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ORIGINAL ARTICLE

Molecular identification of *Bacillus thuringiensis* var. *israelensis* to trace its fate after application as a biological insecticide in wetland ecosystems

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Bacillus thuringiensis var. *israelensis*, biopesticide, PCR, ribotyping, Vectobac-G®, wetland reserve.

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Abstract

Aims: To determine the fate of viable *Bacillus thuringiensis* var. *israelensis* (Bti) spores dispersed in the environment, using a universally applicable molecular detection methodology.

Methods and Results: Soil samples were spread on growth medium, after a temperature selection of the spores. A PCR amplification of the *cry4Aa* and *cry4Ba* insecticidal genes was applied on the colonies. Ribotyping was performed subsequently. This combined molecular method proved to be very specific for Bti, which was easily differentiated from the other *B. thuringiensis* serovars. A site regularly treated with Vectobac-G® was chosen within the 'Bolle di Magadino' natural reserve, and monitored throughout 1 year for the detection of Bti spores. The results showed that the numbers were relatively high after insecticidal applications (1.4×10^5 CFU g^{-1}), and decreased approx. 10-fold after 220 days. A successive treatment induced a new increase.

Conclusions: The results show that yearly repeated use of Vectobac-G® does not seem to have a major ecological impact on the 'Bolle di Magadino' natural reserve. Bti spores followed a trend leading to their eventual disappearance from the ecosystem, despite the seasonal application of this biological insecticide for more than a decade.

Significance and Impact of the Study: The molecular identification of Bti cells through the PCR analysis of the delta-endotoxins genes coupled to ribotyping, is an innovative method, that has enabled the identification of this organism into wetland environments.

Introduction

Bacillus thuringiensis var. *israelensis* (Bti) (Goldberg and Margalit 1977) is a Gram-positive, spore-forming bacterium, used worldwide as a biopesticide against mosquito and black fly larvae, belonging to families Culicidae and Simuliidae, respectively. During sporulation Bti produces insecticidal proteins, which are deposited within the sporangium as crystalline aggregates (Crickmore *et al.* 1998; Helgason *et al.* 1998). They are composed of different delta-endotoxins (encoded by the genes *cry4Aa*, *cry4Ba*, *cry10Aa* and *cry11Aa*), and haemolytic factors (encoded by *cyt1Aa* and *cyt2Ba*). These genes are located on a plasmid of 127.9 kb, present in few copies per cell (Berry *et al.* 2002). The crystalline aggregates are dissolved in the mid-gut of mosquito larvae. The polypeptides are activated by proteases followed by receptor-mediated formation of pores at the gut epithelial membrane. The loss of controlled permeability leads to the death of the larvae (Schnepf *et al.* 1998).

Bacillus thuringiensis (Bt) belongs to the *Bacillus cereus* (Bc) group, along with the species *B. cereus* (*sensu stricto*),

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Bacillus mycoides, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus anthracis* (Lechner *et al.* 1998). *Bacillus thuringiensis*, *B. cereus* and *B. anthracis* exhibit pathogenic properties, and genomic data show a high degree of similarity (Rasko *et al.* 2005). However, the pathogenic properties and the target organisms differ greatly. *Bacillus thuringiensis* (Bt) is primarily an insect pathogen. Some *B. cereus* strains can cause intestinal infections in humans. *Bacillus anthracis*, which can be distinguished from the other two by microbiological and biochemical tests, is the causative agent of the potentially lethal disease anthrax.

Within a mosquito control programme in a natural wetland reserve called 'Bolle di Magadino', Bti has been used on a regular basis since 1987 to control the flood water mosquito *Aedes vexans*. This natural reserve, 600 ha in size, is located in southern Switzerland, and is surrounded by residential areas. The larval breeding sites are treated with the Bti preparation, Vectobac-G® (Valent BioSciences, Libertyville, IL, USA) by helicopter. The long-term regular treatments following flooding of the larval habitats constitute an ideal situation for testing molecular methods to monitor the fate of Bti in soil (Chappuis 2002).

