Cloning and Analysis of the First cry Gene from Bacillus popilliae

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An 80-kDa parasporal crystal protein was detected in protein extracts of sporangia of Bacillus popilliae isolated from a diseased larva of the common cockchafer (Melolontha melolontha L.). Amino acid analysis of tryptic peptides revealed significant homology to the Cry2Aa endotoxins of Bacillus thuringiensis. The gene cryBP1 (cry18Aa1), which codes for the parasporal crystal protein, was found in a putative cry operon on the bacterial chromosome, which contains at least one further (smaller) open reading frame, orf1. The 706-amino-acid-long CryBP1 (Cry18Aa1) protein has a predicted molecular mass of 79 kDa and shows about 40% sequence identity to the Cry2 polypeptides of B. thuringiensis. In the light of published observations which suggest that the parasporal crystal proteins of B. popilliae are slightly toxic to their grub hosts, we propose the following survival strategy of B. popilliae. As an obligate pathogen of grubs, B. popilliae germinates in the gut of a grub and the parasporal crystal proteins are released and activated. The activated protein does not cause colloid osmotic lysis but instead damages the gut wall somehow to allow the vegetative cells to enter the hemolymph more easily. By becoming a parasite, B. popilliae can continue to proliferate efficiently while the living grub provides a food supply. This process is in contrast to that of B. thuringiensis, which rapidly kills the insect and is then limited to growth on the larval carcass.

The gram-positive, spore-forming bacterium Bacillus popilliae Dutky (14) is the obligate causal agent of milky disease of scarab larvae. The name of the disease comes from the milky-white appearance of the normally clear hemolymph, which is due to the extremely high number of spores generated in the hemolymph (2 x 10^9 to 5 x 10^9 spores per larva) during the infection of the grubs by B. popilliae (21; for reviews, see references 7, 26, and 37). B. popilliae preparations have been used for more than 50 years in the United States for suppression of Japanese beetle larvae (Popillia japonica Newman) (for a review, see reference 25). In fact, this bacterium was the first microorganism registered in that country as an insecticide.

For biological control of grubs, only B. popilliae spore preparations can be used, since vegetative cells rapidly lose viability in the soil. Despite considerable research aimed at achieving efficient in vitro production of spores, only very low levels have ever been attained in the laboratory. The lack of methods to efficiently induce sporulation in vitro has been the major factor preventing more research and development projects for these bacteria (38).

During sporulation, B. popilliae forms parasporal crystals in a manner reminiscent of Bacillus thuringiensis, which is well-known as a specific microbial pesticide for larvae of varieties of Lepidoptera, Diptera, and Coleoptera (23). However, the role of the parasporal crystals in B. popilliae pathogenesis remains unclear. Weiner isolated parasporal bodies from B. popilliae NRRL 23098, a pathogen of the Japanese beetle, and compared different routes of inoculation. Significant toxicity was detected when the intact or solubilized parasporal bodies were injected into hemolymphs but not if the grubs were fed with the parasporal bodies (44).

Splitsstoeesser et al. studied the infection process of the European chafer Amphimallon majalis after inoculation with B. popilliae. They showed that the B. popilliae spores germinate in the guts of the grubs and subsequently traverse the epithelial, basal lamina, and capsular barriers as vegetative rods to enter the hemolymph, despite the defense system of regenerative nidi, epithelial cells, and hemocytes. Once in the hemolymph, vegetative rods are able to grow, evade phagocytosis by the hemocytes, multiply through several cycles, and eventually sporulate (35, 36). Splitsstoeesser et al. believed the process to be similar in other scarab species, but they did not discuss the role of the parasporal crystals.

This communication describes work on B. popilliae subsp. melolonthae H1, which is a pathogen for larvae of the common cockchafer (Melolontha melolonthae L.) and is able to form parasporal crystals which mostly consist of a 79-kDa protein. The gene cryBP1 (cry18Aa1), which encodes the parasporal crystal protein, was cloned and sequenced. It is the second open reading frame of a putative operon containing at least two genes and resembles the cry2 and cry11 operons of B. thuringiensis. The structural and sequence similarities between these operons from B. thuringiensis and B. popilliae suggest that these peptides have related functions. This relationship will be discussed with the published data about the etiologies and courses of the corresponding insect diseases.

MATERIALS AND METHODS

Collection of grubs. The grubs of the common cockchafer (M. melolontha) were collected from the soil in a field near Heidelberg, Germany, and maintained in our laboratory.

