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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\*<sup>[5]</sup>

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Fabienne Vigneux<sup>‡</sup>, Robert Zumbihl<sup>‡</sup>, Grégory Jubelin<sup>‡</sup>, Carlos Ribeiro<sup>‡1</sup>, Joël Poncet<sup>§</sup>, Stephen Baghdiguian<sup>¶</sup>, Alain Givaudan<sup>‡2</sup>, and Michel Brehélin<sup>‡3</sup>

From the <sup>‡</sup>Ecologie Microbienne des Insectes et Interactions Hôte-Pathogène, Unité Mixte de Recherche 1133, Institut National de la Recherche Agronomique-Université de Montpellier II, 34095 Montpellier, France, <sup>§</sup>Unité Mixte de Recherche CNRS 5203, INSERM U661, Université de Montpellier I et II, 34094 Montpellier, France, and <sup>¶</sup>Institut des Sciences de l'Évolution, Unité Mixte de Recherche 5554 CNRS-Université de Montpellier II, 34095 Montpellier, France

*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 hemolytic effect on mammalian red blood cells of this toxin have been described (Ribeiro, C., Vignes, M., and Brehélin, M. (2003) *J. Biol. Chem.* 278, 3030–3039). We show here that this toxin, *Xenorhabdus*  $\alpha$ -*xenorhabdolyisin* (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), plasmatocytes 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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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ249320.

<sup>1</sup> Recipient of an Organization for Economic Cooperation and Development (OECD) fellowship. Present address: Universidade dos Açores, Rua Mae de Deus, Ponta Delgada, Portugal.

<sup>2</sup> To whom correspondence may be addressed: Ecologie Microbienne des Insectes et Interactions Hôte-Pathogène, UMR 1133, INRA-UMII, Place Eugène Bataillon 34095 Montpellier, France. Tel.: 33-467-144-672; Fax: 33-467-144-679; E-mail: givaudan@univ-montp2.fr.

<sup>3</sup> To whom correspondence may be addressed: Ecologie Microbienne des Insectes et Interactions Hôte-Pathogène, UMR 1133, INRA-UMII, Place Eugène Bataillon 34095 Montpellier, France. Tel.: 33-467-144-672; Fax: 33-467-144-679; E-mail: brehelin@univ-montp2.fr.

**TABLE 1**  
Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>E. coli</i>		
SURE 2	e14 <sup>-</sup> (McrA <sup>-</sup> )Δ( <i>mcrCB-hsdSMR-mrr</i> )171 <i>endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuCD</i> ΔTn5 (Kan <sup>r</sup> ) <i>uvrC</i> [F' <i>proAB lacI<sup>q</sup>Z Ω M15 Tn10</i> (Tet <sup>r</sup> )]	Stratagene
Top10	F- <i>mcrA</i> ( <i>mrr-hsdRMS-mcrBC</i> ) Δ 80 <i>lacZ</i> M15. <i>lacX74 deoR recA1 araD139(araA-leu)</i> 7697	Invitrogen
CFP201	<i>galU galK rpsL endA1 nupG</i> MC4100 <i>sheA::Tn5-2.1</i> null mutant	Ref. 10
<i>X. nematophila</i>		
F1	<i>X. nematophila</i> , wild type	Laboratory collection
<i>xaxAB9</i>	F1 <i>xaxA::Ω Cm</i>	This work
<b>Plasmid</b>		
pJQ200KS	Gm <sup>r</sup> <i>sacRB mob oriV</i> (p15A replicon)	S. Forst
pHP45-Ω Cm	Ap <sup>r</sup> Cm <sup>r</sup> interposon Ω Cm	Ref. 11
pBBR1MCS-5	Medium copy mobilizable vector; Gm <sup>R</sup>	Ref. 12
pBBxaxAB	2558-bp region overlapping <i>xaxAB</i> cloned into the XbaI-Sall sites of pBBR1MCS-5	This work
pAB6	pBBxaxAB with a 1-kilobase deletion overlapping the <i>xaxA</i> and <i>xaxB</i> coding region	This work
pAB7	3.5-kilobase BamHI fragment from pHP45-Ω Cm cloned into pAB6 (contains <i>xaxAB::Ω Cm</i> )	This work
pAB9	5.3-kilobase Sall-XbaI fragment from pAB7 cloned into pJQ200KS (contains <i>xaxAB::Ω Cm</i> )	This work
pBBxaxA	1455-bp region overlapping <i>xaxA</i> cloned into the XbaI-Sall sites of pBBR1MCS-5	This work
pBBxaxB	1358-bp region overlapping <i>xaxB</i> cloned into the XbaI-Sall sites of pBBR1MCS-5	This work
pBAD-B-Myc-His	Low copy vector derived from pBR322; Amp <sup>R</sup>	Invitrogen
pXaxA-Myc-His	PCR product of 1224 bp cloned into the NcoI-XbaI of pBAD-B-Myc-His	This work
pHB6-HA-His	High copy vector, Amp <sup>R</sup>	Roche Applied Science
pXaxB-HA-His	PCR product of 1050 bp cloned into the HindIII-NotI of pHB6-HA-His	This work

