Rapid discrimination of *cytK-1* and *cytK-2* genes in *Bacillus cereus* strains by a novel duplex PCR system

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Abstract

Bacillus cereus is the causative agent of gastrointestinal diarrhoea. At least three known enterotoxins may be involved in this syndrome: nonhaemolytic (Nhe) enterotoxin, Hbl enterotoxin and cytotoxin K. Two different forms were recently described for cytotoxin K, encoded by *cytK-1* and *cytK-2* genes. The CytK-1 toxin appeared to carry a high toxicity, but there is currently no method available to rapidly detect and discriminate the *B. cereus* strains able to produce this CytK-1 form. In this study, a duplex PCR assay was developed and validated on 162 known cytotoxin-containing strains. This PCR method is the first molecular tool to provide rapid detection and discrimination of *cytK-1*- and *cytK-2*-carrying *B. cereus* strains.

Introduction

The gastrointestinal diseases associated with diarrhoeal syndromes caused by *Bacillus cereus* are generally attributed to at least three enterotoxins (Lund *et al*., 2000). While the haemolytic enterotoxin Hbl and the nonhaemolytic enterotoxin Nhe were discovered a long time ago, the necrotic enterotoxin cytotoxin K was only isolated in 2000 and incriminated in an outbreak of severe gastroenteritis causing the death of three patients in France in 1998 (Lund *et al*., 2000). Recently, this toxin was exhaustively characterized by Fagerlund *et al*. (2004), who showed that cytotoxin K could have two different forms, either CytK-1 or CytK-2, according to the *B. cereus* strain. These forms exhibited 89% amino-acid sequence homology. One important difference between the two protein forms was their biological effect, as CytK-1 was highly toxic towards human intestinal Caco2 cells and Vero cells compared with CytK-2. It is reasonable to assume that CytK-1 toxin represents a more severe hazard for consumers than CytK-2. Therefore, it is important to be able to discriminate *cytK-1* strains from *cytK-2* strains encountered in foods.

Bacillus cereus is a ubiquitous spore-former that cannot be totally eliminated. It is an especially major problem in convenience foods and mass catering. Ingestion of *B. cereus* spores, cells or toxins can have different consequences including absence of symptoms, emetic syndrome (because of emetic toxin) or diarrhoeal syndrome (because of enterotoxins). Furthermore, the disease presents variable severity, ranging from a relatively mild short outbreak in most cases to highly severe disease in other rarer cases, such as the France outbreak in 1998 for which the CytK-1 toxin form was responsible. Therefore, it is important to develop methods for rapidly discriminating hazardous strains from nontoxic strains. Suitable methods exist for detecting emetic toxin producers (Ehling-Schulz *et al*., 2004, 2005) and Hbl/Nhe enterotoxin producers (Buchanan & Schultz, 1992, 1994; Dietrich *et al*., 1999; Hansen & Hendriksen, 2001; Guinebretiere *et al*., 2002; Corona *et al*., 2003; Moravek *et al*., 2004; Dietrich *et al*., 2005). A validated PCR method is able to detect *cytK* gene carriers (Guinebretiere *et al*., 2002) but does not give any information on the encoded CytK forms. To date, no method available to detect and discriminate the two types of *cytK* strains has been described. We

report a PCR system that rapidly amplifies either partial *cytK-1* gene or partial *cytK-2* gene, based on gene sequence differences and specially designed primers. This method was validated on 162 strains and represents a suitable method for discriminating *cytK-1* strains from *cytK-2* strains.

Materials and methods

Strains

Reference strains for the development of the PCR assay were the food poisoning strain NVH 391-98 used as a positive control for *cytK-1* gene, the food poisoning strain NVH 1230-88, the *Bacillus cereus* type strain ATCC 14579T and the *B. thuringiensis* type strain CIP 53137T used as positive controls for *cytK-2* gene, and the *B. weihenstephanensis* type strain WSBC 10204, the *B. mycoides* type strain CIP103472 and the *B. pseudomycoides* type strain DSM 12442 used as negative controls for *cytK* gene. One hundred and fifty-six independent strains from the *B. cereus* group (*sensu lato*) were used to validate the PCR system. Most of these strains had been previously characterized for their toxin content (Guinebretiere *et al*., 2002). Some outgroup strains were also included to assess the specificity of the PCR assay: *B. subtilis* type strain CIP 52.65T, *Paenibacillus polymyxa* type strain ATCC 842T, *Clostridium botulinum* NCTC 7273, *Clostridium perfringens* S40, *Staphylococcus aureus* CIP 57.10, *Listeria inocua* CIP 80.12 and *Erwinia carotovora* CFBP 1349. All strains are listed in Table 2.

