 and 1 + 1 = 10), with ‘low’ toxicity. In the presence of either input (only one of wNheC and rNheC), it reports ‘True’ or ‘1’ answers, with ‘high’ toxicity. The AND gate producing a carry C output, is based on the readout of an ELISA that specifically detects the concentration of free NheC in solution. Both XOR and AND gates implement their tasks independently and in parallel, performing precise two-input-two-output computation. In addition, we constructed a half-subtractor device performing the subtraction of inputs requiring the combination of another XOR gate and an INHIBT gate producing difference D and borrow C, Fig. 2c and e. The design of half-subtractor is based on that two mAbs (1C2 and 1E11) recognize different epitopes of NheB resulting in different reactions in cytotoxic assay and ELISA.[3b] In the XOR gate, either 1C2 or 1E11 report ‘high’ values in single-antibody ELISAs, but fail to produce a positive result in the sandwich (double-antibody) ELISA. The INHIBIT gate

(1C2 ANDNOT 1E11) is based on the fact that mAb 1E11, but not 1C2, can neutralize toxicity.

Furthermore, examples for the *associate property*, which applies equally to addition and multiplication, and the *distributive property* are shown in Fig. 3a to c. Also, two Boolean rules to reduce equations into their simplest forms are presented in Fig. 3d and e, the corresponding proofs are shown in Fig. S7. The ability of performing such fundamental algebraic operations will be essential to simplify logic circuits and to provide diagnostic information.

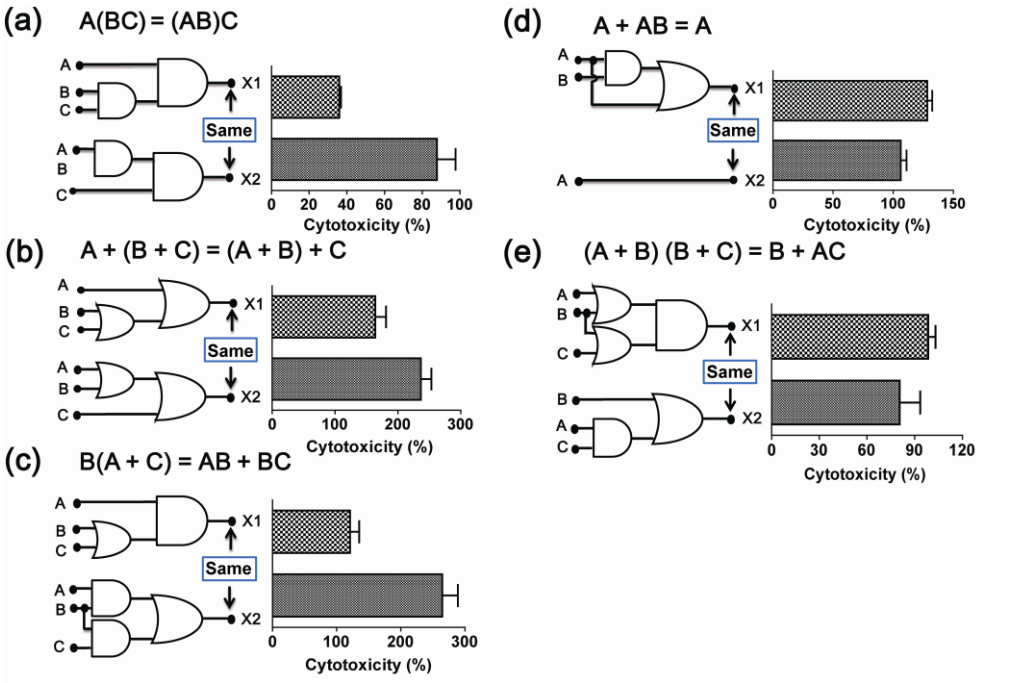


Fig. 3 Cytotoxicity for the *Associate property* of multiplication (a) and addition (b). a) X1, rNheA plus MHI 1761, and X2, MHI 1672 plus rNheC. b) X1, MHI 1491 plus rNheA and MHI 1761, and X2, MHI 1491 plus MHI 1672 and rNheC. c) Cytotoxicity for the *distributive property*. X1, MHI 1491 plus rNheA and C, X2, MHI 1491 plus MHI 1672 and MHI 1761. Associated cytotoxicity of equation (d) and the *simplification* of a product-of-sum (e). d) X1, MHI 1672 plus 1491, and X2, MHI 1491. e) X1, MHI 1491 plus 1672 and 1761, and X2, MHI 1491 plus NheB, rNheA and C.

In conclusion, the use of a multi-component bacterial toxin to control cellular logic gates creates a new input format that could be beneficial for advanced biological computation. The distinct advantages provided by the Nhe-based cellular logic systems, allow the setup of a programmable system combining bacterial toxins and cells to perform both combinatorial and sequential logic operations. Moreover, this library of logic gates was used to demonstrate basic Boolean algebraic properties of addition and multiplication

(*associative* and *distributive properties*) as well as two Boolean rules for *simplification*. It could be envisioned that this platform can be expanded to include other toxins and proteins to perform sophisticated operations.

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Electronic supplementary information (ESI) available: Experimental details and supporting figures and tables. See DOI: 10.1039/c3cc48100j

Notes and references

1 a) Y. Benenson, Nat. Rev. Genet., 2012, 13, 455; b) P. Siuti, J. Yazbek, T. K. Lu, Nat. Biotechnol., 2013, 31, 448.

2 a) E. Katz, V. Privman, Chem. Soc. Rev., 2010, 39, 1835; b) K. Macvittie, J. Halámek, V. Privman, E. Katz, Chem. Commun., 2013, 49, 6962.

3 a) A. Prokup, J. Hemphill, A. Deiters, J. Am. Chem. Soc., 2012, 134, 3810; b) K. Zhu, R. Dietrich, A. Didier, G. Acar, E. Märtlbauer, Chem. Commun., 2013, 49, 9314; c) E. Katz, J. Wang, M. Privman, J, Halámek, Anal. Chem., 2012, 84, 5463.

4 a) M. A. TerAvest, Z. Li, L. T. Angenent, Energy Environ. Sci., 2011, 4, 4907; b) M. A. Arugula, N. Shroff, E. Katz, Z. He, Chem. Commun., 2012, 48, 10174; c) T. S. Moon, C. Lou, A. Tamsir, B. C. Stanton, C. Voigt, Nature, 2012, 491, 249; d) Z. Li, M. A. Rosenbaum, A. Venkataraman, T. K. Tam, E. Katz, L. T. Angenent, Chem. Commun., 2011, 47, 3060; e) A. Tamsir, J. J. Tabor, C. A. Voigt, Nature, 2011, 469, 212.

5 a) Z. Xie, L. Wroblewska, L. Prochazka, R. Weiss, Y. Benenson, Science, 2011, 333, 1307; b) W. Weber, M. Fussenegger, Curr. Opin. Chem. Biol., 2011, 15, 414; c) K. Rinaudo, L. Bleris, R. Maddamsetti, S. Subramanian, R. Weiss, Y. Benenson, Nat. Biotechnol., 2007, 25, 795; d) S. Ausländer, D. Ausländer, M. Müller, M. Wieland, M. Fussenegger, Nature, 2012, 487, 123.

6 a) P. Wei, W. W. Wong, J. S. Park, E. E. Corcoran, S. G. Peisajovich, J. J. Onuffer, A. Weiss, W. A. Lim, Nature, 2012, 488, 384; b) K. Zhu, U. Acaröz, E. Märtlbauer, Chem. Commun., 2013, 49, 5198.

7 a) K. A. Riccione, R. P. Smith, A. J. Lee, L. You, ACS Synth. Biol., 2012, 1, 389; b) J. Estrada, R. Guantes, Mol. Biosyst., 2013, 9, 268; c) T. Pawson, Nature, 1995, 373, 573.

8 a) I. G. Romero, I. Ruvinsky, Y. Gilad, Nat. Rev. Genet., 2012, 13, 505; b) K. Chen, N. Rajewsky, Nat. Rev. Genet., 2007, 8, 93.

9 S. W. Hicks, J. E. Galán, Nat. Rev. Microbiol., 2013, 11, 316.

10 a) L. P. S. Arnesen, A. Fagerlund, P. E. Granum, FEMS Microbiol. Rev., 2008, 32, 579; b) A. Matarante, F. Baruzzi, P. S. Cocconcelli, M, Morea, Appl. Environ. Microbiol., 2004, 70, 5168.

11 G. de Ruiter, E. Tartakovsky, N. Oded, M. E. van der Boom, Angew. Chem., 2010, 122, 173.

12 T. Lindbäck, S. P. Hardy, R. Dietrich, M. Sødring, A. Didier, M. Moravek, A. Fagerlund, S. Bock, C. Nielsen, M. Casteel, P. E. Granum, E. Märtlbauer, Infect. Immun., 2010, 78, 3813.

13 N. Krause, M. Moravek, R. Dietrich, E. Wehrle, J. Slaghuis, E. Märtlbauer, Int. J. Food Microbiol., 2010, 144, 322.

14 U. Heilkenbrinker, R. Dietrich, A. Didier, K. Zhu, T. Lindbäck, P. E. Granum, E. Märtlbauer, PLoS ONE, 2013, 8, e63104.

Supporting information --- ---

Experimental Section

B. cereus strains and culture conditions.

The wild-type *B. cereus* reference strain for Nhe production NVH 75/95 was designated MHI 1491 in this work (1). The mutant strains producing only parts of the three components of Nhe complex were designated as MHI 1761 (no expression of NheA) and MHI 1672 (no expression of NheC), genetic and expression profiles of these strains have been previously demonstrated by PCR, ELISA, and cell proliferation assay (2). For the production of the intact Nhe toxin or functional active Nhe components, CGY medium plus 1% glucose was used as previously described (3). Briefly, *B. cereus* was inoculated into 20 mL CGY medium plus 1% glucose, and cultured in a water bath for 17 h at 32 °C. Subsequently 0.1 mL of the culture liquid was transferred into 20 mL CGY medium plus 1% glucose and incubated for another 6 h at 32 °C. 200 µL EDTA (1mM) was added at the harvesting time, the supernatants were collected by centrifugation for 20 min at 3 000 g, at 4 °C, then passed through 0.22 µm filter to get the cell-free supernatants for further use. More details of the strains used in this work are listed in Table S2.

Mammalian cell lines and culture conditions.

A549, Caco-2 and IPEC-J2 cell lines were provided by the German Collections of Microorganisms and Cell Cultures (DSMZ). HEp-2 and Vero cell lines were purchased from the European Collections of Cell Cultures. Cells were cultured at 37 °C and 7% CO_2 in cell culture media as recommended by the suppliers (4).

