Deoxyribonucleic Acid Relatedness and Phenotypic Study of the Genus *Xenorhabdus*

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A collection of 30 clones derived from 20 luminous and nonluminous *Xenorhabdus* strains was studied by using deoxyribonucleic acid (DNA) relatedness and electron microscopy, as well as growth factor requirement, nutritional ability, and other biochemical tests. Nonluminous *Xenorhabdus* cells each contained a crystal-like structure, whereas the cells of luminous strains each contained an accumulation of amorphous material associated with mesosomes and microtubules of unknown function. Two DNA relatedness groups of nonluminous strains were delineated, one of which corresponded to *Xenorhabdus nematophilus*, a species associated with the nematode *Steinernema feltiae* (= *Neoplectana carpocapsae*). The other group contained yellow-pigmented bacteria associated with *Steinernema bibionis* and new *Steinernema* species. No definite phenotypic characteristic (except pigmentation) separated the two DNA relatedness groups of nonluminous *Xenorhabdus* strains. Four DNA relatedness groups were delineated among the luminous clones studied. These four groups could be separated by biochemical tests. Unfortunately, different clones derived from the same strain belonged to different DNA relatedness groups. This happened with the type strain of *Xenorhabdus luminescens*.

Among the nematodes belonging to the order Rhabditida, two families, the Steinernematidae and the Heterorhabditidae, are composed of organisms whose larvae can penetrate living insects and kill them while developing in their bodies (24). These nematodes contain bacteria in their digestive tracts. The nematode genus *Steinernema* (now including *Neoplectana* [29]) is associated with the bacterial species *Xenorhabdus nematophilus*, whereas the nematode genus *Heterorhabditis* is associated with the bacterial species *Xenorhabdus luminescens* (19, 23, 25, 28). Although other bacteria are associated with *Steinernema* spp. (22; N. Boemare, Ph.D. thesis, Université des Sciences, Montpellier, France, 1983), the presence of *Xenorhabdus* spp. is essential for the mass production of the nematodes used in insect pest control projects (5; Boemare, Ph.D. thesis).

The genus *Xenorhabdus* Thomas and Poinar 1979 has been assigned to the family Enterobacteriaceae (28).

The purposes of this work were to (i) characterize the species of *Xenorhabdus* by electron microscopy, (ii) characterize the species of *Xenorhabdus* by using tests commonly used to describe species in the Enterobacteriaceae, (iii) identify growth factor requirements and determine the nutritional abilities of *Xenorhabdus* strains, and (iv) delineate the species of *Xenorhabdus* by deoxyribonucleic acid (DNA)-DNA hybridization.

MATERIALS AND METHODS

Bacterial strains. All strains were purified by picking a single colony on a nutrient agar (Difco Laboratories, Detroit, Mich.) plate. When more than one colonial form were produced by a strain, different colonies were picked and grown separately as different clones. The *Xenorhabdus*

strains and clones used in this study are listed in Table 1. The 81 non-*Xenorhabdus* reference strains used in the DNA relatedness study included 67 strains listed by Grimont et al. (16) (all strains except *Cedecea* sp. strain 621-75 and *Kluyvera* sp. strains 406-78 and 411-78), as well as *Edwardsiella tarda* 3592-64, *Enterobacter agglomerans* 2780-70, *Enterobacter amnigenus* 1319-79 and 1325-79, *Enterobacter cloacae* 1347-71, *Enterobacter gergoviae* 7601, *Escherichia hermannii* 980-76, *Escherichia vulneris* 2898-73, *Ewingella americana* 1468-78, *Serratia fonticola* 4556-71, *Serratia marcescens* 868-57, *Yersinia kristensenii* 1476, *Yersinia enterocolitica* 497-70, and *Yersinia pseudotuberculosis* P62. These 81 strains represented 73 DNA relatedness groups.

Morphological characterization. Two-day-old cultures in tryptocasein soy broth (Institut Pasteur Production, Marnes-la-Coquette, France) at 30°C were examined with an optical microscope after wet mounting and Gram staining. A 2-day-old colony of strain 4 on tryptocasein soy agar (Institut Pasteur Production) was touched with a carbon-coated specimen grid, negatively stained with 2% (wt/vol) phosphotungstic acid, and examined with a Hitachi model HU 11CS electron microscope. Five-day-old cultures of strains 3, 4, 14, and 17a in tryptocasein soy broth at 28°C were fixed with 2.5% (wt/vol) glutaraldehyde for 2 h at 6°C, postfixed in 1% (wt/vol) osmium tetroxide for 30 min, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate.

Characterization as primary or secondary form. A total of 19 clones were characterized as primary or secondary forms by examining colonies grown on nutrient agar (Difco) supplemented with 0.004% (wt/vol) triphenyltetrazolium chloride and 0.025% (wt/vol) bromthymol blue (1) or by detecting the production of inhibitory substances against *Serratia marcescens* 5 and *Serratia plymuthica* 392, using the method of Akhurst (2).

