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Updating the H-antigen classification of *Bacillus thuringiensis*

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M.-M. LECADET, E. FRACHON, V. COSMAO DUMANOIR, H. RIPOUTEAU, S. HAMON, P. LAURENT AND I. THIÉRY. 1999. The classification of *Bacillus thuringiensis* strains has been revised and updated based on flagellar antigens which have been in use for many years. Sixty-nine serotypes and 13 sub-antigenic groups have now been identified, giving 82 serovars among the 3500 *B. thuringiensis* isolates of the IEBC Collection. The number of serovars has gradually increased with the total number of strains. The biochemical characters used have also been investigated and their value assessed for identification of *B. thuringiensis* at the subspecies level. A crystal analysis was carried out in terms of morphology, δ-endotoxin profiles and larvicidal activity for the newly identified serovars. It was found that atypical crystals, some with novel components, are becoming more common. No insect susceptible to these serovars has been discovered among known target species. The number of cross-reacting H-antigens among *B. cereus* strains is increasing and may be of biological significance.

INTRODUCTION

The discovery of *Bacillus thuringiensis* strains with activity against certain species of Diptera (Goldberg and Margalit 1977; de Barjac 1978) and Coleoptera (Krieg *et al.* 1983) suggested possible new insecticidal treatments. This led to considerable attention being focused on this entomopathogenic bacterium, the activity spectrum of which was initially thought to be limited to insects in the order Lepidoptera. In the last 10 years there has been a continuous worldwide search for natural isolates with activity against economically important target insects (Martin and Travers 1989; Bernhard *et al.* 1997; Chaufaux *et al.* 1997).

Today, several tens of thousands of isolates, probably more than 50 000 (Sanchis *et al.* 1996) obtained from numerous screening procedures (Kalfon and de Barjac 1985; Ohba 1996), are distributed among various private and public collections, and are considered to be potential 'reservoirs' for novel toxins.

There are several reasons for the increased interest in *B. thuringiensis*. For example, emergence of insect populations resistant to many chemicals has been rapid and there is a fear that resistance to the widely used *B. thuringiensis* or *B. sphaericus* toxins may develop. The surprising diversity of *B. thuringiensis* δ-endotoxin genes (Höfte and Whiteley 1989; Lereclus *et al.* 1993), and evidence of their possible multiplicity within individual strains (Sanchis *et al.* 1988; Delécluse *et al.* 1989), make tentative classification based on pathotypes impossible. Thus, it has become increasingly necessary to have simple and reliable tools for classifying *B. thuringiensis* strains according to significant criteria.

Of the phenotypic methods used, H-serotyping and biochemical characters have both contributed to the establishment of a useful classification of *B. thuringiensis* isolates. The differentiation of these strains into serovarieties was developed on the basis of flagellar antigens by de Barjac and Bonnefoi (1962), and has been used ever since (de Barjac and Frachon 1990). New H-serotypes are numbered and registered at the International Entomopathogenic *Bacillus* Centre (IEBC) Collection (Burges *et al.* 1982) at Institut Pasteur, Paris, France. However, it is necessary to know whether such a system is still representative of the diversity of *B. thuringiensis* and whether it is relevant given the genetic exchanges that are known to occur between subspecies and with the closely related species, *B. cereus*.

This work updates the H-classification for which the number of serotypes has steadily increased. It also reports on the

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present status of biochemical characters. The limitations of these methods were considered and compared with other approaches, including molecular methods, in terms of taxonomic value.

MATERIALS AND METHODS

Origin of the strains

The *B. thuringiensis* strains included in this study were sent for identification to the IEBC (Unité des Bactéries Entomopathogènes at Institut Pasteur, France), a WHO Collaborating Centre. These strains, obtained from many research groups throughout the world (more than 22 countries), constitute the basis of the IEBC Collection registered under No. 590 in the World Data Centre on Microorganisms (Japan). They are listed in a catalogue, which is updated every 2 years.

H-serotyping and designation of new serotypes

Serotype was determined by tube agglutination tests, using diluted H-specific antisera, as previously described by de Barjac (1981) and recently reviewed by Thiéry and Frachon (1997). Reference H-antisera, representative of the various serovars, were used at dilutions of 1/200–1/25 600, to determine the agglutination titre defined as the lowest dilution that agglutinated the antigenic suspension tested. A modified protocol from Thiéry and Frachon now uses the Micronic® polycarbonate 96 tubes in plastic Micronic® boxes (Polylabo, France) to perform the preliminary determination of antigenic-antisera agglutination (antisera dilutions 1/100–1/400). This gains time and sera supply. Depending on this first result, titration is performed until dilution 1/25 600 of the reacted antisera is reached. Any *B. thuringiensis* strain not detected by any of the reference antisera available at the time is considered to be a potential new serotype. An antiserum directed against an antigenic suspension of such a strain was prepared and tested against H-antigens of all known serotypes to test whether or not they were agglutinated by the new antiserum. If a cross-reaction with other antigenic factors occurred, the antiserum saturation technique was used (de Barjac 1981) to identify possible antigenic subfactors determining distinct serovars within a known serotype.

