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Molecular Biology of the Symbiotic-Pathogenic Bacteria *Xenorhabdus* spp. and *Photorhabdus* spp.

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INTRODUCTION

Xenorhabdus and *Photorhabdus* are two genera of bacteria that symbiotically associate with specific nematodes. The nematode-bacterium pair is capable of invading and killing the larval stage of numerous insects (10). Both *Xenorhabdus* and *Photorhabdus* spp. are motile gram-negative bacteria belonging to the family *Enterobacteriaceae* (21, 22). The general features of the life cycles of these bacteria are quite similar, as shown in Fig. 1. The similarities include habitation in the gut of entomopathogenic nematodes, growth in the hemolymph of larval stage insects, and pathogenic potential toward the infected insect. However, upon closer examination, substantial differences between the biology of these bacteria become apparent. Most notably, the bacteria symbiotically associate with different families of nematodes, which undergo distinct reproductive cycles. We have chosen to point out the differences in the biology of *Xenorhabdus* and *Photorhabdus* spp. by presenting two separate life cycles, as shown in Fig. 1.

Xenorhabdus and *Photorhabdus* spp. are carried as symbionts in the intestine of the infective juvenile stage of nematodes belonging to the families Steinernematidae and Heterorhabditidae, respectively (112). The nematodes enter the digestive tract of the larval stage of diverse insects and subsequently penetrate into the hemocele of the host insect. The nematode can also gain access to the hemocele via the respiratory spiracles or by penetrating directly through the insect cuticle (11, 112). Upon entrance into the hemocoel, the nematodes release the bacteria into the hemolymph. Together, the nematodes and the bacteria rapidly kill the insect larva, although in most cases the bacteria alone are highly virulent (11). Protein insect toxins have been found to be elaborated from both bacteria (8, 25, 50). Within the hemocoel of the larval carcass, the bacteria grow to stationary-phase conditions while the nematodes develop and sexually reproduce. Nematode reproduction is optimal when the natural symbiont (*Xenorhabdus* or *Photorhabdus* spp.) dominate the microbial flora, suggesting that the bacteria can serve as a food source and/or provide essential nutrients that are required for efficient nematode proliferation (10, 11,

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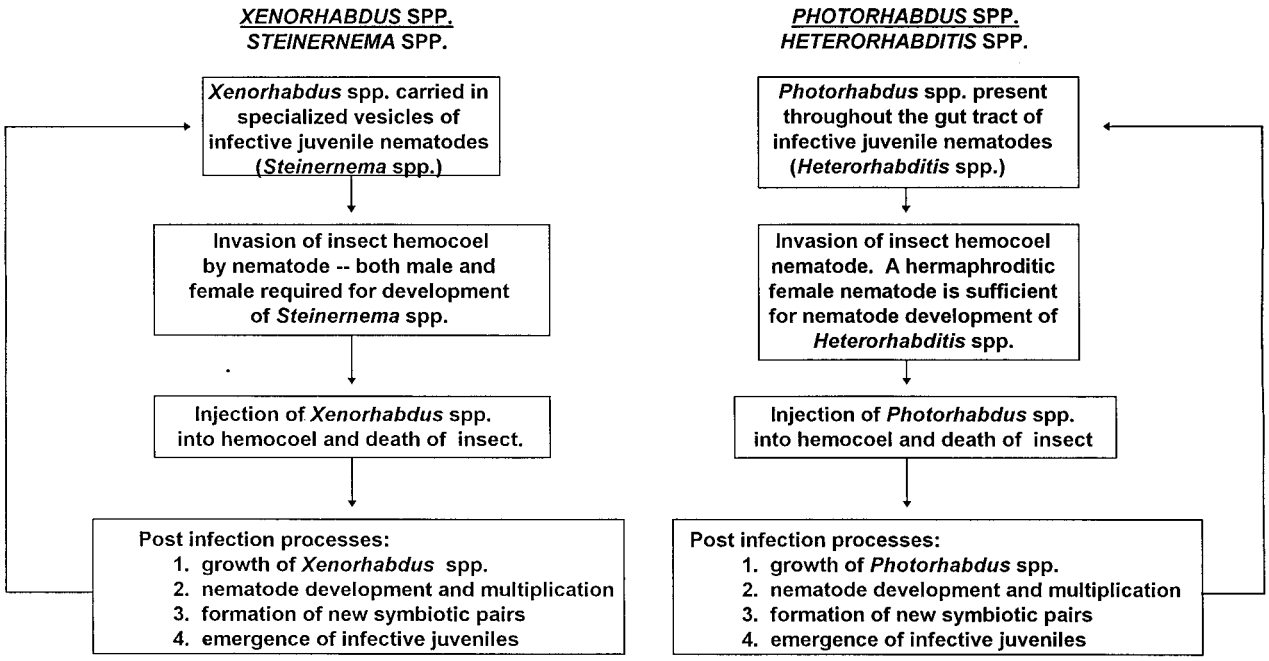

FIG. 1. Life cycles of bacterium-nematode symbioses. This diagram shows the major features of the *Xenorhabdus-Steinernema* and *Photorhabdus-Heterorhabditis* life cycles. While the general features are similar, the cycles are drawn separately to emphasize that many of the details are distinctly different, as discussed in the text.

112). During the final stages of development, the nematodes and bacteria reassociate and the nematodes subsequently develop into the nonfeeding infective juvenile stage. The infective juveniles, carrying the bacteria in their intestinal tracts, emerge from the insect carcass in search of a new insect host.

While both *Xenorhabdus* and *Photorhabdus* spp. are symbionts of entomopathogenic nematodes and function as pathogenic agents toward susceptible insect larvae (85), several salient features of their life cycles are distinctly different from each other (Fig. 1). *Xenorhabdus* spp. are carried in specialized vesicles formed by an outpocketing of the gut of *Steinernema* nematodes, while *Photorhabdus* spp. are located throughout the gut of *Heterorhabditis* nematodes (19). The reproductive cycle of the respective nematodes is also quite different. The infective juvenile nematodes of the *Steinernema* family exist as amphimictic males and females (112). For development to proceed, both a male and a female must infect the host hemocele. In contrast, *Heterorhabditis* nematodes develop into hermaphroditic females and subsequently into amphimictic males and females (147). Thus, infection of the host by a single hermaphroditic *Heterorhabditis* infective juvenile is sufficient to initiate nematode development and proliferation within the hemocoel. It is of interest that these two entomopathogenic nematodes have been proposed to be derived from different ancestral lineages (112). Finally, although the mechanism by which the insect host is killed is not well understood, recent observations suggest that the molecular events that lead to insect death may differ for *Xenorhabdus* and *Photorhabdus* spp.

All of the *Xenorhabdus* isolates, and almost all of the *Photorhabdus* isolates studied so far have been obtained from nematodes harvested from soil samples, although a rigorous and systematic study looking for the free-living form has not yet been conducted. Free-living forms of the bacteria have not yet been isolated from soil or water sources. These findings suggest that the symbiotic association may be essential for the survival of the bacteria in the soil environment. The bacteria, in turn, are essential for effective killing of the insect host and are required for the nematode to efficiently complete its life cycle (7, 85).

Both *Xenorhabdus* and *Photorhabdus* spp. can be grown as free-living organisms under standard laboratory conditions. Growth in vitro is probably supported by the rich nutrient supply of the laboratory growth media and the lack of competition that normally exists in the soil environment. As the bacteria enter the stationary phase of their growth cycle, they secrete several extracellular products, including a lipase(s), phospholipase(s), protease(s), and several different broad-spectrum antibiotics (2, 10, 21, 106) that can be assayed in the culture media. These products are believed to be secreted in the insect hemolymph when the bacteria enter stationary-phase conditions. The degradative enzymes break down the macromolecules of the insect cadaver to provide the developing nematode with a nutrient supply, while the antibiotics suppress contamination of the cadaver by other microorganisms. Cytoplasmic inclusion bodies, composed of highly expressed crystalline proteins, are also produced by both bacteria during stationary-phase growth (35, 36).

Another intriguing property of *Xenorhabdus* and *Photorhabdus* spp. is the formation of phenotypic variant forms (1, 20, 21, 79) that can be isolated at low and variable frequencies during prolonged incubation under stationary-phase conditions. The variant forms, or so-called phase II cells, are altered in many properties and are not found as natural symbionts in the nematode. Phase I cells are the form of the bacteria that naturally associate with the infective juvenile nematode. In *Photorhabdus* spp., the levels of lipase and protease activity and the amounts of secreted antibiotics found in the extracellular medium of stationary phase grown phase II cells are greatly reduced relative to those of the phase I cells. A similar phenotypic pattern exists in *Xenorhabdus nematophilus*, except that the lipase activity is weak in phase I cells and is more strongly produced in phase II cells (21).

While *Xenorhabdus* and *Photorhabdus* spp. are similar in numerous characteristics, they differ in several salient features. *Xenorhabdus* spp. are found specifically associated with entomopathogenic nematodes in the family Steinernematidae while *Photorhabdus* spp. associate only with the nematode group Heterorhabditidae. A primary property distinguishing *Photorhabdus* from *Xenorhabdus* spp. is the ability of the former to emit light under stationary-phase culture conditions and in the infected host insect (116). On the other hand, *Xenorhabdus* spp. are catalase negative, which is an unusual property for bacteria in the family *Enterobacteriaceae* (21, 52). Although *Xenorhabdus* and *Photorhabdus* spp. are related phylogenetically, it is becoming increasingly more apparent that common phenotypic traits shared by these bacteria may be determined by distinct underlying molecular and biochemical pathways. We will present information that has accumulated over the past several years supporting the contention that these bacteria may represent an example of convergent molecular evolution in which many phenotypic properties required for the symbiotic/pathogenic lifestyle of *Xenorhabdus* and *Photorhabdus* spp. were acquired from disparate genetic origins.

In this review, we focus primarily on recent studies that describe various aspects of the pathogenicity, cell surface properties, and molecular biology of *Xenorhabdus* and *Photorhabdus* spp. New information concerning antibiotic and bacteriocin production, characterization of crystalline proteins, and novel aspects of the bioluminescence and phase variation is also presented. Several previous reviews have discussed the biology (7, 10, 11, 15), physiology (105, 106), and taxonomy (10) of both genera, as well as specific topics such as bioluminescence in *Photorhabdus* spp. (63, 64). Physiological and molecular biological studies suggest that these genera are distinctly different in numerous characteristics. An exciting and fertile area for future work will be to elucidate the different molecular biological processes and biochemical products that allow these bacteria to adapt to life in the gut of the nematode and the hemolymph of a broad spectrum of insects.

PHYLOGENY AND TAXONOMY

Phylogeny

Both oligonucleotide cataloging (48) and 16S rRNA sequence analysis (120) have placed the *Xenorhabdus*/*Photorhabdus* group within the gamma subdivision of the purple bacteria (proteobacteria). By oligonucleotide cataloging, it was estimated that *Xenorhabdus*/*Photorhabdus* constituted a taxonomic unit equivalent to, and possibly distinct from, the family *Enterobacteriaceae*, into which it has been placed after phyletic analysis by several laboratories (see below). 16S rRNA data recently published by Rainey et al. (120) support this contention, showing a close relationship with other members of the gamma proteobacteria, which, interestingly include many other organisms specialized as symbionts and/or pathogens of eukaryotes. Thus, while the placement of the genera *Xenorhabdus* and *Photorhabdus* within the *Enterobacteriaceae* seems firm, it should be noted that some uncertainty still exists with regard to this issue. Rainey et al. (120) discuss the appropriateness of including the group within the family *Enterobacteriaceae*, while on the other hand, Janse and Smits (82), on the basis of fatty acid analyses, suggested that the differences were sufficient for them to be separated from the *Enterobacteriaceae*. Farmer (52) and Farmer et al. (53), have pointed out that the inability of *Xenorhabdus* spp. to reduce nitrate, coupled with their catalase-negative property, is a reason to question their inclusion in the *Enterobacteriaceae*. They also note that *Photorhabdus* species are nitrate reduction negative, that they produce characteristic yellow or red pigments not common in the *Enterobacteriaceae*, and, of course, that they are bioluminescent, a property that is otherwise unknown in the *Enterobacteriaceae*. Also under discussion is the appropriateness of giving genus status to *Photorhabdus* (see the discussion in reference 120).

Taxonomy

The taxonomy of the *Xenorhabdus*/*Photorhabdus* group has had a brief and somewhat confusing history since the recent naming of the organisms in the mid-1960s. From the early studies, it was concluded that these bacteria are closely related to the family *Enterobacteriaceae* and share many properties with their enteric neighbors. However, the names chosen for both the bacteria and their nematode hosts have occasionally changed, leaving the situation somewhat difficult to understand. Furthermore, as new strains have been isolated and characterized, the situation has become more complex. To help the reader sort out some of the difficulties, we have summarized the names of both the bacteria and their nematode hosts since their first characterization (Table 1). The presently used names are at the bottom of the table, and previously used names are indicated as predecessors above them. A brief discussion of this table follows.

One of the first published discussions of bacteria associated with entomopathogenic nematodes appeared in 1959 (47), although the bacteria were neither fully characterized nor named. Given that the nematodes studied were in the *Neoaplectana* (also known as the *Steinernema*) group, it seems likely that the bacteria were *Xenorhabdus* spp. and would have been given the designation *X. nematophilus*. It was noted in this report that the bacteria produced antibiotic activity and inhibited the putrefaction of the insect carcasses. The nematode was referred to as DD-136 (now named *Steinernema carpocapsae*) and was later used by Poinar and Thomas (115) as the source material for isolation of a bacterium referred to as *Achromobacter nematophilus.*

According to the recommendation of Hendrie et al. (73), the genus *Achromobacter* was disallowed, and the symbionts were left without a name when some of the existing type strains of symbiotic luminous bacteria, such as Hb (ATCC 29999) and NC-19 (ATCC 29304), were isolated (86, 117). This situation continued until the proposal of the genus name *Xenorhabdus* by Thomas and Poinar (137) to accommodate both the nonluminous (*X. nematophilus*) and the luminous (*X. luminescens*) symbionts. It was recognized at this time that the luminous symbionts were obtained only from heterorhabditid nematodes, while the nonluminous *X. nematophilus* isolates were found associated with neoaplectanid nematodes (137). *Xenorhabdus* was placed in the family *Enterobacteriaceae* on the basis of extensive phyletic analysis, although some traits are notably different from those of the other members of the *Enterobacteriaceae* (*Xenorhabdus* spp. are catalase negative and do not reduce nitrate to nitrite; *Photorhabdus luminescens* strains are nitrate reduction negative, pigment positive, and bioluminescence positive). A quick look at the description of the species in *Bergey's Manual* (52) reveals that *Xenorhabdus* spp. are much less metabolically diverse than many of their enterobacterial neighbors.