Biochemical and physiological characterization. All tests

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TABLE 1. Origins of the *Xenorhabdus* strains studied

Species	Strain designations			Origin ^a
	This paper	CDC	Sender	
<i>X. nematophilus</i>	1a	9168-81	CCEB 786	CCEB < ATCC 19061 < G. O. Poinar; <i>Steinernema feltiae</i> ^b
	1b	9012-80	ATCC 19061 ^T	G. M. Thomas; different clone from the same strain ATCC 19061 ^T
	1c	1184-80	ATCC 19061 ^T	ATCC; different clone from the same strain ATCC 19061 ^T
	2a	9013-80	A11	G. M. Thomas; <i>Steinernema</i> sp., Georgia
	2b		A11	Colonial variant from strain 2a
	3		262	N. Boemare; <i>Steinernema feltiae</i> DD136, France
	4		291	N. Boemare; <i>Steinernema feltiae</i> Plougastel, France
	5	1427-81	301	N. Boemare; <i>Steinernema feltiae</i> Plougastel, France
	6		246	N. Boemare; <i>Steinernema feltiae</i> DD136, France
	7		288	N. Boemare; <i>Steinernema feltiae</i> Plougastel, France
<i>Xenorhabdus</i> sp.	8		297	N. Boemare; <i>Steinernema feltiae</i> DD136 imported from the United States
	9	1426-81	286	N. Boemare; <i>Steinernema</i> species 1, Brittany, France
	10	2411-80	R	G. M. Thomas; <i>Steinernema</i> sp., Massachusetts
	11a	9015-80	X-II	G. M. Thomas; unidentified nematode, Poland
	11b		X-II	Colonial variant of clone 11a
	12	1425-81	276	N. Boemare; <i>Steinernema</i> species 2, Aquitaine, France
	13		269	N. Boemare; <i>Steinernema</i> sp. close to <i>Steinernema bibionis</i> , Normandy, France
	14		278	N. Boemare; <i>Steinernema bibionis</i> , Lorraine, France
	15		293	N. Boemare; <i>Steinernema</i> species 2, Picardy, France
	16	9014-80	DN	G. M. Thomas; <i>Steinernema bibionis</i> , Denmark
<i>X. luminescens</i>	17a	9167-81	Hb	G. M. Thomas; <i>Heterorhabditis bacteriophora</i> , Australia
	17b	9016-80	Hb	Same origin as clone 17a
	17c		Hb	Colonial variant of clone 17a
	17d	1185-80	ATCC 29999	ATCC < G. M. Thomas Hb; different clone from strain Hb
	18a	2294-80	Hb B	G. M. Thomas; <i>Heterorhabditis</i> , Georgia
	18b		Hb B	Colonial variant of clone 18a
	19	2295-80	Hm	G. M. Thomas; undetermined <i>Heterorhabditis</i> , Georgia
	20a	2296-80	NC-19	G. M. Thomas, ATCC 29304; <i>Heterorhabditis heliothidis</i>
	20b	1195-80	ATCC 29304	ATCC; same source as clone 20a
	20c		CCEB 954	CCEB < W. M. Brooks NC-19; same source as clone 20a

^a The name of the sender is given first. The symbol < indicates from whom the sender received the strain. ATCC, American Type Culture Collection, Rockville, Md.; CCEB, Culture Collection of Entomogenous Bacteria, Prague, Czechoslovakia.

^b A newly proposed nomenclature of insect-parasitic nematodes (union of the genera *Steinernema* and *Neoplectana*) has been followed (29).

done at the Institut Pasteur (IP) were incubated at 30°C unless otherwise stated. The tests done at the Centers for Disease Control (CDC) were incubated at 25°C unless otherwise stated. Growth factor requirements were determined by the method of Clowes and Hayes (10). Carbon source

utilization tests (IP) were done with API-CH, API-AO, and API-AA strips (API System, La Balme-Les-Grottes, France). A description and an evaluation of these strips have been published previously (14). The defined medium used with the API-CH, API-AO, and API-AA strips, which was

provided by API System, was supplemented with nicotinic acid and *para*-aminobenzoic acid (1 µg/ml each), as well as serine, tyrosine, and proline (20 µg/ml each). The strips were examined for growth after 2, 4, and 8 days.

Biochemical reactions were determined at the CDC by using the method of Edwards and Ewing (12), as recently updated (13). The biochemical reactions determined at the IP included the following: the Voges-Proskauer test (27), hydrolysis of gelatin (film methods [21]), production of H₂S from glucose-lactose-iron agar (Institut Pasteur Production), growth in peptone-water (10 g of Bacto-Peptone [Difco], 1 liter of distilled water, pH 7) containing 0, 2, 6, or 8% (wt/vol) NaCl, the β-glucuronidase test (20), and the γ-glutamyl-transferase test (15). Luminescence was tested by suspending 1 loopful of a Mueller-Hinton agar culture in 10 ml of distilled water in a scintillation vial and immediately counting in a scintillation counter with a fully opened window setting (4). All other tests done at IP were performed by using procedures described elsewhere (17).

DNA relatedness. The procedure used at CDC included in vivo labeling of DNA with ³²P and DNA hybridization by the hydroxyapatite (HA) method (8, 9). The temperature at which 50% of the reassociated DNA is eluted by 0.14 M phosphate buffer [*T_{m(e)}*] has been defined previously (8). The value called $\Delta T_{m(e)}$ is the difference between the *T_{m(e)}* of the

homologous reaction and the *T_{m(e)}* of the heterologous reaction. $\Delta T_{m(e)}$ is an estimate of the divergence between two DNAs (7).