A micromethod performed in 96-well microplates has recently been described (Laurent *et al*. 1996). This reduces the amount of serum required and saves time. The titration value was recorded using a magnifying glass or a Multi-Skan 352 MS apparatus (Life Sciences International; Labsystems OY, Finland) with a system to detect the wells in which agglutination took place.

Determination of biochemical characters

Standard biochemical tests are usually performed using classical methods as described by Sneath (1986) in *Bergey's Manual* and recorded by Smibert and Krieg (1994). During recent years, micromethods derived from the biochemical identification galleries have been introduced, especially regarding sugar utilization. The API identification systems for *Bacillus* sp. (API 20E + 50CHB; BioMerieux, Marcy l'Etoile, France), as reported by Logan and Berkeley (1984), are progressively replacing the classical methods that are still used as an alternative when necessary.

Presence and morphology of crystals

Sporulating cultures of the *B. thuringiensis* reference strains were produced in the standard *B. thuringiensis* medium (UG) containing Bactopeptone (7 g l^{-1}), glucose and salts as previously reported (Lecadet and Dedonder 1971). The presence of crystals, and their morphology, was recorded during sporulation, before and after cell lysis after 24 and 48 h, by direct examination of these cultures under the phase contrast microscope ($\times$1000), and confirmed by staining with Coomassie Brilliant Blue (0·25% solution in 50% ethanol and 7% acetic acid) as described by Sharif and Alaeddinoglu (1988) with the following modification: before coloration, bacterial smears on slides were washed with a 50/50 mixture of ethanol and acetone (Frachon unpublished). This staining procedure has proved very useful in many cases for confirming the presence of a parasporal inclusion.

SDS-PAGE analysis

The protein profiles of crystal components from the *B. thuringiensis* reference strains were determined by SDS-PAGE analysis, as described by Laemmli (1970), using 10% or 12·5% acrylamide separating gels. Samples (5–15 μg) of washed spore-crystal mixtures or purified crystals, prepared as described by Thomas and Ellar (1983), were placed in 2 $\times$ concentrated sample buffer and heated at 100 °C for 10 min, as previously described (Lecadet *et al*. 1992) and loaded onto the gel immediately before electrophoresis.

RESULTS

Updating the serological classification

After more than three decades, classification of *B. thuringiensis* strains according to H-serotypes is still an efficient way of classifying strains on the basis of stable and specific characters, thereby preventing confusion with the thousands of isolates available worldwide.

The present state of this classification is shown in Table 1.

By the end of 1998, 69 serotypes and 82 serovars were recorded and named. A previous classification status published by de Barjac and Frachon (1990) listed 27 serotypes and 34 serovars. Thus, 42 serotypes, or 48 serovars, were identified during this period, indicating a large increase.

Table 2 shows changes in the number of serovars relative to the total number of strains identified at the IEBC collection. Despite rapid increases since 1992, it is clear that the ratio has remained almost constant over the years, with a value of 2–2·5%. Therefore, the number of serotypes or serovars increases with the total number of isolates, leading to a relatively steady increase in the number of new H-antigenic structures. This suggests the stable development of these characters, resulting in a useful phenotypic classification which provides guidelines for the thousands of isolates in worldwide collections (Table 1). The new serovars were identified among strains from at least 22 countries: 11 of the serovars originated from Spain and the Azores, seven were from China, six from South Korea and three from South America (Brazil and Argentina). Many studies on serovar identification and preliminary characterization are now available or in progress.

Biochemical characters

The traditional phenotypic tests were long considered to be the key for the formal description of taxa at the species or subspecies level (Vandamme *et al.* 1996). However, de Barjac and Frachon (1990) showed with the 27 H-serotypes and the 34 serovars known at that time, that "most serovars cannot be distinguished by biochemical characters". Therefore, how useful are biochemical characters for *B. thuringiensis* strain identification, given the large number of serovars identified? The use of miniaturized systems such as the commercially available API galleries, complemented by classical methods when necessary, is an effective way of comparing large numbers of strains, given the large number of characters. This work is mostly concerned with the determination of more than 80 characters for the 40 new serotypes identified since 1990. Several important characteristics were identified by this evaluation (Table 3).

The biochemical characters were roughly grouped into three major categories. The first consisted of characters positive for all serovars, as indicated in Table 3. The second grouped together those characters that are generally negative. This category includes the use of many sugars (35 of the 50 substrates of the API 50 CH galleries), and several enzymes, such as lysine decarboxylase (LDC), ornithine decarboxylase (ODC), tryptophan deaminase (TDA), β-galactosidase and indole production. Only one (H24a24b) of the 3500 *B. thuringiensis* isolates of the IEBC collection contained β-galactosidase and fermented galactose, whereas the other known strains of this serovar, and strains of serovar *novosibirsk* (H24a24c), did not. Indole production was not detected in any of the *B. thuringiensis* strains of the collection.

The third category grouped together characters which are taxonomically useful because they act as discriminant factors between serovars. The main factors in the group were: the presence of arginine dihydrolase (ADH) or enzymes for reduction of nitrates, urease, and the ability to ferment sucrose, mannose, cellobiose or salicin. Also included in this group is the production of acetyl-methyl-carbinol (AMC) that is negative only in a few cases. These are precisely the elements of the key previously described (de Barjac and Frachon 1990). Such characters were used to compare the various antigenic subgroups of the serotypes tested, and to compare several isolates for particular serovars.