The diversity of *Xenorhabdus* types became apparent in the early 1980s (4, 9, 21, 69), when several subspecies of *X. nematophilus* were proposed on the basis of differences in morphological properties. About this time, the nematodes also were reassigned, with *Neoaplectana* being abandoned and replaced

TABLE 1. Chronology of *Xenorhabdus*/*Photorhabdus* bacteria and their nematode hosts

Yr	Bacterial symbiont	Nematode host
1959	Bacteria seen; not named	*Neoaplectana* sp. strain DD-136[a]
1966	*Achromobacter* spp.[b] (ATCC 19061)	*Neoaplectana* sp. strain DD-136
1971	*Achromobacter nematophilus*	*Neoaplectana* sp. strain DD-136
1977	Unnamed symbiont Hb (ATCC 29999)	*Heterorhabditis bacteriophora*
	Unnamed symbiont NC-19 (ATCC 29304)	*Chromonema* (*Heterorhabditis*) *heliothidis*
1979	*Xenorhabdus nematophilus*[c]	*Neoaplectana* spp.
	Xenorhabdus luminescens[c]	*Heterorhabditis* spp.
1983	*X. nematophilus*[d]	*Neoaplectana bibionis*, *N. feltiae*; *N. glaseri*, *Neoaplectana* spp., *Steinernema kraussei*
	X. luminescens	*Heterorhabditis* spp.
1983	*X. nematophilus* subsp. *nematophilus*	*N. feltiae* (synonym *N. carpocapsae*)[e]
	X. nematophilus subsp. *poinarii*	*N. glaseri*
	X. nematophilus subsp. *bovienii*	*N. bibionis*
1986	*X. nematophilus* subsp. *beddingii*	*Steinernema* sp. strain M; *Steinernema* sp. strain N
1987	*X. luminescens* (nonluminous isolate)	*Heterorhabditis* spp.
1989	*X. luminescens* (human isolate)[f]	No known nematode host
1992	*X. nematophilus* SK-1	*Steinernema kushidai*[g]
1993[h]	*Xenorhabdus nematophilus*	*Steinernema carpocapsae*
	Xenorhabdus bovienii	*Steinernema feltiae*, *Steinernema kraussei*, *Steinernema affinis*, *Steinernema intermedia*
	Xenorhabdus poinarii	*Steinernema glaseri*
	Xenorhabdus beddingii	*Steinernema* sp. strains M and N
	New unnamed symbionts (six)[h]	*Steinernema* spp.
	Photorhabdus luminescens[h,i]	*Heterorhabditis* spp.

[a] Reference 47 (see also references to earlier work of Dutky cited in this article).
[b] Symbionts named by Poinar and Thomas (115); *Achromobacter* was disallowed as a genus in 1974 (73).
[c] Genus *Xenorhabdus* proposed by Thomas and Poinar (137).
[d] *X. nematophilus* strains were recognized as being associated with many different neoaplectanid nematodes.
[e] It was recognized that several different subgroups of *X. nematophilus* existed and that they were associated with different nematodes in the neoaplectanid group. *Neoaplectana* was recognized as a later synonym for the previously established genus *Steinernema* (141a). After 1986, *Steinernema* was used exclusively in preference to *Neoaplectana.*
[f] Farmer et al. (53) described several *X. luminescens* strains isolated from wounds of human patients.
[g] Reference 146.
[h] Akhurst et al. (1993) separated the *Xenorhabdus* spp. via DNA-DNA hybridization and proposed that several new species might be found when more strains are examined; they also proposed that the luminous bacteria be given a separate genus status as the genus *Photorhabdus.*
[i] It seems likely that the clinical isolates of Farmer et al. (53) will be described as a new species on the basis of DNA-DNA hybridization results (type strain ATCC 43950).

by the name *Steinernema*. As more strains of bacteria were studied and more data accumulated (1986 to 1992), the subspecies were given species status (22), with DNA-DNA hybridization being the technique of choice for delineating various species. Recently, on the basis of the DNA-DNA hybridization, Boemare et al. (22) proposed that the luminous bacteria should be placed in a separate genus; this is the present state of affairs as regards the taxonomy, although there is still some controversy over the split of the genus *Xenorhabdus*. Rainey et al. (120), on the basis of 16S rRNA sequence analysis, have argued that there may not be adequate separation of the two groups to warrant genus status for the luminous isolates now placed in *P. luminescens*. Part of the problem appears to be that the DNA-DNA hybridization methods are well defined and useful for species separation (129) but less so for delineation of genera. As pointed out by Rainey et al. (120), however, the results of their own work are dependent on the statistical methods used to analyze the data, and the data set is at present rather small. The hybridization studies of Boemare et al. (22) show quite clearly that the *Xenorhabdus* and *Photorhabdus* groups form significantly different homology groups, and the issue of genus or species assignment should be resolved as more strains are isolated, studied, and sequenced.

Of note in this regard is the recent identification by Farmer et al. (53) of human clinical specimens in the group *X.* (*P.*) *luminescens*. Five separate isolates were obtained and classed as *X. luminescens*. DNA-DNA hybridization showed these five isolates to be closely related to each other but sufficiently different from other *Photorhabdus* spp. that they may in fact constitute a new species. These are the only members of the *Xenorhabdus*/*Photorhabdus* group to be isolated as saprophytes (i.e., with no nematode host), and the major identifying property that allowed their identification was that of bioluminescence (53). They form a yellow pigment on tryptic soy agar and show a very unusual pattern of hemolysis on sheep blood agar plates. No 16S rRNA sequence comparisons have been reported with these isolates.

Although molecular methods (16S rRNA sequence comparisons) have not yet completely resolved the taxonomic issues, it has been possible to develop group-specific probes of several types. 16S rRNA probes were developed for the four *Xenorhabdus* spp. and for *P. luminescens* (118). Of 27 strains tested, 24 could be assigned to a specific species by using these probes. Using a different approach, Wimpee et al. (141) showed that the luciferase (*luxA*) gene probe was positive for all strains of *P. luminescens* tested and showed no hybridization with any of the *Xenorhabdus* species. Interestingly, we have also used the genes coding for pigment formation in *P. luminescens* and shown that they exhibit no cross-hybridization with any *Xenorhabdus* strains (119). As more genes are isolated and sequenced, it will be of interest to conduct cross-hybridization studies between the *Xenorhabdus* and *Photorhabdus* isolates, as well as between pairs of species isolated from different environments. In terms of searching for free-living forms of *Photorhabdus* and *Xenorhabdus* spp., it may be that colony blots with such function-specific probes could be very valuable, especially

for the identification of phase II cells, but as the amount of variability within a species is not yet known, this speculation cannot be verified, with the possible exception of the *lux* genes, which appear to be highly conserved among all *P. luminescens* isolates studied.

A side note here concerns the isolation of two nonluminous bacteria as symbionts from *Heterorhabdus* nematodes. One was identified as a nonluminous *P. luminescens* strain (9), while the other was identified as a pigmented nonluminous *Providencia* strain (81). The former example has been examined in our laboratory by hybridization studies with the cloned *lux* genes as a probe (102), and no cross-reactions were seen, indicating that they did not contain the *lux* genes. This was in agreement with light measurements with a very sensitive photometer, which showed no detectable luminescence under any conditions of growth or incubation. These studies serve to point out that luminescence is not a trait required for successful symbiosis of the *Heterorhabdus* nematodes and leave open the possibility that more new species will be identified in the future in this symbiotic niche.

Another molecular approach was used by Janse and Smits (82), who analyzed whole-cell fatty acid patterns. These workers concluded that *X. nematophilus* could be clearly differentiated from *X.* (*P.*) *luminescens* and proposed that the groups might constitute different genera. In contrast to the accepted view, these workers suggested that the *Xenorhabdus* spp. might be sufficiently different from other enterobacteria to possibly exclude them from the *Enterobacteriaceae.*

Given the present state of affairs, one might profitably ask, "What are the phenotypic characteristics that unite the *Xenorhabdus*/*Photorhabdus* group and distinguish it from other closely related bacteria?" As more strains of this group have been studied, the answer to this question has become more elusive rather than less, but some of the traits of the group are given in Table 2. The relevance of this information relates to whether these bacteria have a life cycle independent of the host nematodes and whether they might have been previously isolated and identified. As Farmer et al. (53) point out, it is not an easy matter to identify these bacteria, because they are biochemically inactive in comparison with many other members of the *Enterobacteriaceae* (e.g., many of the traits used to distinguish the genera that form the *Enterobacteriaceae* are negative for the *Xenorhabdus*/*Photorhabdus* group). For example, the human clinical isolates remained unidentified for many years until it was discovered that they were dimly luminescent (53). This becomes even more of a problem with the phase II isolates, in which one or more of the traits listed in Table 2 as variable can become negative, and the bacteria are much more difficult to identify with certainty.

To carry these questions further, one may ask which factors distinguish the *Xenorhabdus* from the *Photorhabdus* types and where might one look for important differences. Here we depart from the views held by many of our colleagues and suggest that some of the features that have traditionally been used to unite these genera may actually be used to distinguish between them (Table 3), possibly suggesting that these bacteria are significantly different when details of the traits are examined. That is, at the phenetic level, the traits seem similar, but at the genetic level, they are quite distinctive. This would put these organisms in a very unusual position in terms of their evolution. One might view the niche they inhabit as one available and hospitable to bacteria of the gamma proteobacteria types of organisms, thus explaining the similarities seen via the 16S rRNA approach. Furthermore, the need for other functions that favor successful exploitation of the niche might have led to convergent evolution as the bacteria acquired protease, lipase, crystals, pigments, antibiotics, and other properties. While functionally convergent, however, the genes acquired have significant structural differences based on different origins, leading to bacteria which appear significantly different when examined at the molecular level. If this scenario proves to be true, one might also expect some major differences between *Photorhabdus* spp., depending on when, and from which organisms, they acquired their various genes. Molecular studies will certainly reveal the details of gene origin, but if the proposed pathway is correct, this system represents one in which lateral gene transfer has played a major and important role in the successful exploitation of specific symbiotic niches.

In summary, the phylogeny of these organisms is relatively undisputed in the sense that they are clearly placed in the gamma group of proteobacteria. The taxonomic details of whether they might constitute a group separate from the *Enterobacteriaceae* are still in question, as is the issue of whether *Xenorhabdus* and *Photorhabdus* are definitely deserving of genus status. As more isolates in both genera are obtained, it seems likely that more species will be established, and our view of the group will change accordingly. Finally, it is not easy to identify these organisms if encountered outside the nematode niche, and whether such a saprophytic niche exists is still a valid question. One might note in this regard that other symbionts such as *Rhizobium* spp. clearly exist in soil environments but are difficult to isolate as free-living organisms since they exist in very low concentrations (often less than 10 cells per g of soil) relative to the predominant microorganisms that exist in soil (130).

TABLE 2. Distinguishing traits of *Xenorhabdus* and *Photorhabdus* spp.

Property type	Trait
Ecological	Isolation from entomopathogenic nematodes[a]
	Xenorhabdus spp. (found associated with steinernematid nematodes)
	Photorhabdus spp. (found associated with heterorhabditid nematodes)
	Very pathogenic for insects
	Propensity to form phase variants
Phenotypic (that remain constant)	Gram negative
	Resistant to penicillin and beta-lactam antibiotics
	Negative for nitrate reduction to nitrite
	Mol% G+C of DNA = 43–44
Phenotypic (that can change when phase variants are formed)	Intracellular protein inclusions common
	Large cells (4 μm and larger) (phase II cells are smaller)
	Motile by peritrichous flagella
	Photorhabdus cells are bioluminescent
	Positive for hemolysis of blood agar cells[b]
	Positive for antibiotic production[c]
	Positive for several extracellular enzymes[d]
	Pigmentation of colonies
	Dye uptake and reduction[e]

[a] The human clinical isolates are the only free-living members so far isolated (53).

[b] The human clinical isolates displayed a very unusual pattern of hemolysis referred to as *X. luminescens* hemolysis (53).

[c] Almost all strains produce antibiotic activity against a broad range of test bacteria.

[d] Strains are characteristically positive for protease, lipase, and amylase.

[e] Absorption of the dye bromothymol blue and reduction of triphenyltetrazolium chloride.

TABLE 3. Characteristics distinguishing *Xenorhabdus* from *Photorhabdus* spp.

Character	Values in:	
	Xenorhabdus spp.	*Photorhabdus* spp.
Isolation from *Steinernema* spp.	+	−
Isolation from *Heterorhabditis* spp.	−	+
Bioluminescence	−	+
Catalase	−	+
Pigment[a]	Unknown	Anthraquinone
Antibiotics[b]	Xenorhabdins, xenocoumacins, indoles	Hydroxystilbenes, anthraquinones
Intracellular crystal size (kDa)[c]	22, 24	10, 11
Protease[d]	Unknown	Serine protease
Lipase[e]	Unknown	Lip-1
Toxin size (kDa)	31[f]	>700[g]

[a] While some *Xenorhabdus* strains are pigmented, the yellow or red color characteristic of *Photorhabdus* spp. is not seen, and no anthraquinones have been isolated.
[b] Antibiotic production is a group trait, but the compounds produced are quite different between *Xenorhabdus* spp. (xenocoumacins, xenorhabdins, and indole derivatives) and *Photorhabdus* spp. (hydroxystilbene derivatives).
[c] Bintrim (18) has shown that the crystalline protein genes from *Photorhabdus* spp. are not homologous with those from *Xenorhabdus* spp.
[d] Patterns of protease activity and expression (21) suggest that these proteins are probably distinctly different between the two genera.
[e] Reference 139.
[f] Reference 8.
[g] Reference 25.

PATHOGENICITY

Xenorhabdus and *Photorhabdus* spp. are released from the nematode into the host hemolymph within 5 h of invasion, and the larvae are generally killed within 48 h (11). The efficiency with which the bacteria-nematode complex kills the larvae depends on the insect species infected, the immunological and physiological state of the insect, and the particular bacterial species (11). To grow within the hemolymph, the bacteria must be able to tolerate the host defensive response, evade recognition as nonself, or suppress the host nonself response. Since *Xenorhabdus* and *Photorhabdus* spp. proliferate within the insect hemolymph as part of their natural life cycle, it appears that the bacteria are well adapted to evade or tolerate the insect defensive response. The pathogenic potential of the bacteria-nematode complex has been exploited for its usefulness as a biological pest control agent (87).

