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Cloning and Expression of the First Anaerobic Toxin Gene from *Clostridium bifermentans* subsp. *malaysia*, Encoding a New Mosquitocidal Protein with Homologies to *Bacillus thuringiensis* Delta-Endotoxins

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A gene (*cbm71*) encoding a 71,128-Da mosquitocidal protein (Cbm71) was obtained by screening a size-fractionated *Xba*I digest of total genomic DNA from *Clostridium bifermentans* subsp. *malaysia* CH18 with two gene-specific oligonucleotide probes. The sequence of the Cbm71 protein, as deduced from the sequence of *cbm71*, corresponds to that of the 66-kDa protein previously described as one of the mosquitocidal components of *C. bifermentans* subsp. *malaysia*. Cbm71 shows limited similarities with *Bacillus thuringiensis* delta-endotoxins, especially in the four first conserved blocks. However, Cbm71 was not immunologically related to any of the Cry toxins and thus belongs to a novel class of mosquitocidal protein. The *cbm71* gene was expressed in a nontoxic strain of *B. thuringiensis*, and Cbm71 was produced during sporulation and secreted to the supernatant of culture. Trichloroacetic-precipitated supernatant preparations were toxic for mosquito larvae of the species *Aedes aegypti*, *Culex pipiens*, and *Anopheles stephensi*.

The genus *Clostridium* is a heterogeneous group including approximately 100 known anaerobic species. Most efficiently degrade polysaccharides and proteins, producing a variety of industrially important products; for example, acetone, butanol, and ethanol are produced by *Clostridium acetobutylicum*. A second important subgroup comprises the human disease-causing clostridia such as *Clostridium tetani* and *Clostridium perfringens*. More than 90% of the work on the genus *Clostridium* addresses industrial biotechnology and medical aspects (21).

The discovery of a novel serovar of *Clostridium bifermentans*, *C. bifermentans* subsp. *malaysia* CH18 (7), has broadened the field of research to the biological control of insects. This strain is the first identified anaerobic bacterial strain highly toxic to mosquito larvae (31). Although the genus *Clostridium* contains species pathogenic for higher animals, *C. bifermentans* subsp. *malaysia* CH18 is totally innocuous to mammals and a variety of aquatic nontarget organisms (30, 34). This is a valuable feature for a biological insecticide.

Increasing resistance of mosquitoes to chemical insecticides necessitates the development of other methods of control. Currently, the major alternative is based on bacterial toxins produced by *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* (for a review, see reference 25). The targets of these toxins belong to the genera *Anopheles*, *Culex*, and *Aedes*, vectors of severe human diseases. However, in the last 3 years, field populations of insects showing resistance to the *B. sphaericus* bacterial toxins have been observed (26, 27, 29). Although no resistance to *B. thuringiensis* subsp. *israelensis* has been described, there is a need for new insecticides.

This has led to work to identify novel bacterial toxins with different structures and modes of action so as to minimize the risk of developing insect resistance.

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C. bifermentans subsp. *malaysia* toxins are highly toxic to *Anopheles* species. Its toxicity against *Anopheles stephensi* is about 10 times higher, but its toxicity against *Culex pipiens* or *Aedes aegypti* is 10 times lower, than that of *B. thuringiensis* subsp. *israelensis* (31).

This *C. bifermentans* subsp. *malaysia* toxicity is expressed during the sporulation stage and decreases considerably with cell lysis (5). In vitro incubation for 2 h at 37°C with proteinase K also destroys the toxicity (23). Thus, the toxicity is presumably due to a toxic protein that is susceptible to proteases released when the cell lyses. Unlike *B. thuringiensis* and *B. sphaericus*, *C. bifermentans* subsp. *malaysia* produces no inclusions associated with toxicity (5), and the *C. bifermentans* toxic components have not been isolated. However, biochemical analysis suggests that three proteins of 66, 18, and 16 kDa are involved in the toxicity (22). These proteins are not immunologically related to *B. thuringiensis* or *B. sphaericus* toxins and may therefore belong to a novel class of insecticidal toxins (22).

The roles and specificities of these proteins in the overall *C. bifermentans* subsp. *malaysia* mosquitocidal activity have not been clearly determined. One reason for this is that they aggregate into a complex, and thus it was not previously possible to purify them, even by ion-exchange chromatography, without loss of toxicity. Furthermore, they are unstable, being inactivated by physical filtration, sonication, or a cycle of freezing-thawing (22). In view of the technical difficulties associated with biochemical analysis, we are cloning the genes encoding the 66-, 18-, and 16-kDa proteins.

This paper reports the cloning and expression of the gene encoding the 66-kDa protein, designated *cbm71*. The deduced protein, Cbm71, has regions similar to the four first blocks conserved in all Cry1, Cry3, and Cry4 *B. thuringiensis* delta-endotoxins (14).

Expression of *cbm71* in *B. thuringiensis* resulted in a mosquitocidal protein in the culture medium. This is thus the first reported example of a secreted or excreted mosquitocidal toxin derived from an anaerobic bacterium.