lytic for sheep red blood cells (SRBC)<sup>4</sup> but is not hemolytic for rabbit red blood cells (RRBC). C1 lyses cells via a mechanism involving protein toxins, as it is susceptible to heat and trypsin (8), and a peptide cytotoxin called  $\alpha$ -xenorhabdolysin ( $\alpha$ X) 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.

## 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 × 7.5-mm inner diameter column (TSKgel, G3000SW TosoHaas) with elution in 0.25 ml/min phosphate buffer (20 mM, pH 7) containing 300 mM NaCl. Under these conditions positive fractions were eluted in a single peak. These fractions were used as purified cytotoxin. The cytotoxin was named *Xenorhabdus*  $\alpha$ -xenorhabdolysin (Xax).

In some experiments bacterial extracts (cytosolic fraction) were produced by producing lysates of *Escherichia coli* (3 freeze-thaw cycles), which were then centrifuged (16,000 × g for 20 min), and the resulting supernatant was filtered (0.22

μm). The toxin was also purified as described above from these *E. coli* extracts.

**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 V. 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 *Photobacterium 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).

<sup>4</sup> The abbreviations used are: SRBC, sheep red blood cells;  $\alpha$ X,  $\alpha$ -xenorhabdolysin; Xax, *Xenorhabdus*  $\alpha$ -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-, benzyloxycarbonyl; fmk, fluoromethyl ketone; HU, hemolytic units.

TABLE 2

## Primer sets used in this study

Restriction enzyme sites are underlined.

Primer	Sequence	Construct
Bsal1fw	GCAGT <u>CGACT</u> TTAATGATGCGCTGAAGCTG	PBBxaxB
Bxba1rev	GCAT <u>CTAGAG</u> CGGGTATATCCGTTGGAGAG	pBBxaxB/AB
ABsal1fw	GCAGT <u>CGACT</u> GCCACTAATATAGGTGGTGATG	pBBxaxA/AB/pAB6
AF1xba1rev	GCAT <u>CTAGACT</u> GCCTGATTCATGGCTTTA	pBBxaxA
EAXBam-rev	CGGGAT <u>CC</u> TTTCAGCCAGCTGCTTATCAG	pAB6
AMHncol1fw	GCACCATGGAGAACGATATGTCATCAAAT	pXaxA-Myc-His
AMHxba1rev	GCAT <u>CTAGAA</u> TGTGATAGAGTTTTTTATATTCAGCC	pXaxA-Myc-His
B6HAHind3fw	GCA <u>AA</u> GC <u>TT</u> GATGTCGACAATACCCTCTCA	pXaxB-HA-His
B6HANot1rev	GCAGCGGCCCGCTTTCAGCTTATTGTAC	pXaxB-HA-His

**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, *xaxAB9***—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, Sall and BamHI restriction sites. The PCR fragment, digested with Sall 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 chloramphenicol-resistant  $\Omega$  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 Sall 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 pHB6-HA-His (Roche Applied Science) in-frame with the Myc-His tag or the HA-His tag, respectively, creating XaxAMyc-His and XaxBHA-His (Tables 1 and 2). All constructs were checked by DNA sequencing (MilleGen, Toulouse, France) before being used to transform the *E. coli* TOP10 and XL1blue strains. Recombinant polyhistidine-tagged proteins were purified on a Protino Ni-IDA 2000 column according to the manufacturer's instruction (Macherey-Nagel, Germany) (see supplemental Fig. S2).

**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) =  $2 \times 10^{(OD - 0.72)}$ .

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  $\mu$ l of a SRBC suspension to this supernatant, incubated the mixture at 37 °C for 1 h, centrifuged it at 10,000  $\times$  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 F-7, 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).<sup>5</sup> 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 $\times$  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% CO<sub>2</sub> 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 (10<sup>5</sup> cells/coverslip) in a moist

<sup>5</sup> R. Zumbihl, unpublished data.