Strains and media. B. popilliae subsp. melolonthae H1 was isolated from a diseased grub of the common cockchafer (M. melolontha L.) found near Heidelberg, Germany. It was grown on blood agar (39 g of Columbia agar base [Oxoid] per liter to which 50 ml of sheep blood was added after autoclaving) or brain heart infusion broth (Difco) at 28°C. Escherichia coli XL-1 Blue MRF+ (Stratagene), the plasmid vector pBC SK+ (Stratagene), and pUC19 were used for cloning and sequencing. E. coli strains were grown at 37°C in Luria broth (34) supplemented with ampicillin (100 µg/ml), tetracycline base (12.5 µg/ml), or chloramphenicol (25 µg/ml) when necessary.
Preparation of B. popilliae cultures and spores. B. popilliae sporangia, the spore-bearing mother cells which contain parasporal crystals, were pasteurized by heating them at 75°C for 20 min, plated onto blood agar supplemented with 10 μg of vancomycin per ml, and incubated at 29°C for 2 days. B. popilliae cells were then washed off the agar dishes with 0.9% saline and transferred to brain heart infusion medium.

For the preparation of B. popilliae sporangia, about 10^7 vegetative cells suspended in 10 μl of 0.9% NaCl were injected into the hemolymph of each common cockchafer grub. After the appearance of milky disease (4 to 6 weeks after infection), when the hemolymph is full of spores of B. popilliae, the grub bodies were opened and the hemolymph was drained from the grubs. The B. popilliae spores harvested were centrifuged down with a table centrifuge, washed three times with 0.9% NaCl, and stored at 4°C.

SDS-PAGE of the sporangial protein extracts. Suspensions of sporangia containing parasporal crystals were mixed with equal volumes of 2× Laemmli sample buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.2% bromophenol blue) and heated at 98°C for 10 min. After a short centrifugation, the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (29).

In situ protein digestion and determination of the N-terminal sequences of the tryptic peptides of the parasporal crystal protein. The dominant protein band from the SDS-polyacrylamide gel was cut out of the gel and chopped into small pieces of 2 by 2 mm^2, which were washed with water and destained with 50% (vol/vol) acetonitrile. The gel pieces were equilibrated with 100 μl of 100 mM NH_4HCO_3 (pH 8.0). After addition of 50 pmol of trypsin, the reaction mixture was incubated at 37°C for 14 h. Peptide fragments were then extracted from the gel pieces twice with 100 μl of 100 mM NH_4HCO_3 (pH 8.0) and once with 100 μl of acetonitrile. The supernatants were combined and concentrated. The peptides were separated by reverse-phase high-performance liquid chromatography (Vydac C_18, model TP218 column, 1.6 by 250 mm; bead size, 5 μm), and peak fractions were collected manually. The peptide fractions were subjected to Edman degradation with an Applied Biosystems sequencer (model 473A).

DNA manipulation. DNA restriction, DNA fragment ligation, and subcloning were performed by standard methods (34). Isolation of DNA fragments from agarose gels was carried out by electroelution (18). B. popilliae total DNA was isolated by the method described by Meade et al. (33).

Plasmid isolation from B. popilliae. A standard alkaline lysis method (34) was employed to isolate plasmids from B. popilliae subsp. melolonthae H1, except that the samples were incubated on ice for 45 min after addition of solution III.

Colony hybridization and Southern hybridization. Colony hybridization and Southern hybridization were carried out by standard methods (34). Prehybridization was carried out for 1 to 4 h at 60°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt’s solution, 0.5% SDS, and 50 μg of denatured herring sperm DNA per ml. Hybridization was performed overnight at 60°C after addition of a 32P-labeled DNA probe. Membranes were then washed twice with 2× SSC-0.1% SDS at room temperature for 10 min each and twice with 0.1× SSC-0.1% SDS at 60°C for 15 min each. Labeling of the DNA fragments was performed by nick translation with a nick translation kit from Boehringer Mannheim and the recommended protocol. For small DNA fragments (200 to 1,000 bp), the reaction was performed at 15°C for 15 min instead of 15°C for 35 min. We found that this change gives much better hybridization results, probably because this treatment prevents the cutting of the DNA fragment into very small pieces and improves the stability of the DNA/DNA hybrids.

