The Genus *Xenorhabdus*

Entomopathogenic Bacteria for Use in Teaching General Microbiology

David Bermudes, Kenneth Nealson, and Raymond Akhurst

The bacteria in the group *Xenorhabdus* form a rather tight cluster in the Enterobacteriaceae, showing a close relationship to the genera *Escherichia* and *Salmonella*. But in contrast to their enteric relatives, *Xenorhabdus* species alternate between a symbiotic existence within the guts of soil nematodes, and a pathogenic growth stage inside of any number of different insects (Figure 1). Many of the details of the nematode/bacteria/insect system, including aspects of its potential use in insect control, are described in a recent symposium volume (Gaugler and Kaya 1990).

In our laboratory research during the past few years, the authors have been isolating and studying various species of insect-pathogenic bacteria in the genus *Xenorhabdus*. These appear to be excellent subjects for laboratory studies of microbiology, pathology/parasitology, and symbiosis.

In this paper, we present a brief description of these fascinating bacteria and their life cycle, followed by a protocol that can be easily adapted for classroom teaching.

*Xenorhabdus* species are mutualistically symbiotic with nematodes in the groups *Heterorhabditis* and *Steinernema*, which are known pathogens of insects. The bacteria are carried as non-growing symbionts in the gut tract (often in a specialized vesicle; Figs 2-4) of the nematode. The foraging nematodes locate the prey insects and penetrate to the hemolymph, acting as vectors for the injection of their symbiotic bacteria. These bacteria do not kill the insects if ingested, but do so quickly if they are introduced to the hemolymph, either via nematode infection or via syringe injection of laboratory cultures. Once into the hemolymph, the bacteria grow rapidly,
produce a variety of extracellular products, and participate in the killing of the insect. Bacteria injected into the insect in the absence of nematodes are very effective in killing, often with a mean lethal dose (LD50) of only a few cells per insect prey.

Active nematode/bacterium isolates can be found worldwide (Africa, Asia, Australia, Europe, the Pacific region, and North and South America), so there is little reason to believe that they are anything but ubiquitous. In North America, Heterorhabditis and its associated bacterium, X. luminescens, are found much more commonly than Steinernema spp. and their various associated species of Xenorhabdus.

Because of the wide distribution of these organisms, the availability of materials needed to work with them, the rapidity of the process, and the ease of manipulation, we feel that this system is an excellent one for the teaching of various aspects of symbiosis, parasitism, and pathogenesis, and we offer here an experimental protocol that is inexpensive, enjoyable, and instructive.

In addition to its use for teaching some basic features of pathogenesis and symbiosis, this system has other unique features. The isolated bacteria have many unusual properties that lend themselves well to laboratory study. These features are detailed in Table 1, and discussed briefly below.

Most members of the group Xenorhabdus luminescens (which are symbionts of the nematodes in the group Heterorhabditis), are bioluminescent, and viable infections result in insects that are visibly glowing. The luminous system is similar to that of other known luminous bacteria, and offers the opportunity to examine the physiology and biochemistry of bacterial bioluminescence in the laboratory.

In addition to bioluminescence, almost all Xenorhabdus isolates studied to date produce one or more antibiotic compounds, several of which have been purified and identified. These are broad spectrum antibiotics which can be used to demonstrate the production and activity of antibiotics in the laboratory. Extracellular Enzyme production is another general feature of members of the group Xenorhabdus; they produce one or more proteases, and usually produce a potent lipase activity. A protocol for the purification of one of the proteases has been published (Schmidt et al. 1989). Most

<table>
<thead>
<tr>
<th>Xenorhabdus luminescens</th>
<th>Antibiotic Production</th>
<th>Extracellular Enzyme Production (lipase, protease)</th>
<th>Intracellular Protein Crystals</th>
<th>Pigment Production</th>
<th>Insecticidal Toxin Production</th>
<th>Phase Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioluminescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotic Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular Enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigment Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insecticidal Toxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase Variation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unlike the Bacillus thuringiensis crystals, the crystals from X. luminescens have not been shown to have insect-toxic properties.

