Diversity and differential distribution of IS231, IS232 and IS240 among *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides*

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***Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* are very closely related bacteria, generally considered as subspecies of *B. cereus sensu lato*. Different transposable elements have been isolated from *B. thuringiensis*, including IS231, IS232 and IS240 and their variants. The distribution of these three insertion sequences (IS) within the *B. cereus* group has been investigated in 90 strains of *B. thuringiensis* (representing 61 serovars), in 30 reference strains of *B. cereus* and in 33 strains of *B. mycoides*. Since these IS elements are delimited by well-conserved and specific inverted repeats, the use of primers corresponding to these ends allowed their amplification by PCR. The results showed that IS231 is the most abundant element in the three taxa, whereas IS232 is apparently exclusively associated with *B. thuringiensis*. Hybridization and *Dra*I RFLP analysis of the PCR products confirmed and extended knowledge of the heterogeneity previously observed among iso-IS231 elements. Moreover, a similar diversity was observed among iso-IS240 elements. This contrasted with the relative homogeneity displayed by iso-IS232 elements. No specific association appeared to exist between any particular iso-element and a specific strain or serotype.**

Keywords: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, insertion sequences, PCR

INTRODUCTION

The genus *Bacillus* is generally recognized as being more heterogeneous than most other bacterial genera. Within the genus, the *Bacillus cereus* group has been for a long time a subject of controversy. This group comprises the *B. cereus*, *B. thuringiensis*, *B. mycoides* and *B. anthracis* taxa.

B. cereus sensu stricto is an ubiquitous soil bacterium also found as a contaminant in dairy products, rice and meat. Some strains produce extracellular enterotoxins and cause food poisoning in man, and other toxins such as phospholipase C, haemolysins and emetic toxins can also be produced. *B. thuringiensis* is characterized by the synthesis of highly specific insecticidal toxins and is one of the most widely used biopesticides (Priest *et al.*, 1994). *B. mycoides* does not produce any toxin, is a saprophytic

organism and is identified unambiguously by its unique rhizoid colony morphology and its lack of motility. Finally, *B. anthracis* is the causative agent of anthrax in humans and animals. It can be differentiated from the other members of the group by its gamma phage susceptibility, penicillin sensitivity or lack of motility and haemolytic activity (Sneath, 1986; Priest, 1993). Its virulence depends on the combined presence of two plasmids: pXO1, bearing the toxin genes, and pXO2, carrying the capsule genes.

On the basis of their habitats, pathogenicity and gross morphological characteristics, these organisms are considered as four separate species. However, recent studies indicate that they may be more appropriately considered as subspecies of a single generic species, *B. cereus*. Indeed, comparative analysis of 16S rRNA sequences of representatives of the four taxa showed that they are virtually identical and within the variation expected of a single species (Ash *et al.*, 1991). Moreover, recent comparisons of several strains of *B. thuringiensis* and *B. cereus* by pulsed-field gel electrophoresis and multi-

Abbreviations: IR, inverted repeat; IS, insertion sequence; MEE, multi-locus enzyme electrophoresis.