PCR. PCR was carried out with Vent DNA polymerase (New England Biolabs) in a 50-μl reaction mixture including 1 μg of total DNA of B. popilliae subsp. melolonthae H1, each primer at a final concentration of 1 μM, each deoxynucleoside triphosphate at a final concentration of 200 μM, and 2 U of Vent DNA polymerase in 1× Vent polymerase reaction buffer. The reaction mixture was overlaid with 60 μl of mineral oil. The conditions for the amplification with a PCR device (Trio-Thermoblock [Biometra]) were as follows. The initial denaturation step was at 93°C for 5 min; a further 35 cycles of denaturation at 93°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s were performed. The following two oligonucleotides were synthesized and used without further purification as primers for PCR (see Fig. 3): TTTAATGATCAIGTIGA (A/G)GA(C/T/TTT) and TTGGATATTCGCIAT(A/G)AT(C/T)TC.

Nucleotide sequence accession number. The nucleotide sequence of the putative cryBP1 operon is available in the GenBank database under accession no. X99049.

RESULTS

Analysis of the crystal protein of B. popilliae subsp. melolonthae H1. The final stage of sporulation, namely, the lysis of the sporangium and the liberation of the mature spore, which is common to most Racillu species, has not been observed upon sporulation of B. popilliae subsp. melolonthae H1. One spore and one parasporal crystal can be seen in one sporangium of B. popilliae subsp. melolonthae H1. Kaya et al. reported that a few B. popilliae strains which cause the so-called blue disease in Cyclocephala hirta can form sporangia with multiple parasporal bodies (24).

The parasporal crystal protein of B. popilliae subsp. melolonthae H1 was harvested as parasporal crystals enclosed in the sporangia together with the spores. Under the procedures employed for SDS-PAGE of the sporangial protein extracts, it was possible to differentially solubilize the parasporal crystals, leaving the spores unaffected. This process was checked by phase-contrast microscopic examination, which showed that all the sporangia lost their parasporal crystals while the spores remained intact and the cell walls of sporangia were also visible (data not shown).

SDS-PAGE of sporangial protein extracts revealed a predominant polypeptide band of about 80 kDa (Fig. 1). This polypeptide was digested in situ by trypsin, and the peptide fragments that formed were extracted from the gel and separated by reverse-phase high-performance liquid chromatography. The N-terminal sequences of six proteolytic fragments were determined (Fig. 2B). With the amino acid sequences of the fragments, the SwissProt database was screened with the program MPsrch (39). Fragment 35 (EYIAEYSNALSTYDVQRDGF) and fragment 43 (NNLITTFNDOVEDFLQON) showed significant similarity similarity to the B. thuringiensis Cry2 polypeptide (45, 47), which are toxic to the larvae of some lepidopterous and dipterous insects. Based on the Cry2A amino acid sequence, we suggested that fragment 43 is closer to the N-terminal end of the B. popilliae parasporal crystal protein than fragment 35.

Amplification of a DNA fragment from B. popilliae total DNA encoding part of the parasporal crystal protein. For the isolation of a DNA fragment encoding part of the putative parasporal crystal protein which is marked by polypeptide fragments 43 and 35, two amino-acid-sequence-based degenerate oligonucleotide primers were designed for use in PCR amplification, with B. popilliae total DNA as the template (see Materials and Methods). A 283-bp-long fragment which was cloned into plasmid pUC19 and sequenced was obtained from the reaction mixture. The sequence data of this fragment showed significant similarity to a variety of B. thuringiensis cry genes, especially to the cry2 genes with >60% amino acid identity at the protein level (Fig. 3).

The B. popilliae cry gene. With the above-described PCR product as a probe, Southern analysis was performed with B. popilliae total DNA digests. A 5.3-kb EcoRI DNA fragment was detected by the Southern hybridization. Restriction fragments ranging in size from about 4 to 6 kb were recovered by electroelution after agarose gel electrophoresis and cloned into the EcoRI site of plasmid pBC SK+. Colony hybridization with the 283-bp PCR fragment as a probe yielded one positive clone, pBP5.3, which contained the 5.3-kb EcoRI fragment

FIG. 1. SDS-PAGE of the sporangial protein extracts of B. popilliae subsp. melolonthae H1.
detected by the Southern analysis of *B. popilliae* genomic DNA.

**Sequence analysis.** Sequencing data obtained with a general sequencing primer for pBC SK+ indicated that the cry-like gene was situated in proximity to one end of the 5.3-kb EcoRI fragment. About 3 kb of both strands was sequenced starting with the insertion site of the *B. popilliae* DNA fragment mentioned above. We detected two open reading frames which encode polypeptides composed of 175 and 706 amino acids with estimated molecular masses of 19.6 and 79 kDa, respectively (Fig. 2). The EMBL and GenBank databases (release 96) were screened for sequences homologous to those of the polypeptides encoded by the two above-described open reading frames with the program BLASTX (4).