To our knowledge, former investigations to trace Bt in the field were carried with selective growth media, followed by colony counts or H-flagellar serotyping, using noncommercial antibodies (Eskils and Lövgren 1997; Hendriksen and Hansen 2002; Hajaij *et al.* 2005). The aim of the present study was to develop a standardized and universally applicable molecular method for the detection of Bti, which could be directly applied to colonies grown on agar medium.

Materials and methods

DNA extraction and PCR of *cry4Aa* and *cry4Ba* genes

Bacillus strains (Table 1) were grown on nutrient Columbia Agar Base (Oxoid) supplemented with 5% sheep erythrocytes, for 16 h at 30°C. Colonies were taken with a 10-μl loop, resuspended in 200 μl of distilled water, and incubated for 10 min at 100°C to disrupt the cells. After centrifugation for 10 min at 15 000 ***g*** and 4°C, the supernatant was used for PCR. The amplification was performed using the primers Un4(d) (5′-GCATATGATGTAGCGAAACAAG CC-3′) and Un4(r) (5′-GCGTGACATACCCATTTCCAG GTCC-3′), producing a unique, identical fragment of 439 bp from both the *cry4Aa* and *cry4Ba* genes (Ben-Dov *et al.* 1997). PCR conditions were as described by Ben-Dov *et al.* (1997). The presence of the expected fragment was checked on a 0.8% agarose gel.

Ribotyping and analysis of patterns

Genomic DNA was prepared according to the method by Ausubel *et al.* (1989). Ribotyping was carried out with 37 of the *Bacillus* strains (Table 1). After evaluation of several restriction enzymes, alone or in combination (*Asn*I, *Pvu*II, *Ban*I + *Hae*II, and *Asn*I + *Pvu*II), *Pvu*II was chosen for this study.

Ribotyping was performed as described by Grimont and Grimont (1986), with some modifications. After complete digestion of RNA, genomic DNA samples were digested with restriction endonuclease *Pvu*II (Roche, Basel, Switzerland), following the recommendations of the manufacturer. The DNA digest was separated overnight by electrophoresis on a 0.8% agarose gel. The fragments were transferred to a positively charged nylon membrane by overnight Southern blotting, and then fixed for 15 min at 80°C. The blotted genomic digests were probed with peroxidase-labelled pKK3535 plasmid, which is a pBR322-derivate plasmid carrying the *rrnB* ribosomal RNA operon of *Escherichia coli* (Brosius *et al.* 1981). Prehybridization and overnight hybridization were carried at 42°C. Washing conditions and detection of fragments were performed according to the manufacturer (ECL Direct Nucleic Acid Labelling and Detection System, Amersham, Buckinghamshire, UK).

Degrees of similarity between ribotyping profiles were calculated and cluster analysis (UPGMA) was performed using a Jaccard coefficient (GelCompar™, Version 4.1, Applied Math software, Austin, TX, USA).

Soil sampling

A site regularly treated with Vectobac-G®, was selected in the centre of the natural wetland reserve. Composite soil samples were obtained by mixing five aliquots (at 2 cm depth) from 1 m^2. A total of five composite samples were collected at this site between August 2000 and June 2001, and stored at −20°C until processed. During this period the natural reserve had to be treated three times with Bti, at a concentration of 13 kg ha^{-1} of Vectobac-G®. The applications were carried out on 25 April, 3 May 2000 and 13 June 2001. The first series of soil sample was taken 120 and 112 days following the treatments. The last sample was collected 8 days after the third Bti application. One composite control soil sample was taken from a wetland site with a similar ecosystem, situated approx. 30 km east from the 'Bolle di Magadino' natural reserve, and never treated with Bti.

Bacteria belonging to the genus *Bacillus* were isolated from soil samples following the method of Ohba and Aizawa (1978), with some modifications. Briefly, 0.5 g of soil was added to 5 ml of 0·9% NaCl, incubated for

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Table 1 *Bacillus* strains used for the PCR and ribotyping tests