Table 1. *B. thuringiensis*: relevant characteristics and IS distribution

Serotype	Serovar	Strain number	Source/ reference*	IS231†				IS232†		IS240†	
				P231-1	H231A	P231-2	H231V	P232	H232A	P240	H240A
01	<i>gelechiae</i>	Bt1	A. Lövgren	1650		0		2200		0	
01	<i>thuringiensis</i>	Berliner 1715	IEBC	1650	+	0		2200	+	0	
01	<i>thuringiensis</i>	BGSC 4A2	BGSC	1650	+	0		0		0	
01	<i>thuringiensis</i>	BGSC 4A8	BGSC	1650		0		0		0	
01	<i>thuringiensis</i>	H1.1	IEBC	1650		0		2200		0	
01	<i>thuringiensis</i>	T01 016	IEBC	1650		0		2200		0	
01	<i>thuringiensis</i>	T01 027	IEBC	1650		0		2200		0	
02	<i>finitimus</i>	T02 001	IEBC	0		0		0		0	
03a, 3b	<i>kurstaki</i>	BGSC 4D14	BGSC	1650		0		2200		0	
03a, 3b	<i>kurstaki</i>	BGSC 4D18	BGSC	1650		0		2200		0	
03a, 3b	<i>kurstaki</i>	BGSC 4D2	BGSC	1650		0		2200		0	
03a, 3b, 3c	<i>kurstaki</i>	Bt128	Y. Chen	1650		0		2200		0	
03a, 3b, 3c	<i>kurstaki</i>	HD-1 cry – B	D. Lereclus	0		0		0		0	
03a, 3b, 3c	<i>kurstaki</i>	T03A001	IEBC	1650	+	0		2200		0	
03a, 3b, 3c	<i>kurstaki</i>	T03A016 (HD-1)	IEBC	1650		0		2200		0	
03a, 3c	<i>alesti</i>	BGSC 4C1	BGSC	0		0		0		0	
03a, 3c	<i>alesti</i>	Bt5	A. Lövgren	1650		0		2200		0	
03a, 3c	<i>alesti</i>	T03 001	IEBC	0		0		0		0	
03a, 3d	<i>sumiyoshiensis</i>	T03B001	IEBC	0		0		0		0	
03a, 3d, 3e	<i>fukuokaensis</i>	T03C001	IEBC	1650		1650		0		0	
04a, 4b	<i>dendrolimus</i>	T04A001	IEBC	0		0		0		0	
04a, 4b	<i>sotto</i>	BGSC 4E1	BGSC	0		0		0		0	
04a, 4b	<i>sotto</i>	T04 001	IEBC	1650		0		2200		0	
04a, 4c	<i>kenyae</i>	BGSC 4F1	BGSC	1650		0		2200		0	
04a, 4c	<i>kenyae</i>	T04B001	IEBC	1650		0		2200		0	
05a, 5b	<i>galleriae</i>	BGSC 4G4	BGSC	1650		0		2200		0	
05a, 5b	<i>galleriae</i>	BGSC 4G5	BGSC	1650		0		2200		0	
05a, 5b	<i>galleriae</i>	Bt3	A. Lövgren	1650		0		2200		0	
05a, 5b	<i>galleriae</i>	T05 001	IEBC	1650	+	0		2200	+	0	
05a, 5c	<i>canadensis</i>	BGSC 4H1	BGSC	0		0		0		0	
05a, 5c	<i>canadensis</i>	T05A013	IEBC	0		0		0		0	
06	<i>entomocidus</i>	BGSC 4I2	BGSC	1650		0		2200		0	
06	<i>entomocidus</i>	BGSC 4I3	BGSC	1650		0		2200		0	
06	<i>entomocidus</i>	BGSC 4I5	BGSC	1650		0		2200		0	
06	<i>entomocidus</i>	T06 001	IEBC	1650		0		2200		0	
06	<i>subtoxicius</i>	T06A001	IEBC	1650		0		2200		0	
07	<i>aizawai</i>	T07 001	IEBC	1650		0		2200	+	0	
07	<i>aizawai</i>	T07 029	IEBC	1650		0		2200		0	
08a, 8b	<i>morrisoni</i>	BGSC 4K1	BGSC	1650		0		0		0	
08a, 8b	<i>morrisoni</i>	BGSC 4K3	BGSC	1650		1950		0		850	
08a, 8b	<i>morrisoni</i>	T08 018	IEBC	1650		1950		0		850	
08a, 8c	<i>ostrinae</i>	T08A001	IEBC	0		0		0		850	+
08b, 8d	<i>nigeriensis</i>	T08B001	IEBC	0		0		0		0	
09	<i>tolworthi</i>	T09 001	IEBC	1650		0		0		0	
10a, 10b	<i>darmstadiensis</i>	T10 001	IEBC	1650		0		2200	+	0	
10a, 10b	<i>darmstadiensis</i>	T10 011	IEBC	1650		0		0		850	
10a, 10c	<i>londrina</i>	T10A001	IEBC	0		0		0		0	
11a, 11b	<i>toumanoffi</i>	T11 001	IEBC	1650		0		0		0	
11a, 11c	<i>kyushuensis</i>	T11A001	IEBC	1650		0		0		0	
12	<i>thompsoni</i>	T12 001	IEBC	0		0		0		(850)	+
13	<i>pakistani</i>	T13 001	IEBC	1950	–	0		0		0	
14	<i>israelensis</i>	4Q2-72	IEBC	1650	(+)	1950	+	0		850	+

Table 1. (cont.)

Serotype	Serovar	Strain number	Source/ reference*	IS231†				IS232†		IS240†	
				P231-1	H231A	P231-2	H231V	P232	H232A	P240	H240A
14	<i>israelensis</i>	4Q2-81	IEBC	0		0		0		0	
14	<i>israelensis</i>	BGSC 4Q4	BGSC	1650		0		0		850	
14	<i>israelensis</i>	H14	IEBC	1650		0		0		850	
15	<i>dakota</i>	T15 001	IEBC	1650		0		0		0	
16	<i>indiana</i>	T16 001	IEBC	1650		0		0		850	
17	<i>tohokuensis</i>	T17 001	IEBC	1650		0		0		850	
18a, 18b	<i>kumamotoensis</i>	T18 001	IEBC	1650		0		0		0	
18a, 18c	<i>yosoo</i>	T18A001	IEBC	0		0		0		850	
19	<i>tochigiensis</i>	T19 001	IEBC	0		0		0		0	
20a, 20b	<i>yunnanensis</i>	T20 001	IEBC	0		0		2200	+	850	
20a, 20c	<i>pondicheriensis</i>	T20A001	IEBC	1650		0		0		0	
21	<i>colmeri</i>	T21 001	IEBC	0		0		0		0	
22	<i>shandongensis</i>	T22 001	IEBC	0		0		0		0	
23	<i>japonensis</i>	T23 001	IEBC	0		0		0		0	
24a, 24b	<i>neoleonensis</i>	T24 001	IEBC	(3200) 2000 (1600)	(+) (+) (+)	0		0		0	
24a, 24c	<i>novosibirsk</i>	T24A001	IEBC	(3200) 2000 (1600)	(+) (+) (+)	0		0		0	
25	<i>coreanensis</i>	T25 001	IEBC	0		0		0		0	
26	<i>silo</i>	T26 001	IEBC	1650		0		0		0	
27	<i>mexicanensis</i>	T27 001	IEBC	0		0		0		800	—
28a, 28b	<i>monterrey</i>	T28 001	IEBC	1900 1600	— +	0		0		0	
28a, 28c	<i>jegathesan</i>	T28A001	IEBC	0		0		0		(1900) 850	+ +
29	<i>amagiensis</i>	T29 001	IEBC	0		0		0		0	
30	<i>medellin</i>	T30 001	IEBC	1650		0		0		850	+
31	<i>toguchini</i>	T31 001	IEBC	0		0		2200	+	0	
32	<i>cameroun</i>	T32 001	IEBC	0		0		0		850	—
33	<i>leesis</i>	T33 001	IEBC	1650		1950	+	0		0	
34	<i>konkukian</i>	T34 001	IEBC	0		0		0		0	
35	<i>seoulensis</i>	T35 001	IEBC	0		0		0		0	
36	<i>malaysiensis</i>	T36 001	IEBC	1650		1950		0		850	
37	<i>andalousiensis</i>	T37 001	IEBC	3000 2000	+ (+)	0		0		850	+
38	<i>oswaldocruzi</i>	T38 001	IEBC	1950		1950	+	0		0	
39	<i>brasiliensis</i>	T39 001	IEBC	0		0		0		0	
40	<i>huazhongensis</i>	T40 001	IEBC	1650		0		0		0	
41	<i>sooncheon</i>	T41 001	IEBC	0		0		0		0	
42	<i>jinghonjiensis</i>	T42 001	IEBC	0		0		2200		0	
43	<i>guiyangiensis</i>	T43 001	IEBC	1650		0		0		850	
44	<i>higo</i>	T44 001	IEBC	1650		1950		0		0	
45	<i>roskildiensis</i>	T45 001	IEBC	0		0		0		0	

