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Symbiosis and Pathogenicity of Nematode-Bacterium Complexes

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Abstract

The present knowledge on the properties of the entomopathogenic nematode-bacterium complexes is reviewed. The bacterial ectosymbionts, *Xenorhabdus* and *Photorhabdus*, are closely associated to their host nematodes, *Steinernema* and *Heterorhabditis*, respectively, despite their intestinal localization. The specificity of each symbiont for the nematode species is clearly established by taxonomic studies from freshly harvested infective juveniles from the nature, and by laboratory gnotobiological experiments. Both bacterial genera belong to Enterobacteriaceae, but are nitrate-reductase negative. *Photorhabdus* are catalase positive, and are the only known terrestrial bacterium able to emit light similarly as the marine bioluminescent bacteria. These symbioses are a good model of co-evolution where four biotic levels have to be investigated: defense reactions of the insect target, nematode pathogenicity, bacterium pathogenicity, and symbionts lysogeny. The high entomopathogenicity noticed for both bacterial genera is the most relevant point which has to be correlated with the pathogenic action of these complexes. Some associations show an obvious co-operation between symbiotic partners for killing the insects. Bacteria are unable to through the natural barriers of the insects (intestine or tegument), but are highly pathogenic by inoculation. In all cases the nematode acts as a vector, inoculating bacteria in the insect hemolymph. During the

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nematode reproduction, and before the recruitment of the symbionts by the infective juveniles escaping the insect cadaver, the helminthic-bacterium association is protected from bacterial contaminants by an important production of several antimicrobial barriers (antibiotic molecules, bacteriocins and phages). *Xenorhabdus* and *Photorhabdus* occur in two forms as the result of a phase variation. Depending on strains, phase I is characterized by protoplasmic inclusions, surface appendages, and produces antimicrobial molecules and exoenzymes, while phase II does not show such properties or express them very weakly. The genetic mechanism of such a phenomenon is presumably regulated differently in both genera depending on environmental conditions. Phase I provide better conditions for nematode reproduction and are always isolated from natural samples. Phase II appears after *in vitro* cultures and during the reproduction in insects. The truth is certainly in the following syllogism: properties have been defined for phase variation, these properties seem to be necessary for symbiosis, so phase variation of bacterial partners should be an essential property of the symbiosis. *Photorhabdus* and *Xenorhabdus* possess several common properties which are probably elements of a convergent evolution necessary for the association with two phylogenetically different entomopathogenic nematodes.

Keywords: Gnotobiology, co-evolution, pathology, invertebrate immunology, bacteriological phase variation, taxonomy, *Steinernema, Heterorhabditis, Xenorhabdus, Photorhabdus*

1. Introduction

The Steinernematidae and the Heterorhabditidae are widely available for use in biological control. Our knowledge about the Steinernematidae arose from the beginning of this century with the discovery *of Steinernema* (= *Aplectana*) *kraussei* by Steiner (1923), and the first *Heterorhabditis* described by Poinar (1976) and Khan et al. (1976). All these nematodes are characterized by their mutualistic relationship with bacteria of the genera *Xenorhabdus* for Steinernematidae (Thomas and Poinar, 1979) and *Photorhabdus* for Heterorhabditidae (Boemare et al., 1993). After their penetration in the body cavity of the insect, nematodes breach the defense reactions, release their symbiotic bacteria inducing a septicemia and the target dies. Because these nematodes serve as vectors of bacteria, we have to adopt the term of entomopathogenic nematodes rather than "entomophilic", "entomogenous", "entomophagous" or "insect-parasitic nematodes" (Gaugler and Kaya, 1990). In fact these nematodes occupy a position between predators and microbial agents.

They can be mass reared and stored on a scale only imaginable with some microbial pathogens. They possess extreme virulence for insects at a non-specific broad host range (Gaugler and Kaya, 1990). Among 128 insect species tested in laboratory conditions, only 28 belonging mainly to Diptera were shown to be somewhat resistant to *Steinernema carpocapsae* strain DD136

(Laumond et al., 1979). In addition they are safe for mammals. These attributes have encouraged several companies to attempt to develop steinernematid and heterorhabditid as biological control agents (Georgis and Grewal, 1994).

2. Specificity of Symbiosis

Taxonomic studies of symbionts and their host nematodes have defined that almost each species of nematode possesses a specific symbiont species. This specificity was analyzed by using gnotobiological experiments. The gnotobiology is a part of the microbial ecology which studies the relationships between animals and their associated microbial populations. To undertake such studies the main tool is the use of germ-free animals reared on artificial media substituting the nutrients normally provided by the microbial partners. Gnotobiological terms are the following: an axenic animal is an animal living and reproducing free from any micro-organism; a mono-, di-, tri-, .., poly-xenic animal is an animal living and reproducing after an association with one, two, three, ..., several defined micro-organisms; a gnotoxenic animal is an animal which carries well defined micro-organisms. When the association is made with the natural microflora previously identified we obtain holoxenic animals and, when it is made with foreign microflora (from another host species) we obtain heteroxenic nematodes. For maintaining monoxenic animals, as the axenic rearing, they have to be transferred in sterile conditions to prevent any contamination. In fact, gnotobiological experiments show that the specificity is often defined at the level of strain, whereas bacteriological and nematological methods are unable to recognize differences between strains.

