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The xaxAB Genes Encoding a New Apoptotic Toxin from the Insect Pathogen *Xenorhabdus nematophila* Are Present in Plant and Human Pathogens*§*

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*Xenorhabdus nematophila*, a member of the Enterobacteriaceae, kills many species of insects by strongly depressing the immune system and colonizing the entire body. A peptide cytotoxin has been purified from *X. nematophila* broth growth, and the cytolytic effect on insect immunocytes and hemolysis on mammalian red blood cells of this toxin have been described (Ribeiro, C., Vigneux, M., and Brehelin, M. (2003) J. Biol. Chem. 278, 3030–3039). We show here that this toxin, *Xenorhabdus α*-xenorhabdolysin (Xax), triggers apoptosis in both insect and mammalian cells. We also report the cloning and sequencing of two genes, xaxAB, encoding this toxin in *X. nematophila*. The expression of both genes in recombinant *Escherichia coli* led to the production of active cytotoxin/hemolysin. However, hemolytic activity was observed only if the two peptides were added in the appropriate order. Furthermore, we report here that inactivation of xaxAB genes in *X. nematophila* abolished the major cytotoxic activity present in broth growth, called C1. We also show that these genes are present in various entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, in *Pseudomonas entomophila*, in the human pathogens *Yersinia enterocolitica* and *Proteus mirabilis*, and in the plant pathogen *Pseudomonas syringae*. This toxin cannot be classified in any known family of cytotoxins on the basis of amino acid sequences, locus organization, and activity features. It is, therefore, probably the prototype of a new family of binary toxins.

Entomopathogenic bacteria are widely used as crop protection agents. Most studies on these bacteria have focused on the properties of insecticidal toxins, with a view to improving them. These pathogens are able to overcome insect immune responses (1–2), which parallel those of mammals to some extent. Insects do not have adaptive immunity and are easy to handle, making them a powerful tool for studies of host innate immunity and for the identification of bacterial virulence factors (3). The genomes of invertebrate pathogens represent a potentially extensive reservoir of virulence genes that have evolved over long periods to overcome the innate immune responses of their hosts. This virulence gene pool may act as a source of virulence factors for transfer into human commensal or pathogenic bacteria (4).

*Xenorhabdus nematophila* (Enterobacteriaceae) is a Gram-negative bacterium that is transported into insects by the entomopathogenic nematode *Steinernema carpocapsae*. Once inside the insect, it secretes various extracellular factors, including antibiotics, lipases, proteases, and toxins, which are involved in insect killing (5). In particular, *X. nematophila* grows within the body of the insect and must, therefore, be able to escape the immune response, but little is known about the way in which it does this. Cellular immunity comes into play immediately after the penetration of a foreign body into the insect hemocoel, and *X. nematophila* must escape these potent cellular reactions. Hemocytes are the immunocytes of insects, and cellular immune reactions against bacteria involve cells of several different lineages. In our insect model, *Spodoptera littoralis* (Lepidoptera), plasmacytoid cells build nodules that isolate clumps of bacteria and necrotic insect tissues, and granular hemocytes are the professional phagocytes (6). Cytotoxic factors targeting these immune system cells are good candidates for the molecules mediating immunosuppression. In *X. nematophila* grown in liquid culture, various cytotoxic activities principally targeting the hemocytes have been identified (7–9). One of the major cytotoxic activity present in *X. nematophila* broth growth, C1 (8), targets the phagocytes. C1 is also hemo-
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**TABLE 1**

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| **E. coli**       |             |           |
| SURE 2            | e14-′(Mca−A)Δ(mcrCB-hsdSMR-mrr)171 endA1 supF44 thi-1 gyrA96 relA1 lac recB recF sbcC unuC56Tn5 (Kan)′ uvrC [F′ proAB lacI21 F− ΔM15 Tn10 (Tet)] | Stratagene |
| Top10             | F- mcrA (mrr-hsdRMS-mcrBC) Δ80lacZ, M15 lacY74 deoR recA1 araD139 (araA-lexA0)7697 galU galK rpsL endA1 napG | Invitrogen |
| CFP201            | MC4100 she4: Tet r2.1 null mutant | Ref. 10 |
| **X. nematophila**|             |           |
| F1                | X. nematophila, wild type | Laboratory collection |
| xaxAB9            | F1 xaxA::F1 Cm | This work |

**Plasmid**

| pQ200KS           | Gm′ sacR B mob oriV (p15A replicon) | S. Forst |
| pHP45-Ω Cm        | Ap′ Cm′ interposon Ω Cm | Ref. 11 |
| pBBR1MCS-5        | Medium copy mobilizable vector; Gm′ | Ref. 12 |
| pBBxaxAB          | 2504-bp region overlapping xaxAB cloned into the XbaI-Sall sites of pBBR1MCS-5 | This work |
| pAB6              | pBBxaxAB with a 1-kilobase deletion overlapping the xaxA and xaxB coding region | This work |
| pAB7              | 3.5-kilobase BamHI fragment from pHP45-Ω Cm cloned into pAB6 (contains xaxAB::Cm) | This work |
| pAB9              | 5.3-kilobase Sall-XbaI fragment from pAB7 cloned into pQ200KS (contains xaxAB::Cm) | This work |
| pBBxaxA           | 1550-bp region overlapping xaxA cloned into the XbaI-Sall sites of pBBR1MCS-5 | This work |
| pBBxaxB           | 1558-bp region overlapping xaxB cloned into the XbaI-Sall sites of pBBR1MCS-5 | This work |
| pBAD-B-Myc-His    | Low copy vector derived from pBR322; Ampr | Invitrogen |
| pXaaA-Myc-His     | PCR product of 1224 bp cloned into the Ncol-XbaI of pBAD-B-Myc-His | This work |
| pH86-HA-His       | High copy vector, Ampr | Roche Applied Science |
| pXaaB-HA-His      | PCR product of 1050 bp cloned into the HindIII-NotI of pHB6-HA-His | This work |

