

# The bacterial symbiont *Xenorhabdus poinarii* (Enterobacteriaceae) is harbored by two phylogenetic related host nematodes: the entomopathogenic species *Steinernema cubanum* and *Steinernema glaseri* (Nematoda: Steinernematidae)

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## Abstract

*Xenorhabdus* symbionts were isolated from infective juveniles of *Steinernema cubanum*, an entomopathogenic nematode isolated in western Cuba. A polyphasic approach, including phenotypic tests, restriction polymorphism analysis of PCR-amplified 16S rRNA genes and DNA-DNA hybridizations with determination of the  $\Delta T_m$ , was used to characterize this *Xenorhabdus* isolate. All methods converged to the conclusion that the isolate was a strain of *Xenorhabdus poinarii*. Until today, this species was only isolated from *Steinernema glaseri* and was considered to be specific to this nematode species. From the closely phylogenetic relatedness of *S. cubanum* and *S. glaseri*, which is supported by morphological and genotypic similarities, one can assume that the divergence of these two species is relatively recent. The speciation of the respective symbiotic bacteria may be in course, but at the present time, phenotypic and genotypic divergence is not sufficient to delineate two *Xenorhabdus* species. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** *Xenorhabdus poinarii*; *Steinernema cubanum*; 16S rRNA gene polymorphism; DNA-DNA hybridization; Phenotypic data; Symbiosis

## 1. Introduction

*Xenorhabdus* species are Gram-negative enterobacteria, symbiotically associated to entomopathogenic nematodes of the family Steinernematidae that are

used in the biological control of insect pests. In fundamental terms, the symbiosis between both partners is a fascinating model of co-speciation. The bacteria are carried monoxenically in a differentiated vesicle [1] within the gut of infective juveniles (IJs) and are released into the insect hemocoel after the nematode invasion. Death of the insect occurs within 48 h. The bacteria multiply, establishing favorable conditions

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Table 1  
Origin of the strains used in this study and associated 16S rDNA genotype

Strain designation <sup>a</sup>	Host nematode origin	Geographical origin	Source <sup>b</sup>	16S rDNA genotype <sup>c</sup>
Isolate studied				
CU01	<i>S. cubanum</i>	Cuba	E. Arteaga	3
<i>Xenorhabdus nematophilus</i>				
AN6 (ATCC 19061) <sup>T</sup>	<i>S. carpocapsae</i>	USA (Georgia)	R. Akhurst	1
A24	<i>S. carpocapsae</i>	Russia	R. Akhurst	2
F1	<i>S. carpocapsae</i>	France	E. Bonifassi	2
<i>Xenorhabdus poinarii</i>				
G6 (ATCC 49121) <sup>T</sup>	<i>S. glaseri</i>	USA (North Carolina)	R. Akhurst	3
NC33	<i>S. glaseri</i>	USA (North Carolina)	R. Akhurst	3
SK72	<i>S. glaseri</i>	USA (Florida)	N. Simões	3
<i>Xenorhabdus beddingii</i>				
Q58 (UQM 2872) <sup>T</sup>	<i>Steinernema</i> sp. <sup>d</sup>	Australia (Queensland)	R. Akhurst	5
<i>Xenorhabdus bovienii</i>				
T228 (UQM 2211) <sup>T</sup>	<i>S. feltiae</i>	Australia (Tasmania)	R. Akhurst	6
USFL52	<i>S. feltiae</i>	USA (Florida)	G. Smart	6
Si	<i>S. intermedium</i>	USA (South Carolina)	R. Akhurst	7
F3	<i>S. affine</i>	France	E. Bonifassi	8
SK2	<i>S. kraussei</i>	Czech Republic	Z. Mráček	8
F5	<i>S. feltiae</i>	France	E. Bonifassi	8
<i>Xenorhabdus japonicus</i>				
JP02	<i>S. kushidai</i>	Japan	N. Ishibashi	18
<i>Xenorhabdus</i> sp.				
SaV	<i>S. arenarium</i>	Russia	R. Akhurst	9
Other Enterobacteriaceae				
<i>Proteus vulgaris</i> CIP 58.60 <sup>T</sup>			Institut Pasteur, Paris	31

<sup>a</sup>Names are given according to the usual nomenclature of the strains as described in previous works, except for the new designations which indicate first the international code of the home country followed by a number (CU = Cuba; JP = Japan; USFL = USA, Florida).

