Prokaryotes (2006) 6:451–494
DOI: 10.1007/0-387-30746-x_16

CHAPTER 3.3.16

The Genera *Photorhabdus* and *Xenorhabdus*

NOEL BOEMARE AND RAYMOND AKHURST

Introduction

The genera *Photorhabdus* and *Xenorhabdus* are members of the family Enterobacteriaceae that encompass the intestinal bacterial symbionts living in commensalism with entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema*, respectively (Akhurst and Boemare, 1990; Forst et al., 1997). Most of them are pathogenic for insects when injected into the hemocoel. In addition, some nonsymbiotic strains of *Photorhabdus* have been identified as opportunistic pathogens for humans (Farmer et al., 1989; Peel et al., 1999). Various insect and vertebrate symbionts are members of the γ-subclass of Proteobacteria, which contains a wide spectrum of animal and human pathogens, such as members of the families Enterobacteriaceae, Legionellaceae, Pasteurellaceae, Vibrionaceae, and the genera *Pseudomonas* (sensu stricto) and *Acinetobacter* (Stackebrandt, 1999). Symbionts of EPNs are phylogenetic neighbors of an important group of endosymbionts of insects (the RDP tree of the {Bergey's Web site}). Most of the insect endosymbionts cluster according to the phylogeny of their eukaryotic hosts, which may indicate coevolutionary events (Stackebrandt, 1999). A similar cospeciation (See Habitat/Cospeciation in this Chapter) also was observed in the case of EPNs (Akhurst, 1983; Boemare et al., 1997a; Forst et al., 1997).

However, though most of the insect symbionts are endocytobionts and not cultivable, *Xenorhabdus* and *Photorhabdus* have the distinct advantage of cultivability on standard bacteriological media. Moreover, their hosts can be easily axenized. Consequently, pure cultures of axenic nematodes and bacteria are available for combination in gnotobiological experiments that demonstrate the viability and performance (both qualitative and quantitative) of the bacterial/helminthic symbioses (Boemare et al., 1997a). Other bacterial–animal symbioses involving cultivable Proteobacteriaceae have been described: some marine animal light-organ symbionts (Nealson et al., 1990) such as the model *Vibrio fischeri* (associated with the squid *Euprymna scolopes*) and *Aeromonas veronii* bv. *sobria* (symbiont of the medicinal leech *Hirudo medicinalis*). In this latter, despite the diverse bacterial flora found along the tegument of the leech, the *A. veroni* bv. *sobria* are typically found as a pure culture in the gut, suggesting that these symbionts possess a special capability that enables them to proliferate in the medicinal leech (Graf, 2000). *Vibrio harveyi*, often commensal with marine animals, may be pathogenic for the black tiger prawn, *Penaeus monodon* (Manefield et al., 2000), as are the terrestrial luminous *Photorhabdus* for insects.

Taxonomy

Initially only one genus, *Xenorhabdus*, was described as encompassing all the symbionts of the entomopathogenic nematodes. Those strains initially considered as belonging to the species *Xenorhabdus luminescens* clearly form a DNA-relatedness group that is distinct from all the other *Xenorhabdus* strains (Akhurst et al., 1996; Boemare et al., 1993). These DNA data, together with the significant differences in phenotypic characters between "*X. luminescens*" and the other *Xenorhabdus* species (Akhurst and Boemare, 1988; Boemare and Akhurst, 1988), differences in fatty acid composition (Janse and Smits, 1990), and chemotaxonomic data (Suzuki et al., 1990) led to the transfer of *X. luminescens* into a new genus, *Photorhabdus*, as *Photorhabdus luminescens* comb. nov. (Boemare et al., 1993). Nevertheless, comparison of 16S rDNA sequences of the type strains of *Photorhabdus* and *Xenorhabdus* species indicate the close phylogenetic relationship of these two genera (Rainey et al., 1995). The Ribosomal Dataset Project (RDP) tree established that the two genera branch deeply in the family Enterobacteriaceae without any common ancestor. They are neighbors of *Proteus vulgaris* and *Arsenophonus nasoniae*, and between *Salmonella*, *Erwinia*, *Serratia*, and several endosymbionts of insects (*Hafnia*, *Rahnella* and *Yersinia*; Liu et al., 1997;

the RDP tree of the {Bergey's Web site}). All *Xenorhabdus* strains could be clearly distinguished from all strains of *Photorhabdus* by the 16S rDNA signature sequences; *Xenorhabdus* have a TTCG sequence at positions 208–211 (*E. coli* numbering) of the 16S rDNA, whereas *Photorhabdus* have a TGAAAG sequence at positions 208–213 (Szállás et al., 1997).

There are also biological arguments to distinguish these two genera. In all the microbial ecological surveys undertaken in the five continents (more than 100 independent reports), a *Xenorhabdus* isolate has never been recovered from *Heterorhabditis* or a *Photorhabdus* from *Steinernema*. Moreover, by testing the specific interaction with the nematode host, gnotobiological experiments demonstrate that *Photorhabdus* isolates do not support culture of any *Steinernema* species in vitro (Akhurst, 1983), although in some combinations, they support culture of non-host *Heterorhabditis* spp. (Akhurst and Boemare, 1990; Han et al., 1990). Similarly *Xenorhabdus* spp. do not support culture of *Heterorhabditis* (Akhurst, 1983).

For identifying the diversity of the *Photorhabdus* and *Xenorhabdus*, a fast method using the polymorphism of the 16S rRNA genes was described by Brunel et al. (1997). Fischer-Le Saux et al. (1998) applied this polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method (Fig. 1), which gives an excellent estimation of the diversity of the genus by using efficient restriction endonucleases after first checking that they provide the most discriminative restriction patterns within the given sample. It allows analysis of a large sample of different isolates giving a series of genotypes, each one corresponding to a specific restriction pattern. Based on the total amplified sequence of the 16S ribosomal RNA genes, the method provides reference restriction patterns for identifying other isolates.

According to rule 65 (2) of the *International Code of Nomenclature of Bacteria*, generic and subgeneric names, which are modern compounds of two or more Latin or Greek words, have the gender of the Greek or Latin word used as the last component of the compound. Consequently, *Photorhabdus* and *Xenorhabdus* (genera with names ending in *rhabdus* [from *rhabdos*, the Greek word for rod] are feminine) become in modern Latin feminine words, explaining the feminine species and subspecies names when they refer to adjectives (Euzéby and Boemare, 2000). Thus, "*X. nematophilus*" and "*X. japonicus*" become *X. nematophila* and *X. japonica*, and the new species of *Photorhabdus*, *P. temperata* and *P. asymbiotica*. Otherwise when the species names refer to an author, they take the gender of the author, such as *X. bovienii*, *X. poinarii*, *X. beddingii*, *P. luminescens* subsp. *laumondii* and *P. luminescens* subsp. *akhurstii*.

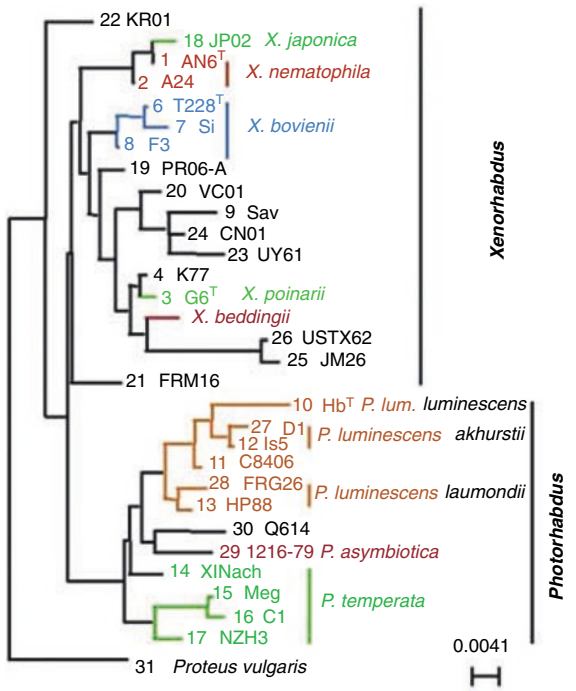


Fig. 1. Phylogenetic tree from PCR-RFLP 16S rDNA genotypes *Xenorhabdus* and *Photorhabdus*. The neighbor-joining method was applied to 30 defined genotypes, from a total of 117 strains. The number of the genotype according to Fischer-Le Saux et al. (1998) is followed by the name of the representative strain.

Habitat

Life Cycle

Symbionts of EPNs live in two different habitats during their life cycle: they survive in the gut of the free-living stage of their nematode host, and they are inoculated into and multiply in the body cavity of insects, thus creating a pure monoxenic culture (See Habitat and Ecology sections of the chapters Photorhabdus and Xenorhabdus). So the life cycle of the EPN symbionts is unique because it involves both symbiosis of nematodes and pathogenesis of insects, as summarized in Fig. 2. The infective juveniles (also named "dauer" larvae), which are the nematodes' third stage, search (cruiser species) or wait (ambusher species; Gaugler, 1993) for an insect prey (step 1). When the target insect is found they penetrate by natural openings (mouth, anus, spiracles) or directly through the tegument (mainly *Heterorhabditis*). During penetration, the nematodes exsheath the second stage cuticle, which was retained during their period in the soil, before

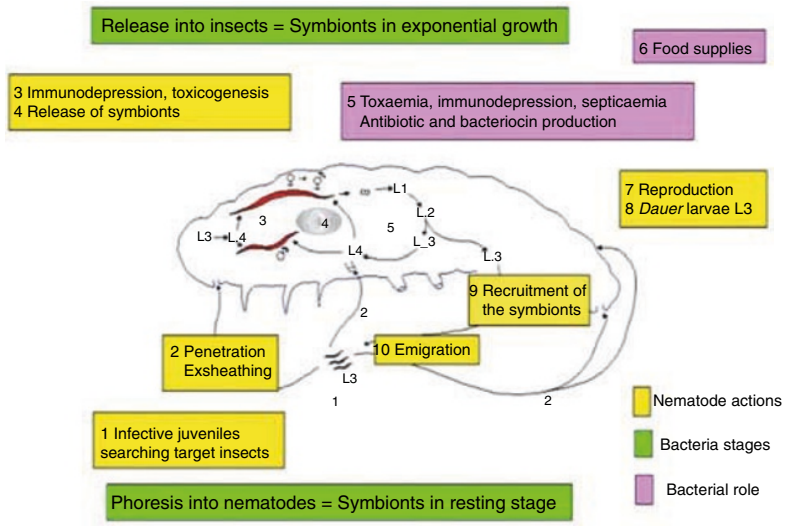


Fig. 2. Life cycle of entomopathogenic nematodes and their bacterial symbionts.

entering into the body cavity of the insects (step 2). They transform into the fourth and adult stages and during this time induce a toxicogenesis (Boemare et al., 1983b; Boemare et al., 1982), releasing an immune depressive factor (step 3) active against antimicrobial peptides produced by the insects (Götz et al., 1981). This immune depressive factor is presumably a protease that facilitates release of the nematode's bacterial symbionts (step 4). The bacteria multiply, and at the final stage, the insects die due to the septicemia (step 5). Depending on the insect, nematode and bacterial symbionts, the pathogenic process can be the result of the action of one partner or both together (see Xenorhabdus/Ecology in this Chapter). Sometimes a toxemia induced by the symbiont precedes the resulting septicemia (Forst et al., 1997). At the end of the bacterial multiplication, production of a large variety of antimicrobial compounds (see Photorhabdus and Xenorhabdus in this Chapter) prevents microbial contamination, mainly from the insect intestinal microflora. By using the food supplies provided by the bacterial biomass and the metabolized insect tissues (step 6), the nematodes reproduce in the insect cadaver (step 7) for one, two or three generations. Thus, bacteria create suitable conditions for the development of their nematode host in the insect cadaver. At the end of the parasitism, the recruitment of some bacterial cells by the dauer larvae of the nematodes (step 9), before leaving the insect (step 10), maintains the perenniality of the symbiosis through the generations.

Physiology

Phase Variation

A highly significant feature of bacteria of the genera *Photorhabdus* and *Xenorhabdus* is a phase variation that occurs during the stationary period of the growth cycle (Akhurst, 1980). It can be easily detected by two major properties: dye adsorption and antibiotic production. Only phase I of the symbionts has been detected in nature, but under in vitro conditions, a variable proportion undergoes profound change affecting colony and cell morphologies, motility, secondary metabolites, endo- and exo-enzymes (including respiratory enzymes; Akhurst, 1980; Boemare and Akhurst, 1988; Givaudan et al., 1995; Smigielski et al., 1994b). The timing and extent of the phase change is largely unpredictable, but the rate of change from phase I to phase II is generally greater than the reverse. In contrast to *Photorhabdus* where no reversion has yet been reported, the revertants obtained with *X. nematophila* (Akhurst and Boemare, 1990; Givaudan et al., 1995) indicate that the variation in the genus *Xenorhabdus* better fits the classical definition of phase variation. Indeed, phase variation in bacteriology is commonly accepted as a reversible genetic event usually mediated by DNA instability such as DNA inversion. Usually such a phenomenon affects one or a small number of gene products and is reversible at a significant frequency. In the case of *Photorhabdus*, many phenotypic traits are altered in phase II variants and no reversion has been confirmed. Some described "revertants" came from intermediate variants (Krasomil-Osterfeld, 1995), but not from true phase II variants, which have a complete loss of phase I phenotypic traits. Consequently the use of the phrase "phase variation" for *Photorhabdus* phenotypic shifts is not exactly appropriate, but is commonly used to refer to an alternative balance in expression/nonexpression of physiological traits from the same genome.

In general, variation in phase-related characters of *Photorhabdus* and *Xenorhabdus* has been

Table 1. Phase characters of *Photorhabdus* and *Xenorhabdus*.

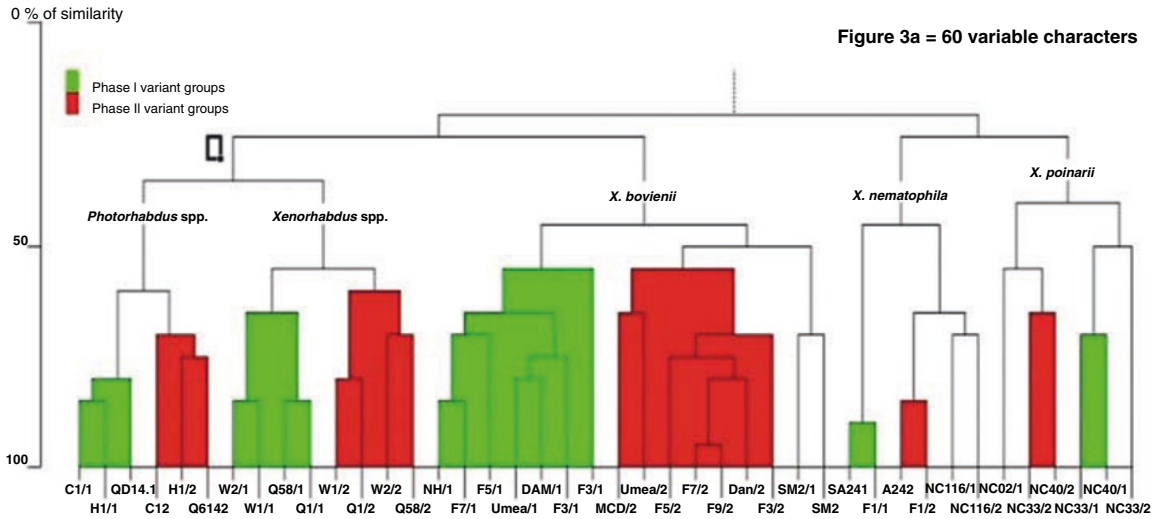
	Phase I variants	Phase II variants
Colonial characteristics		
— Morphology	mucoid	smooth
— Stickiness	+	–
— Dye adsorption	+	–
— Pigmentation	+	— or different[1]
— Motility	+[2]	–
— Swarming	+[2]	–
Ultrastructural elements and cytological properties		
— Protoplasmic crystalline inclusions	+	d
— Flagella	+[2]	–
— OpnA, CpnB	+[2]	–
— Fimbriae (or pili)	+[2]	–
— Glycocalyx thickness	+	w
— Insect hemocyte agglutination	+	–
— Erythrocyte agglutination	+	–
Enzymatic activities and secondary metabolites		
— Respiratory enzymatic activity	w	+
— Bioluminescence	+[1]	w
— Antibiotic production	+	d
— Lecithinase	+	–
— Tween-esterases	d	+
— Proteases	+	d

reported qualitatively ("+" and "–"). However, for every character that can be quantified (e.g., luminescence and antibiotic production), it is clear that the difference between phases is a matter of magnitude, not presence or absence. It is highly probable that this holds true for all phase-related characters.

Table 1 summarizes the characters affected by phase variation. The bacterium isolated from the infective stage (dauer larvae) was named the phase I variant (Boemare and Akhurst, 1988). Phase I colonies are mucoid and stick to the loop when streaked on plates, produce antibiotic molecules (Akhurst, 1982a), adsorb dyes when incorporated into agar (e.g., the neutral red in MacConkey agar). Phase II appears spontaneously during stationary growth period from in vitro culture and during nematode rearing on artificial diets. Phase II colonies are not mucoid, do not adsorb dye and do not produce antibiotics.

Numerical analyses of phenotypic data for the two phase variants of multiple *Xenorhabdus* and *Photorhabdus* strains correspond to the genotypic definition of species (Fig. 3). However, these analyses also show that phase variation is a general phenomenon throughout the genera that results in conspecific strains grouping by phase status before grouping by species (compare Fig. 3a with Fig. 3b, where some phase-related characters are deleted from the analysis). Phase variation is a general phenomenon at the infra-specific level for both genera. It does not affect the phenotypic clustering of the species, although phase variants are more similar inside each species. However, depending on the phase characters affected for each strain, clustering by phase variants or by strains may be different. Thus, some strains have phase variants differentiated by many characters (e.g., strains F1 and A24 of *X. nematophila*, strains Q614 and HI of *Photorhabdus*; Fig. 3b), others have only a few characters distinguishing the two phase variants (strains SK2 of *X. bovienii* and NC116 of *X. nematophila*; Fig. 3a).

The variation between phases in phenotypic data has been the source of some discrepancies between results published by different laboratories. Consequently, before starting the analysis of such phenotypic characters, we have to recommend an accurate screening of clones. Thus, for a taxonomic study, the two extreme clones of each strain expressing the largest number of different phenotypic responses to bacteriological tests, designated as phase I and phase II variants, must be selected. Clones that are intermediate (i.e., expressing some phase I and some phase II characters) should be eliminated from a taxonomic study, particularly for the genus *Photorhabdus*. Practically, before any mathematical treatment devoted to the taxonomy of species, the physiological data is compiled to check any positive response of related clones to a strain, and to be consistent with the definition of all the potentialities of the given strain. This is also a reason to take great care to harvest isolates expressing all the strain properties during the isolation from the nematode host. Conversely, these intermediate expressions are of great interest for genetic studies.



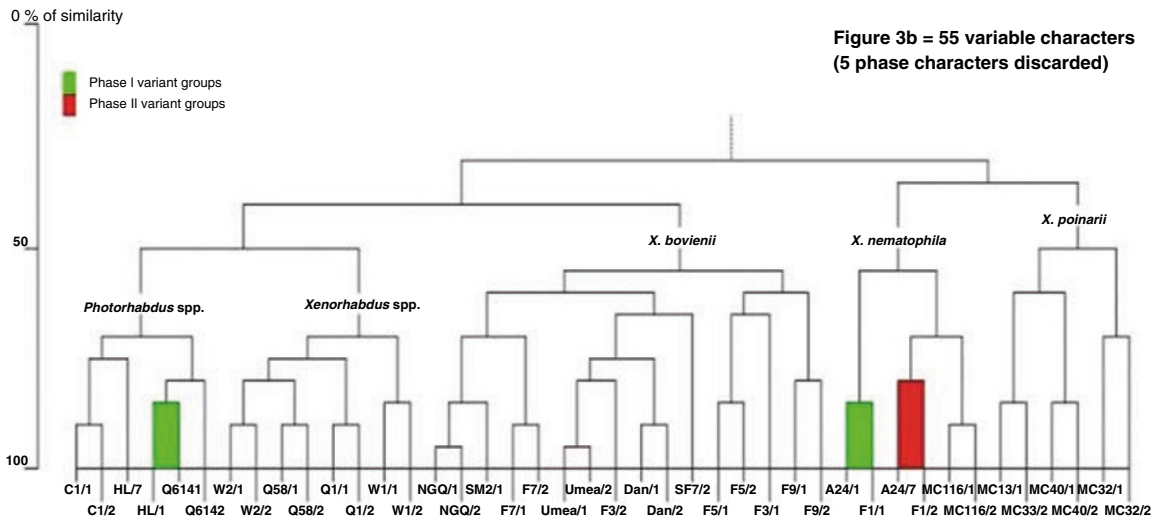


Fig. 3. Numerical analysis of phenotypic data from *Xenorhabdus* and *Photorhabdus* phase variants. All the data are treated as two-state (0,1) characters. Characters that were positive or negative for all isolates (i.e., that were not discriminative) were deleted (180/240). Similarity coefficients were calculated with the Jaccard coefficient (Sneath and Sokal, 1973). To construct dendrograms, the internode lengths were calculated for each coefficient by complete linkage clustering (furthest-neighbor sorting strategy: FNS) using the GENSTAT program (Alvey et al., 1980). However, simple linkage (nearest-neighbor clustering), group-average sorting (UPGMA), weighted clustering (WPGMA), unweighted and weighted centroid clusterings were also tested (not shown) and gave comparable results. The dendrograms obtained with FNS best illustrated the universality of the phase variation concept occurring in both genera. Codification of isolates: N/1 = phase I variant of strain N; N/2 = phase II variant of strain N. (a) Dendrogram obtained with the 60 characters that were not common to both phases of all strains. Note that for no strain, except *X. bovienii* strain SK2 and *X. nematophila* strain NC116 where phase characters are poorly expressed, did the two phase variants cluster together before clustering with another strain. (b) Dendrogram obtained from the same data set with 55 characters, the 60 previous characters minus 5 identified as being the principal phase-related characters. They were: antibiosis, dye-binding, phospholipase, protoplasmic inclusions, pigmentation. When these phase-related characters were deleted from the analysis, both phase variants of each strain clustered together before clustering with another strain (except in *Photorhabdus* spp. and *X. nematophila*, groups where some strains expressed additional phase-related characters). Updated from data published by Akhurst and Boemare (1988).

