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Characterization of *Bacillus thuringiensis* Strains in the Vietnam *Bacillus thuringiensis* Collection

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In the Vietnamese Collection of Microorganisms, the *Bacillus thuringiensis* collection is abundant and diverse. Established since 1993, the *Bacillus thuringiensis* Collection, currently includes 119 reference strains belonging to 78 different serovars received from Korea, Japan, France, USA and more than 1080 strains isolated from various samples collected in several areas across Vietnam. Among them, 601 strains produce parasporal inclusions of different morphological characteristics such as rhomboidal, cuboidal, spherical, amorphous and heterogeneous crystals. H-serotyping of 442 crystalliferous strains showed that they belong 19 serotypes. Most of them belong to *Bacillus thuringiensis* subsp. *kurstaki* (132 strains), *aizawai* (74 strains), *morrisoni* (74 strains). Bioassays revealed that 127 strains are active against *Plutella xylostella* (Lepidotera), 33 strains are active against *Aedes aegypti* (Diptera) and 5 strains are active against *Tribolium castaneum* (Coleoptera). PCR was used to detect genes encoding crystal proteins for 185 strains: the genes found are *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B*, *cry1*C, *cry1D*, *cry1E*, *cry1F*, *cry2A*, *cry3A*, *cry4B*, and *cyt2* (Table 6) The Vietnamese *Bacillus thuringiensis* collection is continuously being developed.

Introduction

The Institute of Biotechnology (IBT) is the leading research institute in biology and biotechnology in Vietnam. One important mandate of IBT is to collect and curate microorganism strains. The Vietnam Collection of Microorganisms (VCM) was established in 1993 by IBT on the basis of the Collection of the former Department of Microbiology of the Biology Institute (founded in 1975). At first, there were about 1000 strains belonging to various groups of microorganisms such as bacteria, mold, yeast, actinomycetes. Since then, the Vietnam Collection of microorganisms has been continuously developed in cooperation with other Vietnamese and overseas collections. The VCM is considered one of the largest in Vietnam. Its primary mandate is to maintain and preserve microorganism strains and, recently an attempt has been made to develop a GeneBank.

Bacillus thuringiensis is the most abundant microorganisms in this collection, with more than 1000 strains collected from several different sources (Table 1). At present, the Vietnam *Bacillus thuringiensis* Collection includes 78 subspecies reference-strains and more than 1000 of well-characterized local isolates. This is a valuable biological resource for research, education and various applications (1) .

TABLE 1. Introduction of the Vietnam *Bacillus thuringiensis* Collection

Source (Country)	Number of *Bt* strains	*Bt* strain's Site in the VCM	Year obtained
Korea	4	Reference strains	2000
Japan	5	Reference strains	2001
France	32	Reference strains	2000
USA	78	Type strains	2002-2003
Vietnam	1080	Isolates	From 1975 to present

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The characteristics of isolates in the Vietnam collection of *Bacillus thuringiensis* isolation

Several *Bt*-based bioinsecticides are available on the Vietnamese market, most of which are imported. Using imported products may bring foreign and recombinant bacteria into the local ecosystem. This may also causes the change of local microorganisms distribution (1). Accordingly, it is necessary to study the distribution and the contribution of the local native *Bacillus thuringiensis* bacteria. *Bt* has been isolated from numerous types of samples collected across Vietnam, including 550 soil, 185 leaf and 45 insect-cadaver samples. Isolation procedures were implemented following a method improved from Ohba and Aizawai (1986) (10) (Table 2).

TABLE 2. Characteristics of *Bacillus thuringiensis* isolates in Vietnam.

