Characterization of the type strain of *Bacillus thuringiensis* subsp. *cameroun* serotype H32

Victor Manuel Juárez-Pérez a, Philippe Jacquemard b and Roger Frutos a,*

a BIOTROP-IGEPAM, CIRAD, 2477 Avenue du Val de Montferrand, B.P. 5035, 34032 Montpellier Cedex 1, France, and b CIRAD-CA, CIRAD, 2477 Avenue du Val de Montferrand, B.P. 5035, 34032 Montpellier Cedex 1, France

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Abstract: The strain 273 B, the type strain of a H serotype of *Bacillus thuringiensis* not yet characterized: *B. thuringiensis* subsp. *cameroun*, serotype H32, was isolated from soil samples collected in Cameroon. This strain produces cuboidal parasporal bodies composed of two major proteins of 53 kDa and 35 kDa. N-terminal sequences of the major proteins share no homology with published sequences. Only the 35 kDa protein is susceptible to digestion by trypsin. A complex array of 9 plasmids was revealed.

Key words: *Bacillus thuringiensis* subsp. *cameroun*; Serotype H32; H serotype; Crystal proteins

Introduction

*Bacillus thuringiensis* (Bt) is a spore forming, Gram-positive soil bacterium characterized by the production during sporulation of parasporal inclusion bodies. Parasporal inclusion bodies, referred to as 'crystals', are composed of one or several insecticidal crystal proteins (ICPs) showing toxicity to several groups of insects [1] and nematodes [2]. ICPs, originally classified into five classes comprising according to their host range and homology degree [3], are now distributed in seven different groups. ICPs are currently classified in CryI, CryII, CryIII, CryIV, CryV and CryVI proteins with specificity to lepidoptera, lepidoptera-diptera, coleoptera, diptera [1], lepidoptera-coleoptera [4] and nematodes [2] respectively. The last group is composed by the Cyt proteins which show non-specific cytolytic activity [5]. This diversity in host range is complemented by an heterogeneity in size and sequence [3].

New types of insecticidal crystal proteins, with molecular masses of 34 kDa and 40 kDa, were recently reported [6] and reports of new types of ICPs will probably continue due to screening programs currently underway worldwide. In accordance with this remark we report here the characterization of strain 273 B, the type strain of *Bacillus thuringiensis: B. thuringiensis* subsp. *cameroun*, serotype H32. This strain produces cuboidal crystals containing two very atypic ICPs...
Materials and Methods

Bacterial isolates

The strain 273 B, the type strain of Bacillus thuringiensis subsp. cameron, was isolated in 1990 from soil samples collected in Cameroon following a procedure previously described [7]. B. thuringiensis subsp. israelensis IPS-82 (BTI) and B. thuringiensis subsp. tenebrionis DMS-2803 (BTT) were obtained from Institut Pasteur (Paris, France). B. thuringiensis subsp. kurstaki (BTK) was isolated from a commercial formulation of Bactospine® and B. thuringiensis subsp. thompsoni (Hn C) from our laboratory collection.

Determination of the H serotype of strain 273 B

The H serotype was determined by Dr. H. De Barjac, International Entomopathogenic Bacillus Center, Unité des Bactéries Entomopathogènes, Institut Pasteur, Paris, France.

Growth conditions

Bacterial strains were cultured in an orbital shaker in 250 ml of ‘Milieu Usuel’ (MU) [8] at 28°C and 200 rpm until cell lysis was complete (about 96 h).

Purification of parasporal inclusion bodies

After completion of cell lysis, spores, parasporal bodies and cell debris were pelleted by centrifugation at 12000 × g for 30 min. The pellet was resuspended in 125 ml of NaCl 1 M, vortexed vigorously to obtain a spore-rich foam which was eliminated with a spatula. The suspension was centrifuged again in the same conditions and the pellet was resuspended in 15 ml of sterile distilled water. Parasporal bodies were separated from spores and debris by centrifugation through a sodium bromide discontinuous gradient [9]. Purified crystal were washed three times with sterile distilled water and stored at -20°C.

Light microscopy and scanning electron microscopy

The strain 273 B was grown in MU medium at 28°C until phase-bright spores and parasporal inclusion bodies were observed in phase contrast microscopy. A sample of purified inclusion bodies was extensively dialysed against sterile distilled water glued on coverslips and dehydrated before coating with iron-platinum. Samples were observed using a DMS 950 ZEISS scanning electron microscope.