Phase I variants provide and protect essential nutrients for the nematodes by killing and metabolizing the insect host and producing a range of antimicrobial agents. Although phase II variants may also kill the insect host and are capable of colonizing the dauer vesicle of *Steinernema* or the anterior part of the intestine of *Heterorhabditis*, they are less effective in providing growth conditions for the nematodes (Akhurst, 1980; Akhurst, 1982a; Akhurst and

Boemare, 1990) and have never been found associated with naturally occurring nematodes. In addition some *Photorhabdus* phase II variants may be deleterious to their original *Heterorhabditis* (Ehlers et al., 1990).

Convergence

Photorhabdus-Heterorhabditis and *Xenorhabdus-Steinernema* symbioses are widely divergent. These symbioses are believed to use different mechanisms to maintain superficially similar associations. The similarities in their patterns of infectivity, life cycle and mutualism with nematode should be considered to result from evolutionary convergence. Indeed the symbiotic, pathogenic, and phase variation properties, which are the conditions for such associations (Boemare et al., 1997a), do not necessarily imply the same physiological mechanisms. Information gained from current genetic studies will probably clarify the picture of and help explain the convergent evolution of *Photorhabdus–Heterorhabditis* and *Xenorhabdus–Steinernema* symbioses (Boemare and Akhurst, 1994).

Applications

These bacteria are now recognized as potentially having great importance beyond their associations with nematodes (See Applications sections of Photorhabdus and Xenorhabdus). Recently, the use of *Photorhabdus* and *Xenorhabdus* genes encoding for entomotoxins to create transgenic plants for crop protection was proposed (ffrench-Constant and Bowen, 1999). The particular properties of these bacteria in fundamental research terms (such as cellular exportation, exoenzymatic activities, production of many special metabolites, pathogenic processes, ability to differentiate into multicellular populations and colonize different micro-niches) were inducements for the Pasteur Institute and INRA (France) to sequence the whole genome of *Photorhabdus*, which will soon be available to the international community (Kunst et al., 2000; the http://www.pasteur.fr/recherche/unites/gmp/Gmp_projects.html#pl {Pasteur Web}). We believe that these two bacterial genera will provide in the coming years new insights for microbiology in terms of genetics and physiology, particularly on pathogenic mechanisms and specific metabolic pathways of prokaryote–eukaryote interaction. Presumably researchers also will be able to address shortly an evolutionary history for these mechanisms.

Genus *Photorhabdus*

Phylogeny

Only 16S rRNA gene sequence data are available for analyzing the phylogeny of *Photorhabdus*. However, these provide solid trees regardless of the clustering method used (neighbor joining, parsimony or maximum likelihood) for understanding the position of the genus in the radiation of the Enterobacteriaceae. Thus, 16S rDNA sequences from representative strains of *Photorhabdus* (Fischer-Le Saux et al., 1999b; Szállás et al., 1997) were compared to homologous sequences of several species of Enterobacteriaceae. Binary similarity values determined for *Photorhabdus* ranged above 96%, whereas the similarity values for *Photorhabdus* compared to *Xenorhabdus* and other Enterobacteriaceae strains were between 94–96% and 92–95%, respectively. PCR-RFLP analysis (Fischer-Le Saux et al., 1998) and sequence analysis (Fischer-Le Saux et al., 1999b; Szállás et al., 1997; Fig. 4) also verified the existence of three major 16S rRNA clusters that corresponded to the DNA-DNA relatedness groups (see Taxonomy in this Chapter). Depending on the out-group inserted and on dispersion of other related bacteria (Stackebrandt et al., 1997; Stackebrandt and Goebel, 1994), the *P. luminescens* subsp. *luminescens* may aggregate differently with other subgroups or even with *P. asymbiotica*. Nevertheless, sequence analyses of 16S rDNA show that all *Photorhabdus* strains branch deeply within the radiation of the family Enterobacteriaceae, and have a specific TGAAAG sequence at positions 208–213 (*E. coli* numbering). The nearest phylogenetic neighbor as demonstrated by PCR-RFLP (Brunel et al., 1997) and sequencing of 16S rRNA (Fischer-Le Saux et al., 1999b; Rainey et al., 1995; Suzuki et al., 1996; Szállás et al., 1997) is *Xenorhabdus*, then Proteus.

It appears that the genus *Photorhabdus* evolved after the main radiation of *Xenorhabdus* species occurred (Forst et al., 1997). This hypothesis, originally derived from the analysis of only seven strains (Rainey et al., 1995), was verified when the 16S rDNA analysis was extended to a balanced number of datasets from *Photorhabdus* and *Xenorhabdus* in comparison to other genera and families (Fischer-Le Saux et al., 1999b; Suzuki et al., 1996; Szállás et al., 1997).

Taxonomy

Nomenclature and Family Linking Poinar et al. (1977) isolated a bacterium from a previously unknown nematode, Heterorhabditis bacteriophora (Poinar, 1976), which was apparently similar to a *Xenorhabdus* sp. but noticeably

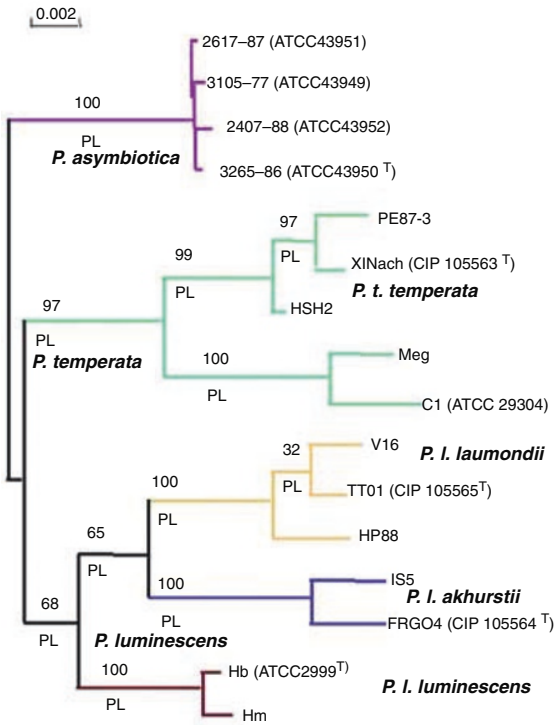


Fig. 4. Phylogenetic tree of *Photorhabdus* spp. from 16S rDNA sequences obtained by the neighbor-joining method (Saitou and Nei, 1987) using a bootstrap approach (Felsenstein, 1985) to determine the reliability of the topology obtained (numbers given above the nodes). Those clusters also obtained by parsimony (Kluge and Farris, 1969) and by maximum likelihood (Felsenstein, 1981) are indicated by a "P" and an "L" below the nodes, respectively. The accession numbers are given after the strain names. Internode values are only weak at the linkage of both subspecies *laumondii* and *akhurstii* with *luminescens*. Here no out-group has been inserted in the analysis to establish the diversity inside the group of *Photorhabdus*. Depending on the out-group inserted and on dispersion of other related bacteria (Stackebrandt et al., 1997; Stackebrandt and Goebel, 1994), the *P. luminescens* subsp. *luminescens* may aggregate differently with other subgroups or even with *P. asymbiotica*. Such proximity can be also observed by phenotypic analysis (see Fig. 6). The bar indicates a distance of 0.002 substitutions per site (s/s). (Modified from Fischer-Le Saux et al., 1999b).

luminous. They proposed inclusion of this luminous species in the genus as *X. luminescens* (Thomas and Poinar, 1979). However, the genus Photorhabdus was created in 1993 to accommodate this species as *Photorhabdus luminescens* comb. nov. (Boemare et al., 1993). Thus, due to the rules of bacterial nomenclature, the type species was named *Photorhabdus luminescens*, creating an unavoidable pleonasm.

By DNA/DNA hybridization, *Photorhabdus* is only 4% related to *Escherichia coli*, the type species of the type genus for the family. However, *Photorhabdus* possesses the enterobacterial common antigen (Ramia et al., 1982). These data indicate that, although most biochemical tests used to differentiate the Enterobacteriaceae are negative for *Photorhabdus* and although this genus is only distantly related to the core genera of this family (Farmer, 1984), *Photorhabdus* should be retained in the Enterobacteriaceae. At present time the genus includes three species with four subspecies (Boemare and Akhurst, 2000; Fischer-Le Saux et al., 1999b).

THE POLYPHASIC APPROACH TO SPECIES DELINEATION Bacteriologists cannot delineate species by crossbreeding, a method used by other biologists, but rather by comparing the total DNA of strains hypothesized to be closely related. Indeed, DNA-DNA hybridization is the key method to delineate bacterial species inside a well-distinguished group (Wayne et al., 1987). Consequently a species in bacteriology is an artificial concept. It is defined as a group of strains sharing approximately 70% or greater DNA/DNA relatedness associated with 5°C or less change in melting temperature (δTm). However, bacterial taxonomists recognize that these values are indicative rather than absolute (Vandamme et al., 1996). Based on data obtained using the S1 nuclease method, Grimont (1980) made the definition more robust by stating that strains having 80% reassociation with δTm below 5°C did belong and those having less than 60% reassociation with δTm more than 7°C did not belong to the same species. For reassociation values between 60 and 80%, or δTm values between 5 and 7°C, strains should be carefully studied to delineate species.

Thus, by using the hydroxyapatite (HA) method to analyze DNA-DNA *Photorhabdus* heteroduplexes, two DNA-relatedness groups associated with nematode *Heterorhabditis* and one containing strains found in human clinical specimens (Farmer et al., 1989) were recognized (Akhurst et al., 1996). Recently, restriction patterns obtained after amplification of the 16S rDNAs allowed identification of 12 genotypes among *Photorhabdus* strains (Fischer-Le Saux et al., 1998). By hybridizing the DNA of some representative strains of each ribosomal genotype, and by using the S1 nuclease method, the previous three genomic groups were confirmed (Fischer-Le Saux et al., 1999b; Fig. 5). They exhibited between them DNA-DNA hybridization values lower than 42% with δTm higher than 8.7°C. As reported before (Grimont et al., 1980), DNA reassociation values obtained with the S1 method were lower than those obtained with the HA method. Moreover, the phylogenetic trees inferred from the complete 16S rDNA sequence analysis (using neighbor joining, parsimony and maximum likelihood) delineate the same clusters as both DNA-DNA hybridization

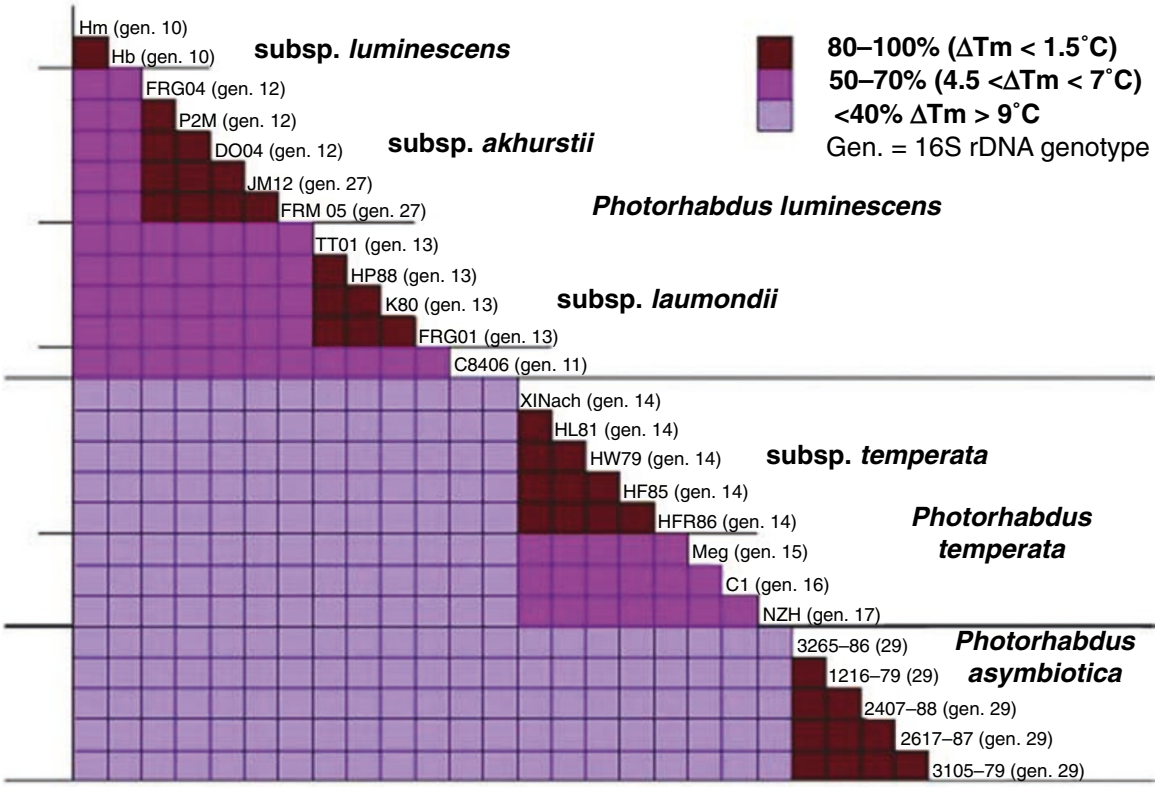


Fig. 5. Interpretation of the DNA/DNA binding ratio data showing the levels of relatedness of *Photorhabdus* strains. Three clusters of DNA-reassociation values were delineated: (i) higher than 80% associated with δTm lower than 1.5°C correspond to relatedness values between members of the same subspecies (dark pink boxes); (ii) 50–70 % with >δTm 4.5–7°C correspond to levels of relatedness occurring among members of different subspecies within a species (pink boxes); and (iii) lower than 40% with δTm higher than 9°C correspond to reassociation values between members of different species (mauve boxes). The 16S rRNA genotype designations according to Fischer-Le Saux et al. (1998) are indicated between brackets after the strain names. (Modified from Fischer-Le Saux et al., 1999b.)

methods (Fig. 4). All members of these genomic groups clustered with high bootstrap confidence values.

Clustering of phenotypic data by multivariate correspondence analysis (Fig. 6) shows a good correlation with the previous genomic analyses (Figs. 4 and 5) and helped the taxonomist to recognize discriminative characters between the species. We note for instance (Table 2) that the maximum growth temperature, DNase, urease and indole production, utilization of mannitol and of DL-lactate, and esculin hydrolysis may be specific diagnostic characteristics depending on the species.

Symbols: +, 90–100% of strains are positive; [+], 76–89% are positive; d, 26–75% are positive; [+], 11–25% are positive; =, 0–10% are positive, w (e.g., [+]w), indicates a weak reaction.

Abbreviations of type strains: ATCC, American Type Culture Collection (Rockville, Md.); CIP, Collection de l'Institut Pasteur (Paris, France); ^{T}, type strain. [a]All tests were done at 28°C ± 1°C unless otherwise noted. [b]Numbering of genotypes according to Fischer-Le Saux et al. (1998). (Modified from Fischer-Le Saux et al., 1999b.)

Consequently, species and subspecies among *Photorhabdus* were delineated by applying a polyphasic approach, combining 16S rDNA, DNA-DNA hybridization and phenotypic data (Fischer-Le Saux et al., 1999b). The species are: a) *P. luminescens* containing the type strain and the symbiotic strains of ubiquitous and/or warm-region nematodes (max. growth at 35–39°C), b) *P. temperata* containing exclusively the symbiotic strains of temperate region nematodes (max. growth at 33–35°C) and c) *P. asymbiotica* originating from clinical samples from the United States. On the basis of 16S rDNA sequencing, the new Australian clinical strains (Peel et al., 1999) will probably constitute a new species (R.J. Akhurst et al., unpublished observation). The one non-luminous strain (Akhurst and Boemare, 1986c) probably is yet another species (Akhurst et al., 1996).

Within *P. luminescens* and *P. temperata*, subgroups of strains shared very high DNA-DNA hybridization values and δTms lower than 1.5°C,

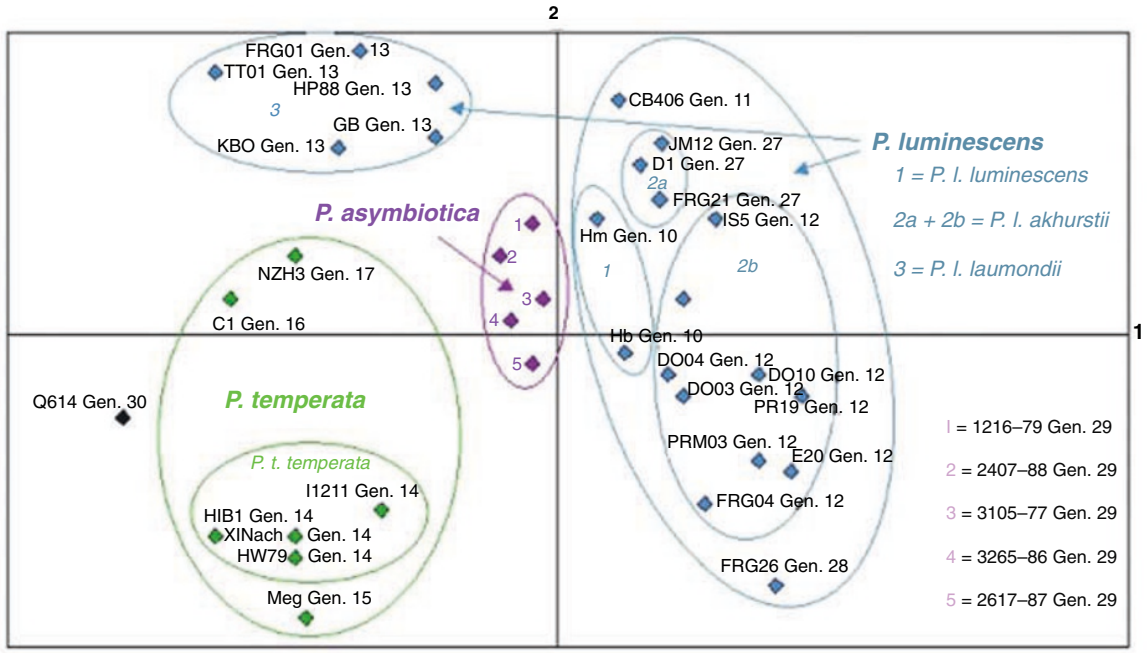


Fig. 6. Multivariate correspondence analysis of phenotypic data from *Photorhabdus* strains. This Q-R reciprocal analysis used the software Statlab (SLP®) taking into consideration only the physiological and biochemical characters scored as variable. The strains are positioned in the A space defined by the two first components. Strains with their PCR-RFLP genotype number (Fischer-Le Saux et al., 1998) are indicated at their position in the mathematical space. The corresponding species and subspecies are encircled. Only one unclassified strain is noted; Q614 (black spot) is a wild-type non-luminous *Photorhabdus* (Akhurst and Boemare, 1986c). Analysis of the inverted matrix (R analysis) shows that the first component is mainly supported by variables of growth temperature and DNase, explaining that the "warm" strains of *P. luminescens* subsp. *luminescens* and *P. luminescens* subsp. *akhurstii* are on the right side of the representation while the "temperate" strains are on the left side. Note that *P. asymbiotica* (pink spots) is a "consensual group" in the middle of the space (see also Fig. 7) near *P. luminescens luminescens* (cf. remark in Fig. 4 legend). Analysis used data published by Fischer-Le Saux et al. (1999b).

and were separated by stable 16S rDNA branching (Figs. 4 and 5). Consequently, *P. luminescens* was divided into three subspecies: *P. luminescens* subsp. *luminescens* (containing the type strain and strains associated with nematodes from the Brecon subgroup of *H. bacteriophora*), *P. luminescens* subsp. *laumondii* (containing strains associated with the HP88 subgroup of *H. bacteriophora*) and *P. luminescens* subsp. *akhurstii* (containing strains associated with *H. indica*). Similarly, a subspecies within *P. temperata* containing strains associated with the Palaearctic subgroup of *H. megidis*, and containing the type strain, was proposed as *P. temperata* subsp. *temperata*. As more strains become available, other subspecies will be defined in *P. temperata* especially for the Nearctic subgroup of *H. megidis*.

Genus Characteristics Cells of this genus (Thomas and Poinar, 1979; Boemare et al., 1993) are asporogenous and rod-shaped (0.5–2 by 1–10 ∝m). Cell size is highly variable within and between cultures with occasional filaments up to 30 ∝m long. In the last stage of exponential growth and during the stationary growth period, spheroplasts may occur with an average of 2.6 ∝m in size (10–20% of cell population), resulting from the partial disintegration of the cell wall. Proteinaceous protoplasmic inclusions are synthesized inside a high proportion of cells (50–80%) during the stationary period. Cells are Gram negative, motile by means of peritrichous flagella, and facultatively anaerobic, having both a respiratory and a fermentative type of metabolism. Optimum growth temperature is usually ca. 28°C; some strains grow at 37–38°C. Most strains produce pink, red, orange, yellow or green-pigmented colonies on nutrient agar, and especially on rich media (tryptic soy agar, egg yolk agar). Luminosity (usually detectable by the dark-adapted eye) varies in intensity within and between isolates and may only be detectable by a photometer or scintillation counter in some isolates; only one non-luminous isolate is known among approximately 150 reported strains. Spontaneous phase shift occurs in subcultures, inducing the appearance of phase II clones (see Physiology/Phase Variation). The latter are characterized by considerably less neutral red adsorption on MacConkey agar, production of

Table 2. Characteristics that differentiate *Photorhabdus* species.