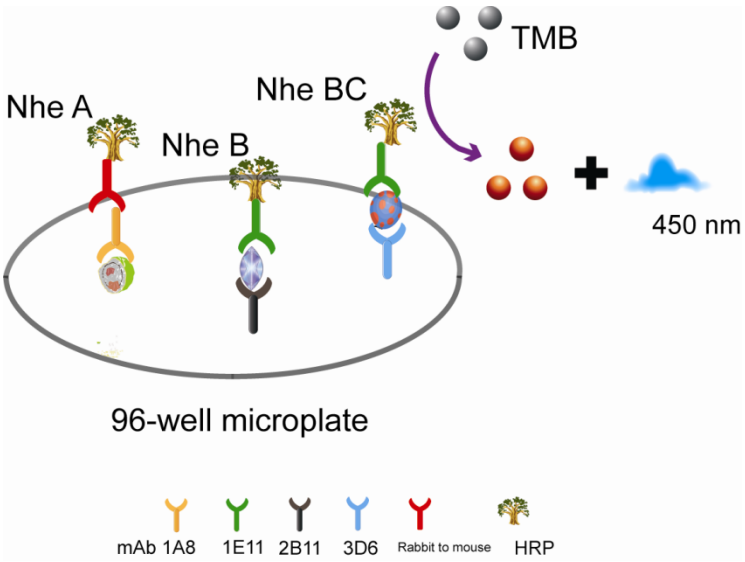
Cell proliferation assay.

Cytotoxic effects of the different components of Nhe complex from *B. cereus*, and the recombinant components of Nhe were tested on diverse cells under simultaneous incubation conditions with the addition of WST-1 (water-soluble tetrozolium salt, Roche Diagnostics) and PI (propidium iodide, Fluka) to the culture media, subsequently the cellular viability was determined as endpoint titer by Tecan photometer and VICTOR™ Multilabel Plate Reader as previously described (2). Briefly, serial dilutions of the Nhe components or the tripartite complex were placed into 96-well microplates (0.1 mL/well) and 0.l mL cell suspension (A549, Caco-2 and HEp-2, 2×10^4 cells/well; IPEC-J2, 5×10^3 cells/well; and Vero, 1×10^4 cells/well) were added immediately afterwards. The growth

media and dilution solutions consisted of MEM or DMEM supplemented with additional nutritional factors as claimed by the suppliers. After 24 h incubation of the test mixture at 37 °C in a 7% CO_2 atmosphere, the morphology of the cells was checked by light microscopy. Moreover, the mitochondrial activity of viable cells was detected by colorimetric measurement at 450 nm after the addition of WST-1, and the fluorescence of PI bound to DNA was measured by setting excitation and emission wavelength to 535 nm and 617 nm, respectively. The dose dependent curves were used to evaluate the 50% inhibitory concentrations of the tested targets of interest by linear interpolation. The highest dilution of reference strain MHI1491 that resulted in 50% dead cells was defined as 100% cytotoxicity.

PCR and ELISA.

The *nhe* genes of the three components in each strain were detected according to the previous report (2). On the protein level, detection of the Nhe component was performed using different formats of ELISA, as shown in Scheme S1. NheA was detected by an indirect ELISA using monoclonal antibody (mAb 1A8) as the primary antibody, meanwhile, two mAbs 1E11 and 2B11, which recognize different epitopes of NheB, were employed for the sandwich ELISA (5). No free NheC can be detected in solution, because it forms a stable complex with NheB (6). Therefore, a complex ELISA was established to detect this toxin component. In this ELISA, the NheC specific mAb 3D6 was used as the capture antibody, and the horseradish peroxidase (HRP) labeled mAb 1E11 against NheB was used as detection antibody.



Scheme S1. Schematic representation of different formats of ELISA used for the detection of Nhe components produced by *B. cereus*. NheA, indirect ELISA (mAb 1A8), NheB, sandwich ELISA (mAbs 1E11 and 2B11) and NheBC, complex ELISA (mAb 3D6 and 1E11), respectively.

Strain (MHI)		***nhe*** gene	**Nhe proteins** A	B	C			
1	1477	+	+	+	+	1		
2	1489	+	+	+	+	1		
3	1493	+	+	+	+	1		
4	1496	+	+	+	+	1		
5	1503	+	+	+	+	1		
6	1504	+	+	+	-	0	0	1
7	1507	+	+	+	+	1		
8	1522	+	+	+	+	1		
9	1527	+	+	+	+	1		
10	1541	+	+	+	+	1		
11	1543	+	+	+	+	1		
12	1556	+	-	-	-	0	0	0
13	1647	+	+	-	-	0	0	1
15	1668	+	+	+	+	1		
16	1670	+	-	+	+	0	1	0
17	1692	+	+	+	+	1		
18	1698	+	-	-	-	0	0	0
19	1699	+	-	-	-	0	0	0
20	1700	+	-	-	-	0	0	0
21	2963	+	+	+	+	1		
22	2965	+	+	+	+	1		
23	2967	+	+	+	+	1		
24	2968	+	-	+	+	0	1	0
28	2970	+	+	+	+	1		
29	3038	+	+	+	+	1		
30	3086	+	+	+	+	1		
	Total	**30**	**24**	**25**	**24**	**22**	**2**	**2**

Table S1. Comparison of the actual toxicity of the Nhe complex, produced by diverse *B. cereus* strains (= input X1), with their gene and protein profile; *nhe* genes were detected by PCR; proteins NheA, B, and C were detected by ELISA based on monoclonal antibodies; toxicity is presented as 0/1 result of the Buffer gate; 0/1 result of the AND gates provides additional information about the toxin profile.

	Strains	Nhe components			References
B. cereus	MHI 1672[a]		B		*2, 7*
	MHI 1672	A	B		*7*
	MHI 1761		B	C	*7*
	MHI 1491	A	B	C	*1,2,7*
E. coli	LMG 194	rNhe A			*8*
	LMG 194			rNhe C	*5, 8*

Table S2. Strains of *B. cereus* and *E. coli* and the associated Nhe-components used in this study.

Note: The *B. cereus* reference strain for Nhe complex NVH 75/95 was designated MHI 1491.

MHI 1672[a], Nhe B was purified by immunoaffinity chromatography (IAC) from the supernatant of MHI 1672. *E. coli* was used as the vector to produce the recombinant NheA and C, respectively.

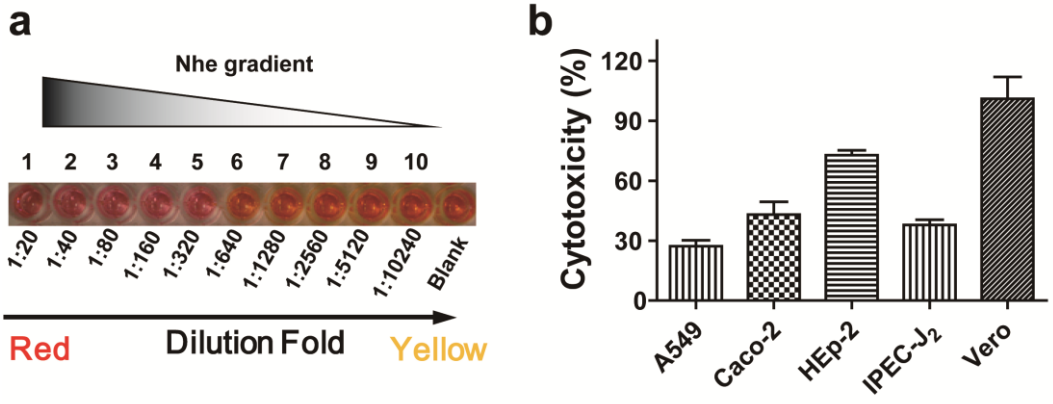


Fig. S1 (a) Photograph of Nhe complex-treated cells with WST-1. Well 1-11, 1×10^4 Vero cells were inoculated into each well; Wells 1-10, Nhe was added in a two-fold serial dilution, starting at 1:20. Well 11, blank control without Nhe complex. (b) Susceptibility of several cell lines to Nhe complex. Five different cell lines, A549, Caco-2, HEp-2, IPEC-J_2 and Vero cells were treated with Nhe complex to trigger full cytotoxicity.

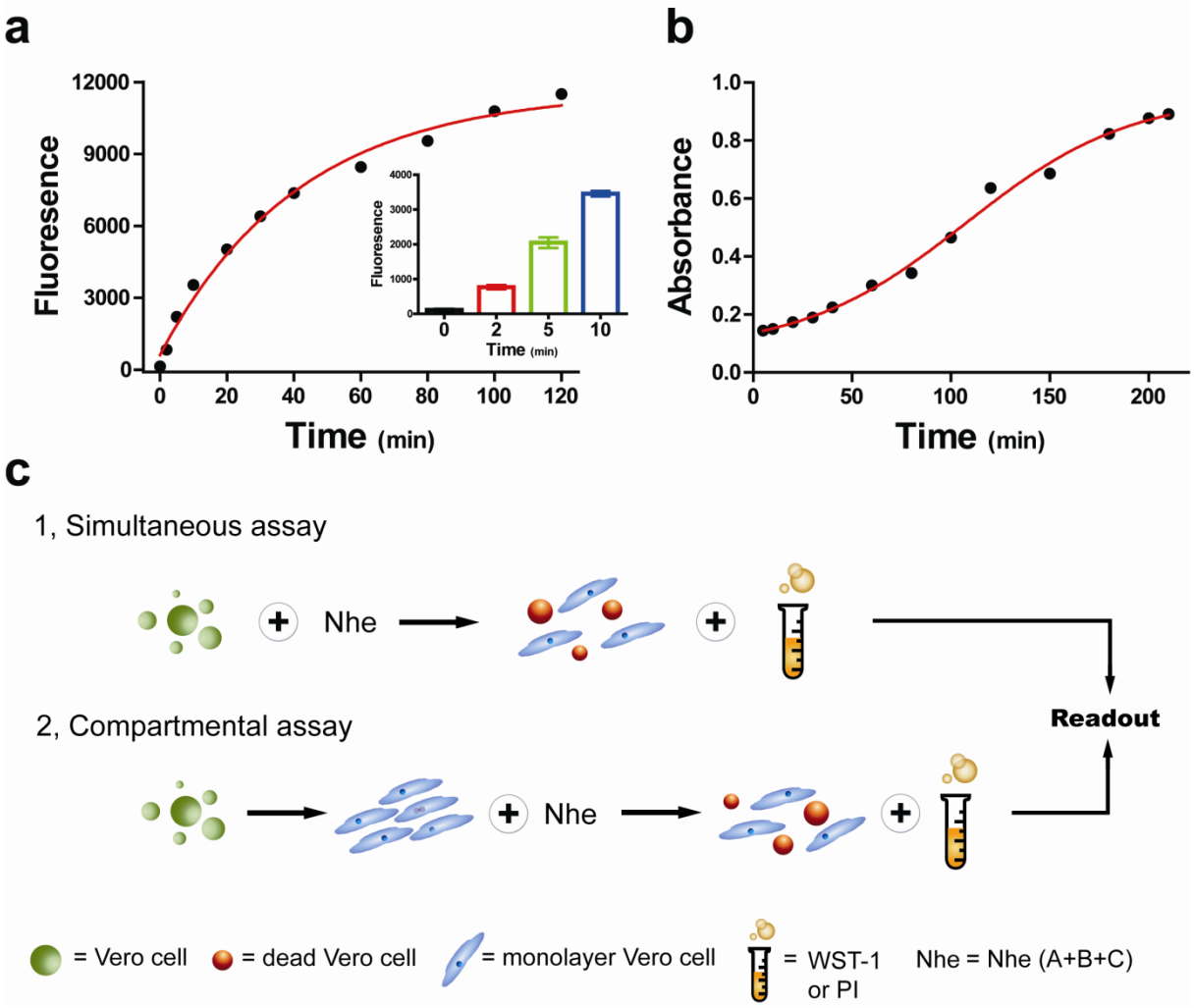


Fig. S2 Time-dependent cytotoxicity of the Nhe complex to Vero cells. A monolayer of cells was treated with Nhe complex for different time periods ranging from 0 min to 120 min (PI measurement, a) and from 0 min to 200 min (WST-1 measurement, b). The inset shows the PI response during the first 10 min. (c) Scheme of simultaneous and compartmental assays used in this work.