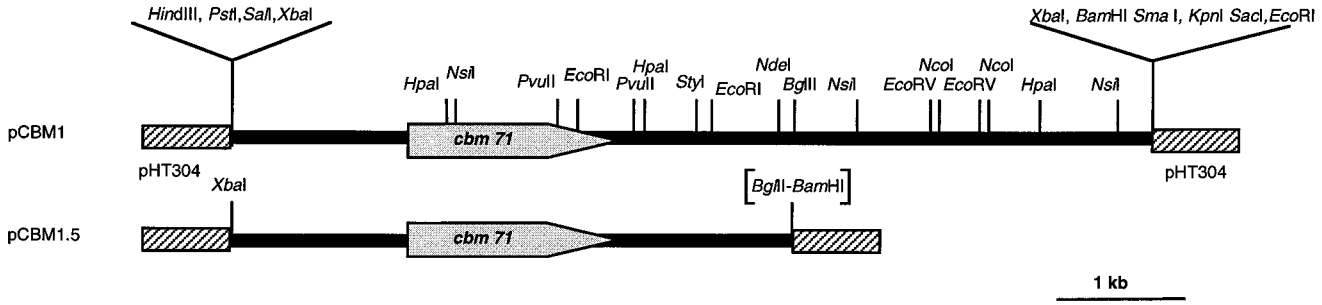

FIG. 1. Restriction maps of pCBM1 and pCBM1.5 containing the *cbm71* gene. Hatched boxes represent the pHT304 vector, and arrows show the position and the direction of transcription of *cbm71*. Sites in brackets have been lost.

MATERIALS AND METHODS

Bacterial strains, media, and plasmid. *C. bifermentans* subsp. *malaysia* is from the collection of the Unité des Bactéries Entomopathogènes (Institut Pasteur), where it is listed as strain CH18. *Escherichia coli* TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15] was used for cloning experiments. The crystal-negative *B. thuringiensis* SPL407 (serotype H1) was used as a model aerobic gram-positive host (18).

C. bifermentans subsp. *malaysia* was grown anaerobically in TGY medium (5) under 5% H_2–5% CO_2–90% N at 34°C. *E. coli* was cultured at 37°C in Luria broth (LB). *B. thuringiensis* was cultured in nutrient broth media (Difco, Detroit, Mich.). Ampicillin (100 μg/ml) and erythromycin (25 μg/ml) were added as required.

The shuttle vector pHT304 (2) was used as a cloning vector. *E. coli* was transformed with plasmid DNA following $CaCl_2$ treatment as previously described (17). *B. thuringiensis* was transformed by electroporation as described by Lereclus et al. (18) except that cells were grown in LB medium with shaking at 37°C until the optical density at 650 nm was 0.8.

DNA procedures. *C. bifermentans* total DNA was isolated as described by Delécluse et al. (8). Large amounts of plasmid DNA were prepared from *E. coli* by using a Qiagen kit (Qiagen GmbH), and small amounts were prepared from *E. coli* and *B. thuringiensis* by using an RPM kit (Bio 101, Inc., Vista, Calif.). *C. bifermentans* plasmids were extracted by alkaline lysis and further purified on CsCl gradients.

Chromosomal linear DNA was eliminated by DNase (Plasmid-Safe ATP-dependent DNase) treatment as described by the manufacturer (TEBU, Madison, Wis.). DNA fragments used in plasmid construction were isolated from agarose gels by using a Prep-a-Gene kit (Bio-Rad, Hercules, Calif.). Oligonucleotides used for sequencing and PCR experiments were prepared by Eurogentec.

All enzymes were used as recommended by the manufacturers. Nucleotide sequences were determined and analyzed as described by Delécluse et al. (10). Hybridization experiments were performed on Hybond-N$^+$ filters (Amersham). The oligonucleotides were labeled with fluorescein by using the ECL (enhanced chemiluminescence) 3′ Oligolabeling system (Amersham). For mapping, the 8-kb *Xba*I cloned fragment from pCBM1 was labeled by using the ECL direct nucleic acid labeling system (Amersham).

Cloning of the *C. bifermentans cbm71* gene. Short NH_2-terminal and internal amino acid sequences of the 66-kDa protein from *C. bifermentans* subsp. *malaysia* were determined (Laboratoire de Microséquençage des Protéines, Institut Pasteur, Paris, France), using a model 473 automatic sequencer (Applied Biosystems), after transfer of the protein to Problot membranes (Applied Biosystems); oligonucleotides corresponding to the amino acid sequences MNTNIFS THLEFSKG and NNDEWIYGEPDSSNI, respectively, were synthesized by the methoxyphosphoramidite method (Unité de Chimie Organique, Institut Pasteur). The sequences of the oligonucleotides were chosen according to *Clostridium* codon usage (35).

dI residues were included at each degenerate position (where there are more than two codons for one amino acid). The resulting oligonucleotide probes were 66A, corresponding to the NH_2 terminus from positions 1 (M) to 9 (H) (ATG AAT ACI AAT ATI TTT TCI ACI AA), and 66B, complementary to the sequence encoding amino acids from positions 3 (D) to 11 (D) (TG IGG TTC ICC ATA IAT CCA TTC ATC), and were labeled with fluorescein. Both probes hybridized to an 8-kb *Xba*I DNA fragment of *C. bifermentans* subsp. *malaysia* total DNA. *C. bifermentans* subsp. *malaysia* total DNA was digested with *Xba*I, and fragments of between 7 and 9 kb were ligated into the *Xba*I site of pHT304 (pretreated with alkaline phosphatase). TG1 cells were transformed, and 400 recombinant colonies were picked and screened for hybridization with probes 66A and 66B. Ten clones were identified as giving similar signals with the two probes. All contained the same plasmid, designated pCBM1 (Fig. 1). pCBM1.5 was obtained by eliminating a 3.8-kb *Bgl*II-*Bam*HI fragment from pCBM1 while still hybridizing with probes 66A and 66B.

