2 Biology, Taxonomy and Systematics of *Photorhabdus* and *Xenorhabdus*

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2.1. Introduction

2.1.1. Habitat and position in the Proteobacteria

Xenorhabdus and *Photorhabdus* are two bacterial genera mutualistically associated with infective juveniles of entomopathogenic nematodes from the genera *Steinernema* and *Heterorhabditis*, respectively. Most *Xenorhabdus* and all *Photorhabdus* are pathogenic for insects when injected into the haemocoel. In addition, non-symbiotic strains

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of *Photorhabdus asymbiotica* have been identified as opportunistic pathogens for humans (Farmer *et al.*, 1989) and another group of *Photorhabdus*, not yet identified at the level of a species, causes infections needing antibiotherapy (Peel *et al.*, 1999).

All isolations made during the last 20 years, from wild nematodes (*c.* 1000), established the presence of *Xenorhabdus* in *Steinernema* and *Photorhabdus* in *Heterorhabditis* juvenile intestines (Forst *et al.*, 1997). When sometimes other bacterial strains were found associated with *Steinernema* (Lysenko and Weiser, 1974; Aguillera *et al.*, 1993; Elawad *et al.*, 1999), it was shown that they were contaminants of the cuticle (Bonifassi *et al.*, 1999) and in all cases the symbiont was found in the infective juvenile gut. In *Heterorhabditis*, *Ochrobactrum* spp. (Babic *et al.*, 2000) and *Providencia rettgeri* (Jackson *et al.*, 1995) have sometimes been isolated and were probably also located between the cuticle and the juvenile sheath.

Xenorhabdus and *Photorhabdus*, as with other insect and vertebrate symbionts, are members of the γ-subclass of Proteobacteria. Both are negative for nitrate-reductase, and *Xenorhabdus* is negative for catalase, two major positive characters of the Enterobacteriaceae. Nevertheless, they have the enterobacterial common antigen (Ramia *et al.*, 1982). Moreover, if we look at the tree of the ribosomal data-set project (Larsen *et al.*, 1997), both genera branch deeply in the family of the Enterobacteriaceae without any common ancestor. Their nearest neighbour is the genus *Proteus*.

2.1.2. Fundamental and applied interests

The study of symbiont properties, such as cellular exportation, exoenzymatic activities, special metabolites, pathogenic processes, capabilities to differentiate into multicellular populations for the colonization of different habitats, are bringing new insights for microbiology. This is particularly true for the study on pathogenic mechanisms and specific metabolic pathways. Presumably researchers will soon also be able to propose an evolutionary history for these mechanisms with the comparative data given by these bacterium–invertebrate interactions.

The foremost applied interest in studying these bacteria concerns nutritional requirements for improving mass production of the nematodes for biological control of insects. We know that the quality of the symbiont inoculum, in terms of viability and preservation from microbial contamination, must be maintained for effective commercial production (Ehlers *et al.*, 1990, 1998). The second interest of these bacteria is to use their secondary metabolites which have a commercial potential in the pharmaceutical and agroforestry industries (Webster *et al.*, 1998). The third interest is the occurrence of protein toxins identified in *Photorhabdus* (Bowen *et al.*, 1998) that enhanced the use of the bacteria alone for biological control. Programmes to insert toxin genes into plant genomes for crop protection have been proposed, as for *Bacillus thuringiensis* ('Bt'). In addition, to counter resistance development in target insects consuming these transgenic 'Bt' plants, the insertion of both toxin genes (from *B. thuringiensis* and *Photorhabdus*) into the same plant has been suggested (ffrench-Constant and Bowen, 1999).

2.2. Biology of *Xenorhabdus* and *Photorhabdus* Bacteria

2.2.1. Life cycle – a vegetative multiplication in insects and a quiescence in nematodes

Parasitism starts when nematodes enter by natural openings (mouth, anus, spiracles) or directly through the integument (mainly *Heterorhabditis* nematodes) of the insects. *Steinernema* spp. induce a toxicogenesis (Boemare *et al.*, 1982, 1983b) and produce an immune depressive factor active against antimicrobial peptides from the insects (Götz *et al.*, 1981); nothing is known for *Heterorhabditis* in this respect. Infective juveniles of both genera release their bacterial symbionts in the host body cavity and develop into fourth-stage juveniles and adults. The insects die mainly due to a septicaemia. Sometimes a bacterial toxaemia precedes the resulting septicaemia (Forst *et al.*, 1997). Nematodes reproduce in the insect cadaver and feed on the symbiont biomass and insect tissues metabolized by the bacteria.

There are two different physiological states in the life cycle of the symbionts corresponding to two different ecological niches. The first is a phoretic state in the resting stage of the nematode host: *Xenorhabdus* occurs naturally in a special intestinal vesicle of *Steinernema* infective juveniles (Bird and Akhurst, 1983), while *Photorhabdus* are mainly located in the anterior part of *Heterorhabditis* infective juvenile guts (Boemare *et al.*, 1996). The second state is a vegetative state when the bacteria multiply inside the insects after inoculation in the haemolymph. Consequently, the symbionts alternate between a poor and a rich nutrient existence. If we compare with several other bacteria that are symbiotic with animals (see Forst and Clarke, Chapter 3 this volume), the life cycle of these bacteria is unique: they are symbiotic with nematodes and pathogenic with insects.

2.2.2. Virulence of bacteria and contribution of nematodes

In general terms, except for the rare strains which are pathogenic by ingestion, the bacterial infection starts after the penetration of the nematode into the body cavity of insects. No development in the insect gut has been reported. Depending on the three partners of this interaction (insect, nematode, bacteria), the pathogenic process may be the result of some insect resistance according to insect species, the action of one of the partners (bacteria or nematode), or the action of both partners (bacteria and nematodes).

