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Improving the Insecticidal Activity against Resistant *Culex quinquefasciatus* Mosquitoes by Expression of Chitinase Gene *chiAC* in *Bacillus sphaericus*▽

Yajun Cai,[1,2] Jianpin Yan,[1] Xiaomin Hu,[1] Bei Han,[1] and Zhiming Yuan[1]*

State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China,[1] *and Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*[2]

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Expression of a chitinase gene, *chiAC*, from *Bacillus thuringiensis* in *B. sphaericus* 2297 using the binary toxin promoter yielded a recombinant strain that was 4,297-fold more toxic than strain 2297 against resistant *Culex quinquefasciatus*. These results show that this chitinase can synergize the toxicity of the binary toxin against mosquitoes and thus may be useful in managing mosquito resistance to *B. sphaericus*.

Insects of dipteran species such as *Culex* spp., *Anopheles* spp., and *Aedes* spp. are responsible for the transmission of many infectious disease agents. Directing an attack against these mosquitoes is recognized as one of the more effective approaches for eradicating the threat from infectious diseases (4). As a gram-positive, spore-forming, aerobic, entomopathogenic bacterium, *Bacillus sphaericus* has been successfully used for mosquito control in the last decade. Its activity against target mosquito larvae is mainly due to the crystal toxin, commonly referred to as the binary toxin, as it consists of equimolar amounts of two proteins of 51 and 42 kDa. (1). However, high-level resistance against *B. sphaericus* binary toxin after intensive treatment has developed in mosquitoes (6), with resistance ratios ranging from 35- to 150,000-fold in the laboratory (5, 8) and from 10- to 100,000-fold in the field (15). Experiments testing binary toxin binding to the mosquito larval midgut in vitro have demonstrated that resistance of mosquitoes to *B. sphaericus* binary toxin has occurred mainly because of elimination of the toxin-binding site on the midgut brush border membrane fraction (1, 6). The appearance of high-level of resistance in mosquitoes is a threat to the future application of *B. sphaericus* as a mosquito control agent.

On the basis of the sequence of the *chi* gene from *B. thuringiensis* subsp. *israelensis* (GenBank accession no. AF526379), two pairs of primers, chiAC-1–chiAC-3 and chiAC-2–chiAC-3, were designed for amplifying the *chiAC* open reading frame by PCR with different enzyme digestion sites introduced from strain T04A001 (3) (Table 1) as described elsewhere (17). After digestion with EcoRI and HindIII, the PCR fragment amplified with primers chiAC-1 and chiAC-3 was ligated with EcoRI/HindIII-digested plasmid pET28a and the resulting plasmid was transformed into *Escherichia coli* BL21. The recombinant E-pETC21 was selected on LB agar supplemented with kanamycin (50 μg/ml). The 70-kDa ChiAC fusion protein was purified for preparation of rabbit anti-ChiAC with a His-Bind Resin chromatography kit by following the procedure provided by the manufacturer (Novagen) (9).

For the expression of *chiAC* in *B. sphaericus*, a binary toxin promoter was amplified as described elsewhere (14) with primers pbinary-1 and pbinary-2. The amplified binary toxin promoter (~0.5 kb) was digested with BamHI and SphI and then introduced into BamHI/SphI-digested vector pBU4, resulting in recombinant plasmid pBb. The open reading frame of ChiAC, obtained by PCR with primers chiAC-2 and chiAC-3, was inserted into pBb at the SphI and HindIII sites after digestion with SphI/HindIII, giving recombinant shuttle vector pBbC.

Plasmids pBbC and pBU4 were transformed into *B. sphaericus* 2297 (serotype H25, from the Institute Pasteur) by electroporation as described elsewhere (7). Recombinant strains 2297-pBU4 and 2297-pBbC were selected on LB agar supplemented with tetracycline (12.5 μg/ml) and then confirmed by PCR and plasmid restriction enzyme digestion. All *B. sphaericus* strains were grown in LB medium at 30°C with shaking (200 rpm); the cells and supernatant of cultures at different growth stages were collected by centrifugation and stored at −20°C for protein analysis and bioassays.

Recombinant *B. sphaericus* 2297-pBbC grew and developed normally in LB medium and produced a typical parasporal body during sporulation. Sodium dodecyl sulfate-poly-

TABLE 1. Primers used in this study

Name	Sequence[a]	Position	Restriction site
chiAC-1	5′-GGGGAATTCATGGCT ATGAGGTCTC-3′	250–265	EcoRI
chiAC-2	5′-GGGGCATGCATGGCT ATGAGGTCTC-3′	250–265	SphI
chiAC-3	5′-GGGAAGCTTCTAGTTT TCGCTAATGAC-3′	2263–2280	HindIII
pbinary-1	5′-GGGGATCCGTCAACA TGTGAAGATT-3′	4–20	BamHI
pbinary-2	5′-GGGCATGCGCTTTCTT CATCTCCTTA-3′	478–495	SphI

[a] Restriction sites are underlined.

* Corresponding author. Mailing address: Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China. Phone: 86-27-87197242. Fax: 86-27-87198120. E-mail: yzm@pentium.whiov.ac.cn.