Isolation of DNA and Southern blot analysis

DNA was prepared as described previously (Guinebretiere & Nguyen-The, 2003), except that incubation with proteinase K was reduced to 1 h and the RNAse treatment step was omitted. DNA was quantified by absorbance at 260 nm on an MBA2000 spectrophotometer (Perkin-Elmer, Courtaboeuf, France).

Strains that were negative in PCR experiments were submitted to Southern blotting to check for the absence of *cytK* gene. Positive controls were included for each probe tested. Ten micrograms of genomic DNA was digested to completion with *Eco*RI and electrophoresed overnight on a 1% agarose gel at 15 V. Southern blotting was carried out according to standard protocols (Sambrook *et al*., 1989). A probe was generated by PCR amplifications of *cytK* gene fragments with CKF/CKR primers, and ^{32}P-labelling was performed with Ready-to-Go DNA labelling beads (Amersham Pharmacia Biotech, Orsay, France) according to the manufacturer's recommendations. Membranes were hybridized at 60 °C for 2 h in Rapid hybridization buffer (Amersham Pharmacia Biotech), then washed according to supplied protocols and exposed to a Phosphor-Imager screen (Storm; Molecular Dynamics, Bondoufle, France) before revelation.

Primer design

The *cytK* gene sequences available from databanks (AJ277962, AJ318875, AJ318876, AJ318877 AAJM0100 0030.1, AE017001.1) were aligned using Clustal W 1.8 software (Thompson *et al*., 1997) and Multalin version 5.4.1 software (Corpet, 1988) to identify sequence mutations, and particularly low conserved regions between the two sequence forms *cytK-1* and *cytK-2*. As the PCR system was based on amplification of either *cytK-1* DNA fragment or *cytK-2* DNA fragment in a single PCR (duplex PCR), the respective primer pairs had to run exclusively on the targeted gene form (*cytK-1* form or *cytK-2* form). Therefore, we designed primer pairs CK1F/CK1R and CK2F/CK2R used for amplifying *cytK-1* DNA fragment and *cytK-2* DNA fragment, respectively, to target low conserved regions. Targets of each primer pair were therefore specific, particularly on the 3′ end region (Fig. 1a). To discriminate *cytK-1* DNA fragment from *cytK-2* DNA fragment, primers pairs were designed so that the resulting DNA fragments were of different length (Fig. 1b).

Primers candidates were selected according to criteria such as %GC, T_m and primer interactions, and according to their compatibility in duplex PCR (Henegariu *et al*., 1997). Sequences of primers were analysed using the BLAST algorithm (Altschul *et al*., 1997) to prevent nonspecific targets and anticipate the specificity of the PCR assay. Table 1 lists the selected primers, their characteristics and the resulting amplicons.

CytK-specific PCR

A step-by-step protocol was first applied as described previously (Henegariu *et al*., 1997). The final PCR mixture (15 μL) contained 50 ng of DNA template, 0.2 mM dNTP mix (Eurogentec, Seraing, Belgium), 2.5 mM $MgCl_2$, 0.25 μM of each primer (CK1F, CK1R, CK2F, CK2R), 0.75 U of AmpliTaq polymerase (Perkin-Elmer) and 1.5 μL of 10 × AmpliTaq buffer (Eurogentec).

Thermal cycling was carried out in a PCR 9700 thermocycler (Perkin-Elmer) with the following run: a starting cycle of 5 min at 94 °C, followed by 30 cycles of 15 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, and a final extension of 7 min at 72 °C.

PCR products were separated on 1.5% agarose gel with the DNA Smart Ladder SF molecular weight marker (Eurogentec). Gels were stained with ethidium bromide and digitized using a gel imager (Bioblock, Illkirch, France). The *cytK-1* and *cytK-2* forms were identified according to the length of the observed fragment.

