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Physiological and molecular detection of crystalliferous *Bacillus thuringiensis* strains from habitats in the South Central United States

AO Ejiofor and T Johnson

Department of Biological Sciences, P.O. Box 9536, Tennessee State University, 3500 John Merritt Boulevard, Nashville, TN 37209, USA

Gram-positive, endospore-forming *Bacillus thuringiensis*-like strains were isolated from 95 of 413 samples collected at the 0–5 cm depth of noncultivated soils and stagnant or dried-up ponds as well as from dust from stored grain products in South Central United States. Based on the production of parasporal crystals, 25 isolates were identified as *B. thuringiensis* after examining 227 *B. thuringiensis*-like colonies. The greatest proportion of samples yielding *B. thuringiensis* were from the dust from grain storage. The sodium acetate selective medium, heat processing, and crystal staining used in the initial screening revealed diverse populations of *B. thuringiensis*, which were categorized into distinct crystal morphological groups. Sugar fermentation, antibiotic sensitivity, growth characteristics and PCR studies showed diversity among the isolates that were distributed among 25 of the 58 known strains. The most frequently isolated strains were *kurstaki*, *aizawai*, *morrisoni*, *thuringiensis*, *sotto* and *kenyae* that together represented more than 90% of the characterized isolates. PCR analysis using 30 family primer pairs for *cry* and *cyt* genes showed that the frequency of the *cry1* gene (62%) was predominant followed by the *cry2* genes (30%), and the rest (8%) were other *cry* gene types, such as *cry3*, *cry4*, *cry10*, *cry11*, *cry14*, *cry15*, *cry20*, *cry24* and *cry26*. Both *cyt1* and *-2* genes were also detected. Several isolates showed PCR products on the gel that were not consistent with the expected sizes of nucleotides targeted by the primers. These were suggestive of nonspecific amplifications and were not used in the characterization process.
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Introduction

Bacillus thuringiensis is an aerobic, Gram-positive, endospore-forming soil bacterium. During sporulation, it produces a parasporal bipyramidal protein toxin called insecticidal crystal protein (ICP) or δ-endotoxin. Plasmid-borne *cry* and *cyt* genes control the production of these proteins [2,3]. The proteins are toxic to the larvae of dipteran, lepidopteran and coleopteran insects [21]. They are also toxic to some hymenoptera, homoptera, and mallophaga, as well as to many nematodes, flatworms and Sarcomastigophora [17].

More than 130 crystal proteins based on *cry* gene sequences and amino acid homologies have been described [4,9]. The insecticidal properties of these toxins are of great importance to agriculture and public health worldwide. Many strains were obtained from Africa, Asia, Europe, New Zealand, Latin America and the United States [5,14,18-20,26,29]. However, information about the distribution of *cry* genes is still limited and does not cover many distinct geographic areas. There is, therefore, the need to search for novel and more potent strains with wider host ranges, especially in parts of the world that have not been adequately sampled. The South Central States of the United States constitute one such area. Our search for crystalliferous strains in this region has yielded some isolates. In this report, the physiological and molecular characteristics of the isolates, as well as the distribution of *cry* gene types among them are presented. Results of comparative studies with the known strains of *B. thuringiensis* subsp. *israelensis* BTI (ATCC 35646) and *kurstaki* BTK (ATCC 33679) are also presented.

Correspondence: Dr AO Ejiofor, Department of Biological Sciences, P.O. Box 9536, Tennessee State University, 3500 John Merritt Boulevard, Nashville, TN 37209, USA
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Materials and methods

Isolation of B. thuringiensis *subspecies*

B. thuringiensis subspecies were isolated from samples collected from the surface (0–5 cm soil depth) of uncultivated fields that have no history of treatment with *B. thuringiensis* products; stagnant or dried-up ponds in Tennessee, Mississippi, and Alabama; and agricultural cooperative granaries in Nashville, Tennessee. Samples from the fields were mostly topsoil that was a mixture of humus, rock particles and microorganisms. Those from the ponds were a mixture of humus and silt especially when the ponds were drying out. Samples from the granaries were dust from stored grains. The samples were processed by an adaptation of the acetate selective method [34]. One gram of each sample was incubated for 4 h at 30°C and 250 rpm in a baffled Erlenmeyer flask containing 20 ml of 0.30 M sodium acetate (pH 6.8). This selectively suppressed *B. thuringiensis* spores that germinated when plated on a rich agar medium after incubating 2 ml of the sample at 80°C for 10 min. Three hundred microliters of each heat-treated sample was then spread-plated and incubated for 24 h on plates of a medium containing (per liter): 3 g tryptone; 2 g tryptose; 1.5 g yeast extract; 0.05 M sodium phosphate (pH 6.8); 0.005 g

MnCl and 15 g agar. Developing colonies were grown for 24 h at 30°C on LB agar plates. Smears of these were stained by a differential spore/crystal stain containing 5% aqueous malachite green, 2% aqueous aniline blue black (buffalo black), 0.25% aqueous safranin and 0.2% aniline blue black in 50% acetic acid. Stained organisms were viewed by light microscopy at 1500×.