Species or sub-species	*Photorhabdus luminescens*	*P. luminescens luminescens*	*P. luminescens laumondii*	*P. luminescens akhurstii*	*Photorhabdus temperata*	*P. temperata temperata*	*Photorhabdus asymbiotica*
Isolated from	*Heterorhabditis spp.*	*Heterorhabditis bacteriophora* group Brecon	*Heterorhabditis bacteriophora* group HP88	*Heterorhabditis indica*	*Heterorhabditis* spp.	*H. megidis* Palaearctic group	Human blood and/or wounds
Type culture collection	ATCC 29999 DSM3368	ATCC 29999 DSM3368	CIP 105565	CIP 105564	CIP 105563	CIP 105563	ATCC 43950
PCR-RFLP 16S rDNA genotype	10, 11, 12, 13, 27	10	13	12, 27	14, 15, 16, 17	14	29
Max. growth T°C	35–39	38–39	35–36	38–39	33–35	34	37–38
Indole	+	+	+	d	[–]	–	–
DNase	[–]	–	+	–	+	+	–
Urease, Christensen's	d	–	[+]	d	d	[–]	+
Esculin hydrolysis	+	+	+	d	[+]	d	+
Tryptophan deaminase	–	–	d	–	[+]	d	–
Simmons' Citrate	d	+	d	d	d	d	+w
Annular hemolysis (sheep blood agar)	d	+	[–]	+	d	+w	+
Annular hemolysis (horse blood agar)	d	+	–	d	[+]	+	+
Acid production from:							
Mannitol	d	dw	–	+	[–]	–	–
Trehalose	[+]w	+w	[+]w	[+]w	d	+	[+]
Utilization of:							
L-Fucose	dw	d	–	[+]	d	+	–
DL-Glycerate	–	d	–	–	+	+	d
L(-)Histidine	d	+	[+]w	d	[+]	+	d
myo-Inositol	+	+	+	+	[+]	d	d
DL-Lactate	dw	–	–	d	–	–	–
D-Mannitol	d	+	–	+	[–]	–	–

antibiotics, and other properties usually exhibited by wild clones (named "phase I variants") freshly isolated from the natural environment. Cells do not reduce nitrate and are proteolytic for gelatin, positive for catalase and negative for oxidase, *O*-nitrophenyl-β-D-galactopyranoside (ONPG), Voges-Proskauer, arginine dihydrolase, lysine and ornithine decarboxylase. Proteolytic for gelatin. Most strains will hemolyze sheep and/or horse blood, some producing an unusual annular hemolysis on sheep blood at 25°C. All strains are lipolytic on Tween 20, and many are lipolytic on Tweens 40, 60, 80 and/or 85, as well. Cells produce acid from glucose (without gas), fructose, D-mannose, maltose, ribose, and *N*-acetylglucosamine, but only weakly from glycerol. Fumarate, glucosamine, L-glutamate, L-malate, L-proline, succinate and L-tyrosine are utilized as sole carbon and energy sources. Biochemical identification of *Photorhabdus* is summarized in Table 2. The mol% G+C of the DNA is 43–45 (*P. luminescens* strain Bd).

The American Type Culture Collection has the type strain (Hb) under accession number {ATCC 29999}. Unfortunately, it appears that the type strain of both the genus (*Photorhabdus*) and the species (*P. luminescens* strain Hb) is not a good representative of the *Photorhabdus* isolates, in as much as none of the numerous new isolates have been found to belong to this taxon. So far, strain Hm remains the only one closely related to the type strain Hb. The European Molecular Biology Laboratory (EMBL) accession number of the 16S rRNA gene sequence of the strain DSM 3368 (paratype of {ATCC 29999}; Rainey et al., 1995) is X82248.

Photorhabdus Luminescens The species characteristics of *Photorhabdus luminescens* (lu.mi.nes'cens. M.L. pres. part. *luminescens*, luminescing; for its luminescence; Thomas and Poinar, 1979; Boemare et al., 1993) are listed in Table 2. Cells are large rods (2–6 by 0.5–1.4 ∝m). There are two phase variants (I and II), both luminous, but phase I has luminescence more than 100-fold greater than phase II. Maximum growth in nutrient broth occurs at 35–39°C. Strains are indole positive. Whereas some strains acidify mannitol, most are weak acid producers from fructose, *N*-acetyl-glucosamine, glucose, glycerol, maltose, mannose, ribose and trehalose. Protein inclusions found in the protoplasm of phase I cells are poorly produced in phase II cells. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *Heterorhabditis bacteriophora* (Brecon and HP88 subgroups), and of Heterorhabditis indica. *Photorhabdus luminescens* is divided in three subspecies. Accession numbers of type strain Hb and of the 16S rRNA gene sequence are given above.

Photorhabdus Luminescens subsp. *Luminescens* The subspecies characteristics of *Photorhabdus luminescens* subsp. *luminescens* (Fischer-Le Saux et al., 1999) are listed in Table 2. Maximum growth in nutrient broth occurs at 38–39°C. Cells are positive for esculin hydrolysis, weakly positive for indole, and negative for DNase, tryptophan deaminase and urease. They produce annular hemolysis on sheep and horse blood agars. Cells use mannitol, not DL-lactate, as sole source of carbon and energy. This subspecies is symbiotically associated with nematodes from the Brecon subgroup of *H. bacteriophora*, the type species of the genus *Heterorhabditis* (Poinar, 1976). See accession numbers of type strain Hb and of the 16S rRNA gene sequence above.

Photorhabdus Luminescens subsp. *Laumondii* The subspecies characteristics of *Photorhabdus luminescens* subsp. *laumondii* (lau.mon'di.i. M.L. gen. n. *laumondii* of Laumond: referring to Dr. C. Laumond, a major contributor to the use of entomopathogenic nematode/bacterial complexes for insect pest control; Fischer-Le Saux et al., 1999) are listed in Table 2. Maximum growth in nutrient broth occurs at 35–36°C. Cells are positive for esculin hydrolysis, indole and DNase, mostly positive for urease and variable for tryptophan deaminase. They show total hemolysis on sheep and horse blood agars (the *Photorhabdus* annular reaction is rare) and do not use L-fucose, DL-glycerate, DL-lactate or mannitol.

Symbiotically, this subspecies (isolated in South and North America, southern Europe and Australia) is associated with nematodes of the HP88 subgroup of *H. bacteriophora*, which is evidenced by the binding of the satellite DNA probe of the nematode strain HP88 (provided by the team of Dr. C. Laumond; Grenier et al., 1996).

Type strain TT01 is held in the Collection of l'Institut Pasteur under accession number CIP 105565. The EMBL accession number of the 16S rRNA gene sequence is {AJ007404}.

Photorhabdus Luminescens subsp. *Akhurstii* The subspecies characteristics of *Photorhabdus luminescens* subsp. *akhurstii* (ak.hurs'ti.i. M.L. gen. n. *akhurstii* of Akhurst: referring to Dr. R. Akhurst, a major contributor to the bacteriological symbionts of entomopathogenic nematodes) are listed in Table 2. Maximum growth in nutrient broth occurs at 38–39°C. Esculin hydrolysis is positive, tryptophan deaminase and DNase negative, urease and indole variable. Annular hemolysis is observed on sheep blood agar, and in some strains, on horse blood

agar. Utilization of DL-lactate as sole source of carbon is variable and weak when positive. Mannitol is used (producing acid) and DL-glycerate not used.

Symbiotically, this subspecies is associated with the nematode *H. indica* isolated in warm regions; the first strain (strain D1) was isolated from Australia (Darwin, Northern Territory) by Dr. R. Akhurst.

Type strain FRG04 is held in the Collection of l'Institut Pasteur under accession number CIP 105564. The EMBL accession number of the 16S rRNA gene sequence is AJ007359.

THE SUBSPECIES *PHOTORHABDUS TEMPERATA* The species characteristics of *Photorhabdus temperata* (tem.pe.ra'ta, L. fem. part. adj. *temperata*, moderate, so named because this species grows at moderate temperature; Fischer-Le Saux et al., 1999) are listed in Table 2. Cells are large rods (2–6 by 0.5–1.4 ∝m). Two phase variants occur, both of them highly luminous. Maximum growth in nutrient broth occurs at 33–35°C. Strains are positive for DNase, mostly positive for esculin hydrolysis and tryptophan deaminase, mostly negative for indole, and variable for urease. Acid is produced from fructose, *N*-acetyl-glucosamine, glucose, mannose and ribose; acid production from glycerol and maltose is weak. Protein inclusions are in protoplasm of phase I and II cells, but poorly produced in phase II cells. Annular hemolysis often occurs on sheep and horse blood agars. Most strains use DL-glycerate, and not DL-lactate as sole source of carbon. However, a few strains use mannitol.

The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *Heterorhabditis megidis*, of the NC subgroup of *H. bacteriophora* and of *H. zealandica*.

Type strain XlNach is held in the Collection of l'Institut Pasteur under accession number CIP 105563. The EMBL accession number of the 16S rRNA gene sequence is {AJ007405}.

PHOTORHABDUS TEMPERATA SUBSP. *TEMPERATA* The subspecies characteristics of Photorhabdus temperata subsp. temperata (Fischer-Le Saux et al., 1999) are listed in Table 2. Cells are large rods (2–6 by 0.5–1.4 ∝m). Two phase variants and occasionally several intermediate forms occur, all of which are luminous. Maximum growth in nutrient broth occurs at 34°C. Cells are indole negative, DNase positive, esculin hydrolysis and tryptophan deaminase variable, and mostly urease negative. Annular hemolysis occurs on sheep and horse blood agars in most isolates. Strains use DL-glycerate and L-fucose, and not DL-lactate and mannitol, as the sole source of carbon.

The natural habitat is in the intestinal lumen of entomopathogenic nematodes of the Palaearctic subgroup of *H. megidis*. See above for accession numbers of the type strain XlNach and 16 rRNA gene sequence.

PHOTORHABDUS ASYMBIOTICA The species characteristics of *Photorhabdus asymbiotica* (a.sym.bio'ti.ca., Gr. pref. *a*, not; M.L. adj. *symbioticus*, *-a*, *-um*, living together; M.L. fem. adj. asymbiotica, not symbiotic; Fischer-Le Saux et al., 1999) are listed in Table 2. Cells are rod shaped (2–3 by 0.5–1.0 ∝m) and produce a yellow or brown pigment. Maximum growth in nutrient broth occurs at 37–38°C. No phase I isolates have been detected, and isolates never adsorb dyes and sometimes weakly produce antibiotics. Cells are negative for lecithinase on egg yolk agar, positive for urease, esculin hydrolysis and Christensen's citrate, but weakly positive for Simmons' citrate. They are negative for tryptophan deaminase, indole and DNase. Acid is produced from fructose, *N*-acetyl-glucosamine, glucose, maltose, mannose and ribose, but only weakly from glycerol. Protein inclusions are poorly produced. Tween 40 esterase is variable. Annular hemolysis occurs on sheep and horse blood agars. Cells do not use L-fucose, DL-lactate or mannitol.

Natural habitat is uncertain. All isolates are obtained from human clinical specimens. Strain 3265-86 (ATCC 43950) is the designated type strain, as suggested previously by Farmer et al. (1989).

The EMBL accession number of the 16S rRNA gene sequence (Szállás et al., 1997) is {Z76755}.

Habitat

In natural conditions, cells of symbiotic *Photorhabdus* are carried in the intestinal lumen of the free-living dauer stage of *Heterorhabditis* (often referred to as the infective juvenile) and insects infected by these nematodes. The bacterial cells are stored and do not multiply in the gut of the dauer host; this is a nonfeeding stage. When infective juveniles infect an insect, they release their symbionts into the body cavity of the insect prey (Fig. 2). Exceptionally, a few *Photorhabdus* strains may be experimentally infective per os (Ragni et al., 1996), some of them possessing entomotoxins acting orally (Bowen et al., 1998b; Guo et al., 1999; see Applications in this Chapter). Nevertheless, natural *Photorhabdus* septicemia without nematode help has not yet been reported from insect sampling. However, it is notable that such collections would be difficult to recover from nature. Whatever the mode of penetration,

when *Photorhabdus* gain entry into the body cavity of the insects, the bacterial cells multiply inducing toxemia and septicemia and the insect dies. Entomopathogenic nematode-infected insect cadavers do not putrefy and the nematode reproduction occurs in a sort of "bag" where the integrity of the cuticle of the insect cadaver seems to be a major barrier to external saprophages. In addition, several antimicrobial barriers acting in the body cavity of the insect contribute by suppressing microbial competitors. *Photorhabdus* produces both antibiotics (see Physiology in this Chapter) possessing a wide spectrum of activity (Akhurst, 1982a; Li et al., 1995; McInerney et al., 1991a; McInerney et al., 1991b; Paul et al., 1981; Richardson et al., 1988; Webster et al., 1998) and bacteriocins acting against closely related species (Baghdiguian et al., 1993; Boemare et al., 1992; Boemare et al., 1997a). These products have been identified from in vitro cultures, and also from in vivo analyses (Hu et al., 1998b), strongly indicating their role in the prevention of bacterial and fungal putrefaction of cadavers. Consequently the carcass is a sort of natural monoxenic microcosm that produces an apparent "mummification." Nematodes reproduce inside and feed on the insect remains as metabolized by the symbiotic bacteria and also on the bacterial biomass. When the dauers escape the insect cadaver to search for new prey, they carry the symbiont in their gut, ensuring the vertical transmission of the mutualistic association. Although the nematode hosts are the natural vectors of symbiont propagation in the insects, in nutritional terms the *Photorhabdus* associated with nematodes might be considered entomophilic, rather than nematophilic, microorganisms.

However, *P. asymbiotica* is associated with human clinical specimens, not nematodes. These human clinical isolates are the only free-living members so far isolated from the genus. The life cycle of the clinical strains of *Photorhabdus* is much less certain. They have been isolated from five clinical sources in the United States (Farmer et al., 1989) and five recently in Australia (Peel et al., 1999). Isolations were variously made from tissue, blood and sputum samples. No definite route of infection has been established; two patients reported spider bites prior to the infection and a third reported the possibility of a spider bite, but no definite connection between bite and infection has been made. Although some patients may have been immunocompromised, this was definitely not the case for at least two of the Australian patients. These clinical isolates were all cultivable on standard media at 37°C. Three of the four tested isolates exhibited the annular hemolysis on sheep blood agar at 25°C. By the time they were identified, all were phase II cultures (see Physiology/Phase Variation in this Chapter); it is not known whether they were originally isolated in phase II form.

Cospeciation When the bacterial taxonomic results are compared with the taxonomic data of the host nematodes, a relatedness of the two taxonomic structures is noted, and a phenomenon of cospeciation between bacterium and nematode genera is implied (Table 3). The apparent exceptions do not fundamentally modify the concept because they are essentially the result of taxonomic confusions in the subgroups of *Heterorhabditis bacteriophora*. This species is widely distributed throughout the world, and with the use of more precise methods, nematological taxonomists will probably delineate subspecies. A phenomenon always seems to be in progress: the coevolution between both partners of the symbiosis. How is the specific association between bacteria and nematode maintained? It is likely that signal compounds are involved in the recognition for both partners, and there are indications of a specific attachment by the bacteria to the host cells. When leaving the insect cadaver, dauer larvae reinitiate the symbiosis by the recruitment of symbiont cells, which do not multiply inside the larvae primarily because of the lack of suitable nutrients. So this special association, where bacteria are not digested and are actually very well

Table 3. Species correspondence between *Photorhabdus* and *Heterorhabditis* nematodes.

Heterorhabditis	Symbiont	Secondary guest[1]
H. bacteriophora subgroup Brecon[2]	*Photorhabdus luminescens luminescens*	
H. bacteriophora subgroup HP88[3]	*Photorhabdus luminescens laumondii*	*Ochrobactrum* spp.
H. indica	*Photorhabdus luminescens akhurstii*	*Ochrobactrum anthropi*, *O. intermedium*
H. zealandica	*Photorhabdus temperata*	
H. bacteriophora subgroup NC (synonym *H. heliothidis*)	*Photorhabdus temperata*	
H. megidis Nearctic group (Ohio, Wisconsin)	*Photorhabdus temperata*	
H. megidis Palaearctic group	*Photorhabdus temperata temperata*	*Providencia rettgeri*
Clinical opportunistic strains (vector candidates: spiders?)	*Photorhabdus asymbiotica*	

preserved, suggests a special behavior of the dauer larvae, and a special quiescent physiology of the bacteria.

An exciting report of Poinar (1993) suggested that *Heterorhabditis* evolved from a marine ancestor. On the basis of biological, taxonomic and ecological arguments the genus *Heterorhabditis* would come from a *Pellioditis*-like-ancestor in an arenicolous marine environment. As it seems likely that its symbiotic bacterium would have obtained the *lux* genes by horizontal genetic transfer from marine bacteria, the symbiosis may have originated at the seashore interface. *Photorhabdus luminescens* subsp. *akhurstii* isolated from *H. indica*, restricted to the sandy beaches of the Caribbean basin (Constant et al., 1998), may be a good example. In other parts of the world where complexes of *H. indica* and *P. luminescens* subsp. *akhurstii* also have been isolated, they were found under (or linked to) the sea that in geological times covered the Northern Territory in Australia, Nile Delta in Egypt and Negev Desert in Israel, except in India where they were found inland.

On some rare occasions other bacteria have been isolated from *Heterorhabditis* with *Photorhabdus*, for instance the genus *Ochrobactrum* (Babic et al., 2000) or the genus *Providencia* (Jackson et al., 1995). For all the nematodes sampled and on the basis of 16S RNA genotype and phenotypic properties, the *Ochrobactrum* isolates were correlated with their geographical origin, whereas the corresponding *Photorhabdus* symbiont was only correlated with their host's genotype (Babic et al., 2000). This shows the absence in the former case, and the presence in the latter case, of a cospeciation. Moreover the *Ochrobactrum* (and *Providencia*) probably came from an intercuticular location in contrast to *Photorhabdus*, which comes from the intestine of the host-nematode (see Isolation/Isolation of Other Isolates in this Chapter).

Isolation

Isolation of Symbiotic and Nonsymbiotic Strains of *Photorhabdus* Three methods have been used for isolating *Photorhabdus* from nematodes. The "hanging drop" uses a sterile drop of insect hemolymph to which surface-disinfected dauer-stage *Heterorhabditis* are added (Poinar and Thomas, 1966b). The nematodes exsheath their old cuticles in the hemolymph drop and commence development, releasing their symbiont, which can be subcultured after about 24 h. A second method is to collect under sterile conditions a drop of insect hemolymph from an insect 24 h after infection by *Heterorhabditis*, and to streak it onto nutrient agar. The third method is to crush about 100 surface-disinfected dauer-stage *Heterorhabditis* and to streak the macerate onto nutrient agar (Akhurst, 1980). This last method is the most rigorous method for assessing the microflora of the intestine of entomopathogenic nematodes, provided that a suitable control on the effectiveness of the surface disinfection is employed. The three methods have revealed the occurrence of *Photorhabdus* in every *Heterorhabditis* sp.

Human clinical isolates of *P. luminescens* have been variously obtained from open wounds, fluid aspirated from unerupted lumps, blood and sputum. These clinical isolates can be cultured at 37°C.

Isolation of Other Bacteria from Heterorhabditis The presence of secondary bacteria occurring together with the natural symbiont in some samplings of *Heterorhabditis* is noteworthy (see Habitat/Cospeciation in this Chapter). For example, in the case of *H. indica* (Babic et al., 2000), it is easy to discriminate *Ochrobactrum* by simple bacteriological tests (for positive oxidase and nitrate-reductase, for oxidative metabolism) from secondarily developed (see Physiology/Phase Variation in this Chapter) *Photorhabdus* variants (Wouts, 1990), small colony variants (Hu and Webster, 1998a), or intermediate forms of phase variants (Gerritsen et al., 1995). It was believed that these bacteria are carried in the space between the L2 and L3 cuticles and are isolated from infective juveniles when they are not adequately exsheathed during the disinfection of the nematode larvae. The conclusion that these bacteria are occasionally present in the nematodes without any role in the association is quite reasonable (Babic et al., 2000).

Identification

The taxonomic work necessary to characterize the species requires a long time for collecting the largest possible sample of strains to ensure adequate representation of the biodiversity of the genus and to permit conduct of the polyphasic analyses (see Taxonomy in this Chapter). Bacteriologists have to propose practical methods to identify new isolates more rapidly. This problem is not easy with *Photorhabdus*, which exhibits few positive responses with the classical phenotypic tests used for Enterobacteriaceae. Even if most of the responses are negative, some phenotypic tests must be conducted to confirm the genus identification.

The conventional methods for other Enterobacteriaceae are used to identify the *Photorhabdus* strains, but all biochemical tests are routinely conducted at 28°C and checked after 3–5 days of incubation. Discriminative characters are summarized in Table 2. Light microscopic examina-

tion of the rods at beginning of the stationary period of a culture is particularly notable: there are large, motile (by peritrichous flagella), Gram-negative rods (average 3–5 by 1–1.5 ∝m), sometimes up to 10 ∝m long, containing highly diffractive protoplasmic inclusions.

Photorhabdus strains are easily distinguished phenotypically from *Xenorhabdus* spp. because luminescence and catalase, both physiologically very significant characters, are positive for Photorhabdus and negative for *Xenorhabdus*. Urease in most *Photorhabdus* strains is positive, and assimilation of DL-lactate is negative for *Photorhabdus*; annular hemolysis on sheep blood agar at 25°C is only observed with *Photorhabdus* strains.

