Lysogeny and Bacteriocinogeny in Xenorhabdus nematophilus and Other Xenorhabdus spp.

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Induction by mitomycin or high-temperature treatment resulted in the production of bacteriocins and phages in both phases of Xenorhabdus nematophilus A24, indicating lysogeny. Phage DNA purified from X. nematophilus A24 hybridized to several fragments of DraI-digested A24 chromosomal DNA, confirming that the phage genome was incorporated into the bacterial chromosome. Bacteriocins and phages were detected in cultures of most other Xenorhabdus spp. after mitomycin or high-temperature treatment. Xenorhabdus luminescens K80 was not lysed by these treatments, and no phages were seen associated with this strain. However, bacteriocins were detected in limited quantities in all Xenorhabdus cultures, including X. luminescens K80, without any induction. X. nematophilus A24 bacteriocins were antagonistic for other Xenorhabdus species but not for A24 or other strains of X. nematophilus.

Xenorhabdus spp. (members of the family Enterobacteriaceae) are symbiotically associated with entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae (3, 25). The nematodes act as vectors, transporting their bacterial symbionts into the hemocoel of the insect host. Xenorhabdus spp. contribute to the symbiotic relationship by providing nutritional requirements for their nematode partners (23).

Xenorhabdus strains spontaneously produce two colony form variants (1) which have been called phase variants (6). Phase 1 variants adsorb dyes on agar plates, produce lecithinase, emit light (in bioluminescent strains only [6]) and have cytoplasmic paracrystalline inclusions (7, 18), while these properties are either absent or greatly reduced in phase 2 (6, 18). Phase 1 variants also produce nonprotein agar-diffusible antibiotics that minimize the secondary invasion of the insect cadaver by other microorganisms, while phase 2 variants produce no such compounds (2). Some of these antibiotics have been identified as indole derivatives (19, 24), trans-stilbenes (19, 24), xenorhabdins (16), and xenocounacins (17).

Antibiotic activity has also been attributed to elements like the phage tails that were detected in Xenorhabdus luminescens. These elements, interpreted as virulent for Bacillus cereus, were designated defective bacteriophages (22). Subsequently, a bacteriophage that was lytic for phase 1 but not for phase 2 of X. luminescens was reported (21). It has been hypothesized that this phage was the cause of phase variation in Xenorhabdus spp. (20). However, the lytic activity of this phage occurred without any induction, indicating that the phage may have originated from another host. To test the hypothesis that lysogenic phages were present in Xenorhabdus spp., we attempted to induce lysis by several treatments and determine the nature of the elements causing lysis.

MATERIALS AND METHODS

Bacteria and growth conditions. We used phase 1 and phase 2 variants of Xenorhabdus nematophilus A24 (Australia) and F1 (France), Xenorhabdus bovienii Dan (Denmark), Xenorhabdus beddingtoni Q58 (Australia), and X. luminescens K80 (Guadeloupe, French Antilles). Phase 1 and phase 2 of Xenorhabdus strains are indicated by a slash and a numeral 1 or 2, respectively, to the strain designation (6). At each subculture, phase status was assessed on nutrient agar supplemented with 25 mg of bromothymol blue and 40 mg of triphenyltetrazolium chloride per liter; phase 1 colonies are blue, and phase 2 colonies are maroon red (1). Xenorhabdus strains were grown in Luria broth (LB) or on nutrient agar (1.5% [wt/vol]) at 28°C.

Twenty-one strains of various bacterial species from our laboratory (Table 1), which were routinely checked for purity and species characteristics, were used as indicator strains. These strains were grown in LB at 37°C.

Induction of lysis in bacterial cultures. Mitomycin (0.5 μg ml−1; Sigma) was added to logarithmic-phase growth (A600 = 0.5) cultures of Xenorhabdus spp. LB (14). Lysis was also induced by heating Xenorhabdus spp. cultures at 45°C for at least 40 min and subsequently incubating overnight at 28°C.

Purification of bacteriocins and phages. Bacteriocins and bacteriophages were purified from X. nematophilus A24/1 and A24/2. For each phase, 1 liter of lysed culture was centrifuged at 6,000 × g (10 min, 4°C), and the supernatant was sterilized by passage through a 0.45-μm pore-size Millipore filter. The filtrate was then centrifuged at 52,000 × g (90 min, 4°C). The resulting pellet was resuspended in 8 ml of 10 mM phosphate buffer (pH 6.8) and clarified by low-speed centrifugation at 4,000 × g (10 min, 4°C). The final suspension was fractionated on a Servacel DEAE 52 cellulose column (1.5 by 6 cm), washed previously with phosphate-buffered saline and eluted with 400 ml of an NaCl gradient (0 to 0.5 M in 10 mM sodium phosphate buffer). Samples corresponding to all of the peaks were checked by electron microscopy for the presence of bacteriocins and/or phages.

