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Phenotypic and Morphometric Characterization of *Bacillus thuringiensis* Parasporin–1 Producers: A Comparison with Insecticidal Cry–rotein Producers

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This study determined phenotypic and morphometric characteristics of the 8 *Bacillus thuringiensis* strains producing parasporin–1 (PS1), anti–cancer Cry proteins, for comparison with *B. thuringiensis* insecticidal Cry–protein producers. Aspect ratios of the spores of PS1 producers were significantly smaller than those of the insecticidal Cry–protein producers. Motility was not associated with the PS1 producers. The API tests revealed that all of the eight PS1 producers fall into the *Bacillus cereus* group. They proved hemolysis–negative on sheep blood agar, and resistant to ampicillin but susceptible to bacitracin. The gene *atxA* encoding the anthrax toxin was not detected in PS1 producers and insecticidal Cry–protein producers. All of the PS1 producers contained the SG–749 fragment, a DNA marker specific for the *B. cereus* group. Overall results showed that the eight PS1 producers can be allocated to the *B. cereus* group. However, they differed in several classic key characters from the known subgroups existing in the *B. cereus* group, including *Bacillus anthracis*.

INTRODUCTION

Bacillus thuringiensis was first isolated in Japan as an insect pathogen from diseased larvae of the silkworm, *Bombyx mori*, by Ishiwata (1901). It produces proteinaceous parasporal inclusions during sporulation. Parasporal inclusions often contain δ–endotoxins that exhibit specific insecticidal activity against several orders of insects, including Lepidoptera, Diptera and Coleoptera (Glare and O'Callaghan, 2000). The δ–endotoxins consist of two major families, Cry and Cyt proteins. The Cry proteins have selective toxicities against midgut epithelial cells of susceptible insects. This makes *B. thuringiensis* a promising agent in biological control of agriculturally and medically important insect pests.

Although *B. thuringiensis* as a species has long been assigned to an insect pathogen, previous studies have also provided a hypothesis that the majority of *B. thuringiensis* populations occurring in natural environments are not pathogenic for insects (Ohba and Aizawa, 1986a; Ohba *et al*., 2000; Lee *et al*., 2003; Yasutake *et al*., 2006; Armengol *et al*., 2007). Thus, the question arises as to whether parasporal inclusions of non–insecticidal *B. thuringiensis* have any biological activity against non–insect invertebrates and vertebrates (Ohba *et al*., 1988). In this regards, it is of particular interest to note that certain *B. thuringiensis* isolates produce non–insecticidal parasporal proteins with a unique activity preferentially killing human cancer cells (Mizuki *et al*., 1999, 2000; Yasutake *et al*., 2006; Uemori *et al*., 2007; Jung *et al*., 2007). Currently, these anti–cancer proteins, designated parasporins (PS), are categorized into four groups (PS1, PS2, PS3 and PS4) on the basis of amino acid sequence homology (see The Committee of Parasporin Classification and Nomenclature website at http://parasporin.fitc.pref.fukuoka.jp/index.html).

At present, the existing PS1–producers are all presumptively allocated to *B. thuringiensis* in the light of the morphological features of colony, vegetative cell, sporangium, and endospore, and the formation of noticeable parasporal inclusions. However, very little is known about the other microbiological characteristics of the organisms. The objective of this study was to examine the parasporin–1 (PS1) producers isolated from Japan and Vietnam for phenotypic and morphometric characterization, in comparison with insecticidal *B. thuringiensis* strains.

MATERIALS AND METHODS

Organisms and culture conditions

B. thuringiensis strains used in this study are listed in Table 1. A strain of *B. cereus*, IAM 1029 (Ohba *et al*., 1981), was also used in PCR experiments. The media routinely used were nutrient broth and agar which contained (g/l in distilled water): meat extract, 10; polypeptone, 10; NaCl, 2; agar (for solid medium), 20; pH 7.6. Bacteria were grown on the media at 27˚C.

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Table 1. *Bacillus thuringiensis* strains used in this study

