Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

A real-time PCR method to quantify spores carrying the *Bacillus thuringiensis* var. *israelensis cry4Aa* and *cry4Ba* genes in soil

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Keywords
Bacillus thuringiensis var. *israelensis*, biocontrol agent, detection, quantification, sediment, soil, TaqMan real-time PCR.

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2010/0022: received 7 January 2010, revised 26 March 2010 and accepted 31 March 2010

doi:10.1111/j.1365-2672.2010.04741.x

Abstract

Aim: To develop a rapid real-time PCR method for the specific detection and quantification of *Bacillus thuringiensis* var. *israelensis* (Bti) spores present in the environment.

Methods and Results: Seven soil samples as well as one sediment sample obtained from various regions of Switzerland and characterized by different granulometry, pH values, organic matter and carbonate content were artificially inoculated with known amounts of Bti spores. After DNA extraction, DNA templates were amplified using TaqMan real-time PCR targeting the *cry4Aa* and *cry4Ba* plasmid genes encoding two insecticidal toxins (δ-endotoxins), and quantitative standard curves were created for each sample. Physicochemical characteristics of the samples tested did not influence DNA extraction efficiency. Real-time PCR inhibition because of the presence of co-extracted humic substances from the soil was observed only for undiluted DNA extracts from samples with very high organic matter content (68%). The developed real-time PCR system proved to be sensitive, detecting down to 1×10^3 Bti spores per g soil. One-way analysis of variance confirmed the accuracy of the method.

Conclusions: Direct extraction of DNA from environmental samples without culturing, followed by a specific real-time PCR allowed for a fast and reliable identification and quantification of Bti spores in soil and sediment.

Significance and Impact of the Study: The developed real-time PCR system can be used as a tool for ecological surveys of areas where treatments with Bti are carried out.

Introduction

The *Bacillus cereus* group contains Gram-positive, endospore-forming, rod-shaped bacteria of the species *Bacillus thuringiensis*, *Bacillus anthracis*, *B. cereus* (sensu stricto), *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis* (Lechner *et al.* 1998; Vilas-Bôas *et al.* 2007). The first three species, for which extensive genomic data are available, are highly related genetically (Glare and O'Callaghan 2000; Helgason *et al.* 2000; Rasko *et al.* 2005). Nevertheless, they differ considerably in their pathogenic potentials and disease spectrum. While *B. anthracis* causes the potentially lethal disease anthrax, *B. cereus* is an opportunistic pathogen causing several types of intestinal infections in humans, whereas *B. thuringiensis* (Bt) is an insect pathogen (Jensen *et al.* 2003; Vilas-Bôas *et al.* 2007). Although *B. anthracis* can be distinguished from *B. cereus* and *B. thuringiensis* by both microbiological and biochemical tests, Bt is phenotypically distinct from *B. cereus* only by its ability to form insecticidal protein crystals during spore formation (Glare and O'Callaghan 2000; Vilas-Bôas *et al.* 2007); these endotoxins are toxic to specific insects depending on the bacterial strain.

Bacillus thuringiensis var. *israelensis* (Bti) (Goldberg and Margalit 1977) is used worldwide as a biopesticide against mosquito and black fly larvae (WHO 1999), belonging to

the families *Culicidae* and *Simuliidae*. The Bti intracellular insecticidal protein crystal aggregates produced during sporulation are composed of different delta-endotoxins (encoded by the genes *cry4Aa*, *cry4Ba*, *cry10Aa*, and *cry11Aa*) and haemolytic factors (encoded by *cyt1Aa* and *cyt2Ba*) (Crickmore *et al.* 1998). The genes encoding these proteins are located on a 127·9 kb plasmid (Berry *et al.* 2002). The activation of the toxins in the alkaline midgut environment of mosquito larvae leads to the disruption of membrane integrity and eventual cytolysis of the epithelial cells and the consequent blocking of intestinal functions. The germination and multiplication of ingested spores may cause septicaemia followed by the death of the larvae (Schnepf *et al.* 1998; WHO 1999; Bravo *et al.* 2007).