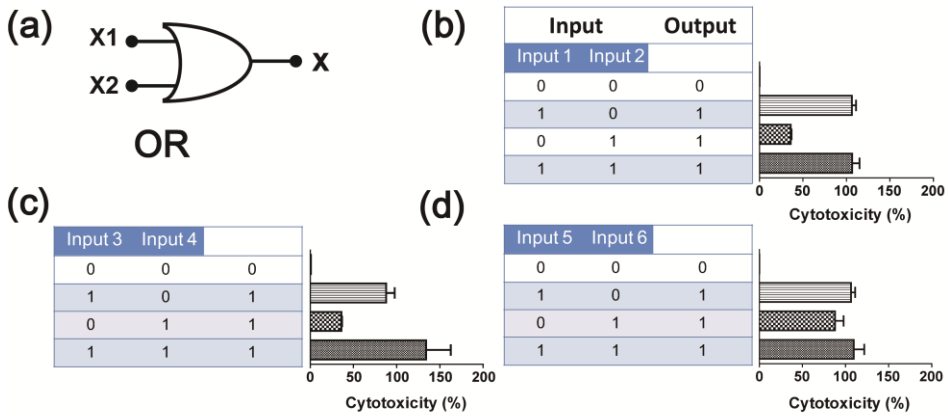


Fig. S3 Nhe-based cellular OR gates. Equivalent circuit (a) and truth tables of the cellular OR gates and the associated cytotoxicity. b) OR gate of NheA, NheA (input 1) and rNheA (input 2), c) OR gate of NheB, NheB of MHI 1672 (input 3) and NheB of MHI 1761 (input 4), and (d) OR gate of NheC, NheC (input 5) and rNheC (input 6). Each gate contained the complementary Nhe components to trigger full cytotoxicity.

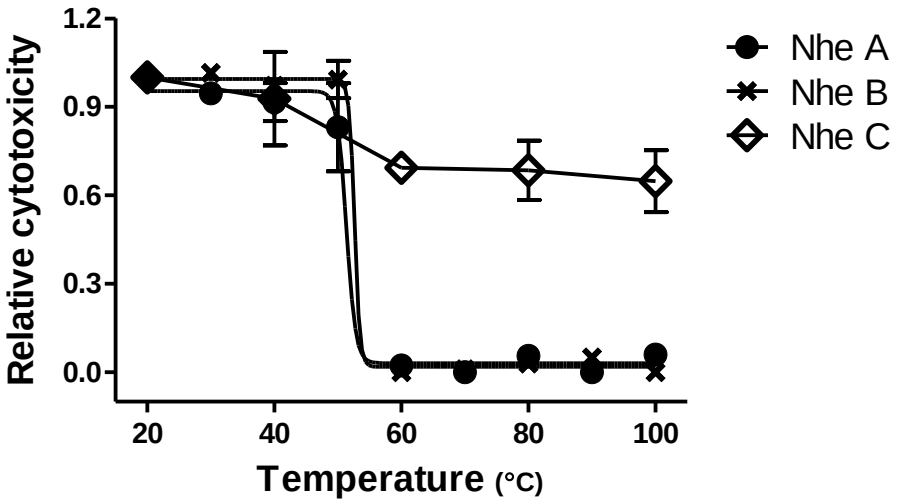


Fig. S4 Thermostability of each component in the tripartite Nhe complex. The Nhe complex was heated for 10 min from 20 °C to 100 °C. The relative cytotoxicity was defined as the ratio of the cytotoxic titer of the heated components to that of the control.

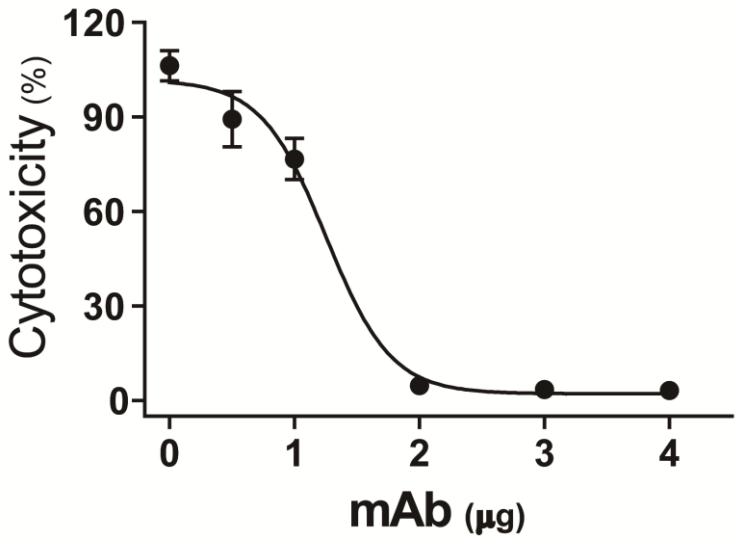


Fig. S5 Inhibition of cytotoxicity by mAb (1E11) against NheB; the antibody was used to construct an INHIBIT logic gate.

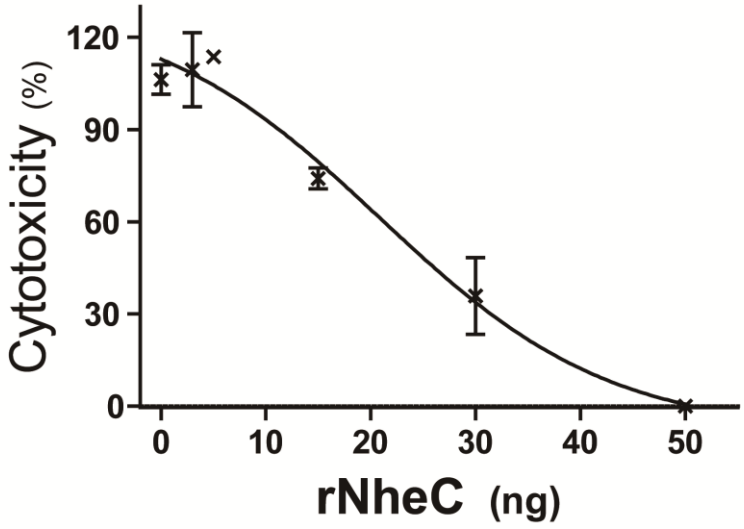


Fig. S6 Inhibition of cytotoxicity by excess rNheC; an excess amount of rNheC was used to construct an INHIBIT logic gate.

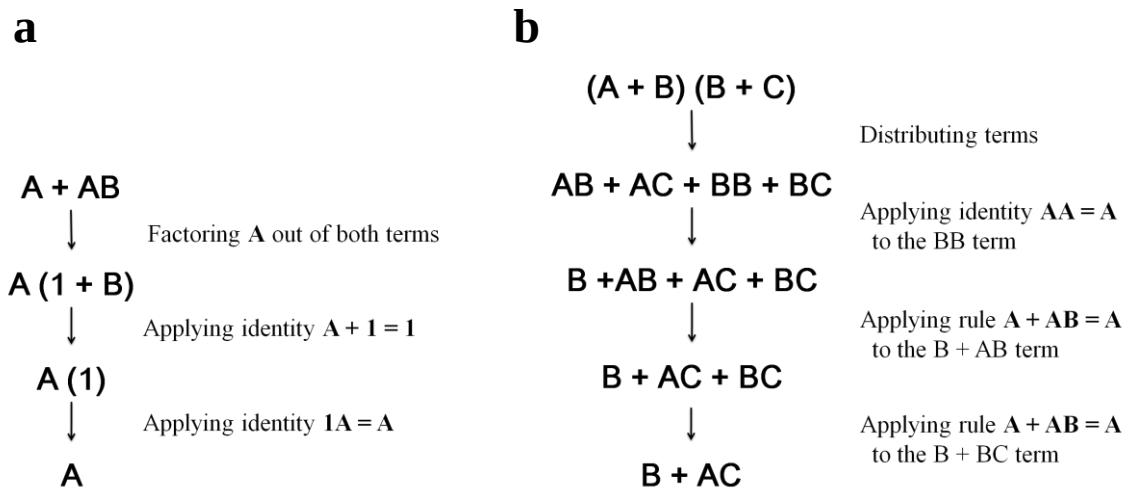


Fig. S7 The proofs of the two Boolean rules for simplification in the Nhe-based cellular logic system.

References

1. T. Lund, P. E. Granum. *FEMS Microbiol Lett.* 1996, *141*, 151-156.
2. E. Wehrle, M. Moravek, R. Dietrich, C. Bürk, A. Didier, E. Märtlbauer, *J. Microbiol. Methods* 2009, *78*, 265-270..
3. D. J. Beecher, A. C. L. Wong. *Infect Immun.*, 1994, *62*, 980-986.
4. A. Didier, R. Dietrich, S. Gruber, S. Bock, M. Moravek, T. Nakamura, T. Lindbäck, P. E. Granum, E. Märtlbauer, *Infect. Immun.* 2012, *80*, 832-838.
5. K. Zhu, R. Dietrich, A. Didier, G. Acer, E. Märtlbauer, *Chem. Commun*. 2013, *49*, 9314-9316.
6. U. Heilkenbrinker, R. Dietrich, A. Didier, K. Zhu, T. Lindbäck, P. E. Granum, E. Märtlbauer, *PLoS ONE* 2013, *8*, e63104.
7. T. Lindbäck, S. P. Hardy, R. Dietrich, M. Sødring, A. Didier, M. Moravek, A. Fagerlund, S. Bock, C. Nielsen, M. Casteel, P. E. Granum, E. Märtlbauer, *Infect. Immun.* 2010, *78*, 3813-3821.
8. T. Lindbäck, A. Fagerland, M. S. Rodland, P. E. Granum. *Microbiology* 2004, *150*, 3959-3967.