Such discriminant characters (e.g. ADH, urease, sucrose and mannose) may differ between serovars of the same serotype, but with different sub-antigenic characterization. Isolates from a single serovar may also differ from each other in one or two characters. For example, in serovar *morrisoni* (H8a8b), six isolates were identical for the major characters AMC, CIT utilization, NO_3 reduction, sucrose and mannose utilization, but differed in ADH production. Two of the six strains were ADH^+, the others being ADH^- (including the reference strain *morrisoni*), and these two strains have activity against Coleoptera. Only one of the six strains was positive for mannose. Many more samples must be compared before it will be possible to conclude that most of the discriminant characters are the same within a given serovar. However, the discriminant characters for the serovar *kurstaki* appear to be homogeneous.

Finally, from these investigations conducted mainly with newly characterized serovars, it is concluded that the biochemical key is still of value, but it cannot be used in isolation to differentiate between or within serovars. This procedure used in conjunction with H-serotyping does help to distinguish between several possibilities when serotyping is unclear.

There are also technical considerations concerning reliability of micromethods, particularly the API systems. Such methods are effective for many characters, particularly for sugar utilization, and the results are consistent with those obtained by classical methods. For a very small number of characters (two or three), there may be some ambiguity. This is the case for urease, AMC production and sometimes also for ADH. Differences in the substrates used for the two methods may account for conflicting results. It is therefore advisable to use classical methods as well as micromethods in a few cases.

Crystal morphology and δ-endotoxin profiles

The morphology and biochemical features of bacterial species are among the classical phenotypic properties used to define

Table 1 Classification of *Bacillus thuringiensis* strains according to the H serotype

H antigen	Serovar	Abbreviation	First mention and/or first valid description
1	*thuringiensis*	THU	Berliner 1915* ; Heimpel and Angus 1958
2	*finitimus*	FIN	Heimpel and Angus 1958
3a, 3c	*alesti*	ALE	Toumanoff and Vago 1951 ; Heimpel and Angus 1958
3a, 3b, 3c	*kurstaki*	KUR	de Barjac and Lemille 1970
3a, 3d	*sumiyoshiensis*	SUM	Ohba and Aizawa 1989
3a, 3d, 3e	*fukuokaensis*	FUK	Ohba and Aizawa 1989
4a, 4b	*sotto*	SOT	Ishiwata 1905 ; Heimpel and Angus 1958
4a, 4c	*kenyae*	KEN	Bonnefoi and de Barjac 1963
5a, 5b	*galleriae*	GAL	Shvetsova 1959* ; de Barjac and Bonnefoi 1962
5a, 5c	*canadensis*	CAN	de Barjac and Bonnefoi 1972
6	*entomocidus*	ENT	Heimpel and Angus 1958
7	*aizawai*	AIZ	Bonnefoi and de Barjac 1963
8a, 8b	*morrisoni*	MOR	Bonnefoi and de Barjac 1963
8a, 8c	*ostriniae*	OST	Ren *et al*. 1975
8b, 8d	*nigeriensis*	NIG	Weiser and Prasertphon 1984
9	*tolworthi*	TOL	Norris 1964 ; de Barjac and Bonnefoi 1968
10a, 10b	*darmstadiensis*	DAR	Krieg de Barjac and Bonnefoi 1968
10a, 10c	*londrina*	LON	Arantes *et al*. (unpublished)
11a, 11b	*toumanoffi*	TOU	Krieg 1969
11a, 11c	*kyushuensis*	KYU	Ohba and Aizawa 1979
12	*thompsoni*	THO	de Barjac and Thompson 1970
13	*pakistani*	PAK	de Barjac, Cosmao Dumanoir, Shaik and Viviani 1977
14	*israelensis*	ISR	de Barjac 1978
15	*dakota*	DAK	De Lucca, Simonson and Larson 1979
16	*indiana*	IND	De Lucca, Simonson and Larson 1979
17	*tohokuensis*	TOH	Ohba, Aizawa and Shimizu 1981
18a, 18b	*kumamotoensis*	KUM	Ohba, Ono, Aizawa and Iwanami 1981
18a, 18c	*yosoo*	YOS	Lee, H. H. *et al*. 1995
19	*tochigiensis*	TOC	Ohba, Ono, Aizawa and Iwanami 1981
20a, 20b	*yunnanensis*	YUN	Wan-Yu, Qi-Fang, Xue-Ping and You-Wei 1979*
20a, 20c	*pondicheriensis*	PON	Rajagopalan *et al*. (unpublished)
21	*colmeri*	COL	De Lucca, Palmgren and de Barjac 1984
22	*shandongiensis*	SHA	Wang Ying *et al*. 1986
23	*japonensis*	JAP	Ohba and Aizawa 1986
24a, 24b	*neoleonensis*	NEO	Rodriguez-Padilla *et al*. 1988
24a, 24c	*novosibirsk*	NOV	Burtseva, Kalmikova *et al*. 1995
25	*coreanensis*	COR	Lee H. H. *et al*. 1994
26	*silo*	SIL	de Barjac and Lecadet (unpublished)
27	*mexicanensis*	MEX	Rodriguez-Padilla and Galan-Wong (unpublished)
28a, 28b	*monterrey*	MON	Rodriguez-Padilla *et al*. (unpublished)
28a, 28c	*jegathesan*	JEG	Seleena, Lee, H. L. and Lecadet 1995
29	*amagiensis*	AMA	Ohba (unpublished)
30	*medellin*	MED	Orduz, Rojas, Correa, Montoya and de Barjac 1992
31	*toguchini*	TOG	Hodirev (unpublished)
32	*cameroun*	CAM	Jacquemard, 1990* ; Juarez-Perez *et al*. 1994
33	*leesis*	LEE	Lee H. H. *et al*. 1994
34	*konkukian*	KON	Lee H. H. *et al*. 1994
35	*seoulensis*	SEO	Lee H. H. *et al*. 1995
36	*malaysiensis*	MAL	Ho (unpublished)
37	*andaluciensis*	AND	Aldebis, Vargas-Osuna and Santiago-Alvarez 1996
38	*oswaldocruzi*	OSW	Rabinovitch *et al*. 1995
39	*brasiliensis*	BRA	Rabinovitch *et al*. 1995
40	*huazhongensis*	HUA	Dai Jingyuan *et al*. 1996