According to Bucher (1960), when the LD_{50} is < 10,000 cells the bacterium may be considered as entomopathogenic. Most *Xenorhabdus* strains are highly pathogenic for larvae of the greater wax moth, *Galleria mellonella*, with LD_{50}s < 100 cells (Akhurst and Boemare, 1990). However, *X. poinarii* has little pathogenicity for *G. mellonella* when injected alone (LD_{50} > 5000 cells), although it is highly pathogenic when co-injected with axenic (germ-free) *Steinernema glaseri*, its natural host (Akhurst, 1986b). Axenic *S. scapterisci* and its *Xenorhabdus* symbiont alone are also not pathogenic to *G. mellonella*. The combination of both partners re-establishes the pathogenicity of the complex towards *G. mellonella* (Bonifassi *et al.*, 1999).

All *Photorhabdus* strains examined to date have been reported to be entomopathogenic, the LD_{50} usually being < 100 cells when injected into *G. mellonella*

(Fischer-Le Saux *et al.*, 1999b). Consequently *Photorhabdus* have to be considered as highly entomopathogenic. In addition some of them are entomopathogenic by ingestion (ffrench-Constant and Bowen, 1999). 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).

2.2.3. Antimicrobial barriers preserve the symbiosis

There is a large production of anti-microbial compounds at the end of *Xenorhabdus* spp. *in vitro* multiplication (Paul *et al.*, 1981; Akhurst, 1982; McInerney *et al.*, 1991a,b; Sundar and Chang, 1993; Li *et al.*, 1996, 1997). It was demonstrated that they were also produced in insects (Maxwell *et al.*, 1994). Similarly there is production of antimicrobial compounds from *Photorhabdus* spp. *in vitro* (Paul *et al.*, 1981; Richardson *et al.*, 1988; Li *et al.*, 1995; Hu *et al.*, 1997) and *in vivo* (Hu *et al.*, 1998). All these molecules show a large scale of antibiotic activity (see Webster *et al.*, Chapter 5, this volume). Conversely, bacteriocins produced by both genera are active against closely related bacteria such as other species of *Xenorhabdus, Photorhabdus* and the nearest genus *Proteus* (Boemare *et al.*, 1992; Thaler *et al.*, 1995). So antimicrobial barriers play an important role to protect the specificity of the symbiosis by eliminating microbial competitors (Boemare *et al.*, 1993b; Thaler *et al.*, 1997).

2.2.4. Gnotobiology

Nematodes reproduce in a state of natural monoxenic microcosm (containing only one bacterial species) occurring during the insect parasitism and offer a sort of monoxenic sanctuary inside their gut during the resting stage. These two features are exceptional in terms of microbial ecology and are at the origin of the definition of the concept of natural monoxeny occurring with the entomopathogenic nematodes (Bonifassi *et al.*, 1999). After the death of an organism, the putrefaction normally proceeds from the intestinal microflora of the dead organism. This is not true with parasitism by entomopathogenic nematodes: the insect cadaver is essentially a mummified bag containing only *Xenorhabdus* or *Photorhabdus* cells that work with nematodes on insect tissues and provide their own biomass for nutrition of the nematodes. When juvenile larvae are escaping the insect cadaver, they recruit some symbiont cells in their intestine for the next infection assuring continuation of the mutualistic association. During long-term starvation in the resting infective juveniles, about 100 cells of *Xenorhabdus* or *Photorhabdus* are maintained. Two possibilities have been hypothesized: either bacteria are digested by starving juveniles and there is a threshold of about 100 bacterial cells that are maintained (Selvan *et al.*, 1993), or bacteria are not digested. The latter, where bacteria are not digested and are well preserved, supposes a special behaviour of the infective juveniles, and a special quiescent physiology of the bacteria.

Another interesting feature is that the reproduction of nematodes on artificial diet is possible. This point was at the origin of the mass production of the nematodes for biological control. As we are able to axenize nematodes and to cultivate the bacteria on nutrient agars, combinations have been tested to establish the reliability of the symbiosis. Such gnotobiological experiments show that some combinations with non-symbiotic bacteria, as *E. coli*, allow development of the nematodes

(Ehlers *et al.*, 1990). Steinernematidae and Heterorhabditidae belong to Rhabditida, a nematode group adapted to feed on various microorganisms. They retained the ancestral microbivorous behaviour allowing them to feed and reproduce on microorganisms other than their own symbiont. However, these non-symbiotic bacteria do not support long-term experimental associations (Akhurst and Boemare, 1990; Ehlers *et al.*, 1990; Han and Ehlers, 1998). Similarly, *Photorhabdus* isolates do not support *in vitro* culture of any *Steinernema* species or *Xenorhabdus* spp. of any *Heterorhabditis* (Akhurst, 1983; Akhurst and Boemare, 1990; Han *et al.*, 1990; Gerritsen and Smits, 1993). Consequently some non-symbiotic bacteria are able to create provisional associations with these nematodes, but the naturally occurring symbiotic bacteria provide the most efficient development. This paradox underlines our inadequate understanding about the needs provided by most of the bacteria for the nutritional requirements of the microbivorous nematodes, and for the special nutritional or hormonal substances provided by the symbionts for entomopathogenic nematodes.