Growth characteristics and antibiotic sensitivity of the isolates

Growth characteristics of the isolates were studied in shake flask cultures at 30°C at 250 rpm for 24 h by determining biomass production through turbidimetric measurements. A seed culture was developed in a 250-ml baffled Erlenmeyer flask containing 50 ml LB broth supplemented with 1% glucose by inoculating it with a loopful of a colony of each isolate that was grown on agar plates of the LB medium and stored on slants at 4°C. One milliliter of the seed culture was used to inoculate a fresh 100-ml LB broth in a 500-ml baffled Erlenmeyer flask. Samples (1-ml) were taken hourly, the turbidity was determined in triplicates at 660 nm and the mean and standard error of means were calculated. Data sets were compared by performing the F test at $p=0.05$. Where necessary, dilutions were made before spectrophotometry, and the dilution factor was used in the calculation of the final spectrophotometric readings.

Carbohydrate fermentation, gelatin hydrolysis and antibiotic sensitivity studies were carried out according to general microbiological procedures [8]. Tubes of phenol red fermentation broth containing lactose, sucrose, maltose, mannitol, mannose or glucose were inoculated with a loopful of cells. Tubes of gelatin were stab-inoculated. Tubes were incubated at 30°C for 24 h and the gelatin tubes were stored in a refrigerator for 24 h before checking them for hydrolysis. Those that did not solidify on refrigeration were considered hydrolyzed.

Antibiotic-impregnated discs (BBL Sensi-Discs Becton Dickinson, Sparks, MD), were used to test for the sensitivity of the isolates. The antibiotics and their concentrations (micrograms per disc) were amoxicillin (30), oxytetracycline (30), ampicillin (10), neomycin (5), chloramphenicol (30), tetracycline (30), erythromycin (15), vancomycin (30), rifampin (5), methicillin (5), streptomycin (10) and bacitracin (10). A lawn of each isolate was made on a plate of antibiotic sensitivity agar (Difco Laboratories, Detroit, MI) with a sterile glass rod. The discs were placed on the plate that was incubated at 30°C for 24 h, after which the diameters of the zones of inhibition were measured.

Molecular characterization of isolates

Extraction of genomic DNA from the crystalliferous isolates: Total DNA was obtained from 16- to 18-h cultures of the isolates grown in LB broth supplemented with 0.1% glucose. Cells were harvested from 5-ml samples by sequential centrifugation of 1-ml aliquots of culture and washed with TES buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl). Washed cells were resuspended in 200 μl lysing buffer containing TE (25 mM Tris–HCl [pH 8.0], 10 mM EDTA), 25% sucrose and 10 mg/ml lysozyme, and incubated at 37°C for 1. The rest of the procedure was an adaptation of the protocol for small-scale DNA preparation [33]. Briefly, the procedure involved centrifugation of the lysate followed by extraction of the DNA with a 25:24:1 mixture of phenol, chloroform and isoamyl alcohol. The extract was washed with chloroform to remove phenol. RNA was then removed by RNase digestion and the DNA was precipitated with ice-cold 100% ethanol. The precipitate was resuspended in 200 μl of water and the DNA was quantified at 260 nm. Ratios of readings at 260 and 280 nm were used to determine the degree of removal of residual phenol and RNA.

Amplification of extracted DNA: Sets of forward (+) and reverse (−) primers were designed with Lasergene software for Windows (DNASTAR, Madison, WI) for each of the target *cry* and *cyt* genes whose nucleotide sequences were available in GeneBank. The MegAlign program of the software was used to align the nucleotide sequences of members of each *cry* and *cyt* family genes and the PrimerSelect program was used to select and optimize the primer pairs from highly conserved regions of the aligned sequences. The selected primers were 17 to 24 bases long, since specificity is at least partly dependent on primer length. Fifteen to 24 bases are reliable lengths [13]. Repetitive sequences were avoided within the template nucleotide sequences. Intraprimer homology beyond three base pairs were also minimal to avoid self-homology and primer dimer formation at the 3′ end of either primer. Furthermore G-C content was held at 45% to 55% to avoid polypurine and polypyrimidine stretches that could promote nonspecific annealing and obtain a T_m in the range of 56–62°C. Finally, mispriming was controlled at the 3′ terminal position and the primers were optimized with the PrimerSelect program. The primers were then synthesized by the Ransom Hill Bioscience Laboratories, Ramona, CA and tested by amplifying representative *cry* genes of each family before application on the DNA of the isolates. For example, *cry4* family primer was tested against BTI known to carry *cry4Aa1* genes. The primers, their product lengths and locations are shown in Table 1. These primers were designed with high sensitivity and specificity as a priority.