The procedures used at IP included in vitro labeling of DNA with tritium-labeled nucleotides and DNA hybridization by the S1 nuclease-trichloroacetic acid method (18). ΔT_m values were determined by the method of Crosa et al. (11). It has been shown that $\Delta T_{m(e)}$ values determined by the HA method and ΔT_m values determined by S1 nuclease methods are similar for a given pair of DNAs (18).

RESULTS

DNA relatedness. The results obtained when DNAs from nonluminous *Xenorhabdus* strains or clones 1a, 1c, 9, and 16 were labeled and tested against unlabeled DNAs from 31 *Xenorhabdus* strains or clones are shown in Table 2. The results obtained with labeled DNAs from luminous *Xenorhabdus* strains or clones 17a, 17b, 17d, 20b, and 20c tested against the same collection of 30 unlabeled DNAs are shown in Table 3. The results obtained with the HA method at CDC agree very well with the results obtained with the S1 nuclease method at IP, although the relative binding ratios yielded by the S1 nuclease method were lower than the values yielded by the HA method, especially when the DNA pairs were from different species.

TABLE 2. Reassociation of DNAs from nonluminous *Xenorhabdus* species with DNAs from luminous and nonluminous *Xenorhabdus* species, as determined by the S1 nuclease method at 60°C and the HA method at 60°C

Source of unlabeled DNA (clone)	% Reassociation with labeled DNA from:					
	<i>X. nematophilus</i> strain 1a		<i>X. nematophilus</i> strain 1c (HA method)	<i>Xenorhabdus</i> sp. strain 9		<i>Xenorhabdus</i> sp. strain 16 (HA method)
	S1 nuclease method	HA method		S1 nuclease method	HA method	
<i>X. nematophilus</i>						
1a	100 (0) ^a	100		21		
1b		86	78 (0)			35
1c	83 (0.5)	99	100 (0)	21 (13.0)	51 (12.0)	58
2a	91 (1.5)	91	83 (0.5)	21 (15.5)	43 (10.0)	41
2b	87 (0.5)			23 (17.0)		
3	91			26 (14.5)		
4	91 (1.0)			42 (10.0)		
5	93 (2.5)	97		27 (15.0)	41	
6	86 (1.0)			23 (14.5)		
7	105 (0)			22 (14.5)		
8	102 (1.0)			27 (16.0)		
<i>Xenorhabdus</i> sp.						
9	25 (16.0)	36		100 (0)	100 (0)	
10	29		41	74 (2.0)	87 (1.0)	81 (3.0)
11a	26		51	69 (1.0)	78 (0.5)	80 (1.0)
11b	23			71 (1.5)		
12	25	37		92 (2.0)	95 (0)	
13	32			96 (1.5)		
14	33			77 (5.0)		
15	27		45	81 (1.0)		
16	23 (15.0)	33		76 (+0.5)	87 (0.5)	100 (0)
<i>X. luminescens</i>						
17a	9 (16.0)	22		7 (20.0)		
17b		22	44 (6.5)		28	23
17c	8			9 (22.0)		
17d	9 (12.5)	37		7	25	21
18a	10	32	37		24	23
18b	11			11 (20.0)		
19	10	21	40	9 (23.5)	22	24
20a	10	29	32	9 (20.5)	23	24
20b		17	36 (9.0)		24	22
20c	12 (28.0)			10 (10.0)		

^a The values in parentheses are ΔT_m values (in degrees Celsius).

TABLE 3. Reassociation of DNAs from luminous *Xenorhabdus* species with DNAs from luminous and nonluminous *Xenorhabdus* species, as determined by the S1 nuclease method at 60°C and the HA method at 60°C

Source of unlabeled DNA (clone)	% Reassociation with labeled DNA from <i>X. luminescens</i> strain:					
	17a		17b (HA method)	17d (HA method)	20b (HA method)	20c (S1 nuclease method)
S1 nuclease method	HA method					
<i>X. nematophilus</i>						
1a	7 (18.5) ^a	16				9 (9.0)
1b		16	18	22	17	
1c	6 (18.0)	16	16	27	23	11 (15.5)
2a	10 (18.5)	21	20	28	30	14 (8.0)
2b	9					9
3	10					9
4	9					9
5	10	14	18			8
6	8					8
7	9					8
8	11					9
<i>Xenorhabdus</i> sp.						
9	8 (18.5)	12	13			10 (15.0)
10	9		22	42 (8.5)	28	9 (17.5)
11a	16		19	38 (11.5)	25	9
11b	8					9
12	11	14	15			7
13	11					10
14	8					7
15	6					9
16	8 (13.0)	29	32	45 (9.0)	35	9 (>20)
<i>X. luminescens</i>						
17a	100 (0)	100				44 (12.5)
17b		100	100 (0)	72 (6.5)	60 (11.5)	
17c	100 (0.5)	100				44 (11.5)
17d	61 (6.5)	70	57	100 (0)	65 (10.0)	40 (10.5)
18a	103 (0.0)		99 (1.0)	71 (7.0)	58 (9.0)	39
18b	66 (6.0)					44 (11.0)
19	72 (8.5)	70	53	74 (7.0)	53 (8.0)	45 (11.0)
20a	49 (11.0)	61	48	63 (11.0)	100 (0)	101 (0.0)
20b		47	51	63 (10.0)	100 (0)	
20c	48 (10.5)					100 (0)

^a The values in parentheses are ΔT_m values (in degrees Celsius).