Electron microscopy. Purified suspensions of bacteriocins

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and phages from *X. nematophilus* and lysates of cultures of other *Xenorhabdus* spp. were negatively stained with 1% phosphotungstate. The samples were examined with an electron microscope (JEOL 200).

**Antibiotic activity of bacteriocin suspensions.** The in vitro activity of the purified *X. nematophilus* A24/1 and A24/2 bacteriocins was tested by spotting suspensions from the second peak of the DEAE 52 column onto lawns of indicator bacteria. Indicator strains from 24-h LB cultures were spread onto nutrient agar (1.5% [wt/vol]) plates, and then 15-μl aliquots of $10^9$ to $10^{-5}$ dilutions of the bacteriocin suspensions 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 inhibition of the indicator bacterium.

**Partial characterization of bacteriocin suspensions.** The purified bacteriocin suspensions were treated with pronase E (Sigma; 5 U ml$^{-1}$, 37°C, 30 min) or trypsin (Bohringer), chymotrypsin (Sigma), or papain (Sigma) (5 U of each ml$^{-1}$, 25°C, 30 min) or by heating (100°C, 30 min). The antimicrobial activity of the treated suspensions was assayed as described above.

**Antibiotic activity of noninduced cultures.** The antimicrobial activity of *X. nematophilus* was tested by a modification of the in vitro method of Akhurst (1). Nutrient agar (1.5% [wt/vol]) was spot inoculated with *X. nematophilus* A24/1 and phages from *X. nematophilus* and lysates of cultures of other *Xenorhabdus* spp. were negatively stained with 1% phosphotungstate. The samples were examined with an electron microscope (JEOL 200).
and A24/2 and incubated at 28°C for 24 to 120 h. The plates were exposed to chloroform for 1/2 h to kill X. nematophilus cells and left standing for 1 h to allow evaporation of the chloroform. Plates were then overlaid with soft nutrient agar (0.6% [wt/vol]) containing an indicator bacterium and incubated for a further 24 h. Antibiotic activity was scored by measuring the diameter of inhibition zones.

**Extraction, purification, and isolation of phage DNA.** X. nematophilus A24/1 and A24/2 cultures were lysed by induction with mitomycin and phage DNA purified from the lysates using the plaque lambda method (14, 26). To prevent any host DNA contamination from lysed bacteria, the lysate was first treated with DNase and RNase (1 μg ml⁻¹ [wt/vol], 30 min, 37°C) before the concentration step. NaCl was added to 0.5 M, and the phages particles were precipitated by mixing in polyethylene glycol 6000 to 8% (wt/vol). The precipitate was resuspended in a small volume of SM buffer (14) (NaCl, 5.8 g liter⁻¹; MgSO₄·7H₂O, 2 g liter⁻¹; 1 M Tris-HCl [pH 7.5], 50 mM liter⁻¹; 2% [wt/vol] gelatin, 5 ml liter⁻¹) and checked by electron microscopy for the presence of phages. The suspension (500 μl) was treated with 5 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS) and 5 μl of 0.5 M EDTA (pH 8.0) for 15 min (68°C) and then extracted with phenol-saturated SM buffer, phenol-chloroform (1:1), and chloroform to remove the bacteriocins and phage proteins (26). Phage DNA was precipitated by the addition of isopropanol and resuspended in Tris-EDTA (14).

**Extraction of Xenorhabdus genomic DNA.** Genomic DNA of X. nematophilus spp. was prepared from 3 ml of bacterial culture. The bacterial pellet was lysed by the addition of 400 μl of lysozyme solution (50 mM glucose, 10 mM EDTA, 25 mM Tris base [pH 8.0], 4 mg of lysozyme per liter) and then 10% (vol/vol) SDS. The mixture was heated for 2 min at 65°C and further purified by one phenol and one chloroform extraction. The upper phase (400 μl) was collected and incubated for 2 h at 37°C in the presence of proteinase K (50 mg ml⁻¹). The DNA was further purified by one phenol and one chloroform extraction and then by ethanol precipitation.