Protein analysis. Recombinant *B. thuringiensis* strains were grown in nutrient broth supplemented with erythromycin, with shaking, at 30°C for up to 23 h, corresponding to the mid-sporulation stage. Cultures were centrifuged at 19,500 × *g*, and the supernatant was collected and treated with trichloroacetic acid (to a final concentration of 10% [wt/vol]) to precipitate soluble proteins.

The samples were incubated for 30 min on ice and centrifuged at 20,300 × *g*. The pellet was washed with 95% ethanol and resuspended in one-quarter of its original volume in H_2O. The protein concentration of each preparation was measured by the Bradford assay (Bio-Rad), using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide (12 and 10%) gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (16). Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Hybond C Super; Amersham) and detected immunologically by using an ECL Western blotting (immunoblotting) kit (Amersham) as recommended by the manufacturer. The antiserum raised against total *C. bifermentans* subsp. *malaysia* protein extracts was prepared as described by Nicolas et al. (22).

Bioassays on mosquito larvae. Total cultures, washed pellets, and trichloroacetic acid-precipitated supernatants (STCA) of recombinant *B. thuringiensis* strains were diluted in glass petri dishes containing 2 ml of deionized water and 0.5 mg of yeast extract and tested in duplicate against 20 second-instar larvae of *Anopheles stephensi* ST15, *Culex pipiens pipiens* Montpellier, and *Aedes aegypti* Bora-Bora. Ten different dilutions were used. Mortality was scored after 48 h of exposure at 25°C. Bioassays were repeated three times and LC_{50}s and LC_{90}s (concentrations giving 50 and 90% mortality, respectively) were determined by probit analysis.

Nucleotide sequence accession number. The nucleotide sequence data shown in Fig. 2 are available in the EMBL nucleotide sequence database under accession number X94146.

RESULTS

Cloning and sequence analysis. The gene encoding the 66-kDa protein from *C. bifermentans* subsp. *malaysia* was obtained on an *Xba*I fragment by using probes 66A and 66B, corresponding to the NH_2-terminal and internal amino acid sequences of this protein, respectively, as described in Materials and Methods. The restriction map of the *Xba*I fragment was established (Fig. 1). One plasmid, pCBM1, was used for further experiments.

The position of the gene encoding the 66-kDa protein (hereafter designated *cbm71*) was determined by both hybridization and PCR experiments. The *cbm71* gene was mapped to a 4.2-kb *Xba*I-*Bgl*II fragment (Fig. 1). Plasmid pCBM1.5, containing only this fragment of *C. bifermentans* DNA, was constructed by digestion of pCBM1 with *Bgl*II and *Bam*HI and ligation of the compatible ends. The direction of transcription of *cbm71* was determined by PCR experiments, and the gene was sequenced on both strands.

The base composition was 25% dG+dC, showing that *C. bifermentans* subsp. *malaysia* belongs to the group of extremely low dG+dC clostridia. There is a single open reading frame of 1,842 bp, encoding a polypeptide of 614 residues with a predicted molecular mass of 71,128 Da (Fig. 2). Nucleotides 1 through 45 encode an amino acid sequence identical to the determined 66-kDa N terminus (MNTNIFSTHLEFSKG) except that amino acid in position 9 is a histidine in the deduced sequence instead of the glutamine in the determined sequence.