Gnotobiology: Axenic and gnotoxenic nematode rearing

If on the point of view of the pathology these nematodes are able to infect a broad host range of insects, in terms of symbiosis the relationship between nematode and symbiont is very close. Today *Steinernema* axenic rearing is possible, but a substitute diet has not yet been discovered for *Heterorhabditis*. Living axenic nematodes mean that a complete life cycle with progeny can be obtained without any digestive contribution of micro-organisms. Axenic *Steinernema* are not produced on artificial diet in a yield comparable to the natural conditions. However they are viable during several generations. For *Heterorhabditis* we are just able to disinfect eggs and combine them immediately with their symbionts to obtain sure monoxenic associations. Several examples were reported with this kind of experiments (Boemare et al., 1983a; Gerritsen et al., 1992) which mainly have established the specificity of

each symbiont for its host and the difficulty for establishing heteroxenic associations. When these heteroxenic associations are viable during several generations, most of the examples show that it is with a closely related bacterial strain. But at this stage we have to compare the yield of reproduction, the quality in physiological and pathological terms, from those of the holoxenic animals. The best is the retention test of the symbiont, meaning that we have to probe during several generations the keeping of symbionts in the resting stage L3 (Akhurst and Boemare, 1990).

Gnotobiology: Ecological niches and artificial cultures of symbionts

If such gnotobiological experiments are necessary to define more precisely symbiotic relationships, this is because alternatively we are able to cultivate the symbionts by themselves. The major feature on this respect is the intestinal location of *Xenorhabdus* and *Photorhabdus* in the juveniles L3. These bacteria can be cultivated on nutritive agars and are studied separately from their natural host. This is very convenient for defining some of their in vitro properties. However we consider that they have the status of obligate symbionts, because they are essential for their nematode host which cannot multiply correctly without their physiological contribution. Within some Steinernematidae, *Xenorhabdus* are located in a special vesicle (Bird and Akhurst, 1983), and within Heterorhabditidae, *Photorhabdus* are housed in the anterior part of the gut.

Symbiont multiplication does not apparently occur in the juveniles and bacteria are not yet released in the insect gut before entering together with nematodes into the insect hemolymph. Some unpublished data estimated that 100–200 bacterial cells occur by *Steinernema*, meaning that they are in a sort of starvation (like their L3 host larvae). All the experiments reported today of symbiont isolation from nematodes indicate the presence of *Xenorhabdus* or *Photorhabdus*. According to the method of Akhurst (1980), by external axenization of juvenile larvae, crushing, and streaking on plates the obtained suspensions, they can be isolated. But occasionally, other bacterial species can be also isolated (Boemare et al., 1983a; Aguillera et al., 1993). When they are isolated from a hemolymph hanging drop according to Poinar and Thomas' technique (1966), or after collecting a hemolymph drop from two days parasitized insects, the symbionts are growing and can be duplicated faster. This demonstrates that insect hemolymph is the best medium in terms of selection and enrichment to elicit growth of these bacteria. Although these Enterobacteriaceae are borne in the nematode gut, they multiply in natural conditions essentially in the body cavity of the parasitized insect.

Bacteriological background

Xenorhabdus and *Photorhabdus* are chemoheterotrophic bacteria with respiratory and fermentative metabolism, and belong to the family of Enterobacteriaceae (Thomas and Poinar, 1979; Grimont et al., 1984). We consider that they are atypical Enterobacteriaceae because most of *Xenorhabdus* and *Photorhabdus* are nitrate-reductase negative (as only some strains of *Erwinia* and *Yersinia*), and in addition *Xenorhabdus* are catalase negative (some strains of Shigella dysenteriae O group 1 are the few other examples in this family). Several bacteriological data (Table 1), the poor DNA relatedness, and biological properties, strongly suggested the separation of *Xenorhabdus* and *Photorhabdus* into two genera (Boemare et al., 1993). However the sequence comparisons of 16S rRNA genes have a higher value to establish phylogenetic relationships between bacterial genera. Thus such analyses, from 4 strains of *Xenorhabdus* and 3 strains of *Photorhabdus*, were unable to establish as clear a separation between these two "sister" genera, but confirmed a good clustering among many other Enterobacteriaceae (Rainey et al., 1995).

Table 1. *Photorhabdus* and *Xenorhabdus* major discriminative characters

	Xenorhabdus	*Photorhabdus*
Bioluminescence	–	+
Catalase	–	+
Entomopathogenicity	d	+
Urease	–	d
Indole	–	d

+ = positive; – = negative; d = varies with strain or biovar.

For the delineation of species and measurement of intraspecies relationships, the "*Ad hoc* Committee of approaches to bacterial systematics" (Wayne et al., 1987) established that approximately 70% or greater DNA-DNA relatedness with 5°C or less ΔT_m, and phenotypic characteristics, are appropriate. On this basis some species of *Xenorhabdus* can be distinguished by phenotypic data (Akhurst and Boemare, 1988) and DNA relatedness (Boemare et al., 1993). More generally, taxonomic studies have defined clear groups with *Xenorhabdus* spp., but it was awkward with *Photorhabdus*, which is apparently a more homogeneous genus (Akhurst et al., 1996).