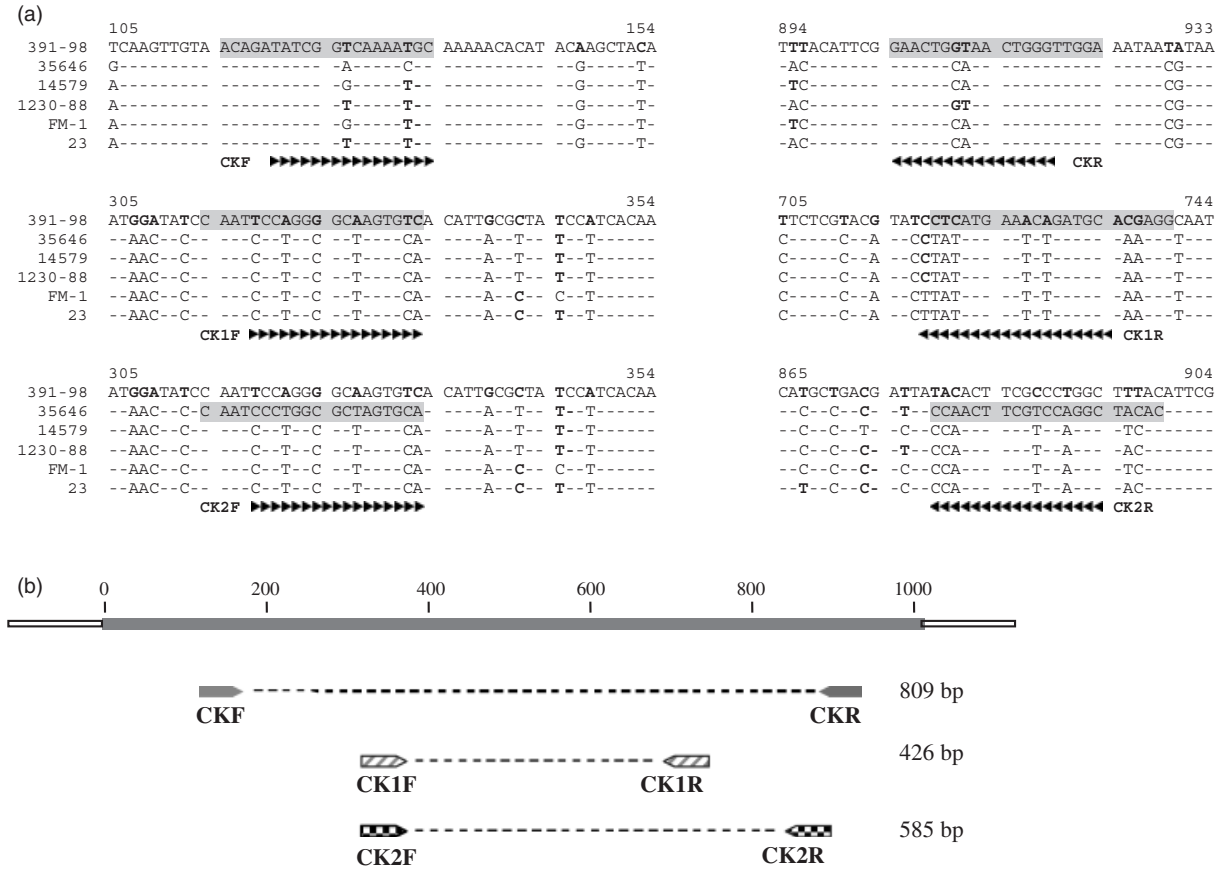

Fig. 1. (a) Alignment of partial forward sequences for *cytK* gene, showing point mutations (A, T, G or C) and conserved bases (- - -) by reference to NVH 391-98 sequence. Only sequence regions containing primers fixation sites (on grey background) are shown. The sequences were obtained from databanks, with accession numbers AJ277962 (strain NVH 391-98), AJ318875 (strain FM-1), AJ318876 (strain NVH 1230-88), AJ318877 (strain 23), AAJM01000030.1/EAO56270 (strain ATCC 35646), AE017001/AAP08097 (strain ATCC 14579). Base 1 corresponds to the first base in the *cytK* gene according to bank sequence annotations. Symbols '▶' and '◀' point the primer direction. (b) Schematic diagram of *cytK* gene showing the approximate position at which each of the primers hybridizes to *cytK* gene and the sizes of the resulting PCR products. Base 1 corresponds to the first base in the *cytK* gene-coding region.

Amplified products from strains ATCC 14579, NVH 391/98, NVH 1230/98, CIP 53.137T, NVH 883/00, INRA AF2, INRA 32, INRA A3, INRA I23, INRA C42, INRA C3, PHLS F1942/85, PHLS F4429/71, F4433/73, AFSSA 98HMPL63 and F0284/78 were sequenced (MWG-Biotech, Ebersberg, Germany) and identified using the BLAST algorithm (Altschul *et al.*, 1997) to check the specificity of the duplex PCR system.