Analysis of proteins by SDS-PAGE and immunodetection

The protein content of purified parasporal bodies was analysed on SDS-PAGE according to Laemmli [10]. Proteins were separated on 12% polyacrylamide gels. The following standards were used for the determination of molecular mass: myosin (212 kDa), α2-macroglobuline (170 kDa), Β-galactosidase (116 kDa), phophorylase b (94 kDa), transferrin (76 kDa), albumin (67 kDa), glutamic dehydrogenase (53 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) soybean trypsin inhibitor (20 kDa) and α lactoalbumin (14.4 kDa). After transfer onto PVDF membrane [11] (Millipore Immobilon-P membrane) proteins were incubated with polyclonal antibodies raised against solubilized crystal proteins from the strain 273 B.

Digestion of crystal proteins with trypsin

A sample of purified inclusion bodies (aprox. 3 mg) was concentrated by centrifugation at 12500 g for 10 min, then resuspended in 1 ml of solubilization buffer (Na₂CO₃ 100 mM, pH 10.6; DTT 10 mM) and incubated for 30 min. at 4°C. Insoluble particles were removed by centrifugation. The supernatant was dialysed overnight against trypsinization buffer (Tris-HCl 20 mM pH 8.6; NaCl 0.2 M). The solution was then incubated at 37°C for 2 h with 150 μl of trypsin (1 mg ml⁻¹), with a ratio trypsin-ICPs of 1/20. Digestion products were separated from insoluble fraction by centrifugation and the supernatant was analyzed by SDS-PAGE as described above.

Electrophoretic analysis of plasmid patterns

Plasmids were extracted essentially as described by Kronstad et al., [12]. Strains were pre cultured overnight at 37°C and 200 rpm in spizizen medium supplemented with 1% glucose, 1%
yeast extract and 0.1% casamino acids. Plasmid patterns of strain 273 B was compared to those of other *B. thuringiensis* type strains using agarose gel electrophoresis. Plasmid were separated in 0.6% agarose gels in TBE buffer [13].

**Results**

*Determination of the H serotype of strain 273 B*

The strain 273 B was assayed against existing H antisera and was found to be unrelated to any known H serotype. This strain was assigned to a H serotype not yet reported, serotype H32, which corresponds to the subspecies *cameroun*. The strain 273 B is the type strain of this novel H serotype and is registered at the International Entomopathogenic Bacillus Center under number T32 001.

*Morphology of parasporal inclusions*

Observation of sporulated cells with phase-contrast microscopy showed the presence of large cuboidal parasporal bodies which is always released independently from the spore after cell lysis (Fig. 1a). Examination with scanning electron microscopy confirmed the presence of large cuboidal crystals of 640 nm in size (Fig. 1b). An interesting feature is the presence of deep cavities on the sides (Fig. 1b). The size of crystals produced by the strain 273 B is close to that of inclusion bodies characteristic of CryI IA [14] or crystals from *B. thuringiensis* subsp. *thompsoni* [6], which have respective sizes of 500 nm and 700 nm.

*Protein composition of purified parasporal bodies*

Protein contents of purified parasporal bodies from strain 273 B is shown in Fig. 2. Two major proteins with molecular masses of 53 kDa and 35 kDa can be easily observed in the protein pattern (Fig. 2 lane D). The protein contents of parasporal bodies of strain 273 B is varied greatly from that of other type strains (Fig. 2 lanes A,B,C and E). Other proteins with molecular masses of 134 kDa, 91 kDa, 77 kDa, and 27 kDa can be observed repeatedly in the protein contents of crystals from strain 273 B. However, these proteins are present in much lower amount than the two major proteins. In order to determine whether these proteins correspond to known Cry toxins, proteins were transfered onto PVDF membranes
and incubated with a mixture of polyclonal antibodies raised against CryIA(a), CryIA(b), CryIA(c), CryIB, CryIC, CryIE and CryIIA but no response was detected.