Each amplification process was carried out in a 100-μl reaction mixture containing 250 ng DNA, 10 mM of dNTP mixture, 1.0 μM of each primer, and 0.5 U/μl of *Taq* DNA polymerase in a GeneAmp PCR System 2400 DNA thermal cycler (Perkin-Elmer, Norwalk, CT). The reagents were in a PCR kit (Gibco BRL, Gaithersburg, MD). There was an initial 4 min of denaturation at 94°C and 35 cycles of amplification with a 1 min denaturation at 94°C, 45 s annealing at 45°C, and 2 min extension at 72°C. The product was automatically held at 4°C at the completion of the cycles until electrophoresed in Tris–acetate–EDTA buffered agarose gel (1.0%) at 70 V and stained with ethidium bromide. A large capacity multipurpose electrophoretic assembly, model MPH (International Biotechnologies–Eastman Kodak, New Haven, CT) was used in the investigations presented in Figure 2d and e. It had two comb positions each with 16 wells giving a total of 32 lanes. In the investigations presented in Figure 2a, b, c and f, Hoefer HE 33 minihorizontal submarine unit (Pharmacia Biotech, San Francisco, CA) with one comb position giving a total of 16 lanes was used. Two gel slabs were placed side by side to give 32 lanes. A 50-bp DNA ladder (Gibco BRL) was used as a molecular weight marker in the first lanes while positive control DNA from the PCR kit was placed in the last lanes.

Results

Based on the selective isolation method, 95 isolates of *B. thuringiensis*-like organisms were obtained from 227 *B. thuringiensis*-like colonies that grew from the 413 samples. Of these,

Table 1 PCR amplification results for BTI, BTK and the representative *B. thuringiensis* isolates