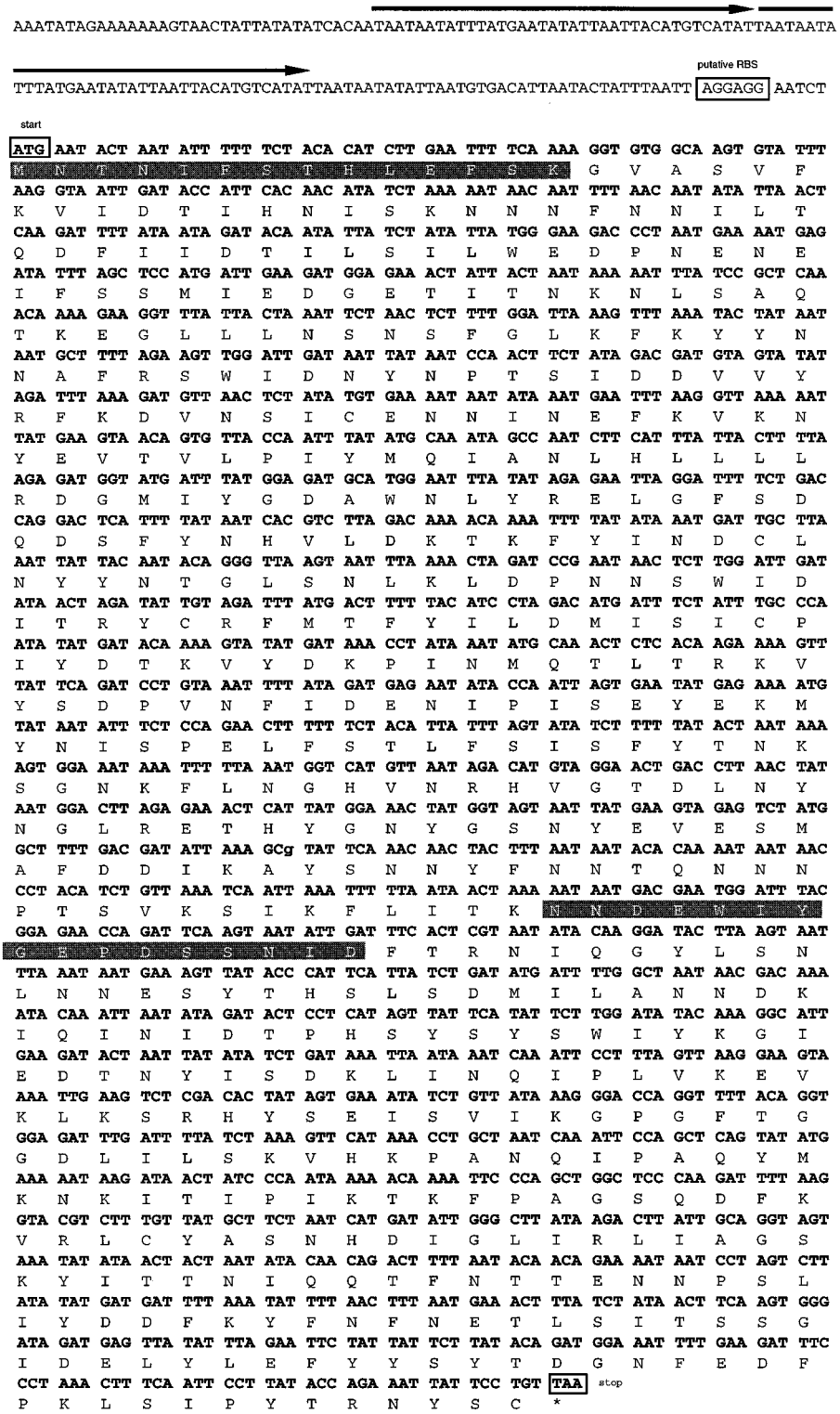

FIG. 2. Nucleotide sequence of the 1,842-bp fragment corresponding to the *cbm71* gene and its upstream region. A putative ribosome binding site (RBS) is indicated. Arrows indicate both 37-bp perfect direct repeats. Gray blocks represent the NH_2-terminal and internal amino acid sequences as determined by microsequencing.

