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Chromosome-Directed PCR-Based Detection and Quantification of *Bacillus cereus* Group Members with Focus on *B. thuringiensis* Serovar israelensis Active against Nematoceran Larvae

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***Bacillus thuringiensis* serovar israelensis is a wide-spread soil bacterium affiliated with the *B. cereus* group (Bcg) and is widely used in biocontrol products applied against mosquito and black fly larvae. For monitoring and quantification of applied *B. thuringiensis* serovar israelensis and its effect on indigenous *B. thuringiensis* serovar israelensis and Bcg assemblages, efficient and reliable tools are essential. The abundance and properties of *B. thuringiensis* serovar israelensis strains in the environment traditionally have been investigated with cultivation-dependent techniques, which are hampered by low sensitivity and the morphological similarity between *B. cereus* and *B. thuringiensis*. Currently available PCR-based detection and quantification tools target markers located on plasmids. In this study, a new cultivation-independent PCR-based method for efficient and specific quantification of *B. thuringiensis* serovar israelensis and Bcg is presented, utilizing two sets of PCR primers targeting the bacterial chromosome. Sequence database searches and empirical tests performed on target and nontarget species, as well as on bulk soil DNA samples, demonstrated that this diagnostic tool is specific for *B. thuringiensis* serovar israelensis and Bcg. The method will be useful for comparisons of Bcg and *B. thuringiensis* serovar israelensis abundances in the same samples. Moreover, the effect of *B. thuringiensis* serovar israelensis-based insecticide application on the total Bcg assemblages, including indigenous populations, can be investigated. This type of information is valuable in risk assessment and policy making for use of *B. thuringiensis* serovar israelensis in the environment.**

The bacterium *Bacillus thuringiensis* (Bt) is one of the grand successes in microbial pest control (1). After its first discovery in 1901 and description by Berliner in 1911, the first biocontrol product based on Bt, Sporein, for control of the European corn borer (*Ostrinia nubilalis*), was commercially available already in 1938 (1). The crystalline, proteinaceous δ-endotoxins of Bt are formed during sporulation, and their lethal effect is manifested only after larval ingestion of spores or free crystals (2, 3). Bt is a member of the *Bacillus cereus* group (Bcg), which consists of the Gram-positive rod-shaped spore-forming bacterial species *B. cereus*, Bt, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (4, 5). However, a multidatatype phylogenetic analysis of Bcg strains isolated from various sample types, including environmental, clinical, food, and diary sources, did not reflect current Bcg taxonomy well. This finding indicated that the phylogeny of this bacterial group is more complicated than reflected by the division into the current six species (6).

The bacterium *B. thuringiensis* serovar israelensis produces toxins active against nematoceran larvae and has been widely used for biocontrol of mosquitoes and black flies (3). *B. thuringiensis* serovar israelensis was described in 1976 (7) and has been shown to have a worldwide distribution. It is generally found in soil but also has been isolated from insects, plants, and mushroom compost (8, 9). *B. thuringiensis* serovar israelensis-based biocontrol products are used mainly in inundation biological control, where, due to the quickly reduced activity of the crystal toxins (10) and because they are only formed during sporulation, the product needs to be reapplied in order to keep the abundance of larvae below a critical level.

For a long time, investigations of the abundance of Bt in the environment relied on cultivation on selective media (11, 12), subsequently in combination with identification of *B. thuringiensis* serovar israelensis with PCR (13), serotype characterization (14, 15), and/or amplified fragment length polymorphism genotyping (14). Cultivation approaches are comparatively insensitive, laborious, and hampered by the morphological and phenotypic similarities between *B. cereus* and Bt. The *cry* genes, which together with other plasmid-borne genes encode the δ-endotoxins, have been used as markers differentiating between *B. thuringiensis* serovar israelensis and *B. cereus* (16, 17). The genes also have been targets for PCR-based monitoring of *B. thuringiensis* serovar israelensis populations in wetlands (18). However, the plasmid has a high potential for horizontal transfer and can be lost in the environment (19–21) and can be picked up by strains of *B. cereus* (22). Therefore, primers differentiating *B. cereus* and *B. thuringiensis* serovar israelensis on the chromosomal level are needed for

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comparing their populations in various habitats and *B. thuringiensis* serovar israelensis product-treated as well as untreated environments. Furthermore, the *cry* genes often are associated with transposable elements (5). This makes plasmid-borne genes comparatively unstable and less reliable as genetic markers. Hence, development of more robust methods has a strong potential to improve detection and quantification of Bcg bacteria in the environment.

In this study, a cultivation-independent PCR-based method, using new primers targeting the bacterial chromosome, was developed for quantification of *B. thuringiensis* serovar israelensis and Bcg in the environment. Phylogenetic analysis based on published genomes of Bcg strains was used to identify useful chromosomal loci. The developed PCR primers were validated for target specificity as well as for PCR amplification efficiency and potential inhibition using soil DNA extracts.

MATERIALS AND METHODS

Soil samples. Soil samples were collected in May 2013 from five different sites, two forest swamps (FS1, lat 60.296645, long 16.842440; FS2, lat 60.291774, long 16.830497; latitude and longitude coordinates) and three wet meadows (WM1, lat 60.220125, long 16.752357; WM2, lat 60.248941, long 16.784031; WM3, lat 60.249198, long 16.783335), all located in the River Dalälven floodplains in central Sweden. With the exception of WM1, all sites have been subjected to mosquito control with *B. thuringiensis* serovar israelensis since the year 2000 within the Biological Mosquito Control project (23). At each site, five 10-cm-deep soil cores (diameter, 2.5 cm) were taken at evenly distributed positions within 1 m^2 and pooled. Soil samples were transported in cooling boxes to the laboratory, where they were stored at 4°C until further processing and experiments. Samples were homogenized by passing through a 4-mm sieve and their dry weight determined.