2.2.5. Intraspecific biodiversity

Phase variation can be easily detected by dye adsorption and antibiotic production (Akhurst, 1980) for any symbiotic strain. Only phase I of the symbionts have been isolated from wild nematodes in natural conditions. After *in vitro* subculture a variable proportion of clones is affected in colony and cell morphologies (Boemare and Akhurst, 1988), motility (Givaudan *et al.*, 1995), production of endo- and exo-enzymes, respiratory enzymatic activities (Smigielski *et al.*, 1994), and secondary metabolites (Akhurst, 1980, 1982). Revertants have been obtained with *X. nematophila* (Akhurst and Boemare, 1990; Givaudan *et al.*, 1995) indicating that variation in *Xenorhabdus* fits the classical definition of phase variation. In bacteriology, phase variation is commonly defined as a reversible genetic event usually mediated by DNA instability such as DNA inversion occurring at a significant frequency. In the case of *Photorhabdus* no reversion has been confirmed. In fact, the terminology of phenotypic conversion should be more appropriate. For *Xenorhabdus* and *Photorhabdus*, 'phase variation' is commonly used to refer to an alternative balance in expression/non-expression of physiological traits from the same genome. For every character which can be quantified (e.g. luminescence, antibiotic production) the difference between phases is quantitative, and it is highly probable that this is also true for other phase-related characters emphasizing the occurrence of a genetic regulation (see Forst and Clarke, Chapter 3 this volume).

2.2.5.1. Physiological significance

Phase I variants provide 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, they are less effective in providing growth conditions for the nematodes (Akhurst, 1980, 1982; Akhurst and Boemare, 1990). In addition, some *Photorhabdus* phase II variants may be deleterious for their nematode partner (Ehlers *et al.*, 1990; Gerritsen and Smits, 1993). There is no satisfactory explanation for the ecological role of phase II: is it a survival form, ancestral bacterial form of the symbiont, or a shift in relationship with the life cycle of the nematode?

2.2.5.2. Consequences for taxonomic studies

This phenotypic conversion has been the source of dissimilarities between results published by different laboratories. It occurs at an intra-specific level for all *Xenorhabdus* and *Photorhabdus* but does not affect the phenotypic characters that discriminate the species (Akhurst and Boemare, 1988).

When testing a bacteriological property, even if only one variant is positive among other variants of the same strain that are negative, the response has to be compiled as positive for the given strain. In fact for taxonomic purposes we have to be consistent with the definition of all the potential expression of the studied strain.

2.3. Taxonomy and Systematics

Initially only one genus, *Xenorhabdus*, encompassed all the symbionts of the entomopathogenic nematodes (Thomas and Poinar, 1979). The species previously named '*Xenorhabdus luminescens*' clearly formed a DNA relatedness group distinct from all other *Xenorhabdus* strains (Boemare *et al.*, 1993a; Akhurst *et al.*, 1996) and had also significant differences in phenotypic characters (Fischer-Le Saux *et al.*, 1999b). This resulted in the transfer of *X. luminescens* into a new genus, *Photorhabdus*, as *Photorhabdus luminescens* comb. nov. (Boemare *et al.*, 1993a). *Xenorhabdus* strains are clearly distinguished from 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, while *Photorhabdus* have a TGAAAG sequence at positions 208–213 (Rainey *et al.*, 1995; Szállás *et al.*, 1997).

A fast method using the polymorphism of the 16S rRNA genes (Brunel *et al.*, 1997; Fischer-Le Saux *et al.*, 1998) gives an excellent estimation of the diversity of the genera by using efficient restriction endonucleases providing the most discriminative restriction patterns (Fig. 2.1). Both genera have a particular cell morphology showing, mainly in phase I variants, protein inclusions inside the protoplasm that are highly characteristic (Fig. 2.2).

The names of the bacteria are modified from the previous descriptions to comply with bacteriological nomenclature. Thus, according to rule 65 (2) of the Code of Nomenclature of Bacteria, *Photorhabdus* and *Xenorhabdus*, genera ending by *rhabdus* (from *rhabdos*, rod in Greek as a feminine word), becomes in modern Latin a feminine word (Euzéby and Boemare, 2000). Consequently, '*X. nematophilus*' and '*X. japonicus*' become *X. nematophila* and *X. japonica*, and the new species of *Photorhabdus*, *P. temperata* and *P. asymbiotica*. Details related to the description of the genera and the species are described in Boemare and Akhurst (2000, 2001) and Akhurst and Boemare (2001).

2.3.1. Genus *Xenorhabdus*

A comprehensive phenotypic study led to the elevation of the subspecies previously described (Akhurst, 1983) to species status, as *X. nematophila* (*X. nematophilus*), *X. bovienii*, *X. poinarii* and *X. beddingii* (Akhurst and Boemare, 1988). *Xenorhabdus japonica* (*X. japonicus*), symbiotically associated with *Steinernema kushidai*, was described later (Nishimura *et al.*, 1994), meaning that today five species are recognized. DNA/DNA hybridization (Suzuki *et al.*, 1990; Boemare *et al.*, 1993a; Akhurst *et al.*, 1996) and 16S rDNA analyses (Suzuki *et al.*, 1996; Brunel *et al.*, 1997; Szállas *et al.*, 1997; Fischer-Le Saux *et al.*, 1998) validated the inclusion of five species in the genus *Xenorhabdus*.

However, DNA/DNA hybridization analyses and multivariate analyses of phenotypic data 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*), but too few to warrant a decision on their taxonomic status (Bonifassi *et al.*, 1999; Fischer-Le Saux *et al.*, 1998).