The nonluminous *Xenorhabdus* strains (strains 1 to 16) comprised two DNA relatedness groups (Table 2). The first group contained 11 clones (clones 1a to 8) derived from eight strains. These clones were 83 to 100% (S1 nuclease method) or 78 to 100% (HA method) related to the type strain of *X. nematophilus* (strain 1) at 60°C. The same levels of relatedness were observed at 75°C (HA method). The ΔT_m values were 0.0 to 2.5°C. Some variation in relatedness was observed among the three clones derived from strain 1. However, reassociated DNAs were very stable. The two clones derived from strain 2 (clones 2a and 2b) gave nearly identical relative binding ratios. This first group corresponded to *X. nematophilus* sensu stricto since it contained the type strain of that species. Of the eight strains of *X. nematophilus* sensu stricto, seven were isolated from *Steinernema feltiae* and one was from an unidentified *Steinernema*.

The second group contained nine clones derived from eight strains previously labeled *X. nematophilus*. These clones were 69 to 100% (S1 nuclease method) or 78 to 100% (HA method) related to strain 9 at 60°C and 80 to 100% (HA method) related to strain 16 at 60°C. The same levels of relatedness were observed at 75°C (HA method). The ΔT_m values were below 3.0°C. Clones derived from the same strain behaved alike in DNA hybridization tests. Of the eight strains in this group, two were isolated from *Steinernema*

bibionis, one was isolated from a *Steinernema* species close to *Steinernema bibionis*, one was isolated from a new species (species 1), two were isolated from another new species (species 2; new species of *Steinernema* according to C. Laumond [personal communication to N.B.]), one was isolated from *Steinernema* sp., and one was isolated from an unidentified nematode. This group constituted a new genomic species since the levels of DNA relatedness with *X. nematophilus* sensu stricto were 21 to 42% (S1 nuclease method) and 33 to 58% (HA method) at 60°C and 9 to 30% (HA method) at 75°C, with ΔT_m values ranging from 10.0 to 16.0°C. Both groups of nonluminous *Xenorhabdus* strains (strains 1 to 16) were only 6 to 16% (S1 nuclease method) or 13 to 45% (HA method) related to luminous *Xenorhabdus* strains (strains 17 to 20) at 60°C, with ΔT_m values between 6.5 and 28.0°C. The levels of relatedness between luminous and nonluminous *Xenorhabdus* strains at 75°C were 3 to 27% (HA method).

The collection of 10 clones derived from four luminous *Xenorhabdus* strains (labeled *X. luminescens*) was composed of four DNA relatedness groups (Tables 3 and 4). Different clones that supposedly were from the same strain diverged significantly in DNA relatedness. Of the four clones derived from the type strain of *X. luminescens* (strain 17), clones 17a, 17b, and 17c seemed quite similar in DNA

TABLE 4. Reassociation of DNA from *X. luminescens* strain 19 with DNAs of other selected strains and clones of *X. luminescens*, as determined by the S1 nuclease method at 60°C

Source of unlabeled DNA (clone)	Relative binding ratio with strain 19 DNA (%)
19	100 (0.0) ^a
18b	89 (0.5)
17c	54 (7.0)
17d	67 (7.0)
18a	59 (6.0)
20a	40 (10.5)
20c	39 (9.5)

^a The values in parentheses are ΔT_m values (in degrees Celsius).

relatedness. However, clone 17d showed only 61% (S1 nuclease method) or 57 to 72% (HA method) relatedness to the other clones of strain 17 at 60°C, with a ΔT_m value of 6.5°C. The levels of relatedness between clone 17d and clones 17a and 17b at 75°C were 44 to 51% (HA method). Of the two clones derived from strain 18, one (clone 18a) was highly related to clones 17a and 17b, whereas the other (clone 18b) was highly related to strain 19 (Table 4). The three clones derived from strain 20 (clones 20a, 20b, and 20c) were highly similar in DNA relatedness studies.

The levels of DNA relatedness between *Xenorhabdus* and other members of the *Enterobacteriaceae* (73 DNA relatedness groups studied) ranged from 0 to 14% (S1 nuclease method) and 2 to 15% (HA method) at 60°C.

Morphological characterization. The cells of all of the *Xenorhabdus* strains examined were gram-negative, motile, peritrichous, nonsporeforming, straight rods about 1.5 to 3 by 0.8 to 1.1 μm . Older cultures (3 to 5 days) contained bacilli of varying sizes (up to 8 μm long) and spheroplasts 1.4 to 2 μm in diameter. Most bacilli and occasional spheroplasts contained refringent inclusions (all strains examined). An electron micrograph of negatively stained strain 4 is shown in Fig. 1. Strain 4 is clearly peritrichous.

Electron microscopic examination of ultrathin sections showed that the cells of nonluminescent *Xenorhabdus* strains 3, 4, and 14 each contained a crystal appearing as a lozenge measuring 0.4 by 0.2 μm and constituted of numerous arrays (Fig. 2). Luminous *Xenorhabdus* strain 17a contained dense material with a rectangular shape (e.g., 0.6 by 0.7 or 0.5 by 0.2 μm) or crescent shape (up to 0.5 μm thick) that was seemingly attached to the cell membrane (Fig. 3). In addition, tubular structures about 120 by 20 nm arranged in packs of about 5 to 50 tubules were observed in bacilli or spheroplasts.