Block I

```
Cry1AA     153   YQVPLLSVYVQAANLHLSVLRDVSVFGQRW
Cry3A      197   YEVLFLTTYAQAANTHLFLLKDAQIYGEEW
Cry4A      202   YNILVLSSYAQAANLHLTVLNQAVKFEAYL
Cry7AA     180   YEIPLLTVYAQAANLHLALLRDSTLYGDKW
Cry8A      191   HEVLLLAVYAQAVNLHLLLLRDASIFGEEW
Cry9A      186   AQILLLPSFASAAFFHLLLLRDATRYGTNW
Cry10A     195   YRIPTLPAYAQIATWHLNLLKHAATYYNIW
Cbm71      141   YEVTVLPIYMQIANLHLLLLRDGMIYGDAW
                 ••• ** ** • ••••••••••••••••  •
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Block II

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Cry1AA     226   WVRYNQFRRELTLTVLDIVALFSNYDSRRYPIRTVSQ-LTREIYTNPV
Cry3A      270   WVNFNRYRREMTLTVLDLIALFPLYDVRLYPKEVKTE-LTRDVLTDPI
Cry4A      289   WNTYNTYRTKMTTAVLDVVALFPNYDVGKYPIGVQSE-LTREIYQVLN
Cry7AA     253   WINYNRFRREMILMALDLVAVFPFHDPRRYSMETSTQ-LTREVYTDPV
Cry8A      264   WLNYHQFRREMTLLVLDLVALFPNYDTHMYPIETTAQ-LTRDVYTDPI
Cry9A      258   WLEFHRYRREMTLMVLDIVASFSSLDITNYPIETDFQ-LSRVIYTDPI
Cry10A     275   WNMYNTYRLEMTLTVLDLIAIFPNYDPEKYPIGVKSE-LIREVYTNVN
Cbm71      218   WIDITRYCRFMTFYILDMISIFPIYDTKVYDKPINMQTLTRKVYSDPV
                 **   •• • ••  ***** *•• ••   •   *  • ••• ••*•••
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Block III

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Cry1AA     452   FSWQHRSAEFNNIIPSSQITQIPLTKSTNLGS
Cry3A      499   LTWTHKSVDFFNMIDSKKITQLPLVKAYKLQS
Cry4A      520   FAWTHSSVDPKNTIYTHLTTQIPAVKANSLGT
Cry7AA     479   FSWTHRSAEYYNRIYPNKITKIPAVKMYKLDD
Cry8A      508   FVWTHTSADLNNTIYSDKITQIPAVKGDMLYL
Cry9A      498   YGWTHKSLARNNTINPDRITQIPLTKVDTRGT
Cry10A     492   FSWTHTSVDFQNTIDLDNITQIHALKALKVSS
Cbm71      433   YSWIYKGIEDTNYISDKLINQIPLVKGSKIES
                 •••  * ••  • •    • •••••• •• •
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Block IV

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Cry1AA     519   SQRYRVRIRYAST
Cry3A      566   SQKYRARIHYAST
Cry4A      583   QQSYFIRIRYASN
Cry7AA     546   SQKYRVRVRYATN
Cry8A      575   SQRYRVRIRYAST
Cry9A      565   RQQYRIRVRYAST
Cry10A     556   SRQYQVRIRYATN
Cbm71      516   SQDFKVRLCYASN
                 •• ••••• ••••
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FIG. 3. Comparison of the deduced amino acid sequence of Cbm71 with those of the conserved blocks I to IV of *B. thuringiensis* delta-endotoxins. Dots and asterisks indicate identical and functionally similar residues, respectively, between Cbm71 and at least three delta-endotoxins (accepted conservative-replacement groupings are I, L, V, and M; D and E; Q and N; K and R; T and S; G and A; and F and Y).

Nucleotides 1122 through 1167 encode NNDEWIYGEPDS SNI, identical to the determined internal sequence.

A putative ribosome binding site (AGGAGG) with a ΔG of −79.7 kJ/mol, calculated by comparison with the *Bacillus subtilis* consensus sequence as described by Tinoco et al. (32), was found five bases upstream from the initiation codon. Similar sequences have been found in several *Clostridium* species (35). No typical promoter or terminator was identified upstream or downstream from the coding region, respectively. In contrast, there is a 37-bp perfect direct repeated sequence 48 bp upstream from the start codon (Fig. 2), but with no significant similarity with other bacterial repeated sequences.

The amino acid sequence of the *cbm71* gene product was compared with those of other known toxins and proteins in Swiss-Prot and PIR-Prot databanks. It is about 30% similar to sequences of *B. thuringiensis* toxins. The similarities are restricted to the four first blocks conserved in most delta-endotoxins (Fig. 3). Block I and block IV are the most similar (88 and 58% similarity with Cry1, respectively). Cbm71 contains no block V, and block III is relatively dissimilar to the consensus. The homologies with *B. thuringiensis* toxins indicate that Cbm71, although coming from a non-*B. thuringiensis* organism,

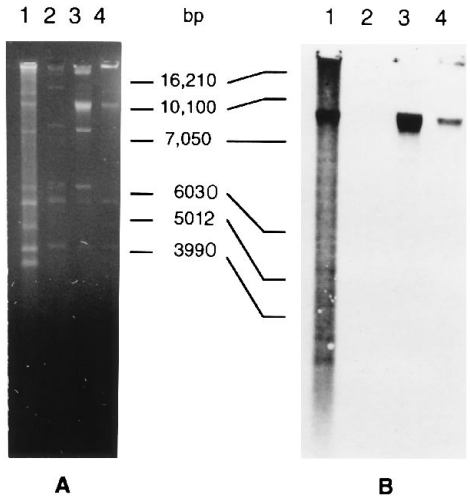

FIG. 4. Analysis of CCC plasmid DNA, before and after Plasmid-Safe treatment, and total DNA of *C. bifermentans* subsp. *malaysia* CH18. (A) 0.7% agarose gel stained with ethidium bromide. (B) Autoradiograph of DNA transferred to nylon filters and hybridized with the labeled 8-kb *Xba*I pCBM1 fragment. Lane 1, total *C. bifermentans* subsp. *malaysia* CH18 DNA; lane 2, CCC plasmid preparation treated with Plasmid-Safe; lanes 3 and 4, nontreated CCC plasmid preparation. The numbers between the panels represent CCC size markers.

may belong to the Cry toxin family. Therefore, *cbm71* was proposed in the new *B. thuringiensis* nomenclature (6) as *cry16A* (4a). No significant similarity was found with other toxins or other *Clostridium* proteins.

Hydropathy analysis of Cbm71 was performed by using the Kyte-Doolittle program (15), with a window length of 17 residues. Cbm71 is hydrophilic, with only four major hydrophobic peaks, all in the 300 N-terminal residues (data not shown).

***cbm71* gene mapping.** *C. bifermentans* subsp. *malaysia* contained five plasmids of approximately 20, 15, 8.5, 7, and 4 kb and no large plasmid (Fig. 4A, lanes 3 and 4). Preparations of total DNA (Fig. 4B, lane 1), covalent closed circular (CCC) plasmid DNA containing chromosomal DNA (Fig. 4B, lanes 3 and 4), and CCC plasmid treated with a Plasmid-Safe kit to eliminate all chromosomal DNA (Fig. 4B, lane 2) were tested for hybridization with the 8-kb *Xba*I fragment cloned in pCBM1. The *Xba*I fragment was found in chromosomal but not plasmid DNA.

Expression of *cbm71* in a crystal-negative strain of *B. thuringiensis*. Plasmids pCBM1 and pCBM1.5 were introduced by electroporation into the crystal-negative strain of *B. thuringiensis* 407 (in which sporulation toxin genes are generally well expressed) to study whether expression of the *cbm71* gene in aerobic gram-positive hosts was associated with mosquitocidal activity. Plasmid pHT304 was also introduced as a negative control. Recombinant cells sporulated well in nutrient broth and lysed after 28 to 30 h of growth.