2.3.1.1. Main characters of the genus

Xenorhabdus cells are asporogenous, rod-shaped cells (0.3–2 × 2–10 μm), and Gram negative. They are facultatively anaerobic, with both respiratory and fermentative types

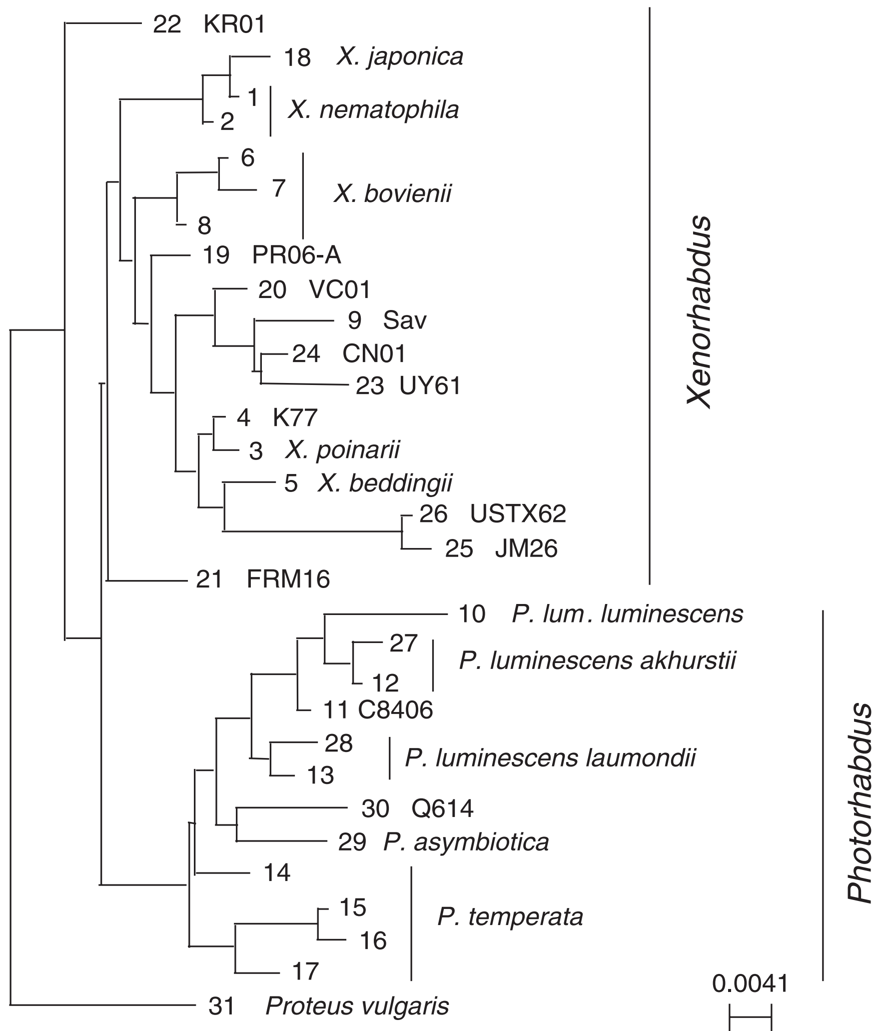

Fig. 2.1. The neighbour-joining method was applied to 30 defined genotypes from a total of 117 strains. The number of the genotypes according to Fischer-Le Saux *et al.* (1998) is followed by the name of the strain or the species (from Boemare and Akhurst, 2000). Scale bar: number of substitutions per site.

of metabolism. The optimum temperature is usually 28°C or less; a few strains grow at 40°C. Acid (no gas) is produced from glucose, and some other carbohydrates are poorly fermented. Catalase is negative and nitrate is not reduced to nitrite. The genus is negative for most tests used to differentiate Enterobacteriaceae. Phase shift occurs to varying degrees in growth stationary cultures (Boemare and Akhurst, 1988). Phase I variants produce protoplasmic crystalline inclusions in stationary period cultures (Boemare *et al.*, 1983a), are motile by means of peritrichous flagella and may swarm on 0.6–1.2% agar (Givaudan *et al.*, 1995), and are lecithinase positive on egg yolk agar. Phase II variants lack dye adsorption (Akhurst, 1980), antibiotic production (Akhurst, 1982), protein inclusions and some other characteristics of phase I variants. Lipase is detected with Tween-20® (Sigma, St Louis, USA) and most strains hydrolyse Tweens-40, 60, 80 and 85; lipolytic responses are more positive with phase II than phase I variants. Some phase II strains are motile, but this is generally a property of phase I variants. Most strains are positive for deoxyribonuclease and protease. Biochemical identification of *Xenorhabdus* within the family Enterobacteriaceae and the main characters of the species are summarized in Tables 2.1 and 2.2. *Xenorhabdus* are only known from the intestinal lumen of entomopathogenic nematodes of the Family Steinernematidae and insects infected by these nematodes.

2.3.1.2. *Xenorhabdus beddingii*

The upper threshold for growth is 39°C. Both variants are motile (Givaudan *et al.*, 1995). Phase I is highly unstable, producing a very stable phase II. The strains hydrolyse aesculin and the pigmentation is lightly brown. They are associated with two undescribed species of *Steinernema* from Australia (Akhurst, 1986a), one of which may be *S. longicaudum* which was described from China.

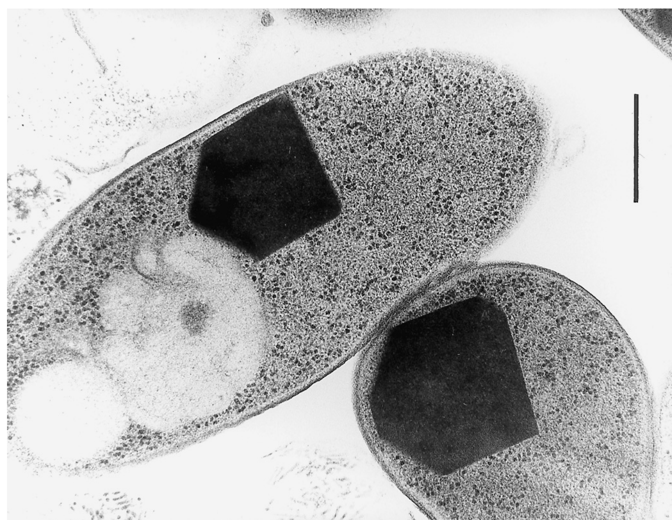

Fig. 2.2. Protoplasmic inclusions in *Photorhabdus temperata temperata*. Transmission electron microscopy after fast freeze fixation and cryo-substitution. After uranyl acetate and lead citrate dying, crystalline inclusions are highly contrasted (black areas) and the 'myelinic membranes' (clear areas) are poorly contrasted. The functional significance of these structures is unclear. Scale bar = 0.5 μm.