Abbreviations: ATCC, American Type Culture Collection (Rockville, MD); CIP, Collection de l'Institut Pasteur (Paris, France); UQM, Culture Collection of the University of Queensland, Department of Microbiology (Brisbane, Australia); <sup>T</sup>, type strain.

<sup>b</sup>For one part, the symbiont cultures were provided by: Dr E. Arteaga, Estación de Sanidad de los Cítricos, Ministerio de Agricultura, Ciudad Habana, Cuba; Dr R. Akhurst, CSIRO, Division of Entomology, Canberra, ACT 2601 Canberra, Australia; Dr E. Bonifassi, INRA, Laboratoire de Biologie des Invertébrés, 06606 Antibes Cedex, France. For the other part, the symbiont strains were isolated by the Montpellier laboratory from the nematodes provided by: Prof. N. Simões, Universidade dos Açores, 9502 Ponta Delgada Codex, Portugal; Prof. G. Smart, University of Florida, Nematology Department PoBox 1106, Building 970, Hull Road, FL 32611-0620, Gainesville, FL, USA; Dr Z. Mráček, Institute of Entomology, Branisovská, 31, 37005 Ceske Budejovice, Czech Republic; Prof. N. Ishibashi, University of Saga, Konjocho-1, 840 Saga, Japan.

<sup>c</sup>Numbering of the 16S rDNA genotypes was previously defined [5].

<sup>d</sup>New species of *Steinernema* not yet described.

for nematode reproduction by providing nutrients and inhibiting growth of other microorganisms [2]. The invading nematodes reproduce within the prey cadaver producing new infective juveniles which escape from the cadaver to infect new hosts. Both symbiotic partners can be cultivated separately: on one hand Steinernematidae are reared axenically on an artificial diet, on the other hand pure cultures of *Xenorhabdus* bacteria grow on nutrient media. Thereby, gnotobiological experiments have demonstrated in most cases that nematode reproduction is

optimal with the native symbiont. In fact, taxonomic studies of symbionts and their host nematodes have defined that almost each species of *Steinernema* possesses a specific symbiont species. *Xenorhabdus nematophilus* is the symbiont species of *Steinernema carpocapsae*, *Xenorhabdus poinarii* of *Steinernema glaseri*, *Xenorhabdus beddingii* of another unnamed *Steinernema* species [2] and *Xenorhabdus japonicus* of *Steinernema kushidai* [3]. *Xenorhabdus bovienii* is the only species associated with several host species: *Steinernema affine*, *Steinernema intermedium*, *Steiner-*