Homogenized soil from site WM1 (not treated with *B. thuringiensis* serovar israelensis) was autoclaved, and the absence of Bcg cells was confirmed by dilution to 1:100, heating to 65°C for 40 min, and plating in triplicates on T3 agar (24) supplemented with 25 mg $liter^{-1}$ DelvoCid Instant (DSM, Düsseldorf, Germany) (25). After 24 h at 30°C, plates were checked for the absence of Bcg-like colonies (rugose, ice crystal-like appearance and a diameter of >1 mm).

Bacterial strains. A collection of 68 *Bacillus* strains was established (Table 1). The collection covers representatives of both Bcg species, including different Bt serovars and *B. thuringiensis* serovar israelensis strains isolated from commercial biocontrol products (AM65-52 and DSM 5724), and soil in Sweden (strains 06:11, 06:12, 08:36, 08:37, and 08:38). Strains were grown and maintained on T3 agar (24). Spores of *B. thuringiensis* serovar israelensis strain AM65-52 used in the commercial *B. thuringiensis* serovar israelensis-based product Vectobac-G (Valent Biosciences, Libertyville, FL) were obtained by inoculating liquid T3 medium followed by incubation at 28°C for 5 days (26). The cultures were checked for spores and crystals by phase-contrast microscopy. The density of spores was determined by cultivating 30 μl of 10^5 dilutions on T3 agar after vegetative cells in the spore solution had been eliminated by heating to 75°C for 15 min.

DNA extraction. For DNA preparation from *Bacillus* strains, bacteria were cultivated on Luria-Bertani agar (LB) and incubated at 30°C overnight. Bacterial cells were suspended in Tris-EDTA and lysed by boiling. DNA-containing extracts were separated from cell debris by centrifugation (27) and stored at −20°C. Soil DNA was extracted from 250-mg subsamples using the PowerLyzer PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. A FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA) was used for bead beating the soil samples (6,000 rpm for 45 s). The quality of soil DNA extracts was examined by electrophoresis (1% [wt/vol] agarose gels and ethidium bromide staining were used throughout). The DNA concentrations of extracts were determined with Pico-Green (Invitrogen, Carlsbad, CA) using a Qubit fluorometer (Invitrogen).

Primer design. In order to locate chromosome regions suitable for designing Bcg- and *B. thuringiensis* serovar israelensis-targeting primers, comparative analysis of available annotated Bt genomes and one *B. cereus* genome (*Bacillus cereus* G9842 [NCBI accession number CP001186], Bt HD-771 [CP003752], *B. thuringiensis* serovar israelensis HD-789 [CP003763], and *B. thuringiensis* serovar kurstaki HD-73 [CP004069]) was undertaken using the software Genious 6.0.5 (28). Aligned contigs were visually scanned for regions containing high mutation rates (29), i.e., showing high net nucleotide differences. Within the identified regions, putative primer pairs targeting Bcg (Bcg1_for/rev) and *B. thuringiensis* serovar israelensis (Bti1_for/rev) were designed. In a first check that the putative primers target Bcg-specific (i.e., *B. cereus*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and different Bt serovars) or *B. thuringiensis* serovar israelensis-specific signatures, BLAST sequence homology searches using concatenated primer sequences were performed in the whole-genome shotgun contig (WGS) database of NCBI (30) restricted to *Bacillus* spp. The BLAST sequence homology searches were run with the default settings and the number of hits set to 500 for Bcg- and *B. thuringiensis* serovar israelensis-specific primers (26 February 2015).

Primer specificity tests. Primer specificity was evaluated experimentally by applying two different approaches. In the first, PCR amplification of genomic DNA from the *Bacillus* strain collection was assessed with the primer pairs Bcg1_for/rev and Bti1_for/rev (Tables 1 and 2). As a control, *cry4Aa* and *cry4Ba* genes targeting primers Un4(d)/Cry4R (Cry4) (17) were assessed as well. The reaction volume was 20 μl, containing 1 μl bacterial DNA extract, 1× Fermentas DreamTaq green PCR master mix (Thermo Fisher Scientific, Waltham, MA), and 0.3 μM each primer (Life Technologies, Carlsbad, CA). PCR was performed with a C1000 thermal cycler (Bio-Rad, Hercules, CA) with a 3-min initial activation at 95°C, followed by 35 cycles of 30 s of denaturation at 95°C; 30 s of annealing at 60°C for the Bti1 primer, 65°C for the Bcg1 primer, and 67°C for the Cry4 primer; 1 min of extension at 72°C; and finally an extension step for 10 min at 72°C. PCR products were visualized by electrophoresis.

The quality of *Bacillus* DNA was checked by PCR using 0.5 μM universal bacterial primers SD-arch-0519-aS-15 and SD-Bact-0785-bA-18 (31) by following the PCR conditions described above with the addition of 0.1 mg ml^{-1} bovine serum albumin (BSA) and annealing at 52°C with 32 cycles.

In the second approach, the specificity of primers was checked by determining the identity of PCR products amplified with the Bti1 and Bcg1 primer pairs from soil DNA. The same PCR conditions were used as those described above, and 0.6 mg ml^{-1} BSA (GE Healthcare, Piscataway, NJ) was added. The annealing temperature was lowered to 56 and 63°C for the *B. thuringiensis* serovar israelensis- and Bcg-specific PCR, respectively, and the number of cycles was increased to 40. Amplification products were cloned using the TOPO TA cloning kit and One Shot TOP10 chemically competent *Escherichia coli* cells (Life Technologies) according to the manufacturer's recommendations. Colony PCR with 0.5 μM M13 forward (−20) and M13 reverse primers was performed on 12 randomly selected clones for each soil sample, using the conditions described above but with an annealing temperature of 55°C and 25 cycles. PCR products were checked for quality by electrophoresis, followed by sequencing (Macrogen, Republic of South Korea) using M13 forward (−20) and M13 reverse primers. The clones were divided into sequence homology groups using the software Bioedit, version 7.1.3.0 (32). BLAST sequence similarity searches with representative sequences from each homology group were performed in the WGS database of NCBI (30), restricted to *Bacillus* spp. The default settings were used with the number of hits set to 500 for *B. thuringiensis* serovar israelensis and Bcg primer fragments, and sequences with 100% compliance were retrieved (26 February 2015).