Correlation between symbionts and hosts taxonomies

These two sister genera share several common properties apparently linked with nematode symbiosis, but all the present recorded bacteriological and gnotobiological data indicate that they are basically different. It has been suggested that similarities between *Xenorhabdus* and *Photorhabdus* are the result of a convergent evolution between two different bacterial genera (Akhurst and Boemare, 1994; Boemare and Akhurst, 1994) associated with two phylogenetically different nematode genera, *Steinernema* and *Heterorhabditis*, respectively (Poinar, 1993).

X. nematophilus is the symbiont species of *S. carpocapsae, X. poinarii* of *S. glaseri, X. beddingii* of another unnamed *Steinernema* sp (Akhurst and Boemare, 1988). For the other *Xenorhabdus* associated with *S. anomalae, S. rarum, S. ritteri,* differences in phenotypic and DNA-DNA hybridization have been noticed. However, only one bacterial strain was isolated for each nematode species which does not allow the definition of new *Xenorhabdus* spp. In the case of *S. scapterisci,* different associated bacterial genera have been also reported (Aguillera et al., 1993). *Xenorhabdus bovienii* is the only species in which bacteriological studies undertaken have not led to distinguish parallel differences with those of their four host species: *S. affine, S. intermedium, S. kraussei, S. feltiae* (Table 2). Studies in progress will allow the definition of further sub-species at this level. However, using probes hybridizing against the region at positions 455 through 480 of 16S rRNA, it was reported that a *Xenorhabdus* strain D-1.1 isolated from *S. affine,* was a distinctive species (Pütz et al., 1990). This strain should be compared with other *Xenorhabdus* strains isolated from the same host. However, the comparative studies have clearly revealed the limitations of 16S rRNA polymorphism analyses in the determination of relationships at the strain level, for which DNA-DNA hybridization experiments still constitute a superior method (Stackebrandt and Goebel, 1994). At this stage we do not possess enough data to establish all the species of *Xenorhabdus.*

3. *Xenorhabdus/Steinernema* and *Photorhabdus/Heterorhabditis*: Models of Co-Evolution

These helminthic-bacterium complexes are a fascinating model of co-evolution which comprises three biotic interactions: relationship between the insect and the parasite, symbiosis between the nematode host and its bacterial symbiont, and the interaction between the bacterium and its phage. So to investigate these three interactions, we have to concentrate the physiological studies at four biotic levels: (i) the study of the insect defense reactions, (ii)

Table 2. Some correspondences between nematodes[1] and symbionts

Hosts : Steinernematidae[2]			Bacteria
1 *Steinernema carpocapsae* Weiser 1955			*Xenorhabdus nematophilus* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
DD136	*Carpocapsa .pomonella*	VA, USA	DD136
Mexican	*Carpocapsa .pomonella*	Allende, Mexico	Mex
Agriotos	*Agriotes lineatus*	St.Petersburg, Russia	A24
All	*Vitacea polistiformis*	GA, USA	All
K27, Breton	*Otiorrhynchus sulcatus*	Plougastel, Bretagne, France	F1
2 *Steinernema glaseri* Steiner 1929			*Xenorhabdus poinarii* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
NC34, NC40	Soil	NC, USA	NC40
NC33	Soil	NC, USA	NC33
G6		NC, USA	G6 (UQM 2216)
3 *Steinernema feltiae* Filipjev, 1934			*Xenorhabdus bovienii* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
T335	*Otiorhynchus sulcatus*	Tasmania, Australia	T335
K60	Soil	Berry, France	F5
K53	Soil	Champagne, France	F7
4 *Steinernema affine* Bovien, 1937			*Xenorhabdus bovienii* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
DK	Bibionidae	Danemark	Dan
K47	Soil	Picardie, France	F3
5 *Steinernema kraussei* Steiner, 1923			*Xenorhabdus bovienii* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
SK2	Tenthreidae	Czeck Republic	SK2
6 *Steinernema intermedium* Poinar, 1985			*Xenorhabdus bovienii* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
SC	Soil	SC, USA	Si

Table 2. Continued

7 *Steinernema kushidai* Mamiya, 1988			*Xenorhabdus japonicus* Nishimura et al., 1994
Strain	Origin	Location	Strain
Hamakita	*Anomala cuprea*	Shizuoka, Japan	SK1
8 *Steinernema anomalae* Kozodoi, 1984			*Xenorhabdus* sp.
Strain	Origin	Location	Strain
Voronez	*Anomala dubia*	Voronezh, Russia	Sav
9 *Steinernema rarum* Doucet, 1986			*Xenorhabdus* sp.
Strain	Origin	Location	Strain
K77	*Heliothis* sp.	Cordoba, Argentina	K77
10 *Steinernema scapterisci* Nguyen & Smart, 1990			*Xenorhabdus* sp. and other gen. Aguillera et al., 1993
Strain	Origin	Location	
Uruguay	*Scapteriscus* sp.	Uruguay	
11 *Steinernema cubanum* Mracek, Arteaga, Boemare, 1994			*Xenorhabdus* sp.
Strain	Origin	Location	Strain
Pinar	*Pachnaeus litus*	Pinar del Rio (Troncoso)	Cub1
12 *Steinernema* sp.			*Xenorhabdus beddingii* Akhurst & Boemare, 1988
Strain	Origin	Location	Strain
Q58		Qld., Australia	Q58 (UQM 2872)