Genomic DNA was digested with restriction enzyme DraI as recommended by the enzyme manufacturer (Boehringer Mannheim).

**Phage probes, DNA transfers, and hybridizations.** Nonradioactive phage DNA probes were prepared by using the technical instructions of the digoxigenin kit from Boehringer Mannheim. They were hybridized to DraI-digested genomic DNAs of X. nematophilus A24 and X. luminescens K80 that had been transferred onto Schleicher & Schuell nitrocellulose filters by Southern blotting (14). The hybridization was undertaken at 68°C without formamide. Since no difference in the restriction patterns of the two phases of Xenorhabdus genomic DNA have been found (4, 13), experiments to determine whether phage DNA was located on the Xenorhabdus chromosome were conducted only with the phase 1 variant.
RESULTS

Lysis of X. nematophilus A24 cultures. Lysis was induced in both phases of X. nematophilus A24/1 and A24/2 cultures 3 to 5 h after the addition of mitomycin. Lysis was also induced by heating cultures at 45°C for at least 40 min and subsequently incubating them overnight at 28°C.

Electronic microscopy examination of the lysates. A variety of components were detected by electron microscopy after induction of lysis in X. nematophilus (Fig. 1). There were bacteriocinlike phage particles such as headless rods with extended striated bases (ca. 170 nm in length) and a base plate to which fibers were attached (Fig. 1a and c), contracted sheaths revealing an inner core, segments of empty contracted sheaths, loose cores (Fig. 1a and b), and complete or empty phage heads (Fig. 1a and d). Intact phage particles (ca. 220 nm from head to base plate; Fig. 1e and f) were also detected.

Purification of phages and bacteriocins. Bacteriocins and bacteriophages were purified from X. nematophilus A24/1 and A24/2 by DEAE column chromatography. Two peaks were obtained; electron microscopic examination showed that the first peak contained some intact phages, phage heads, and phage tails, and the second peak contained only bacteriocins with extended sheaths.

Antimicrobial activity of bacteriocin suspensions. When the antimicrobial activity of suspensions from the second DEAE peak were tested in vitro, the only strains inhibited were Xenorhabdus spp. (other than X. nematophilus) and two Proteus species (Table 1). There was no detectable difference in the antimicrobial activity of bacteriocins from phase 1 and phase 2 of X. nematophilus.

Induction by mitomycin increased the antimicrobial activity of bacteriocins enormously. X. luminescens and X. beddingtonii were up to 10,000-fold more sensitive to induced cultures of A24/1 and A24/2 than they were to noninduced cultures used as controls (Table 1).

Sensitivity to the bacteriocin suspensions was eliminated by treating the purified suspensions with pronase E or by heating at 100°C for 30 min. Trypsin, chymotrypsin, and papain had no effect on the antibacterial activity of the suspensions.

Antibiotic activity of noninduced cultures. The antibiotic activities of noninduced cultures were very different from those of the bacteriocin suspensions from those cultures. A broad spectrum of bacterial strains was susceptible to the antimicrobial activity of the noninduced cultures (Table 2), but strains that were sensitive to the bacteriocins (Table 1) were not sensitive to noninduced cultures.

Moreover, activity could be detected in phase 1 cultures only (Tables 2 and 3). Even when cultures were incubated for up to 120 h before being tested, no antibiotic activity could be detected in phase 2 (Table 3).

Hybridization of A24 phage DNA with genomic DNAs. When phage DNAs purified from X. nematophilus A24/1 and A24/2 lysates were electrophoresed, they produced a band above the 23-kb marker that did not correspond with plasmid of X. nematophilus (Fig. 2a).

DNA purified from phage isolated from X. nematophilus A24/1 hybridized to 18 bands of DraI-digested chromosomal DNA of X. nematophilus A24/1 (Fig. 2b). There was neither homology to A24 plasmid bands nor to X. luminescens K80 chromosome DNA (Fig. 2b).

Lysis of other Xenorhabdus spp. cultures. Other isolates of Xenorhabdus spp., which lysed after mitomycin or heat treatment, were X. nematophilus F1/1 and F1/2, X. bovienii Dan1 and Dan2, and X. beddingtonii Q58/1 and Q58/2. Each of these isolates yielded high levels of bacteriocins with the same morphologies as those of the A24 bacteriocins. DNA phages were also detected in the X. nematophilus F1, X. bovienii Dan, and X. beddingtonii Q58 lysates. DNAs obtained from the purified phages produced a single band of >23 kb on 0.7% agarose gel.