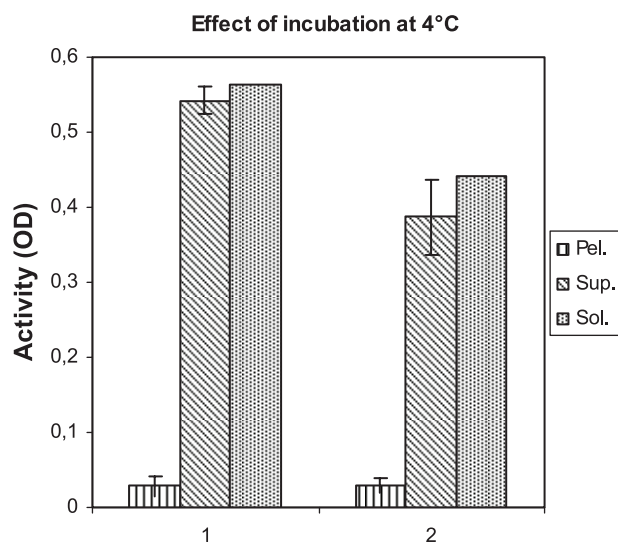


FIGURE 1. SRBC suspensions were incubated for 1 h at 4 °C in 2 different concentrations of purified Xax in PBS. Sol., titers of the Xax solutions used: experiment 1 (OD = 0.565), titer of the Xax solution, 0.34 HU; experiment 2 (OD = 0.441), titer, 0.14 HU. After centrifugation, the SRBC pellet was suspended 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  $\mu$ l 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 ( $5 \times 10^{-3}$  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(I/L)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 tRNA 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 (Psynr\_3990, Psynr\_3989 and PSTPO4287, PSTPO4571) 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 PSEEN 4369 similar to XaxA and XaxB family (supplemental Fig. S1), respectively, were detected in the genome sequence of *P. entomophila*. Both genes are located at a synteny break point between the genomes of *P. entomophila* and the other *Pseudomonas* spp. We also searched for homologues of this pair of genes in the bacterial genomes that are currently being sequenced. Significant similarities were found in TblastN searches between *Plu1961* and *Plu1962* and two predicted proteins from *Y. enterocolitica* (*E* =  $1.5e-43$  and  $4.1e-83$ , respectively) (Fig. 2) and from *Proteus mirabilis* HI4320 (*E* =  $6.3e-9$  and  $7.5e-57$ ) (www.sanger.ac.uk/). Finally, we detected homologues to this pair of genes (*plu1961–1962*), as expected, in the incomplete genome sequence of a *X. nematophila* strain (*E* =  $5e-96$  and