III. DISCUSSION

In modern computer engineering and related industries, silicon based semiconductors represent the basic materials of electronic elements including transistors and light-emitting diodes (LEDs). Despite their defined electrical properties such building blocks are largely incompatible with biological systems. Biological components may be contained in intracellular compartments, undergo intracellular trafficking or can be anchored to the fluidic cell membrane and are involved in biochemical reactions, which might be time- and space-resolved. In theory, any cellular component could be an alternative brick for the construction of a biocomputer. For practicability reasons, such as handling, stability and capability of information storage, so far only a few biological components including DNA, RNA and proteins, have been used for this purpose. For example, DNA oligonucleotide based biochemical reactions have been fruitfully used in logic gates, circuits and devices (Pei et al., 2010; Wang et al., 2012). Either single or double-stranded DNA fragments or probes can be easily designed and fabricated, and DNA strands can specifically interact with their complementary strands (Qian and Winfree, 2011). Modification of gene sequences in promoters and/or coding regions is another possible choice to rewrite cellular processes in a heritable fashion (Turan et al., 2013). For example, a bacteriophage serine integrase mediated transcriptor has been invented to control the information flow of RNA polymerase along DNA (Bonnet et al., 2013). Logic gates and sequential logic support autonomous cell to cell communication at the gene level, and can catalyze deletion or inversion of DNA between aligned sites. Interestingly, the transfer of circular DNA strands of bacterial plasmids has been implemented for the multicellular computing in a Boolean XOR fashion (Goñi-Moreno et al., 2013). Given their natural versatility such as modularity, small size and high affinity to a broad range of molecules, RNA based biological logic circuits and biocomputing systems have attracted attention during the recent years (Isaacs et al., 2006). For example, researchers have engineered a class of aptamers with affinity to endogenous proteins, which enable rewiring of different signal pathways in mammalian cells (Culler et al., 2010). In addition, a cellular classifier enables identification of human cancer cells by six endogenous miRNA molecules (Xie et al., 2011). Applying Boolean logics an apoptotic gene can selectively be triggered in

these cancer cells.

Enzymes play a prominent role in protein based biochemical logic gates, for example, to build biosensors or as model systems to implement different Boolean logic tasks. The enzyme molecule can be directly used to sensor analytes of interest, by inhibiting enzymatic activity in the presence of an antagonist or under harsh environmental conditions. For instance, the widely used horseradish peroxidase (HRP) can be specifically inhibited by Cu(I) in solution (Xianyu et al., 2013). Based on this phenomenon, an AND logic gate has been performed to assay Cu(II) in the presence of reducing agents. Based on their strict substrate specificity, enzymes may also be used to construct 'metabolic logic', where the input is the respective substrate and the output is the catalyzed product, which generates a colorimetric, fluorometric or electrochemical signal (Katz and Privman, 2010). For example, a multi-enzyme digital biosensor based on the coordinated action of eight Boolean AND logic gates has been developed for simultaneously processing five biomarkers of traumatic brain injury and soft tissue injury (Halamek et al., 2010). In addition, various enzymes can be combined to design an enzyme cascade performing sophisticated computations including associative memory (MacVittie et al., 2012; MacVittie et al., 2013).

Besides enzymes, antibodies have also been utilized to construct logic gates. As a first example, an immune-keypad lock for the detection of 3-nitro-L-tyrosine was developed with the help of HRP and urease (Halámek et al., 2010). In the present thesis, we used different mAbs to perform an OR logic gate for the simultaneous detection of seven proteins of *B. cereus* (Zhu et al., 2013c). Also we extended this tube immunoassay to single and sandwich antibody tests to identify mutant proteins from different *B. cereus* strains. Moreover, an INHIBIT logic gate has also been developed based on a mAb (1E11), which specifically neutralizes Nhe and therefore prevents Nhe cytotoxicity to mammalian epithelial cells (Zhu et al., 2014). Lastly, two sandwich immunoassays combined with cellular assays have been used to construct a half-adder and a half-subtractor (Zhu et al., 2014). In addition, Boolean logic cannot only improve the detection of multiple targets, but also holds the sophisticated power to simplify the selection of markers in complex biological systems (Zhu et al., 2013c). In principle, the format of this Boolean logic-based immunoassay could also be used for the detection of other multiple contaminants such as the residues of antibiotics or mycotoxins in food samples. Therefore, the antibody based logic system represents a

versatile platform for the determination of multiple targets of interest and to perform sophisticated logic operations.

Additionally, we can create modified or artificial antibodies, which do not exist in nature. For example, it is possible to produce high affinity antibodies by reasonable design of antigens (Cai et al., 2013) or to generate recombinant antibodies with multiple functions (Spiess et al., 2013). In theory, the number of pairs of antigen and antibody is unlimited, thus providing an abundant resource for the construction of Boolean logic gates and devices. Combination of different antibodies allows the construction of antibody cascades, i.e. one antibody can recognize its corresponding antigen and can also serve as an antigen for a second antibody and so on, to be used for complex computing. It could be envisioned that multivalent antigens and their corresponding antibodies can serve as proper candidates for the construction of defined complexes similar to self-assembling protein complexes designed to construct a desired symmetric structure (King et al., 2012).

During the last few years, cell-based computing systems have gained more and more attention. Two general approaches have been proposed to reshape or reprogram cellular behavior. The most widely used procedures represent transcription or translation control devices in a trigger-inducible manner (Culler et al., 2010; Tamsir et al., 2011). Transcriptional activation can be regarded as a Boolean 'Buffer' gate, and transcriptional repression as a 'NOT' gate, respectively. Using trigger-controlled transcription factors diverse Boolean logic operations such as NOT, NAND and N-IMPLY gates were realized in single mammalian cells (Auslander et al., 2012). Also, other arithmetic functions, e.g., half-adder and half-subtractor, enable individual cells to perform predictable metabolic activities. Interestingly, some physical factors such as light can be used as input signals as well (Tabor et al., 2009; Ye et al., 2011). Blue light (approx. 480 nm) sensed by the photopigment melanopsin can be converted into an input for cellular signaling cascades, which finally cooperate with transcription factors to induce transgene expression (Ye et al., 2011). The expressed glucagon-like peptide 1 was able to reduce the blood glucose levels in a type 2 diabetic db/db mouse model. As mentioned in the introduction, the expression of HlyII by clinical isolates of *B. cereus* is strictly controlled by the specific transcriptional regulator HlyIIR and the global Fur regulator (Budarina et al., 2004; Harvie et al., 2005). The pathways of HlyIIR and Fur are dependent on the concentrations of glucose and iron ion,

respectively. Since both HlyIIR and Fur are negative regulators of the expression of HlyII, combining glucose and iron as the input signals and the expression of HlyII as output reflects a natural NOR Boolean logic gate.

Furthermore, exogenous proteins are directly utilized to specifically target cellular components, either through protein-protein interactions or protein triggered genetic information flows. Many bacteria particularly pathogenic bacteria produce a variety of virulence factors to modulate host factors or to hijack certain signaling pathways in cells (Ribet and Cossart, 2010) (Schiavo and van der Goot, 2001). Even direct activation of nociceptors and induction of pain in mice by α-haemolysin (β-PFT) and an N-formylated peptide from *S. aureus* have been demonstrated (Chiu et al., 2013). Therefore, a part of the current thesis was focused on the effects of exogenous proteins secreted by bacteria on host cells. We firstly exploited both α-PFTs (Nhe and Hbl) and β-PFT (CytK) to construct a cellular logic circuit for the simultaneous detection of these three toxins based on their pore-forming capabilities on cell membranes (Zhu et al., 2013b). This logic circuit consists of a set of concerted AND and OR Boolean logic gates. Additionally, we developed a combinatorial AND gate and a sequential logic circuit based on the intrinsic sequential binding order of the tripartite Nhe complex (Zhu et al., 2014). This interaction takes place at the environment-cell interface, and the available data allow the setup of a model including the steps from toxin secretion by the bacterial cells (Sec pathway) to binding of the Nhe complex to the cell membrane (Fagerlund et al., 2010), as shown in Figure 4.

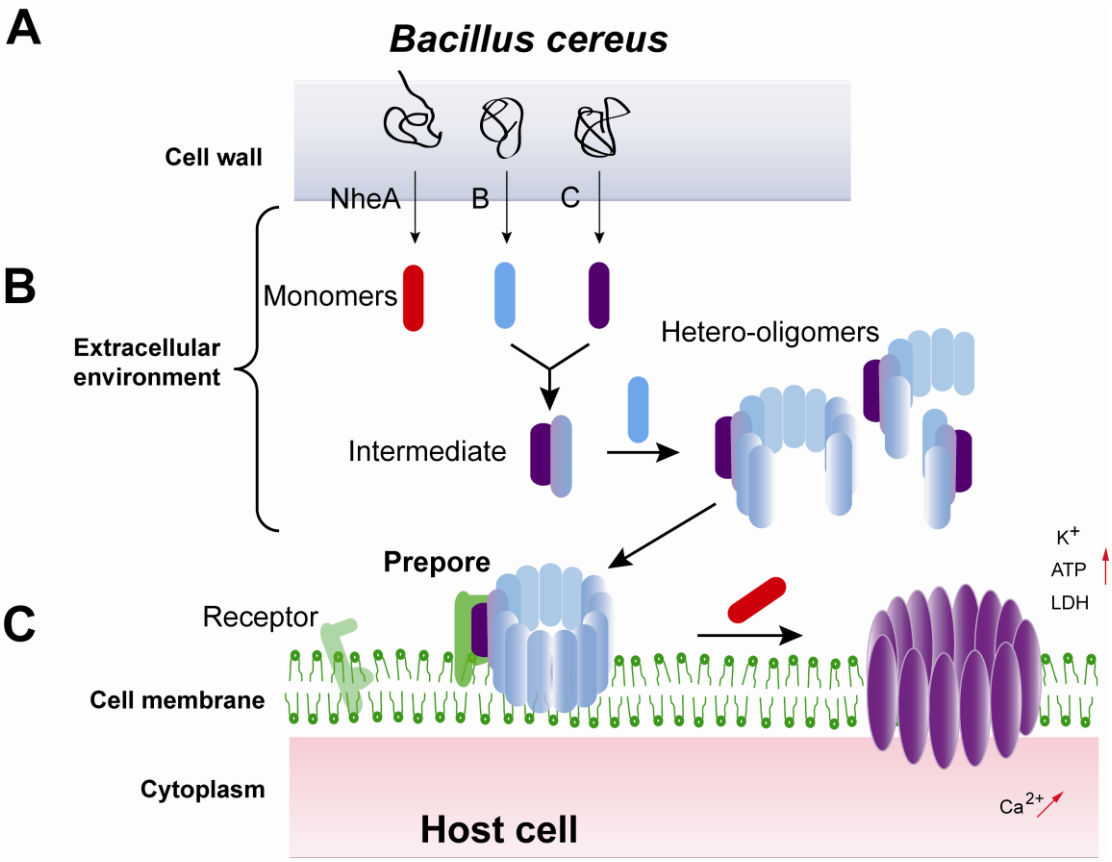


Figure 4 Proposed scheme of mode of action of Nhe. The monomers of the tripartite Nhe components are secreted via the Sec translocation pathway (A), then NheB and -C self-assemble to hetero-oligomers in the extracellular environment (B) and finally the full pore is formed on the cell membrane (C).