As assessed by microscopy, the *B. thuringiensis*(pCBM1) and *B. thuringiensis*(pHT304) transformants contained no parasporal inclusion during sporulation, whereas aggregates were present in pCBM1.5-transformed sporangia containing refractile spores (Fig. 5). However, purification of these bodies was not possible with conventional techniques such as sucrose gradients, and thus the toxicity of these aggregates could not be tested.

Expression of *cbm71* at the mid-sporulation stage (20 to 23 h of culture) was analyzed by Coomassie brilliant blue staining and by Western blotting. Proteins in STCA preparations, washed pellet, and total culture extracts were analyzed by SDS-

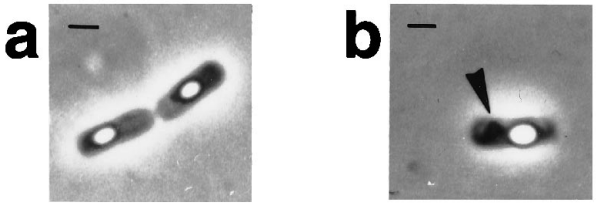

FIG. 5. Phase-contrast micrographs of pCBM1 (a)- and pCBM1.5 (b)-transformed *B. thuringiensis* sporangia. The arrowhead indicates a dark aggregate. The bars represent 1 μm.

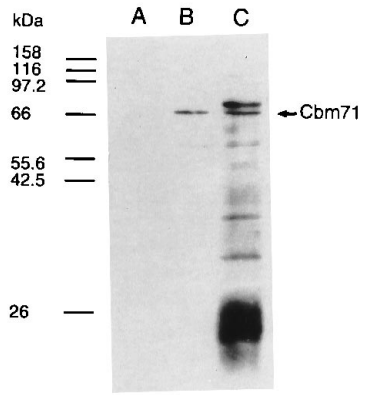

FIG. 6. Production of Cbm71 in the various *B. thuringiensis* strains. The STCA samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies raised against *C. bifermentans* subsp. *malaysia* toxic extract. Lanes: A, *B. thuringiensis*(pHT304); B, *B. thuringiensis*(pCBM1.5); C, *B. thuringiensis*(pCBM1). Cbm71 is indicated by an arrow, and molecular mass markers are shown on the left.

PAGE and Coomassie brilliant blue staining. All extracts, including those corresponding to the vector pHT304 alone, contained a wide variety of proteins, from 130 to 10 kDa. However, no major component associated with *cbm71* was identified.

In contrast, Western blotting with antibodies raised against total *C. bifermentans* subsp. *malaysia* toxic extract revealed two 66- to 68-kDa proteins in the STCA of cells containing pCBM1 (Fig. 6, lane C). STCA of pCBM1.5 transformants contained a single 66-kDa protein (Fig. 6, lane B). No protein reacting with *C. bifermentans* subsp. *malaysia* antiserum was observed in the STCA from cells containing pHT304 (Fig. 6, lane A). No proteins reacting with *C. bifermentans* subsp. *malaysia* antiserum were detected in washed pellet samples or total culture of any of the clones (data not shown).

Larvicidal activity of Cbm71 toxin in the two different constructions. STCA preparations from *B. thuringiensis* 407(pCBM1) and *B. thuringiensis* 407(pCBM1.5) were assayed for mosquitocidal activity by using *Aedes aegypti*, *A. stephensi*, and *Culex pipiens* larvae. STCA from *B. thuringiensis* 407(pHT304) was tested in the same conditions as a negative control and was totally inactive. Washed pellets were also assayed in the same conditions and were totally inactive (data not shown). In contrast, both pCBM1 and pCBM1.5 STCA samples were toxic, with LC_{50}s and LC_{90}s of around 150 and 300 μg/ml, respectively, against all three species tested.

Cbm71 had the same activity spectrum as *C. bifermentans* subsp. *malaysia*, with highest toxicity against *A. stephensi* and lowest toxicity against *Aedes aegypti* (31) (Table 1). However, the toxicities for the three different species of mosquito were similar, suggesting that the activity is nonspecific. No major difference in toxicity was found when we compared precisely (with standard error) the values for pCBM1.5- and pCBM1-transformed cells.

DISCUSSION

We report the cloning and characterization of a novel gene from *C. bifermentans* subsp. *malaysia*. The *cbm71* gene encodes a protein of 71,128 Da involved in mosquitocidal activity.

The deduced amino acid sequence of Cbm71 is similar to sequences of mosquitocidal toxins from *B. thuringiensis*, and the name Cry16A was proposed to indicate its relationship with the Cry-like toxin family. This Cry-like toxin is the first one found in an anaerobic, non-*B. thuringiensis* organism. The regions of strongest similarity were within the toxic domain of *B. thuringiensis* delta-endotoxins, i.e., the amino-terminal part (20), especially within blocks I to IV. The sequence of block V and the carboxy terminus of delta-endotoxins are absent from Cbm71. Thus, Cbm71 is most similar to Cry3 proteins, which are also truncated in the C-terminal region, although Cry3 toxins contain block V (20). Possibly, the absence of the C-terminal region conserved in *B. thuringiensis* delta-endotoxins (Cry1) and the small number of cysteine residues contribute to the instability of Cbm71.

Cbm71 is the first-described anaerobic bacterial protein showing similarities with *B. thuringiensis* toxins. It is likely that *C. bifermentans* subsp. *malaysia* and *B. thuringiensis* mosquitocidal toxins have a common ancestor. The perfect 37-bp direct repeats and the presence of an open reading frame similar to the gene for the resolvase of transposon Tn*1546* from *Enterococcus faecium* (described in the Swiss-Prot database; EMBL accession number M97297 [4]) 300 bp upstream from the *cbm71* gene (data not shown) may indicate a possibility of acquisition of genes by transposition as previously suggested (11). Analysis of the whole transposon and total sequencing of the region surrounding *cbm71* may allow elucidation of the mechanism of mobility of this *Clostridium* gene.