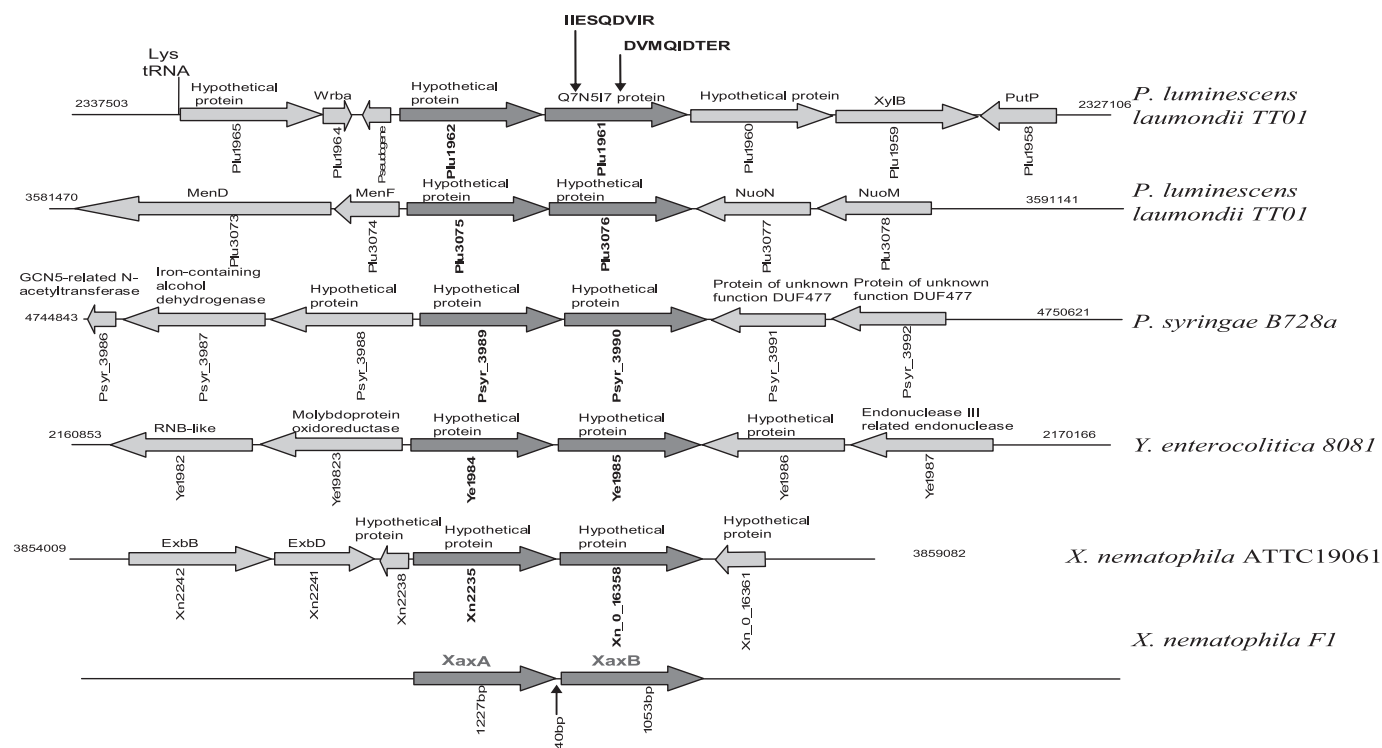


FIGURE 2. Predicted genetic organizations of *xaxAB* hemolysin loci in the genomic sequences of various Gram-negative bacteria. All genes in the chromosomal sections are indicated by gray arrows, except for *xaxAB* homologues, which are shown in dark gray. Genomic organizations were compared using genomic 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\_enterocolitica), 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* TTO1 (Q7N5I7). *RNB*, exoribonuclease II.

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* F1 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- $\beta$ -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.

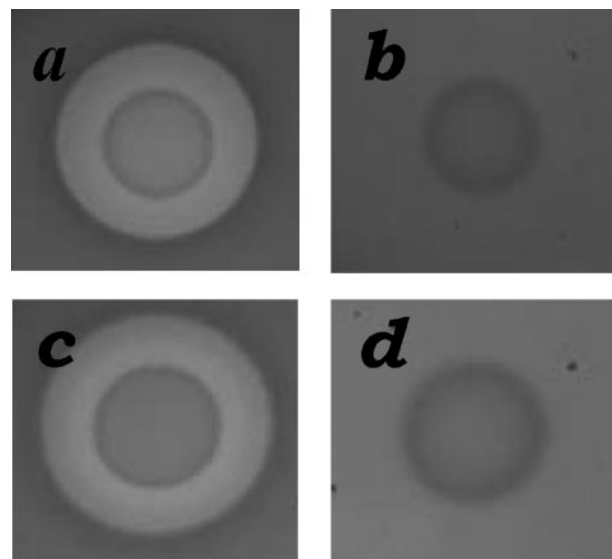


FIGURE 3. Detection of hemolysin activity from *X. nematophila* XaxAB in various recombinant *E. coli* strains. Aliquots (15  $\mu$ l) of cultures of the indicated strains were spotted onto sheep red blood agar plates containing gentamicin (30 mg/liter) and isopropyl 1-thio- $\beta$ -D-galactopyranoside (0.2 mM) and incubated at 28  $^{\circ}$ 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).