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Production and Purification of Toxin**

The strains and plasmids used in this study are listed in Table 1. *X. nematophila* (strain F1, laboratory collection) were grown in Luria Bertani broth at 28 °C. In these conditions cytotoxic C1 production was maximal in 20-h-old cultures (8). The cytotoxin was prepared as described (9) with a modification of the final step (reverse phase high performance liquid chromatography (HPLC)) that was replaced by a HPLC gel filtration on 60 cm column (reverse phase high performance liquid chromatography (HPLC)).

Partial interpretation of spectra and by means of MASCOT searches (in-house server, Swissprot and Trembl merged data base).

**Tandem Mass Spectrometry—Nanoelectrospray mass spectrometry** was performed offline on a quadrupole time-of-flight mass spectrometer (QSTAR Pulsar-i, Applied Biosystems, Foster City, CA) fitted with a Protana nanospray inlet system (Protana, Odense, Denmark). Spectra were recorded with Analyst QS software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage, 900 V; curtain gas, 25; declustering potential, 45−75 V; focusing potential (FP), 265 V; declustering potential 2, 15 Peptides were fragmented in the collision cell using nitrogen gas on the doubly charged ions detected, with an individually optimized collision energy profile (30−55 V). Capillaries (Protana, Odense, Denmark) were loaded with the samples according to the following procedure; each aliquot after trypsin cleavage was solubilized in 5 μl of 1% formic acid, desalted on Poros 20 R2 (Applied Biosystems), packed into a gel-loader pipette tip, and eluted with 1.5 μl of 50:50:1 methanol/water/formic acid (13). The loaded capillaries were placed in the source tip holder. Molecules were identified by manual partial interpretations of spectra and by means of MASCOT searches (in-house server, Swissprot and Trembl merged data base).

**Plasmid Derivatives and Gene Cloning—** *E. coli* Sure 2 (Stratagene) was used as an intermediate host for cloning experiments. The oligonucleotide primer sets used (Table 2) were designed from an alignment of several hemolysin loci from other bacteria including *Photorhabdus luminescens, X. nematophila,* and *Yersinia enterocolitica*. Standard PCR with each primer set was performed in a 50-μl reaction volume with a Gene Amp 2400 thermocycler system (PerkinElmer Life Sciences). For cloning we generated xaxAB, xaxA, and xaxB fragments with flanking XbaI and Sall sites by PCR. These fragments were inserted into pBBR1MCS-5 to generate pBBxaxAB, pBBxaxA, and pBBxaxB, respectively (Table 1). All constructs were checked by DNA sequencing (MilleGen, Toulouse, France).

lytic for sheep red blood cells (SRBC) but is not hemolytic for rabbit red blood cells (RRBC). C1 lyases cells via a mechanism involving protein toxins, as it is susceptible to heat and trypsin (8), and a peptide cytotoxic called α-xenorhabdolysin (αX) has been purified from a culture medium with C1 activity (9).

We report here the molecular characterization of this cytotoxin produced by *X. nematophila* and show that it has both necrotic and apoptotic activities in insect hemocytes and mammalian cells. The genes encoding this toxin were also identified in various plant and human pathogens.

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*The abbreviations used are: SRBC, sheep red blood cells; αX, α-xenorhabdolysin; Xax, Xenorhabdus α-xenorhabdolysin; RRBC, rabbit red blood cells; PBS, phosphate-buffered saline; ORF, open reading frame; HA, hemagglutinin; TUNEL, terminal dUTP nick-end labeling; DAPI, 4',6-diamidino-2-phenylindole; OD, optical density; z-, benzylloxycarbonyl; fmk, fluoromethyl ketone; HU, hemolytic units.*
Nucleotide Sequence Accession Number—The sequence of the X. nematophila xaxAB locus has been assigned GenBank™ accession number DQ249320.

Construction of the xaxAB-null Strain, xaxAB—Deletion of a 1-kilobase region overlapping xaxA and xaxB ORFs was realized in two steps. The first one was to amplify the first 480 bp of xaxA from pBBxaxAB (Table 1) as the template and by using primers ABsal1fw and EAXBam-rev (Table 2), which contain, respectively, SalI and BamHI restriction sites. The PCR fragments, digested with SalI and BamHI, were then cloned into the corresponding sites of the pBBxaxAB, which contain only the last 770 bp of xaxB and yields plasmid pAB6. Then a chloromphenicol-resistant Ω cassette (11) with transcriptional and translational terminators was inserted into the unique BamHI site within the disrupted xaxAB operon of pAB6 to yield plasmid pAB7. The xaxAB region carrying the Cm-interposon was, therefore, purified by digestion of plasmid pAB7 with SalI and XbaI and cloning into the corresponding sites of the pJQ200KS, resulting in plasmid pAB9 (Table 1). Experiment mating and exconjugant selection were done as previously described (14).