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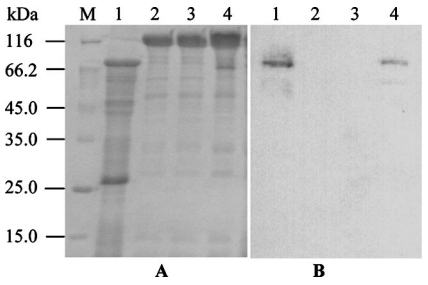

FIG. 1. Protein analysis of expression of *chiAC* in recombinant *B. sphaericus* by SDS-PAGE (A) and Western blotting (B). Lane M, markers; lane 1, T04A001; lane 2, 2297; lane 3, 2297-pBU4; lane 4, 2297-pBbC.

acrylamide gel electrophoresis (SDS-PAGE) and Western blot (9) analysis showed that recombinant 2297-pBbC could express an ~70-kDa chitinase during bacterial growth, and no corresponding band was detected in parental strain 2297 or control strain 2297-pBU4 (Fig. 1). SDS-PAGE and extracellular quantitative enzyme activity assays (3) indicated that ChiAC protein was expressed in recombinant *B. sphaericus* 2297-pBbC in LB medium throughout the vegetative growth and sporulation stage (Fig. 2), and the highest level of extracellular chitinase activity (360 U/ml) was observed at late sporulation and the level subsequently decreased (Fig. 3).

Using the standard method recommended by the World Health Organization (13), the toxicity of recombinant and native *B. sphaericus* to second- and third-instar larvae of a susceptible and a resistant laboratory *C. quinquefasciatus* colony (SLCq and RLCq1, respectively) was evaluated (8, 16). The bioassay results (2) showed that the 50% lethal concentrations (LC_{50}s) of parental strain 2297, control strain 2297-pBU4, and recombinant strain 2297-pBbC for susceptible *C. quinquefasciatus* were 7.52, 5.64, and 5.96 ng/ml, respectively, at 48 h, while the LC_{50}s of 2297, 2297-pBU4, and 2297-pBbC for resistant *C. quinquefasciatus* were >100,000, >100,000, and 23.23 ng/ml, respectively. The ratios of mosquito colony RLCq1 resistance to 2297 and 2297-pBbC were 18,300- and 3.90-fold, respectively, compared with the susceptible colony SLCq. The expression of ChiAC in *B. sphaericus* 2297 increased the strain's toxicity against resistant *C. quinquefasciatus* by about 4,290 times compared to that of parental strain 2297 (Table 2).

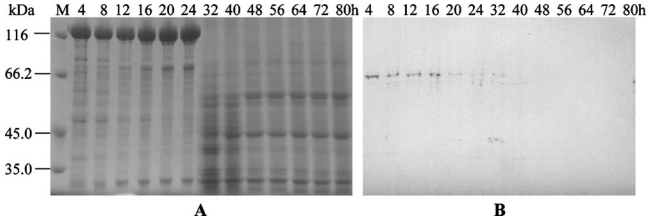

FIG. 2. Time-lapse analysis of expression of *chiAC* in recombinant *B. sphaericus* 2297-pBbC by SDS-PAGE (A) and Western blotting (B). Lane M, markers; lanes 1 to 13, bacterial cellular extracts taken from the cultures at 4, 8, 12, 16, 20, 24, 32, 40, 48, 56, 64, 72, and 80 h.

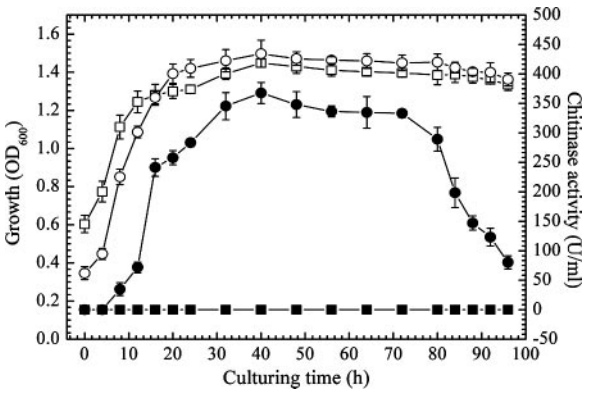

FIG. 3. Kinetics of chitinase production during bacterial growth. Open squares, growth of 2297; open circles, growth of 2297-pBbC; filled squares, chitinase activity of 2297; filled circles, chitinase activity of 2297-pBbC. OD_{600}, optical density at 600 nm.

The higher toxicity of strain 2297-pBbC to resistant *C. quinquefasciatus* compared to that of strain 2297 may be a consequence of synergy between the chitinase and binary toxin produced during sporulation (10, 11). After strains are digested by target mosquito larvae, the chitinase secreted by 2297-pBbC might degrade the chitin linkage in the peritrophic matrix; thus, the binary toxin can come into contact with target cells easily, resulting in cytopathogenicity without the specific toxin-receptor binding step.

A plasmid stability study revealed that foreign plasmid pBbC was not stable in recombinant strain 2297-pBbC under nonselective growth conditions. Within 7 days, the recombinant lost plasmid pBbC rapidly and only 0.01% tetracycline-resistant colonies, which harbored the foreign plasmid, were observed. Therefore, it is important to develop additional technologies to enhance the stability of the integrated chitinase gene by homologous recombination processes (12) to generate highly toxic *B. sphaericus* strains for mosquito control.

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TABLE 2. Toxicity of native and recombinant strains of *B. sphaericus* for *C. quinquefasciatus*

Strain[a]	Toxicity (ng/ml) against *C. quinquefasciatus* (95% CL)[c]				Resistance ratio[b]
	Susceptible colony		Resistant colony		
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	
2297	7.52 (6.21–9.23)	59.6 (39.4–109)	$>1 \times 10^5$	$>1 \times 10^5$	18,300
2297-pBU4	5.64 (4.68–6.62)	14.9 (12.0–20.7)	$>1 \times 10^5$	$>1 \times 10^5$	18,200
2297-pBbC	5.96 (4.73–8.00)	67.0 (36.9–172)	23.2 (19.9–27.0)	54.7 (44.5–74.1)	3.90

[a] Final whole cultures of native and recombinant strains.
[b] The resistance ratio at the LC_{50} is shown.
[c] 95% CL, 95% confidence limit.

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