Cloning and Expression of the Binary Toxin Gene from *Bacillus sphaericus* IAB872 in a Crystal-Minus *Bacillus thuringiensis* subsp. *israelensis*

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Received: 31 October 2000 / Accepted: 8 December 2000

**Abstract.** *Bacillus sphaericus* IAB872 has high toxicity against susceptible *Culex* spp. and medium larvicidal activity against binary toxin-resistant *Culex* spp. Sequence analysis revealed that the sequence of the binary toxin gene from IAB872 was totally identical to that of the reference strain 2362. The recombinant plasmids were introduced into an acrystalliferous *B. thuringiensis* strain 4Q7, and the resulting transformants produced parasporal inclusion composed of 51 kDa and 42 kDa proteins during sporulation. SDS-PAGE and Western blot further confirmed that *B. thuringiensis* transformants were able to express the binary toxin in a high level. Toxicity bioassays showed that they performed high toxicity to susceptible *Culex* spp. larvae, but no toxicity to resistant *Culex* larvae. It was, therefore, suggested that other unknown toxins perhaps existed in the wild strain IAB872 except the binary toxin. Their modes of action might be different from that of binary toxin, and they were possibly responsible for the activity of the wild strain IAB872 against resistant mosquito larvae.

**Bacillus sphaericus** is an aerobic, spore-forming bacterium with terminal swelled sporangium and spherical spore. Presently, 9 of 49 serotypes of *B. sphaericus* (H1, H2, H3, H5, H6, H9, H25, H26, and H48 respectively) have mosquito-larvicidal activity to a certain extent [16]. Most of the highly toxic strains are classified in serotypes H5, H6, H25 and H48; for instance, 2362, 1593, C3-41, IAB59 and IAB872 [9, 20]. They are more toxic to *Culex* spp., less toxic to *Anopheles* species, and poorly or not toxic to *Aedes* larvae. All highly toxic strains produce the binary toxin composed of equimolecular amounts of proteins of 51.4 kDa and 41.9 kDa during the early stage of sporulation, which are assembled into parasporal crystalline inclusion existing alongside the spore. Both 51.4 kDa and 41.9 kDa peptides are required for larvicidal toxicity, and they act as a binary toxin [3, 4]. Other kinds of larvicidal toxins are Mtx (mosquitocidal toxins), which are present in part of high-toxicity strains and all of low-toxicity strains. Mtx toxins express during the vegetative phase of growth before the onset of sporulation and are unstable and proteolytically degraded as the cell enters the stationary stage. *mtx* genes encoding 100 kDa, 35.8 kDa, and 31.8 kDa proteins differ from the binary toxin gene and are not homogeneous with the latter [1].

Different toxic *B. sphaericus* strains have different mosquitocidal activities against susceptible and binary toxin-resistant larvae. C3-41, serotype H5, like most of high-toxicity strains of *B. sphaericus*, has high toxicity against susceptible *Culex quinquefasciatus* colony and no toxicity against the resistant colony; Strain LP1-G, belonging to serotype H3, has medium activity against both susceptible and resistant mosquito colonies. However, IAB872, belonging to serotype H48, performs high larvicidal activity against susceptible mosquito larvae like C3-41 and medium toxicity against resistant larvae [18]. During the last decade, *B. sphaericus* mosquitol larvicidal formulation has been widely used for mosquito control in many countries all over the world. After the intensive application of these formulations, high-level resistant mosquito larvae were recorded in some regions of China, India, France, and Brazil. The appearance of resistance in the mosquito has a severe influence on the...
further effective application of *B. sphaericus* as a kind of mosquito-larvicidal agents. Therefore, the mechanism and the control of resistant mosquitoes is becoming an active research field of biological control. Because IAB872 has toxicity against both susceptible and resistant mosquito larvae, it will perhaps become a potential natural strain to delay and control the appearance of resistant mosquitoes in future application. Accordingly, it is necessary to find the difference of the binary toxin genes between IAB872 and other strains.

The binary toxin genes from many *B. sphaericus* strains have been located and cloned in order to understand their genetic character and their toxicity [2, 5, 6, 11, 13, 19]. It has been proved that the binary toxin gene from IAB872 is located on a 3.5 kb *Hind* III fragment of its chromosomal DNA [15, 18]. In this paper, the binary toxin gene of IAB872 was cloned, sequenced, and expressed in a crystal-minus *B. thuringiensis* strain. In addition, the toxicity of *B. thuringiensis* transformants is also discussed.

Materials and Methods

**Bacterial strains and plasmids.** *B. sphaericus* IAB872 and the acrystalliferous strain 4Q7 of *B. thuringiensis* subs. *israelensis* were obtained from the Institute Pasteur, France; the latter was used as a recipient strain in the transformation experiments. *Escherichia coli* TG-1 [(lac-proAB) SupE thi hsd D F’(ral)36 proA’ proB’ lacI lacZ M15] was used as cloning host. The shuttle vector pBU4 for cloning was a gift from A Delecluse and contains tetracycline and ampicillin resistance determinants [7]. The plasmid pCW-1 contains the binary toxin gene from *B. sphaericus* C3-41 [19] and was used as a source of a 2.4-kb probe for hybridization detection. Plasmids pBS-1 and pBS-2 were constructed by ligating a 3.5 kb *Hind* III DNA fragment of total genomic DNA from *B. sphaericus* IAB872 into *Hind* III-cut, phospha-tase-treated vector pBU4, but in the opposite directions.