Production and Purification of Tagged Peptides—For the production of recombinant hemolysin, we generated xaxA and xaxB fragments with flanking HindIII, NcoI, NotI, and XbaI sites by PCR and inserted them into pBAD-B-Myc-His (Invitrogen) or pBBxaxAB (Table 1) as the template and by using primers ABsal1fw and EAXBam-rev (Table 2), which contain, respectively, SalI and BamHI restriction sites. The PCR fragment, digested with SalI and BamHI, was then cloned into the corresponding sites of the pBBxaxAB, which contain only the last 770 bp of xaxB and yields plasmid pAB6. Then a chloromphenicol-resistant Ω cassette (11) with transcriptional and translational terminators was inserted into the unique BamHI site within the disrupted xaxAB operon of pAB6 to yield plasmid pAB7. The xaxAB region carrying the Cm-interposon was, therefore, purified by digestion of plasmid pAB7 with SalI and XbaI and cloning into the corresponding sites of the pJQ200KS, resulting in plasmid pAB9 (Table 1). Experiment mating and exconjugant selection were done as previously described (14).

Hemolytic Activity and Titration of the Toxin Solutions—SRBC and RRBC were obtained from BioMérieux (France) as a 50% suspension. They were extensively washed in pH 7.2 phosphate-buffered saline (PBS) and were diluted in this buffer to give a 5% suspension. We tested the hemolytic activity of bacteria grown on trypticase soy agar supplemented with 5% SRBC. Hemolysis was observed as a clear zone surrounding colonies. Hemolysis was also assessed in 5% SRBC or RRBC suspensions as described (9). The titer of a toxin solution can be calculated from the absorbance value obtained according to the following formula, deduced from numerous absorbance determinations with serial dilutions of toxin (9): titer (in HU) = 2 × 10^{OD_540 - 0.72}.

In some experiments we assessed osmotic protection by adding polyethylene glycol 6000 to the toxin solution at a final concentration of 30 mM before incubation with the SRBC. In other experiments, before testing for hemolysis we incubated the toxin solutions alone for 1 h at 60 °C or for 1 h at 37 °C with trypsin (30 units) or with SRBC ghosts as described (9).

In a series of experiments, SRBC were incubated for 1 h at 4 °C in toxin solutions. The mixture was centrifuged at 4 °C, and the absorbance of the supernatant was measured. We then added 50 μl of a SRBC suspension to this supernatant, incubated the mixture at 37 °C for 1 h, centrifuged it at 10,000 × g, and determined the absorbance at 540 nm of this second supernatant. The pellet obtained after incubation at 4 °C was rapidly rinsed, suspended in PBS, and incubated at 37 °C for 1 h, and its absorbance was measured. Protein concentration was determined by the Bradford method.

Immunoprecipitation and Immunoblotting—Recombinant purified epitope-tagged XaxAMyc-His and XaxBHA-His were incubated in Nonidet P-40 buffer (150 mM NaCl, 1% Nonidet P-40, 50 Mm Tris-HCl, pH 8) at 4 °C with monoclonal mouse anti-c-Myc (clone 9E10, Upstate Biotechnology) or monoclonal anti-HA (clone F7-2, Santa Cruz Biotechnology) antibodies at 4 °C for 2 h. As controls, XaxAMyc-His was incubated with CifHA-His, a recombinant polyhistidine HA-tagged irrelevant protein (15), and XaxBHA-His was incubated with recombinant polyhistidine Myc-tagged Cif (CifMyc-His). Immune complexes were then collected with protein-G-Sepharose (GE Healthcare) and washed four times with Nonidet P-40 buffer. Immunoprecipitates were mixed with 2× Laemmli buffer and separated by 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted using anti-Myc antibodies after immunoprecipitation with monoclonal anti-HA or immunoblotted with polyclonal anti-HA (Sigma H6908: rabbit anti-HA tag, affinity-isolated antibody) after immunoprecipitation with anti-Myc antibodies followed by mouse or rabbit secondary antibodies conjugated with horseradish peroxidase. Signals were generated by the enhanced chemiluminescence reaction (GE Healthcare) and detected using x-ray film.

Insects, Hemocyte Monolayer Preparation, Human Cell Lines, and Test for Cytotoxic Activity—Insect (larvae of S. littoralis) rearing and hemocyte monolayer preparation were described (9). HeLa cells were cultured at 37 °C in a 5% CO2 atmosphere in RPMI supplemented with 10% fetal calf serum. Cytotoxic activity was tested on hemocyte monolayers and human cell lines on 12-mm glass coverslips (105 cells/cover slip) in a moist atmosphere on 12-mm glass coverslips (105 cells/cover slip) in a moist

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5 R. Zumbihl, unpublished data.
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FIGURE 1. SRBC suspensions were incubated for 1 h at 4 °C in 2 different concentrations of purified Xax in PBS. Sol, titers of the Xax solutions used: experiment 1 (OD = 0.565), titer of the Xax solution, 0.34 HU; experiment 2 (OD = 0.441), titer, 0.14 HU. After centrifugation, the SRBC pellet was suspended in PBS, incubated for 1 h more at 37 °C, and centrifuged again, and the OD of released hemoglobin (Pel) was measured. A fresh SRBC suspension was added to the supernatant of the first incubation, incubated for 1 h more at 37 °C, and centrifuged, and the OD was measured (Sup.). Results are the means of three experiments in duplicate. Note that almost all hemolytic activity was recovered in supernatants of the incubations at 4 °C.

chamber at 23 °C (insect) or 37 °C (human) for 10 min to 2 h. Cell mortality was checked by adding 2 μl of trypan blue dye (0.04% final in PBS) and 5 min more of incubation. Specimen preparation for transmission electron microscopy observation was described in Ribeiro et al. (9).

Test for Apoptosis—We assessed apoptosis in target cells by TUNEL staining (kit from Roche Applied Science), after incubating cells (hemocyte monolayers or HeLa cell line) for 12 h with toxin solution (5 × 10−3 HU) in modified Eagle’s medium and RPMI, respectively. We checked that TUNEL-positive cells were actually apoptotic by adding z-VAD-fmk (a pan-caspase inhibitor) or z-DEVD-fmk (caspase 3-specific inhibitor) to the medium and comparing the proportion (after angular transformation) of TUNEL-positive cells with that in experiments carried out in the absence of inhibitor.