The persistence, the possible proliferation, and the environmental accumulation of human-spread Bti may have a negative impact on nontarget organisms and increase the risk of resistance development in mosquito larvae (Glare and O'Callaghan 2000; Tilquin *et al.* 2008). Assessment of environmental impact and safety depends on the availability of fast and reliable quantitative identification techniques to monitor and follow the environmental persistence and spread of the biopesticide (Glare and O'Callaghan 2003).

In recent years, real-time PCR-based methods have become the most common techniques for the sensitive detection and quantification of environmental microorganisms (Ryu *et al.* 2003; Castrillo *et al.* 2007; Savazzini *et al.* 2008). Successful detection and quantification of the target organism require the overcoming of some common problems associated with environmental DNA-based methods, such as cell lysis efficiency, DNA adsorption to soil particles, and PCR inhibition because of co-extracted humic substances (Kuske *et al.* 1998; Fykse *et al.* 2003; Cook and Britt 2007). De Respinis *et al.* (2006) developed a highly specific method based on cultivation of Bti spores followed by molecular detection of the *cry4Aa* and *cry4Ba* genes using conventional PCR and ribotyping. While the described approach allowed a very specific detection and quantification of Bti in soil, at the same time, it was very expensive and time-consuming. To overcome these limitations, we describe here the development of a rapid and cost-effective *cry4Aa*- and *cry4Ba*-targeted TaqMan real-time PCR technique that does not require bacterial cultivation and allows the quantitative detection of *B. thuringiensis* var. *israelensis* spores directly from environmental samples. Because of its ease, rapidity, cost-effectiveness, and sensitivity, this method has the potential of rendering ecological surveys of protected wetland ecosystems more readily available, which in turn would encourage a constant monitoring of the Bti spores concentration in soil.

Materials and methods

Bacterial strains and growth conditions

The 38 reference strains of *Bacillus* spp. used in this study are listed in Table 1. Vegetative cells were grown overnight at 30°C on Columbia agar base (Oxoid, Basel, Switzerland) supplemented with 5% sheep erythrocytes.

A pure suspension of *B. thuringiensis* var. *israelensis* spores was derived from the commercial product Vectobac-G® (Valent Biosciences, Libertiville, FL) by re-suspending the preparation in water. The absence of vegetative cells was checked by microscopy after coomassie blue staining (Rampersad *et al.* 2002). Spores were then quantified by classical plate counting using blood agar plates. Colony-forming units per ml of suspension (CFU ml^{-1}) were then calculated after overnight incubation at 30°C. The spore suspension was stored at 4°C.

Soil/sediment samples

Samples from seven soils and one sediment, taken in Switzerland (Table 2), were selected on the basis of their physicochemical characteristics to test the influence of their properties on the efficacy of the methodology. Reference soil samples from Fribourg, Jura, and Neuchâtel were kindly provided by the Soil and Vegetation Laboratory, Institute of Biology, University of Neuchâtel (Switzerland). Because in the wetland reserve 'Bolle di Magadino' regular treatments with the Vectobac-G® have been carried out since 1988, all samples from this area were autoclaved at 121°C for 30 min, to eliminate Bti spores and free DNA (Burgmann *et al.* 2003). The absence of Bti spores after heat treatment was checked by plating on blood agar. All samples were dried and passed through a 2-mm mesh sieve for removal of large debris and homogenization.

DNA extraction from pure bacterial cultures

Total DNA from vegetative cells of the 38 strains of *Bacillus* spp. was extracted using the QIAamp DNA Mini kit (Qiagen, Basel, Switzerland), according to the manufacturer's instructions.

DNA extraction from soil/sediment samples inoculated with Bti spores

To assess the detection limit of the method and to create a standard curve, aliquots of 250 mg (dry weight) of each soil and sediment sample were inoculated with 400 μl of Bti spore suspension to obtain a range of 1×10^8 to 1×10^3 spores per g of sample. The concentration of Bti