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*

## New Apoptotic Toxin from Gram-negative Bacteria

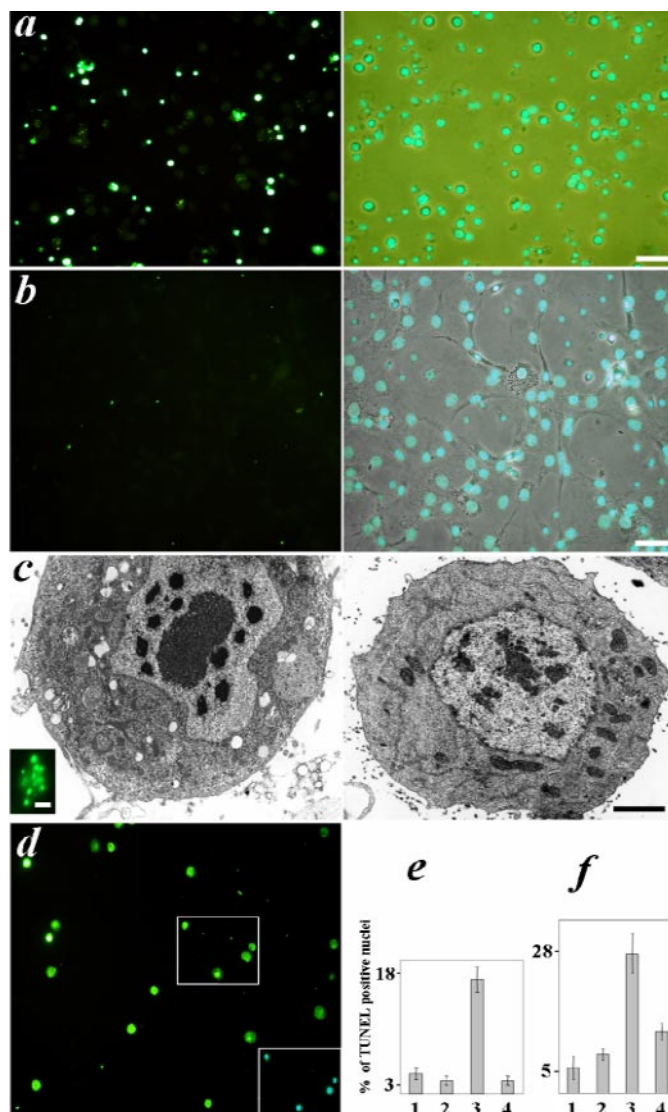
mutation. We introduced pBBxaxAB into CFP201, and the resulting strain, grown on blood agar, also displayed a large halo of discoloration around the colonies, indicating that the genes cloned did indeed encode hemolysin (Fig. 3).

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*, *Photorhabdus*, *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.

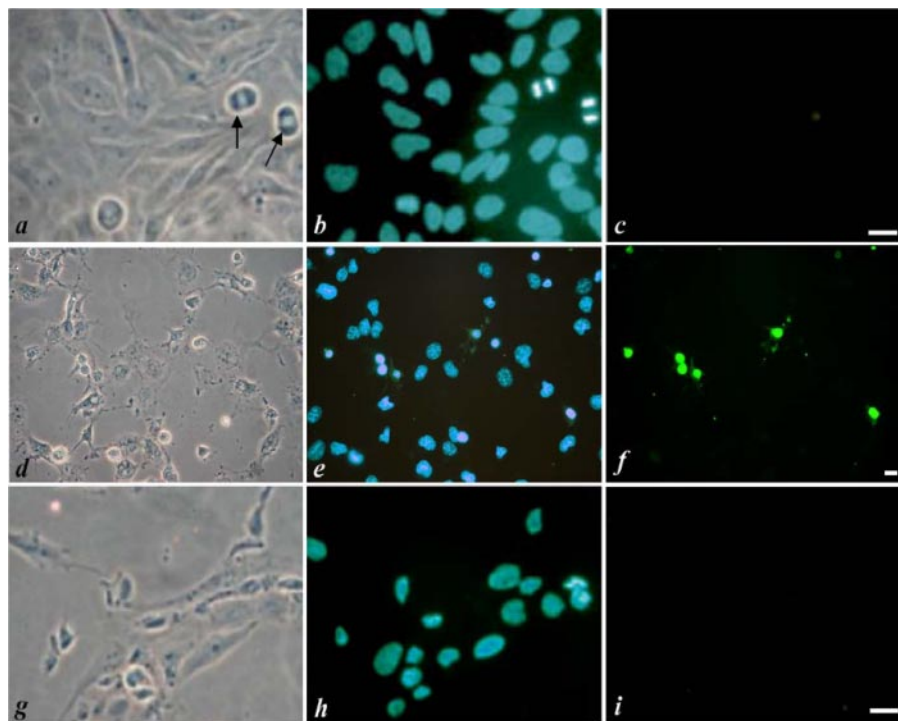
**Xax Is Both Necrotic and Apoptotic in Target Cells**—Insect hemocyte monolayers were incubated for 10 min to 2 h in solutions of Xax purified from *X. nematophila* or with XaxAB purified from *E. coli* SURE (pBBxaxAB). After 1 h of incubation (toxin titer  $10^{-2}$  to 1 HU), many of the hemocytes 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-VAD-fmk, to the incubation medium of Xax-treated hemocytes (Fig. 4e). Very few cells became apoptotic in the presence of z-VAD-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).