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† Size, in bp, of the PCR products obtained with the different IS primers (P), estimated in 50 bp incremental steps; numbers in parentheses refer to weak PCR bands. These PCR products were hybridized with the corresponding IS (H) and a positive signal is shown as +. Lack of or weak signals are shown as — or (+), respectively. Blanks indicate not determined.

Table 2. *B. cereus*: relevant characteristics and IS distribution

Strain number*	Source/reference†	IS231‡				IS232‡		IS240‡	
		P231-1	H231A	P231-2	H231V	P232	H232A	P240	H240A
ATCC 12826	Wunschel <i>et al.</i> (1994)	0		1900	—	0		0	
ATCC 21281	Carlson <i>et al.</i> (1994)	1650	+	0		2200	+	0	
ATCC 21282	Carlson <i>et al.</i> (1994)	1650	+	0		2200	+	0	
CER 116	IEBC	1650	+	0		0		0	
CER 484	IEBC	0		0		0		850	—

* The following strains were also tested, but gave no PCR product: AHU1219 and AHU1356 described by Wunschel *et al.* (1994); ATCC 6464, ATCC 10987, ATCC 11778, ATCC 10876, Bc41, Bc131, BcNi, BGSC 6E1, BGSC 6E2 and F0837/76 described by Carlson *et al.* (1994); BGSC 6A1, BGSC 6A3 and BGSC 6S1 from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, USA; and CER001, CER002, CER052, CER065, CER068, CER093, CER109, CER111, CER204 and CER467 from the IEBC.

† IEBC, International Entomopathogenic *Bacillus* Centre, Pasteur Institute, Paris, France.

‡ Size, in bp, of the PCR products obtained with the different IS primers (P), estimated in 50 bp incremental steps. These PCR products were hybridized with the corresponding IS (H). A positive signal is shown as +; lack of a signal is shown as —. Blanks indicate not determined.

locus enzyme electrophoresis (MEE) strongly indicate that these two *Bacillus* should be regarded as one species (Carlson *et al.*, 1994; Zahner *et al.*, 1989). Lastly, Andersen *et al.* (1996) have identified a region displaying genetic variability among *B. anthracis*, *B. cereus* and *B. mycoides*. In *B. anthracis* however, this region was shown to carry variable numbers of tandem repeats, and this might simply represent a rare case of hyper-variability for this highly monomorphic bacteria. Considering these results, it seems that the four *Bacillus* taxa are very closely related at the genetic level, even if they display specific phenotypic traits.

Several insertion sequences (IS) and cointegrative transposons have been found structurally associated to the toxin (*cry*) genes in *B. thuringiensis* (Mahillon *et al.*, 1994). This particular organization is thought to facilitate recombination and exchange of plasmids, providing mechanisms to account for the generation of new specificity in crystal-producing strains. Among the mobile elements isolated in *B. thuringiensis* are two transposons belonging to the Tn3 family, Tn4430 and Tn5401, and variants of IS231, IS232 and IS240, belonging to three major prokaryote IS families. Recently, IS231 variants were also found in *B. cereus* and *B. anthracis* (Henderson *et al.*, 1995).

The aim of this work was to establish the distribution of four IS, representatives of three major IS families (IS231A and IS231V from the IS4 family, IS232 classified in the IS21 group, and IS240, a member of the IS6 family) among the different serovars of *B. thuringiensis* and among the reference strains of the closely related *B. cereus* and *B. mycoides*. PCR was used as the main technique of analysis, the presence of conserved and specific inverted repeats (IR) at the end of the IS allowing not only their amplification, but also the amplification of their variants. The diversity and differential distribution of these IS among the different taxa and their

relatedness with entomopathogenic characters are presented.