Table 1 *Continued.*

H antigen	Serovar	Abbreviation	First mention and/or first valid description
41	*sooncheon*	SOO	Lee H. H. *et al.* 1995
42	*jinghongiensis*	JIN	Li Rong Sen *et al.* (in press)
43	*guiyangiensis*	GUI	Li Rong Sen *et al.* (in press)
44	*higo*	HIG	Ohba *et al.* 1995
45	*roskildiensis*	ROS	Hinrinschen, Hansen and Daamgaard (unpublished)
46	*chanpaisis*	CHA	Chanpaisaeng (unpublished)
47	*wratislaviensis*	WRA	Lonc *et al.* 1997
48	*balearica*	BAL	Caballero *et al.* (unpublished)
49	*muju*	MUJ	Seung Hwan Park *et al.* (unpublished)
50	*navarrensis*	NAV	Caballero *et al.* (unpublished)
51	*xiaguangiensis*	XIA	Jian Ping Yan (unpublished)
52	*kim*	KIM	Kim *et al.* (unpublished)
53	*asturiensis*	AST	Aldebis, Vargas-Osuna and Santiago-Alvarez 1996
54	*poloniensis*	POL	Damgaard *et al.* (unpublished)
55	*palmanyolensis*	PAL	Santiago-Alvarez *et al.* (unpublished)
56	*rongseni*	RON	Li Rong Sen (in press)
57	*pirenaica*	PIR	Caballero *et al.* (unpublished)
58	*argentinensis*	ARG	Campos-Dias *et al.* (unpublished)
59	*iberica*	IBE	Caballero *et al.* (unpublished)
60	*pingluonsis*	PIN	Li Rong Sen (in press)
61	*sylvestriensis*	SYL	Damgaard (unpublished)
62	*zhaodongensis*	ZHA	Li Rong Sen (in press)
63	*bolivia*	BOL	Ferré-Manzanero *et al.* (unpublished)
64	*azorensis*	AZO	Santiago-Alvarez *et al.* (unpublished)
65	*pulsiensis*	PUL	Khalique F. and Khalique A. (unpublished)
66	*graciosensis*	GRA	Santiago-Alvarez *et al.* (unpublished)
67	*vazensis*	VAZ	Santiago-Alvarez *et al.* (unpublished)
68	*thailandensis*	THA	Chanpaisaeng *et al.* (unpublished)
69	*pahangi*	PAH	Seleena and Lee H. L. (unpublished)

* First mention or designation of the serovar.

Table 2 Changes in the number of serovars identified with the number of strains evaluated

Year	Number of *Bacillus thuringiensis* isolates*	No. of H-serovars	Percentage of total
1981†	700	20	2·86
1992‡	1600	34	2·13
1992§	2285	42	1·84
1994§	2630	58	2·21
1996§	2970	68	2·29
1998§	3400	80	2·35

* Number of isolates assessed for identification at IEBC.
† de Barjac 1981.
‡ de Barjac and Frachon 1990.
§ Updates IEBC catalogue.

Table 3 Biochemical characters of serovars identified since 1990

Positive characters*	Negative characters†	Discriminant characters‡
Hydrolysis of:	β-Galactosidase	Arginine dihydrolase
starch, gelatin, glycogen,	Indole production	Urease
esculin,	Ornithine decarboxylase	Acetyl-methyl-carbinol production (VP)
N-acetyl-glucosamine	Lysine decarboxylase	Nitrate reduction
	Tryptophan deaminase	Utilization of citrate
	H_2S production	
Fermentation of:	Fermentation of:	Fermentation of:
glucose, fructose, maltose,	galactose, lactose, mannitol	sucrose, mannose, arbutin, salicin,
trehalose, ribose		cellobiose

These are the results for the 46 serovars identified since 1990 and thus, not reported in a previous study (de Barjac and Frachon 1990) namely serotypes H28 to H67, plus seven subgroups.