By allowing the infective juvenile nematode, which carries its specific bacterial symbiont, to invade the insect host, the natural biological events of the infectious cycle can be studied. The pathogenicity of the bacteria alone can be conveniently studied by injecting either *Xenorhabdus* or *Photorhabdus* organisms directly into the hemocoel of the insect host (44, 45). The combined effect of the symbiotic pair on the fate of the insect can be investigated by coinjecting the nematode and the bacteria into the hemocoel (6). A significant advantage of this system is that the symbiotic partners can be obtained individually and subsequently recombined in vitro. It is possible to regenerate the *X. nematophilus-S. carpocapsae* symbiotic partnership by raising newly hatched nematodes, obtained from fertilized eggs of the gravid female, on a pure culture of the bacteria (3, 11). This is possible because rich artificial medium in which *Steinernema* spp. can be grown axenically and proliferate under sterile conditions has been developed (11). The axenic nematodes can then be raised on pure *Xenorhabdus* cultures. The resulting monoxenic infective juvenile nematodes are then able to infect the insect host and release the bacterial symbiont into the hemocoel. By raising the axenic nematodes on mutant *Xenorhabdus* strains, specific bacterial genes can be studied for their role in the infectious process, as well as in the symbiotic association with the nematode. At present, similar approaches are not available for *Heterorhabditis* nematodes, since artificial medium has not yet been developed to raise these nematodes under sterile conditions (11). Therefore, it is presently difficult to study the symbiotic interaction between the nematode and *Photorhabdus* spp. on a molecular level. This difference in the growth phenotype of *Xenorhabdus* and *Photorhabdus* spp. may reflect additional dissimilarities that were already noted in Fig. 1. In this section, we discuss recent studies directed at understanding the mechanisms by which *Xenorhabdus* and *Photorhabdus* spp. survive the host defensive response, proliferate in the hemolymph, and kill the larvae. The tripartite interaction between the bacteria, nematode, and insect (11) and the biochemical aspects of the insect immune system (39) have been presented previously.

Xenorhabdus Pathogenicity

The initial cellular defensive response to bacterial infection is phagocytosis (39). When a large number of bacteria are present in the hemolymph, phagocytosis is augmented by nodule formation. In this process, the cell surface of the activated hemocyte elongates to form filopodia and the hemocytes become more adhesive. Additionally, the bacteria adhere to and are sequestered by the hemocytes, which in turn aggregate with extracellular matrix to form nodules (39). Ultimately, the resulting nodules leave the general circulation by adhering to fatty tissues. The ability of *Xenorhabdus* spp., injected directly into the hemolymph, to kill insects has been tested in the last-instar larvae of the common wax moth, *Galleria mellonella*. In most cases, the bacteria alone are pathogenic. However, Akhurst has shown that while neither *X. poinarii* nor the nematode, *S. glasseri*, alone resulted in larval mortality, the symbiotic pair was highly pathogenic for *G. mellonella* larvae (5). A newly identified *Xenorhabdus* species, *X. japonica*, that is symbiotically associated with the nematode *Steinernema kushidai* was not pathogenic toward *Spodoptera litura* larvae, whereas other *Xenorhabdus* species tested killed this insect host (108, 146). The virulence of *X. japonica* together with its nematode symbiont was not tested in these studies.

G. mellonella larvae have been used extensively for pathogenicity studies. This organism is highly susceptible to bacterial infection; the virulent *Pseudomonas aeruginosa* P11-1 has a 50% lethal dose (LD_{50}) of less than 10^2 bacteria per larva (40). In contrast, the tobacco horn worm, *Manduca sexta*, is more resistant to bacterial infection; the LD_{50} of *P. aeruginosa* P11-1

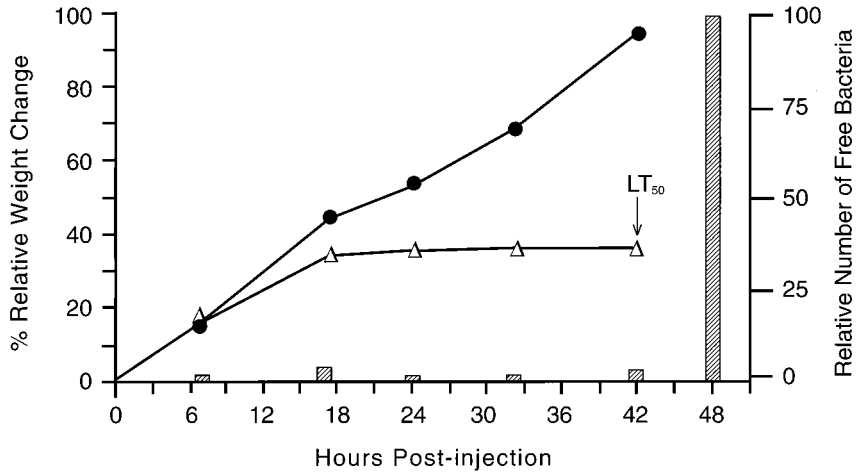

FIG. 2. Inhibition of growth rate and eventual mortality of *M. sexta* given an injection of *X. nematophilus*. Newly molted fifth-instar larvae (2 to 3 g) were given an injection of either 100 μl of Grace's insect medium (●) or 100 μl of medium containing five *X. nematophilus* cells (△). Weight gain was monitored over a 48-h growth period. LT_{50} represents the time at which 50% of the larval population had died. At each time point, the number of free bacteria in the hemolymph was examined by phase-contrast microscopy and plating on selective medium. The relative number of free bacteria observed (hatched bar graph) was normalized against the number of bacteria (10^8 cells per ml) present at 48 h. The averaged results shown are representative of a typical experiment in which eight individuals were treated per experimental condition. The relative number of free bacteria was derived from multiple samplings of the hemolymph of a representative individual. By 48 h, 90 to 100% of the infected larvae had died.

was shown to be 10^5 bacteria per larva of *M. sexta* (40). The resistance of *M. sexta* to bacterial infection is due in part to rapid phagocytosis and nodule formation. *Manduca* larvae are able to survive when as many as 10^6 to 10^7 bacterial cells of either *Serratia marcescens*, *Escherichia coli* D31, or *P. aeruginosa* ATCC 9027 are injected into the hemocoel. In contrast, the larvae are very sensitive to the highly virulent *X. nematophilus*, which can kill more than 90% of the *M. sexta* larvae when a few bacteria are injected into the larvae (Fig. 2). The growth rate of individual *M. sexta* insects given injections of only five *X. nematophilus* cells was found to be markedly reduced relative to that of the untreated larvae. The time at which 50% of the larval population had died (LT_{50}) for the injected larvae was approximately 42 h, and the bacteria were not detectable in the hemolymph as judged by either direct microscopic examination or plating of the hemolymph at regular intervals during this period. This situation may resemble that in *Listeria* spp. (137a) and other intracellular parasites, in which the bacteria enter an intraphagosomal phase, secrete phospholipase and hemolysin to break down the phagosomal membrane, and subsequently live intracellularly in the cytoplasm of the host. In the case of bacteria that enter the hemocoel of the insect larvae, the bacteria may be sequestered in nodules that temporarily leave the general circulation by adherence of the nodule to fat bodies (40). After the insect dies, the *X. nematophilus* concentration increases dramatically (Fig. 2) to high levels (10^8/ml). The lethal events leading to the cessation of feeding and decreased growth rate appear to have occurred before significant numbers of bacteria are present in the hemocoel. Thus, the pathogenic phase of the bacterial life cycle may in fact be separate from the rapid growth phase. This would imply that septicemia is not essential for virulence and that bacterial growth is exponential after the demise of the larva. Taken together, these results emphasize the extreme virulence of *Xenorhabdus* spp. toward the susceptible insect host.

One mechanism that *Xenorhabdus* spp. use to tolerate or evade the humoral defensive response is to inhibit the activation of the insect enzyme phenoloxidase. The activation of phenoloxidase can occur when bacteria are present in the hemolymph (43). The lipopolysaccharide (LPS) *Xenorhabdus* spp. has been shown to prevent the processing of prophenoloxidase into phenoloxidase (44, 45). Phenoloxidase, which is formed by the processing of prophenoloxidase to the active enzyme, converts tyrosine to dihydroxyphenylalanine. The modified phenylalanine binds to the bacterial cell surface, initiating the process of melanization. The precise role of melanization in the insect immune response remains unclear, although it has been suggested to function as an opsonization process promoting adherence to the hemocytes.

The production of several bactericidal proteins (attacins) and peptides (cecropins) is stimulated in many insects following infection with bacteria (28, 54). Attacin was found to alter the permeability properties of the outer membrane of *E. coli* and to inhibit the production of the major outer membrane proteins OmpF, OmpC, and OmpA (28). The ability of *Xenorhabdus* spp. to grow to high concentrations in the hemolymph suggests that the bacteria may be relatively insensitive to the action of the bactericidal proteins or may be able to inhibit either the induction or the function of the bactericidal polypeptides. The presence of the nematode in the hemolymph may also help inactivate the bactericidal proteins. The insect humoral immune system includes nonspecific hemolymph enzymes such as lysozyme, which is induced upon bacterial infection, and can promote bacterial cell wall degradation (39). Other nonspecific antibacterial enzymes (e.g., carbohydrases and proteases) are also present in the hemolymph and can act on and alter the cell surface properties of the invading bacteria (46). *Xenorhabdus* spp. appear to be generally resistant to attack by these enzymes in vivo.

Two different classes of hypovirulent strains of *X. nematophilus* that displayed reduced pathogenicity toward *G. mellonella* have recently been obtained by a chemical mutagenesis approach (41). One class was selected for its enhanced ampicillin resistance, while the second class grew in the presence of the membrane perturbant, polymyxin B. Several cell surface properties of the mutant strains were altered as measured by an increase in hydrophobic character and total LPS content

and a decrease in surface cationic charge and total outer membrane protein content. The levels of specific outer membrane proteins were also altered in the mutant strains (11). The mutant strains adhered to hemocytes more avidly and were more efficiently cleared from the hemolymph than were the wild-type cells. Altering the cell surface properties of the wild-type cells also compromised their ability to kill the insect host. Pretreatment of wild-type cells with enzymes that hydrolyze cell surface components enhanced bacterial adherence to hemocytes and the rapid removal of the bacteria from the hemolymph (46). These findings were consistent with the idea that the cell surface of *X. nematophilus* possesses anti-hemocytic properties that protect the bacterium from the insect defensive system. Part of the anti-hemocytic process for bacterial cells growing in *G. mellonella* may arise from the release of LPS from the bacteria and the subsequent damage to the hemocytes (44). Since the rate of LPS release was shown to be correlated with the reemergence of the bacteria in the hemolymph and injection of purified LPS into *G. mellonella* killed the insects, LPS is considered to be a virulence factor in *X. nematophilus* (44). Hemocyte lysis was proposed to be stimulated by the lipid A moiety of LPS of *X. nematophilus*. The role of LPS (endotoxin) in pathogenicity is of particular interest, since it has also been shown to be a virulence factor in bacterial infection in humans (99).

Avirulent mutant strains have also been isolated by a transposon (Tn*5*) mutagenesis approach (144). These strains were found to possess pleiotropic phenotypes which included a defect in cell motility, an inability to hemolyze sheep erythrocytes, and an absence of a highly expressed 32-kDa protein (78). In addition, the Tn*5* mutant strains attached to insect hemocytes with greater avidity and the outer membrane protein profile was altered relative to the wild-type cells (78). There appeared to be several transposon insertions per chromosome in the various avirulent strains. Taken together with the observation that both the Tn*5* and chemical mutant strains displayed pleiotropic phenotypes, these findings suggest that several genetic loci were affected in the mutant strains. Isolating avirulent mutants that contain discrete chromosomal mutations and complementing these strains with chromosomal clones that restore the wild-type level of pathogenicity should allow one to identify genes that contribute to the high level of virulence of *X. nematophilus* toward the insect target.

The participation of secreted virulence factors, or toxins, in the pathogenicity of *Xenorhabdus* spp. has not been carefully assessed. The virulence of the phase II *Xenorhabdus* cells toward *G. mellonella* has been stated to be similar to that of the wild-type strain (1, 10). However, these studies may not provide a sensitive enough assay for comparing possible differences in the virulence of phase I and phase II cells, since *G. mellonella* larvae possess a relatively weak immune system. A more potent immune system, such as that of *M. sexta*, may provide a more sensitive bioassay to measure differences in the virulence of phase I and phase II cells.

The phase II cells produce lower levels of phospholipase and lipase activity, produce low levels of secreted siderophores, exhibit weak hemolytic activity, and are less motile than the wild-type strain (21, 68). On the basis of these findings, motility, phospholipase and lipase activity, siderophore production, and the ability to lyse blood cells are not likely to be essential virulence determinants in *Xenorhabdus* spp. Akhurst has recently isolated a gene from *X. nematophilus* that produces a 31-kDa polypeptide that is toxic in *G. mellonella* (8). Characterization of this gene product is currently under investigation.

Photorhabdus Pathogenicity

An insecticidal complex elaborated from stationary-phase *P. luminescens* cells has been identified in the cell-free culture medium. The extracellular toxin is believed to be proteinaceous and did not possess protease or phospholipase activity (50). The high-molecular-weight (>700,000) native complex was composed of numerous protein subunits ranging in molecular weight from 23,000 to 200,000 (25). Injection of nanogram quantities of the toxin was sufficient to kill *M. sexta* larvae. Thus, from the data derived from the insecticidal activities studied so far in *P. luminescens* (a multisubunit complex) and *X. nematophilus* (a discrete polypeptide), it is conceivable that these bacteria may make different types of insects toxins.

Recent results of Clarke and Dowds (31) proposed that the lipase activity of *Photorhabdus* sp. strain K122 was a virulence factor for *G. mellonella*. The authors showed that the sterile extracellular culture medium from *E. coli* containing a plasmid encoding the K122 lipase gene was insecticidal whereas the extracellular preparations of the control *E. coli* cells were not toxic to *G. mellonella*. These results suggest that secreted products such as lipase may contribute to pathogenicity in *G. mellonella*. Whether lipase can function as a virulence factor in *M. sexta* and other insects remains unresolved. Study of the K122 strain deleted for the lipase gene should help to clarify the role of lipase in the virulence of *P. luminescens* toward different insect hosts.

Photorhabdus LPS was previously shown to damage the hemocytes of *G. mellonella* (45). However, neither purified LPS nor nonviable whole cells were toxic in *G. mellonella* larvae (31). Mutant strains produced by chemical mutagenesis displayed a pleiotropic phenotype in which several cell surface properties were altered (42). The alterations included an increase in surface hydrophobicity and hemocyte attachment, a reduction in the outer membrane protein content, and an increase in total LPS content. Unlike the hypovirulent mutant strains of *X. nematophilus*, the insecticidal properties of the *Photorhabdus* strains were enhanced relative to the wild-type strain. These results suggest that the role of the cell surface properties in virulence of *Photorhabdus* spp. may be different from that of those in *Xenorhabdus* spp.

In summary, cumulative information suggests that the primary virulence events that lead to the death of the insect host involve multiple factors such as the secretion of insect toxins and other extracellular products, the release of LPS molecules from the bacterial envelope, and the antihemocytic properties of the cell surface. Bacterial septicemia does not appear to be a requisite event for insect fatality as judged by studies with *M. sexta*, as discussed above. The actual molecular events that cause the demise of the insect host infected with *Xenorhabdus* spp. may in fact differ from those that occur during infection with *Photorhabdus* spp. Resolution of the structure and mechanism of action of the respective insect toxins may shed light on this unresolved issue.

