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Purification and Characterization of Xenorhabdicin, a Phage Tail-Like Bacteriocin, from the Lysogenic Strain F1 of *Xenorhabdus nematophilus*

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Xenorhabdicin, the phage tail-like bacteriocins of *Xenorhabdus nematophilus*, and phage head particles, elements produced together after mitomycin induction in *X. nematophilus* lysogenic strain F1 cultures, were separated by DEAE chromatography, examined by transmission electron microscopy, and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis of xenorhabdicin showed two major subunits of 43 and 20 kDa corresponding to the sheath and the inner core, respectively. At least five other minor subunits of 67, 54, 35, 28, and 16 kDa were also characterized. Electrophoresis of the phage head capsids showed a major 40-kDa subunit and two minor 50- and 34-kDa subunits. Bactericidal activity recorded against closely related bacterial species and spontaneously produced by *X. nematophilus* resides in the xenorhabdicin particles and is another antimicrobial barrier to save the symbiotic association.

Xenorhabdus spp. (members of the family *Enterobacteriaceae*) are symbiotically associated with entomopathogenic nematodes of the family *Steinernematidae* (3, 7, 17). Two phase variants which have been characterized by a series of different physiological properties occur spontaneously (6). Among these properties, phase I variants produce chemical, agar-diffusible antibiotics while phase II variants produce no such compounds (2).

A recent study provided the first demonstration of lysogeny concomitant with bacteriocinogeny in *Xenorhabdus nematophilus* A24 (9). Entire phage particles were detected in small quantities (5 to 10%), with larger quantities of bacteriocins and phage heads in cultures of both phases after induction by mitomycin or heat treatment. The *X. nematophilus* prophage was found to be incorporated into the chromosome of both phases of the host species (9). Baghdiguian et al. (5) have shown that the structure of *X. nematophilus* bacteriocins is similar to the structure of rigid bacteriophage tails, which are made up of a contractile sheath, a core, and a baseplate with six caudal fibers. These rigid phage tail-like particles have been shown to be different from the flexible tails of the entire phage particles accompanying bacteriocinogeny (9).

Boemare et al. (9) showed two distinctive types of antimicrobial activity in *X. nematophilus*. The broad-spectrum activity of antimicrobial molecules (2) produced by phase I variants was confirmed, as well as a bactericidal activity from lysates of both variants against a much more limited range of bacteria, mainly those closely related to *X. nematophilus* (9). Basically, both variants of other *Xenorhabdus* spp. were sensitive to *X. nematophilus* lysates. This more limited antimicrobial activity was lost after pronase E treatment, suggesting that it was supported by the proteinaceous bacteriocin elements. When two different entomopathogenic nematodes coinfected an insect, only one species was able to reproduce (4). It was suggested that the first symbiont to establish in the host may initiate the spontaneous production of bacteriocins which would be able to kill other related symbionts and consequently may inhibit multiplication of the second parasite (8).

This study reports, for the first time, the purification of *X. nematophilus* phage tail-like bacteriocins in order (i) to separate the bacteriocins from phage particles, (ii) to verify that the purified bacteriocins support the bactericidal activity of the lysates, (iii) to distinguish the bacteriocins biochemically from phage capsids, and (iv) to provide proof for the possible role of bacteriocins in the vertical transmission of the symbionts to the nematode progeny by eliminating competitors when nematode juvenile larvae are escaping the insect cadaver.

Induction and purification of phage tail-like bacteriocins and phage head particles. Phase I and phase II variants (6) of *X. nematophilus* F1 from *Steinernema carpocapsae* Plougastel (supplied by E. Bonifassi, Institut National de la Recherche Agronomique, Antibes, France) were used as producers of bacteriocins. This lysogenic strain from *X. nematophilus*, very similar to strain A24 used previously (6, 7), was chosen because it produces more bacteriocins than phage particles in order to facilitate biochemical separation. Mitomycin (0.5 $mg \cdot ml^{-1}$) was added in phase I and II Luria broth cultures at the logarithmic stage (A_{600} = 0.5 to 0.6) to induce bacteriocin production. Under these conditions, the entire cellular population of each phase culture was entirely lysed after 3 to 5 h of incubation at 28°C, as demonstrated by negative subculturing from lysates. They were treated with DNase and RNase (10 $mg \cdot ml^{-1}$, 30 min, 37°C), and cell debris was removed by low-speed centrifugation (6,000 × *g*, 10 min, 4°C). Proteins of phase I and II supernatants were precipitated with 10% polyethylene glycol (PEG) and 0.5 M NaCl (18). Precipitates were collected by centrifugation (10,000 × *g*, 15 min, 4°C). Sediments were gently dissolved in buffer B (0.1 M NaCl, 0.01 M phosphate buffer; pH 6.8) added with 0.001 M phenylmethylsulfonyl fluoride. Insoluble material was removed by low-speed centrifugation (5,000 × *g*, 5 min, 4°C), and suspensions were ultracentrifuged (70,000 × *g*, 60 min, 4°C).