It is, however, still challenging that we do not fully understand the structural basis of the self-assembly of this particular protein complex. Also, no information exists about the early cell response after binding of Nhe and future research will be focused on the cellular pathways triggered by the Nhe complex, to comprehensively understand the interaction between *B. cereus* and host cells. It might be envisioned that it will be possible to specifically target cells and to steer the uptake of bacterial molecules into cells. This will help to construct further devices for cellular computation, but also serve for diagnostics and maybe even therapeutic purposes. Towards this end, recently, genotoxins have been selected to modulate host cells for different purposes. For instance, cytolethal distending toxin unit B (CdtB) from *Haemophilus ducreyi* has been fused to the lethal factor of *B. anthracis* toxin to rewire the cell cycle of various human cancer cells to the pathway of apoptosis (Bachran, 2014). Moreover, bacterial effectors can serve as reagents in synthetic

biology to reprogram signaling processes in cells (Moore et al., 2014). Specific effector proteins enable posttranslational modifications, thus providing a strategy to regulate activities in cells (Broberg and Orth, 2010; Ribet and Cossart, 2010). The effector proteins OspF and YopH from *Shigella flexneri* and *Yersinia pestis*, respectively, specifically rewire mitogen-activated protein kinase (MAPK) pathways in both yeast and immune T cells (Wei et al., 2012). The irreversible inactivation of MAPKs by phosphorylation can be used to construct a feedback circuit with novel frequency-dependent input filtering. Analogously, the activities of Certhrax and VIP1-VIP2 are mediated through posttranscriptional modifications by catalyzing the transfer of ADP-ribose to target proteins (Han et al., 1999; Visschedyk et al., 2012). Finally, bacterial proteolytic enzymes can work as 'NOT' logic gates to modify the cell fate by protein degradation, which is independent of the transcription or translation machinery of the host cell (Benenson, 2012; Miyoshi, 2013). For example, modified *E. coli* ClpXP proteases specifically allow tunable degradation of tagged proteins, based on a synthetic gene regulatory network in a *Saccharomyces cerevisiae* strain (Grilly et al., 2007). The degradative enzymes InhA1 and SMase, together with HlyII, are thought to contribute to the survive and escape of *B. cereus* spores in macrophages and to encounter with the host immune system (Tran and Ramarao, 2013). This finding could be regarded as a three-input AND Boolean logic gate for the nongastraintestinal infections caused by *B. cereus*. In addition, the synergistic interactions between SMase and either Nhe or Hbl *in vitro* (Beecher and Wong, 2000a; Doll et al., 2013), are examples of positive feedback among different bacterial toxins and virulence factors with host cells.

Cereulide, Nhe, Hbl and CytK are regarded as the main toxins produced by different *B. cereus* strains. Both the α-PFTs Nhe and Hbl as well as the β-PFT CytK have been simultaneously detected using a cellular logic circuit based on pore formation on cell membranes (Zhu et al., 2013b). As for the emetic toxin, an indirect OR gate based immunoassay has also been set up utilizing a mAb, which detects a specific toxin marker (Zhu et al., 2013c). And a cellular biocomputing assay is under construction for the detection of all the four main toxins of *B. cereus*. In general, the present work demonstrates the successful application of Boolean logic for assaying multiple bacterial toxins and the results suggest that toxins and other virulence factors of bacteria can be used as toolkits for biomolecular computing systems.

IV. ZUSAMMENFASSUNG

Auf Boolescher Logik basierende Assays für die Analyse verschiedener *Bacillus cereus* Toxine

Bacillus cereus ist ein Gram-positives und Sporen bildendes Bakterium aus der *Bacillus cereus* Gruppe, welche auch die phylogenetisch nah verwandten *Bacillus anthracis* und *Bacillus thuringiensis* beinhaltet. *B. cereus* ist sowohl für gastrointestinale als auch nicht-gastrointestinale Syndrome verantwortlich. In ersterem Fall werden Erbrechen und Diarrhö entweder durch das emetische Toxin (Cereulid) oder durch verschiedene Enterotoxine, hauptsächlich durch das nicht-hämolytische Enterotoxin (Nhe), das Hämolysin BL (Hbl) und das Cytotoxin K (CytK) verursacht. Des Weiteren wurde in den letzten Jahren auch von anderen Toxinen und Virulenz Faktoren berichtet: Hämolysin II (HlyII), Certhrax, die vegetativ expremierten insektiziden Proteine (VIPs), der Immune inhibitor A1 (InhA1) und die Sphingomyelinase (SMase). Da das Zusammenspiel der einzelnen Toxine und der anderen Faktoren im Krankheitsfall noch viele Rätsel aufweist, gibt es einen dringenden Bedarf diese potentiellen Targets zu detektieren – sowohl für die Diagnostik als auch für die Lebensmittelsicherheit.

Im ersten Teil dieser Dissertation wird die mathematische Logikfunktion des ODER-Gatters, basierend auf monoklonalen Antikörpern für die simultane Detektion von verschiedenen Toxinen in einem Teströhrchen benutzt. Um den Arbeitsvorgang weiter zu simplifizieren wurde die Boolesche Regel der Vereinfachung angewandt, um ein Marker Toxin aus dem Spektrum der natürlichen Toxine auszuwählen. Desweitern wurde eine zelluläre Schaltkreislogik entwickelt, um die Toxinprofile von *B. cereus* zu entschlüsseln. Dafür benutzten wir als Testsignal die Porenbildung auf der Zellmembran. Dieser neuartige Assay ermöglicht in pathogenen Stämmen verschiedenen Ursprungs die simultane Detektion von sieben verschiedenen Biomarkern. Zuletzt wurde ein zellulärer Logikschaltkreis, basierend auf durch bakterielle Proteine induzierte Cytotoxizität konstruiert, der zu kombinatorischen und sequentiellen logischen Verknüpfungen befähigt ist. Auch fortgeschrittene Elemente wie ein Tastaturschloss, Halb-Addierer und verschiedene elementare Booleschen Eigenschaften wurden auf zellulärer Ebene demonstriert.

Diese Arbeit repräsentiert ein erstes Beispiel für ein auf Boolescher Logik basierendes System unter Benutzung verschiedener bakterieller Toxine. Die Resultate deuten darauf hin, dass bakterielle Toxine und andere Virulenzfaktoren als Bausteine für Bio-Datenverarbeitung genutzt werden können.

SUMMARY

Boolean logic-based assays for the analysis of multiple *Bacillus cereus* toxins

Bacillus cereus is a Gram-positive and spore-forming bacterium of the *Bacillus cereus* group, sharing a closely related phylogenetic similarity with other group members such as *Bacillus anthracis* and *Bacillus thuringiensis*. *Bacillus cereus* is responsible for both gastrointestinal and non-gastrointestinal syndromes. Of the former ones, emesis and diarrhea are caused by either the emetic toxin (cereulide) or different enterotoxins mainly the non-haemolytic enterotoxin (Nhe), haemolysin BL (Hbl) and cytotoxin K (CytK). In addition, other toxins and virulence factors have been reported in the past few years, e.g., haemolysin II (HlyII), Certhrax, vegetative insecticidal proteins (VIPs), immune inhibitor A1 (InhA1) and sphingomyelinase (SMase). Since the relative role of the individual toxins and the other factors in disease is still unknown, there is an urgent demand to detect these potential targets for either diagnostic or food safety purposes.

In the first part of the thesis, we present an OR gate based on monoclonal antibodies for the simultaneous detection of multiple toxins in a single tube. To further simplify the operating procedure, the Boolean rule of *simplification* was used to guide the selection of a marker toxin among the natural toxin profiles. Furthermore, we developed a cellular logic circuit for deciphering the toxin profiles produced by *B. cereus*, using readout techniques based on pore formation on the cell membrane. This new assay enabled the simultaneous detection of seven biomarkers in pathogenic strains from various sources. Lastly, a cellular logic system capable of combinatorial and sequential logic operations based on bacterial protein-triggered cytotoxicity was constructed. Advanced devices such as a keypad lock, half-adder and several basic Boolean properties were demonstrated on the cells.

This represents a first example of a Boolean logic-based system for assaying multiple bacterial toxins. In addition, the results suggest that toxins and other virulence factors of bacteria can be used as toolkits for biocomputing.

V. REFERENCES

Aktories, K. (2011). Bacterial protein toxins that modify host regulatory GTPases. Nature Reviews Microbiology *9*, 487-498.

Andreeva, Z.I., Nesterenko, V.F., Fomkina, M.G., Ternovsky, V.I., Suzina, N.E., Bakulina, A.Y., Solonin, A.S., and Sineva, E.V. (2007). The properties of *Bacillus cereus* hemolysin II pores depend on environmental conditions. Biochimica et Biophysica Acta (BBA) - Biomembranes *1768*, 253-263.

Andreeva, Z.I., Nesterenko, V.F., Yurkov, I.S., Budarina, Z.I., Sineva, E.V., and Solonin, A.S. (2006). Purification and cytotoxic properties of *Bacillus cereus* hemolysin II. Protein Expression and Purification *47*, 186-193.

Andrianantoandro, E., Basu, S., Karig, D.K., and Weiss, R. (2006). Synthetic biology: new engineering rules for an emerging discipline. Molecular Systems Biology *2*, 0028.

Antignani, A., and Fitzgerald, D. (2013). Immunotoxins: the role of the toxin. Toxins (Basel) *5*, 1486-1502.

Asano S. I., N.Y., Bando H., Iizuka T., Yamamoto T. (1997). Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. Applied and Environmental Microbiology *63*, 2638-2642.

Auslander, S., Auslander, D., Muller, M., Wieland, M., and Fussenegger, M. (2012). Programmable single-cell mammalian biocomputers. Nature *487*, 123-127.

Bachran, C.H., R. Leysath, C. E. Sastalla, I. Zhang, Y. Fattah, R. J. Liu, S. Leppla, S. H. (2014). Cytolethal distending toxin B as a cell-killing component of tumor-targeted anthrax toxin fusion proteins. Cell Death and Disease *5*, e1003.

Baida, G.E., and Kuzmin, N.P. (1995). Cloning and primary structure of a new hemolysin gene from *Bacillus cereus*. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression *1264*, 151-154.

Baida, G.E., and Kuzmin, N.P. (1996). Mechanism of action of hemolysin III from *Bacillus cereus*. Biochimica et Biophysica Acta (BBA) - Biomembranes *1284*, 122-124.

Baillie, L., and Read, T.D. (2001). *Bacillus anthracis*, a bug with attitude! Current Opinion in Microbiology *4*, 78-81.