A phylogenetic analysis of the Bcg sequences retrieved from soil was performed on an alignment consisting of one representative sequence

TABLE 1 *Bacillus* strains used in this study[a]

Species	Origin	Bacterial strain[b]	Presence of:		
			Cry4	Bti1	Bcg1
B. cereus group					
B. cereus	Milk	ATCC 14579^T	−	−	+
B. cereus	Unknown	AH 184	−	−	+
B. cereus	Human wound	F 837/76	−	−	+
B. cereus	Milk	ATCC 4342	−	−	+
B. cereus	Blood	ATCC 7064	−	−	+
B. cereus	Powdered milk	ATCC 33018	−	−	+
B. cereus	*Spodoptera frugiperda*	T01 176:C2	−	−	+
B. cereus	Rice	F 3502/73	−	−	+
B. cereus	Soil	ATCC 6464	−	−	+
B. cereus	Cheese spoilage	ATCC 10987	−	−	+
B. mycoides	Soil	DSM 2048^T	−	−	+
B. mycoides	Soil	ATCC 6462^T	−	−	+
B. pseudomycoides	Unknown	DSM 12442	−	−	+
B. pseudomycoides	Soil	CECT 7065^T	−	−	+
B. weihenstephanensis	Pasteurized milk	DSM 11821^T	−	−	+
B. thuringiensis	Soil	06:03	−	−	+
B. thuringiensis	Soil	09:02	−	−	+
B. thuringiensis serovar kenyae	*Corcyra cephalonica*	HD-136	−	−	+
B. thuringiensis serovar alesti	*Bombyx mori*	HD-4	−	−	+
B. thuringiensis serovar aizawai	*Heliothis assulta*	HD-112	−	−	+
B. thuringiensis serovar canadensis	Unknown	HD-224	−	−	+
B. thuringiensis serovar colmeri	Grain dust	HD-847	−	−	+
B. thuringiensis serovar dakota	Unknown	HD-932	−	−	+
B. thuringiensis serovar darmstadiensis	Unknown	HD-146	−	−	+
B. thuringiensis serovar dendrolimus	*Dendrolimus sibericus*	HD-7	−	−	+
B. thuringiensis serovar entomocidus	Unknown	HD-110	−	−	+
B. thuringiensis serovar finitimus	*Malacosoma distria*	HD-3	−	−	+
B. thuringiensis serovar galleriae	*Dendrolimus sibericus*	HD-29	−	−	+
B. thuringiensis serovar indiana	Soya been field	HD-521	−	−	+
B. thuringiensis serovar kuamotoensis	Silkworm litter	HD-867	−	−	+
B. thuringiensis serovar kurstaki	*Pectinophora gossypiella*	HD-1	−	−	+
B. thuringiensis serovar kurstaki	*Ephestia kühniella*	HD-73	−	−	+
B. thuringiensis serovar kyushuensis	*Bombyx mori*	HD-541	−	−	+
B. thuringiensis serovar morrisoni	Unknown	HD-12	−	−	+
B. thuringiensis serovar ostriniae	Unknown	HD-501	−	−	+
B. thuringiensis serovar pakistani	*Cydia pomonella*	HD-395	−	−	+
B. thuringiensis serovar shandogiensis	Unknown	HD-1012	−	−	+
B. thuringiensis serovar sotto	Unknown	HD-770	−	−	+
B. thuringiensis serovar thompsoni	Unknown	HD-542	−	−	+
B. thuringiensis serovar thuringiensis	Unknown	HD-22	−	−	+
B. thuringiensis serovar thuringiensis	*Ephestia kühniella*	HD-2	−	−	+
B. thuringiensis serovar tochigiensis	Silkworm litter	HD-868	−	−	+
B. thuringiensis serovar tohokuensis	Silkworm litter	HD-866	−	−	+
B. thuringiensis serovar tolworthi	Unknown	HD-537	−	−	+
B. thuringiensis serovar toumanoffi	*Galleria mellonella*	HD-201	−	−	+
B. thuringiensis serovar israelensis	Commercial product	DSM 5724^{CM}	+	+	+
B. thuringiensis serovar israelensis	Soil	06:11	+	−	+
B. thuringiensis serovar israelensis	Soil	06:12	+	+	+
B. thuringiensis serovar israelensis	Soil	08:36	+	+	+
B. thuringiensis serovar israelensis	Soil	08:37	+	+	+
B. thuringiensis serovar israelensis	Soil	08:38	+	+	+
B. thuringiensis serovar israelensis	*Culex pipiens*	HD-567 ^{CM}	−	+	+
B. thuringiensis serovar israelensis	Plasmid cured mutant	4Q2-72	+	+	+
B. thuringiensis serovar israelensis	Unknown	Dbt357	−	−	+
B. thuringiensis serovar israelensis	Unknown	HBt1	+	+	+
B. thuringiensis serovar israelensis	Unknown	HBt17	−	−	+
B. thuringiensis serovar israelensis	Soil	Bta2	+	+	+
B. thuringiensis serovar israelensis	Vectobac-G	AM65-52 ^{CM}	+	+	+
B. thuringiensis serovar israelensis	Soil	S128	+	+	+

(Continued on following page)

TABLE 1 (Continued)

Species	Origin	Bacterial strain[b]	Presence of: Cry4	Bti1	Bcg1
B. thuringiensis serovar israelensis	Unknown	LH-1	+	+	+
B. thuringiensis serovar israelensis	Unknown	NB31	+	+	+
Non-*B. cereus* group					
B. amyloliquefaciens	Soil	DSM 7^T	−	−	−
B. circulans	Soil	DSM 11^T	−	−	−
B. licheniformis	Unknown	DSM 1320	−	−	−
B. megaterium	Milk	DSM 32^T	−	−	−
B. polymyxa	Unknown	DSM 36^T	−	−	−
B. sphaericus	Milk	DSM 2898	−	−	−
B. subtilis	Unknown	DSM 10^T	−	−	−

[a] The *Bacillus* strains, including 12 species and 27 subspecies, were tested with the novel PCR-based diagnostics of *B. cereus* group and specifically *B. thuringiensis* serovar israelensis using chromosome-directed primers Bti1 and Bcg1, respectively, as well as the *cry4Aa* and *cry4Ba* genes targeting primers (Cry4) as a control.
[b] A superscript T indicates type strain, and superscript CM indicates a strain used in a commercial product.

from each homology group of clones as well as one representative sequence from the 100% sequence similarity hits. In order to cover the different accession numbers assigned to the 100% sequence similarity hits, one representative for each individual accession number identity at subspecies, species, and genus levels was selected for each homology group. Maximum likelihood as well as neighbor-joining tree analyses were performed based on the Jukes-Cantor model, applying 1,000 bootstrap resamplings and the default settings in the software MEGA 5 (33).