Species	No.*	Strain	Origin	*cry4*†	Ribotyping group‡
B. amyloliquefaciens	Bam9	ATCC 23350^T	Soil	–	ND
B. brevis	Bbr41	ATCC 8246^T§		–	ND
B. cereus	Bce1	ATCC 10876a	Contaminated bottle	–	III
B. cereus	Bce10	ATCC 7004	Pasteurized milk	–	ND
B. cereus	Bce11	LMG 8950	Soil	–	ND
B. cereus	Bce12	LMG 9679	Soil	–	ND
B. cereus	Bce27	TBC016§		–	III
B. cereus	Bce28	GP7§		–	VI
B. cereus	Bce29	ATCC 11778§		–	III
B. cereus	Bce30	GP11§		–	I
B. cereus	Bce43	TGA1§		–	III
B. cereus	Bce45	ATCC 14579^T		–	VI
B. circulans	Bci13	ATCC 11033	Soil	–	VII
B. circulans	Bci14	ATCC 24^T	Soil	–	VI
B. circulans	Bci35	ATCC 24^T§	Soil	–	VI
B. licheniformis	Bli36	ATCC 14580^T§		–	ND
B. megaterium	Bme15	ATCC 14581^T		–	II
B. megaterium	Bme16	LMG 12253	Soil	–	II
B. megaterium	Bme42	DSM 90§	Soil	–	II
B. mycoides	Bmy17	ATCC 6462^T	Soil	–	V
B. mycoides	Bmy18	LMG 9680	Soil	–	V
B. mycoides	Bmy19	LMG 12256	Soil	–	V
B. mycoides	Bmy44	LMG 12410	Soil	–	V
B. polymyxa	Bpo37	ATCC 842^T§		–	ND
B. polymyxa	Bpo38	ATCC 842^T§		–	ND
B. sphaericus	Bsp20	ATCC 14577^T		–	IV
B. sphaericus	Bsp39	ATCC 14577^T§		–	IV
B. sphaericus	Bsp40	DSM 396§	Air	–	VII
B. subtilis	Bsu21	ATCC 6051^T		–	I
B. subtilis	Bsu22	ATCC 7003	Soil	–	I
B. subtilis var. *niger*	Bsu34	ATCC 9372§		–	I
B. thuringiensis	Bt2	Laboratory strain		–	III
B. thuringiensis	Bt32	NRRL B4039§		–	III
B. thuringiensis var. *aizawai*	Bta6	DSM 6099		–	III
B. thuringiensis Berliner	Btb3	ATCC 10792^T		–	III
B. thuringiensis Berliner	Btb31	ATCC 10792^T§		–	III
B. thuringiensis H3a	Bt25	LMG 12268		–	III
B. thuringiensis H4ab	Bt24	LMG 12266		–	III
B. thuringiensis H5ab	Bt23	LMG 12265		–	VI
B. thuringiensis H9	Bt26	LMG 12269		–	III
B. thuringiensis var. *israelensis*	Bti4	DSM 5724	Commercial product	+	III
B. thuringiensis var. *israelensis*	Bti33	DSM 5724§	Commercial product	+	III
B. thuringiensis var. *israelensis*	Vec8	Vectobac-G®	Commercial product	+	III
B. thuringiensis var. *kurstaki*	Btk5	DSM 5725	Commercial product	–	III
B. thuringiensis var. *morrisoni*	Btm52	DSM 6113		–	ND
B. thuringiensis var. *morrisoni*	Btm7	DSM 6112		–	III

T, reference strain; ND, not determined.

*Numeration of the strains.

†Presence (+) or absence (–) of the specific amplicon of *cry4Aa* and *cry4Ba* genes.

‡According to dendrogram of Fig. 1.

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

10 min at room temperature, and suspended with a Vortex mixer several times. The suspension was incubated during 15 min at 80°C, in order to inactivate vegetative cells. Serial dilutions of the suspensions were plated on Columbia Agar Base (Oxoid) supplemented with 5% sheep erythrocytes, and incubated for 48 h at 30°C.

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The colony morphology of *B. thuringiensis* and *B. cereus* cannot be distinguished. Therefore colonies showing the typical morphology of *B. cereus–B. thuringiensis* (i.e. minimum 0.5 cm of diameter; white or grey colour; circular or irregular colonies; granular, milky or mat aspect, haemolytic on blood agar) were counted and designated as Bc/Bt morphology group. To identify Bti isolates, colonies were submitted to the molecular identification. The number of spores was expressed as CFU g^{-1} of dry soil.