METHODS

Bacterial strains. The *B. thuringiensis*, *B. cereus* and *B. mycoides* strains used in this study are listed in Tables 1, 2 and 3, respectively, together with their relevant features. Bacteria were grown overnight on 2YT medium (Brousseau *et al.*, 1993) at 28 °C.

DNA sample preparation and PCR conditions. DNA sample preparation for PCR reactions was adapted from Brousseau *et al.* (1993). The equivalent of a small match-head of a fresh colony or streak was resuspended in 250 µl sterile water and boiled for 10 min. The tubes were immediately centrifuged for 30 s and 200 µl of the supernatant was removed and placed on ice. Each PCR reaction (50 µl) contained 20 µl DNA sample, 10 µl 10 µM primers, 0.5 µl 20 mM dNTP, 0.5 µl 5 mM tetramethylammonium chloride, 0.4 µl (for IS231A and IS240) or 0.6 µl (for IS231V and IS232) polymerase (DynaZym, Life Sciences, 2 U µl⁻¹), and 5 µl 10 × reaction buffer. The reactions were performed in a Perkin Elmer 9600 apparatus: after a 10 min pre-incubation at 96 °C, 30 cycles of 1 min at 92 °C, 1 min at 50 °C and 2 min at 72 °C were performed for IS231A, IS231V and IS240. For IS232, the annealing temperature was 45 °C and the polymerization step lasted for 2.5 min. The reactions were terminated by 10 min at 72 °C.

Sequences of the different oligonucleotides used in this study are shown in Table 4 and compared to the left and right IR of the different iso-elements. Due to heterogeneity in the IR, two distinct IS231 primers were made: P231-1, designed to detect IS231A, IS231G, IS231C, IS231F (one mismatch admitted for the last two variants) and IS231H (assuming its right IR to be very similar), and P231-2, specific to the IS231V and IS231W variants. For IS232, the IR of its A, B and C variants could be matched by the P232 pair of primers, whereas the degenerated P240 primer was chosen to hybridize to the ends of IS240A and B. In addition to these known elements, and because of the conserved nature of the IR, new iso-elements were also expected to be amplified.

Table 3. *B. mycoides*: relevant characteristics and IS distribution

Strain number*	MEE/DNA group†	Source/ reference‡	IS231§				IS232§	IS240§	
			P231-1	H231A	P231-2	H231V	P232	P240	H240A
MYC 002	ND	IEBC	1650		0		0	0	
MYC 003	ND	IEBC	1650	(+)	0		0	0	
MYC 004	ND	IEBC	1650		0		0	0	
MYC 005	ND	IEBC	1650		0		0	0	
NRRL BD-3	D1.01	Nakamura & Jackson (1995)	0		0		0	850	—
NRRL NRS323	D2.06		0		1850	(+)	0	0	
NRRL NRS371	D2.08		0		1850	(+)	0	0	
NRRL NRS321	D2.09		0		1850	(+)	0	850	—
NRRL BD-6	D2.12		(3200)	(+)	0		0	0	
			1950	(+)					
			1650	+					
NRRL NRS1216	D3.02		1650	+	0		0	0	
KBS 1-4	M1.01	Bell & Friedman (1994)	0		2800	—	0	850	(+)
KBS 2-12	M1.02		0		0		0	850	(+)
KNC 3-1	M1.03		0		0		0	850	(+)
KBS 3-1	M1.04		0		0		0	850	(+)
KNC 1-2	M1.05		0		2800	—	0	850	(+)
KBS 1-15	M1.06		0		2800	—	0	850	(+)
KBS 1-5	M2.01		0		1850	(+)	0	850	(+)
KBS 1-16	M2.02		3000	—	1850	(+)	0	850	(+)
KNC 3-15	M2.03		0		1850	(+)	0	(850)	(+)
KNC 2-13	M2.04		0		1850	(+)	0	(850)	(+)
KNC 2-16	M2.05		0		1850	(+)	0	850	(+)
KNC 2-18	M2.06		0		1850	(+)	0	850	(+)
KBS 3-18	M2.07		0		1850	(+)	0	850	(+)
KNC 1–10	M2.08		0		1850	(+)	0	850	(+)

* The following strains were also tested, but gave no PCR product: BD-18, BD-23, BD-2, B-346, B-347, NRS306, NRS319 (DNA group D1) and B-3436 (DNA group D2) described by Nakamura & Jackson (1995), and MYC001 (DNA group D1) from the IEBC.

† Electrophoretic type obtained by MEE, as described by Bell & Friedman (1994); and strain DNA relatedness based on DNA renaturation rates, as described by Nakamura & Jackson (1995). ND, not determined.

‡ IEBC, International Entomopathogenic *Bacillus* Centre, Pasteur Institute, Paris, France.

§ Size, in bp, of the PCR products obtained with the different IS primers (P), estimated in 50 bp incremental steps; numbers in parentheses refer to weak PCR bands. These PCR products were hybridized with the corresponding IS (H). A positive signal is shown as +; lack of or weak signals are shown as — or (+), respectively. Blanks indicate not determined.