Characterization as primary or secondary forms. Clones 1a, 4, 5, 7, 8, 11b, 17a, and 17d, produced blue colonies surrounded by clear zones after 3 to 4 days on nutrient agar containing bromothymol blue and triphenyltetrazolium chloride, whereas clones 3, 11a, 12, 17c, 18a, 18b, 19, 20a, and 20c produced reddish maroon colonies without clear zones; clones 2a and 6 were intermediate, producing reddish colonies with faint halos. Clones 3, 4, 5, 8, 11b, 18b, and 20a produced a substance that inhibited *Serratia marcescens* 5 and *Serratia plymuthica* 392, whereas clones 6, 11a, 17a, 17c, 17d, 18a, 19, and 20c failed to produce any inhibitory substance against the two *Serratia* strains tested. Thus, clones 1a, 4, 5, 7, 8, and 11b are primary forms, and clones 6, 11a, 12, 17c, 18a, 19, and 20c are secondary forms. Clones 2a, 3, 17a, 17d, 18b, and 20a cannot easily be assigned to one form or the other.

Pigment production and luminescence. Colonies of clones 1 to 8 and clone 11b were nonpigmented. Colonies of clones 9 to 16 (except clone 11b) and 20a to 20c produced a yellow pigment when they were grown on nutrient agar (the pigment of clone 10 was yellowish orange), whereas colonies of clones 17a to 19 were rusty.

The liquid scintillation counter was very useful for detecting light emission from bacterial suspensions. Suspending a loopful of a culture of *X. luminescens* (clones 17a to 20c) in 10 ml of distilled water yielded about 10^6 cpm. *X. nematophilus* and *Xenorhabdus* sp. (clones 1a to 16) and a control culture of *Escherichia coli* K-12 produced no light (background noise of about 50 cpm).

Biochemical and physiological characterization. The characteristics of *Xenorhabdus* strains that were neither 100 nor 0% positive are shown in Table 5. The different biochemical patterns observed among luminous *Xenorhabdus* strains and clones are shown in Table 6.

Different clones derived from the same strain showed marked differences in nutritional and biochemical abilities. Clone 1a did not produce acid from fructose and utilized acetate, D-alanine, propionate, L-tyrosine, and valerate but not gluconate, whereas clone 1c showed opposite reactions. Clone 2b utilized 2-ketogluconate, 5-ketogluconate, phenylacetate, propionate, and D-ribose, but did not hydrolyze gelatin (film method), whereas clone 2a utilized none of these substrates but hydrolyzed gelatin (film method). Clone 11b produced white colonies and utilized acetate, amygdalin, L-arabinose, D-arabitol, caproate, glycogen, heptanoate, 3-hydroxybutyrate, isobutyrate, isovalerate, DL-lactate, methylmannoside, methylxyloside, phenylacetate, propionate, putrescine, D-ribose, D-sorbitol, starch, and valerate, but not L-aspartate or L-serine, whereas clone 11a produced yellow colonies and gave opposite reactions.

A growth factor was considered required when no visible growth occurred in its absence in 8 days. A factor stimulated growth when growth occurred in 1 day in its presence and in more than 1 day in its absence. All strains of *X. nematophilus* sensu stricto tested (strains 1a, 1c, 2a, 3, 4, 5, 6, 7, and 8) required nicotinic acid for growth. In addition,

FIG. 1. Electron micrograph of *X. nematophilus* 4. Bar = 1 μm .

FIG. 2. Ultrathin sections of *X. nematophilus* strains 3 (A) and 4 (B) and *Xenorhabdus* sp. strain 14 (C), showing crystalline structures. Bars = 200 nm.

strains 1a and 6 required serine and were stimulated by *para*-aminobenzoic acid. Proline could substitute for nicotinic acid with strain 4.

All strains of *Xenorhabdus* sp. (the second DNA relatedness group) tested (strains 9, 10, 11a, 11b, 12, 13, 14, 15, and 16) required nicotinic acid for growth. In addition, strains 9, 12, and 14 required tyrosine. Serine and *para*-aminobenzoic acid could substitute for tyrosine with strain 14. Strain 11b required serine in addition to nicotinic acid and was stimulated by *para*-aminobenzoic acid. Strain 15 was stimulated by tyrosine. Strains 12 and 13 grew weakly with the growth factors examined in this study (nicotinate plus tyrosine and nicotinate, respectively), and additional requirements could not be determined. Nicotinic acid was required for growth of *X. luminescens* 18a, 18b, and 20c. In addition, strain 18a required tyrosine. Proline could substitute for nicotinic acid with strain 18b, and proline or histidine could substitute for nicotinic acid with strain 20c. The growth of strains 17a, 17c, 17d, and 19 was stimulated by either nicotinic acid or proline. Strain 20c had no growth factor requirement.

The characteristics that did not vary in the collection of strains or clones tested are described below.