Identical results have been obtained from phase I and II variant cultures at each step of purification; the results are described for strain F1. The purification procedure was mon-

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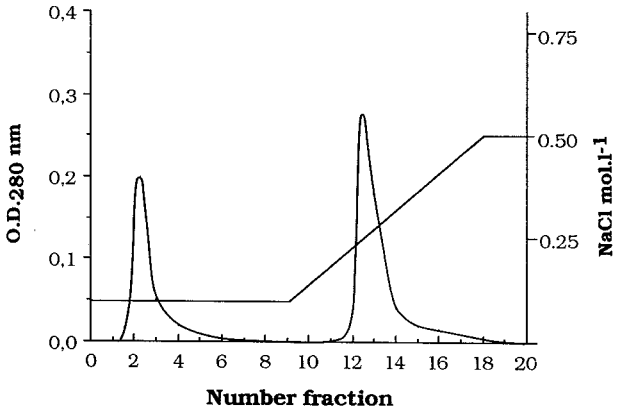

FIG. 1. DEAE chromatography of the 15% sucrose fraction. After DEAE chromatography, one flowthrough fraction (left peak) and one elution fraction (right peak) were obtained. The chromatographic conditions used follow: void volume, 1.4 ml; flow rate, 1.4 ml · min^{-1}; fraction volume, 2.8 ml; sample volume, 1 ml; and protein concentration of the sample, 1 mg · ml^{-1}. $O.D._{280\ nm}$, optical density at 280 nm.

itored by electron microscopic examinations. The pellets dissolved in buffer B were deposited on a 10 to 40% (wt/vol) preformed sucrose density gradient and centrifuged (60,000 × *g*, 120 min, 4°C). An opalescent band at 15% sucrose was collected and dialyzed against buffer B. This dialyzed fraction was chromatographed on a DEAE MemSep (Millipore) column (void volume, 1.4 ml) with an increasing gradient of NaCl (0.1 to 0.5 M) in 0.01 M phosphate buffer (pH 6.8). This procedure generated one flowthrough fraction and one eluted fraction at about 0.2 M NaCl (Fig. 1). When 1 mg of dialyzed fraction protein was applied on a DEAE column, about 33% was found in the flowthrough fraction and 66% was recovered in the eluted fraction.

A sample of the previous 15% sucrose fraction, negatively stained by 1% phosphotungstate and examined by electron microscopy, revealed a mixture of phage tail-like bacteriocin (170 nm long) and phage head particles (50-nm diameter) (Fig. 2a), whereas the electron microscopy observations of DEAE chromatography samples revealed bacteriocins only in the eluted fraction (Fig. 2b) and phage head particles in the flowthrough fraction (Fig. 2c).

Bacteriocin assays for antimicrobial activity. *Xenorhabdus beddingii* Q58 (maintained in our laboratory and initially provided by R. Akhurst, Commonwealth Scientific and Industrial Research Organization) was used as bacteriocin-sensitive indicator bacteria. When 10- or 2-fold serial dilutions of the bacteriocin suspension were applied to a logarithmic-phase growth (A_{600} = 0.5) broth culture of the indicator strain, clearing of the culture was obtained. Subculturing of the lysed indicator culture at the lowest active dilution was not possible, indicating that the purified bacteriocin had a bactericidal effect. Indicator strain from a logarithmic-phase growth (A_{600} = 0.5) culture on Luria broth was mixed (2% vol/vol) into 5 ml of nutrient agar (0.6% [wt/vol]) and spread onto petri dishes. One-microliter aliquots of a 10- or 2-fold serial dilution of the bacteriocin suspension were spotted onto these plates. A clear zone on the bacterial lawn at the drop location after incubation at 28°C for 24 h was interpreted as killing of the indicator bacterium. The bactericidal specific activity (activity units [AU] per milligram) was expressed by the highest dilution of the sample that still kills the indicator strain growth on agar plates for a protein concentration evaluated by the method of Bradford (10). The bactericidal specific activity of the lysate supernatant increased