Beecher, D.J., Olsen, T.W., Somers, E.B., and Wong, A.C.L. (2000). Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-preferring phospholipase C, and collagenase to virulence of *Bacillus cereus* endophthalmitis. Infection and Immunity *68*, 5269-5276.

Beecher, D.J., and Wong, A.C.L. (2000a). Cooperative, synergistic and antagonistic haemolytic interactions between haemolysin BL, phosphatidylcholine phospholipase C and sphingomyelinase from *Bacillus cereus*. Microbiology *146*, 3033-3039.

Beecher, D.J., and Wong, A.C.L. (2000b). Tripartite haemolysin BL: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate. Microbiology *146*, 1371-1380.

Benenson, Y. (2009). Biocomputers: from test tubes to live cells. Molecular BioSystems *5*, 675-685.

Benenson, Y. (2012). Biomolecular computing systems: principles, progress and potential. Nature Reviews Genetics *13*, 455-468.

Bernheimer, A.W., and Grushoff, P. (1967). Cereolysin: production, purification and partial characterization. Journal of General Microbiology *46*, 143-150.

Blöcker, D., Barth, H., Maier, E., Benz, R., Barbieri, J.T., and Aktories, K. (2000). The C terminus of component C2II of *Clostridium botulinum* C2 toxin is essential for receptor binding. Infection and Immunity *68*, 4566-4573.

Bonnet, J., Yin, P., Ortiz, M.E., Subsoontorn, P., and Endy, D. (2013). Amplifying genetic logic gates. Science *340*, 599-603.

Bottone, E.J. (2010). *Bacillus cereus*, a volatile human pathogen. Clinical Microbiology Reviews *23*, 382-398.

Brillard, J., and Lereclus, D. (2007). Characterization of a small PlcR-regulated gene co-expressed with cereolysin O. BMC Microbiology *7*, 52.

Broberg, C.A., and Orth, K. (2010). Tipping the balance by manipulating post-translational

modifications. Current Opinion in Microbiology *13*, 34-40.

Budarina, Z.I., Nikitin, D.V., Zenkin, N., Zakharova, M., Semenova, E., Shlyapnikov, M.G., Rodikova, E.A., Masyukova, S., Ogarkov, O., Baida, G.E., *et al*. (2004). A new *Bacillus cereus* DNA-binding protein, HlyIIR, negatively regulates expression of *B. cereus* haemolysin II. Microbiology *150*, 3691-3701.

Cadot, C., Tran, S.-L., Vignaud, M.-L., De Buyser, M.-L., Kolstø, A.-B., Brisabois, A., Nguyen-Thé, C., Lereclus, D., Guinebretière, M.-H., and Ramarao, N. (2010). InhA1, NprA, and HlyII as candidates for markers to differentiate pathogenic from nonpathogenic *Bacillus cereus* strains. Journal of Clinical Microbiology *48*, 1358-1365.

Cai, X., Tsuchikama, K., and Janda, K.D. (2013). Modulating cocaine vaccine potency through hapten fluorination. Journal of the American Chemical Society *135*, 2971-2974.

Ceuppens, S., Boon, N., and Uyttendaele, M. (2013). Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. FEMS Microbiology Ecology *84*, 433-450.

Ceuppens, S., Rajkovic, A., Hamelink, S., Van de Wiele, T., Boon, N., and Uyttendaele, M. (2012a). Enterotoxin production by *Bacillus cereus* under gastrointestinal conditions and their immunological detection by commercially available kits. Foodborne Pathogens and Disease *9*, 1130-1136.

Ceuppens, S., Rajkovic, A., Heyndrickx, M., Tsilia, V., Van De Wiele, T., Boon, N., and Uyttendaele, M. (2011). Regulation of toxin production by *Bacillus cereus* and its food safety implications. Critical Reviews in Microbiology *37*, 188-213.

Ceuppens, S., Uyttendaele, M., Drieskens, K., Heyndrickx, M., Rajkovic, A., Boon, N., and Van de Wiele, T. (2012b). Survival and germination of *Bacillus cereus* spores without outgrowth or enterotoxin production during in vitro simulation of gastrointestinal transit. Applied and Environmental Microbiology *78*, 7698-7705.

Chitlaru, T., Gat, O., Gozlan, Y., Ariel, N., and Shafferman, A. (2006). Differential proteomic analysis of the *Bacillus anthracis* secretome: distinct plasmid and chromosome CO_2-dependent cross talk mechanisms modulate extracellular proteolytic activities. Journal of Bacteriology *188*, 3551-3571.

Chiu, I.M., Heesters, B.A., Ghasemlou, N., Von Hehn, C.A., Zhao, F., Tran, J., Wainger,

B., Strominger, A., Muralidharan, S., Horswill, A.R., *et al.* (2013). Bacteria activate sensory neurons that modulate pain and inflammation. Nature *501*, 52-57.

Chung, M.-C., Popova, T.G., Millis, B.A., Mukherjee, D.V., Zhou, W., Liotta, L.A., Petricoin, E.F., Chandhoke, V., Bailey, C., and Popov, S.G. (2006). Secreted neutral metalloproteases of *Bacillus anthracis* as candidate pathogenic factors. Journal of Biological Chemistry *281*, 31408-31418.

Culler, S.J., Hoff, K.G., and Smolke, C.D. (2010). Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. Science *330*, 1251-1255.

de Ruiter, G., Tartakovsky, E., Oded, N., and van der Boom, M.E. (2010). Sequential logic operations with surface-confined polypyridyl complexes displaying molecular random access memory features. Angewandte Chemie International Edition *49*, 169-172.

de Ruiter, G., and van der Boom, M.E. (2012). Orthogonal addressable monolayers for integrating molecular logic. Angewandte Chemie International Edition *51*, 8598-8601.

Deng, Q., and Barbieri, J.T. (2008). Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. Annual Review of Microbiology *62*, 271-288.

Didier, A., Dietrich, R., Gruber, S., Bock, S., Moravek, M., Nakamura, T., Lindbäck, T., Granum, P.E., and Märtlbauer, E. (2012). Monoclonal antibodies neutralize *Bacillus cereus* Nhe enterotoxin by inhibiting ordered binding of its three exoprotein components. Infection and Immunity *80*, 832-838.

Dierick, K., Van Coillie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M., and Mahillon, J. (2005). Fatal family outbreak of *Bacillus cereus*-associated food poisoning. Journal of Clinical Microbiology *43*, 4277-4279.

Dietrich, R., Fella, C., Strich, S., and Märtlbauer, E. (1999). Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by *Bacillus cereus*. Applied and Environmental Microbiology *65*, 4470 - 4474.

Dietrich, R., Moravek, M., Burk, C., Granum, P., and Märtlbauer, E. (2005). Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* nonhemolytic enterotoxin complex. Applied and Environmental Microbiology *71*, 8214 - 8220.

Doll, V.M., Ehling-Schulz, M., and Vogelmann, R. (2013). Concerted action of sphingomyelinase and non-hemolytic enterotoxin in pathogenic *Bacillus cereus*. PloS One *8*, e61404.

Doulgeraki, A.I., Ercolini, D., Villani, F., and Nychas, G.-J.E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. International Journal of Food Microbiology *157*, 130-141.

EFSA (2011). EFSA panel on additives and products or substances used in animal feed (FEEDAP); Technical guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal *9*, 2445.

EFSA (2012). Scientific opinion on Toyocerin® (*Bacillus cereus*) as a feed additive for sows, piglets, pigs for fattening, cattle for fattening, calves for rearing, chickens for fattening and rabbits for fattening. . EFSA Journal *10*, 2924

Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Märtlbauer, E., and Scherer, S. (2005). Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. Applied and Environmental Microbiology *71*, 105-113.

Elstner, M., Weisshart, K., Müllen, K., and Schiller, A. (2012). Molecular logic with a saccharide probe on the few-molecules level. Journal of the American Chemical Society *134*, 8098-8100.

Estruch J. J., W.G.W., Mullins M. A., Nye G. J., Craig J. A., Koziel M. G. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proceedings of the National Academy of Sciences of the United States of America *93*, 5389-5394.

Fagerlund, A., Brillard, J., Furst, R., Guinebretiere, M.-H., and Granum, P. (2007). Toxin production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group. BMC Microbiology *7*, 43.

Fagerlund, A., Lindback, T., and Granum, P.E. (2010). *Bacillus cereus* cytotoxins Hbl, Nhe and CytK are secreted via the Sec translocation pathway. BMC Microbiology *10*, 304.

Fagerlund, A., Ween, O., Lund, T., Hardy, S., and Granum, P. (2004). Genetic and functional analysis of the cytK family of genes in *Bacillus cereus*. Microbiology *150*, 2689

- 2697.

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., Thevenot, D., Condron, R., De Reu, K., Govaris, A., *et al.* (2013). Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. International Journal of Food Microbiology *162,* 190-212.

Folcher, M., and Fussenegger, M. (2012). Synthetic biology advancing clinical applications. Current Opinion in Chemical Biology *16*, 345-354.

Ganash, M., Phung, D., Sedelnikova, S.E., Lindback, T., Granum, P.E., and Artymiuk, P.J. (2013). Structure of the NheA component of the Nhe toxin from *Bacillus cereus*: implications for function. PloS One *8*, e74748.

Ghosh, A.C. (1978). Prevalence of *Bacillus cereus* in the faeces of healthy adults. The Journal of Hygiene *80*, 233-236.

Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wächter, M., Kreft, J., and Goebel, W. (1989). A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. Journal of Bacteriology *171*, 744-753.

Gohar, M., Faegri, K., Perchat, S., Ravnum, S., Økstad, O.A., Gominet, M., Kolstø, A.-B., and Lereclus, D. (2008). The PlcR virulence regulon of *Bacillus cereus*. PloS One *3*, e2793.

Gohar, M., Gilois, N., Graveline, R., Garreau, C., Sanchis, V., and Lereclus, D. (2005). A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics *5*, 3696-3711.

Goñi-Moreno, A., Amos, M., and de la Cruz, F. (2013). Multicellular computing using conjugation for wiring. PloS One *8*, e65986.

Gotor, R., Costero, A.M., Gil, S., Parra, M., Gavina, P., and Rurack, K. (2013). Boolean operations mediated by an ion-pair receptor of a multi-readout molecular logic gate. Chemical Communications *49*, 11056-11058.

Granum, P.E. (1990). Clostridium perfringens toxins involved in food poisoning. International Journal of Food Microbiology *10*, 101-111.

Grilly, C., Stricker, J., Pang, W.L., Bennett, M.R., and Hasty, J. (2007). A synthetic gene network for tuning protein degradation in *Saccharomyces cerevisiae*. Molecular systems biology *3*, 127.

Guillemet, E., Tran, S.L., Cadot, C., Rognan, D., Lereclus, D., and Ramarao, N. (2013). Glucose 6P binds and activates HlyIIR to repress *Bacillus cereus* haemolysin hlyII gene expression. PloS One *8*, e55085.