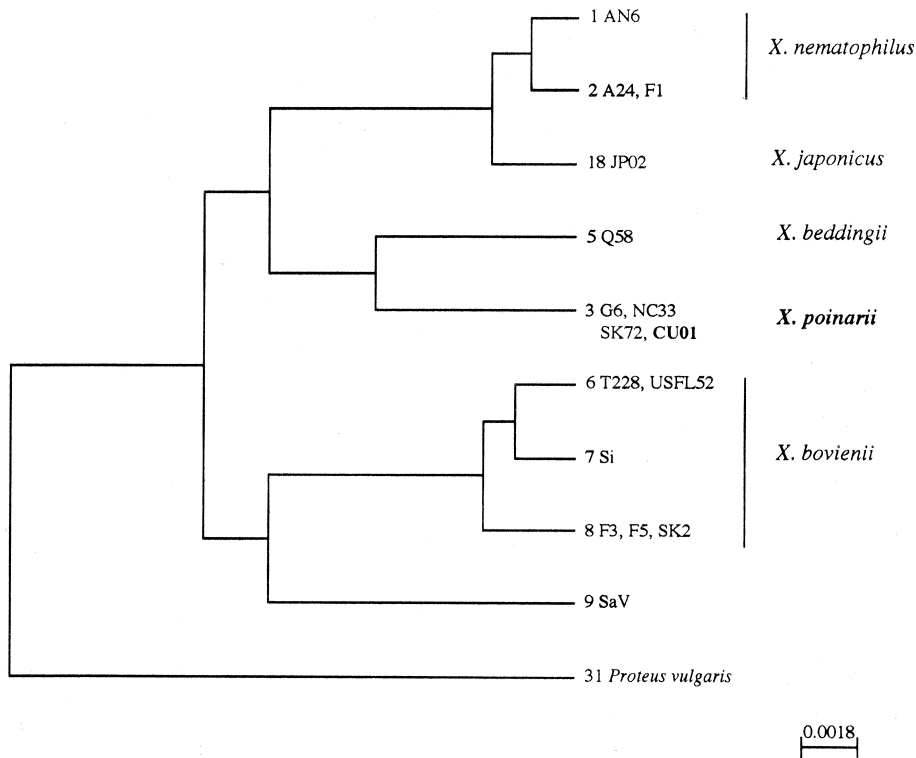


Fig. 1. Phylogenetic tree of the *Xenorhabdus* species inferred from PCR-RFLP analysis of the 16S rDNAs and constructed by the UP-GMA method. The genotype number is followed by the name of the strains exhibiting this genotype. Scale bar = substitution per site.

*nema kraussei* and *Steinernema feltiae*. However, recent restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA genes (rDNA) have evidenced genetic polymorphism within this species, each genotype corresponding more or less to one nematode species [4,5].

A novel species of *Steinernema* [6] was described in Cuba as *Steinernema cubanum*. Its bacterial symbiont was isolated and preliminary phenotypic characterization [6] has indicated a possible new *Xenorhabdus* species. However the bacterial strain isolated presented unusual properties for a *Xenorhabdus* strain as it exhibited catalase and nitrate reductase activities. Usually *Xenorhabdus* strains are considered as atypical members of Enterobacteriaceae because they are devoid of catalase activity and do not reduce nitrate to nitrite [7]. More detailed phenotypic and genotypic data were needed to accurately characterize the symbiont of *S. cubanum*.

Isolation of the true symbiont of *Steinernema* nematodes is a critical step which needs full attention

[8]. We re-isolated the bacterial symbiont from IJs of *S. cubanum* and identified the isolates by using a RFLP analysis of their PCR-amplified 16S rDNA together with reference strains. We found that the *S. cubanum* symbiont had the same RFLP genotype as the *X. poinarii* type strain. To confirm this result, a complete phenotypic characterization and DNA-DNA hybridizations were undertaken. So far, *X. poinarii* species has only been isolated from *S. glaseri*. Its occurrence in another *Steinernema* species is discussed through biogeographical and phylogenetic inferences.

## 2. Materials and methods

### 2.1. *Steinernema cubanum* culture

*S. cubanum* was isolated by E. Arteaga in western Cuba in 1990 and its rearing has been established in the nematode collection of the Institut National de la

Table 2  
Differential phenotypic characteristics of *Xenorhabdus* species

	Isolate CU01	<i>X. poinarii</i>	<i>X. beddingii</i>	<i>X. nematophilus</i>	<i>X. japonicus</i>	<i>X. bovienii</i>
Number of tested strain	1	3	1	3	1	6
Isolation from:						
<i>S. cubanum</i>	+	+	–	–	–	–
<i>S. glaseri</i>	–	+	–	–	–	–
<i>Steinernema</i> species	–	–	+	–	–	–
<i>S. carpocapsae</i>	–	–	–	+	–	–
<i>S. kushidai</i>	–	–	–	–	+	–
<i>S. feltiae</i>	–	–	–	–	–	+
<i>S. kraussei</i>	–	–	–	–	–	+
<i>S. affine</i>	–	–	–	–	–	+
<i>S. intermedium</i>	–	–	–	–	–	+
Maximum growth T (°C)	40°C	40°C	39°C	35°C	35°C	32°C
Pathogenicity against lepidopteran insects	–	–	+	+	–	+
Pigmentation <sup>a</sup>	lb	br	lb	ow	yb	y
Dye adsorption	+ <sup>w</sup>	+ <sup>w</sup>	+	+	+	+
DNase	–	–	+	+	+	+
Lecithinase (egg yolk agar)	–	d <sup>w</sup>	+	+	+	+ <sup>w</sup>
Tryptophane deaminase	–	–	–	–	–	d
Simmons citrate	–	d	+	+	–	d
Esculin hydrolysis	+	–	+	–	–	–
Acid production from						
Myo-inositol	–	–	–	+ <sup>w</sup>	–	d <sup>w</sup>
Ribose	–	–	+ <sup>w</sup>	+ <sup>w</sup>	–	+ <sup>w</sup>
Salicin	–	–	+ <sup>w</sup>	–	–	–
Gluconate	+ <sup>w</sup>	d <sup>w</sup>	+	–	–	d <sup>w</sup>
Utilization of						
DL-Glycerate	–	[–]	+	d	–	d
L-Histidine	+	d	+	–	–	d
Myo-inositol	–	–	–	+ <sup>w</sup>	–	d <sup>w</sup>
Ribose	–	–	+	d	–	[+]
L-Tyrosine	–	[–] <sup>w</sup>	+	–	–	d