CELL SURFACE PROPERTIES

The ability of *Xenorhabdus* and *Photorhabdus* spp. to survive within the host immune system will depend to a considerable extent on the envelope properties of the bacterium. The properties of the cell surface will also strongly influence the symbiotic interactions between the bacteria and the mucosa of the nematode intestine. Recognition of the importance of cell surface properties in the life cycle of *Xenorhabdus* cells has stimulated several laboratories to characterize outer membrane

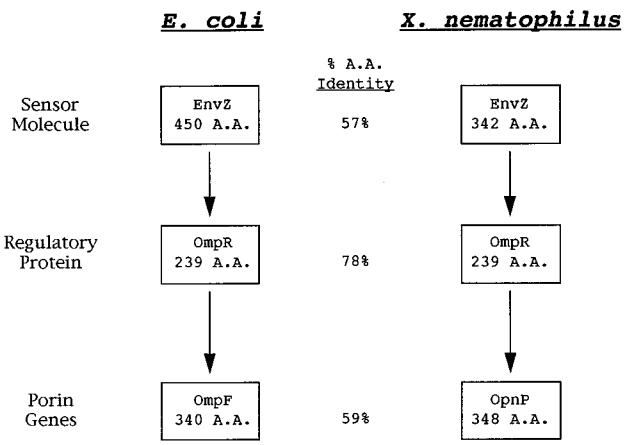

FIG. 3. Comparison of the EnvZ/OmpR signal transduction pathway in *E. coli* and *X. nematophilus*. The proteins are enclosed in boxes, and the number of amino acid residues (A.A.) in each protein is shown underneath. The percent amino acid identity shared by homologous proteins is shown. The OmpC protein of *E. coli* is not shown, since a homologous gene has not yet been identified in *X. nematophilus.*

proteins (62, 90), to analyze the flagella in phase I and phase II cells (68), and to study the properties of the fimbria (pilin) protein of this bacterium (17, 100). The cell surface properties of *Photorhabdus* cells have not been as well studied.

Outer Membrane Proteins of *X. nematophilus*

The outer membrane of gram-negative bacteria functions as a selective diffusion barrier. This specialized membrane contains a limited number of different types of proteins that exist in high copy number (70, 107). Nutrients and other compounds such as penicillin antibiotics passively diffuse across the outer membrane through water-filled diffusion channels or pores. Channels are formed by porin proteins that associate as homotrimers in the outer membrane. In *E. coli*, the regulation and function of two predominant porin proteins, OmpF and OmpC, have been extensively studied (Fig. 3). The channel formed by OmpF is slightly larger and the diffusion rate is approximately 10-fold higher than for the channel formed by OmpC (107). The OmpF protein is expressed under low-osmolarity conditions, while the production of OmpF is repressed and that of OmpC is induced by either higher osmolarity or elevated temperature (60). This adaptive response ensures that the number and type of pores in the outer membrane are adjusted to the growth and survival requirements of the cell. To better understand the adaptive response to environmental change, the major outer membrane proteins, Opn's, of *X. nematophilus* have been characterized (90). The most abundant protein, OpnP, which constitutes more than 50% of the total protein content of the outer membrane, has recently been purified (Fig. 4A). By reconstituting the purified protein in planar lipid bilayers, the general pore properties of OpnP were shown to be closely similar to the values predicted for the OmpF monomer (62). We have chosen a separate nomenclature for the major outer membrane proteins of *X. nematophilus*, although, as will become apparent below, OmpF and OpnP are highly conserved in most respects. The nucleotide sequence of *opnP* has been determined (Table 4). The deduced amino acid sequence of OpnP was found to share 59% identity with OmpF of *E. coli*. However, OpnP, unlike OmpF, is produced at high constitutive levels and is not repressed by high-osmolarity conditions (62).

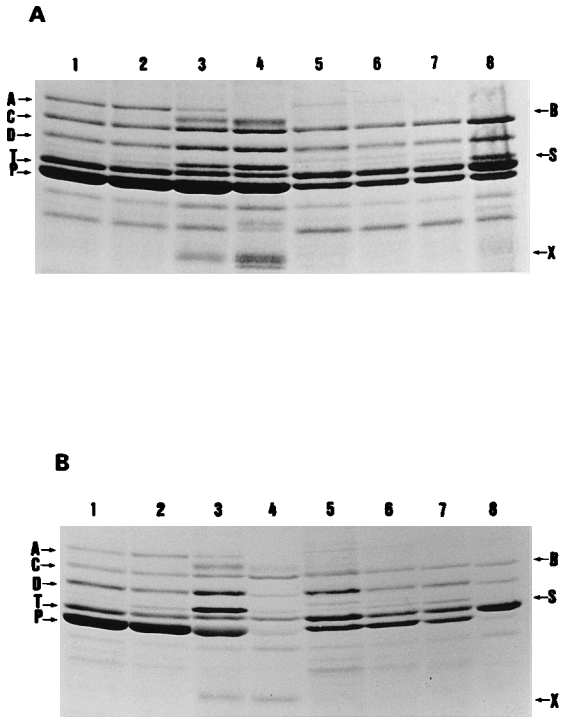

FIG. 4. Characterization of the outer membrane proteins of the wild-type (AN6; lanes 1 to 4) and *envZ* (ANT1; lanes 5 to 8) strains of *X. nematophilus*. (A) Outer membrane proteins stained with Coomassie blue and separated on an SDS-polyacrylamide gel. Opn's were prepared from cells harvested at early-log-phase growth (lanes 1 and 5), mid-log-phase growth (lanes 2 and 6), late-log-phase growth (lanes 3 and 7), and stationary-phase growth (lanes 4 and 8). The positions of OpnA, OpnC, OpnD, OpnT, and OpnP are indicated along the left side of the panel. The positions of the stationary-phase proteins, OpnB, OpnS, and OpnX, are indicated on the right side of the panel. (B) Autoradiograph of pulse-labeling of AN6 and ANT1 with radiolabeled amino acids. Cells were pulse-labeled at the times in the growth curve indicated for panel A.

Pulse-labeling of *X. nematophilus* with radioactive amino acids during progressive stages of bacterial growth (Fig. 4B) showed that the synthesis of OpnP is dramatically reduced as the cell enters stationary-phase growth conditions (Fig. 4B, lane 3). In contrast, the production of other outer membrane proteins, such as OpnS and OpnP (see below), is increased under stationary-phase growth conditions. Since OpnP does

TABLE 4. Sequenced genes of *X. nematophilus* and *P. luminescens*

Gene (strain)	Homologous gene	% Identity	Reference
Xenorhabdus spp.			
ompR (AN6)	*ompR* (*E. coli*)	78	135
envZ (AN6)	*envZ* (*E. coli*)	57	135
opnP (AN6)	*ompF* (*E. coli*)	59	62
asnS (AN6)	*asnS* (*E. coli*)	86[a]	62
aspC (AN6)	*aspC* (*E. coli*)	82[a]	62
xin	*hin* (*S. typhimurium*)	NA[b]	8
Toxin	None	NA	8
Photorhabdus spp.			
rpsO (K122)	*rpsO* (*E. coli*)	86	30
pnp (K122)	*pnp* (*E. coli*)	86	30
lip-1 (K122)	None		139
cipA (NC1)	None		18
cipB (NC1)	None		18

[a] Comparisons based on partial nucleotide sequence analysis.
[b] NA, unpublished sequence for which the percent identity is not available.

not turn over at an appreciable rate (Fig. 4A, compare lanes 1 and 4) and cell division occurs very slowly under these conditions, the OpnP present in cells entering stationary-phase growth was actually synthesized during exponential-phase growth. This implies that de novo synthesis of OpnP is not required for survival during stationary-phase growth. Thus, it appears that one way the cell responds to nutrient limitation during stationary-phase growth is to reduce the synthesis of the highly expressed OpnP protein. As discussed in the next section, study of the regulatory circuitry of the *opnP* gene has revealed interesting similarities and unexpected differences compared with the regulation of *ompF* in *E. coli.*

The outer membrane proteins OpnA, OpnC, OpnD, and OpnT are also produced by *X. nematophilus* during exponential growth at 30°C (Fig. 4A). OpnC, OpnD, and OpnT are produced constitutively at 30°C, while OpnT is not produced in cells grown at temperatures below 27°C (90). During stationary-phase growth, two additional proteins, OpnB and OpnS, are produced and OpnA production is markedly reduced. Pulse-labeling experiments (Fig. 4B) have shown that the synthesis of OpnB and OpnS increases during the transition into stationary-phase growth (lane 3). In *E. coli*, this pattern of regulation is characteristic of genes controlled by stationary-phase sigma factors (74). In this regard, it will be of interest to determine whether the genes that encode OpnB and OpnS are regulated by the stationary-phase sigma factor, σ^s (74).

OpnB is induced during stationary-phase growth but is not produced at elevated temperatures (34°C) or under higher-osmolarity or anaerobic conditions and is not produced by the phase II cells (90). This type of regulation suggests that OpnB is involved in altering surface properties of *X. nematophilus* in response to environmental change.

To fully understand the role of cell surface properties in the adherence to and survival within the nematode gut and in the ability to evade or tolerate the insect host immune system, it will be necessary to elucidate the functions of the Opn's of *X. nematophilus.*

Analysis of Fimbriae, Flagella, and Glycocalyx

Bacterial cell surface adhesins such as fimbriae mediate the attachment to host tissues. Attachment in turn may promote the initial stage of infection in many different microorganisms (92). Fimbriae (pili) of enteric bacteria are generally either rod-like structures approximately 7 nm in diameter (type I) or flexible fibrillae (P pili) with a considerably smaller diameter (27). Fimbriae in *X. nematophilus* are thought to be involved in the establishment of the specific association between the bacterium and the nematode gut. The fimbriae of *X. nematophilus* have recently been analyzed (17, 100). The peritrichously arranged fimbriae were of rigid morphology and were approximately 6 to 7 nm in diameter. The fimbriae were composed mainly of a single subunit with a molecular weight between 16,000 (100) and 17,000 (17). While the fimbriae appear to belong to the type I class, sheep erythrocyte hemagglutination studies revealed that unlike the *E. coli* type I pili, the hemagglutination of sheep erythrocytes by *Xenorhabdus* spp. was resistant to mannose (100). Immunogold-labeling experiments have shown that the 17-kDa fimbria protein is expressed when the bacteria inhabit the gut of the nematode (17). The phase II cells did not produce fimbriae at detectable levels, since there was no positive reaction with antisera directed against the 17-kDa protein and fimbriae were not visible by scanning electron microscopy of negatively stained cells. The apparent absence of fimbriae on the surface of the phase II cells may account, in part, for the poor retention of these cells in the gut of the infective juvenile nematode.

All strains of phase I cells of *Xenorhabdus* spp. so far analyzed were motile in liquid culture and exhibited swarming motility on semisolid agar (68). The molecular weight of the flagellin subunit was shown to be 36,500. In marked contrast, phase II cells of the same strains were found to lack both swimming and swarming ability and did not produce flagella. These results suggested that the presence of flagella and bacterial motility are not essential virulence determinants, since the phase II cells are insecticidal even though they do not produce flagella.

The presence of capsular material on the surface of phase I and phase II cells of *Xenorhabdus* and *Photorhabdus* spp. has been determined by transmission electron microscopy after fixation with glutaraldehyde-lysine (26). The thickness of the glycocalyx layer in the phase I cells of *Xenorhabdus* spp. (142 nm) was approximately threefold greater than that of the phase II cells (49 nm). The same relationship was observed in *Photorhabdus* spp. in which phase I cells possessed a thicker glycocalyx layer (85 nm) than did the phase II cells (40 nm). The greater thickness, and the possible chemical differences of the glycocalyx, of phase I cells relative to phase II cells could contribute to the ability of former cell type to adhere to a greater extent to the intestinal cells of the nematode. Further studies to compare the biochemical composition of the glycocalyx to two phases of the same strain are in progress (26).

In summary, studies on the cell surfaces of *Xenorhabdus* and *Photorhabdus* spp. over the past few years have been instrumental in identifying several components of the cell surface of *X. nematophilus* that may play a role in symbiosis and/or pathogenicity. Seven different major outer membrane proteins of *X. nematophilus* (Opn's) have been identified (90). The gene encoding the major porin protein, OpnP, has been sequenced (135). In addition, pilin subunits have been purified (17, 100) and molecular tools are being developed to study the role of fimbriae in the symbiotic association of the bacterium-nematode complex. Finally, new molecular parameters have been identified to study phase variation in *X. nematophilus*. These include the OpnB and OpnS proteins and the flagellum and pilin subunits.

MOLECULAR BIOLOGY

Intriguing central questions about the life cycle of *Xenorhabdus* and *Photorhabdus* spp. include the following. How do the bacteria survive and proliferate within the larval hemolymph and kill the insect host? What determines the species-specific symbiotic association with the nematode? What are the mechanisms controlling phenotypic plasticity or phase variation? Many of the genetic methods needed to study the molecular mechanisms of these problems have recently become available.

Genetic Approaches to the Study of *Xenorhabdus* and *Photorhabdus* spp.

Xenorhabdus spp. have not been identified as free-living bacteria in nature; rather, they appear to be protected from external environmental conditions as they shuttle between the intestinal vesicle of the nematode and the hemolymph of the host insect. This may explain the relative fragility of these bacteria under standard laboratory conditions (143). For example, the number of CFU obtained after dilutional plating can be dramatically smaller than the number of cells observed by microscopic cell counting (56, 143). This low plating effi-

ciency can be ameliorated by preparing and storing agar plates under dim light conditions, by adding catalase or pyruvate to the growth medium, by diluting the bacteria in appropriate growth medium, and by using gentle plating methods (56, 143).

We have found that *X. nematophilus* tolerates only a narrow range of osmolarity conditions (56) and grows slowly under nutrient broth conditions. Growth is stimulated by the addition of an osmolyte such as 5% sucrose, while cell lysis occurs in the presence of 10% sucrose and when the cells are resuspended in buffer solutions. In contrast, *E. coli* doubles at the same rate in nutrient broth and nutrient broth containing 20% sucrose and does not lyse when resuspended in buffer solutions. The fragility of *Xenorhabdus* spp. has made the transfer of plasmids by electroporation problematic. So far, transformation by electroporation has not been successful, although numerous conditions have been tried (56).

Xenorhabdus spp. appear to possess a restriction modification system that affects the efficiency of transformation by different plasmids. Xu et al. (144) have shown that *X. nematophilus* ATCC 19061 can be efficiently transformed with the broad-host-range vector pHK17 if the plasmid is first passaged through *Xenorhabdus* cells. By using dimethyl sulfoxide-treated cells approximately 10^5 to 10^6 transformants per μg of pHK17 could be obtained. In contrast, when pHK17 was isolated from *E. coli*, the transformation efficiency was reduced more than 250-fold. It was proposed that modification of the plasmid DNA as it was passaged through *Xenorhabdus* cells accounted for the enhanced transformation efficiencies (144). Consistent with this idea is the finding that digestion of *X. nematophilus* chromosomal DNA with several common restriction enzymes is difficult (22, 56).