RESULTS

Purification of the Toxin and Peptide Sequence Determination—Early stationary phase X. nematophila C1 supernatants (8) were collected, and the toxin was purified as described above, with fractions displaying cytolytic activity against insect hemocytes and hemolytic activity against SRBC eluted in a single peak. When SRBC were incubated at 4 °C with purified toxin (see “Experimental Procedures”), all hemolytic activity remained in the supernatant (Fig. 1). Surprisingly, when active fractions from gel filtration were analyzed by C18 reverse-phase chromatography (0–95% acetonitrile gradient and 0.01% trifluoroacetic acid), no peak was detected, and no hemolytic or cytolytic activity was detected even in the void volume. Similarly, no ion was detected when positive fractions were analyzed by matrix-assisted laser desorption ionization or quadrupole time-of-flight in the absence of proteolytic digestion (data not shown).

After trypsin digestion, several apparently doubly charged signals were selected in the BM031219Q-B2 sample for collision-induced decomposition. Only the peak at m/z 536.74 led to an identification (score 45; under our conditions, the significance threshold was 43), using the MS/MS ion search subroutine from MASCOT. This signal was identified as peptide IIESQDVIR from the Q7N5I7_PHOLL protein. Alternatively, the MQ(L/I)D partial sequence could be obtained by manually interpreting the collision-induced decomposition spectrum resulting from the peak at m/z 553.75. Using the sequence query search subroutine of MASCOT, this signal was identified as the DVMQIDTER peptide from the same protein.

Identification of Putative Hemolysin Loci in Different Bacterial Pathogens—The 342-amino acid protein containing both peptide sequences from Xax is a putative protein encoded by gene plu1961 (Fig. 2) in Photorhabdus luminescens TT01 (16), a bacterium closely related to Xenorhabdus. Because the entire genome sequence of P. luminescens has been described, the genomic sequences flanking plu1961 showed that plu1962 was found immediately upstream from plu1961, strongly suggesting that these genes are cotranscribed. This putative hemolysin locus is located downstream from a gene encoding a RNA for lysine in the TT01 genome. Another operon encoding similar proteins (plu3075–3076) (61 and 49% identity to plu1961 and plu1962, respectively) was found elsewhere in the P. luminescens genome, adjacent to the nuoA to nuoN region encoding putative NADH dehydrogenases. The genomic organization of this second putative hemolysin locus was found to be conserved in the genome of a clinical isolate of Photorhabdus asymbiotica isolated from patients in the United States (US3105/77) (www.sanger.ac.uk/Projects/P_asymbiotica/). In the completed genome sequences of bacteria, Plu1961 and Plu1962 display blastP matches (E value between 3e−7 to 3e−33) with two pairs of putative proteins (PsrY_3990, PsrY_3989 and PSTP04287, PSTP04571) predicted from the genome sequences of two pathovars of the plant pathogenic bacteria, Pseudomonas syringae pv. syringae (Fig. 2) and pv. tomato, respectively (17).

Recently, Vodovar et al. (18) reported the genome sequence of an insect pathogen bacterium belonging to the genus Pseudomonas, P. entomophila, and they also showed that this bacterium secretes a diffusible hemolysin activity on sheep blood agar contrary to the other Pseudomonads. Using the Microbial Genome Annotation System (www.genoscope.cns.fr/agc/mage/wwwpkgdb/), two closely linked genes encoding predicted proteins PSEEN4370 and PSEEN4369 similar to XaxA were found in TblastN searches between Plu1961 and Plu1962, indicating that these genes are cotranscribed. This putative hemolysin plu3075–3076 locus is located downstream from a gene encoding a RNA for lysine in the TT01 genome. Another operon encoding similar proteins (plu3075–3076) (61 and 49% identity to plu1961 and plu1962, respectively) was found elsewhere in the P. luminescens genome, adjacent to the nuoA to nuoN region encoding putative NADH dehydrogenases. The genomic organization of this second putative hemolysin locus was found to be conserved in the genome of a clinical isolate of Photorhabdus asymbiotica isolated from patients in the United States (US3105/77) (www.sanger.ac.uk/Projects/P_asymbiotica/). In the completed genome sequences of bacteria, Plu1961 and Plu1962 display blastP matches (E value between 3e−7 to 3e−33) with two pairs of putative proteins (PsrY_3990, PsrY_3989 and PSTP04287, PSTP04571) predicted from the genome sequences of two pathovars of the plant pathogenic bacteria, Pseudomonas syringae pv. syringae (Fig. 2) and pv. tomato, respectively (17).

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1e-130) (xenorhabdus.danforthcenter.org/). As shown in Fig. 2, the putative hemolysin loci, containing two closely linked genes similar to plu1961–62, were found in the same orientation in different genomic contexts in the chromosome of Xenorhabdus, Photorhabdus, P. syringae, and Yersinia. However, these potential virulence genes are not located on or in the vicinity of mobile genetic elements.