Table 1 *Bacillus* strains used in this study

No.	Species	Strains*	Origin of the strain	*cry4*†
1	*Bacillus amyloliquefaciens*	ATCC 23350^T	Soil	–
2	*Bacillus brevis*	ATCC 8246^T‡	Unknown	–
3	*Bacillus cereus*	ATCC 10876a	Contaminated bottle	–
4	*B. cereus*	ATCC 7004	Pasteurized milk	–
5	*B. cereus*	LMG 8950	Soil	–
6	*B. cereus*	LMG 9679	Soil	–
7	*B. cereus*	GP7‡	Soil	–
8	*B. cereus*	ATCC 11778‡	Unknown	–
9	*B. cereus*	ATCC 14579^T	Unknown	–
10	*Bacillus circulans*	ATCC 11033	Soil	–
11	*B. circulans*	ATCC 24^T	Soil	–
12	*Bacillus licheniformis*	ATCC 14580^T‡	Unknown	–
13	*Bacillus megaterium*	ATCC 14581^T	Unknown	–
14	*B. megaterium*	LMG 12253	Soil	–
15	*B. megaterium*	DSM 90‡	Soil	–
16	*Bacillus mycoides*	ATCC 6462^T	Soil	–
17	*B. mycoides*	LMG 9680	Soil	–
18	*B. mycoides*	LMG 12256	Soil	–
19	*B. mycoides*	LMG 12410	Soil	–
20	*Bacillus polymyxa*	ATCC 842^T‡	Unknown	–
21	*Bacillus sphaericus*	ATCC 14577^T	Unknown	–
22	*Bacillus subtilis*	ATCC 6051^T	Unknown	–
23	*B. subtilis*	ATCC 7003	Unknown	–
24	*Bacillus subtilis* var. *niger*	ATCC 9372‡	Unknown	–
25	*Bacillus thuringiensis*	Unknown	Laboratory strain	–
26	*B. thuringiensis*	NRRL B4039	Unknown	–
27	*B. thuringiensis* Berliner	ATCC 10792^T	Unknown	–
27	*B. thuringiensis* H3a	LMG 12268	Unknown	–
29	*B. thuringiensis* H4ab	LMG 12266	Unknown	–
30	*B. thuringiensis* H5ab	LMG 12265	Unknown	–
31	*B. thuringiensis* H9	LMG 12269	Unknown	–
32	*Bacillus thuringiensis* var. *aizawai*	DSM 6099	Unknown	–
33	*Bacillus thuringiensis* var. *israelensis*	DSM 5724	Commercial product	+
34	*B. thuringiensis* var. *israelensis*	Vectobac-G®	Commercial product	+
35	*B. thuringiensis* var. *israelensis*	IP 4444	Unknown	+
36	*Bacillus thuringiensis* var. *kurstaki*	DSM 5725	Commercial product	–
37	*Bacillus thuringiensis* var. *morrisoni*	DSM 6112	Unknown	–
38	*B. thuringiensis* var. *morrisoni*	DSM 6113	Unknown	–

^{T}, type strain.

*ATCC: American Type Culture Collection, MD, USA; DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; LMG: Bacteria Collection Laboratorium Microbiologie, Universiteit Ghent, Belgium; NRRL: Agricultural Research Service Culture Collection, Northern Regional Research Center, Agricultural Research Center, United States Department of Agriculture, Peoria, IL, USA; IP: Institut Pasteur, Paris, France.

†Positive (+) or negative (–) amplification of the 439bp fragment for *cry4Aa* and *cry4Ba* genes.

‡Strain supplied by Prof. Dr. Dittmar Hahn, Department of Biological Sciences, Rutgers University, Newark, NJ, USA.

spores in the suspension was determined as described previously. After inoculation, soil aliquots were mixed using a Vortex device (Vortex-Genie® 2T; Scientific Industries, Bohemia, NY) to homogenously distribute spores in the samples. Before DNA extraction, a DNase treatment step and a short spore germination step were performed to eliminate free DNA and to facilitate cells disruption. DNase treatment was performed according to Turgeon *et al.* (2008). Briefly, 50 μl DNase buffer 10× (400 mmol l^{-1} Tris–HCl, pH8·0, 30 mmol l^{-1} $MgCl_2$, 30 mmol l^{-1} $MgSO_4$) and 200 U/ml DNase I (Roche Diagnostic AG, Rotkreuz, Switzerland) were added to seeded soil/sediment samples which were incubated at 25°C for 30 min on a shaker (200 rev min^{-1}). The enzyme was heat inactivated at 75°C for 20 min. This served also as a heat shock step for spore germination induction. Germination was enhanced by adding 500 μl of tryptic soy broth (TSB) (Oxoid) followed by