IS detection and polymorphism. Each PCR sample (10 µl) was analysed by agarose gel electrophoresis. Sizes of the DNA bands were estimated by comparison to phage λ DNA, cleaved by *EcoRI* and *HindIII*, or to φX174 DNA cleaved by *HaeIII* (Eurogentec). These size estimations were rounded by increment steps of 50 bp. Therefore, although IS231A, IS231V, IS232 and IS240 are 1656, 1964, 2184 and 865 bp long, respectively, their sizes are referred to as 1650, 1950, 2200 and 850 bp, respectively. Positive controls, consisting of total DNA of reference *B. thuringiensis* strains, were added to each test: Berliner 1715 for IS231A and IS232A (Menou *et al.*, 1990; Mahillon *et al.*, 1985), and *israelensis* 4Q2-72 for IS231V (Rezsöházy *et al.*, 1993) and IS240 (Delécluse *et al.*, 1989). Diversity among the different IS-PCR products was estimated by *DraI* (Eurogentec) RFLP.

Hybridization experiments. IS231A and IS232A probes were prepared from pGI622 and pGI612 plasmids, respectively (Mahillon *et al.*, 1987), while integral copies of IS231F, IS231V

and IS240A were amplified by PCR, using the *Pwo* polymerase (Boehringer), on total DNA from strain 4Q2-72 of *B. thuringiensis* subsp. *israelensis*, from which they originated (Mahillon *et al.*, 1994). They were cloned into the *SmaI* site of the pBluescript SK plasmid (Stratagene) and introduced into *Escherichia coli* TG1 (*supE hsdΔ5 thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^q lacZΔM15]*). All IS probes were amplified using the DynaZym polymerase, and recovered from agarose gel with the QIAEX II gel extraction kit (Qiagen).

After migration of the PCR products on agarose gel, the DNA samples were Southern-blotted according to standard techniques (Sambrook *et al.*, 1989). Hybridizations were made using standard conditions of 42 °C with 50% (v/v) formamide.

Parasporal crystal observation. The bacteria were grown on Schaeffer sporulation medium (0.8% Difco nutrient broth, 1 mM MgSO₄, 15 mM KCl, 1 mM CaCl₂, 10 µM MnCl₂ and 1 µM FeSO₄) plates for 5–7 d at 28 °C. The samples were

Table 4. Inverted repeats of IS231, IS232 and IS240 variants and primers used for PCR

Variants or primers	Left IR	Right IR
IS231A/B*/G	5'-CATGCCCATCAACTTAAGAA-3'	5'-CATGCCCATCAACTTAAGAA-3'
IS231C	5'-CATGCCCATCAACTTAAGAA-3'	5'-CATGCCCATCAACTTAAGGA-3'
IS231D	5'-CATGCCCATCAAATTAAGA-3'	5'-CATGCCCATCAACTTAAGAA-3'
IS231E†		5'-CATACCCATCAACTTAAGGG-3'
IS231F	5'-CATGCCCATCAACTTAAGAA-3'	5'-CATGCCCATCAACTTAAGGA-3'
IS231H	5'-CATGCCCATCAACTTAAGGA-3'	ND‡
IS231V/W	5'-CATCGCCATCAAGCTAAGGA-3'	5'-CATCGCCATCAAGCTAAGGA-3'
P231-1 primer	5'-CATGCCCATCAACTTAAGAA-3'	5'-CATGCCCATCAACTTAAGAA-3'
P231-2 primer	5'-CATCGCCATCAAGCTAAGGA-3'	5'-CATCGCCATCAAGCTAAGGA-3'
IS232A/B/C	5'-GTATAAATGCTAACTTAAAT-3'	5'-GTATAAATGTCAAGATAAAC-3'
P232 primers	5'-GTATAAATGCTAACTTAAAT-3'	5'-GTATAAATGTCAAGATAAAC-3'
IS240A	5'-TAGGTTCTGGTGCAAAAAA-3'	5'-AAGGTTCTGGTGCAAAATAA-3'
IS240B	5'-AAGGTTCTGGTGCAAAAAA-3'	5'-AAGGTTCTGGTGCAAAATAA-3'
P240 primer	5'-AAGGTTCTGGTGCAAAWAA-3'§	5'-AAGGTTCTGGTGCAAAWAA-3'

* The first 11 bp of the IS231B right IR are missing (Mahillon *et al.*, 1987).

† IS231E lacks a left IR (Rezsöházy *et al.*, 1992).

‡ IS231H right arm has not been determined (Ryan *et al.*, 1993).

§ W is A or T.

stained with carbolfuchsin and observed using a light microscope.

RESULTS

Experimental set-up

Since most IS display short conserved IR sequences at their ends, their rapid detection can be easily achieved by PCR amplification, using a single oligonucleotide primer. Based on the available DNA sequences of the different *B. thuringiensis* IS, a series of primers were designed for the detection of iso-IS231, IS232 and IS240 (Table 4) and tested on the control strains from which the IS originated.

Using the P231-1 primer in a PCR on *B. thuringiensis* Berliner 1715, a single 1650 bp signal was amplified (Fig. 1, lane 2). Restriction analysis of this PCR product (data not shown) demonstrated that it represented not only IS231A, but also IS231C, both known to be present in this subspecies (Mahillon *et al.*, 1987). With the same DNA sample from Berliner 1715 and using the appropriate primer mix, the PCR gave the expected 2200 bp fragment (Menou *et al.*, 1990) corresponding to IS232 (Fig. 1, lane 4). No signal corresponding to IS231V or IS240 (Fig. 1, lanes 3 and 5, respectively) were observed in the Berliner 1715 samples. Conversely, the PCR with a DNA sample from *B. thuringiensis* subsp. *israelensis* 4Q2-72 gave the 1950 bp fragment expected for IS231V (Rezsöházy *et al.*, 1993) and a 850 bp signal for IS240 (Delécluse *et al.*, 1989) (Fig. 1, lanes 7 and 9, respectively). Moreover, the P231-1 primer allowed the amplification of a 1650 bp signal corresponding to the IS231F variant present in the same 4Q2-72 strain (Fig. 1, lane 6).