* Characters for which tests gave a positive reaction in all *Bacillus thuringiensis* serovars tested, with the exception of H51 which was negative for starch, glycogen and ribose, and H67 which was negative for ribose.

† Characters for which the tests gave a negative reaction with the exception of H65 which was galactose$^+$.

‡ Discriminant characters are those that may differ between serovars.

taxa. This may be valuable for parasporal inclusions which are also responsible for the larvicidal activity of *B. thuringiensis* strains. The presence or absence of crystals is the major criterion for distinguishing between *B. thuringiensis* and *B. cereus*. Therefore, it is very important to be able to detect crystals and to determine their morphology, which, in some cases, may reflect a kind of specificity because that crystal may have various forms, depending on subunit composition. Sporulating cultures of the reference strains of serotypes H30 to H67, as well as of subgroups 10a10c, 18a18c, 24a24c and 28a28c, were examined by microscopy as described in Material and Methods. The protein profiles of crystal components were determined by SDS-PAGE analysis, useful for characterizing δ-endotoxin families; some of them are presented in Figs 1 and 2. As the number of strains and serovars increases, it should be possible to gain more insight into the evolution of such characters.

About 40% of the reference strains produced crystals with classical morphology, either bipyramidal or cuboid (Table 4). The proteins of these crystals are well characterized and are typical of strains active against lepidopteran species (although a precise target has not necessarily been determined). A much smaller percentage of strains had spherical crystals that were closely associated or stacked, like those of *B. thuringiensis israelensis*. This shape was correlated with a particular protein profile. Only one of the 42 reference strains had large flat rectangular crystals with a protein profile typical of the *tenebrionis* strain. However, a large proportion (more than 50%) of the reference strains produced atypical crystals, often heterogeneous in size and shape with protein profiles consisting of many poorly defined components, or novel profiles including polypeptides of 30–50 kDa in size, or larger than 160 kDa, as reported by several authors (Juarez-Perez *et al*. 1994; Burtseva *et al*. 1995; Chaufaux *et al*. 1997). The results of SDS-PAGE analysis referring to crystal proteins from the reference strains of 18 different serovars are shown in Figs 1 and 2. These strains are among those which display atypical and heterogeneous crystals. A great diversity of profiles was observed, some indicating components lower than 60 kDa (Fig. 1, lanes 4, 5, 6 and Fig. 2, lane 1), and others showing multiple components (Fig. 1, lanes 8, 10 and Fig. 2, lane 7) or components ranging between 100 and 65 kDa (Fig. 2, lanes 4, 5, 6); components higher than 160 kDa, as seen in Fig. 2, lane 8 (indicating a polypeptide of about 200 kDa), appeared much less frequently. Most of these different crystal components have not yet been characterized. These figures also indicate that two subgroups of a same serotype may have crystals with very different profiles (Fig. 1, lanes 3 and 4).

In most cases, and typically for serotypes described in the last 10 years, no target insects have been identified. The mosquitocidal activity has been determined for the 40 strains. The *jegathesan* (H28a28c) (Seleena *et al*. 1995), *medellin* (H30) (Orduz *et al*. 1992) and one other strain had high levels of mosquitocidal activity (with the expected protein profiles), and four other strains presented a weak but significant activity against *Culex* or *Aedes* species (Ragni *et al*. 1996). In these four strains, the observed activity was correlated with the presence of components cross-reacting with Cry4B, Cry11 A or CytA (unpublished data). This also makes it impossible to determine the relationship between serovars and pathovars.