Table 2 Origin and characteristics of soil samples used in this study

Sample	Sample number	Typology*	Sampling location	pH (H_2O)	Organic matter (%)	% $CaCO_3$	Granulometry (%)		
							Sand	Silt	Clay
Soils	1	Histosol	Bog, Chaux des Breuleux, Jura	3·8	68·0	0	NA	NA	5–10†
	2	Neoluvisol	Bois de l'Hôpital, Neuchâtel	5·2	5·3	0	24·0	62·9	13·2
	3	Brunisol	Bois de l'Hôpital, Neuchâtel	7·4	10·5	0	24·0	56·0	20·0
	4	Calcosol	Chaumont, Neuchâtel	7·5	13·0	1·1	39·9	38·8	21·3
	5	Carbonated fluvisol	Sarine, Auges de Neirivue, Fribourg	7·5	3·9	2·0	NA	NA	NA
	6	Fluvisol	Bolle di Magadino (Piattone), Ticino	6·3	7·6	0	7·9	78·9	13·2
	7	Fluvisol	Bolle di Magadino (Verzasca inlet), Ticino	5·1	4·3	0	49·7	42·6	7·7
Sediment	8		Bolle di Magadino (Bolla Rossa), Ticino	6·1	5·0	0	26·5	62·4	11·0

NA, not available.
*Pedological reference.
†Determined by tactile test.

incubation at 30°C for 90 min on a shaker. The incubation time allowing germination but not cell division was assessed previously using the spore suspension by spectrophotometry by measuring the decrease in OD at 625nm (Collado *et al.* 2006) and by microscopy [coomassie blue staining (Rampersad *et al.* 2002)]. DNA was extracted using the Fast DNA Spin kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions, with some modifications. Briefly, 100 μl of the spores-soil/sediment suspensions were transferred to 2 ml screw-cap tubes with 0·2 g of ≤106 μm and 0·2 g of 150–212 μm glass beads (Sigma, Buchs, Switzerland). A volume of 978 μl sodium phosphate buffer 0·1 mol l^{-1}, pH 7·5, as well as 122 μl MT buffer (provided with the kit) was added to each tube. Samples were homogenized for three min at speed level 6·0 using a FastPrep-24 instrument (MP Biomedicals). After 15 min centrifugation at 14 000 ***g***, supernatants were transferred to new clean tubes and DNA purified according to the manufacturer's protocol. DNA was eluted with 100 μl TE buffer. Total DNA from the dilutions of pure spores used for soil spiking was extracted as described for seeded samples and was used as positive control. Unspiked soil or sediment aliquots were used as negative controls. All DNA extractions were performed in triplicate.

Set up of the specific and quantitative real-time PCR for Bti

Primers and TaqMan probe

Primers Un4(d) and Un4(r) (Microsynth AG, Balgach, Switzerland) (Table 3) amplify an identical fragment of 439 bases for *cry4Aa* and *cry4Ba* genes located on the 127·9-kb pBtoxis plasmid (Ben-Dov *et al.* 1997). A *Bti*-specific TaqMan probe (Table 3) was designed with the Primer3 (ver. 0.4.0) software (available online: http://frodo.wi.mit.edu/). The probe was labelled at the 5′-end with the fluorescent FAM (6-carboxy fluorescein) reporter dye and at the 3′-end with the TAMRA quencher dye (Microsynth AG). Primers and probe were checked *in silico* for specificity comparing sequences with those present in GenBank database using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Inclusivity and exclusivity tests of the real-time quantitative PCR

TaqMan real-time PCR conditions were optimized using DNA extracted from pure cultures of *B. thuringiensis* var. *israelensis* strain DSM 5724 and the Vectobac-G® strain, respectively.