**DNA manipulations.** Total genomic DNA from *B. sphaericus* was prepared by the methods of Bourgouin et al. [7]. Cloning experiments, restriction enzyme analysis, Southern blotting, and other DNA manipulations were carried out as described by Maniatis et al. [14].

**Cloning of the binary toxin gene from IAB872.** Total genomic DNA from IAB872 was totally digested with *Hind* III restriction enzyme. About 3.5 kb DNA fragments were purified from an agarose gel with Qiagen Gel Extraction Kit (Gene Company) and inserted into *Hind* III-cut, phospha-tase-treated shuttle vector pBU4. Transformed *E. coli* TG-1 recombinant clones were screened by hybridization with the Dig-labeled probe obtained from a 2.4-kb *Sac*I-*Hind* III fragment of plasmid pCW-1.

**Transformation procedure.** Transformation of acrystalliferous *B. thuringiensis* subs. *israelensis* strain 4Q7 was carried out as described by Brain and Schurter [8]. Strain 4Q7 was grown in Luria-Bertani (LB) medium with shaking at 28°C overnight. Fifty ml of fresh LB medium was inoculated with 500 µl overnight culture and incubated with shaking to an optical density at 600 nm of 0.6–0.8. Then, the cell was centrifuged, and the pellet was washed three times with sterile distilled water and finally suspended in a solution containing 1 mM HEPES (pH 7.0) and 10% glycerol. The appropriate volume preparation, generally 200 µl or 400 µl, was added to 1–2 µg plasmid DNA and mixed well, then incubated on ice for 5 min. Electroporation was performed with an ice-cold 0.2-cm electroporation cuvette (BioRad) in a BioRad Gene Pulser apparatus set at 400–800 µF and 1.5–1.7 kV, with the pulser controller at 25 µF. After being placed on ice for another 5 min, the culture was added to 800 µL LB medium and incubated at 37°C for 1 h. Transformed cells were selected on LB plate containing 12.5 g/ml tetracycline, and the recombinant plasmid DNAs were isolated from transformed *B. thuringiensis* strains as described previously [10].

**Protein analysis.** *B. sphaericus* IAB872 and *B. thuringiensis* transformants were grown with shaking at 28°C in MBS medium [12] and LB medium containing 12.5 µg/ml tetracycline, respectively, both until spore formed and released. Spore-crystal mixtures were collected and washed once with deionized water, then suspended in TE-buffer. The spore-crystal suspensions were boiled with the loading buffer, and the soluble mixtures were resolved in a 0.1% sodium dodecyl sulfate-10% polyacrylamide gel, then stained with Coomassie brilliant blue R250 after electrophoresis [7]. If Western blotting was analyzed, the unstained proteins were electroblotted onto nitrocellulose paper and detected with rabbit antiserum directed against 51-kDa and 42-kDa peptides. BM Chromogenic Western Blotting Kit was purchased from Boehringer Mannheim Biochemicals.

**Toxicity bioassays.** The mosquito-larvicidal activities of *B. sphaericus* strain and *B. thuringiensis* transformants were assayed with the third or fourth-instar susceptible and resistant larvae of laboratory-reared *Culex quinquefasciatus* L by the standard method recommended by WHO [17]. The susceptible laboratory colony (SLCq) was established from a laboratory-reared colony maintained at Hubei Academy of Medical Sciences for more than 10 years. The resistant colony was selected with *B. sphaericus* C3-41 for 15 generations and then raised for 23 generations without selection pressure in our laboratory, having a resistance ratio to *B. sphaericus* of 142,000-fold (data not shown). Mortality was recorded after 48 h. Fifty and 90% lethal concentrations (LC50 and LC90) were calculated as final whole culture (FWC) dilution by using probit analysis with a program (E. Frachon, Institute Pasteur, France).

**Results and Discussion**

Approximately 3.5 kb *Hind* III fragments from IAB872 were ligated into pBU4 vector, as described in Materials and Methods, and the *E. coli* recombinants were screened on plates containing 100 µg/ml ampicillin. The recombinant plasmids were cut with *Hind* III enzyme and then transferred onto nylon membrane. 2.4 kb *Sac* I-*Hind* III fragment of plasmid pCW-1, which contains a fragment of binary toxin gene from *B. sphaericus* C3-41, wasDig-labeled and used as the specific probe in Southern blotting. Southern blotting showed that the probe hybridized strongly to 3.5 kb *Hind* III fragment of plasmid pCW-1 as control (Fig. 1, lane 2), and to 3.5 kb insert of one plasmid designated as pBS-1 (Fig. 1, lane 3). The foreign insert of plasmid pBS-1 was extracted from the gel and ligated to the same vector again, and another plasmid pBS-2 was constructed, giving the same size of 9.9 kb as plasmid pBS-1. Digestion of two constructed plasmids with *Sac*I, *Kpn*I, *Eco*RI, *Eco*RV, *Sac*I-*Hind* III revealed that the transcription of inserted genes in plasmid pBS-2 was in the same direction as the