Results and discussion

Characteristics of the duplex PCR system

This PCR system was able to amplify two distinct bands in CytK-positive strains, i.e. either a DNA fragment of 426 bp or a DNA fragment of 585 bp. The first DNA fragment (426 bp) represented the *cytK-1* gene form whereas the second DNA fragment (585 bp) represented the *cytK-2* gene form. In this particular duplex PCR system, only either one or the other DNA fragment could be amplified in a single reaction for each strain, thus determining the cytK group to which the tested strain belonged (*cytK-1* group or *cytK-2* group), as illustrated in Fig. 2b. The duplex PCR was also able to amplify both types of *cytK* DNA fragment (*cytK-1* and *cytK-2* forms) when they were both present in a single reaction, as shown using a mix of DNAs coming from control strains of different *cytK* groups (Fig. 2f). This ensured the efficiency of the method in revealing the possible presence of both gene forms in a strain. As shown for control strains, this PCR system proved to be highly efficient and highly specific. BLASTN analysis showed that there were no targets in the bacterial organisms other than *cytK* gene for the four primers. Thus, the probability that nonspecific bands occurred was very low. Furthermore, highly stringent conditions were applied to the PCR reaction, which enhanced the specificity of the reaction without

Table 1. Characteristics of primers used in this study*

Primer†	Gene	Annealing temperature for the primer couple (°C)	Amplified fragment size (bp)	Sequence (5′ → 3′)	Position on *cytK* gene
CKF	*cytK*	54	809	ACAGATATCGG(G, T)CAAAATGC	115–134
CKR				TCCAACCCAGTT(A, T)(GC)CAGTTC	923–904
CK1F	*cytK-1*	57	426	CAA TTC CAG GGG CAA GTG TC	314–333
CK1R				CCT CGT GCA TCT GTT TCA TGA G	740–719
CK2F	*cytK-2*	57	585	CAA TCC CTG GCG CTA GTG CA	314–333
CK2R				GTG IAG CCT GGA CGA AGT TGG	899–879

*Primers CKF and CKR have been described by Guinebretiere *et al.* (2002).

†F, forward primer; R, reverse primer.

decreasing the yield of the PCR product for each of the targeted loci. The annealing temperature could be increased until 59 °C or decreased until 55 °C without any loss in PCR product yield or specificity (data not shown). No cross-reactivity of the oligonucleotide primers occurred (Figs 2b–e). In particular, there was no cross-reaction between CK2F and CK1R or between CK1F and CK2R. This may be explained by the stringent conditions applied to the reaction and the fact that the targeted sites of primers were significantly different and specific for each *cytK* form (Fig. 1). For the control strains, sequencing of the PCR products always resulted in identification of the *cytK-1* gene form for 426 bp DNA fragments and of the *cytK-2* gene form for 585 bp DNA fragments.

Validation of the PCR system

The reliability and specificity of the present PCR system was assessed on an extended number of bacterial strains. In total, 160 independent *Bacillus cereus* strains as well as strains from different species were analysed (Table 2). Eighty-nine