2.3.1.3. *Xenorhabdus bovienii*

This species grows until 32°C; some strains will grow at 5°C. Some phase II variants may be motile (Givaudan *et al.*, 1995). It is associated with several species of steinernematid nematodes (*S. feltiae, S. intermedium, S. kraussei, S. affine*) from temperate regions (Boemare and Akhurst, 1988; Fischer-Le Saux *et al.*, 1998).

2.3.1.4. *Xenorhabdus japonica*

Strains of the species grow until 35°C. Pigmentation is yellowish brown. It is only associated with *S. kushidai* in Japan. Properties of this species are similar to *X. nematophila*, and *X. japonica* may be a subspecies of *X. nematophila*.

2.3.1.5. *Xenorhabdus nematophila*

The upper threshold for growth in nutrient broth is 35°C. Neither phase is pigmented, but the phase I strongly adsorbs dyes (Akhurst, 1980). Surface appendages and proteins have been described with phase I variants in ultrastructural and molecular terms: occurrence of glycocalyx and fimbriae (Brehélin *et al.*, 1993; Moureaux *et al.*, 1995), a specific outer membrane protein OpnP (Forst *et al.*, 1995), and flagella with the regulation of their synthesis (Givaudan *et al.*, 1995, 1996; Givaudan and Lanois, 2000). Several strains are lysogenic, producing phages and bacteriocins after thermal or mitomycin C induction (Boemare *et al.*, 1992, 1993b; Baghdiguian *et al.*, 1993). This species is only found associated with *S. carpocapsae*.

2.3.1.6. *Xenorhabdus poinarii*

This is the most heat tolerant *Xenorhabdus* species, the upper threshold for growth for several strains being at 40°C. The intensity of pigmentation in phase I varies from light to reddish brown. May be motile phase II variants. In some strains, phase I does not produce antimicrobials; in other strains both phases do. Associated with *S. glaseri* (Akhurst, 1986b) and *S. cubanum* (Fischer-Le Saux *et al.*, 1999a). It is not pathogenic for greater wax moth (*Galleria mellonella)* larvae unless associated with its nematode partner.

Table 2.1. Characteristics differentiating *Xenorhabdus* and *Photorhabdus*, from the nearest genus, *Proteus*, within the Enterobacteriaceae.[a]

	Xenorhabdus	*Photorhabdus*	*Proteus*
Bioluminescence	–	+	–
Catalase	–	+	+
Annular haemolysis	–	d	–
Urea hydrolysis	–	d	+
Indole	–	d	d
H_2S production	–	–	[+]
Nitrate reduction	–	–	+
Acid from mannose [*Xenorhabdus*]	+	+	–

[a] + = 90–100% of strains are positive; [+] = 76–89% are positive; d = 26–75% are positive; [–] = 11–25% are positive; – = 0–10% are positive.

Table 2.2. Characters discriminating *Xenorhabdus* spp.[a]

	X. nematophila	*X. bovienii*	*X. poinarii*	*X. beddingii*	*X. japonica*
Isolated from:					
S. carpocapsae	+	–	–	–	–
S. feltiae, S. intermedium, S. affine, S. kraussei	–	+	–	–	–
S. glaseri, S. cubanum	–	–	+	–	–
Unidentified, *Steinernema* spp.	–	–	–	+	–
S. kushidai	–	–	–	–	+
Upper threshold T°C for growth	35	32	40	39	35
Pathogenicity for lepidopteran insects	+	+	–	+	–
Motility	d	d	d	+	d
Pigmentation	ow	y	br	lb	yb
Simmon's citrate	+	+	+	+	–
Aesculin hydrolysis	–	–	d	+	–
Phenylalanine deaminase	d	[–]	[–]	–	d^{w}
Tryptophan deaminase	–	+	–	$+^{w}$	–
Acid production from:					
myo-Inositol	$+^{w}$	d^{w}	–	–	–
Ribose	–	+	–	+	–
Salicin	–	–	–	+	–
Utilization of:					
Diaminobutane	–	[+]	–	–	–
D,L-Glycerate	+	+	[–]	+	–
L(–)Histidine	–	+	[+]	+	–
myo-Inositol	$+^{w}$	d^{w}	–	–	–
Ribose	–	[+]	–	+	–
L-Tyrosine	–	[+]	–	+	–
Lecithinase (egg yolk agar)	d	d	–	d	d
Lipase (Tween-80)	d	+	$+^{w}$	$+^{w}$	–

[a] All tests were done at 28°C; + = 90–100% of strains are positive; [+] = 76–89% are positive; d = 26–75% are positive; [–] = 11–25% are positive; – = 0–10% are positive. The superscript w (e.g. $[+]^{w}$) indicates a weak reaction; F = fermentative. Pigmentation: ow = off-white; y = yellow; br = brown; lb = light brown; yb = yellowish brown.

2.3.2. Genus *Photorhabdus*

Poinar *et al.* (1977) isolated a bacterium from *Heterorhabditis bacteriophora* (Poinar, 1976), and proposed inclusion of this luminous species in the genus *Xenorhabdus, as 'X. luminescens*' (Thomas and Poinar, 1979). It was later transferred to *Photorhabdus luminescens* comb. nov. (Boemare *et al.*, 1993a).