**FIGURE 4. Detection of apoptosis in treated hemocytes.** *a*, left panel, TUNEL labeling of hemocytes treated with Xax purified from *X. nematophila* at 0.005 HU for 12 h. Apoptotic nuclei appear in green. Right panel, same microscope field where digitized images were merged to superimpose the DAPI-labeled nuclei over the respective phase-contrast field. Only the DAPI-stained nuclei with a small size appear to be TUNEL-labeled. *b*, the same labeling (TUNEL (left panel) and DAPI/phase contrast (right panel)) with control (untreated) hemocyte monolayers. Almost all the untreated cells were TUNEL-negative (left panel) and had nuclei with a typical morphology of living hemocytes. *c*, chromatin condensation and cytoplasmic vacuolation of Xax-treated hemocytes visualized by transmission electron microscopy (left panel). Compare with control (right panel). Inset, DNA fragmentation as evidenced by TUNEL labeling in nucleus of Xax treated hemocytes. *d*, TUNEL labeling of hemocytes treated with XaxAB purified from *E. coli* SURE (pBBxaxAB). Numerous cells appear to be apoptotic, as shown by a positive reaction in TUNEL and DAPI staining (boxed area). Scale bars, 15 μm in *a*, *b*, *d*, and *c* inset and 1 μm in *c*. *e*, pancaspase inhibitor treatment blocks Xax-induced apoptosis (12 h of incubation). 1, untreated cells; 2, cells treated with 20 μM z-VAD-fmk; 3, cells treated with 0.005 HU of Xax; 4, cells treated with 0.005 HU of Xax and 20 μM z-VAD-fmk. *f*, inhibition of Xax-induced apoptosis by caspase 3 inhibitor. 1, untreated cells; 2, cells treated with 100 μM z-DEVD-fmk; 3, cells treated with 0.005 HU of Xax; 4, cells treated with 0.005 HU Xax and 100 μM z-DEVD-fmk. The data shown are the means of three independent experiments, expressed as percentages (y axis). Vertical bars, 95% confidence intervals.

**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



**FIGURE 5. Apoptosis triggered in Xax-treated HeLa cells.** After 48h of culture in RPMI, HeLa cells were incubated for 12 h in absence (control, *a–c*) or in presence (*d–f*) of Xax at 0.005 HU final concentration. In a third set of experiments 20  $\mu\text{M}$  z-DEVD-fmk was added to the Xax solution (*g–i*). *a, d, and g*, phase contrast microscopy. *b, e, and h*, DAPI. *c, f, and i*, TUNEL. In control, cells are in active division (arrows), and the proportion of TUNEL labeled cells is  $3.5 \pm 0.5\%$ . In contrast, in Xax-treated cells the proportion of TUNEL-labeled cells is  $29.4 \pm 4.0\%$  ( $p < 0.001$  compared with control cells). In the presence of the inhibitor the proportion of TUNEL-labeled cells is  $11.1 \pm 1.2\%$  ( $p < 0.001$  compared with Xax-treated cells). The shape of some cells was modified (*g*), but most cells remained TUNEL-negative (*i*) and exhibited a normal nuclei morphology (*h*). Under each experimental conditions the proportions were calculated from 300 cells, and three independent experiments were performed. Scale bar, 5  $\mu\text{m}$ .

**TABLE 3**

**Activity on red blood cells, insect hemocytes, or HeLa cells, of the purified toxin**

$\alpha\text{X}$ , toxin purified from *X. nematophila* broth growth (see 9). Xax, toxin purified from *X. nematophila* broth growth (this study). XaxAB, toxin purified from *E. coli* SURE (pBBxaxAB) (this study). +, active (lysis or apoptosis); –, inactive.