Inclusivity (i.e. detection of target strains) and exclusivity (i.e. nondetection of nontarget strains) (Reekmans

Table 3 Primers and probe used for real-time PCR in this study

Primers/probes	Oligonucleotide sequence (5′–3′)	Oligonucleotide position*	Reference
Un4(d)	GCATATGATGTAGCGAAACAAGCC	589–612 (*cry4Aa*) 2800–2823 (*cry4Ba*)	(Ben-Dov *et al.* 1997)
Un4(r)	GCGTGACATACCCATTTCCAGGTCC	173–198 (*cry4Aa*) 3214–3238 (*cry4Ba*)	(Ben-Dov *et al.* 1997)
Bti_FAM	FAM-GCACAAGCGCGTTATTTGTA-TAMRA	395–414 (*cry4Aa*) 2998–3017 (*cry4Ba*)	This study

*Starting from the first base of the sequence (of the respective *cry* gene) in the GenBank database (*cry4Aa*: accession number AL731825, region 92 986–96 528; *cry4Ba*: accession number AL731825, region 32 597–36 007).

et al. 2009) of the real-time PCR were checked using pure cultures of a set of *Bacillus* reference strains (Table 1). The reactions were performed in a 7500 Fast Real-time PCR System instrument (Applied Biosystems, Rotkreuz, Switzerland). Each reaction consisted of 20 μl of a solution containing 10 μl of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), 0·9 μmol l^{-1} of each primer, 0·2 μmol l^{-1} of the Bti_FAM probe, and 2 μl of template DNA. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 50 cycles composed of 15 s at 95°C and 1 min at 60°C. The data were analysed with the 7500 Fast System SDS Software ver. 1.4.0 (Applied Biosystems, Rotkrenz, Switzerland) with the auto settings used for the baseline.

For TaqMan real-time quantitative PCR, template DNA from spiked and nonspiked samples, as well as from positive control samples, were amplified as previously described for inclusivity and exclusivity testing (Reekmans *et al.* 2009). Moreover, for each soil or sediment sample, the PCR assay included the DNA extracted from spiked samples, the negative controls (unspiked samples), a 'no-template' control, and a positive control consisting of DNA extracted from vegetative cells of the Vectobac-G® strain. For each soil and sediment sample, as well as for the positive control samples, a quantitative standard curve was plotted with the C_t values (threshold cycle) as a function of the spores concentrations. The C_t value corresponds to the cycle number at which the fluorescence emission crosses a threshold within the logarithmic increase phase (Selesi *et al.* 2007). The threshold was set at 0·02. PCR efficacies were calculated with the following formula: $[(10^{-1/\text{slope}}) - 1] \times 100$ (Cordier *et al.* 2007).

Precision testing

The precision of the method was evaluated as described by Savazzini *et al.* (2008) with some modifications. Twenty-five grams of sieved, sterile soil (soil 7; Fluvisol, Bolle di Magadino, Verzasca inlet) were inoculated with a final concentration of 1×10^6 Bti spores per g of soil and dried overnight at 60°C. The samples were thoroughly homogenized and three aliquots of 250 mg (W_1) and 2·5 g (W_2), respectively, were taken for DNA extractions. For the three 250 mg samples, DNA extractions were performed as described previously with the only difference that 400 μl sterile distilled water were added to the samples instead of spores. For the 2·5 g samples, volumes used for DNase treatment and spore germination were adapted accordingly and thus were tenfold higher (i.e. 4 ml water, 500 μl DNase I 200 U/ml, 500 μl DNase Buffer 10× and 5 ml TSB). After amplification of the extracted DNA, quantification of the spores in the six samples was calculated by interpolating the C_t values of the samples with those of the standard curve obtained with known spore concentrations.

Statistical analysis

For each soil and each spore concentration, C_t values were determined in triplicate and the averages and 95% confidence intervals (95% CI) were calculated. Differences among standard curves were tested for significance using one-way analysis of variance (ANOVA) with a significance level set to 0·05 using SPSS (SPSS Statistics for Windows, ver. 17.0; SPSS Swiss SA, Zurich, Switzerland).

For the precision test, data were log transformed and the variance between spores concentration were calculated both for 250 mg and 2·5 g soil samples.

Results

Inclusivity and exclusivity of the real-time PCR

Inclusivity and exclusivity of the real-time PCR were established for pure cultures of *Bacillus* spp. Among the 38 strains tested, amplification products were obtained only from the DNA extracts of the three Bti strains (Table 1), indicating that the methodology is 100% specific for *Bacillus* strains carrying *cry4* genes and 100% exclusive for all the other *Bacillus* spp., including the other members of the *B. cereus* group.