XaxAB from X. nematophila F1 Is a Functional Hemolysin/Cytolysin—To demonstrate that this locus encoded Xax, we first cloned the potential hemolysin locus from X. nematophila and expressed recombinant xaxAB in E. coli (see “Experimental Procedures”). No E. coli transformants were obtained when xaxAB was inserted into high copy number plasmids, such as pUC19. The region encompassing the xaxAB genes was amplified and cloned under control of the plac promoter in a medium copy number plasmid, pBBR1MCS-5 resulting in pBBxaxAB, which was then transferred to the E. coli SURE strain. As expected, in the presence of 0.2 mM isopropyl 1-thio-D-galactopyranoside, the E. coli SURE (pBBxaxAB) strain displayed strong hemolytic activity on sheep blood agar plate, whereas the clone containing vector alone (pBBR1MCS-5) did not (Fig. 3). This suggests that this locus is involved in hemolytic activity. However, several studies have shown that the overproduction of heterologous regulators (19) or the presence of prophagic inserts containing a holin locus from X. nematophila (20), activate silent sheA genes, leading to hemolytic activity when cloned in E. coli laboratory strains.

The sheA null mutant CFP201 was used to investigate whether the X. nematophila hemolysin locus could confer hemolytic activity in this E. coli strain despite the sheA

FIGURE 2. Predicted genetic organizations of xaxAB hemolysin loci in the genomic sequences of various Gram-negative bacteria. All genes in the chromosomal sections are indicated by gray arrows, except for xaxAB homologues, which are shown in dark gray. Genomic organizations were compared using genomic data from P. luminescens laumondii (strain TT01) (genolist.pasteur.fr/PhotoList/), P. syringae pv. syringae B728a (img.jgi.doe.gov), Y. enterocolitica 8081 (www.sanger.ac.uk/Projects/Y_enteroxecolica), and X. nematophila ATCC19061 (maizeapache.ddpsc.org/xeno_blast/). Because X. nematophila sequencing has not yet been completed, ORFs were detected using ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html), and deduced amino acid sequences were then compared using the blastP algorithm at the NCBI. Locus tag numbers and predicted products are mentioned below and above the arrows, respectively. The numbers in the margins indicate the coordinates on the chromosome. Peptide sequences obtained after sequence searches using MASCOT are indicated together with matches with the deduced amino acid sequence of Plu1961 from P. luminescens TT01 (Q7N5I7). RNB, exoribonuclease II.

FIGURE 3. Detection of hemolysin activity from X. nematophila XaxAB in various recombinant E. coli strains. Aliquots (15 μl) of cultures of the indicated strains were spotted onto sheep blood agar plates containing gentamycin (30 mg/liter) and isopropyl 1-thio-β-D-galactopyranoside (0.2 mM) and incubated at 28 °C. Zones of clearing were observed over a 15-h period. a, SURE2 (pBBxaxAB); b, negative control: SURE2 (pBBR1MCS-5); c, CFP201 (pBBxaxAB); d, negative control, CFP201 (pBBR1MCS-5).
mutation. We introduced pBBxaxAB into CFP201, and the resulting strain, grown on blood agar, also displayed a large halo of discoloration around the colonies, indicating that the genes cloned did indeed encode hemolysin (Fig. 3).

The sequencing of this 2514-bp region revealed two closely linked ORFs in the same orientation, separated by 40 bp (Fig. 2). These ORFs encode putative proteins 408 and 376 amino acids long. X. nematophila XaxA and XaxB showed the strongest similarity to plu3075 (61%) and plu1961 (56%), respectively, from P. luminescens. As expected, XaxB carries both peptide sequences determined by tandem mass spectroscopy analysis. No highly conserved domain was detected according to protein sequence alignments of XaxA and XaxB homologues from Xenorhabdus, Photobacterium, P. syringae pv. syringae, and P. entomophila (supplemental Fig. S1).

A prediction of membrane-spanning regions based on Tmpred analysis suggested that XaxA has strong transmembrane helices (total score, 2552), with an external N terminus. Neither XaxA nor XaxB contained a signal peptide, repeats, or membrane helices (total score, 2552), with an external N terminus. The sequencing of this 2514-bp region revealed two closely

linked ORFs in the same orientation, separated by 40 bp (Fig. 2). These ORFs encode putative proteins 408 and 376 amino acids long. X. nematophila XaxA and XaxB showed the strongest similarity to plu3075 (61%) and plu1961 (56%), respectively, from P. luminescens. As expected, XaxB carries both peptide sequences determined by tandem mass spectroscopy analysis. No highly conserved domain was detected according to protein sequence alignments of XaxA and XaxB homologues from Xenorhabdus, Photobacterium, P. syringae pv. syringae, and P. entomophila (supplemental Fig. S1).

A prediction of membrane-spanning regions based on Tmpred analysis suggested that XaxA has strong transmembrane helices (total score, 2552), with an external N terminus. Neither XaxA nor XaxB contained a signal peptide, repeats, or cysteine. Some of the molecular characteristics of XaxAB differ from those of identified families of hemolysins/cytolysins.

**Xax Is Both Necrotic and Apoptotic in Target Cells**—Insect hemocyte monolayers were incubated for 10 min to 2 h in solutions of Xax purified from *X. nematophila* or with XaxAB purified from *E. coli* SURE (pBBxaxAB). After 1 h of incubation (toxin titer $10^{-2}$ to 1 HU), many of the hemocytes had died and swollen and contained large cytoplasmic vacuoles. Transmission electron microscopy examination showed that these vacuoles were dilated cisternae of the rough endoplasmic reticulum (not shown), as previously reported (9). Toxin concentrations below $10^{-2}$ HU triggered very little if any hemocyte necrosis, but some hemocytes became much shrunken and came unstuck from the coverslip. We assessed the possible involvement of apoptosis in hemocyte death by analyzing the effects of low doses of Xax (between $1 \times 10^{-3}$ and $5 \times 10^{-3}$ calculated HU). In these experimental conditions, Xax was found to induce hemocyte apoptosis, as shown by TUNEL analysis and transmission electron microscopy (Fig. 4). These hemocytes displayed typical ultrastructural apoptotic features, such as a rounded shape, nuclear chromatin condensation, and cytoplasmic vacuolation (Fig. 4c) and, in most cases, nuclear fragmentation (Fig. 4, c and d). In contrast, untreated cells were unambiguously TUNEL-negative (Fig. 4b) and had the typical nuclear morphology of living cells, as shown by DAPI staining (Fig. 4b) and transmission electron microscopy (Fig. 4c).

