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

FRÉDÉRIQUE BARLOY,* ARMELLE DELECLUSE, LUC NICOLAS, AND MARGUERITE-M. LÉCADET

Unité des Bactéries Entomopathogènes, Institut Pasteur, 75724 Paris Cedex 15, France

Received 11 January 1996/Accepted 20 March 1996

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 non toxic 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.

*Corresponding author. Mailing address: Unité des Bactéries Entomopathogènes, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 40 61 31 83, Fax: (33) 1 40 61 30 44. Electronic mail address: fbarloy@pasteur.fr.
MATERIALS AND METHODS

Bacterial strains, media, and plasmid. C. bifermentans subsp. malayasia 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 hsdS2F' traD36 proA' proB' lacY1 lacZM15] 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. malayasia was grown anaerobically in TGY medium (5) under 5% H2–5% CO2–90% N2 at 34°C. C. 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 pH304 (2) was used as a cloning vector. E. coli was transformed with plasmid DNA following CaCl2 treatment as previously described (17). B. thuringiensis was transformed by electroporation as described by Lecuèce 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 Delécuèce 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, Madi Sociology). 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 Montpellicer, 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 LC50 and LC90 (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. malayasia was obtained on an XbaI fragment by using probes 66A and 66B, corresponding to the NH2-terminal and internal amino acid sequences of this protein, respectively, as described in Materials and Methods. The restriction map of the XbaI 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 XbaI-BglII fragment (Fig. 1). Plasmid pCBM1.5, containing only this fragment of C. bifermentans DNA, was constructed by digestion of pCBM1 with BglII and BamHI 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. malayasia 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 (MNTNFSTHLFSK) 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 NH₂-terminal and internal amino acid sequences as determined by microsequencing.
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, may belong to the Cry toxin family. Therefore, cbm71 was proposed in the new B. thuringiensis nomenclature (6) as cry16A (4a).}

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.

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. malaysiа 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. malaysiа antiserum was observed in the STCA from cells containing pHT304 (Fig. 6, lane A). No proteins reacting with C. bifermentans subsp. malaysiа 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} and LC_{90} of around 150 and 300 μg/ml, respectively, against all three species tested.

Cbm71 had the same activity spectrum as C. bifermentans subsp. malaysiа, 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. malaysiа. 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. malaysiа 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 Tn1546 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. malaysiа toxin gene is on the chromosome, eliminating the possibility of transfer by plasmid

TABLE 1. Larvicidal activity of B. thuringiensis transformants

<table>
<thead>
<tr>
<th>B. thuringiensis transformant</th>
<th>Mean larvicidal activitya (μg/ml) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. stephensi</td>
</tr>
<tr>
<td></td>
<td>LC_{50}</td>
</tr>
<tr>
<td>407(pCBM1)</td>
<td>129 ± 10</td>
</tr>
<tr>
<td>407(pCBM1.5)</td>
<td>NA</td>
</tr>
</tbody>
</table>

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 resolved 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 a hydrophobic region of the cytoskeleton. Therefore, the difference in the deduced N-terminal sequence, however, do not conform to a translation initiation by ribosomal RNA gene restriction patterns. J. Gen. Microbiol. 138: 1159–1166.