DNA extraction and real-time PCR from soil/sediment samples

Aliquots of soil and sediment samples listed in Table 2 were artificially contaminated with serial dilutions of Bti spores, and DNA was subsequently extracted and amplified with TaqMan real-time PCR. To assess whether the physicochemical properties of either soil or sediment samples had an influence on DNA extraction and PCR amplification, total DNA from pure spores dilutions was also extracted and amplified (positive controls).

As lysis of bacterial spores is extremely challenging, we introduced a germination step prior to chemical and mechanical lysis in which DNA extraction was performed before the first round of cell division could take place. The germination step improved the efficiency of lysis and increased the amount of extracted DNA, resulting in a reduction of the amplification cycles necessary to obtain the desired results (data not shown). Undiluted DNA extracts obtained with this method from spiked soil and sediment samples were directly amplifiable by real-time PCR assays, testifying to the level of purification of the DNA fraction and indicating the absence of amplification inhibitors. The only exceptions were Histosol samples

characterized by a high content of organic matter (68%), for which a tenfold dilution of the extracted DNA samples was necessary to obtain amplification products.

Standard curves were created for each sample by plotting the C_t values as a function of the spore concentration (Figs 1 and 2). A good linear relationship between the log of the Bti spores concentration and the C_t values ($R^2 > 0{\cdot}96$ in all cases) was obtained in each case. The amplification efficiencies varied from 72% (soil sample nr 3) to 91% (soil sample nr 7) across the eight real-time PCR assays. The physicochemical characteristics of the samples tested had no influence on the efficacy of the methodology, as no significant differences were observed among the mean C_t values of the seven soils or sediment samples tested and of the positive control samples (Fig. 1; ANOVA, $P = 0{\cdot}998$). In most soil samples (sample nr 3, 5, 6, and 7), as well as in the positive controls, the TaqMan real-time PCR showed a high degree of sensitivity, with a detection limit of 1×10^3 Bti spores per g soil. The detection limit observed for soil samples nr 2 and 4 and for the sediment sample was 1×10^4 Bti spores per g. In the case of Histosol (Fig. 2, soil sample nr 1), the lowest spore concentration detectable by our PCR amplification method was 1×10^5 Bti spores per g and at this spore concentration, the variability was very high (95% CI = ±5 PCR cycles).

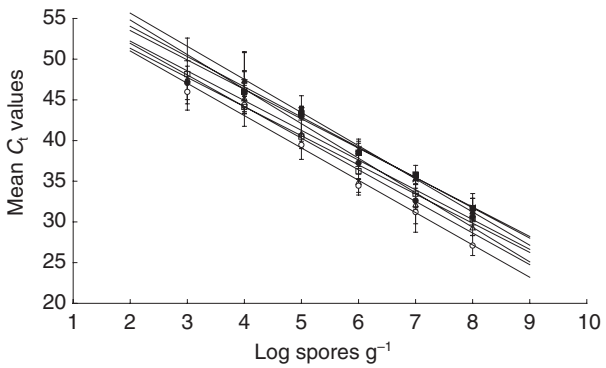

Figure 1 Standard curves for tenfold serial dilutions of Bti spores added to aseptic soil/sediment samples. The C_t values (mean of three replicates) are plotted against the corresponding log concentration of Bti spores. $R^2 > 0{\cdot}97$ in all cases. ANOVA, $P = 0{\cdot}998$. Bars: 95% CI. (●) Positive controls (pure spore dilutions); (○) fluvisol (Piattone); (□) fluvisol (Verzasca inlet); (Δ) carbonated fluvisol; (+) brunisol; (×) neoluvisol; (■) calcosol and (▲) sediment.

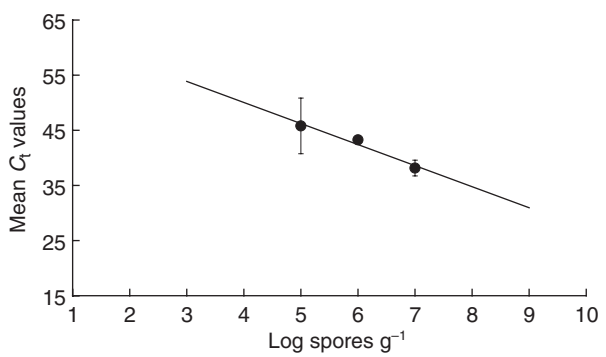

Figure 2 Standard curves for tenfold serial dilutions of Bti spores added to aseptic Histosol samples. The C_t values (mean of three replicates) are plotted against the corresponding log concentration of Bti spores. $R^2 = 0{\cdot}96$. Bars: 95% CI.