Hosts : Heterorhabditidae[3]			Bacteria
1 *Heterorhabditis bacteriophora* Poinar, 1976			*Photorhabdus luminescens* Boemare, Akhurst & Mourant, 1993
Strain	Origin	Location	Strain
Hb	*Heliothidis punctigera*	Brecon, SA, Australia	Hb (ATCC 29999)
NC1	*Heliothis zea*	Clayton, NC, USA	C1 (ATCC 29304)
Hm		Milwaukee, WI, USA	Hm
HP88	*Phyllophaga* sp.	Logan, UT, USA	HP88

Table 2. Continued

2 *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987			*Photorhabdus luminescens* Boemare, Akhurst & Mourant, 1993
Strain	Origin	Location	Strain
H1	*Popillia japonica*	OH, USA	Meg
3 *Heterorhabditis zealandica* Poinar, 1990			*Photorhabdus luminescens* Boemare, Akhurst & Mourant, 1993
Strain	Origin	Location	Strain
T310		Sandy Bay, Tas., Australia	T310
T327		Nicholl's R. Tas., Australia	T327
NZH	*Heteronychus arator*	Auckland, N. Zealand	NZH3
4 *Heterorhabditis* sp.			*Photorhabdus luminescens* Akhurst & Boemare, 1986
Strain	Origin	Location	Strain
HQ614		Bundaberg, Australia	Q614
5 Clinical specimens			*Photorhabdus luminescens* Farmer et al., 1989
		Location	Strain
		CDC, Atlanta, USA	1216/79 (ATCC 43948)
		CDC, Atlanta, USA	2407/88 ATCC 43952)
		CDC, Atlanta, USA	2617/87 (ATCC 43951)
		CDC, Atlanta, USA	3105/77 (ATCC 43949)
		CDC, Atlanta, USA	3265/86 (ATCC 43950)

[1]Some species names have been modified according to the Latin grammar and will be confirmed soon by publications of the COST 819 Working Group I (European network for Science and Technology in the field of the Entomopathogenic nematodes); [2]*Steinernema* is the synonym of *Neoaplectana* (Wouts et al., 1982); [3]Most of these examples are listed according to Poinar (1990). Today *Heterorhabditis*, and their *Photorhabdus* symbionts, are in a complete revision relying on molecular techniques. So the mentioned Heterorhabditidae and their *Photorhabdus* symbionts have to be considered as a provisional information.

the biology and the pathology of nematodes, (iii) the study of pathological and symbiotic properties of the bacteria, (iv) the lysogeny occurring in the bacterial symbionts.

The following description attempts to explain all the potential properties of these helminthic bacterium complexes. It is oversimplified because it depends on each couple prey/parasite. Exceptions can occur during the sequence of the parasitic events. Particularly the immunological responses of the Diptera are more efficient and stop the nematode reproduction, but in many cases they are however unable to prevent the death of the insect host.

Pathology

The first biotic level: Defense reactions of the target

The first biotic level mentioned above, the defense reactions of the target, is a very exciting topic. The nematode broad host range can be explained by the efficient actions developed by the nematodes and their symbiotic bacteria to inhibit the insect defense reactions. When the nematode juveniles L3 enter via insect mouth, anus, trachea and/or tegument, they remove their old L2 cuticle (ex-sheathing) and presumably have a proteolytic activity (proteolytic penetrating factor = PPF) helping their penetration through the gut to the haemocoel (Simoes, 1994).

At this stage, there is a problem of recognition of juveniles as self or non-self by the insect. We have to remember that the recognition of foreign bodies in the insect proceeds by the production of some humoral factors, acting as opsonins. These opsonins are probably lectin like molecules, and different factors produced by the phenol-oxidase system. They direct the recruitment of hemocytes for phagocytosis and encapsulation (Brehélin et al., 1989). This phenol-oxidase system is triggered by endotoxins, some glucans or some serine-proteases such as trypsin. It is known that entomopathogenic organisms are able to depress or to escape the defense reactions in some insect species. The evasion systems can be grouped into two series: avoidance of recognition and/or avoidance of the defense system itself. *Steinernema* and *Xenorhabdus* are capable of these two strategies (Brehélin et al., 1990). Avoidance of recognition was observed without either coagulum formation, or hemocyte degranulation or lysis in contact with the nematode, suggesting that the surface of the cuticle is not recognized as foreign. In the same time, *Locusta* or *Galleria* are normally able to encapsulate foreign bodies which are present into the insect together with the infective nematodes (Brehélin and Boemare, 1988). In addition lipase, which is able to alter the epicuticle, induces hemocyte attachment (Dunphy and Webster, 1988). So in the case of an infestation in *Galleria*, avoidance of *Steinernema* recognition is not a general depression, but the lack of the parasite recognition by the host immune system.