Xu et al. (144) showed that although the type strain, ATCC 19061, could be efficiently transformed, another strain of *X. nematophilus*, IM/1, could not be transformed by the same dimethyl sulfoxide treatment as described above. The reason for the variability in transformation efficiencies of different strains of *X. nematophilus* has not been resolved. To date, the most reliable method to transfer plasmids into *X. nematophilus* has been by conjugal transfer from *E. coli* (56, 145). This has been shown to be an efficient and reproducible method for transferring several broad-host-range plasmids into *X. nematophilus*. We have found that several commonly used conjugatable vectors can be mobilized from the donor cell (e.g., *E. coli* S17-1) into *X. nematophilus.*

In contrast to the technical challenges presented by *Xenorhabdus* spp., manipulation of *Photorhabdus* spp. has been less problematic. *Photorhabdus* cells are not as fragile as *Xenorhabdus* cells and do not lyse readily when resuspended in buffer solutions (102). Electroporation of *Photorhabdus* cells has been achieved (ca. 10^4 transformants per μg) with pBR322 (38). Furthermore, by using a modified $CaCl_2$-$RbCl_2$ method, it has been possible to transform *Photorhabdus* cells with several plasmids used in standard recombinant DNA procedures (63, 64).

Several approaches have been used to create mutant strains of both *Xenorhabdus* and *Photorhabdus* spp. While suicide vectors that do not replicate in *X. nematophilus* have not yet been identified, Xu et al. (145) have recently created a negative selection vector carrying the transposon Tn*5* (kanamycin resistance) and the *sacB* gene (levansucrase) of *Bacillus subtilis*. The production of levansucrase in most gram-negative bacteria is lethal when the cells are incubated on sucrose-containing media. Only bacteria in which the plasmid has been lost and the Tn*5* transposon has inserted into the chromosome will be viable on kanamycin-sucrose plates. Numerous classes of mutant strains of *X. nematophilus* have been identified by this method. Of particular interest was the isolation of three avirulent strains that were defective in their ability to kill *G. mellonella*. The avirulent strains were all nonmotile, possessed low hemolytic properties, and were missing a 32.5-kDa protein that was prevalent in the wild-type strain (78). A similar approach was used to insertionally inactivate the *envZ* gene of *X. nematophilus* (56) and the crystalline protein genes *cipA* and *cipB* of *P. luminescens* (18). To address the question of phase variation, the DNA fragment encoding the invertase (*hin*) gene of *Salmonella typhimurium*, which is involved in phase variation, was used to isolate a clone carrying the homologous gene of *X. nematophilus* (8). The nucleotide sequence of this gene, *xin*, was determined (8) (Table 4). To study the role of invertase in phase variation, a *xin* mutant strain (phase I) was constructed. The phenotype of this strain with respect to phase variation properties and DNA rearrangement was not detectably different from that of wild-type cells. This result indicates that the *xin* gene does not play a role in phase variation in *X. nematophilus.*

Francis et al. (65) have recently developed a lambda delivery system for *X. bovienii*. This bacterium lacks a functional lambda receptor (LamB) and does not support lambda replication. Transferring a *lamB*-bearing plasmid into *X. bovienii* T228 resulted in constitutive expression of LamB on the surface of the bacterium. Infection of *X. bovienii*/pTROY9 with the lambda particle carrying the Tn*10*-derived transposon resulted in enhanced phage absorption over the wild-type strain. Numerous auxotrophic, hemolytic, dye-binding, DNase, protease, and lipase mutants were obtained by this method. The mutation frequency ranged from 0.05 to 0.9%. This method should be applicable to other *Xenorhabdus* spp.

In summary, with the availability of transposon mutagenesis approaches, gene transfer methods, and chromosomal libraries and the identification of genes that are homologous to those found in well-studied enteric bacteria such as *E. coli* and *Salmonella typhimurium*, it is now possible to begin to develop *Xenorhabdus* and *Photorhabdus* spp. as model systems with which to study the molecular biology of numerous biological phenomena such as symbiosis, pathogenesis, phase variation, and adaptation to changes in environmental conditions.

Signal Transduction in *X. nematophilus*

The life cycle of *Xenorhabdus*/*Photorhabdus* species presents a series of challenges to the bacteria, requiring adaptation to different conditions of osmolarity, nutrient level, and, perhaps, predatory stress due to the defense mechanisms of the prey insect (Fig. 2). In the gut of the nonfeeding infective juvenile nematode, nutrient levels are assumed to be low and the bacteria are either not dividing or dividing slowly. When the bacteria are released into the hemolymph, they encounter a nutrient-rich environment. At the same time, they must respond to the assault by the cellular and humoral antibacterial defensive systems. The infection of the insect by *Xenorhabdus* spp. produces a cessation of feeding, a decrease in growth rate, and, ultimately, larval death (Fig. 2). The rapid growth of the bacteria to high concentrations will dramatically alter the composition of the insect hemolymph. Given these changing conditions, it follows that, like *E. coli*, the *Xenorhabdus*/*Photorhabdus* bacteria should have developed the ability to respond to such environmental changes and adapt appropriately to each given stage of the life cycle. Studies of these abilities have begun with a comparison with the well-established mechanisms of adaptation known in *E. coli*, namely, regulation of the genes encoding the outer membrane porin proteins. The expression of the porin genes is modulated in response to changes in the

osmolarity of the growth environment (60) (Fig. 3). *X. nematophilus* has been shown to possess an analogous regulatory system. Most intriguingly, studies of the molecular mechanisms of this regulatory pathway have revealed some significant differences between the signal transduction systems of *Xenorhabdus* spp. and *E. coli*. These studies have already contributed to new insights in the understanding of how the porin gene regulation system functions in *E. coli*, as discussed below.

In the *E. coli* system, the well-studied inner membrane osmolarity sensor protein, EnvZ, undergoes autophosphorylation and subsequently phosphorylates the DNA-binding regulatory protein, OmpR (57, 58, 78, 98, 125). EnvZ and OmpR are members of the large family of signal-transducing molecules referred to as the two-component system of regulatory molecules (61). Modulation of the levels of OmpR phosphate controls the differential expression of the genes that encode the major outer membrane pore-forming proteins, OmpF and OmpC (59, 124, 125, 140). To begin to elucidate the molecular aspects of environmental adaptation, we have recently isolated and sequenced the two-component signal transduction genes, *ompR* and *envZ*, of *X. nematophilus* (135) (Table 4). The OmpR molecules of *X. nematophilus* and *E. coli* were found to share 78% amino acid sequence identity and were highly conserved in the region encompassing the N-terminal site of phosphorylation and the C-terminal DNA-binding domain (Fig. 3). Nucleotide sequence analysis revealed that EnvZ of *X. nematophilus* ($EnvZ^{X.n.}$) is composed of 342 amino acid residues, which is 108 residues shorter than EnvZ of *E. coli* ($EnvZ^{E.c.}$). Amino acid sequence comparison showed that the cytoplasmic domains, containing the ATP-binding and autokinase activity of EnvZ, shared 57% sequence identity (Fig. 3). In contrast, the large hydrophilic periplasmic domain of $EnvZ^{E.c.}$ was missing in $EnvZ^{X.n.}$ and was replaced by a divergent hydrophobic region. Remarkably, $envZ^{X.n.}$ was able to complement an *envZ*-minus strain of *E. coli* and properly regulate *ompF* and *ompC* expression in response to changes in medium osmolarity (135). These results indicated that the EnvZ protein of *X. nematophilus*, which lacks the hydrophilic periplasmic domain, was able to sense changes in the osmolarity of the growth environment and properly regulate the levels of OmpR phosphate in *E. coli*. As mentioned above, OpnP is not osmoregulated in *X. nematophilus*; however, the above results indicate that the $EnvZ^{X.n.}$ protein is able to osmoregulate the *ompF* gene of *E. coli*. The molecular explanation for this difference in the regulation of *ompF* and *opnP* genes in *E. coli* and *X. nematophilus*, respectively, will be addressed below.

Using an interposon inactivation approach, we have created an *envZ*-minus strain of *X. nematophilus* (136) (Fig. 4A, lanes 5 to 8). The OpnP level in this strain was reduced by more than 60% relative to that of the wild-type strain, indicating that *envZ* regulates OpnP. The production of several stationary-phase-regulated Opn's was also significantly reduced in the *envZ* null strain. In contrast, all other biochemical and physiological properties examined in the *envZ* strain were indistinguishable from those in the wild-type strain. These findings provide the basis for the notion that the EnvZ protein of *X. nematophilus* is a complex, multifunctional sensor involved in both the production of OpnP during logarithmic-phase growth and the stationary-phase regulation of OpnB and OpnS. At present, the molecular details of the sensing mechanisms of $EnvZ^{E.c.}$ and $EnvZ^{X.n.}$ are not clearly understood. It is interesting that a common feature of cells grown under high-osmolarity conditions and under stationary-phase conditions is a significant decrease in cell volume. It is conceivable that both EnvZ proteins are sensing similar physical parameters (e.g., inner membrane fluidity) that are generated by different physiological events (high osmolarity versus stationary-phase growth). Most importantly, our results point to the possibility that the membrane domains are involved in the putative membrane-sensing function of EnvZ while the periplasmic domain of $EnvZ^{E.c.}$, which is lacking in $EnvZ^{X.n.}$, is involved in binding specific, as yet unidentified, ligands. Finally, we note that in *Shigella flexneri*, the *ompR*, *envZ*, and *ompC* genes are required for infection in the mouse model system (16).

Since OpnP is the predominant outer protein of *X. nematophilus*, modulation of the level of expression of *opnP* will strongly influence the cell surface properties of this bacterium. To study the regulation of *opnP*, we have isolated and determined the nucleotide sequence of this gene (62). The deduced amino acid sequence revealed that OpnP and OmpF shared 59% identity (Fig. 3). On the basis of secondary-structure predictions, OpnP was found to contain a high degree of β-sheet structure, which is characteristic of porin proteins of gram-negative bacteria (37). Like *ompF*, the *opnP* gene is flanked by the upstream gene, *asnS*, and the downstream gene, *aspC*. A perfect OmpR-binding sequence was present approximately 90 bp upstream of the start of transcription, and a consensus σ^{70} promoter sequence could be identified at the −35 and −10 positions of *opnP*. Moreover, the nucleotide sequences immediately adjacent to the translation start site in *opnP* and *ompF* were almost identical. In *E. coli*, the antisense RNA molecule, *micF* RNA, binds to this region of the mRNA of *ompF*, creating an RNA-RNA hybrid (13). This hybrid is recognized by specific binding proteins that are proposed to stimulate the degradation of *ompF* mRNA (127, 133). The levels of *micF* RNA are elevated under conditions of high temperature (13), intermediate osmolarity (121), or oxidative stress (29). These results suggest that *micF* RNA is present in *X. nematophilus* and may be involved in posttranscriptional regulation of *opnP*. It is important to note that the gene encoding *micF* RNA has been sequenced from several enteric bacteria and shown to be phylogenetically conserved (51).

While the nucleotide sequence of *opnP* and *ompF* is conserved in many features, the upstream intergenic region between *asnS* and *opnP* was found to be 312 bp shorter than the equivalent region between *asnS* and *ompF*. In *E. coli*, an OmpR-binding site located approximately 360 bp upstream of the start of transcription is essential for the repression of *ompF* under high-osmolarity conditions (80, 122). This site was missing in the upstream region of *opnP*. Consistent with this finding is that *opnP* is not repressed by high-osmolarity growth conditions. The nucleotide sequence of *ompF* had previously been determined only in *E. coli*; however, OmpF-like proteins are known to exist in a wide variety of other bacteria. Thus, further characterization of the *opnP* gene should provide new insights into the role that porin molecules play in allowing bacteria to adapt to unique ecological niches and in establishing and maintaining symbiotic associations with the host.

Taken together, these results suggest that the basic elements of the central signal transduction/gene expression system that control some of the most highly expressed proteins in both *E. coli* and *X. nematophilus* have been conserved but component parts of the sensing and regulatory circuits have significantly diverged. While the catalytic domains of the EnvZ molecules and the proximal regulatory regions and structural genes encoding *opnP* and *ompF* were highly conserved, the periplasmic domains of the EnvZ protein of *X. nematophilus* and the upstream repression site of *opnP* have completely diverged from their counterpart sequences in *E. coli*. It appears that the molecular mechanism of the phosphotransfer reactions carried out by the EnvZ molecules and the structure required to form an OmpF-like pore are under strong selective pressure. In

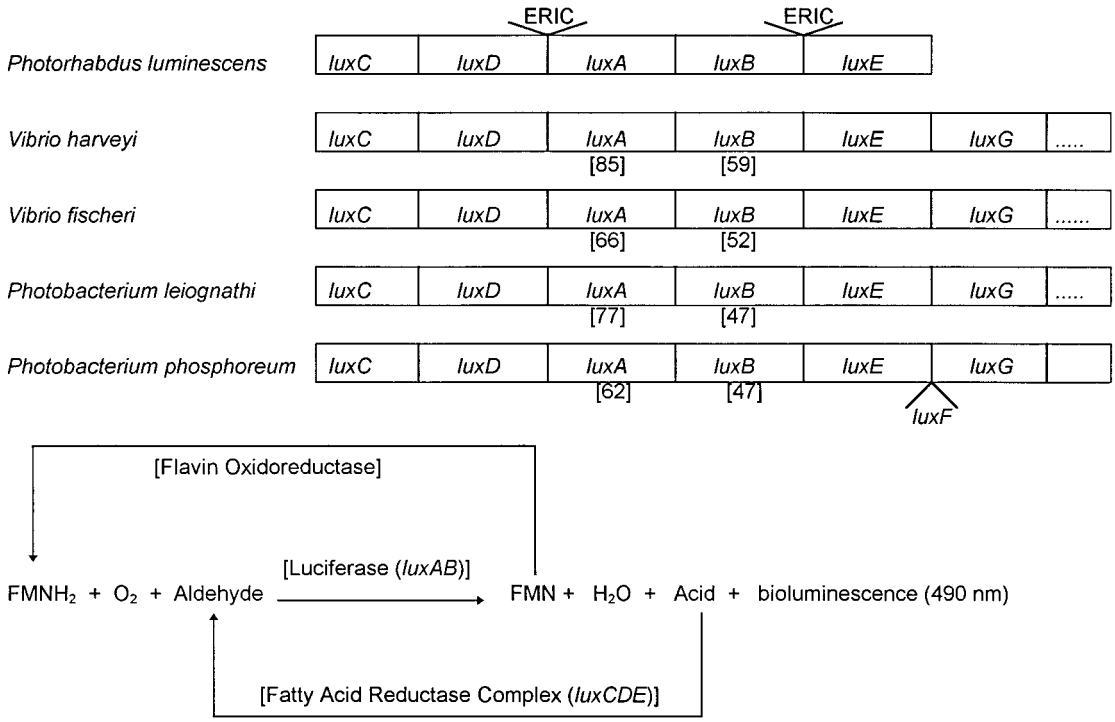

FIG. 5. Bioluminescence genes and gene products known from *P. luminescens*, and their comparison with similar genes from other species of luminous bacteria. The arrangement of the structural genes of *P. luminescens* (including the location of the multiple ERIC sequences) is shown in the top row and is compared with that of similar genes from other luminous bacteria that have been sequenced. The numbers in parentheses indicate the amino acid identity values for the corresponding proteins coded for by the structural genes. Regulatory genes are not included in this diagram, because none has yet been identified from *P. luminescens*. Data for this presentation are taken from references 49, 63, 64, 83, 96, 97, 133, and 142.

contrast, the different ecological niches inhabited by these bacteria have allowed the periplasmic domains of the EnvZ molecules and the upstream regulatory regions that control the expression of *ompF* and *opnP*, respectively, to diverge markedly. This pattern of component molecular evolution may have occurred to allow the respective bacteria to adapt optimally to different environmental conditions.