Results

Molecular identification of *B. thuringiensis* var. *israelensis*

The names and designations of the 46 *Bacillus* strains which were analysed are listed in Table 1. The *cry4* genes were only detected in the three Bti strains, namely Bti4 and Bti33, as well as Vec8, the commercial strain for the production of Vectobac-G®.

Ribotyping, which was carried out with 37 strains (Table 1), allowed the construction of a dendrogram that depicted the similarity degree among the ribopatterns. Twenty-nine different patterns were recognized and clustered in seven groups (Fig. 1). All of the Bt strains, with the exception of *B. thuringiensis* H5ab, could be grouped in cluster III, thus confirming their high degree of relatedness. The three Bti strains Bti33, Bti4 and Vec8 presented identical patterns that differed from the other Bt strains and from all the other spore formers included in the investigation.

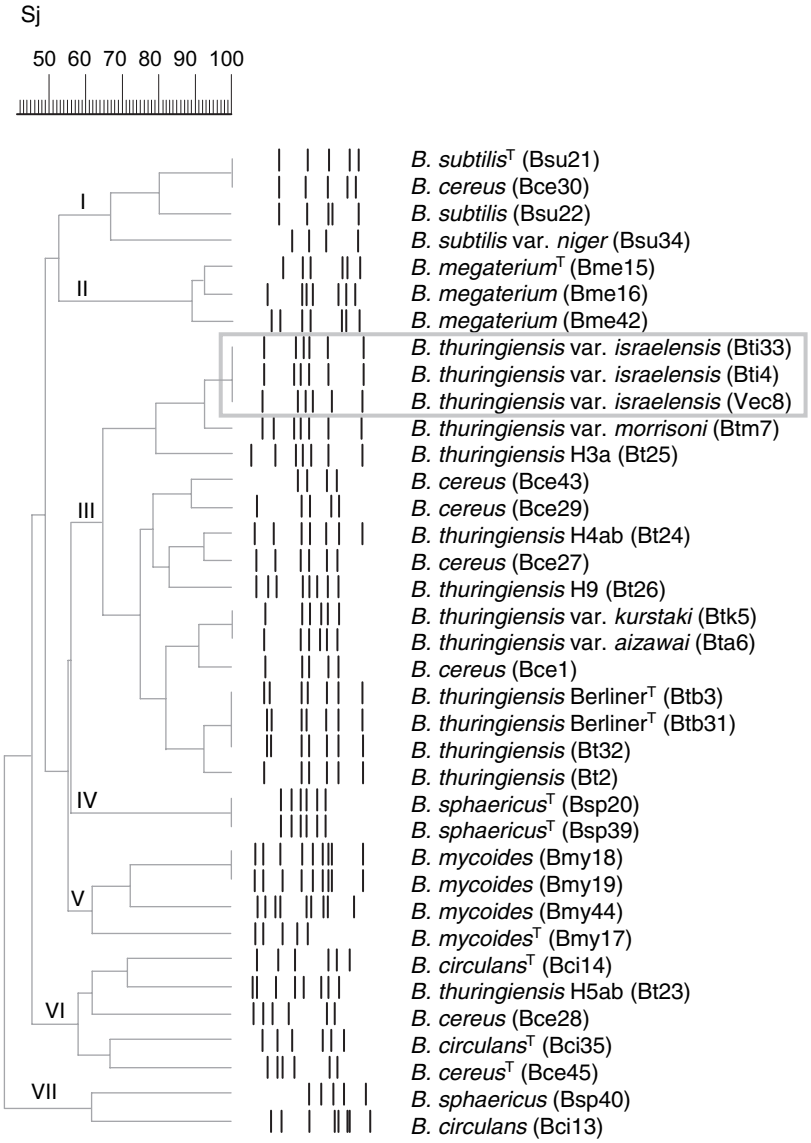

Figure 1 Clustering analysis (UPGMA clustering using a Jaccard coefficient, GelCompar™) of derived ribotyping patterns of 37 *Bacillus* spp. strains. Total DNA was digested using endonuclease *Pvu*II.