Cloning and purification of plasmid DNA standards. For use as a standard in the quantitative real-time PCR (qPCR), a plasmid was constructed containing a fragment amplified by the Bti1 primers from DNA of *B. thuringiensis* serovar israelensis strain AM65-52 and cloned into a pCR4-TOPO vector (Life Technologies) as described above. Positive clones were grown in liquid LB overnight, and plasmids were harvested using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid DNA was quantified with Pico-Green (Invitrogen, Carlsbad, CA) using a Qubit fluorometer (Invitrogen). The plasmid concentration was adjusted to 10^6 copies μl^{-1}, and 10-fold serial dilutions were prepared.

Quantification efficiency. For checking the efficiency of the qPCR with soil extracts, reactions were performed using 10-fold serial dilutions of plasmid DNA with or without addition of 5 ng of soil DNA (extracted from the autoclaved soil from site WM1).

qPCR was performed in 20-μl reaction volumes containing 10 μl of IQ SYBR green supermix (Bio-Rad), 0.5 μM each *B. thuringiensis* serovar israelensis- and Bcg-specific primer pair, 0.1 mg ml^{-1} BSA (GE Healthcare), and 5 μl template, using a CFX 96 real-time system (Bio-Rad). Cycling conditions were 3 min of initial activation at 95°C, followed by 35 cycles of 15 s of denaturation at 95°C and 1 min of annealing and extension at 60 or 65°C for *B. thuringiensis* serovar israelensis- and Bcg-specific amplification, respectively. Following amplification, a melting curve analysis was performed, ranging from 55°C to 95°C with 0.5°C increments for 5 s. For each template dilution, the reactions were performed in triplicates. The threshold line and the sample specific threshold cycle numbers (C_T) were determined with the default parameters of the qPCR instrument software (Bio-Rad CFX Manager, version 3.1). Efficiency values (E) and correlation coefficients (R^2) were calculated by the software for each standard quantification curve of C_T against the number of input copies.

Potential PCR inhibition. From all five soil samples, 5 μl of soil DNA extracts (containing 0, 0.2, 1, 2, 3, or 4 ng DNA μl^{-1}) was added to qPCR vials and amended with 10^6 copies of the *B. thuringiensis* serovar israelensis-specific qPCR plasmid standard, resulting in a total reaction volume of 20 μl (34). qPCR was performed in triplicates using the instrumentation and reaction composition described above, except for adding 0.3 μM each M13 forward (−20) and M13 reverse primer with an annealing temperature of 61°C. Cycling conditions were set at 5 min of initial activation at 95°C, followed by 35 cycles of 40 s of denaturation at 95°C and 40 s of annealing at 61°C, and finally 30 s of extension at 72°C. Following amplification, a melting curve analysis was performed, ranging from 55°C to 95°C with a 0.5°C increment every 5 s.

Statistical analyses. For statistical evaluation of inhibitory effects of soil DNA extracts on PCR, pairwise *t* testing with Welch approximation to the degrees of freedom was applied, using the "stats" package of the R software (version 2.15.1; R Foundation for Statistical Computing, Vienna, Austria). *P* values were manually Bonferroni corrected due to multiple testing.

C_T values from different dilutions of plasmid-based standard curves for Bcg and *B. thuringiensis* serovar israelensis with and without soil DNA extracts were tested for statistical differences by applying parametrical tests using the function "pairwiset.test" (version 2.15.1; R Foundation for Statistical Computing, Vienna, Austria). The residuals were checked for normal distribution.

Nucleotide sequence accession numbers. The sequences from sequence homology groups (SHG) representing Bcg strains detected in soil samples here have been deposited in GenBank under accession numbers KP863940 to KP863943 for SHG1 Bcg to SHG4 Bcg and KP863944 and

TABLE 2 Nucleotide sequences of the novel chromosome-directed primers[a]

Primer	Sequence (5′–3′)	Oligonucleotide position[b] (5′–3′)	Amplified fragment length (bp)
Bti1_for	CAAACATTTCATTCCAATAACA	3152129–3152150	190
Bti1_rev	ATACTGTGTGGGATGCTTATTA	3152310–3152331	
Bcg1_for	AACAGGCTCCATACAATGGTAT	5274038–5274059	250
Bcg1_rev	TGGTAGCGTTTCTTCGTCTTAT	5274262–5274283	

[a] Novel chromosome-directed primers for diagnostics of the *Bacillus cereus* group, and specifically the insecticidal *Bacillus thuringiensis* serovar israelensis, were used in quantitative PCR.
[b] Positions are given relative to the reference genome *Bacillus thuringiensis* serovar israelensis HD-789, GenBank accession number CP003763.