Precision testing

The accuracy of the detection method was evaluated by inoculating a sterile soil sample with 1×10^6 Bti spores per g. Total DNA was extracted from three independent 250 mg samples (W_1) and three independent 2·5 g samples (W_2), and real-time PCR amplification was performed as described previously. The spore concentration in each sample was determined using standard curve C_t values. Mean spore concentrations were 5·56 log and 5·66 log for W_1 and W_2, respectively (Fig. 3). Thus, the concentration of spores detected in the six test samples was slightly lower (about 0·39 log) compared to the amount inoculated into the soil. Variability among the samples was very low, with sample variances of 0·0116

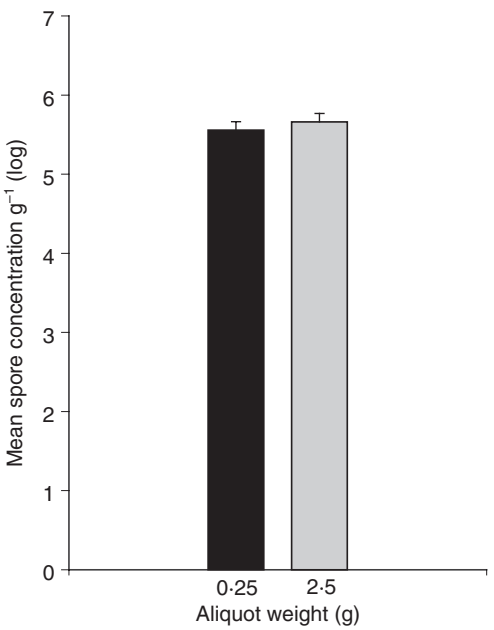

Figure 3 Precision test. Sterile soil sample was inoculated with Bti spores. Spore concentrations were calculated on the basis of the soil nr 7 standard curve. Black column: 250 mg soil sample (W_1); grey column: 2·5 g soil sample (W_2). Bars: standard deviation ($n = 3$).

and 0·0081 for the 250 mg and 2·5 g soil samples, respectively.

Discussion

Quantitative identification techniques, such as real-time PCR-based methods, allow the accurate measuring of the environmental persistence and the spread of biological control agents. This is important for long-term monitoring during and after application and for the ultimate effectiveness of these environmentally safe methods of pest control.

Here, we report the development of a highly specific real-time PCR method for the rapid detection and quantification of *B. thuringiensis* var. *israelensis* spores present in soil and sediment samples. This method is based on TaqMan real-time PCR amplification of a fragment of both *cry4Aa* and *cry4Ba* genes (439 bp). In a previous study, De Respinis *et al.* (2006) developed a specific detection method for Bti spores in soil and sediment. This method however was very time-consuming as it included an *in vitro* growth step, the extraction of total DNA from morphologically selected colonies followed by conventional PCR amplification coupled with ribotyping analysis. To overcome these difficulties, we set out to develop the real-time PCR-based system described here, which allows for a considerably more sensitive, faster, and cost-effective quantification of Bti spores directly from both soil and sediment samples and has the potential of widespread adoption in routine surveys of biopesticide-treated areas.

At the onset, we reasoned that the success of our approach would depend on two key factors: first, the efficiency of cell lysis, which is an essential prerequisite to lower the detection limits (Bürgmann *et al.* 2001); second, the effective removal of soil-derived extraction contaminants, which are known to potently inhibit PCR amplification of extracted DNA. Both cell lysis efficiency and the amounts of DNA co-extracted contaminants depend on the characteristics of the soil sample analysed. As a consequence, we designed our study to include soil and sediment samples from various regions of Switzerland that would cover a broad range of soil characteristics such as a variety of granulometry levels, pH values, and organic matter or carbonate content. We were able to demonstrate that our technique is applicable on samples presenting different chemical and physical features, making it universal and of general interest.