By using the hydroxyapatite method (Akhurst *et al.*, 1996) and the S1 nuclease method (Fischer-Le Saux *et al.* , 1999b) to analyse DNA–DNA *Photorhabdus*

heteroduplexes, two DNA relatedness groups associated with *Heterorhabditis* nematodes and another which contains human clinical specimens (Farmer *et al.*, 1989), were recognized.

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), as described below. 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, Australia, 2000, personal communication). The one non-luminous strain (Akhurst and Boemare, 1986) could be yet another species (Akhurst *et al.*, 1996).

2.3.2.1. Main characters of the genus

Photorhabdus cells are asporogenous, rod-shaped (0.5–2 × 1–10 μm), Gram negative, and motile by means of peritrichous flagella. They are facultatively anaerobic, having both respiratory and a fermentative metabolism. The optimum growth temperature is usually 28°C; some strains grow at 37–38°C. All strains are catalase positive, but do not reduce nitrate. They are negative for many characters of Enterobacteriaceae. They hydrolyse gelatin, and most strains are haemolytic for sheep and horse blood, some producing an unusual annular haemolysis on sheep blood at 25°C. All are lipolytic on Tween-20 and many on Tweens-40, 60, 80 and 85. Glucose is acidified without gas. Fructose, D-mannose, maltose, ribose, and *N*-acetylglucosamine are also acidified. Fermentation from glycerol is weak. Fumarate, glucosamine, L-glutamate, L-malate, L-proline, succinate and L-tyrosine are utilized as sole carbon and energy sources. Spontaneous phase shift occurs in subcultures, inducing the appearance of phase II clones. Phase I variants, which are the wild clones freshly isolated from the natural environment, produce protoplasmic inclusions synthesized inside a high proportion of cells (50–80%) during the stationary period (Fig. 2.2), and are luminous, emitting generally several wavelengths as white light, usually detectable by the dark-adapted eye. The intensity varies within and between strains and may only be detectable by photometer or scintillation counter in some isolates. Only one non-luminous isolate (Akhurst and Boemare, 1986) is known among the approximately 150 reported strains. Phase I variants produce light more than 100-fold greater than phase II variants. Phase I variants produce pink, red, orange, yellow or green pigmented colonies on nutrient agar, and especially on rich media (tryptic soy agar, egg yolk agar). Phase II variants are less pigmented or produce a different pigment. Phase II variants are mainly characterized by the loss of neutral red adsorption on MacConkey agar, and production of antibiotics.

Unfortunately, it appears that the type strain of the genus from *P. luminescens* (strain Hb) is not a good representative of the *Photorhabdus* isolates, because as numerous new isolates are typed, none have been found to belong to this taxon. So far, strain Hm remains the only one closely related to type strain Hb. Differential characters within the Enterobacteriaceae and species characteristics are listed in Tables 2.1 and 2.3.

2.3.2.2. *Photorhabdus luminescens*

The upper threshold for growth in nutrient broth occurs at 35–39°C. Strains are indole positive. Most strains produce weak acid from fructose, *N*-acetylglucosamine,

Table 2.3. Characters discriminating *Photorhabdus* spp.[a]

	Photorhabdus luminescens isolation from: *Heterorhabditis* spp.	*P. luminescens luminescens* isolation from: *H. bacteriophora* group Brecon	*P. luminescens laumondii* isolation from: *H. bacteriophora* group HP88	*P. luminescens akhurstii* isolation from: *H. indica*	*Photorhabdus temperata* isolation from: *Heterorhabditis* spp.	*P. temperata temperata* isolation from: *H. megidis* Palaeartic group	*Photorhabdus asymbiotica* isolation from: human blood and wounds
Upper threshold T °C for growth	35–39	38–39	35–36	38–39	33–35	34	37–38
Protoplasmic inclusions	+	+	+	+	+	+	–
Dye adsorption	+	+	+	+	+	+	–
Antimicrobial production	+	+	+	+	+	+	–
Indole production	+	+	+	d	[–]	–	–
Simmon's Citrate	d	+	d	d	d	d	$+^{w}$
Aesculin hydrolysis	+	+	+	+	[+]	d	+
Urease, Christensen's	D	–	[+]	D	D	[–]	+
Phenylalanine deaminase	[–]	–	D	–	[+]	D	–
Tryptophan deaminase	[–]	–	D	–	$[-]^{w}$	–	–
Acid production from:							
myo-Inositol	d	+	[+]	[+]	d^{w}	[–]	d^{w}
D-Mannitol	d	d^{w}	–	+	[–]	–	–
Trehalose	$[+]^{w}$	$+^{w}$	$[+]^{w}$	$[+]^{w}$	[+]	+	[+]
Utilization of:							
L-Fucose	d	d	–	[+]	d	+	–
D,L-Glycerate	[–]	d	–	–	+	+	d
L(–)Histidine	d	+	$[+]^{w}$	d	[+]	+	d
myo-Inositol	+	+	+	+	[+]	d	d
D,L-Lactate	[–]	–	–	d^{w}	–	–	–
D-Mannitol	d	+	–	+	[–]	–	–
Lecithinase (egg yolk agar)	+	+	+	+	+	+	–
DNase	[–]	–	+	–	+	+	–
Annular haemolysis (sheep blood agar)	D	+	[–]	+	+	$+^{w}$	+
Annular haemolysis (horse blood agar)	d	+	–	d	+	+	+

[a] All tests were done at 28°C; + = 90–100% of strains are positive; [+] = 76–89% are positive; d = 26–75% are positive; [–] = 11–25% are positive; – = 0–10% are positive. The superscript w (e.g. $[+]^{w}$) indicates a weak reaction; F = fermentative.

glucose, glycerol, maltose, mannose, ribose, and trehalose. Some strains acidify mannitol. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *H. bacteriophora* (Brecon and HP88 subgroups) and *H. indica*. This species is divided in three subspecies.