In a final series of experiments we added a pan-caspase inhibitor, z-DEVD-fmk, to the incubation medium of Xax-treated hemocytes (Fig. 4e). Very few cells became apoptotic in the presence of z-DEVD-fmk (5 versus 18% in the absence of this caspase inhibitor), indicating that the inhibitor blocked apoptosis and, therefore, that the apoptosis induced by Xax in hemocytes was caspase-dependent. Very similar results (10% versus 28%) were obtained with z-DEVD-fmk, indicating that caspase 3 is involved at least in part in this form of caspase-dependent apoptosis (Fig. 4f). Interestingly, the apoptotic effect was also observed in HeLa cells treated with Xax purified from *X. nematophila* (Fig. 5, d–f), and this effect was also inhibited by z-DEVD-fmk (Fig. 5, g–i).

**Both XaxA and XaxB Are Required for Hemolysis**—As shown above, XaxAB displayed necrotic, hemolytic (see Table 3), and apoptotic (Figs. 4 and 5) activity. However, if target cells (insect hemocytes or SRBC) were incubated in
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We first incubated suspension of SRBC for 1 h with various concentrations (final concentrations of 1–65 nM) of XaxAMyc-His at 37 °C and then added XaxBHA-His to a final concentration of 65 nM. After incubation for an additional hour at 37 °C, total hemolysis was obtained with a minimal concentration of 8 nM XaxAMyc-His (Fig. 7). We then incubated SRBC with 65 nM XaxAMyc-His at 37 °C and then with various concentrations of XaxBHA-His (from 1 to 65 nM) for 1 h at 37 °C. In these conditions, total hemolysis was also obtained with a minimal concentration of 8 nM XaxBHA-His (Fig. 7). If SRBC were incubated in XaxBHA-His before incubation in XaxAMyc-His or if SRBC were incubated in a mixture of XaxAMyc-His and XaxBHA-His, no hemolysis was observed regardless of the concentrations used (not shown). In the same manner, if the incubation in XaxAMyc-His was achieved at 4 °C (then incubation in XaxBHA-His achieved at 37 °C), no hemolysis was observed (not shown).

Recombinant XaxA and XaxB Interact in Solution and after Membrane Insertion—In agreement with previous results (9), plasma membrane of mammal red blood cells and of insect hemocytes is likely the first target of the toxin. In an attempt to understand the mechanisms of interaction with the plasma lysate from E. coli SURE (pBBxaxA) or (pBBxaxB) strains or with a mixture of these two strains, no activity at all (necrosis, apoptosis, or hemolysis) was detected.

We used sheep broth agar plates to test different combinations of E. coli lysates. Lysates from E. coli SURE (pBBxaxAB) gave strong hemolysis (Fig. 6a). In contrast, lysates from E. coli SURE (pBBxaxA) or (pBBxaxB) strains used separately (Figs. 6, c–d) or as a mixture (Fig. 6e) gave no hemolysis whatever the A/B ratio. To confirm that the presence of both genes is necessary to achieve hemolysin activity, we constructed the pAB6 containing a deletion of a 1-kilobase region overlapping the C-terminal sequence of xaxA and the N-terminal region of xaxB. This plasmid gives no hemolytic activity when harbored by E. coli SURE (data not shown). We then incubated sheep broth broth agar plates for 1 h with a lysate of E. coli SURE (pBBxaxA) and then added a lysate of E. coli SURE (pBBxaxB). Under these conditions, hemolysis was observed (Fig. 6f). If the lysate of E. coli SURE (pBBxaxB) was added before the lysate of E. coli SURE (pBBxaxA), no hemolysis was observed (not shown).

The XaxA/XaxB Ratio Is Equimolar for Maximum Activity—We generated XaxA and XaxB recombinant polyhistidine proteins with c-Myc or HA tags, respectively, to confirm the previous result and to determine the ratio of these two peptides giving maximum activity. The two peptides (XaxAMyc-His and XaxB HA-His) were purified on a Ni-IDA 2000 column, and after SDS-PAGE their molecular weights were as expected (supplemental Fig. S2).

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FIGURE 6. Conditions required for the recovery of hemolysin activity from a mixture of XaaA and XaaB from recombinant E. coli extracts. An aliquot (10 µl) of bacterial lysate from an overnight culture of E. coli grown in LB broth containing 0.2 mM isopropyl-β-D-galactopyranoside was spotted onto the surface of a sheep blood agar plate. The order of addition of hemolysin subunits is indicated below. Zones of clearing were observed over a 15-h period.

FIGURE 7. Ratio of concentrations of XaaAMyc-His and XaaBHA-His for hemolytic activity at 37 °C. His-tagged peptides were produced in E. coli TOP10 and purified on Ni-IDA 2000 column. In one series of experiments (XaaA), SRBC suspensions were incubated with 1–65 nM XaaAMyc-His (1 h) followed by 65 nM XaaBHA-His (1 h). In a second series of experiments (XaaB), SRBC were incubated with 65 nM XaaAMyc-His (1 h) and various concentrations (1–65 nM) of XaaBHA-His were then added (1 h). Absorbance of hemoglobin released after the second incubation was measured at 540 nm.