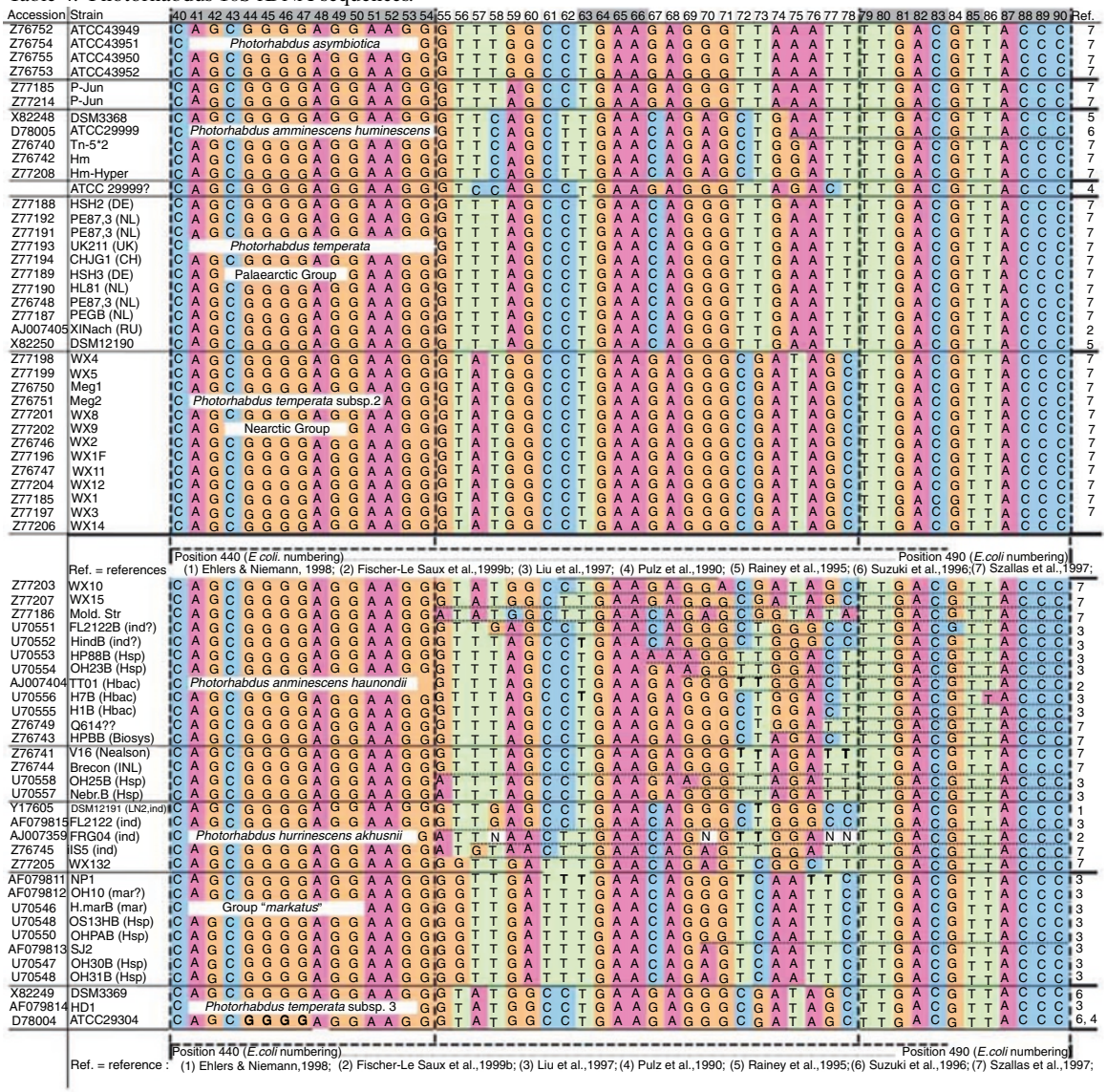
Luminescence does not cause confusion with the light-emitting marine bacteria of other families. *Photorhabdus* is differentiated from *Vibrio*, *Alteromonas* and *Photobacterium* by having peritrichous, nonpolar, flagella and in not requiring sodium ions for growth. Luminescence for most phase I variants (see Physiology/Phase Variation in this Chapter) can be checked in a darkroom after 10 min for dark-adaptation of eyes. To assess absence or weakness of light production in the phase II variants, a scintillation counter, a fluorimeter or a photomultiplier must be used. A loopful of an agar culture of each phase-variant culture is suspended in 10 ml of distilled water in a scintillation vial for immediate counting with a fully opened window setting (Grimont et al., 1984).

Another important phenomenon is the phase variation that essentially appears during in vitro culture (see Physiology/Phase Variation in this Chapter). Details of the techniques for identifying the phase variants have been summarized recently (Boemare et al., 1997b). The essential points are the following. Adsorption of dyes as described by Akhurst (1980) is the most convenient test to characterize the phase variants. MacConkey agar, or better still MacConkey agar without the bile salts, is a good medium for distinguishing phase I (red colonies) and phase II (off-white or yellow) variants (Boemare and Akhurst, 1988). On the nutrient bromothymol blue agar (NBTA) NBTA medium described by Akhurst (1980), the adsorption of bromthymol blue by *Photorhabdus* may be confused by the pigmentation of strains, and the resulting color of the clones can be difficult to distinguish. As most *Photorhabdus* are pigmented, growth on nutrient agar is often sufficient to differentiate clones of the variants, which differ significantly in not only pigmentation but also colony morphology, with phase I mucoid and convex and phase II non-mucoid and flattened. To test antibiotic production by *Photorhabdus*, clones of both variants are spot-inoculated on nutrient agar plates. After growth (generally 48 h later), cultures are killed by chloroform vapor (30 min) and covered by fresh nutrient semisolid agar (0.6%) inoculated with a bacterial indicator such as *Micrococcus luteus* (Akhurst, 1982a). The inhibition halos of the indicator culture denote the phase I variants.

Antibiograms (Bauer et al., 1966) must be done at 28°C and incubated for 3 days to observe clear zones. *Photorhabdus* have large zones of inhibition around disks impregnated with nalidixic acid, gentamycin, streptomycin, kanamycin, tetracycline, and chloramphenicol, but none around penicillin. Resistance to colistin, ampicillin, carbenicillin and cephalothin is variable from strain to strain (Farmer, 1984). It is interesting to note that one patient infected with an isolate sensitive to gentamycin in vitro did not respond to gentamycin treatment (M. Peel, personal communication). The major cellular fatty acids of *Photorhabdus* are $C_{16:0}$ and $C_{18:1}$, with $C_{i\text{-}15}$, $C_{i\text{-}17}$ and $C_{16:1}$ being major components in some strains (Janse and Smits, 1990; Suzuki et al., 1990). Ubiquinone-8 is the respiratory quinone in all strains (Suzuki et al., 1990).

In addition to phenotypic tests, some other simple positive and reliable tests are needed to identify *Photorhabdus* strains. These last four years, molecular probes useful for *Photorhabdus* ribotyping have identified defining restriction patterns specific for each group by using PCR-RFLP of the 16S rRNA genes. Pütz et al. (1990) initiated the use of the variable region of *Photorhabdus* and *Xenorhabdus* 16S rRNA genes to prepare oligonucleotide probes for the ribotyping of both genera, and Ehlers and Niemann (1998a) proposed to extend more precisely this method for *Photorhabdus*. Probes were prepared by PCR and amplified from a primer chosen in the variable region at positions 450–480 (*E. coli* numbering) and a primer from a region highly conserved at the positions 795–755 according to Stackebrandt and Goodfellow (1991). Thus, Ehlers and Niemann (1998a) defined a "type" probe for identifying the type species of the genus, a "tropicus" probe for symbionts from tropical nematodes, and a "temperatus" probe for the symbionts of nematodes from temperate climates. Today more than 22 total and 28 partial 16S-rDNA sequences are available on the gene databases covering most of the diversity of the *Photorhabdus*, allowing one to check the specificity of such variable regions for each species or subspecies or both. Some of the results (Table 4) are in accordance with the definition of a subspecies, with sequences belonging to a given subgroup showing the same variable region. This is the case for the "temperatus" probe, which has a specific sequence identical to sequences of several of the Palaearctic strains and to the sequence of *Pho-*

Table 4. Photorhabdus 16S rDNA sequences.[a,b]



[a]Collected from gene databases (updated April 2000), the sequences have been selected and aligned with algorithms BLAST (Altschul et al., 1997) and CLUSTAL (Thompson et al., 1997). [b]The variable region with its conserved flanking parts is only represented from positions 440 to 490 (*E. coli* numbering).

torhabdus temperata subsp. *temperata*. The "type" probe also should be useful for correcting the discrepancies between several registered sequences (Table 4) to establish the true sequence corresponding to the type species *P. luminescens* subsp. *luminescens*. These discrepancies are due probably to the use of a strain that was not the correct type strain. This will be defined shortly. As only two strains (Hb and Hm strains) have currently been recognized as belonging to the type species (see *P. luminescens luminescens*), this probe does not present a big problem, except to confirm the separate phylogenetic position of the type species. Conversely, the "tropicus" probe is not sufficiently specific because many base substitutions have been detected among the corresponding sequences of the "tropical" strains (Table 4). However, the method seems to be very promising for providing useful tools to identify subspecies when the appropriate sequence of the variable region is chosen accurately. As proposed by Ehlers and Niemann (1998a), "heliothidis" (a probe for identifying another subgroup [not yet identified] of *P. temperata*, particularly the symbiont NC1 of *H. bacteriophora* [= *heliothidis*]), the "Wisconsin" strains (a probe for the clinical strains of *P. asymbiotica* from the United States) and perhaps one for the Australian strains can be provided shortly.

Brunel et al. (1997) described another fast method using the polymorphism of the 16S

rRNA genes for identifying the diversity of the *Photorhabdus* and *Xenorhabdus*. (See Photorhabdus/Taxonomy and Xenorhabdus/Taxonomy in this Chapter) Fischer-Le Saux et al. (1998) applied this PCR-RFLP method (Fig. 1). Based on the total amplified sequence of the 16S ribosomal genes, the method provides reference restriction patterns for identifying other isolates. The reliability of results obtained with these genotypes and those from the previously described probes (Ehlers and Niemann, 1998) is at present uncertain. The latter could be improved by the choice of more specific variable sequences covering all the Photorhabdus biodiversity. Consequently, both molecular probes for and PCR-RFLP analysis of the 16S rRNA genes promise to be efficient tools for rapid identification of *Photorhabdus* isolates.

Cultivation

In vitro subcultures from fresh isolates can be obtained without major difficulty. *Photorhabdus* are mesophilic bacteria able to grow between 15 and 35°C, and some strains at 37–38°C. Subculturing and all biochemical tests should be undertaken around the optimal temperature of 28°C. Usually, nutrient agar or Luria-Bertani agar are sufficient for growth. On minimal media, nicotinic acid, *para*-aminobenzoic acid, proline, tyrosine and serine are required as growth factors, the mix of growth factors varying between strains (Grimont et al., 1984). Minimal medium II (BioMérieux®) contains all the necessary requirements to test utilization of organic compounds.

Preservation

The standard methods of freeze-drying, or low temperature storage (liquid nitrogen or at –80°C) used for Enterobacteriaceae, are also useful for long-term storage of *Photorhabdus* strains; –20°C is unsatisfactory. Cultures do not survive more than few months in broth or on agar plates at room temperature, and phase variation (see Physiology/Phase Variation in this Chapter) is likely to occur in this time. Cultures can be routinely maintained for one month at 15°C, but storage at 4°C is unsuitable; to prevent phase variation, phase I clones have to be subcultured every week from the neutral red dye-adsorbing clones on MacConkey agar.

Physiology

Phase Variation In *Photorhabdus*, the phase variants differ by many characters and this is demonstrated by using a multivariate correspondence analysis of the phenotypic data (Fig. 7). All

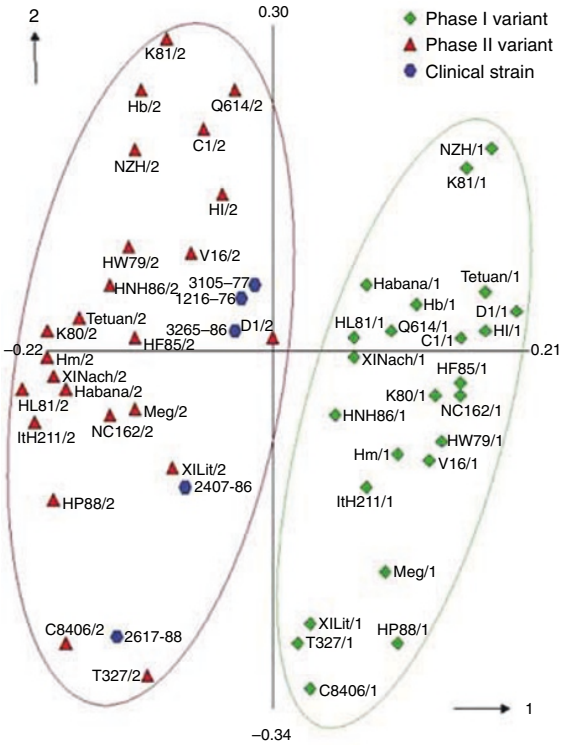


Fig. 7. Multivariate correspondence analysis of phenotypic data from *Photorhabdus* phase variants. All the data published by Akhurst and Boemare (1988) were treated as two-state (0,1) characters. Characters that were positive or negative for all isolates (i.e., that were not discriminative) were deleted (180/240). Here, the phase variants are positioned in the A space defined by the two first components using Q-R reciprocal analyses of the GENSTAT software (Alvey et al., 1980). Correspondence analysis from the contingency table used in Akhurst et al. (1996).

phase I variants are aggregated on the right of the figure, whereas phase II variants are on the left. Note that clinical strains are included in the cluster of phase II variants. No clinical isolates that resemble phase I nematode symbionts have ever been reported. When phase characters are excluded from the numerical analysis, the taxonomic clusters corresponding to the species are very well defined, meaning that phase variation is an infraspecific common phenomenon that does not essentially affect the taxonomy (Fig. 6).

Photorhabdus phase I variants are differently pigmented from phase II variants (e.g., red in phase I and yellow in phase II). Larger than phase II cells, phase I cells also are pleomorphic, comprising rods (80–90%) and spheroplasts (10–20%), and harbor protoplasmic inclusions (Boemare et al., 1983c; Fig. 8). Although the shift from phase I to II is spontaneous, it is remarkable that the wild dauer *Heterorhabditis* almost exclusively harbor phase I *Photorhabdus* (Akhurst and Boemare, 1990). The role of phase

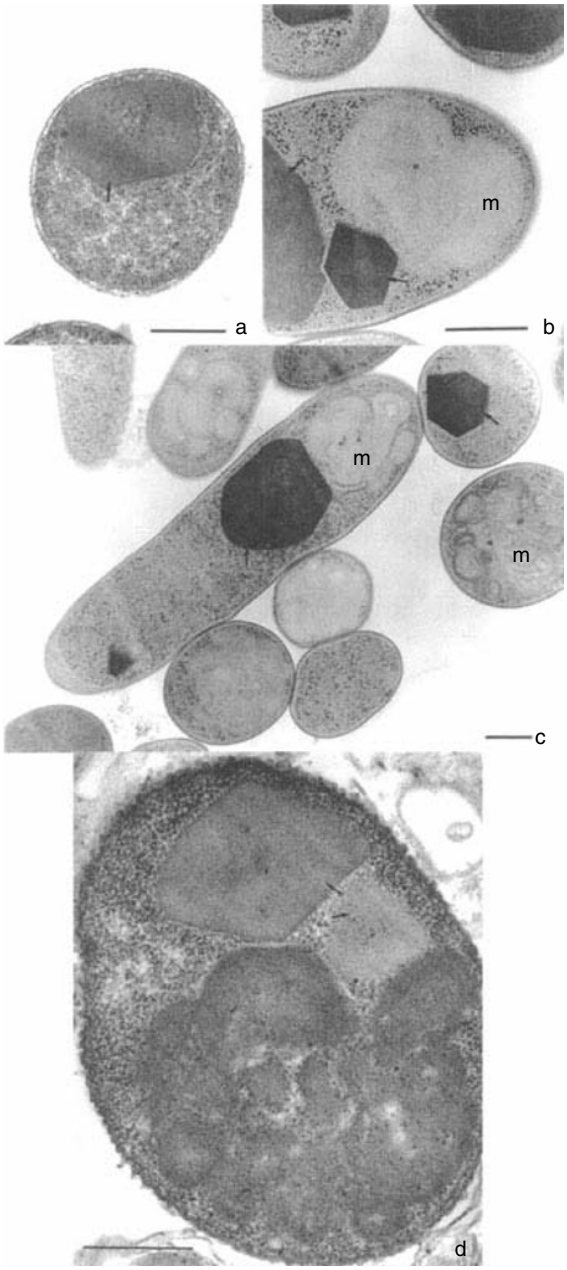


Fig. 8. Protoplasmic inclusions in *Photorhabdus*. (a) Transmission electron micrograph of *Photorhabdus temperata* strain NC1. Culture is in stationary growth on nutrient agar, fixed with 2.5% glutaraldehyde + 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Arrow = crystalline inclusion. Bar = 0.5 ∝m. (b) Fast-freeze fixation and cryosubstitution of *Photorhabdus temperata* strain NC1. Culture is in stationary growth on nutrient agar. Piece of solid agar bearing bacterial colonies was freeze fixed by liquid helium at –260°C on pure copper, cooled, dehydrated by 100% ethanol, and substituted by infiltration with a cryocool for 3 days at –90°C. Sections were contrasted with uranyl acetate and lead citrate. Two types of crystalline inclusions (arrows) are shown. The corresponding structures similar to "myelinic" (m) membranes shown with strain Q614 (Fig. 8d) are poorly contrasted with this method (see Baghdiguian et al., 1993). Bar = 0.5 ∝m. (c) Fast-freeze fixation and cryosubstitution of *Photorhabdus temperata* strain NC1. Culture is in stationary growth on nutrient agar. Specimen preparation was by the method described in legend to Fig. 6b. Membranes (m) of "myelinic" bodies are better visualized than they are in Fig. 8b (see Baghdiguian et al., 1993). Arrows = crystalline inclusions. Bar = 0.5 ∝m. (d) Cell of nonluminous strain Q614 of *Photorhabdus* sp., fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Two types of crystalline inclusions (arrows) are shown in the protoplasm. Significance of the condensed material looking like fingerprints and similar to "myelinic" bodies visible in the two thirds of the protoplasm is not interpreted as yet. Bar = 0.5 ∝m.

II variants is not evident and today the data to support a good explanation for their occurrence is unconvincing.

Phase change occurs during the in vitro stationary period of growth in a highly unpredictable manner (Akhurst and Boemare, 1990). The two phases of *Photorhabdus* differ significantly in respiratory activity (Smigielski et al., 1994b). After periods of starvation, phase II cells recommenced growth within 2–4 h of the addition of nutrients, compared with 14 h for phase I cells, indicating the former had more efficient nutrient uptake ability. Phase II variants may grow a little on complex media previously utilized by phase I variants (Akhurst and Boemare, 1990). However, although reciprocal phase change occurs in *Xenorhabdus*, conversion from phase II *Photorhabdus* to phase I has not been unambiguously demonstrated. Several intermediate colony forms, possessing at least some phase I properties, have been recorded (Gerritsen et al., 1992). However, it is not certain that these colonies were not mixtures of phase I and II cells, rather than truly intermediate. The only confirmed reversion to phase I occurred from intermediate variants not yet established as definitively phase II (Krasomil-Osterfeld, 1995). *Photorhabdus* phase II variants support the growth of their host nematodes very poorly. Consequently the phase traits of *Photorhabdus* are mainly involved in the growth and development of the *Heterorhabditis* nematodes and may be considered to be "symbiotic characters." However, both phases show a similar entomopathogenic effect and share all the other bacteriological properties of members of the genus.

Production of Secondary Metabolites *Photorhabdus* phase I variants produce a variety of secondary metabolites, some of which have antimicrobial properties. The carcass is a sort of monoxenic microcosm where the symbionts eliminate competitive microorganisms by using several antibiotics (see Habitat in this Chapter) possessing a wide spectrum of activity (Li et al.,

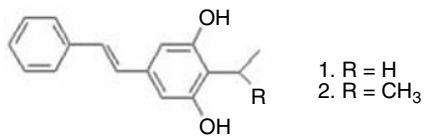
1995; Paul et al., 1981; Richardson et al., 1988). Two chemical groups have been characterized: hydroxystilbenes and polyketides (anthraquinone derivatives; Table 5). Hu et al. (Hu et al., 1997; Hu et al., 1998b) purified these compounds and examined the conditions under which these secondary metabolites are produced both in vivo and in vitro culture using a strain of *P. temperata* that was a symbiont of *H. megidis*. Only one of the two identified stilbenes produced in the extract from nematode-bacterium infected insects is recovered from tryptic soy broth culture. An array of closely related polyketide derivatives is also produced by *Photorhabdus* in the insect larvae of *Galleria mellonella*. These pigments vary in the positioning and/or number of hydroxyl and methoxyl groups around the central anthraquinone ring structure (Hu et al., 1998b), and those that are antimicrobial are mentioned in Table 5. Their occurrence also varies according to in vivo or in vitro production. The available nutrients and the prevailing environmental conditions, undoubtedly have a significant effect on the difference in metabolite composition (Webster et al., 1998). These antibiotics are most commonly active against Gram-positive bacteria, but some are effective against Gram-negative bacteria (see Applications in this Chapter).

Photorhabdus strains are the only terrestrial luminous bacteria known today. The chemical pathway for producing light is similar to the mechanism of marine bacteria (Frackman et al., 1990b; Frackman and Nealson, 1990a). The luciferase catalyzes the reaction in which reduced flavin mononucleotide ($FMNH_2$) and an aldehyde are oxidized to FMN, an acid and H_2O, and light is emitted at 490 nm. Conversely, the fatty acid reductase complex generates an aldehyde and the flavin oxidoreductase reduces $FMNH_2$. No satisfactory demonstration has yet been provided to explain the role of luminescence in this genus. An association between luminescence and virulence factors for the pathogenicity of *Vibrio harveyi* against *Penaeus monodon* has been noted (Manefield et al., 2000).

Bacteriocinogeny Unlike *Xenorhabdus* spp., no temperate phages have been yet characterized in *Photorhabdus* strains, although phage

Table 5. Secondary antibiotic metabolites from *Photorhabdus* strains.

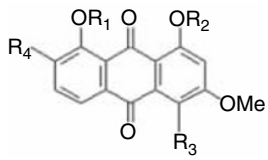
1. *Trans*-stibenes



R substituents	In vivo production	In vitro production	pigmented	active against
3,5-dihydroxy-4-ethyistibene	+	–	–	bacteria, fungi
3,5-dihydroxy-4-isopropyistibene	+	+	+	bacteria, fungi, nematodes

Species: *P. luminescens luminescens* Hb, Hm
P. temperate Nearctic group NC19, C9, HK
(Hu et al., 1998; Li et al., 1995; Paul et al., 1981; Richardson et al., 1988; Sundar and Chang, 1992)

2. Polyketides



R1, R2, R3 substituents	*in vivo* production	*in vitro* production	pigmented	active against
3,8-dimethoxy-1-hydroxy-9,10-anthraquinone	+	+	yellow	bacteria
1,3-dimethody-8-hydroxy-9,10-anthraquinone	+	+	yellow	bacteria
1,8-dihydroxy-3-methoxy-9,10-anthraquinone	+	–	yellow	bacteria
1-hydroxy-2,6,8-trimethoxy-9,10-anthraquinone	+	–	yellow	bacteria
1,4-dihydroxy-2,5-dimethoxy-9,10-anthraquineon	+	–	red	bacteria

Species: *P. luminescens luminescens* Hb, Hm
P. temperate Nearctic group NC19, C9, HK
(Hu et al., 1998; Li et al., 1995; Paul et al., 1981; Richardson et al., 1988; Sztaricskai et al., 1992)

tail-like bacteriocins have been observed by electron microscopy of culture supernatants (Baghdiguian et al., 1993). They are different in size and shape than those of *Xenorhabdus* (Boemare et al., 1992). It is reasonable to think that they play also a role in the competition with closely related bacterial genera, as demonstrated for *Xenorhabdus* (Boemare et al., 1992; Thaler et al., 1995; Thaler et al., 1996; Thaler et al., 1997).