Target genes	Primer sequences	Primer locations	Product length (bp)	Sample DNA amplification by PCR					
				BTI	BTK	BT10	BT20	BT21	BT23
cry1	(+): 22-mer 5′ TGGTCAGGGCATCAAATAACAG 3′	1463...1484	342		+	+		+	
	(−): 20-mer 5′ ATGGCTAAATCCCGCACGAG 3′	1804...1785							
cry2	(+): 21-mer 5′ GGAGCACGGGCCTATTTGGTA 3′	1530...1550	1165		+			?	
	(−): 22-mer 5′ ACAGCGTTTCGGTTAGGGTTCA 3′	2694...2673							
cry3	(+): 22-me 5′ ACAAAAGTACAATTCAGTCAGT 3′	1498...1519	427		+				
	(−): 23-mer 5′ ATGTTTTAGAATACGTCAAGTCC 3′	1924...1902							
cry4	(+): 24-mer 5′AAATTGATGGTACTCTTGCCTCTT 3′	1355...1378	423	+			+		
	(−): 24-mer 5′ TGCGTAATCCGTAACTTCTTGTAG 3′	1777...1754							
cry5	(+): 20-mer 5′ TAAGCAAAGCGCGTAACCTC 3′	2459...2478	322						
	(−): 19-mer 5′GCTCCCCTCGATGTCAATG 3′	2780...2762							
cry6	(+): 22-mer 5′ TGGCGTAGAGGCTGTTCAAGTA 3′	609...630	302						
	(−): 24-mer 5′ TGTCGAGTTCATCATTAGCAGTGT 3′	910...887							
cry7	(+): 24-mer 5′ CAACCAGACCTATTTTATTGGAGT 3′	1222...1245	476						
	(−): 24-mer 5′ ATTTTTACAGCTGGAATTTTGTG 3′	1697...1674							
cry8	(+): 23-mer 5′ AGAAACACAAGATAAAATACTCC 3′	1896...1918	401						
	(−)(24-mer 5′ ATACAGCATCCCCTTCTACAATCT 3′	2296...2273							
cry9	(+): 24-mer 5′ TATTCTCATTTGAAAAATTTAGC 3′	4614...4637	408						
	(−): 20-mer 5′ ATTATAGAATTGCTTACCTT 3′	5021...5002							
cry10	(+): 19-mer 5′ TCGTGGAATGGGCAAAAAC 3′	236...254	404	+			+		
	(−): 21-mer 5′ TATCCCCCTTCAACATCCTCA 3′	639...619							
cry11	(+): 24-mer 5′ TTTGCACCAGATAATACTAAGGAC 3′	1436...1459	485	+			+		
	(−): 24-mer 5′ AACAACTGCGATAAATACCACTCT 3′	1920...1897							
cry12	(+): 19-mer 5′ CTCCCCCAACATTCCATCC 3′	2555...2573	363						
	(−): 24-mer 5′ AATTACTTACACGTGCCATACCTG 3′	2917...2894							
cry13	(+): 21-mer 5′ GCGCCAAGACATAGCATTTTA 3′	798...818	421						
	(−): 21-mer 5′ CACTCCATACGGCCAGCAGAA 3′	1218...1198							
cry14	(+): 22-mer 5′ ATAATGCGCGACCTACTGTTGT 3′	1634...1655	456					?	
	(−): 19-mer 5′ TGCCGTTATCGCCGTTATT 3′	2089...2071							
cry15	(+): 21-mer 5′ ATCTGGGGTTACCGTTTCTGC 3′	1323...1343	430			+		+	
	(−): 20-mer 5′ CGTCGTTGCTGTTCCTCTCC 3′	1752...1733							
cry16	(+): 19-mer 5′ TAAAACTAGATCCGAATAA 3′	786...804	400						
	(−): 17-mer 5′ TCAAAAGCCATAGACTC 3′	1185...1169							
cry17	(+): 24-mer 5′ GATGCCTATATGAACTTGTCTGTG 3′	723...746	426						
	(−): 24-mer 5′ ATCCCATTCTTTATTATCGGTATC 3′	1148...1125							
cry18	(+): 20-mer 5′CCGAGGCGATTTGGATAGAT 3′	690...709	419						
	(−): 21-mer 5′ TGCCGGTGTAAACAAAGAAGG 3′	1108...1088							
cry19	(+): 24-mer 5′ AGGGGAGTCCAGGTTATGAGTTAC 3′	1287...1310	355						
	(−): 24-mer 5′ ATTTCCCTAGTTAGTTCGGTTTTT 3′	1641...1618							
cry20	(+): 20-mer 5′ CAATCCCTGGCTTCACTCGT 3′	985...1004	490						+
	(−): 17-mer 5′ CCGCGGGCATTAGGATT 3′	1474...1458							
cry21	(+): 21-mer 5′ ATACAGGGATAGGATTTCAAG 3′	1112...1132	453						
	(−): 23-mer 5′ ATCCCCATTTTCTATAAGTGTCT 3′	1564...1542							
cry22	(+): 24-mer 5′ CAGATGAGATAGATGGGGATTTGA 3′	866...889	413						
	(−): 23-mer 5′ ATTCGCTTCTATACTTGGCTGTC 3′	1278...1256							
cry24	(+): 19-mer 5′ AGGGGGCGATGGATACGAC 3′	1253...1271	355						
	(−): 21-mer 5′ GGCCCTGCTACAACCGAAACTA 3′	1607...1587							
cry25	(+): 22-mer 5′ CGTTTTCCGCATTATCATTAGG 3′	1499...1520	437						
	(−): 17-mer 5′ ACGCCCCGGCTGTCTTA 3′	1935...1919							
cry26	(+): 23-mer 5′ CGCGCTGTTCAATTATCAAGTGC 3′	3186...3208	362						
	(−): 24-mer 5′ ATATGGAAAGAAAAGGCGTGTGGA3′	3547...3524							
cry27	(+): 24-mer 5′ GTGGCATATAGACTAAGGGAGGAA3′	494...517	387						
	(−): 23-mer 5′ TTGCAGGCCATATAAGAGGTGTT 3′	880...858							
cry28	(+): 24-mer 5′ GTATTGGACCGAGGAGATGAAAGT3′	281...304	466						
	(−): 21-mer 5′ GTACGGCAAAGCGACAGAACA 3′	746...726							
cyt1	(+): 20-mer 5′ CCGATGGGTGCTGTAGTGAG 3′	416...435	277	+					
	(−): 21-mer 5′ CAGTTTGGGCATTTTGGATTG 3′	692...672							
cyt2	(+): 18-mer 5′ ATCCGCCCATAATACAAG 3′	749...766	386	+			+		
	(−): 17-mer 5′ GATACGGTTCACAGACG 3′	1134...1118							

The primers used, their locations and expected product sizes are shown.
+ =amplification. ?=nonspecific amplification; at variance with expected product size. (+)=forward or upper primer. (−)=reverse or lower primer.