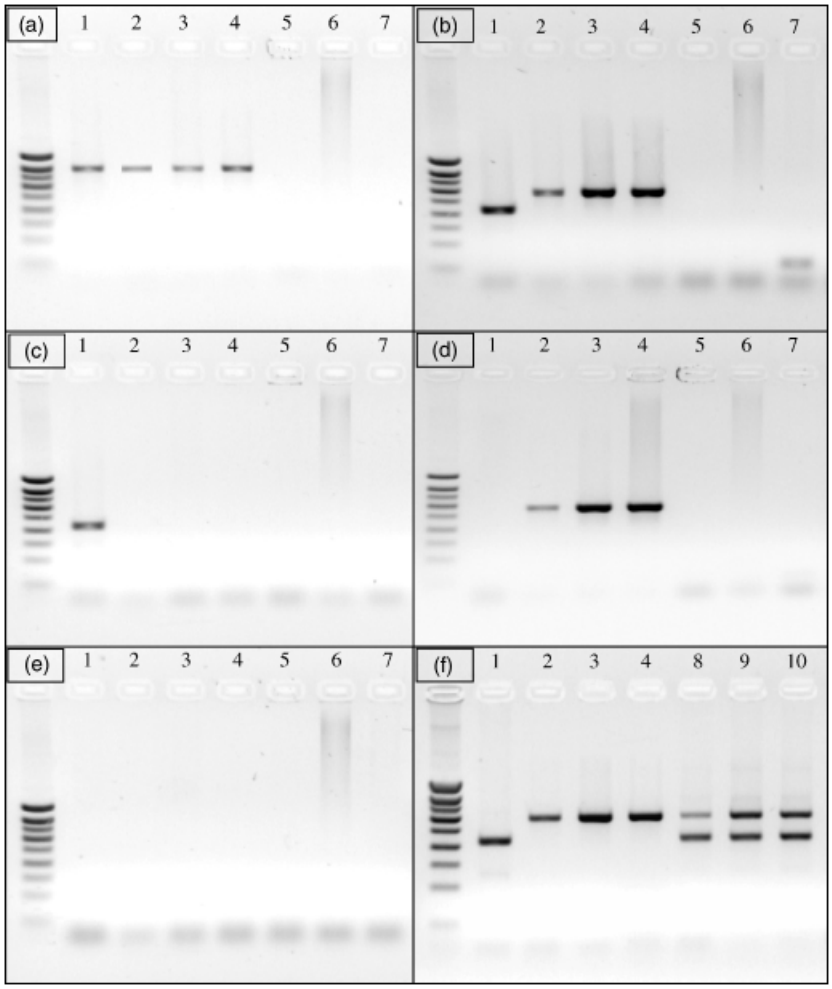

Fig. 2. Amplification products obtained with different combinations of primers in the PCR reaction: (a) primers CKF and CKR; (b) and (f) primers CK1F, CK1R, CK2F and CK2R (duplex PCR); (c) primers CK1F and CK1R; (d) primers CK2F and CK2R, (e) primers CK2F and CK1R. The primer pairs CK1F/CK2R and CK1R/CK2R were also tested and gave the same results as in (e). Lanes 1–10 represent DNAs from the following strains: 1, *Bacillus cereus* NVH 391-98; 2, *B. cereus* NVH 1230-88; 3, *B. cereus* ATCC 14579T; 4, *B. thuringiensis* CIP 53.137T; 5, *B. weihenstephanensis* WSBC 10204; 6, *B. mycoides* CIP 103472T; 7, *B. pseudomycoides* DSM 12442T; 8, *B. cereus* NVH 391-98 and NVH 1230-88 (mixed DNAs); 9, *B. cereus* NVH 391-98 and ATCC 14579T (mixed DNAs); 10, *B. cereus* NVH 391-98 and *B. thuringiensis* CIP 53.137T (mixed DNAs).