Kind of samples	Number of samples with *Bt*/ Number of samples examined (%)	Number of *Bt* isolated/ Number of spore-forming bacteria examined (*Bt* index)
Soils	379/550 (68.91)	763/1861 (0.41)
leaves	104/185 (56.22)	258/662 (0.39)
Insect cadavers	16/45 (35.56)	59/190 (0.31)
Total	499/780 (63.97)	1080/2713(0.4)

The isolation process obtained 2713 colonies with morphological features of the *Bacillus cereus* group, of which 1080 colonies were identified as crystal-producing *Bt*. *Bt* was present in 499 samples (63.97%), and an average Bt-index of 0.4. *Bacillus thuringiensis* was found in all three kinds of samples, including soil, leaves and insect cadavers. However, their prevalence differed depending on kind of samples, i.e. highest (68.9%) in the soil and lowest (35.5%) in insect cadavers (Table 2).

Morphological characteristics of toxic crystal protein

The isolation process resulted in 1080 strains producing crystal proteins with various size and morphological characteristics. Three shapes of crystals were abundant: rhomboidal (63.1%), spherical (11.2%), and cuboidal crystals (4.8%). Some (5.8%) strains produce irregular crystals. Most of isolates produce only one specific kind of crystal, but some (15.1%) are able to produce crystals with various shapes (Figure 1, Table 3).

TABLE 3. Morphology of *Bt* crystal in the Collection.

Crystal shapes	Number of strains	Percentage (%)
Rhomboidal	681	63.1
Spherical	121	11.2
Cuboidal	52	4.8
Heterogeneous	163	15.1
Amorphous	63	5.8

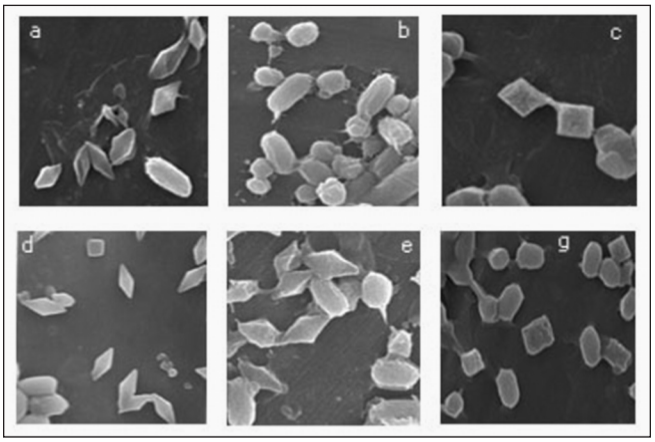

FIG. 1. Scanning electron micrographs of crystal morphology of Bt isolates. a: Strain DB 21-2 isolated from Dien Bien Phu soil sample with rhomboidal crystals; b: Strain HN5-7 isolated from Ha Noi with spherical crystals; c: Strain SH149-11 isolated from Ha Tay province with cuboidal crystals; d: Strain NC29-3 isolated from Thai Nguyen province with cuboidal and rhomboidal crystals; e: Strain TN 1-1 isolated from Thai Nguyen province with rhomboidal and spherical crystals; g: Strain NC27-1 isolated from the Thai Nguyen province with cuboidal and spherical crystals.

Classification characteristics of Bt in the collection

A number of strains (119) belonging to 78 *Bt* subspecies were obtained from various countries, including the USA, France, Japan, and Korea. With these reference strains, a set of antisera was produced for use as a standard serological kit to classify *Bt*. Isolated strains in the VCM were classified according to de Barjac and Bonnefoi (1990) (6) (Table 4).

Of 479 strains classified by flagellar (H) antigen analysis, 442 strains (92.3%) showed positive agglutination with 18 antisera, the remaining 37 strains (7.7%) did not agglutinate with any of 60 antisera used. The results revealed that 27.6% of the isolates belong to serotypes 3a,3b,3c (subspecies *kurstaki*), 15.4% belong to

TABLE 4. H-Serotyping of *Bacillus thuringiensis* isolates in Vietnam Bt Collection.