All tests were done at 28°C unless otherwise mentioned: +=90–100% of strains are positive; [+]=76–89% are positive; d=26–75% are positive; [–]=11–25% are positive; –=0–10% are positive. The superscript w (e.g. +<sup>w</sup>) indicates a weak reaction.

<sup>a</sup>lb, light brown; b, brown; ow, off-white; y, yellow; yb, yellowish brown.

Recherche Agronomique (INRA) at Antibes (France). Nematodes used for our experiments were multiplied on the greater wax moth larvae, *Galleria mellonella*.

## 2.2. Bacterial isolation and reference strains

The bacterial symbiont of *S. cubanum* was isolated from infective stages by the hanging drop technique [9]. The origin of the reference strains used in this study is listed in Table 1. All *Xenorhabdus* strains were cultivated at 28°C on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue

(NBTA medium), in order to control the presence of phase I and phase II variants [10].

## 2.3. PCR amplification and RFLP analysis

DNA extractions were performed according to a rapid protocol using the nucleic acid extraction kit Isoquick (ORCA Research, Bothell, USA) [5]. 16S rDNA of each strain was amplified by PCR under conditions previously described [4]. The following two Eubacteria universal primers were used: 5'-GA-AGAGTTTGATCATGGCTC-3' and 5'-AGGAG-GTGATCCAGCCGCA-3' [11]. Samples of 6–17 µl of the amplified products were digested separately

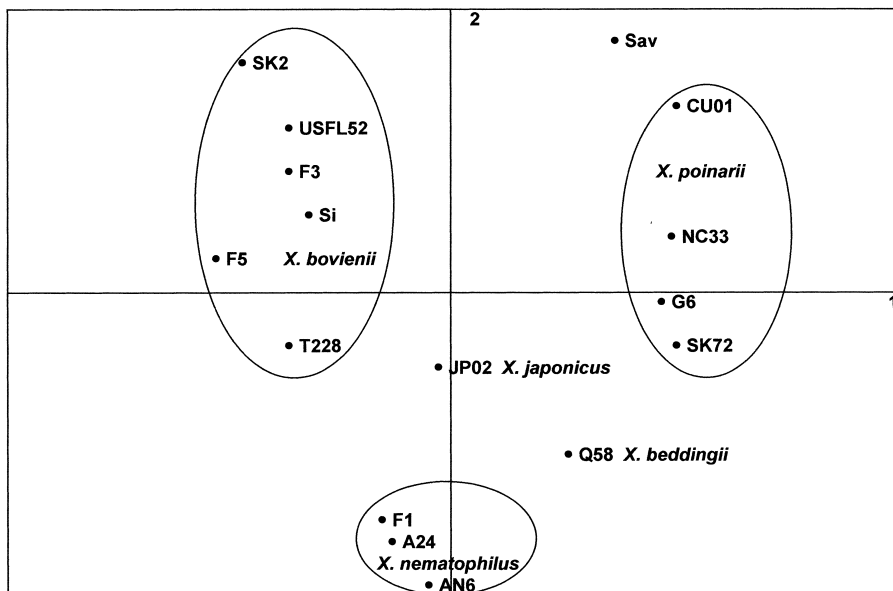


Fig. 2. Schematic representation of the strains in the mathematical space defined by the two first components of a factor analysis (see Section 2) using the phenotypic data (only variable characters are retained). Groups corresponding to a *Xenorhabdus* species are encircled.