Genetics

Genetic studies, undertaken over these last ten years, examined several structural genes and attempted to identify the mechanism of phase variation (for a comprehensive review see: Forst et al., 1997; Forst and Nealson, 1996). Recently the characterization of genes encoding protein toxins that (when ingested) are active against insects was described (Blackburn et al., 1998; Bowen et al., 1998b), and two corresponding toxic proteins have been purified (Guo et al., 1999).

Several strains of *Photorhabdus* harbor megaplasmids for which no role or gene has been identified (Smigielski and Akhurst, 1994a). Megaplasmids in other species are known to contain genetic information necessary for symbiosis, pathogenicity and conjugation. *Photorhabdus* plasmids have not been further characterized or engineered to generate cloning vectors.

Genes and Proteins Several genes have been identified in *Photorhabdus* strains (Table 6). The *lux* operon (*luxCDABE*), which encodes the proteins required for luminescence (Frackman et al., 1990b) has been characterized. The amino acid identity values for the corresponding proteins coded by the structural genes of the marine luminous bacteria *Photobacterium* and *Vibrio* are 62–85% for lux A and 47–59% for *lux B* (Johnston et al., 1990; Meighen and Szittner, 1992). The regulatory genes for the *Photorhabdus lux* operon have not yet been identified (Szittner and Meighen, 1990; Xi et al., 1991).

The operon coding for polynucleotide phosphorylase (Pnp), which degrades mRNA, and for a ribosomal protein, RpsO, was cloned from the strain K122 (Clarke and Dowds, 1994). Regulation of expression of the *rps*O-*pnp* operon is complex. The two genes are induced at low temperature.

A *malB* region with 53.5% identity to the equivalent *E. coli* gene was identified (Dowds, 1997). The maltose regulon of *E. coli* consists of genes involved in maltose uptake. The cloned region encompasses part of the *malE* and *malK* genes and the regulatory region between them. The intergenic region contains the control regions that respond to levels of maltose in the media and also to catabolite repression.

Phase I variants produce a pigment whose color varies according to the strain. The genes coding for the red anthraquinone pigment of the Hm strain have been cloned into *E. coli* (Frackman and Nealson, 1990a).

The genes *cipA* and *cipB* coding for the two types of protein crystals from the protoplasm of *Photorhabdus* (Boemare et al., 1983c; Couche et al., 1987b; Couche and Gregson, 1987a), have been characterized (Bintrim and Ensign, 1998). These genes have separate promoters and when expressed in *E. coli* resulted in inclusion body formation in the cells. Normally these genes are strongly expressed only by phase I variants (Boemare and Akhurst, 1988), so their inactivation induces changes in some other phase-related characters.

The *lip-1* gene of extracellular lipase was also cloned and sequenced (Wang and Dowds, 1993). It encodes a protein of 645 amino acids from which a hydrophobic leader sequence of 24 amino acids is removed during the processing in *E. coli*. Such a processing seems to be necessary for the secretion of other active proteins.

Toxin genes have been cloned from strain W14 (Bowen et al., 1998b). These genes encode large insecticidal toxin complexes with little homology to other known toxins. The toxin purified as a protein complex that has an estimated molecular weight of 1,000,000. It consists of a series of four native complexes at loci named *tca*, *tcb*, *tcc*, and *tcd*. Both tca and tcd encode complexes with high

Table 6. Identified genes and proteins from *Photorhabdus* strains.

Gene	Protein encoded	% identity	with
luxA (4)	luciferase alpha-subunit	85	*Vibrio harveyi*
luxB (4)	luciferase beta-subunit	60	*Vibrio harveyi*
cipA, cipB (1)	protoplasmic inclusions	none	
pnp (3)	polynucleotide phosphorylase	86	*Escherichia coli*
rpsO (3)	ribosomal protein S15	86	*E. coli*
lip-1 (5)	Tween 80 lipase	none	
tca, tcb, tcc, tcd (2)	toxin complexes for insects	not reported	several loci in *Yersina pestis* genome

References: (1) Bintrim and Ensign, 1998; (2) Bowen et al., 1998; (3) Clarke and Dowds, 1994; (4) Szittner and Meighen, 1990; (5) Wang and Dowds, 1993

oral toxicity for insects. Two purified native toxins A and B were found to be active in nanogram concentrations against insects. Two peptides present in toxin B could be processed in vitro from a 281-kDa protoxin by endogenous *P. luminescens* proteases (Guo et al., 1999). A smaller insecticidal protein (ca. 40 kDa) unrelated to the large toxin complex has also been identified in *Photorhabdus* and the cognate gene cloned (East et al., 1999).

The sequencing of the whole genome of strain TT01, which is in progress, will identify new genes, see the (http://www.pasteur.fr/externe{Institut Pasteur}) for further functional genomic studies.

Regulation of Phase Variation Major changes in DNA structure do not appear to account for the phenotypic switches. No differences in plasmid profiles of the two phases have been detected. DNA-relatedness studies and restriction digests of total DNA indicated that the organization of the genome is the same in the two phases (Akhurst et al., 1992; Boemare et al., 1993; Frackman et al., 1990b; Wang and Dowds, 1991; Wang and Dowds, 1993).

DNA has been successfully transferred by means of transformation and conjugation. Modification systems such as methylation are commonly associated with restriction endonucleases that protect the genome from foreign DNA. Endonuclease activities have been detected in a range of *Photorhabdus* spp. and an isoschizomer of XhoI was purified from strain K80 of *P. luminescens* subsp. *laumondii* (Akhurst et al., 1992). Such a restriction-modification system is an obstacle for transformation and conjugation of *Photorhabdus* strains and explains the low frequency of transformants and transconjugants obtained. Nevertheless, Frackman and Nealson (1990a) reported the transformation of strain Hm by using competent cells from a modified $CaCl_2$/RbCl method, and Dowds (1997) used electroporation to transform with pBR322 and pHK17. Conjugation from *E. coli* to *Photorhabdus* strain K122 allowed transfer of the plasmids RP4 and pSUP104 (Dowds, 1997).

The molecular mechanism of phase variation remains uncertain. Some experiments to inactivate genes involved in phase variation have been successful and indicated that phenotypic phase characters can be altered in phase I variants or expressed in phase II variants. For instance inactivation of the crystal protein genes of strain NC1 produced a phase II phenotype (Bintrim and Ensign, 1998). The UV mutagenesis of phase II variants of strain K122 induced expression of phenotypic properties of the corresponding phase I variants (Wang and Dowds, 1993). The lipase gene transcription is initiated at the same site in the two phases (Wang and Dowds, 1993) and lipase mRNAs accumulate to the same extent in the two phases, implying that gene expression in the phase II variant is repressed at a post-transcriptional level. In fact the lipase protein accumulates and is secreted to the same extent in the two phases. However the lipase protein is inactive in the phase II variant, as is the protease (Wang and Dowds, 1993). Lipase and protease are regulated at a post-translational level in the Irish K122 strain of *P. luminescens* (Wang and Dowds, 1993), whereas the *lux* genes are post-transcriptionally regulated in the Hm strain (Hosseini and Nealson, 1995). This contrasts with *X. nematophilus* in which the flagellin genes are not transcribed during phase II (Givaudan et al., 1996). These preliminary studies seem to indicate that phase-specific genes in Photorhabdus are regulated at a post-transcriptional, probably post-translational, level by a common control mechanism, a genetic level different from that in *Xenorhabdus*.

Ecology

Despite the intestinal location of *Photorhabdus*, which allows environmental contamination, their specificity for *Heterorhabditis* is a remarkable feature of this symbiosis (Boemare et al., 1997a; Forst et al., 1997). When microbial ecology studies are undertaken, using a simple and fast method of PCR-RFLP of 16S RNA (Brunel et al., 1997), a clear correspondence between *Photorhabdus* isolates and nematode species can be seen. From a total of 75 isolates identified in the Caribbean region (Fischer-Le Saux et al., 1998), two genotypes were associated only with *Heterorhabditis bacteriophora* and another two only with *Heterorhabditis indica*, although these four genotypes belong to the same DNA/DNA relatedness group.

Within the *Photorhabdus* genus, the maximum growth temperature appears to be a relevant taxonomic character defining a critical value for each subgroup (Table 2). It implies essential physiological adaptations in the enzymatic machinery of the bacteria and must therefore be considered as important for defining taxa. Moreover, if we examine the ecology of the corresponding strains, it is notable that the maximum growth temperature and the host species origin are correlated. Symbionts growing at temperatures up to 35–39°C are harbored by *H. bacteriophora* (Brecon, HP88 groups) and *H. indica* that occur in hot regions. Those that grow only up to 35°C are harbored by *H. megidis* (both its Palaearctic and Nearctic strains) and *H. bacteriophora* (NC group); all of these nematodes live in temperate climates. Thus, temperature tolerance appears to be an important property reflecting

a long-term adaptation to different climatic conditions.

Two ecological niches have been identified for nematode-symbiotic *Photorhabdus*: one is in the insect host as a metabolically active form and the other is in the gut of the nonfeeding dauer nematode as a quiescent form. The occurrence of nonsymbiotic clinical strains possessing the phase II properties of the nematophilic symbionts, and the remarkable differences in respiratory activity between the two phases of the symbiotic strains, suggest that the soil may be a third niche. However, recently reported experiments to test the symbiotic strains' ability to grow and survive in external environments indicate that they disappear within a few days (Morgan et al., 1997). Photorhabdus strains may enter into a nonculturable but viable survival strategy, as do *Aeromonas*, *Vibrio*, *E. coli* and *Salmonella* spp.

Epidemiology

All *Photorhabdus* strains examined to date have been reported to be entomopathogenic, the LD50 usually being <100 cells when injected into hemocoel of the insect *Galleria mellonella* (Akhurst and Boemare, 1990; Akhurst and Dunphy, 1993; Farmer et al., 1989). *Photorhabdus asymbiotica* may also cause some human diseases (see Disease).

To test insect pathogenicity, 10^2, 10^3 or 10^4 cells (total count) from a 24-h broth culture are injected into final instar *Galleria mellonella* or *Spodoptera littoralis* larvae (Lepidoptera). The injected larvae should be placed on dry filter paper in Petri dishes and incubated at 25°C for 3 d. Most *Photorhabdus* spp. will kill <50% at a dosage of 10^2 cells; all will kill <50% at 10^4 cells. Depending on the insect species tested, some differences in pathogenicity between *Photorhabdus* strains and phases may be noted. According to Bucher (1960), when the LD_{50} is $>10^4$ cells, the bacterium may be considered as entomopathogenic. To date, all *Photorhabdus* isolates, regardless of phase, have been reported to infect insects at a $LD_{50} > 10^2$–10^3 cells by injection. Consequently *Photorhabdus* have to be considered as highly entomopathogenic. In addition some of them (strain W14, and an unnamed Italian strain) may be entomopathogenic by ingestion (ffrench-Constant and Bowen, 1999; Ragni et al., 1996). The toxin produced is active on the insect digestive epithelium, not only from the gut lumen, but also from the body cavity (Blackburn et al., 1998; see Applications in this Chapter).

Disease

Photorhabdus has also been isolated from human wounds and blood in the United States (Farmer et al., 1989) and more recently in Australia (Peel et al., 1999). None of the infections were lethal but some required weeks of treatment. Four American patients were elderly people or apparently immunocompromised; the other was a 45-year-old male who had suffered a spider bite. The bacterial strains, isolated from them and kept in the United States Centers for Disease Control and Prevention in Atlanta (CDC), were compared after the description of the symbiotic strains was published, and they were defined as a clinical group in the genus (Farmer et al., 1989), and now belong to *P. asymbiotica* (see Taxonomy in this Chapter). Among the four recent isolations in Australia, one of the Australian patients was definitely a victim of spider bite, and another may have been. None of the patients was immunocompromised; the one elderly patient made the fastest recovery. One case was the result of a disseminated infection, whereas the three others had a bacteremia or skin lesions (Peel et al., 1999). The possibility that spiders may vector infection by nonsymbiotic strains of *Photorhabdus* has yet to be tested.

Applications

Knowledge about the nutritional requirements provided by the symbionts would improve the mass production of the entomopathogenic nematodes for biological control of insect pests. In terms of symbiosis, this is one of the most exciting subjects related to these bacteria. During the industrial process, the quality of the symbiont inoculum, in terms of viability, phase I variant selection, and preservation from any other microbial contamination, must be maintained (Ehlers et al., 1998b; Ehlers et al., 1990). Several programs have been undertaken to define the key factors of the symbiosis; nothing significant has been reported to date. However it should be noted that academic access to this knowledge is difficult to obtain because the know-how of such industrial processes is kept secret. What biochemical compound(s) is/are released in the supernatants in the bioreactors during monoxenic nematode production? A "food signal," a term taken from *Caenorhabditis elegans* physiology, is produced by a German strain of *P. temperata*. Although not produced at the same strength as found in insects, the food signal has been characterized (Strauch and Ehlers, 1998). It induces the exit from the developmentally arrested dauer larvae, explaining the recovery of juveniles when they enter the insects. The food signal could be a special nutrient supplied by the bacteria to the nematode and/or a hormone controlling nematode development.

Some of the secondary metabolites have commercial potential in the pharmaceutical and agro-forestry industries (Webster et al., 1998). Among the isolates examined to date from *P. luminescens* subsp. *luminescens* and *P. temperata*, some of the anthraquinone pigments and the *trans*-stilbenes are antibacterial and the *trans*-stilbenes are antifungal, especially when the isolates are cultivated in vitro. This is of great interest to industry (Table 5).

Moreover, the occurrence of protein toxins produced by some *Photorhabdus* strains and acting orally against several insect species has recently enhanced the interest in these bacteria for biological control (Blackburn et al., 1998; Bowen et al., 1998b; Bowen and Ensign, 1998a; Guo et al., 1999). The toxin genes can be cloned and will be inserted into plant genomes, as with *B. thuringiensis* ("Bt"), used for producing transgenic plants toxic for insects. In addition, to overtake the first resistance observed in the target insects consuming these transgenic Bt plants, the insertion of both (Bt and *Photorhabdus*) toxin genes in the same plant can be envisioned (ffrench-Constant and Bowen, 1999). This second field of industrial projects opens very promising biotechnology programs for the next ten years in plant protection. To date, six patents have been submitted (East et al., 1999; Ensign et al., 1997; Ensign et al., 1998; Kramer et al., 1999b; Ragni et al., 1996).

Genus Xenorhabdus

Phylogeny

Analyses of 16S rDNA sequences show that *Xenorhabdus* is most closely related to *Photorhabdus*. The next nearest phylogenetic neighbors are *Proteus vulgaris* and *Arsenophonus nasoniae* (Brunel et al., 1997; Fischer-Le Saux et al., 1999b; Liu et al., 1997; Suzuki et al., 1996; Szállás et al., 1997). Only limited 16S rDNA sequence data are available at this time, most *Xenorhabdus* species being represented by sequences from only one or two strains. However, *Xenorhabdus* can be distinguished from its nearest phylogenetic neighbor, *Photorhabdus*, by the sequence TTCG at positions 208–211 (*E. coli* numbering) of the 16S rDNA. *Photorhabdus* has a longer version (TGAAAG; Fischer-Le Saux et al., 1999b; Szállás et al., 1997). The PCR-RFLP analysis shows that of the 17 *Xenorhabdus* genotypes identified to date, 8 correspond to 5 species of *Xenorhabdus* and the remainder corresponds to some other, as yet undefined, species (Fig. 9).

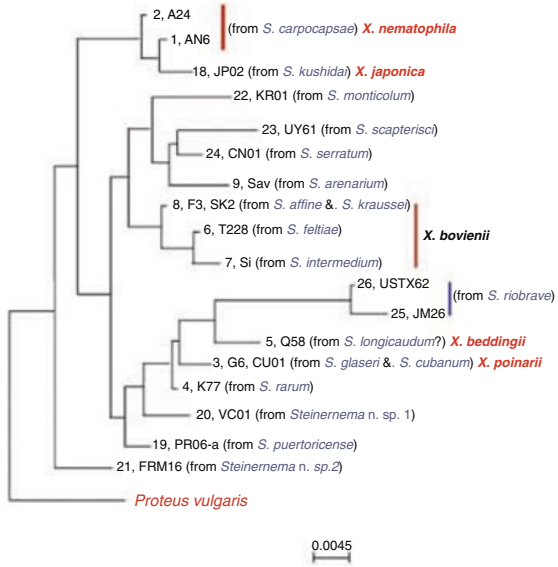


Fig. 9. Phylogenetic tree from PCR-RFLP 16S rDNA genotypes of *Xenorhabdus*. The neighbor-joining method was applied to the 17 defined genotypes, from a total of 65 strains. The number of the genotype according to Fischer-Le Saux et al. (1998) is followed by the name of the representative strain. Defined symbiont species are indicated in red, and host nematodes in blue.

Taxonomy

Nomenclature and Family Linking The first symbiotic bacterium isolated from entomopathogenic nematodes was described as a new species "*Achromobacter nematophilus*" (Poinar and Thomas, 1965). With the rejection of the genus "*Achromobacter*" (Hendrie et al., 1974), this new species could not be accommodated within any existing genus. Thomas and Poinar (1979) described a new genus, *Xenorhabdus*, to accommodate the bacterial symbionts of entomopathogenic nematodes as two species, *X. nematophilus*, symbionts of the family Steinernematidae, and "*X. luminescens*," associated with the Heterorhabditidae; the latter was subsequently reassigned to the genus *Photorhabdus* (Boemare et al., 1993). *Xenorhabdus* have low DNA/DNA relatedness (4%) to the type species of the type genus in the family Enterobacteriaceae (Farmer, 1984) and lack nitrate reductase, which is positive for all other genera in this family. However, they do have the enterobacterial common antigen (Ramia et al., 1982) and phylogenetic analyses based on 16S rDNA (Brunel et al., 1997; Fischer-Le Saux et al., 1999b; Liu et al., 1997; Suzuki et al., 1996; Szállás et al., 1997) confirm their relatedness to the Enterobacteriaceae.

The Polyphasic Approach to Genus and Species Delineation From a phenotypic study of bacterial symbionts of the Steinernematidae,

four groups were recognized within the genus, and the subdivision of *X. nematophila* into subspecies was proposed (Akhurst, 1983). A more comprehensive phenotypic study (Boemare and Akhurst, 1988) led to the elevation of the subspecies to species status, as *X. nematophila* (= *nematophilus*), *X. bovienii*, *X. poinarii* and *X. beddingii* (Akhurst and Boemare, 1988). *Xenorhabdus japonica* (= *japonicus*), symbiotically associated with *Steinernema kushidai*, was described later (Nishimura et al., 1994). DNA/DNA hybridization (Akhurst and Boemare, 2000; Akhurst et al., 1996; Boemare et al., 1993; Suzuki et al., 1990) and 16S rDNA analyses (Brunel et al., 1997; Fischer-Le Saux et al., 1998; Liu et al., 1997; Suzuki et al., 1996; Szállás et al., 1997) validated the inclusion of these five species in, and the exclusion of *P. luminescens* from, the genus (see Taxonomy in this Chapter).

DNA/DNA hybridization analysis indicates that there are (to date) more than the five *Xenorhabdus* species. Data have been recorded for some of the strains that would be assigned to new *Xenorhabdus* species (Boemare and Akhurst, 1988; Bonifassi et al., 1999; Fischer-Le Saux et al., 1998), but too few to warrant a decision on their taxonomic status. A recent multivariate analysis of phenotypic data (Bonifassi et al., 1999) from *Xenorhabdus* strains (Fig. 10) confirmed that some strains should be assigned to several new species (e.g., the symbionts of *S. arenarium*, *S. puertoricense*, *S. riobrave*, *S. scapterisci* and *S. serratum*) that will be defined in due course (Table 7).

Genus Characteristics Cells are Gram-negative, asporogenous, rod shaped, 0.3–2 ∝m by 2–10 ∝m and occasionally have filaments 15–50 ∝m in length. Spheroplasts, averaging 2.6 ∝m in diameter, appear in the last third of exponential growth. Proteinaceous crystalline inclusions develop in a large proportion of cells in stationary phase cultures. Cells move by means of peritrichous flagella, and swarming may occur on 0.6–1.2% agar. These bacteria are facultatively anaerobic, with both respiratory and fermentative types of metabolism. Optimum temperature is usually 28°C or less; a few strains grow at 40°C. Acid (no gas) is produced from glucose; fermentation of some other carbohydrates is poor. Strains are catalase negative, DNAse positive and protease positive. Nitrate is not reduced to nitrite. Most tests used to differentiate Enterobacteriaceae are negative. Lipase is detected with Tween 20 and egg yolk agar; most strains are lipolytic on Tweens 40, 60, 80 and/or 85. Phase shift occurs to varying degrees in stationary phase cultures, giving rise to phase II cells that lack dye adsorption, antibiotic production, protein inclusions and some other characteristics of

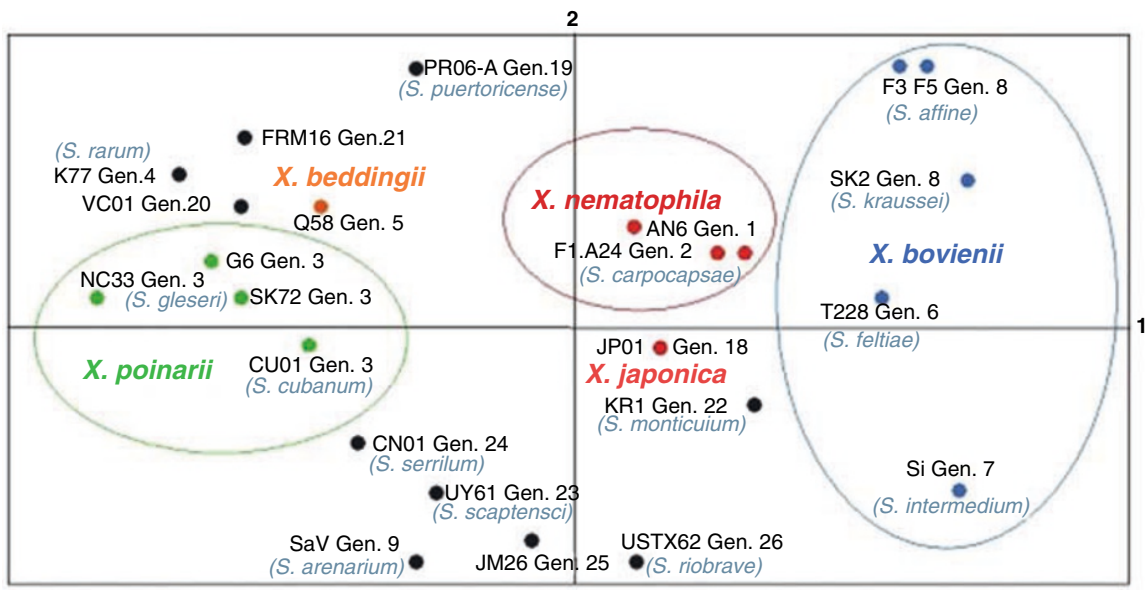


Fig. 10. Multivariate correspondence analysis of phenotypic data from *Xenorhabdus* strains. This Q-R reciprocal analysis used the software Statlab (SLP®) taking into consideration only the physiological and biochemical characters scored as variable. The strains are positioned in the A space defined by the two first components. Strain names with their PCR-RFLP genotype numbers (Fischer-Le Saux et al., 1998) are indicated at their position in the mathematical space. Those species represented by several strains are encircled. Host nematode species are indicated in blue between brackets for the isolated strains. Analysis of the inverted matrix (R analysis) shows that the first component is mainly supported by the growth-temperature variable, which is the reason "warm" strains of *Xenorhabdus* are on the left side while "temperate" strains are on the right side of the representation. Adapted from data published by Fischer-Le Saux et al. (1999a) and Bonifassi et al. (1999).