B. thuringiensis strains	Locality	Source	Reference
PS1 producers			
A1190	Hiroshima Pref., Japan	Soil	Mizuki *et al*. (1999, 2000)
A1965	Fukuoka Pref., Japan	Phylloplane	Uemori *et al*. (2007)
B0186	Fukuoka Pref., Japan	Phylloplane	Uemori *et al*. (2007)
B0195	Fukuoka Pref., Japan	Soil	Uemori *et al*. (2007)
V–31–5	Hanoi, Vietnam	Soil	Yasutake *et al*. (2006)
V–79–25	Hanoi, Vietnam	Soil	Yasutake *et al*. (2006)
V–87–29	Hanoi, Vietnam	Soil	Yasutake *et al*. (2006)
V–92–10	Hanoi, Vietnam	Soil	Yasutake *et al*. (2006)
H–serovar reference strains (insecticidal)			
thuringiensis (H1)			de Barjac and Bonnefoi (1962)
kurstaki (H3abc)			de Barjac and Lemille (1970)
sotto (H4ab)			Bonnefoi and de Barjac (1963)
galleriae (H5ab)			de Barjac and Bonnefoi (1972)
aizawai (H7)			Bonnefoi and de Barjac (1963)
morrisoni (H8ab)			Bonnefoi and de Barjac (1963)
israelensis (H14)			de Barjac (1978)
japonensis (H23)			Ohba and Aizawa (1986b)

Motility test and spore morphometry

Motilities of vegetative cells of the PS1–producing *B. thuringiensis* strains were examined by microscopic observation of 18–h broth cultures. Also, Craigie's tube test (Craigie, 1931) was done to determine the motility of the organisms. Used as the positive controls were the 8 H–serovar reference strains of insecticidal Cry–protein producing *B. thuringiensis* (Table 1).

For morphometric characterization of spores, *B. thuringiensis* strains were grown on nutrient agar at 27˚C for 7 days. Fully sporulated cultures were harvested and suspended in distilled water. Spore suspensions were then smeared on a glass slide and dried at room temperature. This was followed by fixation of smears by gently flame heating. The fixed preparations were observed with an Olympus BX50 phase–contrast microscope (Olympus, Tokyo, Japan) at a magnification of 1500. The images were photographed to measure the length and diameter of 100 individual spores for each strain of *B. thuringiensis*.

Physiological characterization

Physiological characterization of the strains was done with API 50CHB system (BioMérieux, Marcyl 'Etoile, France) according to the manufacturer's instruction. Bacteria were grown on nutrient agar at 27˚C overnight prior to use, then vegetative cells were applied to the test.

Hemolytic activity and antibiotics susceptibility

Hemolysis tests were done by culturing *B. thuringiensis* strains on blood agar containing sheep erythrocytes (Nippon Biotest Lab., Tokyo, Japan). Hemolytic activities were also tested with the supernatant of 24–h broth culture by the method described previously (Kubota *et al*., 2006).

The antibiotic susceptibility of the 16 strains was tested by the disk diffusion method with BD Sensi–disc (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instruction. The test involved 10 antibiotics: ampicillin, kanamycin, gentamicin, streptomycin, tetracycline, chloramphenichol, ciprofloxacin, nalidixic acid, bacitracin, and SXT (sulfamethoxazole+trimethoprim). The assays were done in triplicate.

PCR for the gene *atxA*

Ten *B. thuringiensis* strains, 8 PS1 producers and 2 insecticidal strains, were examined for the presence of *atxA*, a regulatory gene of the *Bacillus anthracis* toxin. This study did not involve *B. anthracis* strains, the positive controls. For PCR experiments, total genomic DNAs were obtained from vegetative cells by the method of Kronstadt *et al*. (1983). A primer set for PCR was designed on the basis of the sequence of *atxA* reported by Adone *et al*. (2002): the forward sequence was 5'–GACATGCTAACACCGATATCC–3', and the reverse was 5'–TGCATTCACCGTTCTTTCC–3'. The oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan). PCRs were performed as previously described (Wasano and Ohba, 1998). The PCR products were analysed on agarose gel electrophoresis for the presence of 1500–bp *atxA*–specific nucleotide (Adone *et al*., 2002).

PCR for the SG–749 fragment specific for the *B. cereus* group

Amplification of SG–749 was done according to the method of Daffonchio *et al*. (1999). The forward sequence (SG–749f) used was 5'–ACTGGCTAATTATG-TAATG–3' and the reverse (SG–749r) was 5'–ATAAT-TATCCATTGATTTCG–3'. The oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan). PCR was carried out as described previously (Wasano and Ohba, 1998).

RESULTS

Microscopic observations revealed that no motilities are associated with vegetative cells of the PS1–producing *B. thuringiensis* strains. In contrast, those of the insecticidal reference strains of *B. thuringiensis* proved actively motile in broth cultures. Likewise, the PS1 producers failed to pass through the Craigie's tube, supporting the above findings.