Genes Sequenced in *P. luminescens*

A number of *Photorhabdus* genes have recently been sequenced (Table 4). These include *pnp* (polynucleotide phosphorylase), *rpsO* (ribosomal protein S15), *lip-1* (lipase), and *cipA* and *cipB* (crystalline proteins). The *luxCDABE* operon, which encodes the proteins required for bioluminescence, has also been sequenced (Fig. 5). Deduced amino acid sequences of *pnp* and *rpsO* have indicated that these proteins share a high degree of sequence identity with their counterparts in *E. coli* while the proteins encoded by *luxCDABE* share sequence similarity with the same genes of marine bioluminescent bacteria such as *Vibrio harveyi*. The nucleotide sequences of *cipA*, *cipB*, and *lip-1* are unique.

Since *Photorhabdus* spp. have been exploited for their usefulness as a biological pest control agent (87), the ability to grow at temperatures below 28°C (the optimal growth temperature in the laboratory) is of considerable interest. To identify proteins that may be important in low-temperature growth, Clarke and Dowds (30) have studied the induction of genes at 9°C in *P. luminescens* K122. N-terminal sequence analysis of a cold-inducible 87-kDa protein revealed that it was nearly identical to that of the cold-induced protein polynucleotide phosphorylase (PNPase) of *E. coli*. PNPase is involved in mRNA turnover in *E. coli*. Clarke and Dowds cloned and sequenced the genes encoding PNPase (*pnp*) and the adjacent ribosomal protein, RpsO. PNPase of *P. luminescens* contains 709 amino acid residues and shares 86% sequence identity with PNPase of *E. coli*. Identical to the situation in *E. coli*, the *rpsO* gene, encoding ribosomal protein S15, was found immediately upstream of *pnp*. The RpsO proteins share 86% amino acid identity. The 244-bp intergenic region between *rpsO* and *pnp* was also highly conserved and contained several regulatory elements. The cold-inducible promoter, P2, was identified in this region. In addition, a putative binding site for the cold-inducible transcriptional regulatory protein, CS7.4, was identified 69 bp upstream of the start of transcription of *pnp* of *P. luminescens* (30). This binding site does not appear to exist in *pnp* of *E. coli*. Furthermore, a stem structure containing 10 bp of nucleotide sequence identity, which is known to be the RNase III cleavage site for the mRNA of *E. coli pnp*, was also found in this region of the *Photorhabdus* gene. Primer extension analysis indicated that the RNase III cleavage site was used in vivo. These results suggested that the posttranscriptional regulation by RNase III was similar for *pnp* of *E. coli* and *P. luminescens*. In contrast, *P. luminescens* utilizes both supercoiled DNA and CS7.4 to transcriptionally regulate *pnp* whereas *E. coli* does not utilize the cold-inducible transcriptional regulatory protein. Clarke and Dowds (30) suggested that to adapt to the constant exposure to low-temperature growth, *P. luminescens* appears to select for the promoters of important genes like *pnp* by positively regulating the promoter with CS7.4. In *E. coli*, which spends much of its life at higher temperatures, cold-inducible transcriptional regulation of *pnp* appears to be coupled to the depletion of energy levels, which

may not occur to the same extent in a psychrotrophic bacterium like *P. luminescens.*

Phase I cells of *P. luminescens* K122 secrete a Tween 80-hydrolyzing lipase during stationary-phase growth. Wang and Dowds (139) found that the specific activity of the Tween 80-hydrolyzing lipase secreted from phase I cells was sixfold higher than that of the phase II cells. To analyze the mechanism of the reduced activity of the phase II lipase, the authors cloned and sequenced the lipase gene, *lip-1*, from phase II cells. The *lip-1* gene encodes a protein of 645 amino acids. The nucleotide sequence of the structural gene did not share similarity with other known lipases. The structure and start of transcription of *lip-1* were shown to be the same for phase I and phase II cells. A consensus σ^{70}-type promoter sequence could be identified at the −10 and −35 positions of *lip-1*. Adjacent promoter sequences that resemble the *uvrB* and *malEFG* promoters of *E. coli* were also identified. Comparison of the expression of *lip-1* in phase I and phase II cells indicated that the levels of *lip-1* mRNA and the amount of secreted LipA protein were essentially equivalent in the two cell types. These intriguing results suggested that a posttranslational mechanism, possibly related to either protein folding or binding of an inhibitory molecule, accounts for the differences in the activity of lipase secreted by the phase I cells and phase II cells. It was also found that sodium dodecyl sulfate (SDS) activated the lipase, as well as the protease, obtained from phase II cells (38). On the basis of these findings, it is conceivable that certain chaperone proteins present in phase I cells are missing in phase II cells and that the activities of several gene products are affected by the absence of this chaperone protein. Interestingly, *E. coli* containing a plasmid encoding *lip-1* of phase II cells secreted an active lipase during stationary-phase growth.

Bintrim has cloned and sequenced the genes *cipA* and *cipB*, encoding the two crystalline proteins of *P. luminescens* NC1 (18). The deduced amino acid sequence of CipA and CipB indicated that the proteins contained 104 and 101 residues, respectively. They shared 24% amino acid sequence identity with each other and did not share similarity with proteins in the GenBank database. Antibodies raised against the respective crystalline proteins were not cross-reactive. The promoters contained a perfect σ^{70} consensus sequence at −35 (TTGA CA) and a nearly perfect −10 sequence (TATAGT). Both gene products were efficiently expressed in *E. coli*, resulting in inclusion body formation in cells carrying the respective clones. An insertional inactivation approach was used to individually create the *cipA*-minus and *cipB*-minus strains. Interestingly, the double-mutant strain could not be obtained, suggesting that at least one crystalline inclusion body protein must be produced to maintain cell viability. The phenotypic properties of the *cip* mutant strains, with respect to dye binding, pigmentation, bioluminescence, protease, lipase, and several other characteristics, were, in general, intermediate between those of the phase I and phase II cells.

ANTIBIOTICS, PIGMENTS, AND INHIBITORS

Antibiotics

In a review article in 1959, Dutky (47) noted that the bacteria associated with insect-pathogenic nematodes produced antibacterial activity that kept the insect carcass from putrefying and was probably important in the successful completion of the life cycle by the nematodes. Antibiotic production is now known to be a very common characteristic of *Xenorhabdus* and *Photorhabdus* species (2, 110), and the compounds produced as antibiotics are quite diverse (94, 95, 123, 132, 134). The role of the antibiotics has not been proven, but it is speculated, in agreement with the views of Dutky, that their importance lies in maintaining dominant cultures of the bacteria during the nematode growth phase in the infected insect and thus avoiding putrefaction of the carcass with resulting poor nematode growth. In general, phase II cells are low or lacking in antibiotic activity (21).

***Xenorhabdus* antibiotics.** A systematic chemical study of the antibiotics produced by the *Xenorhabdus* species (*X. nematophilus*, *X. feltiae*, *X. bovienii*, and *X. poinari*) has not been undertaken, but several different inhibitory compounds have been isolated from various strains of these species and identified. Xenorhabdins (Fig. 6A) are members of the pyrrothine family of antibiotics isolated from *X. bovienii* and other *Xenorhabdus* spp. (94). Xenorhabdins are effective against gram-positive bacteria but less so against gram-negative bacteria. The material was prepared originally by sequential ethyl acetate and acetone extraction of a dried ethanol powder of an original ethanol extract of cells grown on a solid substrate (chicken offal on polyurethane foam) and later by growth on defined media. *Micrococcus luteus* was used as the test organism for purification of the antibacterial activity, which was associated with a yellow fraction with a characteristic UV-visible absorbance spectrum. Several different xenorhabdins were isolated and identified from a few strains so studied (94).

In contrast, the xenocoumacins were purified from the water-soluble fraction as activity that was colorless and had no characteristic UV-visible spectrum. The two different xenocoumacins shown in Fig. 6B were isolated from two different strains (*Xenorhabdus* sp. strain Q-1, which also was used for the purification of xenorhabdins, and *X. nematophilus* A11) and identified (95).

In separate experiments, *Xenorhabdus* spp. were grown on a synthetic complex liquid medium and the growth medium was extracted with ethyl acetate and tested; the antibiotic activity was traced to a series of indole derivatives with potent antibacterial activity (Fig. 6D) (110). It is not clear whether the difference in antibiotics isolated from *X. nematophilus* by the two different groups is due to differences in extraction methods, in the growth media used, or in the strains employed. Sundar and Chang (132) studied indole production in *X. nematophilus* and reported that the activity was maximal during late stationary phase and enhanced by the addition of tryptophan to the growth medium. Addition of the antibiotics caused severe inhibition of RNA synthesis in both gram-positive and gram-negative bacteria. Experiments with *E. coli* strains differing at the *relA* locus led to the conclusion that the mechanism of action of the antibiotic was via an enhancement of the regulatory nucleotide ppGpp (guanosine 3′,5′-bispyrophosphate) synthesis, resulting in the inhibition of RNA synthesis. Maxwell et al. (93) recently reported the stability of antibiotic activity from *G. mellonella* infected with *X. nematophilus*; the chemical nature of these antibiotics was not determined.

***Photorhabdus* antibiotics.** Compounds with antibiotic activity that have been characterized from *P. luminescens* fall into two chemical groups. Paul et al. (110) reported the presence and antibiotic activity of compounds in the hydroxystilbene group (Fig. 6C), as confirmed by Richardson et al. (123) and Li et al. (91). Sztaricskai et al. (134) observed no hydroxystilbenes produced by a strain of *P. luminescens*, a fact that may be explained by the disappearance of these compounds in the very late growth phase (91). These compounds apparently exert their antibiotic activity via the inhibition of RNA synthesis by stimulating the accumulation of ppGpp, as seen for the indole derivatives produced by *X. nematophilus* (131).

Sztaricskai et al. (134), while finding no hydroxystilbene-type

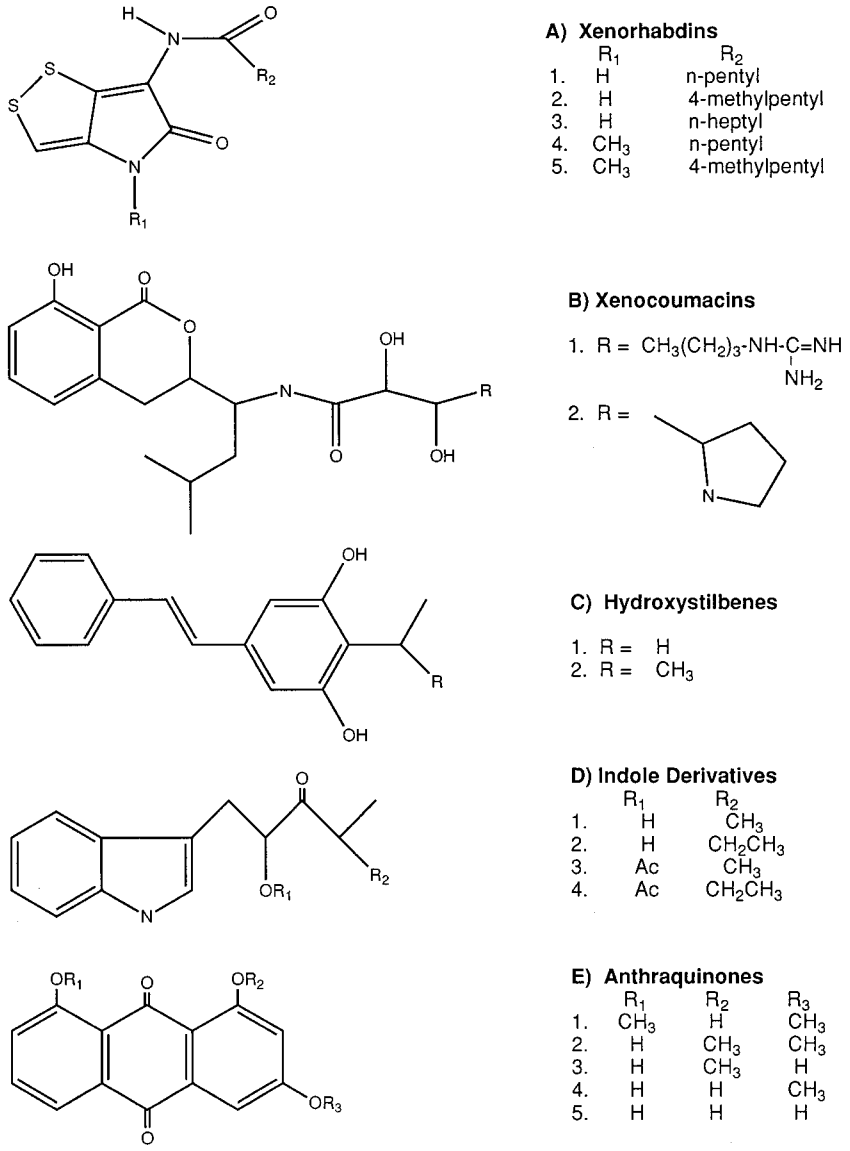

FIG. 6. Antibiotics and pigments from *Xenorhabdus* spp. and *P. luminescens*. The structures of the various antibiotics and pigments that have been identified are shown here. (A) Xenorhabdin antibiotics (94). (B) Xenocoumacin antibiotics (95). (C) Hydroxystilbene antibiotics (91, 110, 123, 131). (D) Indole derivative antibiotics (110, 132). (E) Anthraquinone derivatives, which are colored pigments with some antibiotic activity (91, 110, 123, 134).

antibiotics in an unidentified strain of *P. luminescens*, confirmed the presence of polyketide compounds which possessed antibiotic activity (Fig. 6E). Richardson et al. (123) had reported the polyketide pigments from *P. luminescens* but reported no antibiotic activity of these compounds. The recent report of Li et al. (91) has identified several new polyketide structures, one of which possesses antifungal activity (Fig. 6E). Since these compounds are structurally similar to other known polyketide antibiotics, it is not unreasonable to think that they might have antibiotic activity. A systematic chemical and antibiotic activity analysis of the polyketides from various *P. luminescens* strains has not been published, so the point remains unresolved.

It is fascinating that such a variety of antibiotics has arisen from only a few *Xenorhabdus* and *P. luminescens* strains studied; it would seem that the production of antibiotic activity is of great importance to the symbiosis, although the chemical nature of the compound produced is apparently not conserved among the species. At this point, it seems that polyketides and hydroxystilbenes are the compounds synthesized in *P. luminescens* whereas the indole derivatives, xenocoumacins, and xenorhabdins are produced by various *Xenorhabdus* spp.