This *C. bifermentans* subsp. *malaysia* toxin gene is on the chromosome, eliminating the possibility of transfer by plasmid

TABLE 1. Larvicidal activity of *B. thuringiensis* transformants

B. thuringiensis transformant	Mean larvicidal activity[a] (μg/ml) ± SE					
	A. stephensi		*Aedes aegypti*		*Culex pipiens*	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
407(pCBM1)	129 ± 10	291 ± 109	185 ± 81	314 ± 119	156 ± 10	296 ± 69
407(pCBM1.5)	NA[c]	185 ± 1	140 ± 65	264 ± 69	132 ± 52	264 ± 74

[a] Of total TCA extracts from 20-h cultures. LC_{50} and LC_{90} were determined at 48 h on second-instar larvae of susceptible insects.
[b] For transformant 407(pHT304) concentrations in all cases were >245 μg/ml, levels at which no mortality was obtained.
[c] NA, not available at 48 h. (LC_{50} at 24 h = 174 ± 71 μg/ml).

conjugation. Such chromosomal localization of mosquitocidal toxin genes has been reported for *B. sphaericus* (1) and *B. thuringiensis* (reviewed in references 3 and 19), although in the latter the genes may be carried by a very large plasmid which cannot be discriminated from the chromosome. Despite the similarity with Cry proteins, Cbm71 did not cross-react with antibodies raised against any of the *B. thuringiensis* subsp. *israelensis* or other *B. thuringiensis* toxins (22), confirming that this protein belongs to a novel class of mosquitocidal toxin.

cbm71 was poorly expressed in *B. thuringiensis*, possibly because of the unusually short spacing between the ribosome binding site and start codon or because the *Clostridium* promoter is inefficiently recognized in *B. thuringiensis*. Improvement in the expression of *cbm71* will allow precise analysis of the toxicity and specificity of Cbm71.

A doublet at 68 to 66 kDa [*B. thuringiensis*(pCBM1)] and a single band of 66 kDa [*B. thuringiensis*(pCBM1.5)] were revealed in culture supernatants by Western blotting. Since *cbm71* was the only long open reading frame carried by both clones, presumably Cbm71 corresponds to the lower band of the P68-P66 complex, previously implicated in the toxicity of *C. bifermentans* subsp. *malaysia* (22). Consequently, pCBM1 may contain a second open reading frame corresponding to P68.

Cbm71 was produced and secreted by recombinant *B. thuringiensis* strains. However, inclusions or aggregates were hardly observed in pCBM1.5 transformant clones and were absent from pCBM1 transformants. Cbm71 contains around 35.8% hydrophobic amino acids, which according to Van Holde (33) is close to the limit for a protein to associate in inclusions. Furthermore, Cbm71 does not contain a region corresponding to the COOH part of delta-endotoxins which could be involved in crystal formation. In addition, the apparent secretion of Cbm71 may prevent accumulation of the protein in the cell and therefore inclusion formation. The hydropathy profile indicates that it is a soluble protein with a hydrophobic region at the N terminus. The characteristics of the N-terminal sequence, however, do not conform to a traditional leader peptide (28). Thus, the difference in the deduced size (71.1 kDa) and the size estimated by gel electrophoresis (66 kDa) may be due to cleavage of 30 to 40 C-terminal residues. This type of modification is observed for *E. coli* hemolysin, in which the C-terminal part is cleavable and plays a role in the secretion of the protein (12, 13). Alternatively, the size difference may be due to another posttranslational modification such as glycosylation or unusual electrophoretic behavior of the Cbm71 polypeptide.

B. thuringiensis transformant culture supernatants contained Cbm71 before cell lysis, and thus these cells export the protein as does *C. bifermentans* subsp. *malaysia*. However, the *B. thuringiensis* transformants produced only small amounts of Cbm71 (around 1% of total proteins in STCA preparations). This may lead to overestimation of both LC_{50}s and LC_{90}s. In consequence, the toxicity of recombinant *B. thuringiensis* expressing *cbm71* is difficult to compare with that of recombinant strains of *B. thuringiensis* expressing high amounts of mosquitocidal toxins such as Cry4A, Cry4B, or Cry11A (9, 24). However, even assuming that the best LC_{50}s (129 μg/ml) is an underestimate of ~100-fold, the actual LC_{50} of the toxin would be comparable to those of other individual mosquitocidal toxins such as Cry4A (1.6 to 7.4 μg/ml) (9). Improved expression of *cbm71* and purification of Cbm71 will help elucidate the toxicity of this protein and allow comparison with other mosquitocidal toxins.

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REFERENCES

1. **Aquino de Muro, M., W. J. Mitchell, and F. G. Priest.** 1992. Differentiation of mosquito-pathogenic strains of *Bacillus sphaericus* from non-toxic varieties by ribosomal RNA gene restriction patterns. J. Gen. Microbiol. **138:** 1159–1166.
2. **Arantès, O., and D. Lereclus.** 1991. Construction of cloning vectors for *Bacillus thuringiensis*. Gene **108:**115–119.
3. **Aronson, A. I., W. Beckman, and P. Dunn.** 1986. *Bacillus thuringiensis* and related insect pathogens. Microbiol. Rev. **50:**1–24.
4. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175:**117–127.

4a. **Dean, D., and D. Zeigler.** Personal communication.