TABLE 3 Taxonomic identification of the 100% match hits of the concatenated *Bacillus cereus* group and *B. thuringiensis* serovar israelensis-targeting primer sequences to accession numbers in the WGS database of NCBI

Bacterial species[a]	No. of primer sequences targeting:	
	B. cereus group	*B. thuringiensis* serovar israelensis
B. anthracis	58	
B. cereus	139	2
B. mycoides	5	
B. pseudomycoides	1	
B. weihenstephanensis	4	
B. thuringiensis	12	1
B. thuringiensis serovar aizawai	2	
B. thuringiensis serovar andalousinesis	1	
B. thuringiensis serovar berliner	1	
B. thuringiensis serovar huazhongensis	1	
B. thuringiensis serovar israelensis	2	2
B. thuringiensis serovar kurstaki	2	
B. thuringiensis serovar monterrey	1	
B. thuringiensis serovar morrisoni	1	
B. thuringiensis serovar pakistani	1	
B. thuringiensis serovar pondicheriensis	1	
B. thuringiensis serovar pulsiensis	1	
B. thuringiensis serovar sotto	1	
B. thuringiensis serovar thuringiensis	1	
B. thuringiensis serovar tochigiensis	1	
B. thuringiensis serovar tolworthi	1	
B. gaemokensis	1	
B. manliponensis	1	
B. subtilis	1	
Bacillus sp.	13	3
Total no. of hits	253	8

[a] Species were assigned to accession numbers with 100% sequence similarity to concatenated primer sequences according to the nomenclature adopted from NCBI.

KP863945 for SHG1 *B. thuringiensis* serovar israelensis and SHG2 *B. thuringiensis* serovar israelensis, respectively.

RESULTS

Primer design. Based on whole-genome comparisons, two intergenic regions were selected for designing primer pairs targeting the Bcg and *B. thuringiensis* serovar israelensis, respectively (Table 2). The region flanked by the primers Bcg1_for/rev (Table 2) is located across an intergenic region and a gene predicted as a transcriptional regulator (region 5274038 to 5274283 of reference genome *Bacillus thuringiensis* serovar israelensis HD-789; NCBI accession number CP003763) (35). For the primers Bti1_for/rev, the target is located in an intergenic region (positions 3152129 to 3152331) (35).

The BLAST similarity searches with concatenated Bcg-targeting primer sequences on the WGS database of NCBI recovered 253 hits with 100% sequence similarity for the full query length (Table 3) (26 February 2015). Among them, 237 accession numbers were those of target species, i.e., the Bcg members *B. cereus*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and Bt. A further 13 of the hits were identified as *Bacillus* species, while one hit was with each of the nontarget species *B. subtilis* (strain B7-S; AZNI01000038), *B. gaemokensis* (BL3-6; JOTM01000032), and *B. manliponensis* (BL4-6; JOTN01000018). Among the hits with less than 100% sequence similarity, there were accession numbers identified as *Bacillus* species but none as Bcg members independent of the covered query length. When restricting the BLAST search to the nucleotide database of NCBI, among the 90 retrieved 100% match hits, 87 were assigned to the Bcg (26 February 2015) (data not shown). The remaining three were assigned to *B. bombyseptieus*, *B. toyonensis*, and *B. cytotoxicus* (data not shown).

The corresponding BLAST sequence similarity searches with the *B. thuringiensis* serovar israelensis-targeting primers revealed 100% similarity with accession numbers identified as *Bacillus thuringiensis* serovar israelensis ATCC 35646 (AAJM01000297), *Bacillus thuringiensis* serovar israelensis 4Q7 (JEOC01000010), *Bacillus thuringiensis* IBL 4222 (ACNL01000121), and *Bacillus* sp. strains WBUNB009 (ANFK01000002), L_1B0_8 (JXIT01000096), and L_1B0_5 (JXIS01000041) (Table 3) (25 February 2015). Additionally, there was a 100% match with two accession numbers identified as *Bacillus cereus* VD045 (AHET01000010) and AH676 (ACMQ01000156).

Primer specificity. Among the 68 bacterial strains tested with the primer pairs, the Bti1 and Cry4 primers gave amplification products only with *B. thuringiensis* serovar israelensis strains, although four *B. thuringiensis* serovar israelensis strains (Dbt357, HBt17, HD567, and 06:11) gave no PCR product for either one or both *B. thuringiensis* serovar israelensis primer pairs (Table 1). The Bcg1 primers were positive for all Bcg bacteria but for none of the strains of non-Bcg *Bacillus* species (Table 1). The PCR with universal bacterial primers produced amplicons of the expected sizes for all strains of the collection (data not shown), confirming acceptable quantity and quality of DNA extracts for all of the tested strains.

PCR amplification with the *B. thuringiensis* serovar israelensis- and Bcg-targeting primers from soil DNA extracts produced PCR amplicons of the expected sizes (data not shown). PCR fragments were cloned, and 12 clones per soil sample were arbitrarily selected and sequenced. All 48 clones retrieved for *B. thuringiensis* serovar israelensis contained a fragment with the expected length. Sequences formed two sequence homology groups (SHG), SHG1 and SHG2 *B. thuringiensis* serovar israelensis, which varied in one deleted base. Thirty-five identical clones were designated SHG1 *B. thuringiensis* serovar israelensis (KP863944) (Table 4). These clones are identical to five accession numbers in the NCBI database, identified as *Bacillus thuringiensis* IBL 4222 (ACNL01000121), *Bacillus thuringiensis* serovar israelensis 4Q7 (JEOC01000010), *Bacillus thuringiensis* serovar israelensis ATCC 35646 (AAJM01000297), and *Bacillus* sp. strains L_1B0_8 and L_1B05 (JXIT01000096 and JXIS01000041) (Table 5) (26 February 2015). Two clones (coming from two different soil samples) were designated SHG2 *B. thuringiensis* serovar israelensis (KP863945) (Table 4). The remaining 11 clones had single-base-pair differences at individual positions and were not further analyzed. There were no database accession numbers having 100% similarity with SHG2 *B. thuringiensis* serovar israelensis or any of the clone sequences containing single-base-pair differences.