IS distribution among the *B. thuringiensis* reference strains

With the reaction conditions elaborated for the four IS, 90 *B. thuringiensis* reference strains, representing 61 serovars, were tested. The complete results are listed in Table 1 together with other relevant information. It should be noted that although IS231D and IS231E originated from *B. thuringiensis* subsp. *finitimus* (Rezsöházy *et al.*, 1992), they were not detected by P231-1 or P231-2. The reasons lie in the fact that IS231E lacks a left IR (Table 4), and that the three mismatches present in IS231D left IR, as compared to the P231-1 primer sequence, are located at the 3' end of its IR, therefore preventing any amplification.

IS diversity in *B. thuringiensis*

To estimate the relatedness of the PCR products generated with their respective IS, a selection of them were hybridized with their corresponding IS probes (Table 1). Under the conditions used, the detection threshold was estimated to be 70 % sequence identity, as indicated by the weak signal given by IS231F from *israelensis* 4Q2-72 when detected by IS231A (these elements are 73 % identical).

When hybridized with IS231A, most of the PCR products amplified with the P231-1 primer gave a positive signal, with the exception of the 1950 and 1900 bp bands of subsp. *pakistani* and *monterrey*, respectively. The same PCR products obtained with P231-1 were also tested against the IS231V probe, but none of them gave a positive result (data not shown). Similarly, hybridization using the IS231V probe

Fig. 1. PCR reactions on control strains. Lanes: 1, lambda size marker DNA restricted by *Eco*RI and *Hind*III; 2–5, PCR reactions performed on *B. thuringiensis* Berliner 1715 DNA, with P231-1, P231-2, P232 and P240 primers, respectively; 6–9, PCR reactions performed on *B. thuringiensis* subsp. *israelensis* 4Q2-72 DNA, with P231-1, P231-2, P232 and P240 primers, respectively; 10, ϕ X174 DNA restricted by *Hae*III.

indicated a close relationship between this element and the P231-2-generated PCR products, whereas no signals were observed using IS231A (data not shown). Of the nine IS240-derived PCR products tested for hybridization, (including a weak 1900 bp band), only two bands could not be detected (H27 and H32). Finally, all six IS232 PCR products hybridized strongly with their corresponding probe, indicating that they represented well-conserved copies of this IS.

Although these results indicated a degree of relatedness between these PCR products, more insight was sought by analysis of their restriction fragment polymorphism. *Dra*I restriction profiles of selected PCR products were compared to those obtained with the reference strains (Fig. 2): IS231A/C (lane 1) and IS232 (lane 21) from Berliner 1715, and IS231F (lane 2), IS231V/W (lane 14) and IS240 (lane 28) from *israelensis* 4Q2-72. Note that in strain Berliner 1715, a composite IS231A/C pattern is observed, both IS being present (Mahillon *et al.*, 1987).

The most striking observation is the difference in diversity between the IS232-related PCR products and those from the other elements. Indeed, for the former, five of the six products analysed were identical (Fig. 2, lanes 22–24) or nearly identical (lane 25) to IS232A; only that of serotype 42 (lane 26) displayed a different pattern. For IS231, the situation is more complex. Although each known IS231 pattern can be found in a background different to its original strain, close variants of each IS were also detected. This is particularly well illustrated by IS231C: originally isolated from strain Berliner 1715 (Fig. 2, lane 1), this IS is also detected in strains T03A001, T03C001 and T04B001 (lanes 3–5, respectively), and a variant in strain T18 001 (lane 8). IS231F from strain 4Q2-72 can also be found in strain T17 001 and, with some variation, in T26 001 (lanes 2, 6 and 9, respectively). Similarly, IS231V/W from strain 4Q2-72 are also present in T38 001 and BGSC 4K3 (lanes 15 and 17), but displayed greater variation in strains T33 001 and T44 001 (lanes 16 and 18). All the IS240 patterns tested were dissimilar, with the exception of that from strain T43 001 (Fig. 2, lane 31), also present in strain T20 001, in combination with another iso-IS240 (Fig. 2, lane 29)

IS distribution and diversity among *B. cereus* reference strains

Contrary to *B. thuringiensis*, most of the 30 *B. cereus* strains tested (83%) did not give any PCR products (Table 2). Hybridization of those PCR products that were obtained with their respective probes confirmed their relationship to their parental IS, with the exception of the 1900 bp and 850 bp fragments from strains ATCC 12826 and CER 484, respectively, for which no signal could be observed. *Dra*I restriction analysis of the PCR products (Fig. 2) indicated that whereas the iso-IS240 (CER 484, lane 32) and iso-IS231 (ATCC 21281, lane 11) were novel variants, the iso-IS232 (ATCC 21281, lane 27) and iso-IS231 (CER 116, lane 10) were identical to IS232A, and to an IS231A variant present in strain T11A001 (lane 7), respectively.