However, whereas *Steinernema carpocapsae* are not recognized as non-self, nematodes make wounds and introduce foreign bacteria from their

contaminated tegument and from the gut lumen. We know that a sole sterile injury induces a local healing and a production of immune proteins (such as cecropins, attacins, diptericins) in most insect species. This phenomenon and the phagocytosis are enhanced by foreign bacteria. A correct wound-healing and a destruction of undesirable micro-organisms by the insect are often observed after the infestation. This feature should be checked accurately for each prey/parasite pair, to see if during an infestation provisional axenic conditions are restored by humoral and cellular insect reactions before release of the symbionts. In any event the monoxenic septicemia observed 24–48 h after the nematode penetration would be just the result of the antagonism between symbionts and other foreign microorganisms introduced into haemocoel (see below).

The second biotic level: Nematode pathogenicity

The second biotic level is the study of the nematode pathogenicity itself. The previous immune proteins secreted to kill foreign bacteria should be able to destroy also *Xenorhabdus* when they are released. The developing stages of the nematode secrete an immune-depressive factor against these immune proteins which was evidenced by *in vitro* experiments (Götz et al., 1981). In the natural conditions it is supposed that there is such an occurrence for protecting symbionts, but we need again *in vivo* experiments establishing the exact timing of these possible events. In addition, the complex may elaborate toxins which are able to finalize a non-reversing pathologic process as was demonstrated with axenic *S. carpocapsae* strain DD136 tested against axenic *Galleria* (Boemare et al., 1982; Laumond et al., 1989). The toxin purified from the strain Plougastel of *S. carpocapsae* is a heat sensitive protein of about 70 kDa. Its injection at 13.5 ppm induces grub paralysis 2–3 min later followed by the insect death (Simoes, 1994). But other examples do not support a such toxic effect: the strain AZ27, well identified as *S. carpocapsae* in Azores Islands, was discovered naturally free of symbionts and also unable to kill insects within 10 days (Simoes and Laumond, pers. comm.); germ free *S. glaseri* are not pathogenic (Akhurst, 1986). In case of *Heterorhabditis*, because axenic reproduction experiments were not yet obtained, nothing can be concluded in this respect.

So several results indicate that nematodes may possess pathological properties by themselves, able to destroy the immune system of the insect, and to protect their bacteria. What we can definitively assume is that they play an important part in the pathological process by acting as a living syringe for releasing their symbionts into the haemocoel of the insect host.

The third biotic level: Bacterium pathogenicity

In this particular situation, the release of the symbionts initiates a monoxenic septicemia in the insect hemolymph. For explaining the pathogenicity of the complex, we have to look at the bacteria too. This is the third level corresponding to the pathogenicity of the symbionts. In *Galleria, Xenorhabdus nematophilus* LD_{50} by injection was equivalent to 1–10 viable cells, underlining a high pathogenicity when injected, while *Xenorhabdus poinarii* LD_{50} on the same insect is above 10^4–10^5 viable cells, i.e. non-entomopathogenic (Akhurst and Boemare, 1990). *Xenorhabdus* strain SK1, symbiont of *S. kushidai,* is not pathogenic for *Spodoptera litura* (Yamanaka et al., 1992). More generally, the LD_{50} estimation highly depends on the insect tested (Pye and Burman, 1977; Wulff et al., 1994; Peters and Ehlers, 1994).

The pathogenic action of the symbionts is due to several properties. Ensign et al. (1990) reported the occurrence of an exotoxin of about 40 kDa in *Photorhabdus,* and recently Akhurst (pers. comm.) isolated an exotoxin of about 31 kDa from *Xenorhabdus nematophilus,* both efficient when injected into insects. These preliminary results did not indicate if these exotoxins are active *per os*. The incapability of these bacteria to be pathogenic by themselves when swallowed by insects with food has been reported many times. Experimental attempts of direct ingestion of bacterial broth cultures by insect did not induce any mortality (Boemare et al., 1983a). However, we have to point out that in natural conditions the production of exotoxin is obviously dependent on the bacterial growth and of particular conditions for its expression. Presumably, the exo-enzymatic complex secreted by these bacteria (proteases, lipases, phospholipases) would be also involved in the pathogenic action to facilitate the septicemia (Boemare and Akhurst, 1988). Furthermore LPS of these symbionts, which are the endotoxins of Gram-negative bacteria, can operate at the end of the bacterial growth in the hemolymph (Akhurst and Boemare, 1990).

The co-operation between the 2nd and 3rd levels in the entomopathological process

Several weapons inducing the death of the prey are mentioned above. There are quite frequent features of non recognition, helminthic toxins, an immune depressive factor, and above all the vector role of nematodes for inoculation of the entomopathogenic bacteria *Xenorhabdus* or *Photorhabdus* into the body cavity of insects. Bacteria are also highly virulent with the contribution of exotoxins. When the insect host is quite resistant, septicemia occurs with the active helping of the nematode vector. In many cases, it is quite obvious that both partners co-operate to kill the insect host. With axenic *S. glaseri,* or its sole symbiont *X. poinarii,* no entomopathogenic action was recorded, but when

both partners were re-associated the entomopathogenic action was restored (Akhurst and Boemare, 1990).