Table 7. Characteristics that differentiate *Xenorhabdus* species.

	X. nematophila	*X. bovienii*	*X. poinarii*	*X. beddingii*	*X. japonica*	*Xenorhabdus* sp.	*Xenorhabdus* sp.	*Xenorhabdus* sp.	*Xenorhabdus* sp.
Host nematode	*S. carpocapsae*	*S. feltiae*	*S. glaseri*	*S. longicaudum (?)*	*S. kushidai*	*S. scapterisci*	*S. arenarium*	*S. sarratum*	*S. riobrave*
Number of culture collections	ATCC19061 DSM3370	UQM2210 ATCC35271 DSM4766	UQM2216 ATCC35272 DSM4768	UQM2871 ATCC49542 DSM4764	IAM14265	NR	NR	NR	NR
16S rDNA genotype number	1, 2	6, 7, 8	3	5	18	23	9	24	26
Number of strains or strain designation	6	15	5	2	JP02	UY61	Sav	CN01	USTX62
Simmons' citrate	+	+	+	+	–	+	+	–	–
Esculin hydrolysis	–	–	d	+	–	+	+	–	–
DNase	+	+	–	+	+	+	+w	–	+
Phenylalanine deaminase	d	[–]	[–]	–	dw	NR	–	NR	NR
Tryptophane deaminase	–	+	–	+w	–	–	–	–	–
Indole	–	[–]	–	–	–	+	+	–	–
Growth at 39–40°C	–	–	+	+	–	+	+	+	+
Pigmentation	–	+	+	+	–	+	+	+	+
Utilization of									
Citrate	+	[+]	+	+	–	–	+	–	–
Diaminobutane	–	d	–	–	–	–	+	+w	–
Fumarate	+	+	+	+	+	+	+	+w	–
D-Glucosamine	+	[+]w	[+]w	+	–	–	–	–	–
DL-Glycerate	[+]	[+]	[–]	+	–	–	–	–	–
L(-)Histidine	–	+	[+]	+	–	+	+	–	+
myo-Inositol	+w	–	–	–	–	+	–	+w	+w
DL-Lactate	+	+	+	+	+	+	+	+w	–
L(-)Malate	+	[+]	+	+	–	+	+	+w	–
Maltose	+	+	+	+	+	–	+	+	+
Propionate	[–]	[–]w	+w	+	–	–	–	–	–
D(-)Ribose	[–]	[+]	–	+	–	+w	–	+w	–
Succinate	+	+w	+	+	–	+	+	+w	–
L-Tyrosine	–	[+]	–	+	–	–	–	–	–

Symbols: +, 90–100% of strains are positive; [+], 76–89% are positive; d, 26–75% are positive; [–], 11–25% are positive; –, 0–10% are positive; w (e.g., [+]w) indicates a weak reaction. Abbreviations of type strains: ATCC, American Type Culture Collection (Rockville, Md.); CIP, Collection de l'Institut Pasteur (Paris, France); [T], type strain.

[a]Only five species have been defined in the genus. Several candidates are indicated in the columns 7–10 from which other companion strains are required.

[b]All tests were done at 28 ± 1°C unless otherwise noted.

[c]Numbering of genotypes according to Fischer-Le Saux et al. (1998). (Modified from Fischer-Le Saux et al., 1999a.)

the phase I cells isolated from the natural environment. Biochemical identification of *Xenorhabdus* within the family Enterobacteriaceae is summarized in Table 7. The mol% G+C of the DNA is 43–50 (strain Bd). Strains are only found in the intestinal lumen of entomopathogenic nematodes of the family Steinernematidae and insects infected by these nematodes.

XENORHABDUS NEMATOPHILA The species characteristics of *Xenorhabdus nematophila* (ne.ma.tóphi.la. Modern entomological term *nematode*; Gr. adj. *phila*, loving or having affinity for; M.L. adj. *nematophila* nematode-loving; synonym: *Achromobacter nematophilus*; Poinar and Thomas, 1965; Thomas and Poinar, 1979) are listed in Table 7. No known isolates grow at temperatures in excess of 34°C. Neither phase I nor II colonies are pigmented. Most isolates are sensitive to furazolidone. Strains are found associated only with one species of nematode, *Steinernema carpocapsae*, but this association occurs around the world.

The mol% G+C of the DNA is 43–48 (strain Bd). Type strain number of the holotype is ATCC19061; paratype DSM3370. Genbank accession number of 16S rDNA is {D78009}.

XENORHABDUS BOVIENII The species characteristics of *Xenorhabdus bovienii* (bo.vi.en'i.i. M.L. gen.n. *bovienii*, of Bovien. Named for P. Bovien who first reported the presence of bacteria in the intestinal vesicle of a *Steinernema* species; Akhurst and Boemare, 1988) and *Xenorhabdus nematophilus* subsp. *bovienii* (Akhurst, 1983) are listed in Table 7. No growth occurs at 34°C; some strains will grow at 5°C. Strains are resistant to carbenicillin.

They are associated with several species of entomopathogenic nematode (*Steinernema feltiae*, *Steinernema intermedium*, *Steinernema kraussei* and *Steinernema affine*) in temperate regions.

The mol% G+C of the DNA is 44.3 (strain P1) and 46.9 (strain Bd). Type strain number of the holotype is UQM2210 (phase I of strain T228); paratype numbers are ATCC35271 and DSM4766. Genbank accession number of 16S rDNA is {X82254} and {D78007}.

XENORHABDUS POINARII The species characteristics of *Xenorhabdus poinarii* (poi.nar'i.i. M.L. gen.n. *poinarii*, of Poinar. Named for G.O. Poinar Jr. who made major contributions to the understanding of entomopathogenic nematode/bacterial interactions; Akhurst and Boemare, 1988) and *Xenorhabdus nematophilus* subsp. *poinarii* (Akhurst, 1983) are listed in Table 7. This is the most heat tolerant *Xenorhabdus*, with all strains growing at 36°C and some at 40°C. The intensity of pigmentation in phase I varies from light to reddish brown. In some strains phase I cells do not produce antimicrobials; in some other strains, cells in both phases do.

In the United States, *X. poinarii* was first considered to be associated with only Steinernema glaseri (Akhurst, 1986b). A polyphasic approach, including phenotypic tests, restriction polymorphism analysis of PCR-amplified 16S rRNA genes, and DNA-DNA hybridizations with determination of the δTm, was used to demonstrate that *S. cubanum* also harbors strains of *X. poinarii* (Fischer-Le Saux et al., 1999a). This bacterum is not pathogenic for most wax moth (*Galleria mellonella*) larvae unless associated with its nematode partner.

The mol% G+C is 42.6 (strain P1) and 49 (strain Bd). Type strain number of the holotype is UQM2216 (phase I of strain G); paratype numbers are ATCC35272, DSM4768. Genbank accession number of 16S rDNA is {X82253} (paratype DSM4768).

XENORHABDUS BEDDINGII The species characteristics of *Xenorhabdus beddingii* (bed.din'gi.i M.L. gen. N. *beddingii*, of Bedding. Named for R.A. Bedding who made significant contributions to the development of *Xenorhabdus*/*Steinernema* associations for insect pest control; Akhurst and Boemare, 1988), first named *Xenorhabdus nematophilus* subsp. *beddingii* (Akhurst, 1986a), are listed in Table 7. All isolates grow at 34°C, and some at 38°C. Cells hydrolyze esculin. They are inhibited by cephaloridine and ampicillin. The brown pigmentation is not strong. Phase I is highly unstable, producing the very stable phase II. They are associated with two undescribed species of *Steinernema* from Australia, one of which may be *Steinernema longicaudum*, a described species from China.

The mol% G+C of the DNA is 45.5–50 (strains Bd and P1). Type strain number of the holotype is UQM2871 (phase I of strain Q58); paratype numbers are ATCC49542, DSM4764. Genbank accession number of 16S rDNA is {X82254} (paratype DSM4764).

XENORHABDUS JAPONICA The species characteristics of *Xenorhabdus japonica* (ja.po'ni.ca. M.L. adj. *japonica*, of Japan; Nishimura et al., 1994) are listed in Table 7. Cells do not grow at 37°C. Arginine dihydrolase activity is detected in phase II. Pigmentation is yellowish brown. This species is only known to be associated with *Steinernema kushidai* in Japan.

The mol% G+C of the DNA is 45.9 (strain P1). Type strain number of the holotype is IAM14265. Genbank accession number of 16S rDNA is {D78008}.

Habitat

Xenorhabdus species are insect pathogenic bacteria that occur naturally in the intestinal vesicle of nonfeeding infective stage entomopathogenic nematodes of the family Steinernematidae (Bird and Akhurst, 1983; Bovien, 1937; Poinar and Leutennegger, 1968; Fig. 11). After invading an insect host, the nematode commences development, releasing *Xenorhabdus* into the nutrient-rich hemolymph. The bacteria proliferate, killing the insect host and producing suitable nutrient conditions for nematode growth and reproduction, as well as an array of antibiotics and bacteriocins to minimize competition. As the nutrient source becomes depleted, the immature nematodes develop into dauer juveniles that will transport *Xenorhabdus* to a new nutrient source (Fig. 1; see Introduction in this Chapter).

Cospeciation Between the Bacterial Symbionts and Their Nematode Hosts

When the taxonomic data of *Xenorhabdus* is compared with that of their host nematodes, a close relatedness of the two taxonomic structures is noticed, and a phenomenon of cospeciation between bacterium and nematode genera is shown (Table 8). However, sometimes several species of *Steinernema* can share one species of *Xenorhabdus*. For example, symbiotic species of *S. affine*, *S. feltiae*, *S. kraussei* and *S. intermedium* share *X. bovienii* (Akhurst and Boemare, 1988; Fig. 9), although specific 16S rDNA genotypes could be recognized for each one and subspecies may be defined soon. Two species of nematodes also were reported to harbor *X. beddingii*, but unfortunately the nematode species were not described at that time (Akhurst, 1986a). A recent report describes a similar observation for the symbionts of *S. cubanum* and *S. glaseri* (Fischer-Le Saux et al., 1999a). When *S. cubanum* was described, it was considered to be a related species of *S. glaseri* because some morphological characters were very similar (Mrácek et al., 1994). These morphological similarities make it difficult to distinguish the two species (Hominick

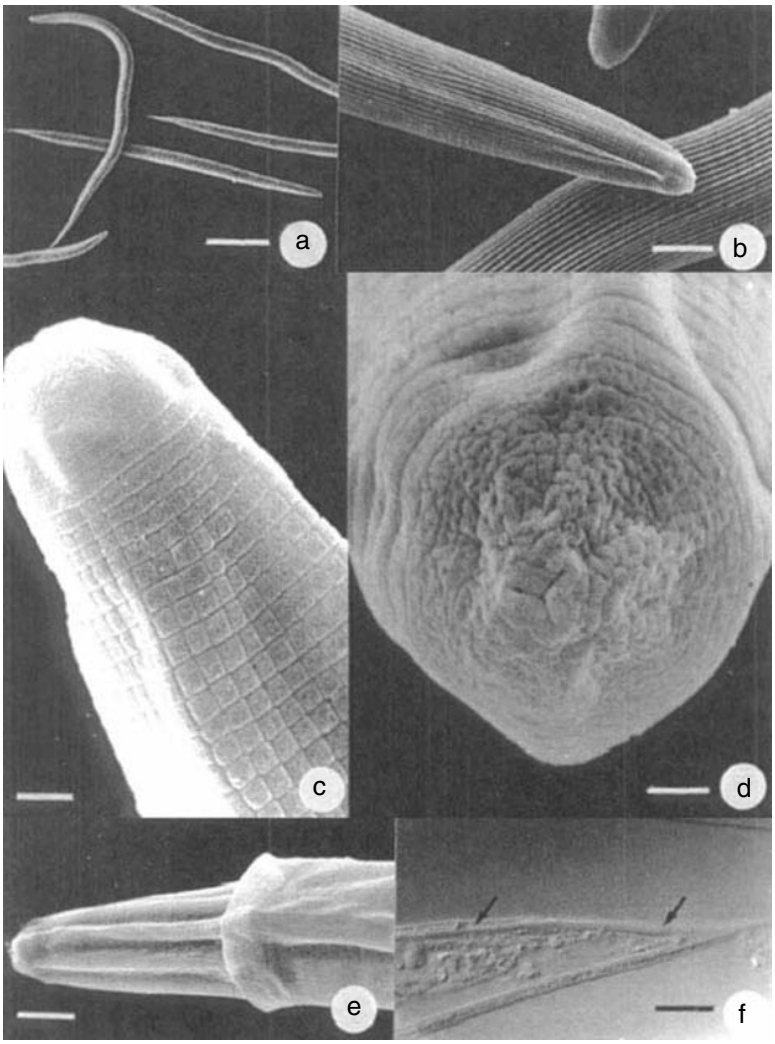


Fig. 11. *Steinernema*, nematode host of *Xenorhabdus*, and anatomical localization of the phoretic bacteria. (a) Scanning electron micrograph of dauer juveniles. Bar = 100 ∝m. (b) Scanning electron micrograph of dauer juveniles. Bar = 10 ∝m. (c) Scanning electron micrograph of dauer juveniles. Detail is shown of the ornamentation of the cuticle. Bar = 2 ∝m. (d) Scanning electron micrograph of dauer juveniles. Bar = 1 ∝m. Detail showing the closed mouth. (e) Scanning electron micrograph of dauer juveniles. Exsheathing of the old cuticle is in progress. To eliminate all the contaminants between the old and new cuticles (Fig. 11f), dauer juveniles must be removed from their old L2 cuticle during axenization. Bar = 5 ∝m (from Bonifassi et al., 1999). (f) Microphotograph of native *Steinernema scapterisci* tail. Light microscopic image, using Nomarski differential interference contrast optics, shows microorganisms (arrows) between L2 and L3 cuticles before the exsheathing (arrows). Bar = 20 ∝m (from Bonifassi et al., 1999). (g) Photomicrograph of the anterior part of the intestine of a dauer larva of *Steinernema feltiae* showing the vesicle containing *Xenorhabdus bovienii* symbionts. Bar = 5 ∝m (from Bird and Akhurst, 1983).

Table 8. Species correspondence between *Xenorhabdus* and *Steinernema nematodes*.

Steinernema	Symbiont	Secondary guest
S. krausset (type species)	*Xenorhabdus bovienii*	
S. abbast	*Xenorhabdus* sp.	*Pseudomonas* (= *Flavimonas*) *oryzthabitans*[1]
S. arenarium (synonym: *S. anomatae*)	*Xenorhabdus* sp.	
S. affine	*Xenorhabdus bovienii*	
S. bicomutum	*Xenorhabdus* sp.	
S. carpocapsae	*Xenorhabdus nematophila*	*Alcaligenes, Pseudomonas, Acinetobacter*[2]
S. cubamon	*Xenorhabdus poinarii*	
S. feltiae	*Xenorhabdus bovienii*	
S. glaseri	*Xenorhabdus poinarii*	
S. intermedium	*Xenorhabdus bovienii*	
S. kushidai	*Xenorhabdus japonica*	
S. longicaudum(?)	*Xenorhabdus beddingii*[3]	
S. monticulum	*Xenorhabdus* sp.	
S. puertoricense	*Xenorhabdus* sp.	
S. rarum	*Xenorhabdus* sp.	
S. riobrave	*Xenorhabdus* sp.	
S. scapterisci	*Xenorhabdus* sp.	*Ochrobactrum, Paracoccus, Xanthomonas*[4]
S. serratum	*Xenorhabdus* sp.	

et al., 1997). The restriction analysis of the intergenic transcribed spacer (ITS) of the ribosomal genes revealed high molecular similarities between *S. cubanum* and *S. glaseri* (Hominick et al., 1997), and the construction of a dendrogram illustrated their close phylogenetic relatedness (Reid and Hominick, 1998). A recent common ancestor could explain high morphological and molecular similarities between these two nematode species. The geographical isolation of the fauna from Cuba could have led to their speciation, which may be relatively recent. Although the bacterial symbionts of the two nematodes at present belong to the same species, the phenomenon of cospeciation may be in progress, and phenotypic and genotypic bacteriological discriminations are not sufficient to delineate two *Xenorhabdus* species. On the basis of these results, one can assume that the previously mentioned exceptions do not fundamentally modify the concept of a possible coevolution.

Questions similar to those asked about *Photorhabdus* (see Cospeciation in this Chapter) are: how do the bacteria and the nematodes maintain their specific association? signal compounds for recognition? specific attachment? In the case of Xenorhabdus, preservation of the bacterial cells from the digestion of the dauer larvae during the free-living phase of the host's life cycle is a function of the nematode's anatomy, i.e., special vesicle in the intestine of the dauer (Bird and Akhurst, 1983; Fig. 11g). This seems to be a clear coevolutionary trait, not appearing in other free-living soil-dwelling nematodes such as species of the family Rhabditida. It may be reasonable to hypothesize that specific attachment by the symbiotic bacteria to such an organ is the mechanism ensuring the maintenance of the symbiosis.

Isolation

Xenorhabdus spp. generally grow well at 25–28°C on nutrient or similar agar (e.g., Luria-Bertani, trypticase soy). Like *Photorhabdus* (see Isolation in this Chapter), *Xenorhabdus* can be isolated from the infective stage nematodes by the hanging drop technique or by maceration (Akhurst, 1980; Poinar, 1966a). For both methods the infective juveniles must first be surface sterilized; this is readily achieved by immersing a small number of live infective stage nematodes (>100), free of debris, in 0.1% merthiolate for 1 h at room temperature and then rinsing thoroughly in several changes of sterile water. In the hanging-drop technique, individual surface-sterilized infective juveniles are transferred to a drop of sterilely change by aseptically collected insect hemolymph on a cover slip that is then inverted over a cavity to prevent desiccation. The drop is incubated (1–3 d) at 25°C until the nematodes commence development. At this time, they void their symbiotic bacteria, which can be subcultured from the hemolymph onto an agar medium (e.g., nutrient agar) 1 d later. A more rapid method involves the maceration by means of a tissue homogenizer of 50–100 surface-sterilized infective juveniles in a nutrient broth. The macerated nematodes (in 10–100 ∝l aliquots) should be plated onto an agar medium immediately and incubated at 28°C for 3 d. The inclusion of suitable controls to confirm that the surface-sterilization procedure has been effective is essential for both methods. *Xenorhabdus* can also be isolated by the less labor-intensive method of collecting hemolymph from an insect (e.g., *G. mellonella*) larva within 24 h of its death caused by *Steinernema*. With this last method, bacteria

other than *Xenorhabdus* may also be isolated; these bacteria may be carried into the host on the exterior of the nematodes or may be picked up into the hemolymph from the insect cuticle. Contamination by other bacteria can be minimized by burying the insect in clean, damp sand, adding a small number of nematodes to the surface of the sand and incubating at 20–25°C until 1–5 nematodes infect the insect. This last method is better suited to reisolation of a *Xenorhabdus* strain rather than to identification of the bacteria specifically associated with a nematode species.

Identification

As discussed for *Photorhabdus* (see Isolation), the use of 16S rDNA probes may be useful to identify the Xenorhabdus species (Fig. 9). Interestingly, several PCR-RFLP 16S rRNA genotypes (Fig. 9; Table 7) can be recognized inside a DNA-DNA relatedness group that may be useful to distinguish subspecies that correlate with the nematode host species (N. Boemare, unpublished observation). This is the case of genotypes 6,7 and 8 inside the *X. bovienii* genomic group (Brunel et al., 1997), which can be more or less related to symbionts of *S. feltiae*, *S. kraussei* and *S. affine* that are not yet distinguishable by DNA-DNA hybridizations (Figs. 9 and 10). If such an approach can be accurately verified, it will provide an amazing result since DNA-DNA hybridization is considered the gold standard method for defining species and subspecies, whereas the ribosomal methods are useful for the genera.

Xenorhabdus are easily distinguished from other Enterobacteriaceae by the absence of catalase and their inability to reduce nitrate. The key characteristics for differentiating *Xenorhabdus* from *Photorhabdus* are luminescence and catalase. The major cellular fatty acids of *Xenorhabdus* are $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{17\text{-cy}}$, and the respiratory quinone system is ubiquinone-8 (Suzuki et al., 1990). Antibiograms scored after 3 d at 28°C show that *Xenorhabdus* are inhibited by streptomycin, neomycin, gentamycin, tetracycline, kanamycin and colistin, but not penicillin. Most strains are resistant to ampicillin and cephaloridine, and to a lesser extent, furazolidone, whereas resistance to chloramphenicol is limited. Resistance to streptomycin, tetracycline and kanamycin after selection has been demonstrated for *X. nematophila*.

Insect pathogenicity is tested by injection of 10^2 and 10^4 cells (total count) from a 24-h broth culture into final instar *G. mellonella* larvae. The injected larvae should be placed on dry filter paper in Petri dishes and incubated at 25°C for 3 d. Most *Xenorhabdus* spp. will kill <50% at a dosage of 10^2 cells; all will kill <50% at 10^4 cells.