Knowing that most *B. thuringiensis* transposable elements have been found associated to *cry* genes (Mahillon *et al.*, 1994), the above five *B. cereus* strains harbouring an IS were investigated for the presence of parasporal crystals in their sporangia. Strains ATCC 21281 and ATCC 21282, those displaying the IS231/IS232 PCR combination, were found to produce small crystal inclusions. Interestingly, these strains have not only been reported as toxic for insects, but hybridization experiments revealed the presence of *cry* genes on their extrachromosomal DNA (Carlson *et al.*, 1994). However, since no inclusions were detected in their sporangia, they were still considered as *B. cereus*. In fact, these crystals have probably been overlooked due to their tiny size. No other *B. cereus* strains investigated produced any detectable crystal inclusion.

IS elements in *B. mycoides*

Due to the limited number of available reference strains of *B. mycoides*, three independent strain collections were obtained. Interestingly, the strain collections provided by L. K. Nakamura (15 strains of world-wide origin; Nakamura & Jackson, 1995) and J. A. Bell (14 strains originating from the USA; Bell & Friedman, 1994) have already been investigated in detail with

Fig. 2. Dral restriction profiles of selected PCR products. Lanes: M, ϕ X174 size marker DNA restricted by *Hae*III; 1, *B. thuringiensis* (*B.t.*) Berliner 1715; 2, *B.t.* 4Q2-72; 3, *B.t.* T03A001; 4, *B.t.* T03C001; 5, *B.t.* T04B001; 6, *B.t.* T17 001; 7, *B.t.* T11A001; 8, *B.t.* T18 001; 9, *B.t.* T26 001; 10, *B. cereus* (*B.c.*) CER 116; 11, *B.c.* ATCC 21281; 12, *B. mycoides* (*B.m.*) MYC 003; 13, *B.m.* NRS1216; 14, *B.t.* 4Q2-72; 15, *B.t.* T38 001; 16, *B.t.* T33 001; 17, *B.t.* BGSC 4K3; 18, *B.t.* T44 001; 19, *B.m.* KBS 1-5; 20, *B.m.* NRS323; 21, *B.t.* Berliner 1715; 22, *B.t.* T03A001; 23, *B.t.* T04B001; 24, *B.t.* T10 001; 25, *B.t.* T31 001; 26, *B.t.* T42 001; 27, *B.c.* ATCC 21281; 28, *B.t.* 4Q2-72; 29, *B.t.* T20 001; 30, *B.t.* T32 001; 31, *B.t.* T43 001; 32, *B.c.* CER 484; 33, *B.m.* BD-3; 34, *B.m.* KBS 1-4.

regards to their DNA relatedness (D1, D2 and D3 subgroups) or MEE and RFLP (M1 or M2 MEE clusters). The results obtained are presented in Table 3. It was particularly noteworthy that, despite their very different origins, no strain gave a PCR signal corresponding to IS232.

Hybridization experiments similar to those performed with *B. thuringiensis* and *B. cereus* indicated DNA relationship (above 70% identity) between most of the PCR products obtained (including the large 3200 bp fragment from strain BD-6) and their corresponding IS (Table 3). No signal could be obtained with the 850 bp

fragment from strains BD-3 and NRS321, nor with the three 2800 bp bands of cluster M1. These results were supplemented by analysis of the PCR restriction polymorphism. As expected, several of them displayed novel *DraI* restriction profiles, including the IS240 band from BD-3 and KBS 14 (Fig. 2, lanes 33 and 34), the 1650 bp band from NRS1216 (lane 13), and the 1850 bp P231-2 products from NRS323 and KBS 1-5 (lanes 19 and 20). Unexpectedly, however, the IS231 pattern seen in *B. mycoides* MYC 003 (lane 12) was apparently identical to those recorded for *B. cereus* CER 116 (lane 10) and *B. thuringiensis* T11A001 (lane 7).

Finally, to investigate the possible correlation between the identified IS elements and *cry* genes, all the *B. mycoides* strains harbouring one IS were analysed for the presence of parasporal crystals in their sporangia. None were detected.

DISCUSSION

IS231 distribution and diversity

IS231 is a member of the IS4 family whose nearest related elements are IS1151 and IS186, from *Clostridium perfringens* and *E. coli*, respectively. So far, a total of ten iso-IS231 have been isolated and characterized. Eight of them (IS231A–H) exhibit a size of about 1650 bp, while the more distantly related IS231V and W variants are about 1950 bp long (Rezsöházy *et al.*, 1993). The present study has shown that these elements are the most widespread IS in the three *Bacillus* species investigated, being found in 64 % of the *B. thuringiensis*, 61 % of the *B. mycoides* and in 13 % of the *B. cereus* strains analysed (Fig. 3).

A second important insight into the biology of IS231 is the observation that the majority of these elements fall into the same two size categories, 1650 and 1950 bp, observed in the above ten variants. The additional

300 bp DNA fragment found in IS231V/W is located upstream of their putative transposase genes. It remains to be seen if this region is conserved in the new 1950-bp-type elements and which structural and/or functional feature, if any, might be indicative of any biological significance. IS231 size is, however, apparently not exclusively limited to these two classes, and several examples of new IS lengths (3200, 2000, 1850 or 1600 bp) were observed, mostly for the PCR products derived from P231-1. Cloning and sequencing of representatives of these IS are currently under way to unravel their singular features.