Guinebretiere, M.H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M.L., Lamberet, G., Fagerlund, A., Granum, P.E., Lereclus, D., *et al.* (2013). *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. International Journal of Systematic and Evolutionary Microbiology *63*, 31-40.

Halamek, J., Bocharova, V., Chinnapareddy, S., Windmiller, J.R., Strack, G., Chuang, M.-C., Zhou, J., Santhosh, P., Ramirez, G.V., Arugula, M.A., *et al.* (2010). Multi-enzyme logic network architectures for assessing injuries: digital processing of biomarkers. Molecular BioSystems *6*, 2554-2560.

Halámek, J., Tam, T.K., Chinnapareddy, S., Bocharova, V., and Katz, E. (2010). Keypad lock security system based on immune-affinity recognition integrated with a switchable biofuel cell. The Journal of Physical Chemistry Letters *1*, 973-977.

Han S., Craig J.A., Putnam C.D., Carozzi N.B., Tainer J.A. (1999). Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. Nature Structural Biology *6*, 932-936.

Hansen, B.M., Hoeiby, P.E., Jensen, G.B., and Hendriksen, N.B. (2003). The *Bacillus cereus bceT* enterotoxin sequence reappraised. FEMS Microbiology Letters *223*, 21-24.

Harvie, D.R., Vílchez, S., Steggles, J.R., and Ellar, D.J. (2005). *Bacillus cereus* Fur regulates iron metabolism and is required for full virulence. Microbiology *151*, 569-577.

Haug, G., Wilde, C., Leemhuis, J., Meyer, D.K., Aktories, K., and Barth, H. (2003). Cellular uptake of *Clostridium botulinum* C2 toxin: membrane translocation of a fusion toxin requires unfolding of its dihydrofolate reductase domain. Biochemistry *42*, 15284-15291.

Heilkenbrinker, U., Dietrich, R., Didier, A., Zhu, K., Lindback, T., Granum, P.E., and

Märtlbauer, E. (2013). Complex formation between NheB and NheC is necessary to induce cytotoxic activity by the three-component *Bacillus cereus* Nhe enterotoxin. PloS One *8*, e63104.

Hoffmaster, A.R., Ravel, J., Rasko, D.A., Chapman, G.D., Chute, M.D., Marston, C.K., De, B.K., Sacchi, C.T., Fitzgerald, C., Mayer, L.W., *et al.* (2004). Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proceedings of the National Academy of Sciences of the United States of America *101*, 8449-8454.

Hotze, E.M., and Tweten, R.K. (2012). Membrane assembly of the cholesterol-dependent cytolysin pore complex. Biochimica et Biophysica Acta (BBA) - Biomembranes *1818*, 1028-1038.

Huseby, M., Shi, K., Brown, C.K., Digre, J., Mengistu, F., Seo, K.S., Bohach, G.A., Schlievert, P.M., Ohlendorf, D.H., and Earhart, C.A. (2007). Structure and biological activities of Beta toxin from *Staphylococcus aureus*. Journal of Bacteriology *189*, 8719-8726.

Isaacs, F.J., Dwyer, D.J., and Collins, J.J. (2006). RNA synthetic biology. Nature Biotechnology *24*, 545-554.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., *et al.* (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature *423*, 87-91.

Jeßberger, N., Dietrich, R., Bock, S., Didier, A., and Märtlbauer, E. (2014). *Bacillus cereus* enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to different human cell lines. Toxicon *77*, 49-57.

Kataev, A.A., Andreeva-Kovalevskaya, Z.I., Solonin, A.S., and Ternovsky, V.I. (2012). *Bacillus cereus* can attack the cell membranes of the alga *Chara corallina* by means of HlyII. Biochimica et Biophysica Acta *1818*, 1235-1241.

Katz, E., and Privman, V. (2010). Enzyme-based logic systems for information processing. Chemical Society Reviews *39*, 1835-1857.

Katz, E., Wang, J., Privman, M., and Halámek, J. (2012). Multianalyte digital enzyme

biosensors with built-in Boolean logic. Analytical Chemistry *84*, 5463-5469.

Kim, Y.-R., Czajka, J., and Batt, C.A. (2000). Development of a fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in nonfat dry milk. Applied and Environmental Microbiology *66*, 1453-1459.

King, N.P., Sheffler, W., Sawaya, M.R., Vollmar, B.S., Sumida, J.P., André, I., Gonen, T., Yeates, T.O., and Baker, D. (2012). Computational design of self-assembling protein nanomaterials with atomic level accuracy. Science *336*, 1171-1174.

Lemichez, E., and Barbieri, J.T. (2013). General aspects and recent advances on bacterial protein toxins. Cold Spring Harbor perspectives in medicine *3*, a013573.

Lindbäck, T., Fagerlund, A., Rødland, M.S., and Granum, P.E. (2004). Characterization of the *Bacillus cereus* Nhe enterotoxin. Microbiology *150*, 3959-3967.

Lindbäck, T., Hardy, S.P., Dietrich, R., Sødring, M., Didier, A., Moravek, M., Fagerlund, A., Bock, S., Nielsen, C., Casteel, M., *et al.* (2010). Cytotoxicity of the *Bacillus cereus* Nhe enterotoxin requires specific binding order of its three exoprotein components. Infection and Immunity *78*, 3813-3821.

Logan, N.A. (1988). *Bacillus* species of medical and veterinary importance. Journal of Medical Microbiology *25*, 157-165.

Lund, T., De Buyser, M., and Granum, P. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Molecular Microbiology *38*, 254 - 261.

MacVittie, K., Halamek, J., and Katz, E. (2012). Enzyme-based D-flip-flop memory system. Chemical Communications *48*, 11742-11744.

MacVittie, K., Halamek, J., Privman, V., and Katz, E. (2013). A bioinspired associative memory system based on enzymatic cascades. Chemical Communications *49*, 6962-6964.

Madegowda, M., Eswaramoorthy, S., Burley, S.K., and Swaminathan, S. (2008). X-ray crystal structure of the B component of Hemolysin BL from *Bacillus cereus*. Proteins: Structure, Function, and Bioinformatics *71*, 534-540.

Mahler H., P.A., Kramer J. M., Schulte P., Scoging A. C., Bär W., Krähenbühl S. (1997). Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. The New

England Journal of Medicine *336*, 1142-1148.

Makarasen, A., Yoza, K., and Isobe, M. (2009). Higher structure of cereulide, an emetic toxin from *Bacillus cereus*, and special comparison with valinomycin, an antibiotic from *Streptomyces fulvissimus*. Chemistry, an Asian journal *4*, 688-698.

Melnick, R.L., Testen, A.L., Poleatewich, A.M., Backman, P.A., and Bailey, B.A. (2012). Detection and expression of enterotoxin genes in endophytic strains of *Bacillus cereus*. Letters in Applied Microbiology *54*, 468-474.

Mikkola, R., Saris, N.-E.L., Grigoriev, P.A., Andersson, M.A., and Salkinoja-Salonen, M.S. (1999). Ionophoretic properties and mitochondrial effects of cereulide. European Journal of Biochemistry *263*, 112-117.

Miles, G., Bayley, H., and Cheley, S. (2002). Properties of *Bacillus cereus* hemolysin II: a heptameric transmembrane pore. Protein Science *11*, 1813-1824.

Miyoshi, S. (2013). Extracellular proteolytic enzymes produced by human pathogenic vibrio species. Frontiers in Microbiology *4*, 339.

Modrie, P., Beuls, E., and Mahillon, J. (2010). Differential transfer dynamics of pAW63 plasmid among members of the *Bacillus cereus* group in food microcosms. Journal of Applied Microbiology *108*, 888-897.

Mols, M., and Abee, T. (2011). *Bacillus cereus* responses to acid stress. Environmental Microbiology *13*, 2835-2843.

Moore, R., Chandrahas, A., and Bleris, L. (2014). Transcription activator-like effectors: a toolkit for synthetic biology. ACS Synthetic Biology, DOI: 10.1021/sb400137b.

Moravek, M., Dietrich, R., Buerk, C., Broussolle, V., Guinebretière, M.-H., Granum, P.E., Nguyen-the, C., and Märtlbauer, E. (2006). Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. FEMS Microbiology Letters *257*, 293-298.

Niazov, T., Baron, R., Katz, E., Lioubashevski, O., and Willner, I. (2006). Concatenated logic gates using four coupled biocatalysts operating in series. Proceedings of the National Academy of Sciences *103*, 17160-17163.

Oda, M., Fujita, A., Okui, K., Miyamoto, K., Shibutani, M., Takagishi, T., and Nagahama, M. (2013). *Bacillus cereus* sphingomyelinase recognizes ganglioside GM3. Biochemical and Biophysical Research Communications *431*, 164-168.

Oda, M., Hashimoto, M., Takahashi, M, Ohmae, Y., Seike, S., Kato, R., Fujita, A., Tsuge, H., Nagahama, M., Ochi, S., et al. (2012). Role of sphingomyelinase in infectious diseases caused by *Bacillus cereus*. PloS One *7*, e38054.

Pei, H., Liang, L., Yao, G., Li, J., Huang, Q., and Fan, C. (2012). Reconfigurable three-dimensional DNA nanostructures for the construction of intracellular logic sensors. Angewandte Chemie International Edition *51*, 9020-9024.

Pei, R., Matamoros, E., Liu, M., Stefanovic, D., and Stojanovic, M.N. (2010). Training a molecular automaton to play a game. Nature Nanotechnology *5*, 773-777.

Perchat, S., Buisson, C., Chaufaux, J., Sanchis, V., Lereclus, D., and Gohar, M. (2005). *Bacillus cereus* produces several nonproteinaceous insecticidal exotoxins. Journal of Invertebrate Pathology *90*, 131-133.

Poopathi, S., Mani, C., Thirugnanasambantham, K., Praba, V.L., Ahangar, N.A., and Balagangadharan, K. (2014). Identification and characterization of a novel marine *Bacillus cereus* for mosquito control. Parasitology Research *113*, 323-332.

Purnick, P.E.M., and Weiss, R. (2009). The second wave of synthetic biology: from modules to systems. Nature Reviews Molecular Cell Biology *10*, 410-422.

Qian L., Winfree E. (2011). Scaling up digital circuit computation with DNA strand displacement cascades. Science *332*, 1196-1201.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., and Cotter, P.D. (2013). The complex microbiota of raw milk. FEMS Microbiology Reviews *37*, 664-698.

Ramarao, N., and Lereclus, D. (2005). The InhA1 metalloprotease allows spores of the *B. cereus* group to escape macrophages. Cellular Microbiology *7*, 1357-1364.

Ramarao, N., and Sanchis, V. (2013). The pore-forming haemolysins of *Bacillus cereus*: a review. Toxins (Basel) *5*, 1119-1139.

Ribet, D., and Cossart, P. (2010). Pathogen-mediated posttranslational modifications: a re-emerging field. Cell *143*, 694-702.