Cultivation

Xenorhabdus species are easily grown in vitro on a range of complex liquid and solid media and in minimal media supplemented with nicotinic acid, *p*-aminobenzoic acid, serine, tyrosine, and/or proline (Grimont et al., 1984). Minimal medium II (BioMèrieux ®) contains all the necessary requirements to test utilization of organic compounds. Nutrient agar is suitable for all strains. They are mesophilic; most grow between 15 and 30°C, but strains growing at 4 or at 40°C have been isolated. All tests for phenotypic characterization of *Xenorhabdus* should be conducted at 28°C. Dye adsorption in most *Xenorhabdus* species can be tested on nutrient agar containing 0.0025% (w/v) bromthymol blue and 0.004% (w/v) triphenyltetrazolium chloride

Table 9. *Xenorhabdus* 16S rDNA sequences.[a]

Accession	Strain	40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55	56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78	79 80 81 82 83 84 85 86 87 88 89 90	Ref.
X82251	DSM4768	C A G C G G G G A G G A A G G C	G T A A G T C T G A A G A G G G C T T A C G A T T G A C G T T A C C C		5
D78009	ATCC19061	C A G C G G G G A G G A A G G C	G T A A G T C T G A A G A G G G C T T A C G A T T G A C G T T A C C C		6
Z76738	riobracis??	C *Xenorhabdus nematophila* G G C	G T A A G T C T G A A C A G G G C T T A C G A T T G A C G T T A C C C		7; 4
Z76737	mex.str.N2-4	C A G C G G G G A G G A A G G C	G T A A G T C T G A A C A G G G C T T A C G A T T G A C G T T A C C C		7
Z77209	103 Nealson	C A G C G G G G A G G A A G G C	G T A A G T C T G A A C A G G G C T T A C G A T T G A C G T T A C C C		7
D78008	IAM14265	C *Xenorhabdus japonica* G G C	G T G G G T C C G A A T A C G G C T C A C G A T T G A C G T T A C C C		6
Z76739	SK-1J	C A G C G G G G A G G A A G G C	G T G G G T C C G A A T A C G G C T C A C G A T T G A C G T T A C C C		7
D78006	DSM4764	C *Xenorhabdus beddingii* G G C	G T G G A C C T G A A T A C G G T T C A C G A T T G A C G T T A C C C		6; 4
X82254	DSM4764	C A G C G G G G A G G A A G G C	G T G G A C C T G A A T A C G G T T C A C G A T T G A C G T T A C C C		5
D78010	DSM4768	C *Xenorhabdus poinarii* G G C	G N N A N C C T G A A T A A G G T T G G C G T T T G A C G T T A C C C		6; 4
X82253	DSM4768	C A G C G G G G A G G A A G G C	G T N A N C C T G A A T A A G G T T G G C G T T T G A C G T T A C C C		5
D78007	ATCC35271	C A G C G G G G A G G A A G G C	A A C A G C G T A A A T A G C G C T G T T G A T T G A C G T T A C C C		6; 4
X82252	DSM4766	C A G C G G G G A G G A A G G C	A A C A G C G T A A A T A G C G C T G T T G A T T G A C G T T A C C C		5
Z77212	Sulc.strain	C *Xenorhabdus bovienii* G G C	A A C A G C G T A A A T A G C G C T G T T G A T T G A C G T T A C C C		7
U70319	S.felB1	C A G C G G G G A G G A A G G C	A A C A G C G T A A A T A G C G C T G T T G A T T G A C G T T A C C C		3
Z77210	S.F.22	C A G C G G G G A G G A A G G C	A A C A G C G T A A A T A G C G C T G T T G A T T G A C G T T A C C C		7
U70318	S.intB	C A G C G G G G A G G A A G G C	A A C A G C G T G A A T A G C G C T G T T G A T T G A C G T T A C C C		3
Z77211	Norv.	C A G C G G G G A G G A A G G C	A A G A G C G T A A A T A G C G N T G T T G A T T G A C G T T A C C C		7
AF079816	XenoOS21	C A G C G G G G A G G A A G G C	A A C A G C G T A A A T A G C G T T G T T G A T T G A C G T T A C C C		3
Z77213	Filip	C A G C G G G G A G G A A G G C	A A C A G C G T A A A T A G C G T T G T T G A T T G A C G T T A C C C		7
U70320	S.rioB	C A G C G G G G A G G A A G G C	G T G A G T C T G A A C A C G G C T C A C G A T T G A C G T T A C C C		3

Position 440 (E.coli numbering) Position.490.(*E.coli* numbering)

Ref = references: (1) Ehlers & Niemann. 1998; (2) Fischer-Le Saux et al., 1999a; (3) Liu et al., 1997; (4) Putz et al., 1990; (5) Rainey et al., 1995; (6) Suzuki et al., 1996; (7) Szallas et al., 1997

[a]The variable region with its conserved flanking parts is only represented from positions 440 to 490 (*E. coli* numbering). Data collected from gene databases (updated April 2000) have been selected and aligned with the aid of algorithms BLAST (Altschul et al., 1997) and CLUSTAL (Thompson et al., 1997). References: (1) Liu et al., 1997; (2) Pütz et al., 1990; (3) Rainey et al., 1995; (4) Suzuki et al., 1996; (5) Szállás et al., 1997.

(Figs. 12a, b). Dye adsorbing phase I colonies of *X. nematophila* will appear dark blue, of *X. bovienii* and other yellow pigmented strains tend to be green, and of *X. beddingii* are more maroon than blue because they do not adsorb so strongly; nonabsorbent phase II colonies in all of these are red (Akhurst, 1980). As *X. poinarii* does not adsorb bromthymol blue, dye adsorption in this species can be assessed on MacConkey agar; adsorbing colonies are dark red (Akhurst, 1986b; Boemare and Akhurst, 1988). Congo red (Francis et al., 1993), and some other dyes can also be used for most *Xenorhabdus* spp. Antibiotic production of phase I variants is tested by spot-inoculating *Xenorhabdus* onto nutrient agar and incubating at 28°C for 3 d. After this time the bacteria are killed by exposure to chloroform vapor for ca. 1 h. After the chloroform has evaporated from the agar, semisolid nutrient agar (0.5%) inoculated with a suitable indicator organism (*Micrococcus luteus* or another Gram-positive species) is poured to form a thin layer. After incubation at 28°C overnight, a halo of inhibition around antibiotic-producing colonies will be evident.

Preservation

Storage in 20% glycerol at temperatures at –70°C or below is very useful for long-term maintenance of *Xenorhabdus* cultures; they do not store well at –20°C. Short-term storage (less than one month) is best conducted at 10–15°C because survival on agar or in broth at 4°C is very poor and when cultures are maintained at temperatures in excess of 15°C, the risk of a proportion of the culture undergoing phase change is significant.

A remarkable feature must be noted. Nematodes of the species *S. feltiae*, kept in collection for more than 10 years, preserve their symbionts; bacterial strains isolated after a such delay were identical, in terms of phenotypic and genotypic characters, to those initially isolated (N. Boemare and F. Grimont, personal communication). Consequently, when the conditions of storage of the nematodes are good, reisolation of the *Xenorhabdus* strain from their dauer larvae may be advised for those species where the association is strongly observed. This is not the case for the *S. glaseri* and *X. poinarii* association, which seems to be more labile.

Physiology

Phase Variation Phase I *Xenorhabdus*, such as those isolated from the nematode's associates or insects infected by the nematodes, form convex, circular colonies with slightly irregular margins and a diameter of 1.5–2 mm after 4 d at 28°C;

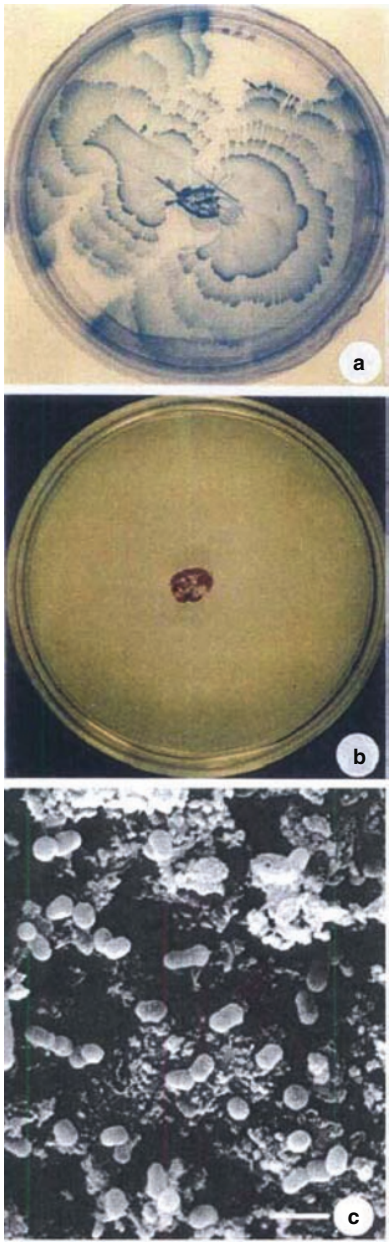


Fig. 12. Motility and adhesion of *Xenorhabdus nematophila*. (a) Behavior of *X. nematophila* F1/1 variants on 1% NBTA agar (24-h-old colony). F1/1 cells induce a swarming colony when inoculated at the center of plates containing 0.6 to 1.2% agar. On 1% agar, periodic cycles of migration (clear zones) and cell division, also named "consolidation," (dark zones) are easily visualized (Givaudan et al., 1995). In addition adsorption of bromthymol blue on NBTA (blue colony), is a characteristic of phase I variants. (b) In contrast phase II variants show a complete lack of swarming motility (Givaudan et al., 1995), do not adsorb the bromthymol blue, but reduce the 2,3,5-triphenyltetrazolium chloride (TTC) to formazan (red colony). (c) Scanning electron micrograph of the colonization by *Xenorhabdus nematophila* strain F1 on sponge substrate coated with the nutrient medium of Wouts (1982), shows adhesion of the cells on solid substrate. Bar = 1 ∝m.

they also have a slightly granular appearance and, in some species, are pigmented (yellow, brown). These colonies adsorb dyes, such as bromthymol blue and neutral red, taking on intense coloration (Fig. 12a, b). Phase II variants generally form similar colonies but flatter and wider (diameter 2.5–3.5 mm after 4 d at 28°C) and with lesser pigmentation; these colonies adsorb dyes only very weakly and no coloration is evident in 4-d colonies. Phase I cells have peritrichous flagella responsible for the motility of the strain (Givaudan et al., 1995) expressed essentially in nutrient broth (Fig. 13a). Phase I, but not phase II, cells have a peritrichous array of fimbriae expressed on solid nutrient agar (Fig. 13b) with diameter of 6.4 nm, morphologically similar to the type I fimbriae of *E. coli* (Binnington and Brooks, 1994; Brehélin et al., 1993; Moureaux et al., 1995). The glycocalyx surrounding *Xenorhabdus* cells is irregular in thickness, with a mean depth of 142 nm and 49 nm in phase I and phase II *X. nematophila*, respectively (Brehélin et al., 1993; Fig. 13c). Both fimbriae and glycocalyx are presumably responsible for the cell adhesion on substrate (Fig. 12c). Phase I cells produce during the stationary period crystalline protoplasmic inclusions characteristic of the genus (Fig. 14b, d).

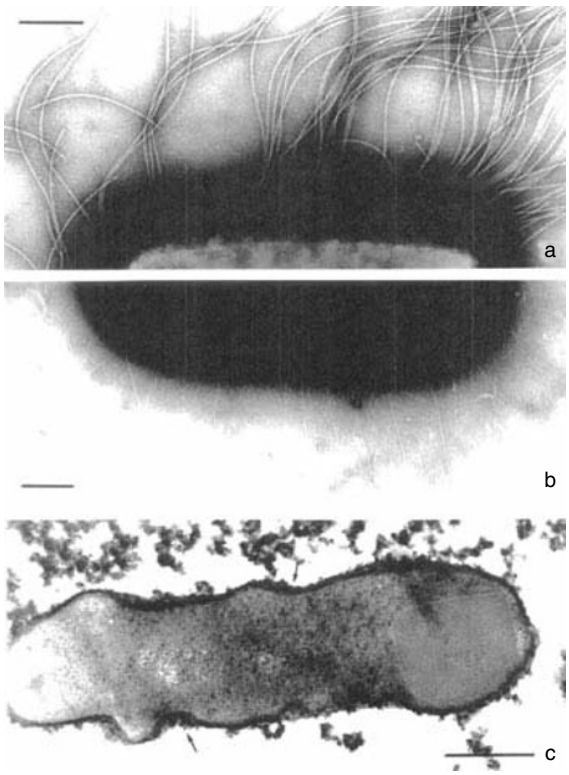


Fig. 13. Surface appendages of *Xenorhabdus nematophila* phase I variants. (a) Transmission electron micrograph of F1/1 cell from nutrient broth culture, showing peritrichous flagella; negative staining with 1% phosphotungstate. Bar = 0.5 ∝m. (b) Transmission electron micrograph of F1/1 cells from nutrient agar at 1.5% previously dried to minimize expression of flagella, showing fimbriae (or pili) around the cell wall. Bacteria were fixed 1.25% glutaraldehyde and 1% paraformaldehyde in 0.015M sodium cacodylate buffer (pH 7.4; 10 min) and were negatively stained with 0.5% phosphotungstate (from Moureaux et al., 1995). Bar = 0.25 ∝m. (c) Transmission electron micrograph of A24/1 cell grown on nutrient agar. Cell fixed with glutaraldehyde-lysine-osmium tetroxide and stained with ruthenium red, uranyl acetate and lead citrate shows significant glycocalyx surrounding the cell wall (arrows). Bar = 0.5 ∝m (from Brehélin et al., 1993).

With some strains of *X. nematophila*, and depending on the insect tested, phase II variants may be less pathogenic than the phase I variants. Thus an analysis of pathogenicity in *Manduca sexta* larvae revealed that the phase II variant of AN6 was significantly less virulent than the phase I variant (Völgyi et al., 1998). The phase II variant of *X. nematophila* strain N2-4 was also avirulent for *M. sexta*. On the other hand, F1/II and 19061/II were as virulent as their respective phase I cells. Consequently phase traits of *Xenorhabdus*, which are mainly involved in the growth and development of nematodes, may be considered as "symbiotic characters," and to some extent depending on the biotic conditions of the infection (infecting strain and susceptibility of the insect), more or less linked with virulence factors. Nevertheless the role of phase II remains uncertain in *Xenorhabdus*, as in *Photorhabdus*.

Swarmer Cells Givaudan et al. (1995) demonstrated that *Xenorhabdus* phase I variants displayed a swarming motility when they were grown on a suitable solid medium (0.6–1.2% agar; Fig. 12a, b). Unlike most phase I variants of different *Xenorhabdus* spp., phase II variants were unable to undergo cycles of rapid and coordinated population migration (swarm and even to swim) over the surface of semisolid agar, particularly those of *X. nematophila*. Optical and electron microscopic observations showed that the nonmotile phase II cells of *X. nematophila* F1 lacked flagella. When flagella from strain F1 phase I variants (Fig. 13a) were purified, the molecular mass of the flagellar structural subunit was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 36.5 kDa. Flagellin from cellular extracts or culture medium of phase II cells was undetectable with antiserum against the denatured flagellin by immunoblotting analysis. This suggests that the lack of flagella in phase II cells is due to a defect during flagellin synthesis as has been demonstrated by genetic studies. (See Genetics in this Chapter)

Production of Secondary Metabolites As for *Photorhabdus*, phase I variants of *Xenorhabdus*

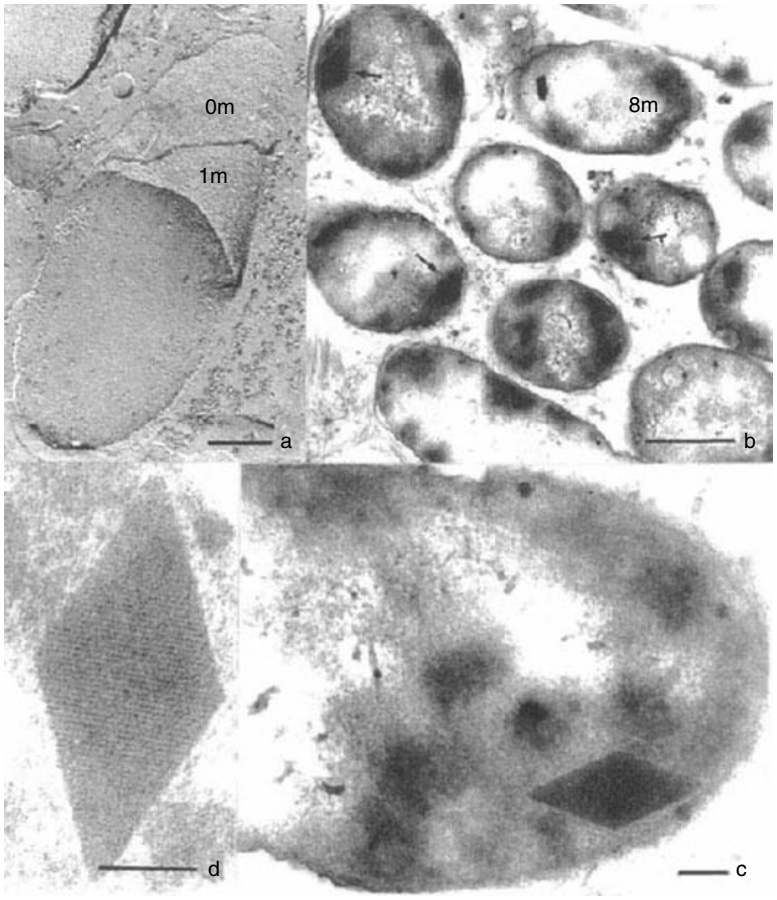


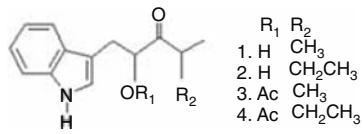
Fig. 14. Ultrastructural traits of *Xenorhabdus nematophila* cells. (a) Freeze-etching of strain F1 cell showing the outer (om) and the inner (im) membranes of the cell wall. Cleavage faces have been displayed, both at the level of cell wall and of plasma membrane. Glutaraldehyde (2.5%) in 0.1 M cacodylate buffer (pH 7.2) was added at 37°C for 1 h. The flask was rinsed with the cacodylate buffer at laboratory temperature, and the cells were gently scraped. The cells were sedimented by low-speed centrifugation, and the pellet was impregnated with 25% glycerol for 20 to 30 min to avoid ice-crystal formation. These samples were again centrifuged, and then were sandwiched between two specimen-holder dishes 3 mm in diameter, in preparation for the double-replica technique. They have been fractured in a Cryofract and both complementary fractures were shadowed by platinium evaporation. The replicas were cleaned in sodium hypochloride, rinsed in double distilled water, dried, and examined in a transmission electron microscope. Bar = 0.4 ∝m. (b) Cultures of 5-d stationary growth of strain F1 fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Crystalline inclusions (arrows) begin by a condensation of amorphous material (am), which crystallizes as rhomboedric bodies. Bar = 0.8 ∝m. (c) Same as b, except bar = 0.1 ∝m. (d) Same as c but shows details of the crystallization of the inclusion. Bar = 0.1 ∝m.

produce a variety of secondary metabolites, some of which have antimicrobial properties (Akhurst, 1982a; Table 10). Four chemical groups have been characterized: indole derivatives (Li et al., 1996; Li et al., 1997; Paul et al., 1981; Sundar and Chang, 1993; Webster et al., 1996), xenorhabdins (McInerney et al., 1991a; Rhodes et al., 1987), xenorxides (oxidized xenorhabdins) and xenocoumacins (Gregson and McInerney, 1989; McInerney et al., 1991b). Maxwell et al. (1994) examined occurrence of the xenocoumacins in vivo (in the insect *Galleria mellonella*) and in vitro (in tryptic soy broth). The level of antibiotic activity was greater under in vivo than in vitro conditions. These experiments demonstrate that *Xenorhabdus* antibiotics may play an important role by preventing microbial contamination in the insect carcass during the development of nematodes. The production of antimicrobials provides at least a partial explanation for the "natural monoxeny" during parasitism.

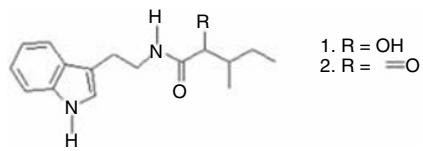
Lysogeny and Bacteriocinogeny The presence of lysogenic phage in and production of bacteriocins by *X. nematophila* was demonstrated by Boemare et al. (Boemare et al., 1992; Fig. 15). Lysis of either phase of *X. nematophila*, *X. bovienii* or *X. beddingii* in response to mitomycin C or heat shock released complete and partial

Table 10. Secondary antibiotic metabolites from *Xenorhabdus* strains.