Table 2 shows the results of morphometric analysis of the spores in 16 strains of *B. thuringiensis*. The spore length of PS1 producers ranged from 0.72 to

Table 2. Spore dimensions of *Bacillus thuringiensis* strains

B. thuringiensis strains	Length (μm)	Diameter (μm)	Aspect ratio*
Parasporin–1 producers			
A1109	0.78 (0.01)	0.64 (0.01)	1.24 (0.02)
A1965	0.79 (0.01)	0.64 (0.01)	1.24 (0.01)
B0186	0.96 (0.01)	0.78 (0.01)	1.24 (0.02)
B0195	0.72 (0.01)	0.77 (0.01)	1.21 (0.02)
V–31–5	0.76 (0.01)	0.63 (0.01)	1.21 (0.02)
V–79–25	0.93 (0.01)	0.79 (0.01)	1.19 (0.01)
V–87–29	0.88 (0.01)	0.73 (0.01)	1.23 (0.01)
V–92–10	0.90 (0.01)	0.78 (0.01)	1.16 (0.01)
H–serovar reference strains			
thuringiensis	0.88 (0.01)	0.57 (0.01)	1.55 (0.02)
kurstaki	1.06 (0.01)	0.62 (0.01)	1.73 (0.02)
sotto	0.85 (0.01)	0.42 (0.01)	2.01 (0.02)
galleriae	1.06 (0.01)	0.62 (0.00)	1.73 (0.02)
aizawai	1.04 (0.01)	0.62 (0.01)	1.67 (0.02)
morrisoni	0.96 (0.01)	0.47 (0.01)	2.08 (0.03)
israelensis	1.06 (0.01)	0.62 (0.01)	1.72 (0.02)
japonensis	1.08 (0.01)	0.64 (0.01)	1.96 (0.01)

* The aspect ratio was calculated by dividing the length by the diameter of each individual spore. Figure in parentheses is the standard error of the mean (n=100).

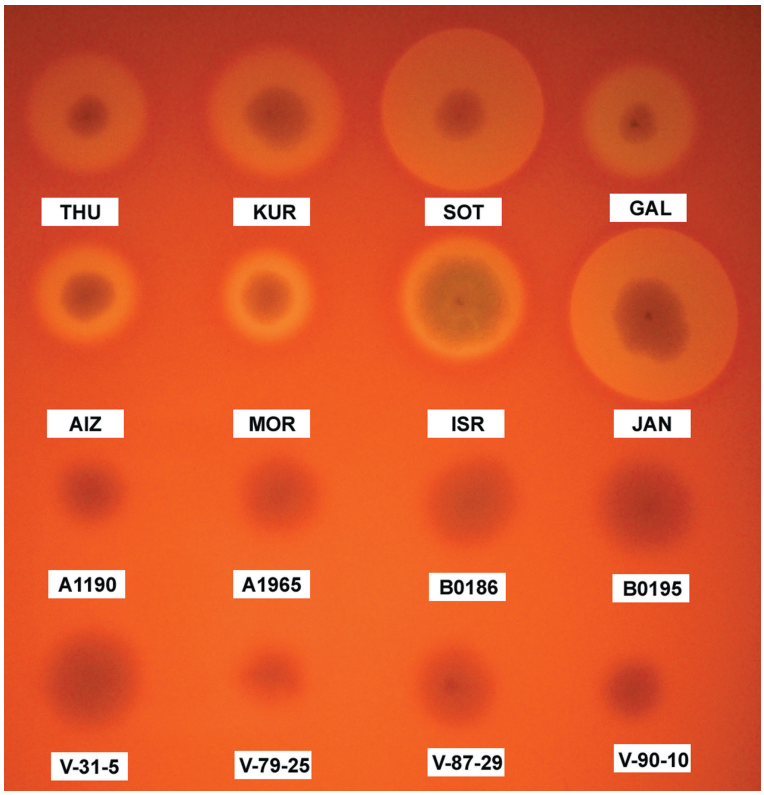

Fig. 1. Hemolysis assay of *Bacillus thuringiensis* strains on sheep blood agar. Hemolysis–positive strains: THU, the type strain of serovar *thuringiensis* (H1); KUR, *kurstaki* (H3abc); SOT, *sotto* (H4ab); GAL, *galleriae* (H5ab); AIZ, *aizawai* (H7); MOR, *morrisoni* (H8ab); ISR, *israelensis* (H14); JAN, *japonensis* (H23). Hemolysis–negative strains: Japanese PS1 producers (A1190, A1965, B0186 and B0195) and Vietnamese PS1 producers (V–31–5, V–79–25, V–87–29 and V–92–10).

0.96 μm, while those of the insecticidal reference strains ranged from 0.85 to 1.08 μm. The diameter of spores measured 0.63–0.78 μm for the PS1 producers and 0.42–0.64 μm for the insecticidal reference strains. A significant difference was observed between the two groups in aspect ratio (length / diameter) of the spore; 1.16–1.24 in the PS1 producers and 1.55–2.08 in the insecticidal reference strains.