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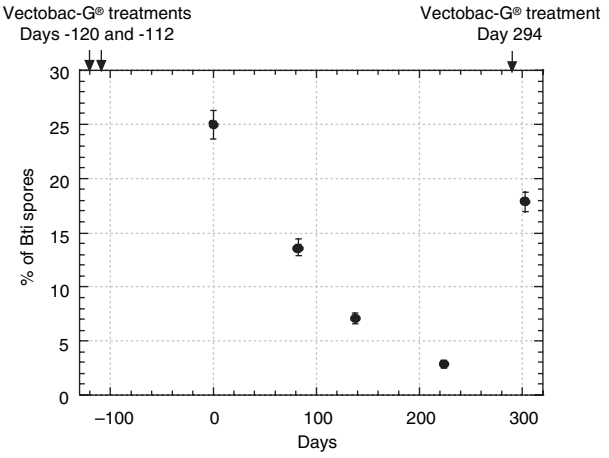

Figure 2 Counts in soil samples of viable spores of *Bacillus thuringiensis* var. *israelensis*, expressed as percentage of total spores from the *B. cereus/B. thuringiensis* morphology group (mean ± 5% errors), at the chosen site of 'Bolle di Magadino' natural reserve. Arrows indicate Vectobac-G® treatments.

Identification of *B. thuringiensis* var. *israelensis* spores in field samples

In the site regularly treated with Vectobac-G®, the number of colonies from viable spores assigned phenotypically to the Bc/Bt morphology group ranged from 5.7×10^5 to 9.4×10^5 CFU g^{-1}. The detection limit was 10^3 CFU g^{-1}.

After growth, colonies were tested by PCR and ribotyping to determine the proportion of Bti spores. A total of 121 colonies with the Bc/Bt morphology group were tested. The presence of the *cry4* genes and the pattern obtained by ribotyping allowed a clear identification of Bti strains. The concentration of Bti spores, identified by PCR and ribotyping ranged from 0.2×10^5 to 1.4×10^5 CFU g^{-1}. Thus, the number of spores from the Bc/Bt morphology group was 7–28 times higher than those of Bti.

Figure 2 shows the percentage of Bti spores based on the formed colonies identified as members of the Bc/Bt morphology group. In-between the Vectobac-G® treatments, the number of Bti spores decreased steadily. Following the treatment at day 294, the number of Bti spores showed the expected increase from 3% to 18% in comparison with the Bc/Bt morphology group.

In the composite soil sample taken from a nontreated wetland control site, no Bti spores could be detected among the 1.8×10^3 CFU g^{-1} colonies of Bc/Bt morphology group.

Discussion

Strains of Bt containing crystal protein genes can be classified using the multiplex PCR method described by Ben-Dov *et al.* (1997). Using the primers for *cry4Aa* and *cry4Ba* genes, it was possible to detect the 439 bp fragment present in the Bti strains. Ribotyping as complementary method allowed for a more stringent discrimination between *Bacillus* spp., to exclude false-negative (Bti cells which have lost the plasmid), and false-positive strains (*Bacillus* spp. which would have incorporated the plasmid).

Ribotyping allows the analysis of DNA polymorphisms in the chromosomal regions containing the highly conserved rRNA genes (*rrn*) (Grimont and Grimont 1986). This method enables classification at the species, subspecies, and even strain level for many bacterial genera (Gillis *et al.* 2001), including the genus *Bacillus* (Priest *et al.* 1994; Joung and Côté 2001, 2002).

The grouping of the 37 *Bacillus* spp. strains into 29 different profiles indicates that this method is suitable for fingerprinting isolates of the genus *Bacillus*. The majority of the Bt strains, including Bti, could be grouped within Cluster III, thus confirming their high degree of relatedness. As Fig. 1 shows, the Bti strains could be easily discriminated within this cluster from the other Bt strains. These results of ribotyping are in agreement with Joung and Côté (2001, 2002), who showed a 92.5% homology within 86 Bt strains.