Similar to the *B. thuringiensis* serovar israelensis clones, all 48 retrieved Bcg clones contained a PCR fragment of the expected size. Among these, 46 had similarities between 0.97 and 1 with base pair differences in 17 positions; however, for two clones the sequences were of insufficient quality (data not shown). Thirty-three clones were divided into four SHG (SHG1 Bcg to SHG4 Bcg, KP863940 to KP863943) (Table 4), while 13 clones revealed sin-

TABLE 4 Distribution of *Bacillus* clones retrieved from soil samples[a]

Soil sample	No. of clones of: SHG1 Bcg (KP863940)	SHG2 Bcg (KP863941)	SHG3 Bcg (KP863942)	SHG4 Bcg (KP863943)	SHG1 *B. thuringiensis* serovar israelensis (KP863944)	SHG2 *B. thuringiensis* serovar israelensis (KP863945)
FS1	9	0	0	0	11	1
FS2	2	3	2	1	8	0
WM2	0	0	1	6	9	0
WM3	3	0	2	4	7	1
Total no. of clones	14	3	5	11	35	2

[a] Soil clones were determined using DNA extracts from soil samples from the River Dalälven floodplains, Sweden. The number of clones retrieved from forested swamps (FS) 1 and 2 and wet meadows (WM) 2 and 3 are listed, as well as their distribution on the sequence homology groups (SHG) of *B. cereus* group (Bcg) and *B. thuringiensis* serovar israelensis.

gle-base-pair differences at individual positions and were not analyzed further. The matches in the searches with representatives of each of the *B. cereus* homology groups were dominated by *B. cereus*, with lesser contributions from other Bcg species, 1 *B. subtilis* strain, and 11 unidentified *Bacillus* strains (Table 5) (26 February 2015). Perfect matches to sequences assigned to *B. anthracis* were found only for SHG1 Bcg, while Bt (including *B. thuringiensis* serovar israelensis) accession numbers were found almost exclusively in SHG3 Bcg. The phylogenetic analyses of the alignment containing sequences with 100% similarities to representatives of the different Bcg homology groups separated them into two main clusters with a bootstrap value of 99 (Fig. 1). Cluster 1 contains all sequences of SHG3 Bcg, including representatives of the accession numbers identified as *B. thuringiensis* serovar israelensis. In cluster 2, all sequences identical to the other sequence homology groups clustered together, including the sequences assigned to *B. anthracis*.

Sensitivity of real-time PCR quantification. The standard curve containing *B. thuringiensis* serovar israelensis plasmid dilutions was linear ($R^2 = 0.997$) over seven orders of magnitude with an amplification efficiency of 91.8% using *B. thuringiensis* serovar israelensis-targeting primers. The melting curve analysis confirmed that amplification was specific (data not shown). With addition of soil DNA extract, the slope of the standard curve changed from −3.536 to −3.318, and small but significant differences were found for 5×10^4, 5×10^3, and 50 copies (Table 6). However, the correlation still was linear over seven orders of magnitude ($R^2 = 0.986$), with an amplification efficiency of 100.2%. The detection sensitivity and amplification efficiency for the Bcg-targeting primers was similar to that for the *B. thuringiensis* sero-

TABLE 5 Numbers and taxonomic identification of the 100% match hits with a representative sequence from each of the *Bacillus* sequence homology groups[a]

Bacterial species	No. of PCR hits for representative sequence of: *Bacillus cereus* group SHG1 (KP863940)	SHG2 (KP863941)	SHG3 (KP863942)	SHG4 (KP863943)	*B. thuringiensis* serovar israelensis SHG1 (KP863944)	SHG2 (KP863945)
B. anthracis	58					
B. cereus	70	9	42	5	5	
B. mycoides	1					
B. weihenstephanensis	3		1			
B. thuringiensis			10	1	1	
B. thuringiensis serovar aizawai			2			
B. thuringiensis serovar andalousinensis	1					
B. thuringiensis serovar berliner			1			
B. thuringiensis serovar huazhongensis			1			
B. thuringiensis serovar israelensis			2		2	
B. thuringiensis serovar kurstaki			2			
B. thuringiensis serovar monterrey	1					
B. thuringiensis serovar morrisoni			1			
B. thuringiensis serovar pakistani			1			
B. thuringiensis serovar pondicheriensis	1					
B. thuringiensis serovar pulsiensis	1					
B. thuringiensis serovar thuringiensis			1			
B. thuringiensis serovar tolworthi			1			
B. subtilis			1			
Bacillus spp.	2		7		2	
Total no. of hits	138	9	73	6	10	0

[a] Soil clones were determined using DNA extracts from soil samples from the River Dalälven floodplains, Sweden. The numbers and taxonomic identification of the 100% match hits (BLAST searches in the whole genome shot-gun contigs database of NCBI) with a representative sequence (corresponding NCBI accession number in parentheses) from each of the sequence homology groups are listed. Species were assigned to accession numbers with 100% sequence similarity to SHGs according to the nomenclature adopted from NCBI.

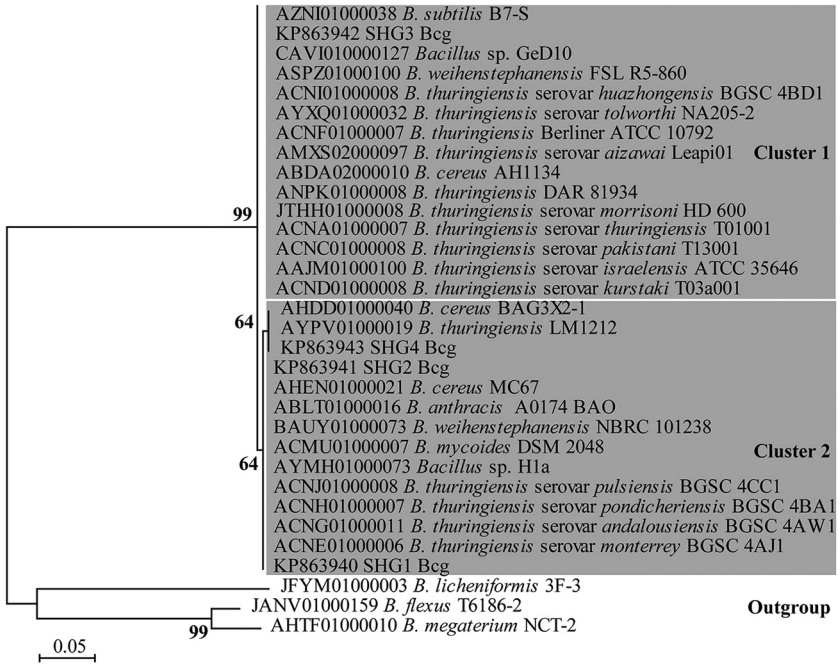

FIG 1 Phylogenetic tree built on NCBI accession numbers with 100% similarity to sequence homology groups formed of soil clones obtained with Bcg1_for/rev primers from targets amplified from soil DNA. Representative sequences from each accession number at subspecies, species, and genus level for each of the sequence homology groups, as well as from different homology groups among clone sequences (SHG1 Bcg to SHG4 Bcg; KP863940 to KP863943), were included. The tree was constructed using maximum-likelihood analysis based on the Jukes-Cantor model of *Bacillus cereus* group 1 (Bcg) sequences from Bcg bacteria with 1,000 iterations of bootstrapping.