No	Subspecies	**H-serotype**	**Agglutination strains**	**Percentage (%)**
1	*alesti*	3a, 3c	35	7.3
2	*sumiyoshiensis*	3a, 3d	6	1.3
3	*kurstaki*	3a, 3b, 3c	132	27.6
4	*fukuokaensis*	3a, 3d, 3e	18	3.8
5	*gallariae*	5a, 5b	7	1.5
6	*aizawai*	7	74	15.4
7	*morrisoni*	8a, 8b	74	15.4
8	*nigeriensis*	8b, 8d	16	3.3
9	*tolwothy*	9	13	2.7
10	*isralensis*	14	7	1.5
11	*indiana*	16	13	2.5
12	*yunnanensis*	20a, 20b	4	0.8
13	*pondicheriensis*	20a, 20c	3	0.6
14	*colmeri*	21	12	2.5
15	*novosibirsk*	24a, 24c	3	0.6
16	*coreanensis*	25	6	1.3
17	*leesis*	33	8	1.7
18	*konkukian*	34	11	2.3
19	Not agglutinated		37	7.7

TABLE 5. Toxicity of *Bacillus thuringiensis* isolates against *Plutella xylostella, Aedes aegypti*, and *Tribolium castaneum,* 3 days after treatment.

Percentage of died insects (%)	**Dosages (spores/ml)**	***Plutella xylostella***		***Aedes aegypti***		***Tribolium castaneum***	
		No. of Bt strains	**Percentage (%)**	**No of Bt strains**	**Percentage (%)**	**No of Bt strains**	**Percentage (%)**
0-20	10^5	49	15.2	33	22.4	39	40.6
	10^7	20	6.2	9	6.1	31	32.3
20-40	10^5	81	25.2	48	32.7	25	26
	10^7	39	12.1	38	25.8	23	24
40-60	10^5	76	23.6	36	24.5	18	18.8
	10^7	127	39.4	43	29.3	22	23
60-80	10^5	68	21.1	21	14.3	11	11.5
	10^7	77	23.9	36	24.5	17	17.7
80-100	10^5	48	14.9	9	6.1	3	3.1
	10^7	59	18.3	21	14.3	5	5.2

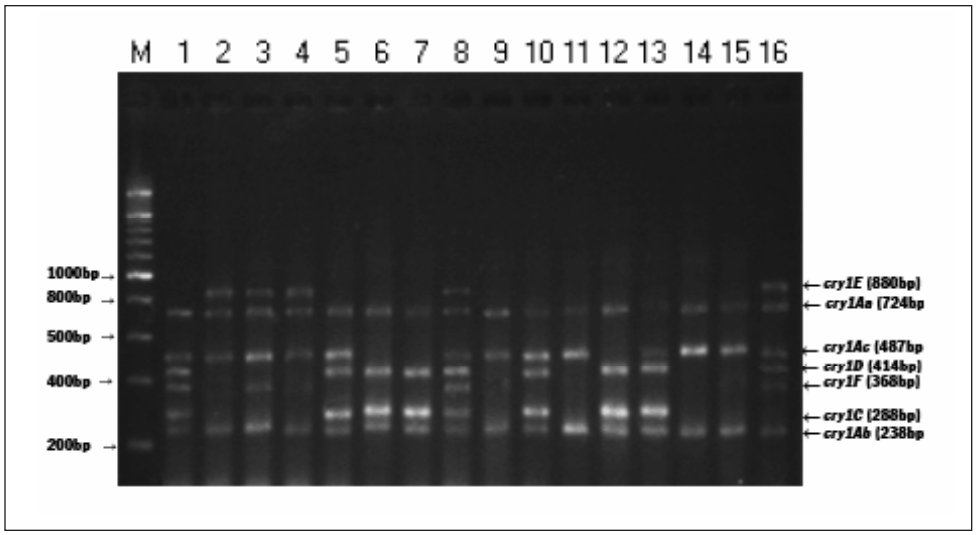

FIG. 2. Agarose gel (1.5%) electrophoresis analysis of multiplex PCR products obtained by using specific primers of Bt isolates. Lane M: Marker, Lane 1: SPJ9-6, Lane 2: DB8-2, Lane 3: HL2-1, Lane 4: DB6-2, Lane 5: SP23-10, Lane 6: BB12-6, Lane 7: SP5-1, Lane 8: NA8-1, Lane 9: QB3-2, Lane 10: SP10-7, Lane 11: DH206-2, Lane 12: TQ3-3, Lane 13: SP14-3, Lane 14: AV132-2, Lane 15: BB85-14, Lane 16: NAK7-4.