Consequently, depending on the insect hosts and on the nematode species, there is a series of different pathological events. They are specific for each couple of opponent species where action and reaction act differently. At this stage of our knowledge there is an urgency to define the *in vivo* exact timing of all these identified actions and reactions.

The fourth biotic level: Symbionts lysogeny

Lysogeny of *Xenorhabdus* and *Photorhabdus* strains is described in another section of this issue (Thaler et al., 1996). The effect of a low production of bacteriocins in natural conditions having a bactericidal action against other related bacteria was reported (Boemare et al., 1992). But the most important consequence of this phenomenon is the possible occurrence of physical or chemical actions, which induces a total lysis of the symbiont population. So this phenomenon may be antagonistic when a mutagenic stress can operate inducing a possible lethal process for the symbiont populations. However in natural conditions, in absence of any mutagenic stress, occurrence of bacteriocins is useful for the symbiosis (Boemare et al., 1994; Thaler, 1994) contributing to compete with closely related bacteria (see below).

Symbiosis

Ecological studies are absolutely necessary to evaluate in nature the estimated laboratory potency of these nematodes. These aspects are studied by people involved in soil ecology, biogeography of the nematodes and ecology of the targets, which establish the conditions of the meeting between the two opponents. Here we suppose that the conditions of infectivity have succeeded. When these ecological conditions are defined, i.e. when the meeting of the two opponents occurs, the pathological conditions mentioned above are going to induce the death of the prey if, of course, the concerned insect populations are susceptible to nematodes. At the moment of the insect death, nematodes are more or less at the adult stage. They have not yet produced their progeny. When parasitism succeeds, we have to consider that physio-pathological conditions have prepared the symbiosis expression. Thus, the next step occurs because the monoxenic conditions have been established. However, other protective mechanisms are necessary to maintain monoxenic conditions at least during the first period of the multiplication.

Antimicrobial barriers

How is the *Xenorhabdus* and *Photorhabdus* monoxenies establishment maintained after the insect death? Entry of other microorganisms, first from insect gut, could be the result of the normal putrefaction occurring in all cadavers. All the dead insects where both nematode genera are multiplying look like mummified. They are as a sort of bag where nematode generations intertwine, consuming the inner contents. Bacteriological tests show the predominance of the symbionts at least during the early stages of the nematode multiplication. *Xenorhabdus* and *Photorhabdus* produce *in vitro*, particularly during the last third part of the log period and the beginning of the stationary period, several antibiotics possessing a large activity against different bacteria, fungi and yeasts (Akhurst, 1982). The characterization of these molecules show a great variety of components depending on species. Those identified to date are indole derivatives (Paul et al., 1981), trans-stilbene derivatives (Richardson et al., 1988), xenorhabdins (McInerney et al., 1991a) and xenocoumacins (McInerney et al., 1991b). So at the time of the insect death, it was suggested that insect gut microflora cannot invade the insect body cavity because (i) the niche is occupied by a large symbiont population, and because (ii) the antibiotics produced during the septicemia can inhibit most of the insect holoxenic micro-organisms. In addition the bactericidal activity of the symbiont bacteriocins, identified by *in vitro* experiments and so-called xenorhabdicin in case of *X. nematophilus* (Thaler et al., 1995), may improve an *in vivo* antagonistic action against closely related bacteria (Boemare et al., 1994). Thus the symbiont transmission to the progeny should be secured by several antimicrobial barriers. These barriers would facilitate the collection of their own symbiont of each nematode species by the dauer larvae leaving the cadaver (Thaler et al., 1996).

Symbionts attachment to their host

But apart from this "negative" effect, studies are in progress to investigate a "positive" mechanism probably due to a specific attachment to the juvenile intestinal epithelium. Fimbriae and glycocalyx have been evidenced in two strains of *X. nematophilus* (Brehélin et al., 1993; Binnington and Brooks, 1994; Moureaux et al., 1995). Gnotobiological studies at the molecular level have been undertaken to evaluate if these structures can be involved in a such adhesion to the special intestinal vesicle of Steinernematidae (Bird and Akhurst, 1983) or along the gut epithelium of Heterorhabditidae.

Nematode nutritional requirements provided by bacteria

The sharing of the meal by the bacteria and the nematodes, which is the visible part of the symbiosis, was prepared by the pathology of the complex.

This symbiosis is essentially a predation because these nematodes kill in a short time their insect prey, and at the same time a commensalism because both partners share common meals. Nematodes need a special "menu", and the most suitable for their reproduction is a medium prepared by their symbionts and the bacterial biomass too. As many other rhabditids, *Steinernema* and *Heterorhabditis* are microbivorous.