The 16 *B. thuringiensis* strains were examined with API 50CH for carbohydrate utilization abilities (Table 3). Marked positive reactions were observed in all or most of the PS1–producing strains, when tested on

Table 3. Physiological characterization of *Bacillus thuringiensis* strains with API 50CH test

	H–serovar reference strains								Parasporin–1 producers							
Carbohydrates	*thuringiensis*	*kurstaki*	*sotto*	*galleriae*	*aizawai*	*morrisoni*	*israelensis*	*japonensis*	A1190	A1965	B0186	B0195	V–31–5	V–76–25	V–87–29	V–92–10
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycerol	w	w	w	w	w	w	+	w	w	w	–	w	–	w	–	+
Erythritol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L–Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ribose	+	+	+	+	+	+	+	+	+	+	–	+	+	w	+	+
D–Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L–Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
β–Methylxyloside	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Galactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D–Fructose	+	+	+	+	+	+	+	+	w	w	–	w	+	–	w	+
D–Mannose	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–
L–Sorbose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dulcitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α–Methyl–D–mannoside	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α–Methyl–D–glucoside	–	–	–	w	–	–	–	–	–	–	–	–	–	–	–	–
N–Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+
Amygdalin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arbutin	+	+	+	+	+	+	+	+	w	–	–	–	–	–	–	–
Esculin	+	+	+	+	+	+	+	+	–	–	–	–	+	w	+	–
Salicin	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–
Cellobiose	+	w	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Melibiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	–	+	–	–	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Raffinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
β–Gentiobiose	w	–	w	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Turanose	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
D–Lyxose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Tagatose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Fucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L–Fucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D–Arabitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L–Arabitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2–Ketogluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5–Ketogluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+, positive; –, negative; w, weak reaction.

Table 4. Antibiotic susceptibility of *Bacillus thuringiensis* strains

B. thuringiensis strains	Antibiotics									
	AM10	K30	GM10	S10	TE30	C30	CIP5	NA30	B10	SXT
H–serovar reference strains										
thuringiensis	++	+	–	–	+	–	–	+	++	+
kurstaki	++	+	–	–	–	–	–	–	+	++
sotto	++	–	–	–	–	–	–	–	+	++
galleriae	++	+	–	–	+	–	–	–	+	++
aizawai	++	+	–	–	–	–	–	–	++	+
morrisoni	++	–	–	–	+	–	–	–	++	++
israelensis	++	–	–	–	–	–	–	–	++	+
japonensis	++	+	–	–	+	+	–	–	++	+
Parasporin–1 producers										
A1190	++	–	–	–	+	–	–	–	–	–
A1965	++	–	–	–	+	–	–	–	–	–
B0186	++	–	–	–	–	–	–	–	–	++
B0195	++	–	–	–	+	–	–	–	–	+
V–31–5	++	–	–	–	+	+	–	–	–	–
V–79–25	++	–	–	–	+	–	–	–	–	–
V–87–29	++	–	–	–	–	–	–	+	–	+
V–92–10	++	–	–	–	+	–	–	–	–	+

Antibiotics: AM10, ampicillin 10 mg; K30, kanamycin 30 mg; GM10, gentamicin 10 mg; S10, streptomycin 10 mg; TE30, tetracycline 30 mg; C30, chloramphenicol 30 mg; CIP5, ciprofloxacin 5 mg; NA30, nalidixic acid 30 mg; B10, bacitracin 10 mg; SXT, sulfamethox

ribose, D–glucose, *N*–acetylglucosamine, maltose, sucrose, treharose, starch, and glycogen. These carbohydrates were also utilized by the insecticidal reference strains. All or most of the insecticidal reference strains produced acids from arbutin, esculin, and salicin, while the PS1 producers did not.

When cultured on sheep blood agar, typical β–hemolysis was induced in all of the insecticidal reference strains, but not in the PS1 producers (Fig. 1). Also, no hemolytic activities were contained in the supernatants from 24–h broth cultures of PS1 producers. As shown in Table 4, the PS1 producers and the insecticidal reference strains were all resistant to ampicillin, and moderately resistant to tetracycline. Kanamycin was moderately inhibitory to the insecticidal reference strains but not to the PS1 producers. Also, the reference strains were resistant to bacitracin, while the PS1 producers were all susceptible to this antibiotic.

In PCR experiments, there was no amplification of the *atxA*, a regulatory gene of the *B. anthracis* toxin, while amplification of the SG–749 segment (750 bp), a DNA marker specific for the *B. cereus* group, occurred in all of the 10 strains used: the 8 PS1 producers; the *B. cereus* strain IAM1092; the reference strain of *B. thuringiensis* serovar *israelensis* (data not shown).