TABLE 6. PCR analysis of the insecticidal crystal protein genes from *Bacillus thuringiensis* isolates.

Specific primer	No. of isolates detected	% of isolates detected
cry1Aa	112	60.5
cry1Ab	114	61.6
cry1Ac	124	67
cry1B	28	15.1
cry1C	43	23.2
cry1D	36	19.5
cry1E	26	14.1
cry1F	20	10.8
cry2A	77	41.6
cry3A	3	1.6
cry4B	16	8.7
cyt2	65	35.1
Not detected	13	7

aizawai and 15.4% belong to *morrisoni*. Subspecies *novosibirsk* and *pondicheriensis* were rare subspecies, with 3 strains (0.6%) of each. Our data indicate that *Bt* isolated in Vietnam is rather diverse in classification. Up to now, there are 82 subspecies of Bt identified all over the world (8), of which 18 subspecies are present in Vietnam. Interestingly, 37 strains of the VCM do not agglutinate with antisera. They may either belong to remaining or new subspecies. The subspecies present in Vietnam, such as *kurstaki, aizawai, israelensis* and *morrisoni,* have been already well-characterized in the world, and have valuable properties for control application.

Insecticidal activity of isolates in the Collection

The most important property of *Bacillus thuringiensis* is their specific insecticidal activity that is amenable to the production of bioinsecticides. For this purpose, it is important to select the most active strains. In our study,

the isolates were assayed to determine their activity against three orders of insects, i.e. Lepidoptera (*Plutella xylostella*), Diptera (*Aedes aegypti*) and Coleoptera (*Tribolium castaneum*). The assays were carried out by dipping leaf discs in *Bt* solutions or by rearing larvae on artificial diets treated with *Bt* (Table 5).

Evaluation of toxicity against *P. xylostella, A. aegypti* and *T. castaneum* was done respectively for 322, 147 and 96 strains. The results showed that 59 strains (18.3%) and 48 strains (14.9%), respectively killed 80-100% *P. xylostella* larvae after 3 days at 10^7 and 10^5 spores/ml. For the target *A. aegypsti*, these numbers were: 21 strains-10^7 spores/ml (14.3%); 9 strains-10^5 spores/ml (6.1%) and for *T. castaneum* larvae: 5 strains-10^7 spores/ml (5.2%), 3 strains-10^5 spores/ml (3.1%).

Gene characterization encoding ICPs of *Bt* isolates

In order to get a toxic gene profile for each strain in the collection and to select strains amenable for bioinsecticide production, PCR was used to detect some toxic genes (Figure 2 and Table 6).

Genes profile was characterized for 185 strains by using 12 specific primer pairs (*cry1Aa, cry1Ab, cry1Ac, cry1B, cry1C, cry1D, cry1E, cry1F, cry2A, cry3A, cry4B, cyt2*): 172 strains contained one or more fragment genes, 13 strains did not contain any gene surveyed. The frequencies of genes were different, i.e. *cry1Ac* accounted for 67%; *cry1Ab*, 61.6%; *cry1Aa*, 60.5% and, *cry3A*, 1.6%.

Conclusion

Though characterization of *Bt* strains in the Vietnam *Bacillus thuringiensis* Collection was limited (not all of toxin genes were studied for complete gene profiles), its result was significant and beneficial. With these results, strains which habour *cry* genes can be selected for production of *Bt* insecticide against Lepidoptera, Coleoptera and tropical Diptera vectors transmitting diseases in Vietnam.

Acknowledgements

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