1. Indole derivatives



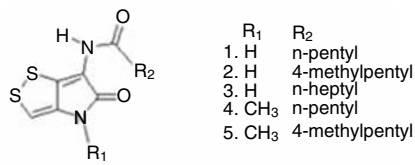
R1, R2, substituents	*in vitro* production	active against
3-(2′-hydroxy-3′-keto-4′-methylpentyl)-indole	+	bacteria
3-(2′-hydroxy-3′-keto-4′-methylhexyl)-indole	+	bacteria
3-(2′-acetoxy-3′-keto-4′-methylpentyl)-indole	+	bacteria
3-(2′-acetoxy-3′-keto-4′-methylhexyl)-indole	+	bacteria



R substituents	*in vitro* production	active against
3-indoleethyl(3′-methyl-2′-hydroxy)pentamide	+	—
3-indoleethyl(3′-methyl-2′-oxo)pentamide (nematophin)	+	bacteria, fungi

Species: *X. nematophila* ATCC 19601, B1, All
X. bovienii DN, B1, BC1, D1
(Li et al., 1996; Li et al., 1997; Paul et al., 1981; Sundar and Chang, 1993; Webster et al., 1996)

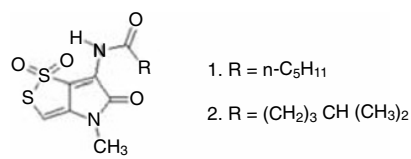
2. Xenorhabdins



N-acyl derivatives of	*in vitro* production	Active against
6-amino-4,6-dihydro-6-oxo-1,2-dithiolo(4,3-b)pyrrole	+	bacteria, insects
6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo(4,3-b)pyrrole	+	bacteria, insects

Species: *Xenorhabdus bovienii*
(Mclnerney et al., 1991; Rhodes et al., 1987)

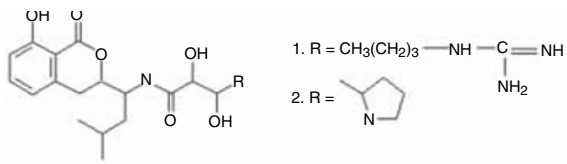
3. Xenorxides



Derivatives of	*in vitro* production	active against
oxidized xenorhabdins	+	bacteria, fungi

Species: *Xenorhabdus bovienii*
(Li et al., 1997)

4. Xenocoumacins



Derivatives of	*in vitro* production	active against
3,4-dihydro-8-hydroxy-1H-2-benzopyran-1-one	+	Stress-induced ulcers, bacteria, fungi

Species: *Xenorhabdus nematophila*
(Gregson and Mclnerney, 1989; McInemey et al., 1991)

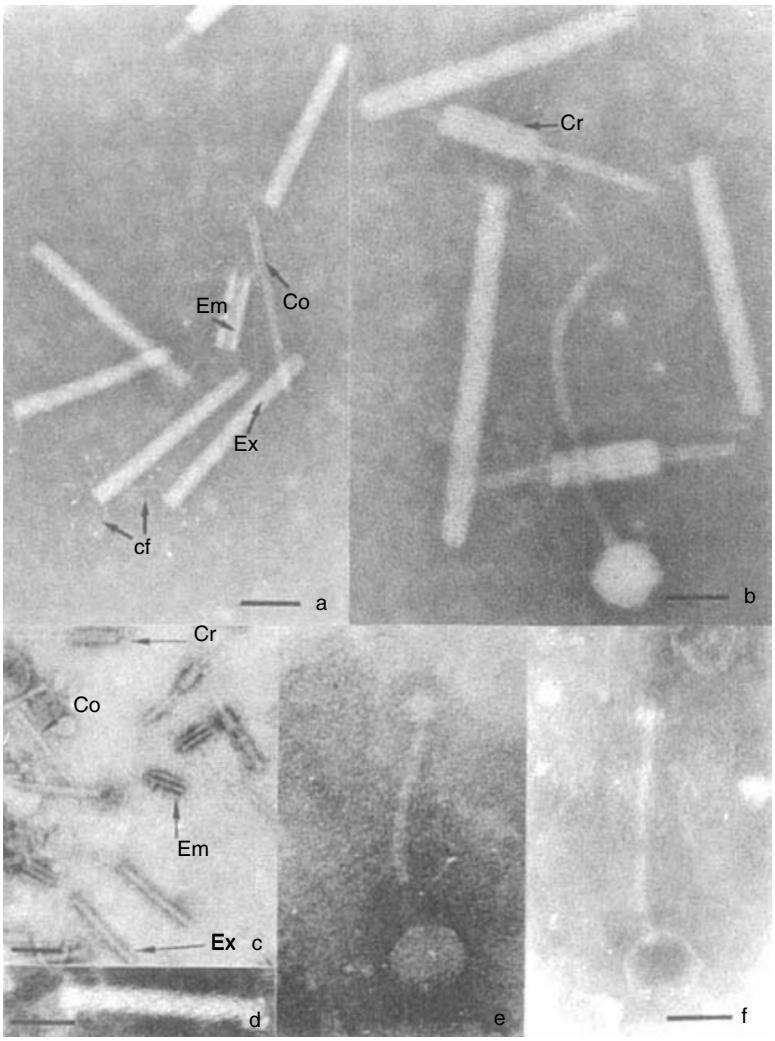


Fig. 15. Electron microscopy of phages (belonging to the family Siphoviridae) and bacteriocins (xenorhabdicin) of *Xenorhabdus nematophila*. Mitomycin C or heat shock treatments induce a phage production at the origin of the culture lysis. At the same time, the production of bacteriocin particles is highly increased. (a) Purified xenorhabdicin particles from mitomycin C-induced culture of strain F1. Bacteriocins with extended sheath (Ex) and caudal fibers on baseplate with adhesive extremities (cf), empty bacteriocins (Em), and loose cores (Co). Bar = 50 nm. (b) Phage and bacteriocin particles in the F1 strain lysate. Cr = bacteriocin with contracted sheath. Bar = 30 nm. (c) Mixture of the different particles of a purified xenorhabdicin suspension of strain A24. Same abbreviations as those in legend to panels 14a and 14b. Bar = 100 nm. (d) Detail showing the surface striation of a complete xenorhabdicin particle of strain A24. Same abbreviations as those in legend to panels 14a and 14b. Bar = 50 nm. (e) Detail of a complete phage of strain A24. Bar = 50 nm. (f) Detail of an A24 phage with empty head. Bar = 50 nm (from Boemare et al., 1992; Baghdiguian et al., 1993; Thaler et al., 1995).

phages. The phage of *X. nematophila* belongs to the family Siphoviridae, as does the λ phage from *E. coli*. The phage head capsid has one major and two minor subunits (Thaler et al., 1995).

The bacteriocins are phage tail-like particles, which in addition to inhibiting non-host *Xenorhabdus*, inhibited *P. luminescens*, *Proteus vulgaris* and *Morganella morgani*. However, none of the other Gram-negative or Gram-positive bacteria tested were inhibited, indicating that the bacteriocins act against closely related genera, in contrast to the antibiotics produced by phase I variants that have a wide spectrum of activity (Boemare et al., 1992; Table 11). Electron microscopic studies of the *X. nematophila* bacteriocin, named xenorhabdicin (Thaler et al., 1995), show that bacteriocin particles have the structure of a rigid phage tail without any "head" (Baghdiguian et al., 1993; Fig. 15). Biochemically, xenorhabddicin was shown to consist of two major protein bands, corresponding to the sheath and core, and five minor bands (Thaler et al., 1995).

The inhibition by several dilutions of a xenorhabdicin suspension was scored 24 h after deposit on a lawn of the bacterial target (+, clear zone at the droplet site, –, no clear zone). The bacteriocin suspension was purified from a culture of X. nematophila A24 (A_{600} = 0.5) after induction by mitomycin C or heat shock and was diluted up to 10^{-5} (Thaler et al., 1995) before assessing bacteriocin activity. When noninduced cultures were centrifuged and the filter-sterilized supernatants were tested similarly, a residual bacteriocin activity was noted for the same susceptible target strains of this table, meaning that a spontaneous bacteriocinogenesis occurs in untreated cultures, albeit at a lower frequency than in induced cultures (Boemare et al., 1992). The xenorhabdicin producer strain and strains belonging to the same species are immune from this antibiotic activity (blue box in the table), strains of other species of *Xenorhabdus* and *Photorhabdus* are susceptible (red box), and the only other genera containing susceptible strains are *Proteus* and *Morganella* (pink box), showing that

Table 11. Antibiotic activity of xenorhabdicin from both variants of *X. nematophila* strain A24.

Indicator strains		Growth inhibition
Citrobacter freundii		–
Enterobacter cloacae		–
Erwinia chrysanthemi		–
Klebsiella pneumoniae		–
Salmonella typhimurium		–
Serratia marcescens		–
Yersinia enterolitica		–
Pseudomonas aeruginosa		–
Pseudomonas testosteroni		–
Micrococcus luteus		–
Micrococcus roseus		–
Streptococcus epidermidis		–
Streptococcus faecalis		–
Bacillus cereus		–
Bacillus megaterium		–
Bacillus subtilis		–
Bacillus thuringiensis		–
Escherichia coli		–
Proteus mirabilis		–
Morganella morganii		+
Proteus vulgaris		+
Photorhabdus luminescens	K80/1	+
Photorhabdus luminescens	K80/2	+
Xenorhabdus beddingii	Q58/1	+
Xenorhabdus beddingii	Q58/2	+
Xenorhabdus nematophila	F1/1	–
Xenorhabdus nematophila	F1/2	–
Xenorhabdus nematophila	A24/1	–
Xenorhabdus nematophila	A24/2	–

this bacteriocin is active only against species closely related to *X. nematophila*.

Genetics

Genetic studies (comprehensively reviewed by Forst and Nealson, 1997; Forst et al., 1996) have been undertaken to analyze the mechanisms and functions of pathogenicity, symbiosis and phase variation. Couche et al. (1987b) demonstrated the presence of one or two small plasmids (3.6–12 kb) in some, but not all, X. nematophila and *X. bovienii* strains. The plasmid profiles did not differ between phases I and II. Smigielski and Akhurst (1994a) reported two megaplasmids (71.8 and 118.5 kb) as well as two additional plasmids (6.5 and 17 kb) in the A24 strain of *X. nematophila*. They also demonstrated that all strains of *X. nematophila*, *X. bovienii*, *X. beddingii* and *X. poinarii* contained megaplasmids (48–<680 kb) and that the megaplasmid profiles of phases I and II were not different. Small or large plasmids of *Xenorhabdus* have not been further characterized or engineered to generate cloning vectors.

Conjugation of plasmids from *E. coli* has been applied successfully for *X. nematophila* and *X. bovienii* (Forst and Nealson, 1996; Francis et al., 1993; Givaudan and Lanois, 2000; Givaudan et al., 1996; Völgyi et al., 2000). In contrast, transformation of *Xenorhabdus* has not been generally successful. Although Xu et al. (1989) reported the transformation of the type strain of *X. nematophila* with a broad host-range vector, they and other workers have been unsuccessful with other strains of *X. nematophila* and other *Xenorhabdus* spp. The increase in transformation efficiency after the vector had been passaged through *X. nematophila* suggested the presence of a restriction modification system. Endonuclease activities have been detected in a range of *Xenorhabdus* spp. and it appeared that their DNA was strongly methylated (Akhurst et al., 1992). *Xenorhabdus bovienii* wild-type strains lack a functional receptor protein (LamB) in the outer membrane and as a result are unable to adsorb the phage λ of *E. coli*. Francis et al. (1993) developed a transposon mutagenesis system for *X. bovienii* by constitutively expressing the LamB protein on the surface of the bacterium allowing them to be infected with l particles carrying the Tn10 transposon. This process produced various dye-binding, lipase, protease, hemolytic, DNase and auxotrophic mutants, i.e., alteration of phase and virulence traits. Unfortunately, use of such material for analysis of phase variation was not further continued (see Genetics/Regulation of Phase Variation in this Chapter). The Tn*5* transposon was used to induce a low frequency of mutagenesis in *X. nematophila* (Xu et al., 1991) and the mini-Tn*10* transposon carried on pLOF-Km^r has been introduced into *X. nematophila* by conjugal transfer (Völgyi et al., 2000).

Table 12. Identified genes and proteins from *Xenorhabdus* strains.

Gene	Protein encoded	% identity	with
opnP (1)	porin	59	*E. coli*
ompR (4)	cytoplasmic regulatory protein	78	*E. coli*
envZ (4)	osmolarity sensor membrane protein	57	*E. coli*
fliC (2)	flagellin	68	*E. coli*
fliD (2)	hook-associated protein	41	*E. coli*
flhC (3)	master regulator	78	*E. coli*
flhD (3)	master regulator	72	*E. coli*
var1 (5)	new protein	none	

References (1) Forst et al., 1995; (2) Givaudan et al., 1996; (3) Givaudan and Lanois, 2000; (4) Tabatabai and Forst, 1995; (5) Völgyi et al., 2000

Genes and Proteins Several genes have been identified in *Xenorhabdus* strains (Table 12). Regulation of outer membrane protein (Opns) genes of *X. nematophila* was shown in response to growth period and growth temperature (Forst

et al., 1995; Leisman et al., 1995). In *E. coli*, the OmpR and OmpC proteins form pores that allow diffusion across the outer membrane. The genes encoding them are regulated by several environmental stimuli. This is mediated by a two-component regulatory system: an EnvZ sensor protein, activated by environmental changes to phosphorylate the OmpR regulatory protein, and the *ompF* and *ompC* genes, regulated by OmpR regulatory protein. The *ompR* and *envZ* two-component signal transduction genes of *X. nematophila* were shown to constitute a single operon regulated by a σ^{70} promoter. One function of EnvZ in *X. nematophila* is to regulate the expression of OmpRs during the stationary period of growth.

Phase I variants of *X. nematophila* strain F1 were motile (see Physiology—swarming in this Chapter), whereas phase II variants were non-flagellated cells that did not synthesize flagellin (Givaudan et al., 1995). A locus containing two open reading frames (ORFs) was identified from phase I cells by using functional complementation of flagellin-negative *E. coli* (Givaudan et al., 1996). The sequence analysis revealed that the first ORF corresponds to the fliC coding for flagellin, and showed a high degree of homology between the N-terminal and C-terminal of *Xenorhabdus* FliC and flagellins from other bacteria. The second ORF, which is in the opposite orientation, encodes a homologue of the enterobacterial hook-associated protein 2, FliD. Both the *fliC* and *fliD* genes of *Xenorhabdus* were required for the complete restoration of *E. coli* motility. A sequence highly homologous to the σ^{28}-consensus promoter was identified upstream from the coding sequences of both genes. The structure of the *fliC* gene and its surrounding region was shown to be the same in both phase variants, but Northern blot analysis revealed that *fliC* and *fliD* were, respectively, not and weakly transcribed in phase II variants. The loci, the transcriptional activators of flagellar genes, *flhDC*, were identified (Givaudan and Lanois, 2000).

Regulation of Phase Variation and Virulence Factors Hypovirulent and avirulent mutants have been produced in *X. nematophila* by chemical and transposon mutagenesis (Dunphy, 1994; Xu et al., 1991). *N*-β-hydroxybutanoyl homoserine lactone (HBHL), the autoinducer of the luminous system of *Vibrio harveyi*, restored virulence to previously avirulent mutants (Dunphy et al., 1997) by increasing lipase activity and lowering phenol oxidase activity in the hemolymph of insects infected with the wild strain, parameters that are associated during pathogenesis. When avirulent mutants are injected, lethality for the insect is restored upon injection with HBHL. Because HBHL, or a closely related analogue, is excreted by the wild-type *X. nematophila*, a role for an HBHL-dependent regulatory system in its virulence process is indicated. The luminescence and virulence of *V. harveyi* against prawns (associated with a toxic extracellular protein) have been suggested to be coregulated under the control of an intercellular quorum-sensing mechanism involving the HBHL and another uncharacterized signaling molecule (Manefield et al., 2000).

Three phenotypically similar variants of *X. nematophila* strain AN6, each containing a single transposon insertion, were obtained by transposon mutagenesis (Völgyi et al., 2000). The insertions occurred at different locations in the chromosome. The variant ANV2 (AN6/1::Tn*10* Amp^r Km^r) neither produced antibiotics nor the outer membrane protein OpnB, but produced lecithinase. The transposon had inserted in a novel gene designated *var1*, which encodes a protein of 121 amino acid residues. Complementation analysis confirmed that the pleiotropic phenotype of ANV2 was produced by inactivation of *var1*. However this *var1* gene does not complement any other phase II variants. These results indicate that inactivation of a single gene can affect multiple phase traits. More generally, all the experiments inactivating some specific phase traits by mutations or overexpression of a structural gene affect one or few functions, but none has been found to affect all the phase-related functions.

Complementation experiments showed that motility and flagellin synthesis of phase II cells cannot be recovered by placing in *trans* the *fliCD* operon from phase I cells (Givaudan et al., 1996). A gene(s) higher in the transcriptional hierarchy of the flagellar regulon, acting in *Xenorhabdus* phase II variants, was investigated. Givaudan and Lanois (2000) constructed *X. nematophila flhD* null mutants showing that flhDC operon controlled flagellin expression. But at the same time this experiment showed that lipolytic and extracellular hemolysin activities also were altered. In addition, the *flhD* null mutant displayed a slightly attenuated virulence phenotype in the insect *Spodoptera littoralis*. Thus, these data indicate that the phenotypic phase traits of *X. nematophila* (such as motility, lipase, hemolysin) participated also in the infectious process in insects. On the other hand, even if the flagellar regulon is considered to be one of the master genes involved in some aspects of phase variation, a super-regulator that controls the total phase mechanism has not been discovered. Nevertheless we should note that, in contrast with *Photorhabdus*, phase variation in *Xenorhabdus* is regulated transcriptionally.

All the analyses to date have shown that different phase variant characteristics are separately controlled. However, it seems unlikely that phase variant phenotypes are all controlled independently of each other. It is probable that a master switch differentially affects a number of other regulatory systems that in turn control one or a small number of phase variant characteristics. In no case has a common regulator (controlling the whole phenomenon) been identified.

Ecology

Xenorhabdus species have been found only in the intestinal tract of infective stage nematodes of the genus *Steinernema* (syn. *Neoaplectana*) and in insects killed by these nematodes. There is a high degree of specificity in these associations, with each nematode species being naturally associated with only one *Xenorhabdus* species. This specificity is determined by the ability of the nematode to retain the bacterium in its intestinal vesicle (Akhurst and Boemare, 1990; Bird and Akhurst, 1983). Although other bacteria in association with *Steinernema* have been reported (Aguillera et al., 1993; Lysenko and Weiser, 1974), closer examinations have always demonstrated that only *Xenorhabdus* have a specific association (Akhurst, 1982b; Bonifassi et al., 1999). For example, gnotobiology of *Steinernema scapterisci* and bacteriological study of its symbiont, confirmed that this nematode harbors a symbiotic species of *Xenorhabdus*, as do other *Steinernema* species. Bacteria, reported previously as associating with this nematode and belonging to several other genera, are contaminants located in the intercuticular space of the infective juveniles (Bonifassi et al., 1999). These bacteria were detrimental to nematode reproduction in *Galleria mellonella*. Combination of the symbiont and its axenic host gave the best yields of infective juveniles (IJs) when produced in this insect and in vitro production on artificial diet.

The infective stage nematodes act as vectors, transporting the bacteria into the insect via natural orifices (mouth, anus and spiracles) and then into the hemocoel (Fig. 2; see Photorhabdus and Xenorhabdus in this Chapter). The infective (dauer) nematode, a third-stage juvenile with closed mouth and anus, recommences development in the hemocoel, releasing its symbiotic bacterium and an inhibitor of the inducible antibacterial enzymes (Götz et al., 1981; Poinar and Himsworth, 1967a). As *Xenorhabdus* multiplies, it provides essential nutrients for nematode maturation and reproduction (Poinar and Thomas, 1966b) and antibiotics (Akhurst, 1982a; Li et al., 1996; Li et al., 1997; McInerney et al., 1991a; Paul et al., 1981).

Xenorhabdus alternate between a nutrient-rich (insect) and nutrient-poor (nematode) existence. Forst and Nealson (1996) hypothesized that phase II may be induced by the nematode gut conditions and better adapted to the nutrient-poor conditions of the intestinal vesicle of the nonfeeding nematode. However, this hypothesis does not account for the fact that the bacteria isolated from field-collected infective stage juveniles are inevitably phase I cells. Smigielski et al. (1994b) found differences in the activity of respiratory enzymes of both phase I and II cells that indicate the greater potential of phase II forms to survive in soil environments than phase I. The lack of a record of isolation of *Xenorhabdus* directly from soil may be due to its slow growth, lack of a suitable selective medium, and/or the difficulty of identifying phase II cells, which has few positive characters in most standard tests for identifying bacteria. However, Morgan et al. (1997) found that *X. nematophilus* declined very quickly in river water and soil, becoming undetectable after 2 d and 7 d, respectively. At this time, there is no satisfactory explanation of the ecological role of phase II forms.

Steinernematidae living in the soil, but specialized as insect parasites, belong to a large group of nematodes in the order Rhabditida that feed on various microorganisms. The Steinernematidae, although adapted to a particular symbiotic bacterium, have retained the ancestral microbivorous behavior allowing them to feed and reproduce on microorganisms other than their own symbiont. In spite of this feeding behavior, however, it has been shown by several researchers that bacteria other than the symbiont are not as favorable as the symbiont for growth and reproduction of *Steinernema* (Akhurst, 1982c; Boemare, 1983a; Ehlers et al., 1990), and not efficient enough to allow the perenniality of a symbiotic association (Akhurst and Boemare, 1990).

Further microbial ecological studies are needed to explain the durability over many generations of the *Xenorhabdus–Steinernema* intestinal symbiotic associations. The concept of the occurrence of a natural monoxeny in these bacterium–helminthic symbioses (Bonifassi et al., 1999) must be accepted, if we consider the following features. In natural conditions, if any bacterial co-associate is carried in the IJs, perhaps the IJs and associated bacteria could kill insects, but the probability of the nematode reproducing inside the insects and providing viable IJs is low. Consequently, the chances of an ecologist isolating polyxenic parasitized insects from the soil are very small due to the scarcity of these occurrences and to the short period of time between infection of an insect and its putrefaction, which would destroy any traces of the association.

Disease

No effects on vertebrates have been demonstrated (Obendorf et al., 1983; Poinar and Thomas, 1967b; Poinar et al., 1982).

Xenorhabdus is an insect pathogen only when delivered into the insect hemocoel, either by their nematode symbiont or by injection; they are not pathogenic when applied per os or topically. Most are highly pathogenic for larvae of the greater wax moth, *Galleria mellonella*, with LD_{50}s of less than 20 cells (Akhurst and Dunphy, 1993). *Xenorhabdus poinarii* has very little pathogenicity for *G. mellonella*, (LD_{50} = 5,000 cells) when injected alone, although it is highly pathogenic when co-injected with axenic *Steinernema glaseri*, its natural host (Akhurst, 1986b). Axenic *S. scapterisci* and its symbiont *Xenorhabdus* strain UY61 alone are also not pathogenic to *G. mellonella*. The combination of both partners re-established the pathogenicity of the complex towards *G. mellonella* (Bonifassi et al., 1999). Pathogenicity of *Xenorhabdus* varies between insects, with *X. nematophila* having an LC_{50} of about 500 for *Hyalophora cecropia* caterpillars, and no effect on maggots of the genus *Chironomus* (Götz et al., 1981). The use of a less susceptible host (e.g., *Manduca sexta*) has enabled detection of differences in pathogenicity between the two phases of a strain (Völgyi et al., 1998).

Applications

Knowledge about the nutritional requirements provided by the symbionts would improve the industrial mass production of the entomopathogenic nematodes for biological control of insect pests. Similar comments made about *Photorhabdus* (see Photorhabdus/Applications in this Chapter) can be applied to *Xenorhabdus*.

Some of the secondary metabolites have commercial potential in the pharmaceutical and agro-forestry industries (Webster et al., 1998). Among the isolates examined to date from *X. nematophila* and *X. bovienii*, some indole, xenocoumacin and xenorhabdin derivatives are antibacterial, and/or antifungal, especially when they are cultivated in vitro. This is of great interest to industry (Table 10).

Moreover, the occurrence of protein toxins identified in some *Xenorhabdus* strains has enhanced interest in the bacteria alone for biological control applications. As with *Photorhabdus* (see Photorhabdus/Applications), programs to insert toxin genes into plant genomes for crop protection have been proposed. Today four patents have been submitted for such exploitation (East et al., 1999; Jarrett et al., 1997; Kramer et al., 1999a; and Smigielski and Akhurst, 1995).

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