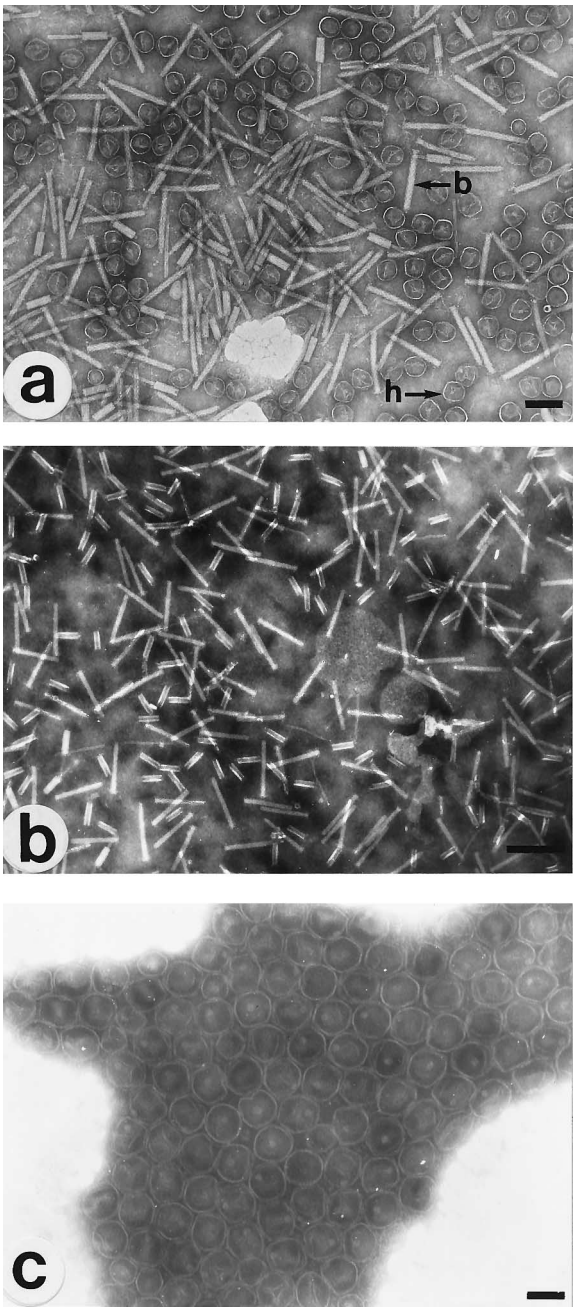

FIG. 2. Electron microscopic controls of the purification steps. Electron micrograph of the 15% sucrose fraction, showing a mixture of phage tail-like bacteriocins (b) and empty phage head particles (h), is presented in panel a. Panel b shows that the DEAE-eluted fraction is constituted of only phage tail-like bacteriocins. In contrast, the DEAE flowthrough fraction consists of pure empty phage head particles (panel c). Scale bars: 100 nm (a), 200 nm (b), and 50 nm (c).

with an apparent purification factor of 450-fold in the DEAE chromatographic elution peak. Neither bactericidal activity nor viral replication was found in the DEAE flowthrough fraction (Table 1).

Biochemical characterization of the phage tail-like bacteriocins. The protein preparations were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the discontinuous system of Laemmli (12). The best

TABLE 1. Purification steps of *X. nematophilus* F1 phage tail-like bacteriocin[a]

Step	Protein concn ($mg \cdot ml^{-1}$)	Bacteriocin activity ($AU \cdot ml^{-1}$)	Sp act ($AU \cdot mg^{-1}$)	Increase in sp act (fold)
Mitomycin-induced lysates	0.094	10^4	1.06×10^5	1
PEG precipitates	0.088	10^5	1.14×10^6	11
15% sucrose fraction	0.126	2×10^6	1.59×10^7	150
DEAE elution fraction	0.063	3×10^6	4.76×10^7	450
DEAE flowthrough fraction	0.142	0	0	0

[a] Steps and calculations are described in the text.

dissociation of protein subunits was obtained with 4% SDS and 10% β-mercaptoethanol at 100°C (10 min). Just after boiling, it was necessary to add 10% (vol/vol) of 0.1 M iodoacetamide to avoid renaturation. Without this precaution, some components were lost in the initial deposit. The phage tail-like bacteriocin was composed of at least seven different bands (Fig. 3, lane 5). From their apparent mobilities and relative abundances in SDS-polyacrylamide gels, we deduced that bacteriocins were composed of two major subunits of about 43 and 20 kDa, two subunits of about 67 and 54 kDa, and three minor subunits of about 35, 28, and 16 kDa. No difference was found between bacteriocins from *X. nematophilus* F1/1 and F1/2. After NaOH-denaturating treatment of the PEG precipitate by the method used by Shinomiya (15) for pyocin denaturation, only one sheath substructure type in the sucrose gradient was isolated. Electrophoresis showed that this component was composed of the 43-kDa major subunit (data not shown). Moreover, pure bacteriocins were treated by 4% SDS at 100°C (10 min) and were applied on a sucrose gradient followed by centrifugation (60,000 × *g*, 120 min. 4°C). The 30% sucrose fraction contained only the inner core (controlled by electron microscopy), and electrophoresis showed that this component was composed of the 20-kDa subunit (data not shown). Therefore, the two major subunits are the sheath monomer (43 kDa) and the inner core monomer (20 kDa).