Pigments

Another common characteristic of the *Photorhabdus* spp. and some *Xenorhabdus* spp. is the propensity to produce pigments that accumulate in the growth medium as secondary metabolites. These give the colonies, and eventually the growth medium, a "cream colored to orangish-red" color as described by Khan and Brooks (86). The pigmentation of colonies can be quite variable, depending on the growth medium used and the age of the culture, so that colonies may vary from cream colored to brick red. Some isolates, notably those in the *X. nema-*

tophilus group, are apparently nonpigmented. Others show weak or variable pigmentation, which may be due to strain variability or to differences in pH of the medium, since at least for *P. luminescens*, the pigment is known to be brightly colored at high pH (>9) but yellow at lower pH. Visual examination of the cultures suggests that pigmentation (like antibiotic activity) develops strongly in late stationary phase, although this has not been quantitatively studied. In general, phase II cells can be identified by their lack of pigmentation (1, 10, 21).

***Photorhabdus* pigments.** Khan and Brooks speculated that the pigment from *P. luminescens* was carotenoid in nature on the basis of its solubility properties, but it has since been shown to be an anthraquinone (polyketide) type of structure (123, 134) (Fig. 6E). The pigments have been isolated and identified from three different strains of *P. luminescens* (91, 123, 134), and in all cases, they were found to be anthraquinones. Several different variations on a central theme were noted, as shown in Fig. 6E, but all were related to a basic polyketide structure similar to that produced by a variety of other organisms and found abundantly in the actinomycetes (75, 84, 109). In a recent survey performed with W. Fenical, we found that all isolates produce anthraquinone-like compounds with UV-visible spectra characteristic of anthraquinones (55). In many cases, two or more different anthraquinones were visualized in a given strain, suggesting that a wide variety of different anthraquinones will be found in this group.

In *Photorhabdus* strains, pigmentation is usually less intense in phase II cells. For example, Boemare and Akhurst (21) reported that most *Photorhabdus* strains converted from pigmented to less pigmented forms upon conversion to phase II. However, considerable variation was seen between bacteria; the phase I cells were seen to vary from off white, almost noncolored, to deep red. Bleakley and Nealson (20) reported that the pigmentation of *P. luminescens* Hm was dependent on the medium and in general was most strongly expressed under conditions which favored other properties associated with the phase I cells, such as bioluminescence and antibiotic production. In recent studies of *P. luminescens*, Gerritsen et al. (67) isolated variants on the basis of pigmentation, beginning with the red phase I cells and isolating colonies that were yellow or white. These variants were not judged to be true secondaries, and the mechanism accounting for their decreased pigmentation was not clear.

When a library of *P. luminescens* DNA was prepared on plasmid pUC18 and expressed in *E. coli*, one recombinant that produced red pigment which accumulated in the growth medium was isolated (119). Like the pigment from *P. luminescens*, the excreted pigment was pH sensitive, being strongly red at pH values of 9.0 or above and more yellow at lower pH values. Unlike the *P. luminescens* pigment, however, the pigment produced by the *E. coli* transformant was unstable after extraction in ethyl acetate and broke down to form a dark-brown residue; no structure was determined. The cloned fragment is now under study to see if the proteins coded for have similarities to known enzymes involved in anthraquinone synthesis in *Actinomyces* isolates (75, 84, 109).

***Xenorhabdus* pigments.** Almost nothing is known of the details of pigmentation in *Xenorhabdus* spp. The strains are characterized as buff or brown and have little or no pigmentation, in contrast to *P. luminescens*. Some species, such as *X. nematophilus*, are apparently nonpigmented. Akhurst and Boemare have noted that pigmentation decreases in phase II variants of *Xenorhabdus* spp., but no systematic studies of the pigments of this organism have been reported.

Inhibitors

Defective phage particles in the *Xenorhabdus*/*Photorhabdus* group were first reported by Poinar et al. (114), who clearly demonstrated the existence of the particles and presented preliminary evidence that the particles might be responsible for killing several strains of sensitive bacteria. Poinar et al. (113) also reported phages that were lysogenic for phase I (but not for phase II) cells of *X. luminescens* and hypothesized that such phages might in fact be responsible for phase variation in *Xenorhabdus* spp.

Subsequent work by Boemare et al. (23) used mitomycin and high temperature to induce phage lysis and demonstrated that most *Xenorhabdus* strains produced detectable levels of bacteriocin activity, which could be ascribed to the defective phage particles. *Photorhabdus* strains produced bacteriocins at low constitutive levels but could not be induced to lyse by mitomycin. The bacteriocin from *X. nematophilus* A24 showed inhibition of *P. luminescens*, *X. beddingii*, and some *Proteus* spp. but no inhibition of other *X. nematophilus* strains. The A24 strain also produced a soluble antibiotic activity during growth, which exhibited a broad-host-range inhibition. While the bacteriocin (defective phage) production was observed in both phase I and phase II cultures, the antibiotic production was seen only in phase I cells. Since antibiotic activity was not checked in the study of Poinar et al. (113), the preliminary report of bacteriocin activity against *B. subtilis* bears repeating.

Boemare and coworkers (14, 23) have pointed out that both *Xenorhabdus* and *P. luminescens* strains continuously produce low levels of defective phage and that they exhibit different sensitivities and susceptibilities to phage induction by mitomycin. They also note that the defective phage induction was responsible for the so-called lattice structures previously seen in *P. luminescens* and hypothesized to be involved with light emission (24). These authors also established that both phase I and phase II cultures produce bacteriocins and defective phages, rendering extremely unlikely the hypothesis that the loss or alteration of phages is responsible for phase variation (113).

From the ecological point of view, it can be hypothesized that the bacteriocins serve a similar function to the antibiotics (i.e., inhibition of competition from other bacteria). In this case, the activity is against the many closely related strains which, while resistant to the antibiotics, would be unacceptable to the nematodes for completion of their life cycle. This hypothesis predicts that closely related strains that are resistant to both antibiotics and bacteriocins would be good candidates for interchangeable symbiotic partners.

SECRETED ENZYMES

Both *Xenorhabdus* and *Photorhabdus* spp. secrete an array of enzymes (21). The enzymatic activities that have been identified so far include general lipases, phospholipases, protease, and DNases. To date, only the gene encoding the Tween 80 lipase has been cloned (139). In general, the production of the extracellular enzymes appears to increase during the late logarithmic and early stationary phase of the bacterial growth cycle, although more rigorous studies must be performed to clarify this point. The production of numerous enzymes that are produced in a growth-phase-dependent fashion is consistent with the idea that these enzymes are involved in providing a nutrient base for the developing nematode within the hemolymph. The hemolymph is a rich source of macromolecules that would provide substrates for the numerous secreted enzymes. It is of interest that the secretion of enzymes from the

phase II cells is markedly reduced for both *Xenorhabdus* and *Photorhabdus* spp. (21, 139), which may explain, at least in part, why nematodes do not reproduce and develop efficiently when the phase II cells dominate the larval carcass (10).

An alkaline metalloprotease produced by *P. luminescens* Hm has been purified and partially characterized (106). The protease had a molecular weight of 61,000 and was not produced by phase II cells. Only a single protease activity was identified for this strain. Others have reported protease activities associated with different *Photorhabdus* strains and different *Xenorhabdus* species (21). Identification of the genes that encode the various protease activities of these bacteria and comparison of their amino acid sequences and biochemical specificities would provide valuable information concerning the question of the evolutionary origin of the protease genes and the function of the enzymes in the provision of a nutrient base for nematode development.

CRYSTALLINE PROTEINS

Xenorhabdus Proteins

A characteristic that distinguishes most *Xenorhabdus* strains from other members of the *Enterobacteriaceae* is the production of crystalline inclusion bodies that are present in stationary-phase cultures of phase I cells but are not produced in exponentially growing cells (35, 36). While the formation of the parasporal crystal protein of *Bacillus thuringiensis* has been well studied for its entomotoxic properties, to date there is no evidence to support the idea that the *Xenorhabdus* inclusion body crystalline proteins can function as insect toxins. Two different types of crystalline inclusion bodies, both of which can be found in a single cell, have been studied in *X. nematophilus*. The large cigar-shaped crystals (type I) are formed by aggregation of a 26-kDa protein, while the smaller, ovoid crystals (type II) are formed by aggregation of a 22-kDa protein. Together, these major crystalline proteins may account for more than 50% of the total SDS-solubilized cellular protein derived from stationary-phase cells. Although both type I and type II inclusion bodies were solubilized and purified by similar methods, the biochemical properties of the respective crystalline proteins that form the inclusion body were found to be significantly different as determined by tryptic mapping analysis, amino acid composition, and immunological cross-reactivity studies. Further immunological analysis revealed that the 26- and 22-kDa proteins were unique to *X. nematophilus* and were not detectable in other *Xenorhabdus* spp.

Interestingly, 26- and 22-kDa proteins are not produced during exponential-phase growth in liquid cultures although both were produced during the late exponential or early stationary phase of growth. The identification and mapping of the genes that encoded these highly expressed proteins will allow one to determine whether they are part of an operon that is regulated from a single promoter or coordinately regulated from separate promoters. In addition, it will be of great interest to elucidate the regulatory mechanism that controls the very high level of expression of the crystalline proteins in a growth phase-dependent fashion. Addressing the important questions, such as the level of regulation of genes encoding these proteins (e.g., transcriptional and/or posttranscriptional), the mechanism of stationary-phase regulation (e.g., is a stationary-phase sigma factor involved), and the biological significance of the production of the crystalline inclusion bodies in *X. nematophilus* make this system a fertile area for future studies.

Photorhabdus Proteins

As described above, *P. luminescens* produces two different crystalline inclusion body proteins, CipA and CipB, composed of 104 and 101 amino acids, respectively (18). To study the biological role of the crystalline proteins, *cipA*-minus and *cipB*-minus strains were constructed. The *cip* strains resembled, for certain characteristics, a phenotype intermediate between those of phase I and phase II cells of *Photorhabdus* spp. In particular, protease, lipase, phospholipase, hemolysin, and siderophore activities were not detectably expressed in the *cip* strains. Interestingly, the pathogenicity of the mutant strains toward *M. sexta* did not appear to be different from that of the wild-type cells. In contrast, while the nematodes could grow and develop when raised on wild-type bacteria, the *cip* strains were unable to support the growth of *Heterorhabditis* nematodes. These tantalizing preliminary observations provide a framework to address the question of the biological role of the crystalline inclusion body proteins in the symbiotic/pathogenic life cycle of *Photorhabdus* spp.

In summary, it appears that crystalline inclusion body proteins play an important biological role in the life cycles of both *Xenorhabdus* and *Photorhabdus* spp. A hint that they may be essential gene products is that with the exception of *X. poinarii*, all *Xenorhabdus* and *Photorhabdus* species and strains studied so far produced inclusion bodies during stationary-phase growth (35) and that it has not been possible to obtain double *cip* mutants of *P. luminescens* (18). Since the molecular properties of the type I and type II crystalline proteins of *X. nematophilus* do not resemble those of CipA and CipB of *P. luminescens* at all, it is apparent that the genes that encode the respective protein products were not derived from a common ancestral gene. These observations further support the notion that these entomopathogenic bacteria have obtained essential genes by a convergent evolutionary pathway.

BIOLUMINESCENCE

It seems likely that luminous bacteria in the genus *Photorhabdus* were first noted by military doctors, who occasionally reported luminous wounds (111). As discussed by Harvey (71, 72), it was taken as a good sign, and it was generally believed that such wounds were likely to heal. Given what we now know about *Photorhabdus* spp., it may well be that the antibiotics produced by such luminous saprophytes as those recently reported by Farmer et al. (53) aided in inhibiting more insidious putrefying bacteria, as they are known to do in the infected insect carcass. The first modern report of bioluminescence was that of unnamed bacterial symbionts of nematodes in 1977 (86, 117); these were later assigned to the group *Xenorhabdus luminescens* (137). The enzyme catalyzing light emission was identified as a typical bacterial luciferase (116); i.e., it uses molecular oxygen to oxidize two substrates (a long-chain aliphatic aldehyde and $FMNH_2$), yielding a blue-green (490-nm) light. Although the luciferase produced typical blue-green light, the bioluminescence of the insect carcass was red shifted, presumably because of the red pigment produced by the bacterial cells that accumulates in the carcass and turns it red. *X. luminescens* luciferase is an alpha-beta dimer similar in size and activity to that of other bacterial luciferases; it can form active hybrids with purified subunits from *V. harveyi* luciferase (126). The enzyme NADH oxidoreductase (which supplies the reduced flavin for the light-emitting reaction) was removed from the luciferase only after treatment with 5 M urea, in contrast to other bacterial systems, in which there is no strong association of the enzymes. In terms of temperature sensitivity,

kinetics of light emission, and substrate inhibition by aldehyde, the luciferase from *P. luminescens* resembles the enzyme from *V. harveyi* more closely than it resembles the luciferases of the other species of marine luminous bacteria (*V. fischeri* [also known as *Photobacterium fischeri*], *Photobacterium phosphoreum*, and *Photobacterium leiognathi*) (133).

In the initial report of colony form variation by Akhurst (1), luminescence was not discussed, but subsequent work (21) showed that phase II variants emitted much less light; approximately 1% of the level emitted by the phase I cells from which they were isolated. However, it was suggested (21) that bioluminescence was not a particularly good characteristic for distinguishing phase I from phase II forms because of variability in luminescence between strains, including one strain of *X. luminescens* (Q-614) that emitted no light at all (9). A study of the luminescence of the phase I and phase II form variants of strain Hm was conducted by Bleakley and Nealson (20), who showed that bioluminescence was very low in the phase II cells and that conditions that favored bioluminescence also favored pigment formation and antibiotic production, traits commonly associated with the phase I cells. A recent study by Gerritsen et al. (67) reported that some (but not all) of the form variants were low in bioluminescence. Taken together, these results suggest that while bioluminescence can be controlled in concert with other traits associated with the phase I-phase II transition, regulation may also occur independently of these traits.

Luminescence is controlled in the marine bacteria by a wide range of factors, all of which appear to operate at the level of transcription of the *lux* operon. These include salinity (osmolarity), oxygen levels, iron, nutrients (catabolite repression), and autoinduction, now referred to as quorum sensing (66, 103). The last mechanism involves the production of a small autoinducer molecule (*N*-acyl homoserine lactone) that accumulates in the medium with cell growth and acts as the specific inducer of the *lux* operon. Thus, conditioned or used medium contains an inducer activity, now known to be a molecule of the *N*-acyl homoserine lactone class that acts to activate, at the level of transcription, the *lux* genes. The mechanism thus operates as a spatial (and bacterial population) sensing mechanism, allowing luminescence to be maximally expressed only at relatively high cell concentrations (101). For *V. fischeri*, the gene involved in autoinducer production (*luxI*) is part of the *lux* operon and the gene that produces the protein (LuxR) needed for activity of autoinducer is located on a separate but closely linked operon. In our laboratory, experiments looking for the accumulation of inducing activity in conditioned medium showed no indication of control of luminescence via autoinduction. Furthermore, experiments by Wang and Dowds (138) and Hosseini and Nealson (77) have suggested that the level of control may be posttranscriptional.