The smaller open reading frame, which encodes a putative peptide of 175 amino acids, was designated orf1 in view of its unknown function but substantial similarity to orf1 of the cry2Aa-cry2Ac operon (45, 47), orf1 of the cry9Ca operon (B. Lambert, accession no. Z37527), and p19 of the cry11Aa operon (2, 10, 12) of *B. thuringiensis*.

The polypeptide encoded by the longer open reading frame, designated cryBP1, has significant sequence similarity to other Cry proteins of *B. thuringiensis* (23) and *Clostridium bifermens* (5). With Cry2, a sequence identity of about 40% was observed (Fig. 3). The distribution of hydrophobicity of CryBP1 seems to be quite similar to that of the Cry3A (30) and Cry1Aa (19) toxins of *B. thuringiensis* (data not shown). The amino acid sequences of all of the six proteolytic fragments of the parasporal crystal protein were found in the CryBP1 amino acid sequence (Fig. 2B). Only one amino acid residue of peptide 44 did not fit (Fig. 2B). Very probably, this discrepancy was due to an error in the peptide sequencing, because no errors were found after careful examination of the DNA sequencing gel autoradiographs.

**Determination of the putative promoter region of the putative cryBP1 operon.** Seventy-six base pairs upstream of the gene cryBP1, we found an open reading frame, orf1 (Fig. 2), which showed sequence similarity only to those open reading frames found in the *B. thuringiensis* cry operons cry2Aa (45), cry2Ac (47), cry9Ca (B. Lambert; accession no. Z37527), and cry11Aa (2, 10, 12). This analogy suggests that cryBP1 and orf1 are in an operon. To prove this hypothesis, data from transcription experiments are needed. However, it is technically very difficult to perform these experiments with *B. popilliae*, because most cry genes are expressed only at a certain stage of sporulation and not in vegetative cells and because the in vitro sporulation of *B. popilliae* is extremely inefficient outside the host insect. By sequence analysis, we found that the upstream sequence of orf1 was quite similar to that of orf1 of the *B. thuringiensis* cry2Aa operon (data not shown). This finding suggests that orf1 has a putative promoter region which is depen-
dent on \( \sigma^{s} \), a \( B. thuringiensis \) analog of \( \sigma^{5} \) of \( B. subtilis \) (6), which is a sporulation-specific sigma factor active from the early to the middle sporulation phase (Fig. 2).

**Location of the cryBP1 gene.** In \( B. thuringiensis \), most cry genes are located on large plasmids. Several researchers have reported the presence of various plasmids in \( B. popilliae \) which are smaller than 15 kbp (1, 11, 15, 40). Macdonald and Kalimakoff compared the plasmid profiles of six \( B. popilliae \) strains by pulsed-field gel electrophoresis and found plasmids in the size range of 3.6 to 9.7 kbp (32). From \( B. popilliae \) subsp. \( melolonthae \) H1, one plasmid of about 7.5 kbp was detected (Fig. 4, lane 2). Southern analysis of \( BamHI \)-restricted total DNA (chromosomal and plasmid DNAs) (Fig. 4, lane 1) and plasmid DNA (Fig. 4, lane 2) was performed with the insert of pBP5.3 as a probe. Whereas a signal was detected with the total DNA digest, none could be seen with the restricted plasmid. This result suggests that the cryBP1 gene is located on the chromosome of the \( B. popilliae \) strain.

**DISCUSSION**

Despite the long history of utilization of \( B. popilliae \) in suppression of the Japanese beetle population, very little is known about the mechanism of its pathogenicity or the factors regulating its host specificity and the cause of insect death. The normal route of infection of grubs by these microbes starts by ingestion of the sporangia during feeding. The spores germinate in the hindguts of grubs, and vegetative cells move to the midguts, where they penetrate the epithelial cells and finally pass to, proliferate in, and sporulate in the hemolymph (36).

The factors triggering \( B. popilliae \) spore formation are not...
known. For the biological control of grubs with \textit{B. popilliae}, only spores can be used because of the rapid loss of viability in soil that occurs with vegetative cells.