A further characteristic of the genus Xenorhabdus is the production of cell-associated pigments, often red or orange in color. In one case, a pigment has been purified (Richardson et al. 1988) and shown to be an anthraquinone, which is yellow at acid pH and dark red at basic pH.

It is possible to use pure cultures of Xenorhabdus to study the relative infectivity and toxicity of these bacteria (for example, to determine the LD50 of the organisms). This can be done in a straightforward way, using Galleria mellonella as the prey, and any pure culture of these bacteria. It is also possible to test isolated fractions of extracts of cultured bacteria for their toxic properties by injection into sensitive hosts (Ensign et al. 1990).

A final interesting feature of Xenorhabdus is its propensity to form spontaneous variants when maintained in culture (Bleakley and Nealson 1988). The factors that control the for-
mation or reversion of these variants, which have been called phase variants, are unknown. However, the organisms offer an excellent system for observing spontaneous variation, since when the variants form, many of the properties discussed above are lost simultaneously (for example, bioluminescence, pigmentation, crystal formation, protease and lipase excretion, and antibiotic production), and can thus be detected in several different ways.

The Heterorhabditis/X. luminescens association offers several advantages over Steinernemal/Xenorhabdus associations for classroom study. Insects killed by the former are more easily identified due to strong pigmentation and bioluminescence. Further, isolated bacterial colonies can also be easily identified by these characteristics. Due to the unambiguity in identifying this system and its common occurrence in North America (Table 2), we have chosen the Heterorhabditis/X. luminescens association as the basis of the protocol for classroom studies described here.

Any of a number of different insect hosts can be used to isolate such entomopathogenic nematode/bacteria from soils. The insect of choice is often the one that is most available, so the larvae of the wax moth, Galleria mellonella, which is commonly available from fishing bait stores and biological supply houses, is often used. This insect is also one of the most susceptible to the nematode/bacterial infection.

We outline below a protocol for the isolation of infected insects, of infective nematodes, and of pure cultures of bacteria. For a beginning course, the process of isolating bacteria directly from the nematodes is considered somewhat difficult, although it can be done, as described by Poinar (1966) or Akhurst (1980). Similarly, reinfestation of insects by ingestion of isolated bacteria is a subtle process, which is not easy to unambiguously accomplish, so it is not included in our protocol. However, for a course more focused on medical microbiology and pathogenesis, one might wish to enlarge this exercise to include these aspects.

Using this protocol, students can isolate infected insects and, subsequently, pure cultures of Xenorhabdus and infective nematodes, reinfest insects with either the bacteria (by injection) or the nematode/bacteria conglomeration, and finally, reisolate the pathogenic bacteria or nematodes. Furthermore, the protocol offers an excellent, fast, and nontraumatic way of teaching many of the basic features of symbiosis and pathogenesis in an efficient, inexpensive, and usually very quantitative way.

In summary, Xenorhabdus is of current interest due to its insecticidal activity, bioluminescence, phase variation, and other characteristics. As a teaching tool, the Xenorhabdus/nematode system offers an excellent method for examining pathogenesis and symbiosis, while the bacteria themselves may be useful for demonstrating many separate bacterial properties to students of general microbiology.

Acknowledgments
We acknowledge the following organizations for their support for parts of the fundamental research described here, and for costs involved with the writing of the article by the authors: 1) National Service Research Award Grant RES 07043 (National Institutes of Health) to DB; 2) Grant N80014-88-0244 (Office of Naval Research), and Grant 88-37263-3501 (U.S. Department of Agriculture) to KHN; and a Shaw Visiting Professorship, from the Milwaukee Foundation, to RJA.

References

Table 2: Recorded Occurrence of Heterorhabditis/X. luminescens in USA.

<table>
<thead>
<tr>
<th>State</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
<td>North Carolina</td>
</tr>
<tr>
<td>Florida</td>
<td>Ohio</td>
</tr>
<tr>
<td>Georgia</td>
<td>Puerto Rico</td>
</tr>
<tr>
<td>Hawaii</td>
<td>South Carolina</td>
</tr>
<tr>
<td>Kentucky</td>
<td>Texas</td>
</tr>
<tr>
<td>Massachusetts</td>
<td>Wisconsin</td>
</tr>
</tbody>
</table>

Would you like to publish in JCST? If so, see our “Manuscript Guidelines” on page 98 of this issue.