FIGURE 8. Heterotypic interactions between XaaAMyc-His and XaaBHA-His in solution. Panel A, XaaBHA-His was incubated with Myc-His tagged proteins and immunoprecipitated (IP) with monoclonal anti HA antibodies. The membrane was first probed with anti HA antibody (panel A, lanes 4–6). Western blot with anti-Myc antibodies. Lanes 1 and 4, solution of XaaBHA-His alone. Lanes 2 and 5, incubation of XaaB HA with CifMyc-His; no additional bands are visible during Western blot with anti-Myc antibodies. Lanes 3 and 6, incubation with XaaAMyc-His. Panel B, XaaBHA-His was incubated with HA-His tagged proteins and immunoprecipitated with monoclonal anti Myc antibodies. The membrane was first probed with polyclonal anti HA antibody (panel B, lanes 4–6). After stripping, the membrane was incubated with anti-Myc antibody (panel B, lane 1–3). Lanes 1 and 4, solution of XaaAMyc-His alone. Lanes 2 and 5, incubation of XaaAMyc-His with CifMyc-His; no additional bands were visible during Western blot with anti-HA antibodies. Lanes 3 and 6, incubation of XaaAMyc-His with XaaBHA-His. HC and LC, heavy chains and light chains. Blots are representative of three independent experiments.

membrane, we generated XaaAMyc and XaaBHA recombinant polyhistidine proteins to undertake immunoprecipitation experiments with the purified recombinant epitope-tagged proteins. In these experiments interactions between XaaAMyc-His and XaaBHA-His were detected. These data indicate that the recombinant XaaAMyc-His and XaaBHA-His proteins are capable of interacting in solution (Fig. 8) as well as after interaction with red blood cells (data not shown) to form XaaAMyc-His-XaaBHA-His complexes. The heterotypic interaction between epitope-tagged proteins is specific as shown in control experiments where no co-precipitation was detected with irrelevant Myc- or HA-tagged Cif proteins (Fig. 8).

XaaAB is Required for the Overall Cytolytic Activity Expressed in the Early Stationary Phase in X. nematophila—Xenorhabdus has multiple hemolytic activities that could lyse mammalian erythrocytes and insect hemocytes (8, 21), only two of which were characterized in the X. nematophila culture supernatants, and they were growth stage-dependent. Extracts from early stationary phase supernatant (termed C1) of wild type X. nematophila were active against insect granulocytes and SRBC, whereas extracts from late stationary phase supernatant (termed C2) lysed insect plasmacytoses and RRBC (8). To know if XaaAB is the only factor responsible for C1 cytolytic activity, we constructed a X. nematophila xaaAB mutant by double allelic exchange, called xaaAB9. Whatever the time of xaaAB9 growth, no SRBC hemolysis was detected in supernatant extracts, whereas a burst of cytolytic activity against insect hemocytes appeared concomitantly with the RRBC hemolytic activity in the C2 extracts after 30 h of growth (data not shown). Therefore, the xaaAB...
mutant displayed no C1 activity but produced a C2 cytotoxic activity at wild type levels.

**DISCUSSION**

The entomopathogenic bacterium *X. nematophila* produces several virulence factors (5) that may enable it to colonize the insect body (22). Two of these factors (C1 and C2) are produced in liquid cultures and target the insect hemocytes (8). A cytotoxin, αX, has been purified from culture medium with C1 activity, and its activity was studied in insect hemocytes and SRBC (9). Starting from the same culture medium, we describe here the purification and nucleotide sequence of a toxin called *Xenorhabdus* Xax. The molecular mass of Xax, deduced from its sequence (78 kDa), is much higher than that reported for Xax (9). The reason of this difference is unknown but is probably related to posttranslational processing. However, αX and Xax have identical biological effects (see Table 3), and the activity of both αX and Xax can be calculated from the absorbance values for the released hemoglobin using the same arithmetic equation.

In addition, we reported here that the xaxAB9 mutant displayed no C1 activity against sheep erythrocytes and insect hemocytes, demonstrating that the same genes encode both αX and Xax.

XhLA, a cell surface-associated hemolysin, was recently characterized, and the corresponding gene was cloned from *X. nematophila* AN6 (21). These proteins are clearly different as they have very different sequences. In addition, XhLA, like its *Photobacterium* homolog PhLA (23), belongs to a family characterized by a two-partner secretion system. No conserved secretion motif, such as LANPL or ANPPNGIs/tChnGCGF (24), was detected in XaxAB.

Like various other bacterial cytotoxins (25), Xax has both necrotic and apoptotic activities in its target cells *in vitro*. Although it is clear from our data that xaxAB genes are required for C1 activity, our preliminary data using TUNEL analysis revealed that the early stationary phase supernatants from the xaxAB9 mutant always induced hemocyte apoptosis. Other *X. nematophila* products may be involved in this observed apoptosis. It has been reported that secondary metabolites such as N-phenethyl-2-phenylacetamide from *X. nematophila* supernatants exhibited apoptotic effects against human cancer cell lines (26). To explain the first phase of apoptosis, which occurs in the absence of Xax, further studies will be necessary to examine the toxic effects of N-phenethyl-2-phenylacetamide against insect hemocytes.