As with \textit{B. thuringiensis}, \textit{B. popilliae} produces parasporal crystals upon sporulation. The \textit{B. thuringiensis} Cry proteins are synthesized and eventually released as protoxin inclusions during sporulation. After ingestion, the gut contents of sensitive larvae dissolve and proteolytically activate these toxins, which then bind to and enter into the midgut epithelia of susceptible insect species, forming transmembrane pores that cause the deaths of larvae by colloid osmotic lysis (27; for a review, see reference 42). It was shown that the Cry3A and Cry1Aa toxins of \textit{B. thuringiensis} have a three-domain structure (19, 30), probably a common structure of these Cry toxins. Domain I consists of a bundle of seven (Cry3A) or eight (Cry1Aa) \(\alpha\)-helices. By site-directed mutagenesis or hybrid toxins, it was shown that domain I inserts itself into the epithelial membranes of the larvae of the sensitive insects and forms pores in them (3, 17, 46). Domain II, which contains three antiparallel \(\beta\)-sheets in both the Cry3A and Cry1Aa toxins and two additional short \(\alpha\)-helices in Cry1Aa, was suggested to be the domain that binds to the epithelial membrane receptors of the sensitive larvae and the determinant of the specificities of the toxins (20, 22, 28, 41). The function of domain III, which was built from two antiparallel \(\beta\)-sheets, is not clear. It has been shown recently that domain III of Cry1Ab-Cry1C and Cry1E-Cry1C hybrids is the major determinant of toxicity to \textit{Spodoptera exigua} and \textit{Mamestra brassicae} (9).

Whereas parasporal crystals from \textit{B. thuringiensis} alone cause the deaths of sensitive larvae by ingestion, intact or alkali-solubilized parasporal bodies from \textit{B. popilliae} had almost no influence if they were fed to the third-instar larvae of Japanese beetles (44) and caused only a transient inhibition of ingestion if they were fed to grubs of the common cockchafer (unpublished observation). Injection of intact or solubilized parasporal bodies caused death in larvae of the Japanese beetle (44), and loss of viability of primary hemocyte cultures of the common cockchafer was observed after addition of the solubilized parasporal crystal protein (31).

The strong sequence similarity and conservation of the hydrophobicity distribution of CryBP1 as compared to \textit{B. thuringiensis} Cry proteins are indications that \textit{B. popilliae} parasporal crystal protein may play a role in the pathogenesis of milky disease. Like the Cry proteins of \textit{B. thuringiensis}, CryBP1 very probably consists of three domains. It has recently been shown that this \textit{B. popilliae} parasporal crystal protein can specifically bind to the brush border membrane vesicles of the common cockchafer (16), which points to its role in the pathogenesis of milky disease. However, as mentioned above, ingestion of \textit{B. popilliae} parasporal crystal protein alone is not toxic enough to kill grubs (44). Furthermore, we noticed that ingestion of even high numbers of vegetative cells of \textit{B. popilliae} by grubs of the common cockchafer does not result in milky disease but that ingestion of spores does (unpublished observation). Nevertheless, \textit{B. popilliae} enters the hemolymph of grubs as vegetative rods (36). These findings lead us to propose the following role for CryBP1 in milky disease of larvae of the common cockchafer, which suggests that \textit{B. popilliae} adopts a survival strategy different from that of \textit{B. thuringiensis}.

The \textit{B. popilliae} spores germinate in the larval gut. At this time, the crystal protein is activated, either by enzymes from the germinating \textit{B. popilliae} spores or by enzymes in the larval gut, and subsequently binds to the brush border membrane vesicles. The activated protein does not cause colloid osmotic lysis but instead damages the gut wall somehow to allow the vegetative cells to enter the hemolymph more easily. The cells now multiply in a parasitic relationship with the host grub, evading phagocytosis. It is not known what triggers sporulation, but eventually the high concentration of sporangia and perhaps the synthesis of toxic substances lead to the death of the grubs. By becoming a parasite, \textit{B. popilliae} can continue to proliferate efficiently while the living grub provides a food supply. This process is in contrast to that of \textit{B. thuringiensis}, which rapidly kills the insect and is then limited to growth on the often young (because they are more susceptible) larval carcasses. For this reason, we refer to CryBP1 as a parasporal rather than an insecticidal crystal protein. To substantiate this hypothesis, further in vivo and in vitro bioassays are needed.

This hypothesis for the infection mechanism of milky disease of grubs may also provide an explanation for why \textit{B. popilliae} is not subjected to autolysis after sporulation. As both spores (later in the form of vegetative cells) and parasporal crystals are important for the infection of grubs with \textit{B. popilliae}, it is likely that they have to be coupled.

Höfte and Whiteley (23) devised a nomenclature system for \textit{B. thuringiensis} cry genes based on the sequence similarity of the cry genes and the toxicity spectrum of the Cry proteins. Recently, an alternative system for the nomenclature of cry genes (not only from \textit{B. thuringiensis}) which relies only on the relative percent identities of the amino acid sequences of the Cry proteins was proposed. According to the latter system, the name of the cryBP1 gene is cry18Aa1 (48).

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