overnight with 5 U of the six following restriction enzymes: *AluI*, *CfoI*, *DdeI*, *HaeIII*, *HinfI* and *MspI*. Restriction fragments were separated by horizontal electrophoresis at  $6 \text{ V cm}^{-1}$  in 3% (w/v) Nusieve or Metaphor agarose gels (Tebu, Le Perray en Yvelines, France) containing 0.5 mg of ethidium bromide per l. Genetic relationships between each pair of strains was evaluated by the presence or absence of DNA restriction fragments of a given length. Dice coefficients were calculated, then genetic distances were estimated by the mathematical model previously defined [12,13] and displayed as a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA).

#### 2.4. DNA hybridizations and thermal stability of duplexes

Total DNA for DNA-DNA hybridizations experiments was extracted from pure cultures of the CU01, NC33 and G6 strains according to the protocol of Brenner et al. [14]. Total DNA from the type strain G6 was labelled *in vitro* by nick translation using tritium-labelled nucleotides (Amersham International, Amersham, UK). Levels of DNA relatedness

were determined by using the S1 nuclease-trichloroacetic acid method [15] at the optimal temperature for DNA reassociation ( $65^\circ\text{C}$ ) for 16 h. Each reaction was carried out in duplicate and values obtained were averaged. The 0% control hybridization was obtained from re-association of the labelled DNA with herring sperm DNA and the 100% control from homologous hybridization. Heterologous hybridization values were normalized using 0% and 100% controls, to determine the percentage of relative DNA binding between two bacterial strains.

The thermal stability of re-associated DNAs was estimated by determining the denaturation temperature ( $T_m$ , temperature at which 50% of the double-stranded DNA was denatured and lysed by S1 nuclease). The  $T_m$  was determined by using the method previously described [16] with the following slight modifications. The temperature of the hybridization mixture was increased from  $65^\circ\text{C}$  to  $95^\circ\text{C}$  in  $5^\circ\text{C}$  increments. The hybridization mixture was not diluted with distilled water (final NaCl concentration 0.42 M) after DNA hybridization was completed. The divergence between DNAs was estimated by calculating the  $\Delta T_m$  value (difference between  $T_m$  of homologous and heterologous duplexes).

## 2.5. Phenotypic characterization

Phenotypic tests were conducted at 28°C by using the same methods as Boemare and Akhurst [10] except for the following tests. Acidification of carbohydrates was determined on API 50 CH strips with API 50 CHE medium (BioMérieux, Craponne, France), using phenol red as pH indicator. Utilization of carbohydrates and of organic and amino acid compounds was tested either on API 50 CH, 50 AO or 50 AA strips with API LRA medium, or on Biotype 100 by using the minimal medium II (BioMérieux). The maximum growth temperature for each strain was determined by inoculating cultures in nutrient broth maintained at a calibrated temperature in a water bath electronically adjusted at 0.1°C (Julabo, Germany) and monitored with an Hg thermometer (graduations 1/20 per °C). Temperatures ranging from 30 to 42°C by 1°C increments were tested. To avoid any pre-selection from the original strains, the inocula for these tests were taken from cultures maintained at 28°C.

The phenotypic relationships among strains were determined by performing a factor analysis using the software Statlab (SLP®, Paris, France) taking into consideration only the physiological and biochemical characters scored as variable.

The pathogenicity of the isolate CU01 was evaluated against *Spodoptera littoralis* larvae. Bacteria for injection experiments were grown overnight in nutrient broth. Different bacterial cell concentrations were obtained by dilution in sterile nutrient broth. Colony forming units (CFUs) of dilutions were assessed by inoculation of 100 µl on nutrient agar in three Petri dishes allowing to calculate the average of CFUs/ml of each dilution. To test the pathogenicity of the bacteria, 20 last instar larvae of *Spodoptera littoralis* were inoculated with 20 µl of each dilution, respectively, and incubated at 23°C with food. Then, the lethal dose to kill 50% of the injected insects (LD<sub>50</sub>) was estimated.