2.3.2.3. *Photorhabdus luminescens* subsp. *luminescens*

The upper threshold for growth in nutrient broth occurs at 38–39°C. Aesculin is hydrolysed and indole is weakly positive. DNase, tryptophan deaminase and urease are negative. Annular haemolysis of sheep and horse blood agars is characteristic of the group. This subspecies does not use D,L-lactate as the sole source of carbon. Mannitol is used as the sole source of carbon and energy. It is symbiotically associated with nematodes from the Brecon subgroup of *H. bacteriophora*, the type species of the genus *Heterorhabditis* (Poinar, 1976).

2.3.2.4. *Photorhabdus luminescens* subsp. *laumondii*

This subspecies has an upper threshold for growth in nutrient broth that is lower than the previous subspecies (35–36°C). Aesculin is hydrolysed, indole and DNase are positive. The tryptophan deaminase is variable and urease is mostly positive. Haemolysis on sheep and horse blood agars is total and the *Photorhabdus* annular reaction is rare. It does not use L-fucose, D,L-glycerate, D,L-lactate, or mannitol. It is symbiotically associated with nematodes of the HP88 subgroup of *H. bacteriophora* isolated in South and North America, southern Europe, and Australia, corresponding to the satellite DNA probe of the nematode strain HP88 (Grenier *et al.*, 1996a,b).

2.3.2.5. *Photorhabdus luminescens* subsp. *akhurstii*

This subspecies, the first one described, has an upper threshold for growth in nutrient broth at 38–39°C. Aesculin is hydrolysed, but tryptophan deaminase and DNase are negative. Urease and indole are variable. Annular haemolysis is observed on sheep blood agar. The use of D,L-lactate as the sole source of carbon is variable and weak when positive. Mannitol is used and acidified, but the D,L-glycerate is negative. It is symbiotically associated with the nematode *H. indica* isolated in tropical and sub-tropical regions.

2.3.2.6. *Photorhabdus temperata*

Colonies are highly luminous. The upper threshold for growth in nutrient broth occurs at 33–35°C. The DNase is positive and most of the strains are indole negative. Aesculin hydrolysis and tryptophan deaminase are mostly positive. Urease is variable. Acid is produced from fructose, *N*-acetylglucosamine, glucose, mannose, ribose, and weakly from glycerol and maltose. Annular haemolysis often occurs on sheep and horse blood agars. This species uses D,L-glycerate, but does not use D,L-lactate as the sole source of carbon. Mannitol is not used by most strains. The natural habitat is in the intestinal lumen of entomopathogenic nematodes of *H. megidis*, of NC subgroup of *H. bacteriophora*, and of *H. zealandica*.

2.3.2.7. *Photorhabdus temperata* subsp. *temperata*

This subspecies often produces several intermediate forms between the two extreme phase I and II variants (Gerritsen *et al.*, 1992). Strains cannot grow above 34°C. Indole

is negative and DNase is positive. Hydrolyses aesculin but tryptophan deaminase is variable. Urease is mostly negative. Annular haemolysis on sheep and horse blood agars is observed in most isolates. The subspecies uses D,L-glycerate and L-fucose, but does not use D,L-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*.

2.3.2.8. *Photorhabdus asymbiotica*

Although this species is not symbiotic with nematodes, it must be mentioned due to its similarities with the symbiotic *Photorhabdus*. The upper threshold for growth in nutrient broth occurs at 37–38°C. Produce a yellow or brown pigment. No phase I isolates have been detected and isolates do not absorb dyes, sometimes weakly produce antibiotics, and are negative for lecithinase on egg yolk agar. They are positive for urease, aesculin hydrolysis, and for Christensen's citrate, but weakly positive on Simmons' citrate. Tryptophan deaminase, indole, and DNase are negative. Acid is produced from fructose, *N*-acetylglucosamine, glucose, maltose, mannose, ribose, and weakly produced from glycerol. Protoplasmic inclusions are poorly produced. Tween-40 esterase is variable. The typical annular haemolysis of *Photorhabdus* of sheep and horse blood agars (Akhurst *et al.*, 1996) was first shown with this species (Farmer *et al.*, 1989). Does not use L-fucose, D,L-lactate, or mannitol. The natural habitat is uncertain. All isolates have been obtained from human clinical specimens.

2.4. Co-speciation Between the Bacterial Symbionts and the Nematode Hosts

When we compare the taxonomic data of *Xenorhabdus* with that of their host nematodes, the close relatedness of the two taxonomic structures reveals co-speciation between bacterium and nematode (Table 2.4). There are *X. nematophila* and *S. carpocapsae*, *X. japonica* and *S. kushidai*, three or four *Xenorhabdus-Steinernema* associations in the course of characterization, *P. luminescens akhurstii* and *H. indica*, *P. temperata temperata* and the palaearctic group of *H. megidis*.

2.4.1. Taxonomic uncertainties do not invalidate the concept of co-speciation

However, sometimes one species of *Xenorhabdus* can share several species of *Steinernema*. As mentioned previously *X. bovienii* is symbiotic for *S. affine*, *S. feltiae*, *S. kraussei* and *S. intermedium; X. beddingii* is harboured by two undefined species of nematodes from which one would be possibly *S. longicaudum* (R. Akhurst, Australia, 2000, personal communication); *X. poinarii* is symbiotic for *S. cubanum* and *S. glaseri*. Conversely, two *Photorhabdus* subspecies are harboured by one species of *Heterorhabditis* (*P. luminescens luminescens* and *P. luminescens laumondii* by *H. bacteriophora*). These reports do not modify the concept of co-speciation. They are essentially the result of taxonomic uncertainties corresponding to the definition of species in bacteriology and nematology. Bacteriologists cannot use the cross-breeding method used by other biologists to delineate species. A species in bacteriology is an artificial concept. It is defined as a group of strains sharing approximately 70% or greater DNA/DNA relatedness with 5°C or less of temperature variation of DNA denaturation (ΔT_{m}) (Wayne *et al.*, 1987). When other criteria will be more accurate, subdivisions would be possible.