25 were Gram-positive, crystalliferous endospore formers, and the crystal morphologies were spherical or ovoid, rhomboid, or bipyramidal. Isolates showed diversity in the relative length, overall thickness and size of their crystals. Of the 25 isolates, 21 were from soil samples and the rest from grain storage dusts. In terms of frequency of isolation, less than 1% of the soil samples yielded *B. thuringiensis*, while about 48% of the grain storage dust yielded the bacterium. Two isolates, designated BT10 and BT20,

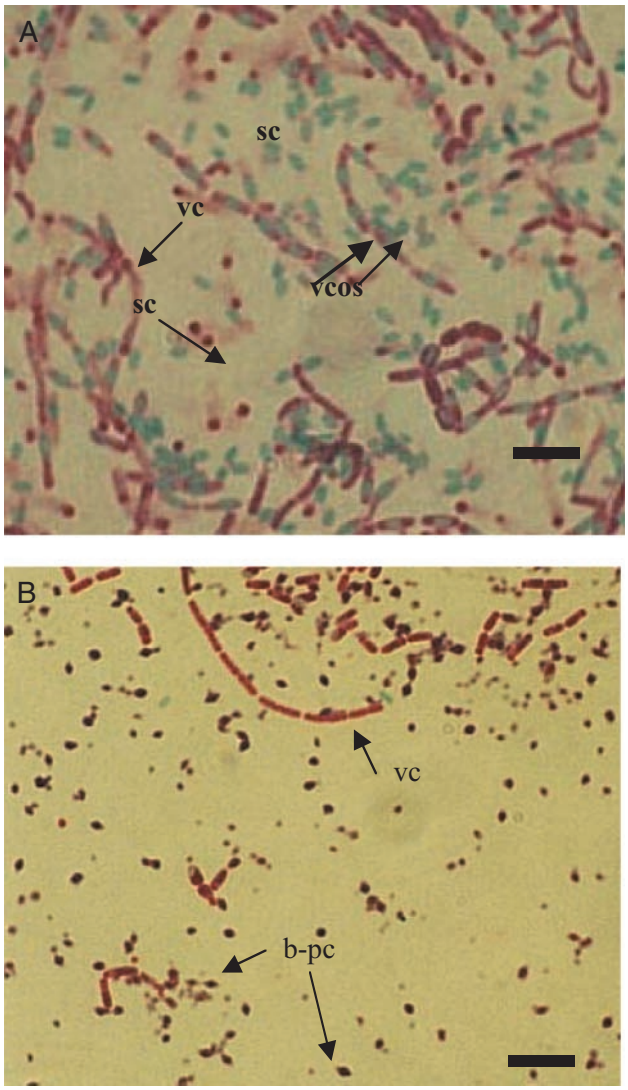

Figure 1 Photomicrograph of typical *B. thuringiensis* isolates showing (A) the spherical crystals of BT20 (subspecies *israelensis*) and (B) the bipyramidal crystals of BT10 (subspecies *sotto*). sc=spherical crystal, vcos=vegetative cells with oval spores, vc=vegetative cells, and b-pc=bipyramidal crystals. Bar=200:m.

showed typical cell and crystal morphologies that were consistent with several groups and are presented in Figure 1A and B. Similar to BTK, BT10 had rhomboid bipyramidal crystals, whereas BT20 had spherical inclusions similar to BTI.

Isolates designated BT10, BT20, BT21 and BT23 were randomly selected for description of the characteristics common to the diverse groups of isolates. BT10, BT21 and BT23 produced acid but no gas in lactose, sucrose, maltose, mannose, glucose and mannitol. BT10 did not hydrolyze gelatin, whereas BTK partially hydrolyzed it, especially at the top of the column. Generally, all isolates showed similar patterns of growth with a 2- to 3-h lag phase and rapid growth in the next 7 to 8 h before the stationary phase. The growth pattern of BT10 was not significantly different from that of BTK ($p>0.05$), whereas each of the remaining isolates had growth patterns distinct from either BTI or BTK ($p<0.05$). The antibiotic sensitivity patterns detected among the isolates also showed slight differences with zones of clearing ranging from 0- to 3.3-cm diameter. BT10, BT21, BT23 and BTK showed greater relationships, whereas BTI and BT20 showed only slightly different patterns (Table 2).