	$\alpha\text{X}$	Xax	XaxAB
SRBC	+	+	+
RRBC	–	–	–
Insect hemocytes necrosis	+	+	+
HeLa cells necrosis	+	+	+
Vacuolation of RER	+	+	+
Maximum lysis <sup>a</sup>	2h	2h	2h
With or without $\text{Ca}^{2+}$	+	+	+
After incubation 60 °C <sup>b</sup>	–	–	–
After trypsin <sup>b</sup>	–	–	–
After ghost incubation <sup>b</sup>	–	–	–
With polyethylene glycol 6000	–	–	–
Incubation at 4 °C <sup>c</sup>	–	–	–
Hemocyte apoptosis <sup>d</sup>	+	+	+
HeLa cells apoptosis <sup>d</sup>	+	+	+

<sup>a</sup> Incubating for a time longer than 2 h did not give stronger lysis results.

<sup>b</sup> The toxin solution was incubated for 1 h at 60 °C or at 37 °C with trypsin or with SRBC ghosts before its addition to the red blood cells suspension.

<sup>c</sup> Incubation of toxin and red blood cells at 4 °C for 1 h followed by centrifugation and incubation of the red blood cells pellet in PBS at 37 °C for 1 h.

<sup>d</sup> see “Results.”

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. 6*a*). In contrast, lysates from *E. coli* SURE (pBBxaxA) or (pBBxaxB) strains used separately (Figs. 6, *c–d*) or as a mixture (Fig. 6*e*) 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 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. 6*f*). If the lysate of *E. coli* SURE (pBBxaxB) was added before the lysate of *E. coli* SURE (pBBxaxA), no hemolysis was observed (not shown).

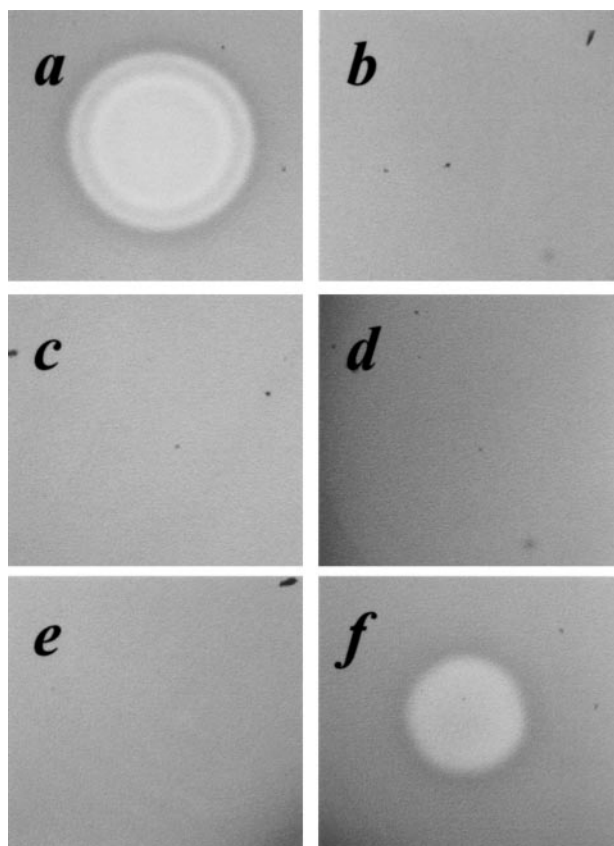
*The XaxA/XaxB Ratio Is Equimolar for Maximum Activity*—We generated XaxA and XaxB recombinant polyhistidine proteins with c-Myc or HA tags, respectively, to confirm the previous result and to determine the ratio of these two

peptides giving maximum activity. The two peptides (XaxA-Myc-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).

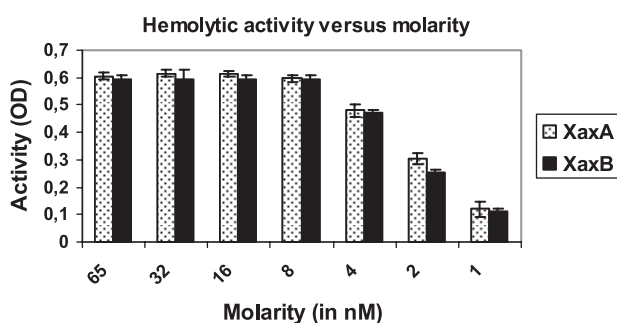
We first incubated suspension of SRBC for 1 h with various concentrations (final concentrations of 1–65 nM) of XaxAMyc-His at 37 °C and then added XaxBHA-His to a final concentration of 65 nM. After incubation for an additional hour at 37 °C, total hemolysis was obtained with a minimal concentration 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