The molecular identification of Bti was successfully applied to soil samples taken from a site in the 'Bolle di Magadino' natural reserve which had been regularly treated with Vectobac-G® for the control of mosquito larvae. As expected, the number of deliberately released Bti spores decreased gradually. An approx. 10-fold decrease was calculated within 220 days. Thus an accumulation of Bti spores due to two to three treatments per year can be excluded. Bt seems to persist in soils as spores and many studies demonstrated that they do, as a rule, not germinate and proliferate in the soil environment (West *et al.* 1985; Akiba 1986; Addison 1993). As far as spores of Bt var. *kurstaki* are concerned, Hendriksen and Hansen (2002) could detect germination only in the rhizosphere and in the gut of invertebrates, thus suggesting that survival of Bt could be a dynamic process involving germination, cell division, and sporulation in specific microhabitats other than soil. Therefore, our investigation was focused on the identification of viable Bti spores only, whereas vegetative cells were eliminated by a heat treatment, which is commonly used for spore selection (Akiba 1986; Smith and Couche 1991; Eskils and Lövgren 1997).

Bacillus thuringiensis is considered to be an ubiquitous micro-organism, although it plays a minor role among bacterial populations in the environment (Bravo *et al.* 1998; Iriarte *et al.* 2000; Porcar and Caballero 2000). The presence of strains of the Bc/Bt morphology group

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depends on the physico-chemical state of the soil. These bacteria require an environment enriched with organic material. This might be the cause why in our control site, very few viable spores of the Bc/Bt morphology group (1.8×10^3 CFU g^{-1}) were detected. Bti spores were below the detection limit. This result is in accordance with the study of Hajaij *et al.* (2005), which did not detect any Bti spores in never treated areas in France. Therefore, most, if not all, of the Bti spores recovered in the soil samples of the treated area 'Bolle di Magadino' might originate from the Vectobac-G® treatments.

After 220 days, the Bti spores decreased to 3.5% of total Bc/Bt morphology group. This is in agreement with other studies showing that an initial decrease of the number of Bt spores was followed by a stabilization (Pedersen *et al.* 1995; Eskils and Lövgren 1997). One important fact is that many studies showed that Bti spores failed to germinate in natural soils (West *et al.* 1985; Akiba 1986). Moreover, Pruett *et al.* (1980) observed a gradual mortality of Bt spores in soil samples, mortality which could be due to UV radiations, as well as predation or competition with autochthonous micro-organisms (Addison 1993). In consideration of the present knowledge, although the Bti spore number did not return to concentrations below the detection limit, our results show that the yearly repeated use of the Vectobac-G® insecticide seems not to have a major ecological impact on this ecosystem.

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References

Addison, J.A. (1993) Persistence and nontarget effects of *Bacillus thuringiensis* in soil: a review. *Can J For Res* **23**, 2329–2342.

Akiba, Y. (1986) Microbial ecology of *Bacillus thuringiensis* VI. Germination of *Bacillus thuringiensis* spores in the soil. *Appl Entomol Zool* **21**, 76–80.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) *Current Protocols in Molecular Biology*. New York: Greene Publishing Associated & Wiley Interscience.

Ben-Dov, E., Zaritsky, A., Dahan, E., Barak, Z., Sinai, R., Manasherob, R., Khamraev, A., Troitskaya, E. *et al.* (1997) Extended screening by PCR for seven *cry*-group genes from field collected. *Appl Environ Microbiol* **63**, 4883–4890.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, M.T.G., Harris, D. *et al.* (2002) Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **68**, 5082–5095.

Bravo, A. Sarabia, S., Lopez, L., Ontiveros, H. Abarca, C., Ortiz, A., Ortiz, M., Lina, L. *et al.* (1998) Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* **64**, 4782–4788.

Brosius, J., Ullrich, A., Raker, M.A., Gray, A., Dull T.J., Gutell, R.R. and Noller, H.F. (1981) Construction and fine mapping of recombinant plasmids containing the *rrn*B ribosomal operon of *E. coli*. *Plasmid* **6**, 112–118.

Chappuis, S. (2002) Approche moléculaire de l'impact de *Bacillus thuringiensis israelensis* en tant que biopesticide. *PhD Thesis* (No. 3377) , University of Geneva, Switzerland.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean D.H. (1998) Revision of the nomenclature of the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**, 807–813.

Eskils, K. and Lövgren, A. (1997) Release of *Bacillus thuringiensis* subsp. *israelensis* in Swedish soil. *FEMS Microbiol Ecol* **23**, 229–237.