Table 2. Characteristic of strains and results of the duplex PCR

		Duplex PCR					Duplex PCR		
Strain†	*cyt K* gene‡	*cytK-1* form‡	*cytK-2* form‡	% Identity§	Strain†	*cyt K* gene‡	*cytK-1* variant‡	*cytK-2* variant‡	% Identity§
Bacillus cereus					*Bacillus cereus*				
PHLS F4430/73	+*	–	+		INRA SL'	–S*	–	–	
PHLS F0285/78	+*	–	+		INRA SZ	–S*	–	–	
PHLS F1942/85	+*	–	+	99	INRA SD	–S*	–	–	
PHLS F2404/79	–S*	–	–		INRA SB'	–S*	–	–	
PHLS F4370/75	–S*	–	–		INRA SM	–S*	–	–	
PHLS F4429/71	+*	–	+	93	INRA SV	–S*	–	–	
PHLS F4432/73	+*	–	+		INRA PA	+*	–	+	
DSM 4282	+*	–	+		INRA PF	–S*	–	–	
NVH 200	+	–	+		INRA A3	+*	–	+	99
NVH 0230/00	+*	–	+		INRA AF2	+	+	–	100
NVH 0500/00	–S*	–	–		NVH 883/00	+	+	–	100
NVH 0861/00	–S*	–	–		INRA C1	–S*	–	–	
NVH 1519/00	–S	–	–		INRA C2	+	–	+	
NVH 1651/00	+*	–	+		INRA C3	+*	–	+	99
NVH 1230/88	+*	–	+	99	INRA C5	+	–	+	
NVH 391/98	+*	+	–	100	INRA C6	+	–	+	
NVH 0075/95	–S	–	–		INRA C13	–S	–	–	
AFSSA 98HMPL63	+*	–	+	99	INRA C15	+	–	+	
PHLS F2769/77	–S*	–	–		INRA C17	+	–	+	
PHLS F0284/78	+*	–	+	98	INRA C18	+	–	+	
PHLS F0352/90	+*	–	+		INRA C20	–S	–	–	
PHLS F4815/94	+*	–	+		INRA C21	–S	–	–	
PHLS F3003/73	–S	–	–		INRA C24	–S	–	–	
PHLS F2081A/98	+*	–	+		INRA C25	–S	–	–	
PHLS F2081B/98	+*	–	+		INRA C26	–S	–	– –	
PHLS F2085/98	+*	–	+		INRA C31	–S	–	–	
DSM 2301	–S*	–	–		INRA C33	+*	–	+	
LMG 17605	+*	–	+		INRA C35	–S*	–	–	
LMG 17604	–S*	–	–		INRA C36	–S*	–	–	
LMG 17615	+*	–	+		INRA C37	+	–	+	
DSM 4222	–S*	–	–		INRA C38	+*	–	+	
DSM 8438	+*	–	+		INRA C39	–S*	–	–	
PHLS F3080B/87	–S	–	–		INRA C41	–S*	–	–	
PHLS 3351/87	–S	–	–		INRA C42	+	–	+	99
PHLS F3752A/86	–S	–	–		INRA C43	+*	–	+	
PHLS F3876/87	–S	–	–		INRA C46	–S*	–	–	
PHLS F3942/87	–S	–	–		INRA C53	+	–	+	
PHLS F4426/94	–S	–	–		INRA C57	+*	–	+	
PHLS F0047/94	–S	–	–		INRA C60	+	–	+	
PFLS F4810/72	–S*	–	–		INRA C62	–S	–	–	
PHLS F0528/94	–S*	–	–		INRA C64	–S*	–	–	
PHLS F5881/94	–S	–	–		INRA C66	+	–	+	
PHLS F4297/83	–S	–	–		INRA C74	–S*	–	–	
PHLS 6921/94	–S	–	–		INRA I2	+*	–	+	
UH IH41064	+*	–	+		INRA I3	–S*	–	–	
UH IH41385	–S	–	–		INRA I4	–S	–	–	
NC 7401	–S	–	–		INRA I5	–S	–	–	
PHLS F0210/76	+*	–	+		INRA I6	–S*	–	–	
PHLS F3465/73	+*	–	+		INRA I7	+*	–	+	
PHLS F3605/73	–S	–	–		INRA I8	–S	–	–	
PHLS F4094/73	+*	–	+		INRA I9	+	–	+	
PHLS F4096/73	+*	–	+		INRA I10	+*	–	+	
PHLS F4346/75	–S*	–	–		INRA I11	–S*	–	–	

PHLS F4431/73	+	–	+		INRA I12	+*	–	+	
PHLS F3371/93	+*	–	+		INRA I13	+*	–	+	
PHLS F3453/94	+*	–	+		INRA I15	– S	–	–	
PHLS F4552/75	– S	–	–		INRA I16	+*	–	+	
PHLS F3350/87	– S	–	–		INRA I17	+*	–	+	
PHLS F4108/89	– S	–	–		INRA I20	– S*	–	–	
ATCC 14579T	+	–	+	99	INRA I21	– S*	–	–	
ATCC 4342	– S	–	–		INRA I22	+*	–	+	
PHLS F4635A/90	– S	–	–		INRA I23	+*	–	+	98
PHLS F4623/90	– S	–	–		*B. pseudomycoides* DSM 12442T	–	–	–	
CIP 58.32	– S	–	–		*B. weihenstephanensis* WSBC 10204T	– S	–	–	
PHLS F4433/73	+*	–	+	93	*B. thuringiensis* CIP 53.137T	+	–	+	99
INRA 1	– S*	–	–		*B. thuringiensis* IEBC T03A001	+	–	+	
INRA 5	– S*	–	–		*B. thuringiensis* IEBC T14007	+	–	+	
INRA 11	– S*	–	–		*B. mycoides* CIP 103472T	– S	–	–	
INRA 15	– S*	–	–		*Paenibacillus polymyxa* ATCC 842T	– S	–	–	
INRA 25	– S*	–	–		*B. subtilis* CIP 52.65T	– S	–	–	
INRA 32	+*	–	+	99	*B. thuringiensis* Bt407	+	–	+	
INRA ZA	– S	–	–		*B. thuringiensis* USDA BtHD1	+	–	+	
INRA BK	– S*	–	–		*B. thuringiensis* Bt tenebrionis	– S	–	–	
INRA BL	– S*	–	–		*B. thuringiensis* Bt ISR	+	–	+	
INRA BY	– S	–	–		*B. anthracis* CEB 9400–40	–	–	–	
INRA BN	– S*	–	–		*Clostridium perfringens* S40	–	–	–	
INRA BC′	+*	–	+		*Clostridium botulinum* NCTC 7273	–	–	–	
INRA BX	– S*	–	–		*Erwinia caratovora* CFBP 1349	–	–	–	
INRA BC	– S*	–	–		*Listeria innocua* CIP 80.12	–	–	–	
INRA BS	– S*	–	–		*Pseudomonas marginalis* CFBP 1538	–	–	–	
INRA SO	– S*	–	–		*Staphylococcus aureus* CIP 57.10	–	–	–	