Axenic rearing of *Steinernema* on artificial diet produces a low level of progeny. Axenic *Steinernema* are able to kill their host, but do not multiply (Boemare et al., 1982). Apparently they are unable to utilise the host tissues and fluids as food sources without the bacterial bioconversion, as they do on artificial diet. Although some non symbiotic bacteria can provide the essential nutrients, none are as suitable as the natural symbionts (Boemare et al., 1983a; Ehlers et al., 1990).

Phase variation of the symbionts

Phase variation is also another common property in both symbiont genera (Boemare and Akhurst, 1988; Nealson et al., 1990). This may indicate that this is a key factor of the symbiosis. It occurs differently in *Xenorhabdus* and *Photorhabdus* but it is apparently necessary for the symbiosis because phase I provides better suitable conditions for the nematode reproduction (Akhurst and Boemare, 1990; Ehlers et al., 1990). In fact all the previous described properties are more or less linked with the phase I variants (depending on strains). More recently Givaudan et al. (1995) have evidenced that the motility of *X. nematophilus* is linked to flagella synthesis occurring only with phase I variants, Moureaux et al. (1995) that these variants, which are piliated, agglutinated insect hemocytes, and Leisman et al. (1995) that outer membranes proteins, OpnA and OpnB, are not produced in phase II variants. Table 3 summarizes the most important facts where colonial properties, ultrastructural elements, cytological properties and enzymatic activities have been analyzed as some of the relevant properties of the symbiosis. The importance to the symbiosis of many of these properties has not yet been established.

The role of phase II variants is not clear and today we have no convincing data which would explain their occurrence. Several hypothesis are given in this issue (see particularly Ehlers and Krasomil-Osterfeld). Phase change occurs during the *in vitro* stationary period in a highly unpredictable manner (Boemare and Akhurst, 1990). Phase variants differ in their assimilation of nutrients and their vitamin requirements (Bonnot and Boemare, unpublished data). Phase II variants might grow a little on complex media previously utilized by phase I variants (Boemare and Akhurst, 1990). Differences in the respiratory activity have been detected between two phases of *X. nematophilus* (Smigielski et al., 1994). After periods of starvation, phase II

Table 3. Phenotypic characters of *Xenorhabdus* and *Photorhabdus* phase variation

	Phase I	Phase II
Colonial properties		
Morphology	mucoid	smooth
Stickiness	+	–
Dye adsorption (1)	+	–
Pigmentation (2)	+	d
Swarming (3)	+	–
Ultrastructural elements and cytological properties		
Protoplasmic inclusions (4)	+	w / –
Flagella (3)	+	–
OpnA, OpnB (5)	+	–
Fimbriae (6, 7)	+	–
Glycocalyx thickness (6)	+	w
Insect hemocytes agglutination (8)	+	–
Erythrocytes agglutination (7, 8)	+	–
Enzymatic activities		
Respiratory enzymes (9)	w	+
Bioluminescence (10)	+	w / –
Antibiotics (11)	+	w / –
Phospholipase(s) (12)	d	w / –
Protease(s) (1)	+	d
Lipase(s) (13)	d	d

+ = positive; – = negative; d = according to strain or biovar; w = weak. (1) Akhurst, 1980; Boemare et al., 1996. (2) no pigmentation for *X. nematophilus*; negative for other *Xenorhabdus* phase II variants, differential pigmentation for *Photorhabdus* variants : Boemare and Akhurst, 1988; Nealson et al., 1990; Boemare et al., 1997. (3) for *X. nematophilus*, few exceptions for other *Xenorhabdus* spp. (Givaudan et al., 1995) and according to strains for *Photorhabdus* (data unpublished). (4) Boemare et al., 1983b; Nealson et al., 1990. (5) Outer membranes proteins of *X. nematophilus* (Leisman et al., 1995). (6) for *X. nematophilus*: Brehélin et al., 1993. (7) for *X. nematophilus*: Binnington and Brooks, 1994. (8) for *X. nematophilus*: Moureaux et al., 1995. (9) for *P. luminescens* and *X. nematophilus*: Smigielski et al., 1994. (10) for *Photorhabdus*; few light can be detected in scintillator counter from phase II variant cultures (Grimont et al., 1984). (11) Akhurst, 1982; some phase II variants can produce a weak antibiosis; Boemare et al., 1997. (12) Boemare and Akhurst, 1988; some negative results in both variants of *Xenorhabdus* and *Photorhabdus*. It is checked as "weak lecithinase" when an opacity is recorded below the colonies; Boemare et al., 1997. (13) Boemare and Akhurst, 1988; sometimes phase II variants are more lipolytic than phase I variants, but they give generally negative or weak responses; Boemare et al., 1997.

cells recommenced growth within 2–4 h from the addition of nutrients, compared with 14 h for phase I cells, indicating a more efficient nutrient uptake ability in the former. The shorter lag period for phase II after addition of nutrients would give it a greater chance than phase I, if they have to compete with other free-living microorganisms outside the insects. Increased membrane potentials have also been noticed, reflecting upon the ability of phase II variant to respond to nutrients, both through growth and nutrient uptake. These experiments suggest that while phase I cells are better adapted to conditions in the insect, phase II cells may be better adapted to other conditions (Smigielski et al., 1994).