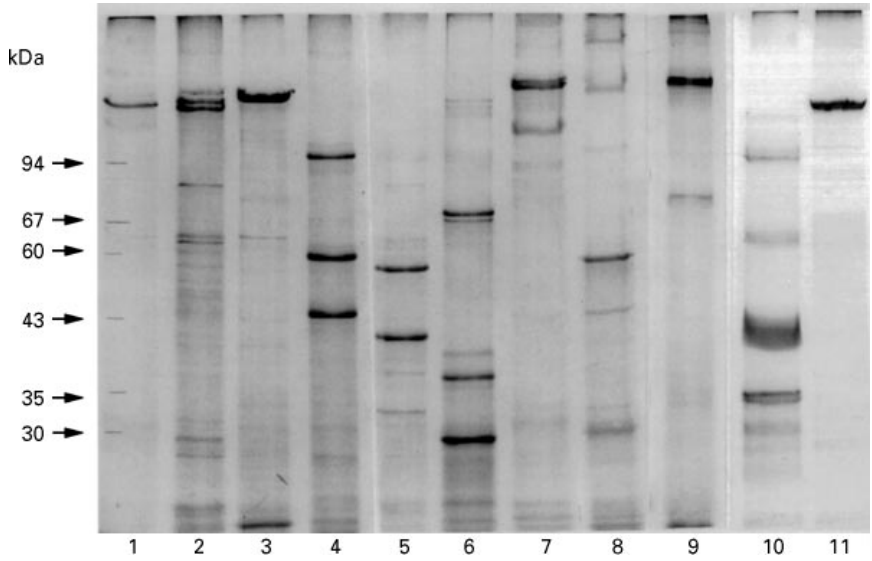

Fig. 1 SDS-PAGE analysis of crystal proteins from the reference strains of differents *B. thuringiensis* (*Bt*) serovars. Lane 1, *Bt* ser. *darmstadiensis* (H10a10b); lane 2: *Bt* ser. *londrina* (H10a10c); lane 3: *Bt* ser. *neoleonensis* (H24a24b); lane 4: *Bt* ser. *novosibirsk* (H24a24c); lane 5: *Bt* ser. *seoulensis* (H35); lane 6: *Bt* ser. *malaysiensis* (H36); lane 7: *Bt* ser. *oswaldocruzi* (H38); lane 8: *Bt* ser. *brasiliensis* (H39); lane 9: *Bt* ser. *huazhongensis* (H40); lane 10: *Bt* ser. *jinghongiensis* (H42); lane 11: *Bt* ser. *guiyangiensis* (H43). Experiment was performed as described in Materials and Methods

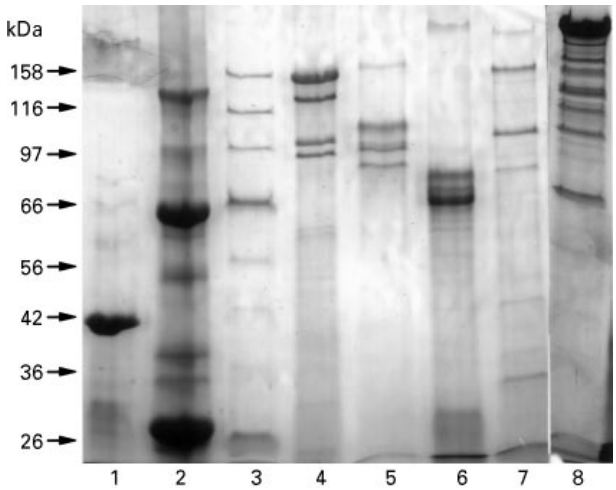

Fig. 2 SDS-PAGE analysis of crystal proteins from the reference strains of differents *B. thuringiensis* (*Bt*) serovars. Lane 1: *Bt* ser. *graciosensis* (H66); lane 2: *Bt* ser. *israelensis* (H14); lane 3: Molecular weight markers; lane 4: *Bt* ser. *azorensis* (H64); lane 5: *Bt* ser. *argentinensis* (H58); lane 6: *Bt* ser. *palmanyolensis* (H55); lane 7: *Bt* ser. *muju* (H49); lane 8: ser. *chanpaisis* (H46). Experiment was performed as described in Materials and Methods

However, there are several correlations involving previously characterized serotypes (before H28).

Several methods of investigation were used, including the detection of several δ-endotoxin gene-products, to obtain information about crystal components.

(i) It is clear from many studies, as reviewed by Delécluse *et al*. (1996), that most of the highly active mosquitocidal strains belong to H14 and all have the same subunit composition. Other very active strains were identified among new serotypes (after H28a28b) as described above. Strains with high or moderate activity were found among well characterized serotypes (H5a5c, H10a10b, H12) and they had various subunit structures (Ragni *et al*. 1996). Other strains with low activity were also detected (Ishii and Ohba 1993). Thus, mosquitocidal strains have been classified within four groups, from highly toxic to low toxicity (Ohba *et al*. 1995; Delécluse *et al*. 1996; Ragni *et al*. 1996).

(ii) It has been regularly observed that new strains with activity against *Spodoptera littoralis* and *S. exigua* belong mostly to two main serotypes, H6 (*entomocidus*) and H7 (*aizawai*), which contain the *cry1C* gene (unpublished). However, it cannot be inferred from this observation that all strains of these serovars have the same activity, or that such activity does not exist in other serotypes. A strain of serotype H1 containing the Cry1C δ-endotoxin and the corresponding gene has recently been identified (data not published). Strains with moderate activity were also detected among various serovars, *kenyae* (H4a4) or *tolworthi* (H9) for example, in which *cry1E* or variants were detected (data not published).

(iii) Another striking example frequently reported is serovar *morrisoni* (H8a8b) that brings together the highly mosquitocidal strain, PG14, which has crystal components similar to those of *B. thuringiensis israelensis*, other strains such as strain *tenebrionis* with activity against Coleoptera and typical subunit composition, and some strains active against Lepidoptera.

Thus, there are many types of relationships between the major known pathotypes and the H classification. However, there are some invariant features which must be kept in mind.

Table 4 Crystal protein profiles and morphology

Protein profile	Percentage of total*	Crystal morphology	Percentage of total*
Common profiles			
'Lepidopteran' like or equivalent:	39	Bipyramidal	30
one or several bands at 130–150 kDa		or	
with or without a component at 65–70 kDa		cuboid	7
'*Bt israelensis* type': 125-135-68-28 kDa	2·5	Spherical to ovoid	5
'Strain *tenebrionis* type'	2·5	Flat-rectangular	2·5
Atypical profiles			
Multiple bands	14	Atypical and	
New profiles including 30–50 kDa components	41	heteromorphic	55

* These are the results for 42 new serovars.