Riccione, K.A., Smith, R.P., Lee, A.J., and You, L. (2012). A synthetic biology approach to understanding cellular information processing. ACS Synthetic Biology *1*, 389-402.

Sastalla, I., Fattah, R., Coppage, N., Nandy, P., Crown, D., Pomerantsev, A.P., and Leppla, S.H. (2013). The *Bacillus cereus* Hbl and Nhe tripartite enterotoxin components assemble sequentially on the surface of target cells and are not interchangeable. PloS One *8*, e76955.

Schiavo, G., and van der Goot, F.G. (2001). The bacterial toxin toolkit. Nature Reviews Molecular Cell Biology *2*, 530-537.

Schweighauser, L., and Wegner, H.A. (2013). Cyclotetraazocarbazole - a multichromic molecule. Chemical Communications *49*, 4397-4399.

Seelig, G., Soloveichik, D., Zhang, D.Y., and Winfree, E. (2006). Enzyme-free nucleic acid logic circuits. Science *314*, 1585-1588.

Serrano, L. (2007). Synthetic biology: promises and challenges. Molecular Systems Biology *3*, 158.

Shaheen, R., Andersson, M.A., Apetroaie, C., Schulz, A., Ehling-Schulz, M., Ollilainen, V.M., and Salkinoja-Salonen, M.S. (2006). Potential of selected infant food formulas for production of *Bacillus cereus* emetic toxin, cereulide. International Journal of Food Microbiology *107*, 287-294.

Shannon, J.G., Ross, C.L., Koehler, T.M., and Rest, R.F. (2003). Characterization of anthrolysin O, the *Bacillus anthracis* cholesterol-dependent cytolysin. Infection and Immunity *71*, 3183-3189.

Shiby, V.K., and Mishra, H.N. (2012). Fermented milks and milk products as functional foods - a review. Critical Reviews in Food Science and Nutrition *53*, 482-496.

Shinagawa, K., Sugiyama, J., Terada, T., Matsusaka, N., and Sugii, S. (1991). Improved methods for purification of an enterotoxin produced by *Bacillus cereus*. FEMS microbiology letters *80*, 1-5.

Shiota, M., Saitou, K., Mizumoto, H., Matsusaka, M., Agata, N., Nakayama, M., Kage, M.,

Tatsumi, S., Okamoto, A., Yamaguchi, S., *et al.* (2010). Rapid detoxification of cereulide in *Bacillus cereus* food poisoning. Pediatrics *125*, e951-955.

Sineva, E., Shadrin, A., Rodikova, E.A., Andreeva-Kovalevskaya, Z.I., Protsenko, A.S., Mayorov, S.G., Galaktionova, D.Y., Magelky, E., and Solonin, A.S. (2012). Iron regulates expression of *Bacillus cereus* Hemolysin II via global regulator Fur. Journal of Bacteriology *194*, 3327-3335.

Siuti, P., Yazbek, J., and Lu, T.K. (2013). Synthetic circuits integrating logic and memory in living cells. Nature Biotechnology *31*, 448-452.

Spiess, C., Merchant, M., Huang, A., Zheng, Z., Yang, N.-Y., Peng, J., Ellerman, D., Shatz, W., Reilly, D., Yansura, D.G., *et al.* (2013). Bispecific antibodies with natural architecture produced by co-culture of bacteria expressing two distinct half-antibodies. Nature Biotechnology *31*, 753-758.

Stark, T., Marxen, S., Rütschle, A., Lücking, G., Scherer, S., Ehling-Schulz, M., and Hofmann, T. (2013). Mass spectrometric profiling of *Bacillus cereus* strains and quantitation of the emetic toxin cereulide by means of stable isotope dilution analysis and HEp-2 bioassay. Analytical and Bioanalytical Chemistry *405*, 191-201.

Stenfors Arnesen, L.P., Fagerlund, A., and Granum, P.E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Reviews *32*, 579-606.

Sweeney, D.A., Hicks, C.W., Cui, X., Li, Y., and Eichacker, P.Q. (2011). Anthrax infection. American Journal of Respiratory and Critical Care Medicine *184*, 1333-1341.

Tabashnik, B.E., Brevault, T., and Carriere, Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. Nature Biotechnology *31*, 510-521.

Tabor, J.J., Salis, H.M., Simpson, Z.B., Chevalier, A.A., Levskaya, A., Marcotte, E.M., Voigt, C.A., and Ellington, A.D. (2009). A synthetic genetic edge detection program. Cell *137*, 1272-1281.

Tamsir, A., Tabor, J.J., and Voigt, C.A. (2011). Robust multicellular computing using genetically encoded NOR gates and chemical 'wires'. Nature *469*, 212-215.

Tamura, H.-o., Noto, M., Kinoshita, K., Ohkuma, S., and Ikezawa, H. (1994). Inhibition of NGF-induced neurite outgrowth of PC12 cells by *Bacillus cereus* sphingomyelinase, a

bacterial hemolysin. Toxicon *32*, 629-633.

TerAvest, M.A., Li, Z., and Angenent, L.T. (2011). Bacteria-based biocomputing with cellular computing circuits to sense, decide, signal, and act. Energy and Environmental Science *4*, 4907-4916.

Tran, S.-L., Guillemet, E., Ngo-Camus, M., Clybouw, C., Puhar, A., Moris, A., Gohar, M., Lereclus, D., and Ramarao, N. (2011). Haemolysin II is a *Bacillus cereus* virulence factor that induces apoptosis of macrophages. Cellular Microbiology *13*, 92-108.

Tran, S.L., Guillemet, E., Gohar, M., Lereclus, D., and Ramarao, N. (2010). CwpFM (EntFM) is a *Bacillus cereus* potential cell wall peptidase implicated in adhesion, biofilm formation, and virulence. Journal of Bacteriology *192*, 2638-2642.

Tran, S.L., and Ramarao, N. (2013). *Bacillus cereus* immune escape: a journey within macrophages. FEMS Microbiology Letters *347*, 1-6.

Tsilia, V., Devreese, B., Baenst, I., Mesuere, B., Rajkovic, A., Uyttendaele, M., Wiele, T., and Heyndrickx, M. (2012). Application of MALDI-TOF mass spectrometry for the detection of enterotoxins produced by pathogenic strains of the *Bacillus cereus* group. Analytical and Bioanalytical Chemistry *404*, 1691-1702.

Turan, S., Zehe, C., Kuehle, J., Qiao, J., and Bode, J. (2013). Recombinase-mediated cassette exchange (RMCE) - a rapidly-expanding toolbox for targeted genomic modifications. Gene *515*, 1-27.

Usui, K., Miyazaki, S., Kaito, C., and Sekimizu, K. (2009). Purification of a soil bacteria exotoxin using silkworm toxicity to measure specific activity. Microbial Pathogenesis *46*, 59-62.

Van der Auwera, G.A., Timmery, S., Hoton, F., and Mahillon, J. (2007). Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs. International Journal of Food Microbiology *113*, 164-172.

Van Doren, J.M., Neil, K.P., Parish, M., Gieraltowski, L., Gould, L.H., and Gombas, K.L. (2013). Foodborne illness outbreaks from microbial contaminants in spices, 1973–2010. Food Microbiology *36*, 456-464.

Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F., and Bourguet, D. (2002).

Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. Applied and Environmental Microbiology *68*, 1414-1424.

Visschedyk, D., Rochon, A., Tempel, W., Dimov, S., Park, H.W., and Merrill, A.R. (2012). Certhrax toxin, an anthrax-related ADP-ribosyltransferase from *Bacillus cereus*. The Journal of Biological Chemistry *287*, 41089-41102.

Wang, Z., Ning, L., Duan, A., Zhu, X., Wang, H., and Li, G. (2012). A set of logic gates fabricated with G-quadruplex assembled at an electrode surface. Chemical Communications *48*, 7507-7509.

Wehrle, E., Moravek, M., Dietrich, R., Bürk, C., Didier, A., and Märtlbauer, E. (2009). Comparison of multiplex PCR, enzyme immunoassay and cell culture methods for the detection of enterotoxinogenic *Bacillus cereus*. Journal of Microbiological Methods *78*, 265-270.

Wei, P., Wong, W.W., Park, J.S., Corcoran, E.E., Peisajovich, S.G., Onuffer, J.J., Weiss, A., and Lim, W.A. (2012). Bacterial virulence proteins as tools to rewire kinase pathways in yeast and immune cells. Nature *488*, 384-388.

Xianyu, Y., Zhu, K., Chen, W., Wang, X., Zhao, H., Sun, J., Wang, Z., and Jiang, X. (2013). Enzymatic assay for Cu(II) with horseradish peroxidase and its application in colorimetric logic gate. Analytical Chemistry *85*, 7029-7032.

Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., and Benenson, Y. (2011). Multi-input RNAi-based logic circuit for identification of specific cancer cells. Science *333*, 1307-1311.

Ye, H., Baba, M.D.-E., Peng, R.-W., and Fussenegger, M. (2011). A synthetic optogenetic transcription device enhances blood-glucose homeostasis in mice. Science *332*, 1565-1568.

Yu, X., Liu, T., Liang, X., Tang, C., Zhu, J., Wang, S., Li, S., Deng, Q., Wang, L., Zheng, A., *et al.* (2011). Rapid detection of vip1-type genes from *Bacillus cereus* and characterization of a novel vip binary toxin gene. FEMS Microbiology Letters *325*, 30-36.

Yuan, Y.M., Hu, X.M., Liu, H.Z., Hansen, B.M., Yan, J.P., and Yuan, Z.M. (2007). Kinetics of plasmid transfer among *Bacillus cereus* group strains within lepidopteran larvae. Archives of Microbiology *187*, 425-431.

Zakeri, B., and Lu, T.K. (2012). Synthetic biology of antimicrobial discovery. ACS Synthetic Biology *2*, 358-372.

Zhu, J., Zhang, L., Li, T., Dong, S., and Wang, E. (2013a). Enzyme-free unlabeled DNA logic circuits based on toehold-mediated strand displacement and split G-quadruplex enhanced fluorescence. Advanced Materials *25*, 2440-2444.

Zhu, K., Acaroz, U., and Märtlbauer, E. (2013b). A cellular logic circuit for the detection of bacterial pore-forming toxins. Chemical Communications *49*, 5198-5200.

Zhu, K., Dietrich, R., Didier, A., Acar, G., and Märtlbauer, E. (2013c). Versatile antibody-sensing Boolean logic for the simultaneous detection of multiple bacterial toxins. Chemical Communications *49*, 9314-9316.

Zhu, K., Shen, J., Dietrich, R., Didier, A., Jiang, X., and Märtlbauer, E. (2014). Ordered self-assembly of proteins for computation in mammalian cells. Chemical Communications *50*, 676-678.

Zou, Q., Li, X., Zhang, J., Zhou, J., Sun, B., and Tian, H. (2012). Unsymmetrical diarylethenes as molecular keypad locks with tunable photochromism and fluorescence via $Cu2^{+}$ and CN^{-} coordinations. Chemical Communications *48*, 2095-2097.

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