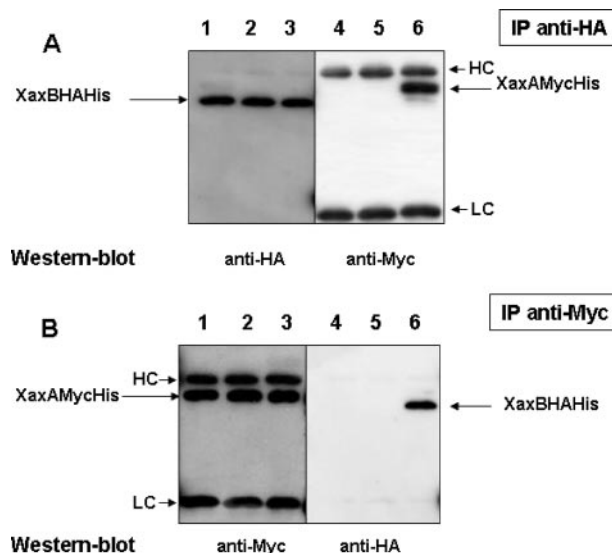


**FIGURE 6. Conditions required for the recovery of hemolysin activity from a mixture of XaxA and XaxB from recombinant *E. coli* extracts.** An aliquot (10  $\mu$ l) of bacterial lysate from an overnight culture of *E. coli* grown in LB broth containing 0.2 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside 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. *a*, positive control, SURE2 (pBBxaxAB). *b*, negative control, SURE2 (pBBR1MCS-5). *c*, SURE2 (pBBxaxA). *d*, SURE2 (pBBxaxB). *e*, bacterial extracts from SURE2 (pBBxaxA) and SURE2 (pBBxaxB) were mixed and immediately spotted onto the agar plate. *f*, SURE2 (pBBxaxA) extract was spotted, and the plate incubated for 1 h before adding SURE2 (pBBxaxB) extract.



**FIGURE 7. Ratio of concentrations of XaxAMyc-His and XaxBHA-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 (*XaxA*), SRBC suspensions were incubated with 1–65 nM XaxAMyc-His (1 h) followed by 65 nM XaxBHA-His (1 h). In a second series of experiments (*XaxB*), SRBC were incubated with 65 nM XaxAMyc-His (1 h) and various concentrations (1–65 nM) of XaxBHA-His were then added (1 h). *x* axis, molarities of the final solutions of XaxAMyc-His and XaxBHA-His. *y* axis, absorbance of hemoglobin released after the second incubation and centrifugation of the SRBC suspension.

membrane, we generated XaxAMyc and XaxBHA recombinant polyhistidine proteins to undertake immunoprecipitation experiments with the purified recombinant epitope-tagged proteins. In these experiments interactions between XaxAMyc-



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

His and XaxBHA-His were detected. These data indicate that the recombinant XaxAMyc-His and XaxBHA-His proteins are capable of interacting in solution (Fig. 8) as well as after interaction with red blood cells (data not shown) to form XaxAMyc-His-XaxBHA-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).

*XaxAB Is Required for the Overall Cytolytic Activity Expressed in the Early Stationary Phase in X. nematophila Supernatant—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 plasmatocytes and RRBC (8). To know if XaxAB is the only factor responsible for C1 cytolytic activity, we constructed a *X. nematophila xaxAB* mutant by double allelic exchange, called xaxAB9. Whatever the time of xaxAB9 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 xaxAB

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,  $\alpha X$ , 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  $\alpha X$  (9). The reason of this difference is unknown but is probably related to posttranslational processing. However,  $\alpha X$  and Xax have identical biological effects (see Table 3), and the activity of both  $\alpha X$  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  $\alpha X$  and Xax.

Xh1A, 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, Xh1A, like its *Photorhabdus* homolog Ph1A (23), belongs to a family characterized by a two-partner secretion system. No conserved secretion motif, such as LANPL or ANPPNGIs/tCnGCGF (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.<sup>6</sup> 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.<sup>7</sup> 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  $\alpha X$  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 cytolysin of *Enterococcus faecalis*, which consists of two subunits, CylL<sub>S</sub> and CylL<sub>L</sub>. When present together in solution, activated CylL<sub>S</sub>' and CylL<sub>L</sub>' have no hemolytic activity (31), like the mixture of

<sup>6</sup> A. Givaudan and C. Ribeiro, unpublished data.

<sup>7</sup> R. Zumbihl, 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 cytolysins, 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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