5. **Charles, J.-F., L. Nicolas, M. Sebald, and H. de Barjac.** 1990. *Clostridium bifermentans* serovar *malaysia*: sporulation, biogenesis of inclusion bodies and larvicidal effect on mosquito. Res. Microbiol. **141:**721–733.
6. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, B. Lambert, D. Lereclus, J. Baum, and D. H. Dean.** 1995. Revision of the nomenclature for the *Bacillus thuringiensis cry* genes. Microbiol. Rev. (submitted).
7. **de Barjac, H., M. Sebald, J.-F. Charles, W. H. Cheong, and H. H. Lee.** 1990. *Clostridium bifermentans* serovar *malaysia*, une nouvelle bactérie anaérobie pathogène des larves de moustiques et de simulies. C.R. Acad. Sci. Sér. III **310:**383–387.
8. **Delécluse, A., J.-F. Charles, A. Klier, and G. Rapoport.** 1991. Deletion by in vivo recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol. **173:**3374–3381.
9. **Delécluse, A., S. Poncet, A. Klier, and G. Rapoport.** 1993. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3922–3927.
10. **Delécluse, A., M.-L. Rosso, and A. Ragni.** 1995. Cloning and expression of a novel gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. Appl. Environ. Microbiol. **61:**4230–4235.
11. **Dürre, P.** 1993. Transposons in clostridia, p. 227–247. *In* D. R. Wood (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Stoneham, Mass.
12. **Felmlee, T., S. Pellett, E. Y. Lee, and R. A. Welch.** 1985. *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. J. Bacteriol. **163:**88–93.
13. **Gray, L., N. Mackman, J. M. Nicaud, and I. B. Holland.** 1986. The carboxy-terminal region of the haemolysin 2001 is required for secretion of the toxin from *Escherichia coli*. Mol. Gen. Genet. **205:**127–133.
14. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
15. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157:**517–525.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
17. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
18. **Lereclus, D., O. Arantès, J. Chaufaux, and M.-M. Lecadet.** 1989. Transformation and expression of a cloned δ-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol. Lett. **60:**211–218.
19. **Lereclus, D., C. Bourgouin, M.-M. Lecadet, A. Klier, and G. Rapoport.** 1989. Role, structure, and molecular organization of the genes coding for the parasporal δ-endotoxins of *Bacillus thuringiensis*, p. 255–276. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
20. **Lereclus, D., A. Delécluse, and M.-M. Lecadet.** 1993. Diversity of *Bacillus thuringiensis* toxins and genes, p. 36–69. *In* P. S. Entwistle, J. S. Cory, M. J. Bailey, and S. R. Higgs (ed.), *Bacillus thuringiensis* and environmental biopesticide: theory and practice. John Wiley & Sons, Ltd., London.
21. **Morris, J. G.** 1993. History and future potential of the clostridia in biotechnology, p. 1–20. *In* D. R. Woods (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Stoneham, Mass.
22. **Nicolas, L., J.-F. Charles, and H. de Barjac.** 1993. *Clostridium bifermentans* serovar *malaysia*: characterization of putative mosquito larvicidal proteins. FEMS Microbiol. Lett. **113:**23–28.
23. **Nicolas, L., S. Hamon, E. Frachon, M. Sebald, and H. de Barjac.** 1990.

Partial inactivation of the mosquitocidal activity of *Clostridium bifermentans* serovar *malaysia* by extracellular proteinases. Appl. Microbiol. Biotechnol. **34:**36–41.
24. **Poncet, S., A. Delécluse, A. Klier, and G. Rapoport.** 1995. Evaluation of synergistic interactions between the CryIVA, CryIVB and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
25. **Porter, A. G., E. W. Davidson, and J.-W. Liu.** 1993. Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol. Rev. **57:**838–861.
26. **Rao, D. R., T. R. Mani, R. Rejendran, A. S. J. Joseph, A. Gajanana, and R. Reuben.** 1994. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. J. Am. Mosq. Control Assoc. **11:**1–5.
27. **Silva-Filha, M. H., L. Regis, C. Nielsen-Leroux, and J.-F. Charles.** 1995. Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). J. Econ. Entomol. **88:**525–530.
28. **Simonen, M., and I. Palva.** 1993. Protein secretion in *Bacillus* species. Microbiol. Rev. **57:**109–137.
29. **Sinègre, G., M. Babinot, J. M. Quermel, and B. Gaven.** 1994. Field occurrence of *Culex pipiens* resistance in southern France, p. 5–8. *In* Proceedings of the VIIIth European Meeting of the Society of Vector Ecology, Barcelona, Spain.
30. **Thiéry, I., S. Hamon, V. Cosmao Dumanoir, and H. de Barjac.** 1992. Vertebrate safety of *Clostridium bifermentans* serovar *malaysia*, a new larvicidal agent for vector control. J. Econ. Entomol. **85:**1618–1623.
31. **Thiéry, I., S. Hamon, B. Gaven, and H. de Barjac.** 1992. Host range of *Clostridium bifermentans* serovar *malaysia*, a mosquitocidal anaerobic bacterium. J. Am. Mosq. Control Assoc. **8:**272–277.
32. **Tinoco, I. J., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246:**40–41.
33. **Van Holde, K. E.** 1977. Effect of the amino acid component and microenvironment on protein structure, p. 1–13. *In* J. R. Whitaker and S. R. Tannenbaum (ed.), Food proteins. Westport, Avi.
34. **Yiallouros, M., V. Storch, I. Thiéry, and N. Becker.** 1994. Efficacy of *Clostridium bifermentans* serovar *malaysia* on target and nontarget organisms. J. Am. Mosq. Control Assoc. **10:**51–55.
35. **Young, M., N. P. Minton, and W. L. Staudenbauer.** 1989. Recent advances in the genetics of the clostridia. FEMS Microbiol. Rev. **63:**301–326.