## 3. Results and discussion

### 3.1. Isolation of *S. cubanum* symbiont

Only one type of individual bacterial colonies was

isolated from the infective stages of *S. cubanum* by the hanging drop technique. This isolate shared the common phenotypic properties with all *Xenorhabdus* reference strains and therefore belonged to the *Xenorhabdus* genus (see Section 3.3): it was named strain CU01. In contrast with previous reported data [6], the bacterial symbiont isolated from *S. cubanum* was devoid of catalase and nitrate reductase activities as most of the *Xenorhabdus* strains are. It seems that the bacterium isolated previously from *S. cubanum* was not the *Xenorhabdus* symbiont but a contaminant. The occurrence of such bacteria is often associated to extended storage periods in a laboratory [17–19]. Another possible explanation is an inadequate disinfection of the nematode tegument microflora before symbiont isolation.

### 3.2. RFLP analysis of the 16S rDNA

In order to define the phylogenetic position of the *Xenorhabdus* strain isolated from *S. cubanum* within the *Xenorhabdus* genus, a PCR-RFLP study of the 16S rDNA was undertaken with several representatives of each defined species. *Proteus vulgaris* was included as an outgroup.

PCR amplification of the 16S rDNA of the isolate CU01 with the universal ribosomal primers produced a single band of about 1600 bp as for all the *Xenorhabdus* strains. The restriction patterns obtained with each of the six endonucleases tested, respectively, were identical to those of the three tested *X. poinarii* strains SK72, G6 and NC33. This combination of patterns was assigned to genotype 3. The restriction patterns obtained with each endonuclease for all the strains studied are shown in Fischer-Le Saux et al. [5] and in Brunel et al. [4].

In all, by combining the restriction patterns for the six endonucleases used, the 16 *Xenorhabdus* strains studied could be grouped into nine different 16S rDNA genotypes. Genetic distances between the 16S rDNA genotypes were calculated and displayed as a dendrogram by using the UPGMA algorithm (Fig. 1). Representatives of each *Xenorhabdus* species formed a separate cluster, clearly delineated from other *Xenorhabdus* strains. The nearest defined species to *X. poinarii*, which included the isolate CU01, was *X. beddingii*. Complete sequence analysis of the 16S rDNA corroborated this latter result [20].

As *S. glaseri* and *S. arenarium* belong to the same morphological group [21] and are phylogenetically related as determined by a RFLP analysis of their internal transcribed spacer of the ribosomal DNA repeat unit [22], we investigated the phylogenetic position of their respective symbionts. The bacterial symbiont of *S. arenarium*, SaV, possessed a specific genotype that did not cluster with any defined species (Fig. 1). Thereby, we found that the symbiotic bacteria of *S. arenarium* and *S. glaseri*, respectively, are not phylogenetically closely related within the *Xenorhabdus* genus.

### 3.3. Phenotypic analysis

Isolate CU01 shared all the common phenotypic properties of the *Xenorhabdus* genus. Cells were asporogeneous, rod-shaped and motile by means of peritrichous flagella. Isolate CU01 was negative for catalase and oxidase activities, nitrate to nitrite reduction, Voges-Proskauer reaction, hydrogen sulfide production, indole production from tryptophane,  $\beta$ -galactosidase activity on *o*-nitrophenyl- $\beta$ -D-galactopyranoside and *p*-nitrophenyl- $\beta$ -D-galactopyranoside, lysine and ornithine decarboxylase activity, and arginine dihydrolase activity. It hydrolyzed gelatin of the Kohn's method and produced antimicrobial compounds. As other *Xenorhabdus* species, isolate CU01 produced acid from fructose, glycerol, *N*-acetylglucosamine, maltose, D-mannose and trehalose, but not from D-adonitol, L-arabinose, cellobiose, dulcitol, lactose, D-mannitol, melibiose,  $\alpha$ -methyl-D-glucoside, raffinose, L-rhamnose, D-sorbitol, sucrose and D-xylose. Isolate CU01 was also able to grow on DL-lactate as sole source of carbon but not on L-fucose and D-mannitol.