Solubilized crystal proteins from strain 273 B were subjected to digestion with trypsin. Analysis by SDS-PAGE of digestion products and non-digested proteins from strain 273 B parasporal bodies showed that the 53 kDa protein is resistant to trypsin whereas the 35 kDa protein is activated in protein of 27 kDa. The similarity in size of the 27-kDa trypsination product with the 27 kDa protein observed in the protein pattern of solubilized parasporal bodies suggests that the latter is a degradation product of the 35 kDa protein. The high intensity of the 27 kDa protein is due to the comigration of the activated ICP with one component of the trypsin solution. Similarly, the smaller bands observed after trypsination are due also to the trypsin solution since they are present when only the trypsin solution is loaded (Fig. 3a lane C). The proteins of 134 kDa, 91 kDa and 77 kDa detected in low amount in the protein contents of parasporal bodies from strain 273 B are no longer present after treatment with trypsin, indicating that they are not resistant to trypsin and therefore are most likely contaminants rather than ICPs.

![Fig. 3. Analysis of the effect of trypsin on solubilized purified parasporal bodies from strain 273 B. (a) Analysis by SDS-PAGE. M, molecular mass markers; A, protein contents of crystals from strain 273 B; B, protein contents of crystals from strain 273 B after treatment with trypsin; C, solution of trypsin; M, molecular mass markers. Protein sizes are given in kilodaltons. (b) Results of immunodetection with polyclonal antibodies raised against crystal proteins from strain 273 B. A, protein contents of crystals from strain 273 B; B, protein contents of crystals from strain 273 B after treatment with trypsin; C, solution of trypsin.](image)

Immunodetection of ICPs before and after treatment with trypsin using polyclonal antibodies raised against solubilized purified crystals from strain 273 B confirmed that the 27 kDa protein is the activated form of the 35 kDa protein (Fig. 3b lane B). The detection of the band of 53 kDa by the antibodies after trypsination also confirmed that the 53 kDa protein is not activated by trypsin (Fig. 3b lane B).

**Comparison of N-terminal sequences with ICPs sequences**

The N-terminal sequence of the 35 kDa and 53 kDa proteins were determined after transfer on PVDF membrane. The first ten amino acids of
the 35 kDa protein are: VREYPDFDS. The methionine is missing at the N-terminal end probably due to cleavage during the sequencing. The sequence found for the 53 kDa proteins is the following: MKKSCDPNPVNO. These sequences were compared to protein sequences derived from nucleotide sequences of ICPs present in data bases and no homology was found, indicating that these crystal proteins are probably new types of toxins.

Electrophoretic analysis of plasmid patterns

Comparison on agarose gels of the plasmid patterns of strain 273 B with those of BTK, BTI, BTI' and Hn C showed different profiles. The strain 273 B produces 9 clearly visible plasmid bands. Seven out of nine show sizes ranging from 4.8 to 113 kb according the size of BTI plasmids.

Discussion

The results reported here on the characterization of strain 273 B, the type strain of a novel H serotype of *B. thuringiensis, B. thuringiensis* subsp. *cameroun* serotype H32 demonstrate the presence of several features characteristic of this new isolate. The cuboidal parasporal bodies produced by strain 273 B show an unusual shape characterized by the presence of hollows on the sides. Although cuboidal crystals have already been reported especially those made by accumulation of CryIIA [14] and the 34-kDa and 40-kDa from *B. thuringiensis* subsp. *thompsoni* [6], the presence of cavities clearly indicates a very specific and original morphology. The protein contents of the parasporal inclusion is also unusual with two proteins of 35 kDa and 53 kDa which are atypic sizes for crystal proteins. The susceptibility to trypsin of only one of the two proteins present in the crystal is another original characteristic of this novel strain. N-terminal sequencing of these proteins showed that they are not related to any known *B. thuringiensis* toxins. The cloning and sequencing of the corresponding genes, which is currently under way [15–18], will yield valuable information on the sequence and on whether the open reading frames correspond to independant units or are organized as an operon as already shown for several *B. thuringiensis* toxin genes.

Although the originality of this novel strain can be seen clearly, there is yet no information on the specificity of the 35 kDa and 53 kDa proteins. Bioassays were conducted on mosquito larvae of *Aedes aegypti*, *Culex pipiens* and *Anopheles stephensi* as well as on larvae of *Spodoptera littoralis*, *Sesamia calamistis* and *Heliothis armigera* and no toxicity was detected. Purified parasporal bodies are currently being tested on different species of lepidoptera and coleoptera as well as on other invertebrates in attempt to identify the natural target of these unusual proteins.

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