All *Xenorhabdus* clones tested grew on nutrient agar at 30°C in 2 days, producing convex or flat colonies about 1.5 to 3 mm in diameter. They were facultatively aerobic. All strains grew at 25 and 30°C in tryptic soy broth (IP).

All strains except strain 15 produced acid in peptone-water from D-glucose (1 to 4 days) and D-mannose (1 to 7

days); strain 15 failed to grow on the peptone-water basal medium (CDC and IP).

All strains of *Xenorhabdus* used the following compounds as sole carbon and energy sources in 8 days (API medium supplemented with nicotinic acid, *para*-aminobenzoic acid, serine, tyrosine, and proline): *N*-acetylglucosamine, fumarate, D-glucose, glycerol, D-mannose, and succinate.

None of the *Xenorhabdus* strains tested produced oxidase (CDC, IP), reduced nitrate to nitrite (although strain 16 gave a faint reaction in one laboratory) (CDC, IP), reduced tetrathionate (IP), or produced H₂S (CDC, IP). Malonate broth was not alkaline (CDC, IP), and phenylalanine deaminase (CDC), tryptophan deaminase (IP), lysine decarboxylase (CDC, IP), ornithine decarboxylase (CDC, IP), and arginine decarboxylase ("dihydrolase") (CDC, IP) were not produced. Growth did not occur in tryptic soy broth at 4, 40, and 42°C or in peptone-water supplemented with 6 or 8% NaCl (IP). Tests for "β-galactosidase" (*o*-nitrophenyl-β-D-galactopyranoside test) (CDC, IP), "β-xylosidase" (IP), and "β-glucuronidase" (IP) were negative. Starch and chitin were not hydrolyzed (IP). Voges-Proskauer and methyl red tests were negative (CDC, IP). Gas was not produced from D-glucose (CDC, IP). Acid was not produced in peptone-water from adonitol (CDC, IP), L-arabinose (CDC, IP), D-arabitol (CDC), cellobiose (CDC, IP), *meso*-erythritol (CDC), D-galactose (CDC, IP), *meso*-inositol (CDC, IP), D-mannitol (CDC, IP), D-melibiose (CDC, IP), α-methyl-D-glucoside (CDC), mucate (CDC), D-raffinose (CDC, IP), L-

FIG. 3. Ultrathin sections of *X. luminescens* 17a, showing accumulation of amorphous material associated with mesosomes (indicated by arrows in A and C) and microtubules (indicated by arrows in B and D). A spheroplast is shown in (D). Bars = 200 nm.

rhamnose (CDC, IP), D-sorbitol (CDC, IP), L-sorbose (IP), D-sucrose (CDC, IP), or D-xylose (CDC, IP). Trabulsi acetate medium (CDC) was unchanged, and esculin was not hydrolyzed (CDC, IP).

No *Xenorhabdus* strain could use any of the following substrates as a sole carbon and energy source (API medium supplemented with growth factors): acetamide, *trans*-aconitate, adipate, adonitol, β -alanine, 3-aminobenzoate, 4-amino-benzoate, 2-aminobutyrate, 3-aminobutyrate, 5-aminovaleate,

α -amylamine, anthranilate, D-arabinose, L-arabitol, azelate, benzoate, benzylamine, betaine, butylamine, butyrate, caprylate, citraconate, DL-citrulline, creatine, *meso*-erythritol, ethanolamine, ethylamine, D-fucose, D-galactose, β -gentiobiose, glutarate, glycine, glycolate, histamin, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, inulin, isoleucine, isophthalate, DL-kynurenine, L-leucine, levulinate, L-lysine, maleate, malonate, D-mandelate, L-mandelate, mesaconate, L-methionine, DL-norleucine, DL-norvaline, oxalate,