This plasticity of metabolic behavior should allow the symbiont to adapt to different niches. It is obvious that the recovery of the symbionts by the L3 escaping the insect cadaver is for the bacteria the beginning of a starvation whereas their release in the insect hemolymph the most suitable situation for their growth. These opposite environmental biotopes probably force adaptive responses where phase variation may take place for saving strain survival. Stress experiments have been reported to induce phase variation. The lack of NaCl in the culture medium causes *Photorhabdus* to phase shift (Krasomil-Osterfeld, 1994). Microaerophilic pressure in unshaken broth for both genera (Boemare and Akhurst, 1990), or culture in anaerobic jar for *Xenorhabdus* (Boemare, unpubl. data), requiring the use of fermentative pathways, induce phase variation. But these experiments cannot explain by themselves mechanism(s) of the phase change. They essentially point out that phase variation is a response to environmental pressures occurring during stationary period when requirements for one phase are consumed or not allowed.

Both phases of one strain have no significant difference in their genome or their plasmid contents (Leclerc and Boemare, 1991; Akhurst et al., 1992; Boemare et al., 1993). Bioluminescence should be a good model to study genetically the phase change because the *Photorhabdus* genes involved in this function are now better documented. Five genes are needed for light production, *luxC, D* and *E* coding for the enzymes of the fatty acid reductase complex which produces the long chain aldehyde substrate for the luciferase whose two units are encoded by the *luxA* and *B* genes (Frackman et al., 1990; Szittner and Meighen, 1990). Their organization is similar as in marine luminous bacteria. The lux gene mRNA accumulates to the same extent in the two phases implying that gene expression in phase II is regulated at a post-transcriptional level (Frackman et al., 1990; Wang and Dowds, 1991). Lipase from the *Photorhabdus* strain K122 is encoded by a gene identified in both variants and the protein is synthesised and secreted in both phases too (Wang and Dowds, 1993). However, the lipase is inactive in phase II. Similar results have been obtained

in studying regulation of the *Photorhabdus* KI22 protease synthesis (Dowds, 1994).

Gene regulation of phase characters in *Xenorhabdus* have not yet been investigated. But preliminary *Photorhabdus* genetic results are in good agreement with weak phenotypic expression observed with most of the phase characters in phase II variants (Table 3). However, it seems difficult to admit that all the phase specific genes are regulated by a sole common control mechanism due to the variety of the involved functions: production of exoenzymes, antimicrobial by-products, pigment, dye-binding external material, internal protoplasmic paracrystalline inclusions, all implying different metabolic pathways. For more details see the recent review of Forst and Nealson (1996), and a review which will be published soon (Forst et al., 1997).

4. Conclusion

This review summarizes the present knowledge. It is limited more or less at the investigations made with *X. nematophilus/S. carpocapsae* and *P. luminescens/H. bacteriophora* models. It is likely that a greater physiological diversity will be found with other bacterium-nematode complexes. However, the occurrence of different association models have been yet documented from a parasite living without symbionts, a parasite with opportunist bacteria, a parasite with a tenuous symbiont as *S. glaseri/X. poinarii*, and a strong association like *S. carpocapsae/X. nematophilus*. In this respect, metabolic plasticity of the symbionts may be an answer for the question of acceptance or non-acceptance of other bacteria. Investigation of food supplies provided by *Xenorhabdus* and *Photorhabdus* is a key factor for our understanding of the significance of these symbioses.

Photorhabdus and *Xenorhabdus* possess several common properties expressed differently. These properties are probably elements of a convergent evolution necessary for the association with two phylogenetically different entomopathogenic nematodes. Among these, we are able to recognize today production of antibiotics, bacteriocins, protoplasmic paracrystalline inclusions, exo-entomotoxins, and the occurrence of two adaptive states resulting from a phase variation. Table 4 summarizes the common properties observed in both genera, but also shows that the molecules, enzymes, proteins and ultrastructural elements are biochemically different in nature. Mechanism(s) of phase variation is/are probably different in both genera as the result of a shift through a complex genetic and metabolic cascade of events starting from environmental signals via membrane transports, gene regulation and protein

synthesis for each appropriate metabolism. The truth is certainly in the following syllogism: properties have been defined for phase variation, these properties seem to be necessary for symbiosis, so phase variation should be an essential property of the symbiosis.

Table 4. Common properties expressed differently in *Xenorhabdus* and *Photorhabdus*

	Xenorhabdus	*Photorhabdus*
Antibiotics	Acetoxylindoles Xenorhabdins Xenocoumacins	Stilbenes
Bacteriocins	Xenorhabdicins	+
Inclusions	+	+
Exotoxins	31 kDa	40 kDa
Exoenzymes	+	+
Plasmids	+	+
Phase variation	+	+

The relationships between nematodes and bacterial symbionts is surely ancient (Poinar, 1993). Phylogenetic studies should define the degree of co-evolution between partners in Steinernematidae and Heterorhabditidae. Progress in our physiological knowledge of these associations will provide tools for improved gnotoxenic animals more effective as biopesticides against agricultural pests.

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