DNA relatedness among IS231 variants was investigated using hybridization with IS231A, IS231F and IS231V probes, as well as *DraI* RFLP analysis of the PCR products. Two important conclusions may be drawn. First, the number of currently known IS231 elements is far from exhaustive. Thus, both new variants, as well as new iso-elements, were present in the three *Bacillus* species tested. Secondly, there is apparently no exclusive association between a particular iso-IS231 and a specific strain. This is not only true within a particular taxon such as *B. thuringiensis*, but across the various *B. cereus* species. The best example is the IS231A variant present in *B. thuringiensis*, *B. cereus* and *B. mycoides* (Fig. 2, lanes 7, 10 and 12, respectively).

IS240 occurrence in the three *Bacillus* taxa

The two nearly identical copies of IS240, belonging to the IS6 family, were originally isolated from the anti-dipteran *B. thuringiensis* subsp. *israelensis*, but their presence was also noted in *B. thuringiensis* subsp. *darmstadtensis* and *morrisoni* (Delécluse *et al.*, 1989). The present study showed that IS240-related sequences can be found in at least 21 % of the *B. thuringiensis* strains. In a similar investigation, Rosso & Delécluse (1997) concluded that IS240-related elements were present in more than 40 % of the *B. thuringiensis* reference strains analysed. Although these different observations could be attributed, in part, to the use of different strains, the main reason for this discrepancy lies probably in the use of different primers.

IS240 distribution is not limited to *B. thuringiensis*. IS240-related PCR fragments were also observed in one *B. cereus* strain and in more than 42 % of the *B. mycoides* strains. In this case, however, a strong correlation was observed between these elements and the origin of the bacterial hosts. Thus, for example, most strains bearing an IS240-like element originated from two USA locations, separated by only 17 km (Bell & Friedman, 1994).

It was also noticeable that IS240 showed at least as much diversity as IS231, as indicated by their hybridization reactions (Tables 1 to 3) and/or variation in their *DraI* restriction profiles (Fig. 2). Preliminary DNA sequencing of one of the 850 bp elements from *B. mycoides* and that from *B. cereus* indicated about 70 % identity between them and against IS240A.

Fig. 3. Distribution of IS231, IS232 and IS240 among the three *Bacillus* taxa. The numbers of strains tested were 90 for *B. thuringiensis* (filled bars), 30 for *B. cereus* (open bars), and 33 for *B. mycoides* (hatched bars).

Specific IS232 distribution

IS232 (Menou *et al.*, 1990) shares similarities with members of an IS family whose archetype is the IS21 element from *Pseudomonas aeruginosa*. Originally observed in *B. thuringiensis* serovars *kurstaki*, *thuringiensis* and *aizawai*, IS232 was shown to be structurally associated with Tn4430 or iso-IS231 (Mahillon *et al.*, 1994). This association has now been encountered in many strains from serotypes H1, H3–H7 and H10, and thus occurs in 90% of the strains from which an IS232 element has been amplified. Among the other serotypes tested, IS232 occurred only three times, in serotypes H20 (*yunnanensis*), H31 (*toguchini*) and H42 (*jinghonjensis*). This uneven IS232 distribution among *B. thuringiensis* serotypes cannot presently be explained.

One of the most striking findings was the complete absence of IS232 from all of the *B. mycoides* strains examined. Moreover, the two *B. cereus* strains where it was detected (ATCC 21281 and ATCC 21282) may be more appropriately designated *B. thuringiensis*, based on their toxicity to mosquitoes and blackflies (Ibuki & Fujiyoshi, 1972a, b), their possession of *cryI* genes (Carlson *et al.*, 1994), the carriage of the IS231A/IS232 combination specifically found in *B. thuringiensis* (see above), and the presence of small parasporal inclusions. Based on these considerations, IS232 is thus apparently exclusive to *B. thuringiensis* and could, therefore, possibly be used as a specific marker for this bacterium. IS232 also appeared to be the most conserved element. Indeed, all the PCR products amplified had the 2200 bp expected size, hybridization of some of these bands gave strong signals, even in stringent conditions, and *DraI* restriction of the PCR products generated confirmed their close relationships (Fig. 2, lanes 21–27). At least two possible, not mutually exclusive, explanations could account for these observations. IS232 could transpose with low efficiency, as previously suggested by its rather weak activity (5×10^{-8}) detected in *E. coli* (Menou *et al.*, 1990). Alternatively, the DNA sequence forming the IS232 IR might vary among the different iso-elements in such a way that their detection by the pair of primers used in this work was not possible. However, the detection of at least one new iso-element (Fig. 2, lane 26) challenges this hypothesis.

Extrachromosomal versus chromosomal location

The differential IS distribution observed in this work could possibly correlate, at least in part, to the fact that most *B. thuringiensis* strains are entomopathogenic and their *cry* genes are generally associated with different IS elements on large plasmids. In fact, no PCR product corresponding to all four IS was amplified (Table 1) from the DNA of the *B. thuringiensis kurstaki* and *B. thuringiensis israelensis* *cry*-negative strains which lacked these conjugative plasmids (strains *kurstaki* HD-1 *cry*-B and 4Q2-81). Preferential IS location on extra-chromosomal molecules would certainly be consistent with these observations. Currently, experiments are in

progress to determine differences in the specificity of IS insertion in the genomes of *B. thuringiensis*, *B. cereus* and *B. mycoides*.

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