The *B. thuringiensis* classification and related problems

The H-serotyping method, as currently defined and used, provides a simple and efficient tool for classifying strains of *B. thuringiensis* species, the characteristic of which is the presence of one or more parasporal inclusions. However, there are at least two problematic situations: (i) strains lacking a parasporal inclusion and thus considered to be *B. cereus* and (ii) 'autoagglutinated strains'. As the number of *B. thuringiensis* strains in collection steadily increases, the number of isolates falling into these two categories also increases in the same proportion.

Some *B. cereus* strains have antigens that cross-react with sera specific for *B. thuringiensis* H-serotypes (de Barjac and Bonnefoi 1973; Ohba and Aizawa 1986). Some of the *B. cereus* isolates of the IEBC Collection were serotyped (Table 5). Ninety-two of the 194 isolates were agglutinated by antisera specific for various *B. thuringiensis* serotypes, of which the most representative were H14, H5, H10, H6 and H27. Such isolates may originate from ancient *B. thuringiensis* strains that have lost plasmid-encoded crystals. They may also be true *B. cereus* with antigens in common with *B. thuringiensis*. This notion is credible, assuming that *B. cereus* and *B. thuringiensis* have common phenotypic characters and are almost identical in terms of phylogenetic characters, as for 16 s RNA sequences (Ash *et al*. 1991). Investigation of more characters, particularly plasmid patterns and additional biochemical characters, is required to determine which of the two alternatives applies.

Similar results were obtained with populations of *B. cereus* strains from natural sources not included in the IEBC collection (Helgason *et al*. unpublished); 55% of the isolates studied had antigens in common with *B. thuringiensis* serotypes.

Serotyping cannot always be performed in true *B. thuringiensis* strains with normal inclusion bodies. 'Autoagglutinated' strains are not typeable. These strains make up almost 3% of the *B. thuringiensis* in the IEBC collection.

Cultures of these strains agglutinated spontaneously, particularly when subjected to the conditions required for the test at all growth stages, thereby making serotyping impossible. The reasons for this are not clear, but this is the only situation in which the H-classification is completely useless. For different reasons, a few *B. thuringiensis* strains, called non-motile strains, also escape H-serotyping (de Barjac and Frachon 1990).

DISCUSSION

The main aim of this paper was to update the *B. thuringiensis* classification based on H-flagellar antigens, and to assess the current validity of the method. We also compared our methods with others currently in use, including molecular techniques, to try to determine the most useful methods for future studies.

There has been a large increase in the number of serovars consistent with the increasing number of *B. thuringiensis* strains of the IEBC collection. The WHO Collaborating Centre for Entomopathogenic *Bacillus* is responsible for making statements on this point. We have thus made this information official to avoid confusion in numbering and naming new serotypes of *B. thuringiensis* as previously suggested by Burges

Table 5 Identification of *Bacillus thuringiensis* (*Bt*) H-serotypes among *B. cereus* strains

	Number of strains	Percentage of total
Total isolates examined	195	
Total of cross-reactions with *Bt* H-antigens:	92	47·2
H10, H14	10 (10·9)*	
H5, H6	8 (8·7)*	
H27	7 (7·6)*	
H20	6 (6·5)*	
H18, H19	5 (5·4)*	
H3a,3b,3c, H41	4 (4·3)*	
H13, H29, H34, H42	2 (2·2)*	
17 other serotypes	1 (1·1)*	
No cross-reaction with *Bt* H-antigens	81	41·5
Auto-agglutinated strains	22	11·3

These are the results for strains identified as *B. cereus* after 1993 and numbered as CER 616 to CER 810 at the IEBC.
* Percentage of total (92) cross-reacting isolates for each of the designated serovars.

et al. (1982). This work also demonstrated that the method efficiently classified *B. thuringiensis* isolates in most situations.

The test tube agglutination method is recommended (Kahn tubes or Micronic® tubes), at least for the titration step of identification. Titration could also be performed using a micromethod (Laurent *et al*. 1996) assuming that suitable automatic equipment is available for reading the plaques.

The set of reference strains and corresponding antisera could be used only for *B. thuringiensis* species as defined in *Bergey's Manual* (Sneath 1986). This requires a meticulous examination for the presence of crystals. There are mutants with no crystals resulting from *in vivo* mutagenesis or from plasmid curing of known *B. thuringiensis* strains. It is clear that such isolates, the origin of which is well established, cannot be considered to be *B. cereus* (de Barjac and Bonnefoi 1973). The situation for natural *B. cereus* isolates of various origins is quite different. If the method is applied to *B. cereus* strains, some ($\geqslant$45%) showed a positive relationship to *B. thuringiensis* H-antigens, whereas the others did not react at all. This is consistent with previous reports (Krieg 1969; Burges 1984; Ohba and Aizawa 1986; Helgason *et al*. unpublished). Further investigation is required as there may be genetic exchanges due to plasmid transfer. However, *B. cereus* is a widely dispersed species including heterogeneous subgroups.It would therefore be sensible to classify *B. cereus* strains possessing *B. thuringiensis* H-antigens into a subgroup *B. cereus*/*B. thuringiensis* H^+ commonly treated as a variety of *B. cereus*. More detailed knowledge of the H-antigen genes would be very useful.