var israelensis primers, and slopes were almost parallel for pure plasmid (−3.322) and plasmid plus soil DNA (−3.363) (Table 6).

Potential PCR inhibition was tested with the addition of five different soil DNA extracts in increasing concentrations. The total amount of soil DNA was lowest in the extract from soil WM1 (20 ng μl^{-1}) and highest from soil FS1 (73 ng μl^{-1}). Consequently, the volume of raw soil DNA extract added to the PCR was 3.65 times higher from soil WM1 than from soil FS1. Extracts from soils FS2 and WM3 gave low, though significant, inhibition already with addition of 5 ng of soil DNA (Fig. 2). PCR inhibition increased with 10 ng and was significant for all samples except WM1. When we added more than 10 ng soil DNA, all extracts caused inhibition.

DISCUSSION

Whole-genome comparisons among four strains from Bcg allowed the identification of Bcg- and *B. thuringiensis* serovar israelensis-specific marker sequences located on the bacterial chromosome. *B. cereus* and Bt are genetically closely related, although they differ in some physiological properties and, notably, insecticidal activity (5). So far, the only way to differentiate between *B. cereus* and Bt was based on the presence of a plasmid carrying the genes responsible for the formation of the insecticidal crystals (5). In this study, we present new Bcg- and *B. thuringiensis* serovar israelensis-specific primers targeting markers located on the chromosome. This method provides comparatively stable and reliable PCR-based differentiation, detection, and quantification of the Bcg and *B. thuringiensis* serovar israelensis bacteria and is suitable for environmental studies. Since the markers are located on the chromosome, the drawbacks of using targets located on exchangeable plasmids (19–21) are avoided. This method will be useful, for example, in studies of effects on indigenous Bcg assemblages of application of *B. thuringiensis* serovar israelensis-based insecticides.

Primer design and evaluation of specificity. BLAST similarity searches on NCBI databases for the Bcg primers gave one match to

TABLE 6 Validation of quantitative PCR targeting the Bcg1 and Bti1 regions of the *Bacillus cereus* group and *B. thuringiensis* serovar israelensis, respectively, with cloned DNA of *B. thuringiensis* serovar israelensis AM65-52[a]

Sample type	E [%]	R^2	C_T at copy no. of:						
			5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	50
Bcg1 plasmid	98.3	0.996	11.48 ± 0.25	16.11 ± 0.08	18.97 ± 0.14	22.03 ± 0.07	25.39 ± 0.13	28.93 ± 0.25	32.23 ± 0.78
Bcg1 plasmid plus soil	100.0	0.994	11.88 ± 0.06	16.21 ± 0.07	19.30 ± 0.64	22.03 ± 0.14	25.33 ± 0.21	28.94 ± 0.03	32.39 ± 1.05
Bti1 plasmid	91.8	0.997	12.74 ± 0.16	16.23 ± 0.04	20.69 ± 0.14	24.03 ± 0.05*	27.20 ± 0.17*	30.82 ± 0.51	33.89 ± 0.48*
Bti1 plasmid plus soil	100.2	0.987	12.85 ± 0.19	16.23 ± 0.07	21.73 ± 0.56	23.70 ± 0.17*	27.07± 0.37*	30.24 ± 0.15	32.67 ± 0.52*

[a] Serial 10-fold dilutions (10^7 to 10) of plasmid DNA or plasmid DNA spiked with 5 ng soil DNA were analyzed. The average efficiency value (E) and correlation coefficient (R^2) for the four different dilution series are indicated. C_T values are the averages from three repetitions ± standard deviations. An asterisk indicates significantly ($P < 0.05$) different copy numbers between dilutions of Bti1 plasmid DNA with and without soil extract addition.

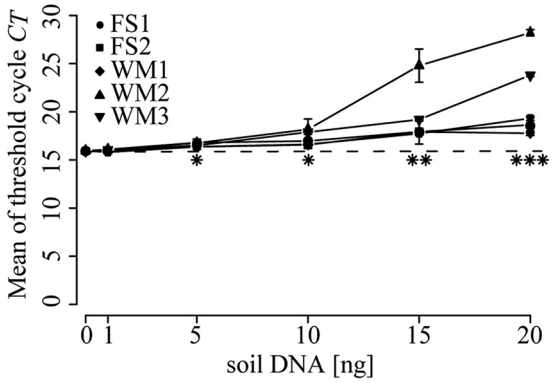

FIG 2 Quantitative real-time PCR analyses for determining potential PCR inhibition by soil DNA extracts. Extracts corresponding to 1, 5, 10, 15, or 20 ng of soil DNA were added to PCR mixtures containing 10^6 copies of the recombinant plasmid pCR4-Topo (Invitrogen). The dashed line indicates the mean C_T of the control without addition of soil DNA. C_T values are the averages from three replicates, and error bars represent standard deviations. Asterisks indicate significant deviations from the control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