Purification of *X. nematophilus* F1 bacteriocins shows clearly that these particles belong to the class of the phage tail-like bacteriocins (14). Their structure revealed an extended or contracted sheath twisting around an inner core and a basal plaque

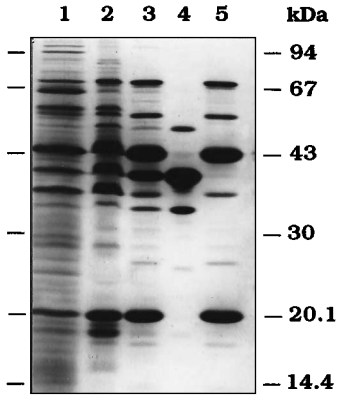

FIG. 3. SDS-PAGE of lysate supernatant from a mitomycin-induced *X. nematophilus* F1 culture (lane 1), of protein extract after 10% PEG treatment (lane 2), of 15% sucrose fraction from sucrose gradient ultracentrifugation (lane 3), of DEAE chromatography flowthrough fraction containing pure empty phage head particles (lane 4), and of DEAE chromatography-eluted fraction containing pure phage tail-like bacteriocins (lane 5). Each well was filled with 10 μg of protein evaluated by the method of Bradford (10).

with six caudal fibers (Fig. 2c). The contractile phage tail bacteriocins of *Pseudomonas aeruginosa* similarly possess a sheath with a 35.8-kDa monomer (15) and a core with an 18-kDa monomer (11). Compared with the previous results, these data are in agreement with the two major bands of the 43-kDa sheath and the 20-kDa inner core of the *X. nematophilus* F1 bacteriocin. In addition, *Xenorhabdus* bacteriocin also shares similarities with the bacteriophage P2 contractile tail in which the major component of sheath and tube have molecular masses of 43 and 19 kDa, respectively (16). According to the producer species name, that is, *X. nematophilus*, and following the usual nomenclature of microbiologists, we propose to name this first identified phage tail-like bacteriocin in the *Xenorhabdus* strain after the generic name of the strain, xenorhabdicin.

Biochemical characterization of the phage head capsids. During the previous purification, we isolated a flowthrough fraction containing phage head particles by DEAE chromatography. After electrophoresis of the DEAE chromatography flowthrough fractions, at least three bands were observed (Fig. 3, lane 4). The phage head particles were composed of a major protein subunit of about 40 kDa and two less important subunits of about 50 and 34 kDa. The molecular mass of the major structural protein of the phage head particles from *X. nematophilus* (40 kDa) is in agreement with the usual molecular weight of the tailed bacteriophage capsomers (1). Moreover, DNA extraction from the phage head particles performed by the QIAGEN lambda kit protocol and by the usual phenol-chloroform extraction failed to reveal any DNA fragment. These results are in agreement with the electron microscopic observations that the flowthrough fraction contains only empty phage heads. Therefore, we have to consider that the isolated phage head particles from DEAE chromatography are essentially empty phage heads.

The bactericidal activity of *X. nematophilus* against the closely related bacteria mentioned previously (9) is due to the purified xenorhabdicin. Attempts to separate phage entire particles in order to find a bacterial target that can be a substitute strain for viral replication are under way. The success of these attempts should allow us to verify if the xenorhabdicin activity acts against viral replication by killing the bacterial target before any viral expression.

Maxwell et al. (13) demonstrated that antimicrobial molecules, such as xenocoumacin molecules produced by phase I of *X. nematophilus*, are maintained during a 10-day insect infection. Xenorhabdicin spontaneously produced in insect cadaver should act as a second antimicrobial barrier in closely related bacteria. In reality, these two antimicrobial barriers (xenocoumacin and xenorhabdicin) save the bacterium helminthic symbiosis in the progeny. Juvenile larvae escaping the insect cadaver harvest in their intestines mainly their own symbionts which have eliminated many contaminants, including the possible occurrence of other closely related bacteria.

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