Many *cry* gene families, including *cry1*, *cry2*, *cry3* and *cry4* have multiple members designated Aa, Ab, Ac ... Ba, Bb, Bc ..., Ca... and so on. However, most of the others have single members, such as *cry*10Aa. *Cry1* family genes are found in subspecies *kurstaki*, *aizawai*, *entomocidus*, *sotto*, *berliner*, *alesti*, *morrisoni*, *wuhanensis*, *gallariae* and *kenyae*. *Cry2* family genes are harbored by subspecies *kurstaki* and *kenyae*, while *cry3* genes are found in subspecies *tenebrionis*, *morrisoni*, *sandiego*, *tolworthi* and *kurstaki*. Subspecies *israelensis*, *jegathesan* and *medellin* contain *cry11* genes, while *cyt1* is found in subspecies *israelensis*, *morrisoni*, *medellin* and *neoleoensis*. Only subspecies *kyushuensis*, *israelensis* and *jegathesan* have *cyt2* genes [8,12]. Most of the other *cry* genes are found in single-member families, while *cry17* and *cry18* genes are found in *Clostridium bifermentans* and *Bacillus popilliae*, respectively. Our isolates yielded electrophoresed PCR products with combinations of *cry* genes. The combinations, when used along with other characters already described, allowed identification of the isolates. Based on these, BT10, which has *cry1* and *14* genes was identified as subspecies *sotto*, BT20 with *cry4*, *10*, and *11* and *cyt1* genes is probably subspecies *israelensis*. Repeated amplification of this sample did not yield *cyt2*. While other combinations indicate that it is subspecies *israelensis*, the absence of *cyt2* suggests more specific tests such as sequencing would be necessary to confirm its exact identity. BT21 with *cry1* and *15* genes was identified as subspecies *thompsoni*, while BT23 was identified as species *fukuokaensis* (Figure 2A–F). Based on this kind of analysis, 40% of the isolates were subspecies *kurstaki*, 26% were *aizawai* and 10% were *kenyae*.

Table 2 Antibiotic sensitivity patterns of BTI, BTK and some of the isolates after 24 h of incubation

Isolate	AMC 30	T 30	AM 10	N 5	C 30	TE 30	E 15	VA 30	RA 5	ME 5	S 10	B 10	K 30	A 30	PB 300
BTI	3.2	3.3	3.3	1	2.4	3.2	2.2	2.1	1.7	2.7	2.5	0.9	2.4	3.4	0.8
BTK	1.1	2.3	0	1	2.5	2.3	2.2	2	1.6	0	2.1	0	2.1	2.6	0.9
BT10	1	2.1	0	0.7	2.4	2.2	2	1.9	1.6	0	2.1	0	1.9	2.6	0.9
BT20	3.2	3.1	3.1	1.1	2.4	3.2	2.5	2.1	1.8	2.6	2.4	0.9	2.4	3.5	0.9
BT21	0	2.3	0	1	1.8	2.2	2.1	1.8	1.4	0	2	0	2	2.6	0.7
BT23	1.1	2.8	0	0.7	2	2.8	2.5	1.8	2.1	0	2	0	1.8	2.7	0.9

Numbers indicate the diameters of zones of inhibition in centimeters.
AMC=amoxicillin, T=oxytetracycline, AM=ampicillin, N=neomycin, C=chloramphenicol, TE=tetracycline, E=erythromycin VA=vancomycin, RA=rifampin, ME=methicillin, S=streptomycin, B=bacitracin, K=kanamycin, A=chlortetracycline, PB=polymyxin B.
Numbers following the antibiotic abbreviations are the concentrations of the antibiotics in micrograms in the impregnated discs.