DISCUSSION

In the present study, none of the PS1–producing organisms retained motility. The findings are consistent with our previous observations (Mizuki *et al*., 1999; Yasutake *et al*., 2006; Uemori *et al*., 2007). This is of interest because motility is usually associated with vegetative cells of the *B. cereus/B. thuringiensis* group, but not *B. anthracis* (Logan, 2005).

One of the most striking aspects in the present study is that the spores of PS1 producers were morphometrically distinct from those of the insecticidal reference strains of *B. thuringiensis*. The difference was significant in diameter and aspect ratio of the spores; PS1 producer's spores were wider in diameter and smaller in aspect ratio than those of the insecticidal reference strains. Consequently, spores of the PS1 producers had an appearance of shortened ellipsoid, more round than those of the ordinary *B. thuringiensis* strains, as shown in our previous studies (Mizuki *et al*., 2000; Uemori *et al*., 2007). Carrera *et al*. (2007) reported that the aspect ratios of spores in *B. anthracis* authentic strains ranged from 1.56 to 1.90. Thus, the difference also exists in spore morphology between *B. anthracis* and PS1–producing *B. thuringiensis*. Likewise, Carrera *et al*. (2007) showed that the spores of authentic strains of *B. cereus* and *B. thuringiensis* had aspect ratios of 1.74 and 2.01, respectively.

Overall profiles of acid production from 50 carbohydrates revealed that the PS1 producers can be allocated to the *B. cereus* group. It also appeared that the PS1 producers are different from the insecticidal reference strains in having no or little ability to utilize arbutin, esculin, and salicin. Our results with the insecticidal reference strains of *B. thuringiensis* are in good agreement with the observation by Logan and Berkeley (1984) that the above three glycosides were metabolised by all of the *B. thuringiensis* strains tested.

It is noteworthy that the PS1 producers exhibited no hemolytic activities against sheep erythrocytes. It has been generally believed that, among the three species of the *B. cereus* group, only *B. anthracis* is non–hemolytic, but the other two, *B. cereus* and *B. thuringiensis*, exhibit strong hemolytic activities (Logan, 2005). In fact, a marked hemolysis was induced by the reference strains of *B. thuringiensis* in the

present study.

Our results showed that the PS1 producers are resistant to ampicillin as well as the insecticidal reference strains. It has been well established that *B. anthracis* is penicillin–susceptible, while *B. cereus* and *B. thuringiensis* are both resistant (Logan, 2005). It should be also noted that the PS1 producers appeared susceptible to bacitracin, while the insecticidal strains resistant. The origin of this difference is presently uncertain. Our findings, however, may provide a tool for discriminating between insecticidal Cry–protein producers and PS1 producers.

PCR experiments detected the SG–749 fragment, specific for the *B. cereus* group (Daffonchio *et al*., 1999), in the PS1 producers. This is in good agreement with another observation in this study that the PS1 producers were allocated to the *B. cereus* group by their physiological characteristics. This paper also examined the presence of *atxA*, a regulatory gene located on *B. anthracis* virulence plasmid pXO1 (Guidi–Rontani *et al*., 1999), in PS1 producers and insecticidal reference strains of *B. thuringiensis*. As mentioned above, *B. anthracis*, a positive control, was not involved in this study. The results suggest that the *atxA* gene is not associated with the present PS1 producers. In this regards, it should be noted that non–pathogenic *B. anthracis* strains, cured of the plasmid pXO1, no longer retain *atxA* gene (Robertson *et al*., 1990).

The PS1 producers used here have been presumptively allocated to *B. thuringiensis*. However, the present results, together with our previous observations (Mizuki *et al*., 1999; Yasutake *et al*., 2006; Uemori *et al*., 2007), highlight the difference in classic key characteristics between the PS1 producers and the other members of *B. cereus* group, including *B. anthracis*, *B. cereus*, and ordinary *B. thuringiensis* strains that produce insecticidal parasporal inclusions. First, the PS1–producing organisms are different from the other members of *B. cereus* group in morphological feature (aspect ratio) of the spores. Second, the organisms are phenotypically similar to *B. anthracis* but different from *B. cereus* and ordinary insecticidal *B. thuringiensis* strains in lacking motility and hemolytic activity. Unlike *B. anthracis*, however, the PS1 producers are resistant to the antibiotic ampicillin.

Future works will include the analysis of the bacterial rRNA and rDNA to examine whether the PS1 producers are genetically monomorphic and constitute a distinct clade when compared with the existing subgroups of the *B. cereus* group.

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