If low concentrations of Xax induce apoptosis during colonization of the insect body by the bacteria, then this process may have physiological implications. Indeed, *X. nematophila* may inhibit the immune system of the insect via the apoptosis-dependent depletion of immunocytes. This kind of destruction in the absence of inflammation may make it possible for the bacteria to proliferate within the insect, with no risk of degradation by the enzymes inevitably released by the host necrotic tissues and without triggering other immune reactions in these tissues. Pretreatment of cells with the z-VAD-fmk pan-caspase inhibitor strongly inhibits the TUNEL reaction induced by Xax. This inhibitory effect was also observed in z-DEVD-fmk-pretreated cells. These results suggested that the apoptosis induced by Xax involved, at least in part, a caspase 3-dependent mechanism. The same apoptotic pathway has been previously reported for N-phenethyl-2-phenylacetamide (26), and it is possible that both toxins play synergistically for activate the same molecules during the progress of apoptosis.

Incubations of SRBC with *E. coli* lysates or with purified tagged peptides showed that Xax is a binary toxin encoded by two genes xaxA and xaxB and that maximum hemolytic activity was obtained with equimolar concentrations of the two peptides. These incubations also showed that the addition of XaxA and then of XaxB, in this strict order, is required for hemolysis. XaxA binds to red blood cells membranes as detected by Western blot experiment. These observations are in agreement with the prediction of membrane-spanning domains in XaxA amino acid sequence. XaxA binding may modify the membrane, making XaxB binding and activity possible. We show here that XaxAMyc-His and XaxBHA-His are able to bind together in presence of RBC membranes. Nevertheless mechanisms are probably more complex since XaxB also binds to membrane. Hemolysis is not possible if XaxA and XaxB are added as a mixture. Such data may be related to the heterooligomerization properties of XaxAMyc-His/XaxBHA-His observed during immunoprecipitation of either XaxAMyc-His/XaxBHA-His in solution (Fig. 8). The entire process starting from the transcription to the export/secretion of mature toxin as well as the elucidation of the molecular mechanisms of toxin activity, are under investigation.

The peptide sequences of Xax do not match those of any known cytotoxins/hemolysins, whatever their origin. However, Xax is a binary toxin that resembles the bi-component leukocidin of *Staphylococcus aureus*, a family of pore-forming toxins composed of two different proteins, S (31–32 kDa) and F (36–38 kDa). However, these toxins are found only in Gram-positive bacteria of the genus *Staphylococcus* or in bacteriophages associated with this bacterium (27). Furthermore, the S and F components are at least 30% identical, a feature absent in Xax. Finally, the addition of a mixture of LukS and LukF (or equivalents) to a red blood cell suspension led to hemolysis (28), whereas a mixture of XaxA and XaxB was inactive.

Xax can also be compared with AB toxins, in which the A moiety bears the toxicity (enzymatic activity), and the B subunit is responsible for toxin binding to the target cell and translocation into the cytoplasm. Some AB toxins form pores, but a low pH is required for efficient pore formation (29). We confirm here the results of Ribeiro et al. (9), showing that αX forms pores in the plasma membrane directly at a pH of 7.4, polyethylene glycol being an efficient osmotic protectant. In addition, AB toxins can bind to target cell membranes (nucleated cells and red blood cells) at 4 °C (30), whereas Xax cannot (Fig. 1).

Finally, Xax can also be compared with the binary cytolsin of *Enterococcus faecalis*, which consists of two subunits, CyIIA and CyII. When present together in solution, activated CyIIA’ and CyII’ have no hemolytic activity (31), like the mixture of

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*A. Givaudan and C. Ribeiro, unpublished data.*

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*L. Zuminh, unpublished data.*
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XaxA and XaxB. However, the hemolysins from E. faecalis and X. nematophila differ in terms of sequence and two other major factors. The first difference concerns the number of genes in each operon; there are eight genes in the cyl operon and only two in the xax operon. The second major difference is that the hemolytic activity of the E. faecalis toxin cannot be detected in liquid cultures and is only observed around colonies growing on blood agar due to the regulation of gene expression by the target cells via one toxin subunit (31). In contrast, active Xax is present in X. nematophila broth growth in the absence of target cells.

Here we show that the closely linked xaxA and xaxB genes are found together in genome sequences from various bacterial pathogens of plants, insects, and humans. The genomic context surrounding the xaxAB loci is conserved only in the genome of P. asymbiotica and in one of the two loci from P. luminescens TTO1. In all other genomic sequences, xaxAB homologues are found in a unique genomic context. However, no characteristic features of genome flexibility, such as phages, transposon-related structures, or genomic islands (Fig. 2), were found in the vicinity of the xaxAB loci in X. nematophila or in the other bacteria. Surprisingly, this hemolysin locus was found to be present in Y. enterocolitica and not in Y. pestis even though Y. pestis, like X. nematophila, spends part of its life cycle in an insect.

Recently, other toxin genes first described in entomopathogenic bacteria have been recovered and found to be functional in mammalian pathogens. Toxin complex genes encoding high molecular weight insecticidal proteins have been identified in P. luminescens (32) and in X. nematophila (33) and were recovered in clinical isolate T83 of Y. enterocolitica (34). Toxin complex genes were more prevalent among clinical strains than in other Yersinia isolates, and their inactivation in Y. enterocolitica T83 resulted in mutants with attenuated virulence in infected mice (34).

In conclusion, we have characterized the prototype of a new hemolysin family, obtained from an entomopathogenic bacterium and having pleiotropic effects on mammalian and invertebrate cells. The presence of latent xaxAB homologues in the genome sequences of well studied bacterial pathogens suggests that the expression of these genes cannot be activated in laboratory conditions. We are, therefore, currently studying the conditions stimulating the production of XaxAB hemolysin during the infection of insects by X. nematophila. XaxAB, like other pore-forming cytolsins, may promote bacterial infection by killing immunocompetent cells. The biological relevance of XaxAB in the virulence of entomopathogenic bacteria in insects and of Y. enterocolitica, P. mirabilis and P. syringae in their respective hosts should also be addressed.

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