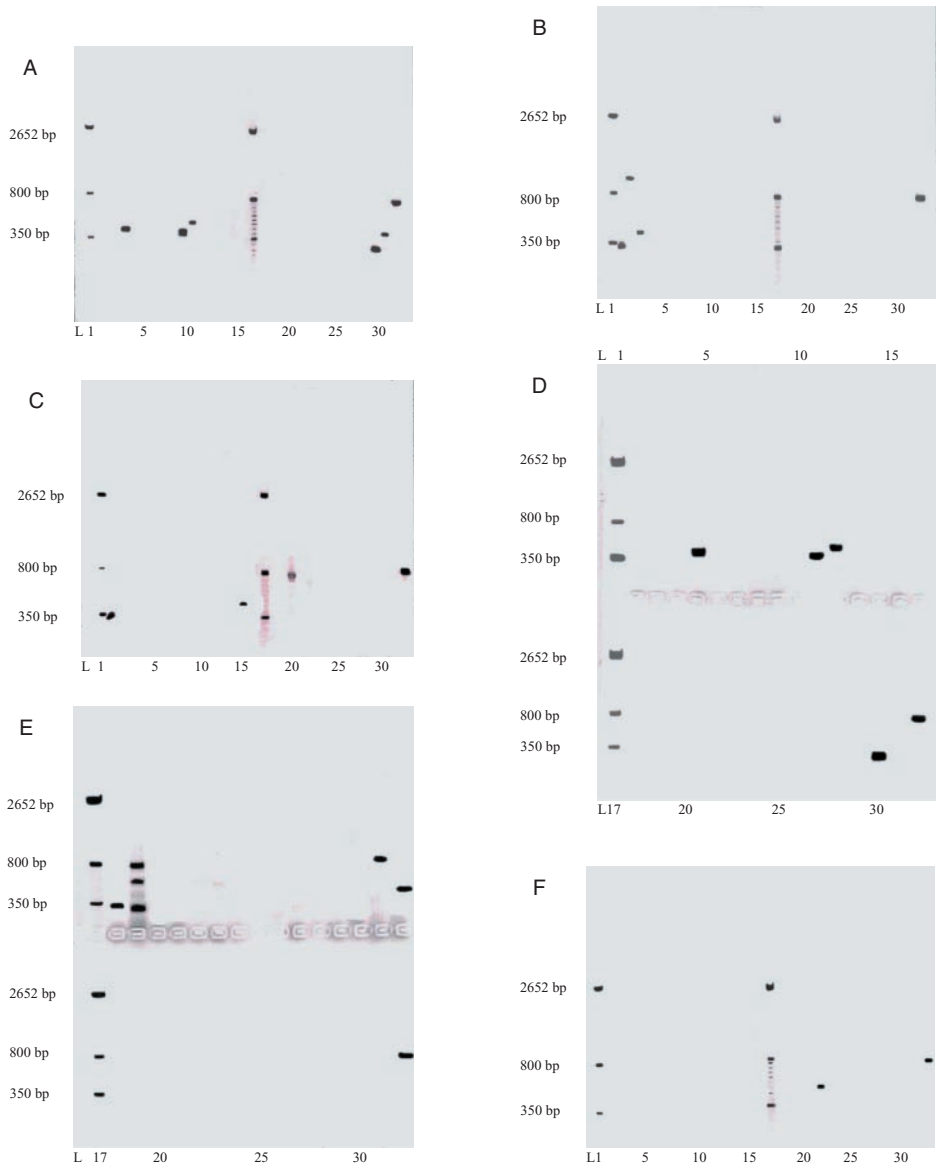

Figure 2 (A) BTI (ATCC 35646) showing amplification of *cry4*, *10*, and *11*, as well as *cyt1* and *2*. Lanes 1 and 17 are 50-bp DNA ladder markers while lane 32 is a positive DNA control. (B) BTK (ATCC 33679) showing amplification of *cry1*, *2*, and *3*. Lanes 1 and 17 are 50-bp DNA ladder markers while lane 32 is a positive DNA control. (C) Isolate BT10 showing amplification of *cry1* and *14* (lanes 2 and 15 respectively). lanes 1 and 17 are 50-bp DNA ladder markers while lane 32 is a positive DNA control. (D) Isolate BT20 showing amplification of *cry4*, *10* and *11* as well as *cyt1*. lanes 1 and 17 are 50-bp DNA ladder markers while lane 32 is positive DNA control. (There were two well locations: upper location had lanes 1–16, and lower location had lanes 17–32). (C) Isolate BT21 showing amplification of *cry1* and *15* (lanes 2 and 16). lanes 1 and 17 are 50-bp DNA ladder markers while lane 32 is a positive DNA control. (There were two well locations: upper location had lanes 1–16, and lower location had lanes 17–32). (F) Isolate BT23 showing amplification of *cry20* (lane 22). lanes 1 and 17 are 50-bp DNA ladder markers while lane 32 is a positive DNA control.

Other subspecies that were present and constituted the remaining 24% were *morrisoni*, *tolworthi*, *thompsoni*, *galleriae*, *kumamotoensis* and *israelensis*. Virtually all of these subspecies except *israelensis* were isolated from the granaries while no soil sample yielded more than one subspecies.

Discussion

Recent reports on the frequency of occurrence of *B. thuringiensis* isolates from natural environments indicate a high possibility of being able to isolate a novel strain [4,5,11,15,16,18-20,23,26,28,29]. Success in isolation of crystalliferous *B. thuringiensis* depends largely on the techniques employed. The sodium acetate selective method used in combination with heat treatment and crystal staining, appeared highly sensitive when compared with other techniques, such as the enrichment technique, because it has a lower limit of detection of about 10^3 bacteria per gram of soil. Immunofluorescence-based techniques that allow direct enumeration [7] have high potential for precision and reliability, but they have a lower detection limit of about 10^5 bacteria per gram of soil. Although soil is considered the primary reservoir of the organism [11,26,28,31,34], only about 1% of the soil samples processed yielded colonies with morphological and molecular characteristics of *B. thuringiensis*.