Studies of the expression of luminescence during the growth cycle of *P. luminescens* Hm revealed that luciferase is synthesized in concert with growth until late in the growth phase (optical density at 560 nm of about 2.0), and then increases rapidly, yielding bright cells. During the early phase of growth, the cells are substrate (aldehyde) limited, while later, the addition of aldehyde has only a two- to threefold effect on bioluminescence. For the phase II cells of strain Hm, luciferase is made at low levels throughout the growth cycle (the amount of luciferase per cell is about 10% of that seen in phase I cells) and the cells are severely aldehyde limited, so that the in vivo light emitted is 0.1 to 1% of the level seen in the wild type (depending on the stage of growth) (77, 106, 126). Colepicolo et al. (33, 34) obtained similar results with the phase I cells of strain Hw, isolated from a human wound (53). For this strain, the luminescence was very dim, with aldehyde limitation at all phases of growth. An unusual result was noted with strain Hw, namely, that not only did the cells tolerate growth in 100% oxygen but also that luminescence was enhanced under these conditions.

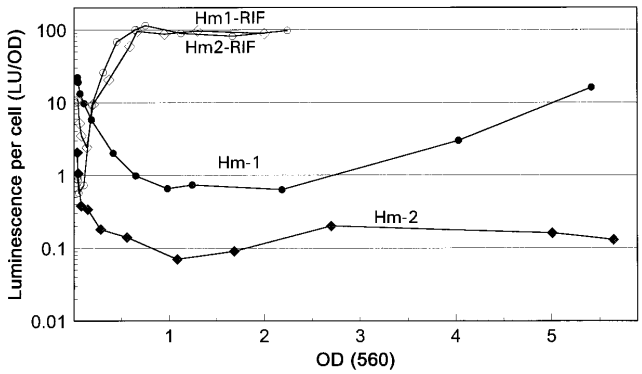

FIG. 7. Effect of addition of 2 μg of rifampin (RIF) per ml on the luminescence of primary (Hm-1) and secondary (Hm-2) forms of *P. luminescens*. Rifampin was added to cultures of both primary and secondary forms of Hm, and luminescence was monitored. Data are plotted as specific activity of bioluminescence (LU/OD) versus growth (optical density at 560 nm [OD_{560}]) to show the growth-dependent nature of the phenomenon. Taken from reference 77 with permission of the publisher.

Hosseini and Nealson (77) studied the effect of addition of low levels of mRNA synthesis inhibitors on the luminescence of strain Hm. In other species of luminous bacteria, addition of rifampin at levels that inhibit growth results in inhibition of the synthesis of the luminous system (104). For strain Hm, rifampin caused premature expression of the *lux* system in Hm phase I cells and a phenotypic reversion of luminescence in the phase II form of Hm (i.e., Hm phase II was stimulated to emit bioluminescence at phase I levels) (Fig. 7). The effect required approximately two generations to occur and was reversible upon the removal of rifampin (77). While these results do not elucidate the mechanism of control, they are consistent with the regulation being at a posttranscriptional level and serve to demonstrate unequivocally that the *lux* genes are functional genes that can be expressed fully even in the phase II culture. To our knowledge, this report constitutes the only example in which a trait identified with the phase I-phase II transition has been shown to be phenotypically reversed. The effect of the rifampin appears to be specific for luminescence, since no effects are seen on pigment production, antibiotic production, or other traits associated with the phase I-phase II transition (77).

The *lux* genes from three strains of *P. luminescens* have been cloned, sequenced, and expressed in *E. coli* by several different groups (32, 63, 64, 83, 97, 133, 142). When these are compared with the genes of the luminous marine bacteria (Fig. 5), it is seen that (i) the gene arrangement of the *lux* operons is similar between all luminous bacteria and (ii) the sequences of the *luxAB* genes show a strong similarity to those of the other luminous bacteria, suggesting that the genes are of a similar evolutionary origin. Meighen and Szittner (97) showed that the nucleotide sequences of the structural genes *luxCDABE* were highly conserved (85 to 90% identity) among the three strains of *P. luminescens* studied. Amino acid alignment of the LuxA and LuxB proteins shows that they have very strong identity with the luciferase from *V. harveyi* (83 to 85% and 58 to 59% identity, respectively [Fig. 5]). The similarity to proteins of the other marine bacteria is still high, but the closest relationship

by far is with *V. harveyi* (Fig. 5). As discussed above, these bacteria are phylogenetically separated from their marine luminous counterparts, raising the possibility that the *Photorhabdus* situation represents one of horizontal gene transfer of the *lux* operon.

A striking feature of the *lux* genes from all three *P. luminescens* strains tested was the existence of multiple ERIC (enteric repetitive intergenic consensus) sequences located between the *luxB* and *luxE* genes of strain Hw, constituting the first report of multiple ERIC sequences in the literature (97). The function of these sequences is not known, but their presence as a result of some involvement in horizontal gene transfer is an interesting possibility. Yet another possibility is that they are involved in some sort of regulation at the posttranscriptional level. Not only were there several such sequences located in that position, but also there was another between *luxD* and *luxA*. The nematode isolates (Hm and ATCC 29999) each had one ERIC sequence in the *luxBE* intergenic region. None of the other luminous bacteria that have been sequenced (Fig. 5) have these sequences. The sequences do not seem to interfere with expression of the cloned genes in *E. coli*, because the cloned isolates are quite bright and are not limited for aldehyde, which would occur if *luxE* were not being expressed properly.

The *lux* genes have been put onto a plasmid (pUC18) with a kanamycin resistance marker (pCGLS-2) and transformed into a phase II variant, yielding luminescent phase II colonies that remain phase II-like with regard to the other features (63). Given that pUC18 is a multicopy plasmid, it is perhaps not surprising that these cells were as bright as the phase I cells. When a transcription terminator is inserted upstream from the inserted genes, they are still expressed until the upstream region is essentially completely removed by deletion, indicating that the *P. luminescens* promoters are functional in *E. coli* (102). In fact, the pattern of late expression of luminescence seen in strain Hm (Fig. 4) is also seen in *E. coli* containing the cloned genes (76).

In summary, it is clear that the *lux* genes are similar to the *lux* genes of other luminous bacteria. The regulation of these genes appears to be at least partially accomplished at a posttranscriptional level, but the mechanism of regulation is not yet clear. Finally, while in some cases the luminescence follows other traits associated with the phase I-phase II transition, it is clear from the rifampin experiments that expression of luminescence can occur independently of the other traits. Because of its ease of measurement, luminescence remains a valuable tool for the study of gene expression in *Photorhabdus* spp., although the purpose of this function, either for the nematode symbionts or the human wound isolates, is not proven.

PHASE VARIATION

Phase variation has been known for many years in *Salmonella* spp., *Neisseria* spp., and other organisms and is thought to involve a mechanism whereby a single switch is used to simultaneously modulate several properties, including regulation of flagellar or pilin gene expression (127a). Such adaptations are thought to be one of several different mechanisms for bacterial pathogens to escape host defense mechanisms (124a). Akhurst (1) first described form variation in *Xenorhabdus* and *Photorhabdus* spp. as a type of variation that involved several factors but could be confidently delineated by changes in just two biochemical properties, namely, the absorption of the dye bromothymol blue and the reduction of triphenyltetrazolium chloride. In subsequent studies (2, 10, 20, 21), several factors were seen to vary simultaneously in the phase II variants. These included protease, lipase, intracellular crystalline proteins, antibiotic production, pigment production, and, for *P. luminescens*, bioluminescence. Akhurst and Boemare (10), however, noted that considerable variation occurred in many of these characteristics, even between phase I cells, and, furthermore, that for some species, the strains tested did not show variation in all characters. Early in the study of phase variation, it was considered that loss of a phage or plasmid might be the cause of the formation of the phase II forms. This has been rendered unlikely for several reasons. First, it is now clear that some *Xenorhabdus* phase II forms can revert to phase I forms with reasonable frequency. Second, it has been shown that no differences are seen with regard to plasmid patterns between phase I and phase II *Xenorhabdus* cells (89). In *P. luminescens*, mRNA for both the *lux* genes is made in both phase I and phase II cultures (138) and the lipase control is at the posttranslational level, indicating no loss of genetic material (139). Furthermore, addition of rifampin leads to total restoration of bioluminescence in phase II variants (77). A second possibility to explain phase variation that has been entertained is that some major DNA rearrangements have occurred. To this end, Akhurst et al. (12) did extensive restriction fragment length polymorphism analysis of phase I and phase II variants of *Xenorhabdus* spp., and concluded that no major DNA rearrangements had occurred in any of the phase II forms studied. At present, it is fair to say that the genes are apparently intact but, by one mechanism or another, their gene products are not expressed, and neither the formation nor the reversion of phase variants is yet understood.

Bleakley and Nealson (20) reported a phase II isolate of *P. luminescens* Hm that showed decreased activity for bioluminescence, antibiotic production, pigment production, and other properties. Because bioluminescence is so easily measured and because it can be detected at very low levels, it was possible to see that the phase II variant possessed about 0.1% of the phase I level of activity. Furthermore, growth conditions that favored high luminescence also favored the expression of other factors involved in the phase I-phase II transition, suggesting that some level of coordinate control existed over these factors. These authors also reported that the phase II form was remarkably stable, with no revertants to phase I being seen. Hurlbert et al. (79) also noted the stability of the phase II forms of *P. luminescens* and showed simultaneous variation of pigment, antibiotic production, and bioluminescence. The stability of phase II forms in *P. luminescens* may be different from that seen in the *Xenorhabdus* species: at this time, no bona fide revertants to phase I have been found in *P. luminescens*, while revertants to phase I are apparently common in at least some of the *Xenorhabdus* species (12, 79).

It is tempting to speculate, on the basis of above observations, that there is a master switch that controls phase transition in *P. luminescens*. This switch should have control overall properties associated with the phase transition and operate at the same level (transcription, translation, etc.) for all functions. However, even in the first paper by Akhurst (1), it was noted that some intermediate phase II-like cultures were seen, which appear to be altered in some of the properties but not others. This, coupled with the cautions of Boemare and Akhurst (10, 21), has led many to believe that the situation is probably much more complex than a simple master switch mechanism controlling all factors. This sentiment is underscored in a recent paper by Gerritsen et al. (67), in which they reported isolation of a series of variants of *P. luminescens* with a range of properties in pigmentation, inclusion granules, bioluminescence, and antibiotic production; all the variants were deficient in dye uptake. Two of the variants were unstable, reverting to phase I after a

few days in culture. While the authors concluded that these were not true phase II forms, it seems likely that the mechanism involved could somehow be connected with the more common phase transition. One possibility that should be considered is that freshly isolated phase variants are more apt to be partial variants (e.g., defective in one or more properties rather than all of them) and to be more likely to revert. Thus, the Hm-2 variant studied by Bleakley and Nealson may be a stable laboratory strain with unusually stable properties. In contrast, the freshly isolated variants described by Gerritsen et al. (67) could be similar to those kinds of variants that might be seen under more natural conditions. In a recent study by Krasomil-Osterfeld, growth in medium of low osmotic strength was used to induce the formation of phase II forms, some of which were shown to readily revert to primary form (88).

With the exception of bioluminescence in *P. luminescens*, almost none of the studies involving phase variation have involved careful quantitation, so it is difficult to characterize the phase variants or their revertants quantitatively. Furthermore, with the exception of bioluminescence and lipase in *P. luminescens*, almost nothing is known about the regulation of the various genes or gene products involved with phase variation. Since bioluminescence appears to be controlled at least partly at the posttranscriptional level and lipase is controlled at the posttranslational level, it might not be surprising to expect a variety of control mechanisms operating at several levels. In this sense, phase variation in *Xenorhabdus*/*Photorhabdus* spp. may offer some very exciting territory for the study of coordinate regulation at different levels in prokaryotic systems.

As a final point, one might ask why these phase variants form at all. Why are they such a dominant feature of both *Xenorhabdus* and *Photorhabdus* spp.? One possibility entertained recently in a paper by Smigielski et al. (128) is that the phase II forms are better suited to survival outside the symbiotic niche, being active with respect to cellular metabolism and respiration. This would fit with other reports that the phase II cells forms grow faster than the phase I cells on defined media (20, 106). Certainly, if the variety of secondary metabolites are not draining cellular energy, the phase II cells would be expected to be more competitive (than phase I cells) outside the symbiotic niche. To this end, it should be pointed out that if phase II forms were common in soils or other environments, they may have been very easily missed, because the usual method for identification relies on those properties that are usually strongly expressed in phase I forms. Colony hybridizations with specific gene probes such as *lux* genes, lipase genes, pigment genes, or others might be a good approach to answering this question.

CONCLUSIONS

The taxonomy and general biology of *Xenorhabdus* and *Photorhabdus* spp. have been studied for nearly two decades. More recently, molecular biological approaches have been used to address several aspects of the unique symbiotic/pathogenic life cycle of these bacteria. The extreme pathogenicity of *Xenorhabdus* and *Photorhabdus* spp. toward susceptible insect hosts is illustrated by the fact that only a few bacterial cells are able to rapidly inhibit the growth and kill the infected larva. Bacterial septicemia does not appear to be an important factor in the demise of the insect. The importance of cell surface properties in the pathogenicity of these bacteria is beginning to be appreciated. It is also clear that protein toxins are likely to play an important role in the virulence of both *Xenorhabdus* and *Photorhabdus* spp. Several fascinating questions concerning the pathogenicity of these bacteria include the following. How do they survive the vigorous attack of the insect immune system? What is the molecular nature and mode of action of the insect toxins? What is the role of endotoxin (LPS) in the mortality of insects infected with either *Xenorhabdus* or *Photorhabdus* spp.? Genetic and molecular tools are now available to address these questions. Of course, complete understanding of the pathogenic process requires that the cellular and humoral response of the insect immune system be considered. This is the subject of other excellent reviews (39).

The general aspects of the symbiotic association between *Xenorhabdus* spp. and *Steinernema* nematodes and between *Photorhabdus* spp. and *Heterorhabditis* nematodes have been appreciated for several years. The specific details of how the symbiosis is established and maintained remain unclear. Whether common themes exist in the symbiotic process of these different bacteria with their respective nematode hosts is not yet known. A central question is how the nematode switches from using the bacteria as a nutrient source to forming a symbiotic association with specific *Xenorhabdus* or *Photorhabdus* species. How do the bacteria and nematode communicate with one another to allow this switch to occur? Finally, as more genes from both bacteria are sequenced, we will be able to formulate a clearer picture concerning the intriguing possibility that *Xenorhabdus* and *Photorhabdus* spp. represent a system of lateral gene transfer and convergent molecular evolution.

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