Table 2.4. Species associations.

Steinernema and *Xenorhabdus*	
S. kraussei	*Xenorhabdus bovienii*
S. abbasi	*Xenorhabdus* sp.
S. arenarium (synonym: *S. anomalae*)	*Xenorhabdus* sp.
S. affine	*Xenorhabdus bovienii*
S. bicornutum	*Xenorhabdus* sp.
S. carpocapsae	*Xenorhabdus nematophila*
S. cubanum	*Xenorhabdus poinarii*
S. feltiae	*Xenorhabdus bovienii*
S. glaseri	*Xenorhabdus poinarii*
S. intermedium	*Xenorhabdus bovienii*
S. kushidai	*Xenorhabdus japonica*
S. longicaudum(?)	*Xenorhabdus beddingii*
S. monticulum	*Xenorhabdus* sp.
S. puertoricense	*Xenorhabdus* sp.
S. rarum	*Xenorhabdus* sp.
S. riobrave	*Xenorhabdus* sp.
S. scapterisci	*Xenorhabdus* sp.
S. serratum	*Xenorhabdus* sp.
Heterorhabditis and *Photorhabdus*	
H. bacteriophora subgroup Brecon	*Photorhabdus luminescens luminescens*
H. bacteriophora subgroup HP88	*Photorhabdus luminescens laumondii*
H. indica	*Photorhabdus luminescens akhurstii*
H. zealandica	*Photorhabdus temperata*
H. bacteriophora subgroup NC	*Photorhabdus temperata*
H. megidis Nearctic group (Ohio, Wisconsin)	*Photorhabdus temperata*
H. megidis Palaearctic group	*Photorhabdus temperata temperata*
Clinical opportunistic strains	*Photorhabdus asymbiotica*

For instance, different genotypes of PCR–RFLP of 16S rDNA have been recognized for *X. bovienii* (Brunel *et al.*, 1997; Fischer-Le Saux *et al.*, 1998), underlying the possibility that bacterial subspecies could be defined shortly based on 16S rDNA sequence, and surprisingly each one would correspond to *S. feltiae, S. affine, S. intermedium,* and *S. kraussei* (Boemare, unpublished data).

Similarly difficulties may also occur with the definition of nematode species as in the case of *S. cubanum* and *S. glaseri*. When *S. cubanum* was described, it was considered to be a related species of *S. glaseri* (Mrácek *et al.*, 1994). Morphological characters and the restriction analysis of the intergenic transcribed spacer (ITS) of the ribosomal genes reveal high similarities between the two species (Hominick *et al.*, 1997).

The only remaining enigma is the NC strain of *H. bacteriophora,* which harbours *P. temperata* and not a subspecies of *P. luminescens* as other symbionts of *H. bacteriophora*. The re-isolation of this group in nature is required to control for possible confusion in previous sampling.

2.4.2. Correlation between symbiont and host heat tolerances

Within both bacterial genera, the upper threshold for growth temperature appears to be a relevant character for distinguishing species (Tables 2.2 and 2.3). If we examine the ecology of the corresponding strains, it is noticeable that this character and the host species origin are correlated. For example, *X. bovienii* and *P. temperata* strains are 'cold' adapted symbionts of nematodes (*S. feltiae* and *H. megidis*) living in temperate cilmates, while *X. poinarii* and *P. luminescens akhurstii* are 'warm' adapted symbionts of nematodes (*S. glaseri, S. cubanum* and *H. indica*) from tropical and sub-tropical areas (Fischer-Le Saux *et al.*, 1999a,b). Thus, temperature tolerance appears to be an important property reflecting long-term adaptation to different climatic conditions in bacterium–helminthic complexes.

2.4.3. Mechanisms of co-evolution

The phenomenon of co-speciation results from co-evolution 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. In the case of *Xenorhabdus*, preservation of the bacterial cells from digestion by the infective juveniles during the free life cycle of the host seems to be ensured by an anatomical sanctuary: the special vesicle in the intestine of the infective juveniles (Bird and Akhurst, 1983). This is a clear co-evolutionary trait, not appearing in other species of free-living soil-dwelling nematodes in the Order Rhabditida. Specific attachment by the symbiotic bacteria to this organ ensures the maintenance of the symbiosis. However, the molecular basis of such associations is unknown, even if bacterial fimbriae and glycocalyx are supposed to be the structures that support the specific attachment during starvation in the host (Brehélin *et al.*, 1993; Moureaux *et al.*, 1995).

Poinar (1993) suggested that *Heterorhabditis* evolved from a marine ancestor. On the basis of biological, taxonomic and ecological arguments, the genus *Heterorhabditis* evolved 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.

2.5. Conclusions and Future Prospects

Further microbial ecology studies are needed to explain the durability over many generations of these intestinal symbiotic associations. The concept of a natural monoxeny in these bacterium–helminthic symbioses must be accepted if we consider the following feature. In natural conditions, if any bacterium is carried in the infective juveniles, perhaps the resulting complex could kill insects, but the probability of the nematode reproducing is low.

Despite the high similarity of their life cycles, *Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* symbioses are widely divergent. The similarities in the patterns of infectivity 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.*, 1997), do

not necessarily imply the same physiological mechanisms. Current genetic studies will probably formulate a clearer picture to explain the convergent evolution of *Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* symbioses.

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