Gillis, M., Vandamme, P., DeVos, P. and Kersters, K. (2001) Polyphasic taxonomy. In *Bergey's Manual of Systematic Bacteriology* ed. Boone, D.R., Castenholz, R.W. and Garrity, G.M. pp. 43–48. New York: Springer.

Goldberg, L.J. and Margalit, J. (1977) A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. *Mosquito News* **37**, 355–358.

Grimont, F. and Grimont P.A.D. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur/Microbiol* **137**, 165–175.

Hajaij M., Carron A., Deleuze J., Gaven B., Setier-Rio M.L., Vigo G., Thiéry I., Nielsen-LeRoux C. *et al.* (2005) Low persistence of *Bacillus thuringiensis* serovar *israelensis* spores in four mosquito biotopes of a salt marsh in southern France. *Microb Ecol* **50**, 475–487.

Helgason, E., Caugant, D.A., Lecadet, M.M., Chen, Y., Mahillon, J., Lövgren, A., Hegna, I., Kvaloy, K. *et al.* (1998) Gene diversity of *Bacillus cereus/B. thuringiensis* isolates from natural source. *Curr Microbiol* **37**, 80–87.

Hendriksen, N.B. and Hansen, B.M. (2002) Long-term survival and germination of *Bacillus thuringiensis* var. *kurstaki* in a field trial. *Can J Microbiol* **48**, 256–261.

Iriarte, J., Porcar, M., Lecadet, M.M. and Caballero, P. (2000) Isolation and characterization of *Bacillus thuringiensis* strains from aquatic environments in Spain. *Curr Microbiol* **40**, 402–408.

Joung, K.B. and Côté, J.C. (2001) Phylogenetic analysis of *Bacillus thuringiensis* serovars based on 16S rRNA gene restriction fragment length polymorphisms. *J Appl Microbiol* **90**, 115–122.

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Joung, K.B. and Côté, J.C. (2002) A single phylogenetic analysis of *Bacillus thuringiensis* strains and bacilli species inferred from 16S rRNA gene restriction fragment length polymorphism is congruent with two independent phylogenetic analyses. *J Appl Microbiol* **93**, 1075–1082.

Lechner, S., Mayr, R., Francis, K.P., Prüss, B.M., Kaplan, T., Wiessner-Gunkel, E., Stewart, G.S.A.B. and Scherer, S. (1998) *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *B. cereus* group. *Int J Syst Bacteriol* **48**, 1373–1382.

Ohba, M. and Aizawa, K. (1978) Serological identification of *Bacillus thuringiensis* and related bacteria isolated in Japan. *J Invertebr Pathol* **32**, 303–309.

Pedersen, J.C., Damgaard, P.H., Eilenberg, J. and Hansen, B.M. (1995) Dispersal of *Bacillus thuringiensis* var. *kurstaki* in an experimental cabbage field. *Can J Microbiol* **41**, 118–125.

Porcar, M. and Caballero, P. (2000) Molecular and insecticidal characterization of a *Bacillus thuringiensis* strain isolated during a natural epizootic. *J Appl Microbiol* **89**, 309–316.

Priest, F.G., Kaji, D.A., Rosato, Y.B. and Canhos, V.P. (1994) Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. *Microbiology* **140**, 1015–1022.

Pruett, C.J.H., Burges, H.D. and Wyborn, C.H. (1980) Effect of exposure to soil on potency and spore viability of *B. thuringiensis*. *J Invertebr Pathol* **35**, 168–174.

Rasko, D.A., Altherr, M.R., Han, C.S. and Ravel, J. (2005) Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* **29**, 303–329.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**, 775–806.

Smith, R.A. and Couche, G.A. (1991) The phylloplane as a source of *Bacillus thuringiensis* variants. *Appl Environ Microbiol* **57**, 311–315.

West, A.W., Burges, H.D., Dixon, T.J. and Wyborn, C.H. (1985) Effect of incubation in non-sterilised and autoclaved arable soil on survival of *Bacillus thuringiensis* and *Bacillus cereus* spore inocula. *N Z J Agric Res* **28**, 559–566.

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