an accession number identified as a non-Bcg strain, *B. subtilis* B7-S (Fig. 1 and Table 3). However, several lines of evidence suggest that B7-S is not correctly identified. To begin with, the size and G+C content of the *B. subtilis* B7-S genome are 5.3 Mb and 35.1%, respectively (36), while for other *B. subtilis* strains deposited in NCBI, genome sizes varied between 4.01 and 4.22 Mb (except for BEST7613, with 7.59 Mb), and G+C content ranged from 43.5 to 45.7%. The *B. cereus* genomes, on the other hand, are very similar to strain B7-S, with sizes ranging from 5.29 to 5.84 Mb and G+C contents from 35.2 to 35.5%. *In silico* DNA-DNA hybridization of *B. cereus* type strain ATCC 14579 (AZNI01000038) with *B. subtilis* B7-S indicates that B7-S is actually a *B. cereus* strain (http://ggdc.dsmz.de) (data not shown). Moreover, no PCR product was obtained from the type strain *B. subtilis* DSM 10 (Table 1). Together, these findings indicate that *B. subtilis* B7-S represents a misclassified entry in the databases. Perfect hits also were found in NCBI databases with *B. toyonensis*, *B. cytotoxicus*, *B. gaemokensis*, and *B. manliponensis* (37–40). Although these novel *Bacillus* species have been proposed to belong to the Bcg, valid species descriptions still are missing. Hence, these strains were not included in our collection for primer specificity tests. Additionally, *B. bombyseptieus* also was a perfect hit for the Bcg primers. This species has been reported to form insecticidal crystals during sporulation, and the released toxins induce host responses when entering the larval midgut of a silkworm (41). The high similarity of *B. bombyseptieus* to *B. thuringiensis* serovar *chinensis* CT-43 (>96.46%) (42) supports its affiliation with the Bcg.

Other primers and probes targeting the chromosome have been designed for specific detection and quantification of the Bcg as well as differentiation among Bcg species (43, 44). However, our BLAST similarity searches on WGS databases in NCBI revealed that these Bcg primers missed a certain proportion of Bcg strains whose genome sequences have been published recently (22 April 2015) (data not shown). Thus, the specificity tests performed in this study indicate that our primers are the first that pick up the total Bcg as it is currently known using a single primer pair.

The target fragment for the *B. thuringiensis* serovar israelensis primers matched accession numbers from WGS databases in NCBI assigned to the nontarget *B. cereus* strains AH676 and VD045. For strain VD045, potential plasmid transfer through conjugation has been reported, and the identification at the species level has been questioned (45). Thus, at least VD045 may actually represent a *B. cereus* strain which is able to take up the plasmid carrying the genes responsible for insect toxicity. In addition, 100% matches were found with accession numbers identified only at the species or genus level, such as Bt IBL 4222 and *Bacillus* sp. strains WBUNB009, L_1B0_8, and L_1B0_5. Interestingly, for three of these strains (IBL 4222, L_1B0_8, and L_1B0_5), contigs were found in the database having annotated genes encoding Cry4Aa and Cry4Ba, which is specific for *B. thuringiensis* serovar israelensis. This finding indicates the affiliation of these strains is with serovar israelensis.

Two of the *B. thuringiensis* serovar israelensis strains in the collection were not amplified with the *B. thuringiensis* serovar israelensis-targeting primers. However, these two strains were not amplified when using *B. thuringiensis* serovar israelensis primers targeting the *B. thuringiensis* serovar israelensis-specific *cry4A*a and *cry4Ba* genes (17) located on the plasmid either (Table 1), implying that their affiliation with serovar israelensis is uncertain. Thus, this result does not really put the *B. thuringiensis* serovar israelensis specificity of the new primers into question.

In PCR amplifications using the soil DNA extracts, the Bcg and *B. thuringiensis* serovar israelensis primers generated amplicons of the expected sizes and sequences. The unequivocal phylogenetic placement of the Bcg1 soil clones into two different clusters containing exclusively Bcg members supports the specificity of the new PCR-based detection tool (Fig. 1). The similarity searches with a group-representative sequence revealed 11 times more perfect matches for SHG3 Bcg than for SHG4 Bcg (Table 5), indicating a stronger representation of SHG3 Bcg in the database. Most clones in SHG1 Bcg were recovered from forested swamps, while most in SHG4 Bcg originated in wet meadows. This indicates that the abundances of the groups is associated with a particular habitat type. Both the Bcg and *B. thuringiensis* serovar israelensis primers generated sequences with single-nucleotide mutations at individual positions and no 100% match to any sequence in the database. It is likely that this finding can partly be explained by random sequencing errors, which cannot be avoided and need to be taken into account.

Extraction, PCR efficiencies, and potential PCR inhibition. The moderate but significant effects of soil extract addition on the plasmid standard curves confirm that coextracted compounds such as humic acids can inhibit PCR amplification (34). This effect also was evident when mixing a fixed amount of plasmid with different amounts of soil DNA extracts (Fig. 2). Potential inhibition varied among the soils, but it also may depend on the DNA extraction protocol used (34). Generally, for soil extracts containing low target concentrations, it is often an advantage to increase the amount of DNA in order to increase the target concentration and lower the detection limit. In the present study, however, if we increase the amount of extract, the risk for PCR inhibition due to coextracted substances also will increase.

Efficient DNA recovery from bacterial material such as spores in soil also is essential for reliable PCR-based quantification. In one study, the recovery of *B. cereus* DNA from soil spiked with *B. cereus* spores varied between 0 and 35%, depending on the number of spores added to the soil, soil type, and DNA extraction kit

used (46). Guidi et al. (17), on the other hand, reported 36 and 46% total DNA recovery from added *B. thuringiensis* serovar israelensis spores. Thus, DNA extraction efficiency is highly dependent on several factors, and additional tests of recovery with appropriate soil types and spore densities are required before using the new primers for quantifying the abundance of the Bcg and *B. thuringiensis* serovar israelensis in the field.

In conclusion, the tests of specificity, sensitivity, as well as DNA recovery of this study reveal that the new cultivation-independent method gives specific and efficient determination of the abundances of Bcg and *B. thuringiensis* serovar israelensis in soil samples. This method will be useful in studies of the biogeography and ecology of the Bcg bacteria in general and specifically for assessing effects on resident Bcg and *B. thuringiensis* serovar israelensis populations in localities treated with *B. thuringiensis* serovar israelensis-based biological control products.

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