†AFSSA, Agence Française de Securité Sanitaire des Aliments, Maison-Alfort, France; CEB, Centre d'Etude du Bouchet, Vert le Petit, France; CFBP, Collection Française de Bacteries Phytopathogènes; DSM, Deutsche Sammlund von Milkroorganismen und Zellkulturen, Germany; INRA, Institut National de Recherche Agronomique, UMR A408, Avignon, France; LMG, Laboratorium voor Microbiologie Universiteit Gent, Gent, Belgium; NVH, The Norwegian School of Veterinary Science, Oslo, Norway; PHLS, Public Health Laboratory Service, London, UK; UH, University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland.

*Results from Guinebretiere *et al*. (2002).

‡+, a PCR product of the expected size was observed; –, no PCR product was observed; – S, negative in PCR and in Southern blotting.

§% identity of amplificon, relative to NVH 391/98 *cytK-1* gene for cytK-1 form and relative to NVH 1230/88 *cytK-2* gene for cytK-2 form (issue from local alignment using BLASTN algorithm, Altschul *et al*., 1997).

of these strains were known for their enterotoxin content (Guinebretiere *et al*., 2002). For the uncharacterized strains, the presence of *cytK* gene was checked by PCR using the previously validated CKF and CKR primers (Guinebretiere *et al*., 2002) and the absence of *cytK* gene was checked by Southern blotting. This approach enabled the reliability of the PCR system to be checked by evaluating the percentage of false-negative and false-positive reactions. The assay turned out to be highly reliable with 0% false-negative reactions and 0% false-positive reactions (Table 2). Concerning the specificity of the duplex PCR system, among the 160 tested strains, we observed only one nonspecific band of low intensity in *Erwinia caratovora*. Its size was more than 1 kb and could not be confused with *cytK* gene. Thus, this had no consequences on the diagnostic reliability of the PCR system. Sequencing the 585 bp PCR product or the 426 bp PCR product for approximately 10% of the positive strains showed that the reaction was highly specific: all 426 bp PCR products were identified to the *cytK-1* form whereas all 585 bp PCR products were identified to the *cytK-2* form (Table 2).

When rigorously applying the conditions described in this study for this duplex PCR system, the method can be easily used on a very large number of strains without significant variation in yield of PCR products and with no significant nonspecificity.

Conclusion

The discovery of two different CytK-1 and CytK-2 forms of Cytotoxin K by Fagerlund *et al*. (2004) and the probable association of the CytK-1 type with the more severe forms of gastrointestinal disease has made it important to produce a method for detecting potential CytK-1-producing strains.

Based on the high level of polymorphism observed between the *cytK-1* gene group and the *cytK-2* gene group, the duplex PCR system presented here allows a rapid, reliable and discriminatory detection of *cytK-1 Bacillus cereus* strains and *cytK-2 B. cereus* strains in a single reaction. This system may provide a significant contribution to a general strategy for discriminating hazardous strains from nontoxic strains when associated with methods for detecting emetic strains and Hbl- and Nhe-producing strains. In addition, this PCR-based detection system can be used to provide insight into the distribution of *cytK-1* strains in the *B. cereus* group *sensu lato* (*B. cereus sensu stricto*, *B. thuringiensis*, *B. mycoides*, *B. weihenstephanensis*, *B. pseudomycoides*) and should explain the apparently rare numbers of *cytK-1* strains or their preferential distribution in specific genetic groups.

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