TABLE 5. Variable characteristics among *Xenorhabdus* strains or clones^a

Characteristic ^b	% Of positive strains or clones		
	<i>X. nematophilus</i> ^c	<i>Xenorhabdus</i> sp.	<i>X. luminescens</i>
Luminescence (All) ^c	0	0	100
Catalase (All)	0	0	100
Indole produced (All)	0	0	30-40 (10 w) ^d
Urea hydrolyzed (All)	0	0	0-20 (0-30)
Citrate (Simmons) (All)	0 (27)	0 (22-33)	20 (50)
"β-Xylosidase" (IP)	0	0	62 (12 w)
Gelatin hydrolyzed (film method, IP)	60 (30)	44 (11)	87 (12)
Gelatin hydrolyzed (tube method, CDC)	67 (33)	100	50 (50)
Tween 40 hydrolyzed (IP)	10 (70)	(11)	12 (62)
Tween 40 growth (IP)	100	22	100
Tween 60 hydrolyzed (IP)	(100)	(55)	50 (12)
Tween 80 hydrolyzed (IP)	10	22 ^e	75
Tributyrin hydrolyzed (IP)	(60)	(55)	25 (25)
DNA hydrolyzed (All)	18 (0-27)	33-44 (0-11)	10-20
Acid produced from:			
Fructose (IP)	50 (40)	11 (67) ^e	12 (50)
Glucose (All)	45-54 (45-54)	67 (33) ^e	50-70 (30-50)
Glycerol (All)	(9)	0 ^e	(0-10)
Maltose (All)	(64) ^f	(44-55) ^e	(70-80)
Mannose (All)	45 (55)	33 (55) ^e	30-60 (40-70)
Salicin (All)	0	0 ^e	(20)
Trehalose (All)	(64-73)	(0-11) ^e	(40) ^f
Growth on tryptic soy agar at 36°C (CDC)	100	0	NT ^g
Growth in tryptic soy broth at:			
15°C (IP) ^h	(22)	12 (37)	29 (14)
37°C (IP) ^h	67	50	86
Growth in peptone-water supplemented with:			
0% NaCl (IP) ^h	22	0	29
2% NaCl (IP) ^h	67 (33)	12	100
4% NaCl (IP) ^h	11 (11)	0	14 (43)
Jordan tartrate (CDC)	0	(100 w)	(33)
Tyrosine hydrolyzed (CDC)	(33)	(33 w)	83 (17)
Utilization of compounds as sole carbon and energy sources (IP)			
Acetate	80	11	100
D-Alanine	60	0	62
L-Alanine	100	89	100
Amygdalin	0	22	0
L-Arabinose	0	22	0
L-Aspartate	100	67	87
Caprate	0	0	25
D-Cellobiose	0	33	12
Citrate	30	55	12
D-Fructose	100	89	100
L-Fucose	0	0	25
Gluconate	20	100	100
D-Glucosamine	80	89	100
L-Glutamate	100	78	100
DL-Glycerate	100	89	100
L-Histidine	100	67	37
myo-Inositol	100	33	100
2-Ketogluconate	40	22	0
5-Ketogluconate	30	0	0
DL-Lactate	100	67	0
D-Lyxose	10	33	0
D-Malate	0	0	25
L-Malate	90	100	100
Maltose	100	78	100
Mannitol	0	33	50
D-Melezitose	0	33	0
D-Melibiose	0	33	0
α-Methyl-D-glucoside	0	33	0
Phenylacetate	20	11	0
L-Proline	100	89	100
Propionate	40	11	50

Continued on following page

TABLE 5—Continued

Characteristic ^b	% Of positive strains or clones		
	<i>X. nematophilus</i> ^c	<i>Xenorhabdus</i> sp.	<i>X. luminescens</i>
Putrescine	30	44	0
Pyruvate	100	89	100
D-Raffinose	0	33	0
L-Rhamnose	0	22	0
D-Ribose	10	67	100
Salicin	0	33	25
L-Serine	90	78	100
Starch	0	22	50
Sucrose	0	44	0
L-Threonine	90	11	50
Trehalose	100	87	100
D-Turanose	0	22	0
L-Tyrosine	60	33	100
Valerate	80	22	25
D-Xylose	0	33	0

^a The values on the table are percentages of positive strains or clones. The percentages of delayed positive (excluding promptly positive) strains or clones are indicated in parentheses, and the percentages of weak reactions are indicated by w. Tests for which only one strain was positive are not listed on the table. These tests are acid production from glycerol (strain 5) and utilization of 4-aminobutyrate (strain 10), D-arabitol (strain 11b), arbutin (strain 14), caproate, glycogen, heptanoate, 3-hydroxybutyrate, isobutyrate, and isovalerate (strain 11b), lactose (strain 14), and methyl-mannoside, methyl-xyloside, and D-sorbitol (strain 11b).

^b The results of the carbon source utilization tests were determined after 10 days; the results of tests for indole production, gelatin hydrolysis (film method), acid production from carbohydrates, and growth at different temperatures and NaCl concentrations were determined after 1 and 7 days; the results of tests for urea hydrolysis, Simmons citrate, gelatin hydrolysis (tube method), Tween hydrolysis, tributyrin hydrolysis, DNA hydrolysis, and tyrosine hydrolysis were determined after 2 and 7 days; Jordan tartrate and "β-xylosidase" tests were examined after 2 and 1 days, respectively.

^c Unless indicated otherwise, the numbers of strains or clones tested at the CDC, IP, and both laboratories (All) were as follows: *X. nematophilus*, 3, 10, and 11 strains, respectively; *Xenorhabdus* sp., 3, 9, and 9 strains, respectively; *X. luminescens*, 6, 8, and 10 strains, respectively. For each characteristic we indicate where the test was performed so that the number of strains or clones tested for each species and each characteristic can be deduced.

^d Discrepancies between laboratories are indicated by two values. This is an underestimate of variability since the number of clones that were studied in duplicate was the number of clones studied at CDC plus the number of clones studied at IP minus the number of clones studied at both laboratories.

^e One strain failed to grow.

^f Two compensated discrepancies.

^g NT, Not tested.

^h Nine strains of *X. nematophilus*, eight strains of *Xenorhabdus* sp., and seven strains of *X. luminescens* were studied.

2-oxoglutarate, pelargonate, phthalate, pimelate, sarcosine, sebacate, sorbose, spermine, suberate, D-tagatose, D-tartrate, L-tartrate, meso-tartrate, terephthalate, trigonelline, tryptamine, D-tryptophan, L-tryptophan, L-valine, xylitol, and L-xylose (IP).