'Autoagglutinated' strains have eluded H-classification. For unknown reasons, H-antigenic suspensions of these strains agglutinate spontaneously in the absence of specific antiserum under the conditions of the test procedure. They are motile strains because it is possible to prepare antigenic suspensions. It would be of interest to determine the factors involved in such a process. This is one of the limitations of the H-classification. The biochemical and genetic basis of the flagellar antigens system in *B. thuringiensis* is totally unknown and should be treated as a priority for investigation.

This work also stressed the difficulties in predicting any correlation between serotypes and pathotypes. However, it was still possible to establish a rough correlation between a pathotype and several well known serotypes or serovars. The diversity and the extreme variability of the δ-endotoxin genes (more than 100 genes have been cloned and more than 22 δ-endotoxin families and subgroups defined by Crickmore *et al*. 1998), and the frequent presence of multiple toxin genes in individual isolates, results in an infinite number of potential gene combinations, making any *B. thuringiensis* classification based on pathotypes impossible. In addition, very few serotypes identified within the last 10 years have a known, specific, target insect.

The value of biochemical characters was also examined in detail. A key was previously established (de Barjac and Frachon 1990) to differentiate groups of strains on the basis of determinant characters, with the aid of computer analysis involving about 80 characters. This rapidly proved to be inefficient for distinguishing between serovars. As the number of strains tested increases, it appears that some of the rare discriminant characters may vary not only between serovars, but also among strains of the same serovar. However, when conflicting serovar identification results are obtained, biochemical characters may provide additional discriminating information. Thus, there are situations in which biochemical

characters may be of value if used in conjunction with other methods.

Other phenotypic methods also have varying levels of success. For example, cellular fatty acid patterns are known to have high taxonomic resolution at the species level. This method, a chemotaxonomic method (Buss *et al.* 1996), with a high degree of automation, successfully differentiates groups within the *B. sphaericus* species (Frachon *et al.* 1991), whereas it cannot distinguish between *B. thuringiensis* subspecies or between *B. thuringiensis* and *B. cereus* (Frachon and Lecadet unpublished). Another approach based on susceptibility to different bacteriophages was developed by Ackerman *et al.* (1995) and resulted in the identification of phage types. As indicated by the authors, there was no correlation between H-antigen serotype and the 25 phagovars determined at that time, which showed frequent cross-reactions with *B. cereus.*

Many genotypic methods, widely used as tools for molecular taxonomy, suggest that there is a very close relationship between *B. thuringiensis, B. cereus* and even *B. anthracis*. Some distinguish, with various levels of success, *B. cereus* and *B. thuringiensis.* Giffel *et al.* (1997) reported possible discrimination between the two species, using specific DNA probes based on particular variable regions of 16 s RNA. Other studies (Bourque *et al.* 1995) suggest that the intergenic spacer region (ISA) is a discriminant probe.

Effective methods of subspecies analysis are needed to provide tools for a molecular biology-based classification. Several attempts using RFLP, DNA colony hybridization, RAPD or ribotyping, as reviewed by Damgaard (1996), have been reported. With regard to ribotyping, there are essentially two valuable approaches for investigating relationships between serotyping and this new method. Priest *et al.* (1994) examined 43 *B. thuringiensis* strains among 10 well known serovars and found a relatively good correspondence between some serovars and ribotype patterns, whereas a significant diversity of these patterns was observed. Another investigation reported by Akhurst *et al.* (1997) detected characteristic profiles for strains belonging to several widespread serovars (THU, KUR, ENT, AZA, MOR, TOL, DAR, TOU, ISR); in addition, their results indicated limited variations among strains within serovars that could be used as a tool to distinguish between strains, particularly between reisolates. In this line, the results of a preliminary investigation to cover the whole classification showed a diversification of ribotype patterns. Nevertheless, these appeared to be relatively constant within the serovars examined (THU, KUR, ISR) (Frachon and Delécluse, unpublished data). However, analysis of many more serotypes and strains within serovars is necessary. It is entirely possible that variability will increase with the number of strains examined. With regard to other molecular techniques, some, including DNA-colony hybridization and random amplified polymorphic DNA (RAPD) analysis, were investigated by Hansen *et al.* (1998). The two methods, which addressed different objectives, were found to be very informative, particularly with respect to screening procedures. Depending on the primers used, RAPD analysis could allow particular isolates to be distinguished within serovars, although profiles appear relatively characteristic of the serovars examined (mainly KUR). Once again, any comparison between these approaches and the phenotypic methods would require the study of many more strains within different serotypes. However, DNA colony hybridization, like RAPD, must be useful for grouping strains, either independently or in conjunction with others. There is a large scope for investigation in this field and much work is required for conclusions to be drawn.

Thus, the serotyping method based on H-antigens is still providing a valuable and reliable tool for discriminating between groups of *B. thuringiensis* strains. Consistent with previous work (Burges 1984; de Barjac and Frachon 1990), we found that serovars cannot be seen as subspecies, but rather as varieties constituting 'clear subdivisional structures' within the species, independently of crystal type. In the future, H-classification will have to be used in parallel with other methods, particularly for *B. cereus* cross-reacting strains and for 'autoagglutinated' strains for which serotyping is impossible. Developing simple molecular approaches should be of great value in these cases.

The need for accurate identification methods to differentiate between the continuously increasing number of *B. thuringiensis* strains requires the use of various complementary approaches, including molecular methods.

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