The detection and identification of known and novel *cry* genes from a large number of isolates is an arduous task. Many researchers have utilized a wide variety of techniques to identify and characterize *cry* gene-carrying *B. thuringiensis*. For example, Brown and Whitely [6] employed molecular characterization to identify two novel crystal protein genes from *B. thuringiensis thompsoni*. They cloned and sequenced the crystal proteins of this strain in *E. coli* and used the electrophoretic mobilities to predict the molecular masses of amino acid sequences. This method has been useful to characterize fully the ICPs of isolates. Recent, many workers have turned to PCR [22,27] or a combination of this technique with other methods, such as serology [25], insecticidal activity [32] and SDS-PAGE for the detection and characterization of *cry* genes from their isolates. Akhurst *et al* [1] used a 16S rRNA gene oligonucleotide probe in PCR experiments, which allowed them to separate *B. thuringiensis* serovars as well as separate them within the serovars *thuringiensis*, *tolworthi* and *kurstaki*. Hansen *et al* [15] combined both molecular and phenotypic characterization to identify isolates from leaves and insects and used PCR for confirmatory purposes. We have combined both molecular and physiological methods to achieve this objective. The basic microbiological and physiological studies showed a high degree of isolation and characterization of the subspecies, as well as detection of the *cry* and *cyt* genes present. DNA sequence homology is now being used as a procedure for confirmation of a novel *cry* gene. It is now required that novel *cry* must have a significant sequence similarity to one or more toxins within the nomenclature or be a *B. thuringiensis* parasporal inclusion protein that exhibits pesticide activity or some experimentally verifiable toxic effect to a target organism for a new name to be assigned to it [10]. The *Bacillus thuringiensis* Stock Culture Center at the Ohio State University is a designated center for that service. All isolates from this work had parasporal crystals. Their toxicity and nucleotide sequence analysis are determined prior to naming their *cry* genes.

The isolation of 95 *B. thuringiensis*-like organisms from the 413 samples suggested that the occurrence of the organism in the South Central United States fits into the overall reports of worldwide distribution of the organism. The distribution of isolates in terms of different habitats was consistent with the findings of other workers that grain storage dusts yield the highest number of *B. thuringiensis* isolates [12,15,16,19,25]. This result indicated that grain storage facilities are more favorable habitats than the others. This could be because many subspecies are natural pathogens of lepidopteran and coleopteran insects that live as pests of agricultural produce. There could be a recycling effect in which naturally occurring *B. thuringiensis* subspecies that live as pathogens of these insect groups undergo repeated cycles of growth, sporulation and spore germination. This would increase the populations of the bacteria in such an environment. Although deliberate efforts were made to exclude samples from those sites known or suspected to have been exposed to *B. thuringiensis* products, the possibilities of carry-over from previous exposures still exist. These possibilities also explain the heterogeneity in the occurrence of subspecies of *B. thuringiensis* in samples obtained from granaries.

Isolates with rhomboid bipyramidal crystals showed closer relationships among themselves in terms of patterns of sensitivity to antibiotics, as did BTI, BT20 and others that have spherical crystals. This was evident in the patterns of sensitivity to amoxicillin, oxytetracycline, ampicillin, tetracycline, methicillin, bacitracin and chlortetracycline. The rest did not show any distinguishable patterns. This is suggestive of a relationship in terms of the plasmids hosted by each of the isolates, as the antibiotic resistance genes are usually plasmid-borne.

The presence and distribution of ICP genes (*cry* genes) in *B. thuringiensis* isolates from some ponds, uncultivated fields and granaries in the South Central United States were evaluated using molecular techniques. These techniques detected 10 different *cry* genes and two *cyt* genes, combinations of which gave up to 25 subspecies of the bacterium. The predominance of subspecies *kurstaki* (40%), *aizawai* (26%) and *kenyae* (10%) in the South Central United States is consistent with the findings of many workers in different parts of the world. Some earlier reports have, however, indicated otherwise, including that of Vankova and Purrini [35] who found isolates of subspecies *thuringiensis*, *morrisoni* and *kurstaki*, in that order, to predominate in natural epizootics in old watermills in Yugoslavia. Norris [30] also found that subspecies *kenyae* predominated in storage products, while DeLucca *et al* [11,12] found the predominant subspecies to be *aizawai* (95%) from 73 dust samples. There is generally a consistent pattern of significant variation in the occurrence of the other subspecies reported here when compared with similar reports from other parts of the world. These subspecies include *morrisoni*, *tolworthi*, *thompsoni*, *galleriae*, *nigeriensis*, *israelensis*, *kumamotoensis*, *pondicheriensis* and *japonensis*. This conclusion is supported by previous reports concerning these subspecies [11,12,24,27,28,31].

The frequency of isolation of *B. thuringiensis* strains suggests that a combination of the techniques used would help in conducting this exercise on a larger scale. This would subsequently help in determining the overall distribution and ecological significance of *B. thuringiensis cry* genes in the South Central United States. Isolates that produced crystals but did not show any amplification of sequences were thought to be unique since they were not detected by the primers for the known sequences used. Their uniqueness could be confirmed by DNA sequencing.

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