DISCUSSION

Genus *Xenorhabdus*. It is not clear whether the luminous and nonluminous species should be maintained in the same genus. DNA relatedness data alone cannot be used to define a genus. Electron micrographs showed major differences between luminous and nonluminous species. No crystal-like structures were seen in the cells of luminous strains, and amorphous structures associated with mesosomes or packs of microtubules have not been observed in nonluminous species. Accumulation of amorphous material in luminous species was misinterpreted as crystals in a preliminary work (6). In fact, this amorphous material can take different shapes, but crystalline arrays have never been observed (as opposed to what can be seen on *X. nematophilus* crystals [6]). In contrast to *X. nematophilus* crystals, the amorphous material in *X. luminescens* is always associated with mesosomes (Fig. 3). Pigment production, luminescence, and catalase production distinguish luminous *Xenorhabdus* species from nonluminous *Xenorhabdus* species.

The genus *Xenorhabdus* can be clearly differentiated from all other genera in the *Enterobacteriaceae* by DNA-DNA hybridization. Furthermore, the cells of *Xenorhabdus* spp. contain structural elements that have never been reported in any other member of the *Enterobacteriaceae* or any other

gram-negative bacterium. The question of inclusion of *Xenorhabdus* in the *Enterobacteriaceae* could be raised. *Xenorhabdus* spp. have all of the characteristics defining the *Enterobacteriaceae* (12) except the ability to reduce nitrate to nitrite. In this property, and in general physiological properties, the genus *Xenorhabdus* is very similar to *Erwinia*

TABLE 6. Different biochemical patterns among luminous *Xenorhabdus* strains and clones

Characteristic	Reactions of clones: ^a			
	17a, 17b, 17c, and 18a	17d	18b and 19	20a, 20b, and 20c
Pigment	R	R	R	Y
Citrate (Simmons)	+	+	+	—
"β-Xylosidase"	+	+	+	—
Tween 40 or 60 hydrolyzed	— or (+)	—	+	+
Tween 80 hydrolyzed	+	—	+	+ or —
Deoxyribonuclease	—	—	+	—
Tributyrin hydrolyzed	—	—	(w)	+
Utilization of:				
D-Alanine	+	—	+	—
Citrate	—	+	—	—
L-Fucose	—	—	+	—
L-Histidine	—	+	+	—
Salicin	—	—	—	+

^a R, Rusty, Y, yellow; +, positive for all clones tested; — or (+), negative or delayed positive reaction; + or —, some clones positive and some clones negative; (w), delayed and weak reaction for all clones tested; —, negative for all clones tested.

amylovora. The enterobacterial common antigen has been recently demonstrated in *Xenorhabdus* species (26).

Nonluminous *Xenorhabdus* species. All of the symbionts recovered from *Steinernema feltiae* (synonyms, *Neoplectana carpocapsae*, *Neoplectana feltiae*) formed a well-defined DNA relatedness group corresponding to *X. nematophilus*. At least three nematode species (*Steinernema bibionis* and two new *Steinernema* species) were associated with the second DNA relatedness group, *Xenorhabdus* sp.

Different clones derived from the same strain were reasonably similar in DNA relatedness tests. However, phenotypic differences between clones (e.g., clones 11a and 11b) were marked and unexplained. After publication of a paper by Akhurst (1) describing morphological and functional dimorphism in *Xenorhabdus* spp., we tried to identify the form (primary or secondary) of some clones. It seems that phenotypically different clones (e.g., clones 11a and 11b) derived from the same strain correspond to different forms. However, we could not match absorption of bromthymol blue (on nutrient agar containing bromthymol blue and triphenyltetrazolium chloride) and production of inhibitory substances (these characterize the primary form) with any specific growth factor requirement, carbon source utilization pattern, or other biochemical test.

The phenotypic instability of nonluminous *Xenorhabdus* strains prevented the identification of any differential test (except yellow pigmentation) to separate the two genomic (DNA relatedness) groups. Recently, Akhurst (3) subdivided *X. nematophilus* into three subspecies, *X. nematophilus* subsp. *nematophilus*, *X. nematophilus* subsp. *bovieni*, and *X. nematophilus* subsp. *poinarii*. *X. nematophilus* subsp. *nematophilus* corresponds unambiguously to our *X. nematophilus* genomic group. *X. nematophilus* subsp. *bovieni* contains yellow-pigmented strains recovered from *Steinernema bibionis*, which hydrolyze Tween 80 but cannot grow at 34, 36, or 40°C (3). Strains fitting the description of *X. nematophilus* subsp. *bovieni* are found in our second genomic species, *Xenorhabdus* sp. However, *Xenorhabdus* sp. also contains strains that were isolated from nematodes other than *Steinernema bibionis* and that have characteristics which do not fit the identification scheme of Akhurst (3). A DNA relatedness study in which the strains of Akhurst (3) are used is urgently needed.

Luminous *Xenorhabdus* species. Some strains labeled *X. luminescens* contained different clones belonging to different DNA relatedness groups. After an exchange of clones between the CDC and IP, four genomic species were clearly delineated. These four species can be separated by using biochemical and nutritional characteristics. The simplest hypothesis to explain the "dimorphism" in *X. luminescens* is that some strains were mixed cultures containing two different luminous *Xenorhabdus* species. An immediate problem is concerned with the type strain of *X. luminescens* strain ATCC 29999, which does not correspond to strain Hb received from G. M. Thomas. A well-characterized clone should be redeposited in the American Type Culture Collection to serve as the type strain. Again, we refrained from naming the four luminous species since too few strains were studied.

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