November 1993 JCST 107
Genus Xenorhabdus Protocol (p. 105-107)

1. Trapping insect-pathogenic nematode/bacteria:
   1.1 Collect 500 g top-soil, avoiding water logged or nematocide or insecticide treated areas
   1.2 Add prey insects to bottom of container, cover with soil
   1.3 Incubate at room temperature, usually up to 4 days (may require up to 7 days)
   1.4 Identify nematode-infected dead insects by development of pigmentation and bioluminescence, and the presence of nematodes inside the insect cadaver

2. Isolation of the bacterium Xenorhabdus luminescens:
   2.1 Surface sterilize dead infected insects by immersion in 70% ethanol for 5 minutes
   2.2 Remove to sterile surface to allow ethanol evaporation
   2.3 Pierce the insect exoskeleton and remove a sample of the body contents with a sterile transfer loop
   2.4 Streak the sample directly on to any standard nutrient agar medium (e.g. LB, tryptone/soy, nutrient agar)
   2.5 Incubate plates at room temperature (20-30°C)
   2.6 After 1-5 days, observe the cultures in total darkness for bioluminescent colonies.
   2.7 Pick and isolate as pure cultures.
   2.8 Store colonies at 10-15° C.

3. Harvesting of infective-stage nematodes:
   3.1 Transfer one infected intact G. mellonella (individuals not used for isolation of bacteria) to dry filter paper in a sterile petri plate (three replicates).
   3.2 Incubate 20-30°C.
   3.3 After one week place moistened filter paper inside the Petri plate lid to maintain humidity, and continue.
   3.4 When nematodes emerge (1-2 weeks after infection), remove the cadaver carefully and add 10 ml dH2O. Dislodge nematodes from petri dish and filter paper using a Pasteur pipet if necessary.
   3.5 A suspension of nematodes from 1 G. mellonella (usually less than 10,000 nematodes/ml) can be stored in the Petri plate for up to several weeks in shallow water at 10-15 °C.

4. Reinfection of Galleria with isolated Xenorhabdus:
   4.1 Inoculate 5 ml LB with a single colony of X. luminescens and incubate at 25-30 °C O/N. Also inoculate 5 ml LB with a non-luminous isolate and with E. coli for negative controls.
   4.2 Dilute the overnight culture 1,000 fold in sterile 1% saline.
   4.3 Using a 1 ml tuberculin syringe with a 26 ga. needle, inject 10-50 ul X. luminescens suspension into each insect (3-5 replicates). Negative controls include injections of saline, non-luminous isolates, E. coli, and dead cells.
   4.4 Incubate at room temperature. Insects injected with X. luminescens should die within 24-48 hr, and should be pigmented and luminous.
   4.5 Isolation of X. luminescens from the cadavers may be repeated to verify identity of bacteria.

5. Reinfection of G. mellonella with isolated nematodes:
   5.1 Place 2-3 G. mellonella larvae in a stoppered test tube and fill the tube with lightly dampened sterile sand.
   5.2 Pipet 0.2 ml of the nematode suspension onto the surface of the sand; replace stopper and tape in place.
   5.3 Incubate and observe as before. Infection and death should occur within 2-3 days, and infected insects should be pigmented and luminous. Both nematodes and bacteria can be reisolated, if desired.

Materials:

Soil (preferably sandy topsoil) Wax moth larvae (or other suitable insect larval form)
Sterile sand (overnight in muffle furnace) Petri plates
Filter paper (9 cm) Stoppered test tubes
Sealable container for soil Nutrient agar plates
(coffee can, plastic bag, etc.) LB broth
Pasteur pipets Sterile 1% saline
Graduated pipets (1 ml) Tuberculin syringes with 26 ga. needles
Tuberculin syringes with 26 ga. needles Stoppered test tubes
Graduated pipets (1 ml) Nutrient agar plates
Tuberculin syringes with 26 ga. needles LB broth
Graduated pipets (1 ml) Sterile 1% saline

108 JCST November 1993