X. luminescens K80/1 and K80/2 did not lyse after mitomycin or heat treatment. However, bacteriocins were detected in low quantities by electron microscopic examination of noninduced cultures of X. luminescens K80/1 and K80/2, as they were in all the other Xenorhabdus isolates. No phage particles were detected.

DISCUSSION

This study provides the first demonstration of lysogeny in Xenorhabdus spp. Phage could only be detected in X. nematophilus A24 cultures after mitomycin or heat treatment, and noninduced cultures of X. nematophilus were immune to the phage. Moreover, the A24 prophage genes were shown to occur on the host chromosome because phage DNA hybridized to chromosomal DNA of the X. nematophilus A24 host cells but not with the A24 plasmid DNA. Lysogeny was also shown to be associated with bacteriocinogeny in X. nematophilus. The bacteriocin and phage elements found in X. nematophilus lysates were very similar to those found in lysogenic strains of Pseudomonas and Erwinia spp. (9–12).

The occurrence of temperate bacteriophage in both phases of X. nematophilus indicates that phase variation in this

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<tr>
<th>Indicator strain</th>
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<tr>
<td></td>
<td>Phase 1</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
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<tr>
<td>Enterobacter cloacae</td>
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</tr>
<tr>
<td>Erwinia chrysanthemi</td>
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</tr>
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<td>Yersinia enterolitica</td>
<td>2</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
<td>3</td>
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<tr>
<td>Pseudomonas testosteroni</td>
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<tr>
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<td>Xenorhabdus nematophilus A24/2</td>
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* 0, no inhibition; 1, <10 mm; 2, <20 mm; 3, <30 mm; 4, <40 mm.
species is not a mechanism for escaping the lytic activity of the phage. The isolation of a phage from *X. luminescens* cultures (21) led to a suggestion that phase variation may be a mechanism for escaping the lytic activity of the phage (20). However, the phage isolated from *X. nematophilus* differs significantly from that reported from *X. luminescens*. The *X. nematophilus* phage was lytic for its host only after induction and was present in both phases, whereas the *X. luminescens* phage was reported to be lytic for its host without induction and only for phase 1. Moreover, although the *X. luminescens* phage DNA was shown to be distinct from the plasmid DNA, its incorporation into the bacterial chromosome was not tested. In contrast, the *X. nematophilus* prophage was incorporated into the chromosome of both phases of the host species.

This study showed two distinct types of antimicrobial activity in *X. nematophilus*. It confirmed the broad spectrum of activity of agar-diffusible antimicrobial agents (2). It also revealed a class of agents with activity against a much more limited range of bacteria, including closely related species. The electron microscopic evidence and sensitivity to protease treatment of this second class of agents show that they are bacteriocins.

Bacteriocins have a much narrower spectrum of antimicrobial activity than most nonprotein antimicrobial agents and commonly function in preventing competition by closely related species (8, 11, 15). Our in vitro test showed that *X. nematophilus* bacteriocins inhibited other *Xenorhabdus* spp. This supports the hypothesis that the dominance of one *Xenorhabdus* species in an insect host, which determines which of any coinfecting nematode species can survive and reproduce (5), is very likely mediated by bacteriocins.

Although this study has only demonstrated that lysogeny occurs in *X. nematophilus* A24, it apparently also occurs in other *Xenorhabdus* strains and species. When cultures of *X. nematophilus* F1, *X. bovienii* Dan, and *X. beddingii* Q58 were induced with mitomycin or heat treatment, lysis occurred and phages and bacteriocins were produced. Electrophoretic examination of phage DNA from any of these strains revealed a DNA band distinct from those of plasmid and chromosomal DNA. However, the location of prophage genes in these strains will have to be determined by hybridizations between phage DNAs and chromosomal DNAs of *Xenorhabdus* spp. Further studies to define the biological activities and antimicrobial activity, size, and biochemical differences between bacteriocin and phage production in the various strains and species of *Xenorhabdus* spp. are being conducted.

Although *X. luminescens* K80 cultures were not lysed after mitomycin or heat treatment, there was a low level of bacteriocins in noninduced cultures, as occurred in other *Xenorhabdus* spp. It is possible that *X. luminescens* is lysogenic but the nature of the phage is different, and different conditions for induction may be required.

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**REFERENCES**