To address the cell lysis efficiency, Bti spore lysis was enhanced by the introduction of a germination step prior to chemical and mechanical lysis. In addition, bead-beating was used for bacterial cells release from the interstices of soil aggregates and cell disruption. This procedure is known to improve the amount of DNA directly recovered from environmental samples, especially when working with organisms that are resistant to disruption, such as endospores or Gram-positive bacteria (More *et al.* 1994; Kuske *et al.* 1998; Miller *et al.* 1999; Kabir *et al.* 2003; Robe *et al.* 2003; Costa *et al.* 2004; Cook and Britt 2007; Lakay *et al.* 2007). As we predicted, combining the germination step with the glass beads disruption step, we were able to eliminate the effect of the presence of soil or sediment on lysis efficiency and consequently on detection limits and specificity. Accordingly, our results showed that physico-chemical characteristics of the samples tested had no influence whatsoever on the DNA extraction efficiency of the Bti spores and the DNA yield was almost identical for both positive controls (i.e. pure spore dilutions) and environmental samples seeded with bacteria. Differences among the mean C_t values of the samples tested were not statistically significant. The mean amplification efficiency of the real-time PCRs was about 83%.

The second challenge we faced in our study is represented by soil components capable of potently inhibiting the PCR amplification, such as humic and phenolic compounds (Sjöstedt *et al.* 1997). Obtaining pure microbial DNA amplifiable by PCR from soil samples is quite challenging (Kuske *et al.* 1998). In our study, the level of DNA enrichment after extraction from soil was sufficient for direct amplification by real-time PCR in all samples with amounts of organic matter lower than 68%. Thus, for these samples, the method was highly efficient and sensitive, showing a detection limit of 1×10^3 Bti spores per g soil. On average, with the real-time PCR conditions used, 47 cycles were necessary to detect such quantity of spores. The detection limit of our technique represents a significant improvement over the technique of De Respinis *et al.* (2006) for the detection of *Bacillus* spores in the environment and therefore for monitoring performance. At the same time, it is comparable to the reported efficiency of real-time PCR-based systems for the detection of other environmental micro-organisms, such as anthrax spores, *Collimonas* cells, and *Sinorhizobium meliloti* cells (Ryu *et al.* 2003; Höppener-Ogawa *et al.* 2007; Trabelsi *et al.* 2009). The only exception was represented by soil nr 1 (Histosol), which presented an organic matter content of 68%, as opposed to an average of 7% present in the other samples we examined. The presence of high levels of PCR-inhibitory organic contaminants in the later rendered tenfold dilution necessary to facilitate the PCR. A dilution step had been previously proven effective in reducing PCR inhibition caused by extraction contaminants (Castrillo *et al.* 2007; Cook and Britt 2007), but has the disadvantage of limiting sensitivity. Thus, as expected, the detection limit we found for soil sample nr

1 was higher compared to those of the other soil samples we studied (1×10^5 *vs* 1×10^3 spores per g).

The quantification of a target strain in soil samples may be influenced by the samples size. Micro-organisms are heterogeneously dispersed in the soil aggregates (Ranjard *et al.* 2003; Robe *et al.* 2003); therefore, the methodology used for bacterial quantification requires some precautions. To overcome this difficulty, a precision test was carried out with known amounts of Bti spores inoculated into sterile soil and subsequently quantified with the developed real-time PCR. The variability among samples was very low, which suggests a high accuracy of the method.

In conclusion, this study led to the development of a highly specific TaqMan real-time PCR for the detection and quantification of *B. thuringiensis* var. *israelensis* spores present in both soil and sediment. The method is specific for Bti strains and sensitive, with a detection limit of 10^3 Bti spores per g soil/sediment. The main advantage of the described method is represented by the direct extraction of DNA from environmental samples, allowing fast, accurate, and less laborious identification and quantification of Bti spores in soil and sediment. This method fulfils the requirements of speed, precision, and relatively low analytical costs to be used as a tool for widespread and long-term surveys monitoring the persistence of bio-insecticidal Bti spores in the environment.

Acknowledgements

We gratefully acknowledge the statistical advice by P. Decristophoris. We thank O. Petrini and C. Strambio De Castillia for helpful advice and critical reading of the manuscript. This work was financially supported by the 'Bolle di Magadino' Foundation (Magadino, Switzerland) and the Swiss Federal Office for the Environment (Bern, Switzerland).

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