Major discriminative characters of *Xenorhabdus* species are listed in Table 2 with the biochemical and physiological characters specific for *X. poinarii* strains and isolate CU01. The most relevant were the negative responses to DNase and lecithinase tests and the ability to tolerate temperatures as high as 40°C. The light brown pigmentation and the weak adsorption of bromothymol blue from NBT medium were also characteristic of *X. poinarii* strains. Similarly to some *X. poinarii* strains, isolate CU01 gave a negative response to the Simmons' citrate reaction. In contrast to the *X. poinarii* strains tested, it

hydrolyzed esculin. However, a positive response to this test has previously been noticed for members of this species [23].

By applying a factor analysis to the phenotypic data, the different *Xenorhabdus* species were well-separated and the three species *X. nematophilus*, *X. bovienii* and *X. poinarii*, which encompassed several strains, formed three separate clusters (Fig. 2). The species *X. nematophilus* corresponded to a tight cluster and strains of *X. bovienii* and *X. poinarii* were more distant. The isolate CU01 was located near the *X. poinarii* strains indicating that it was phenotypically related to this species. One of the determinative characters of factor analysis was the maximum growth temperature. Members of the *X. bovienii* species did not tolerate temperatures higher than 32°C. *X. nematophilus* and *X. japonicus* strains were more tolerant and could grow up to 35°C. At the opposite, *X. beddingii* and *X. poinarii* species were resistant to higher temperatures and could multiply up to 39 and 40°C, respectively.

*Spodoptera* larvae were injected with 100–10 000 cells of isolate CU01. Injection of approximately 1000 cells was necessary to reach the LD<sub>50</sub>. In comparison with other *Xenorhabdus* strains, this value is higher: usually less than 100 cells are able to kill more than 50% of the injected insects (only one cell for strain F1 of *X. nematophilus*). The pathogenic pattern of isolate CU01 corresponded to those of *X. poinarii* strains whose LD<sub>50</sub> is in a range of 1000–10 000 cells [24] and are considered weakly pathogenic for Lepidoptera.

### 3.4. DNA-DNA hybridizations

PCR-RFLP analysis of the 16S rDNA and phenotypic studies both indicated the close relatedness between isolate CU01 and representative strains of *X. poinarii*. In order to confirm these results by DNA-DNA hybridizations, the DNA of the *X. poinarii* type strain (G6) was labelled and respectively hybridized with the total genomic DNA of the isolate CU01 and of strain NC33.

The unstandardized re-association values for homoduplexes (100% control) ranged from 71 to 80%. The levels of re-association in negative control tubes (0%) ranged from 2 to 4%. The  $T_m$  value of homoduplexes in 0.42 M NaCl was 90.1°C. The DNA

relatedness between strain G6 and isolate CU01 from *S. cubanum* was 68%, with a  $\Delta T_m$  value of 3°C. The percentage of relative DNA relatedness between strain G6 and strain NC33, another strain from *X. poinarii* species used as positive control, was 96% with a  $\Delta T_m$  value of 2.4°C. The ad hoc committee on reconciliation of approaches to bacterial systematics defined a bacterial species as a group of strains sharing approximately 70% or greater DNA relatedness with 5°C or less  $\Delta T_m$  [25]. According to these rules the strain CU01 could be considered as belonging to *X. poinarii* species because of its low divergence with the type strain and its relatively high DNA relatedness.

### 3.5. Conclusion

Our three different approaches converged to the same conclusion. The *Xenorhabdus* strain isolated from *S. cubanum* belongs to *X. poinarii*. The high DNA relatedness with the type strain associated to a low  $\Delta T_m$  value was corroborated by a close phylogenetic relationship at the level of the ribosomal gene and common specific phenotypic characters.

From a technical point of view, this study confirmed the validity of the PCR-RFLP of 16S rDNA approach to rapidly identify *Xenorhabdus* isolates at the species level.

The results of this study indicate that the bacterial species *X. poinarii* is associated with two nematode species: *S. glaseri* and *S. cubanum*. Usually, the symbiosis between entomopathogenic nematodes and bacteria is so specific that almost each species of *Steinernema* possesses a specific symbiont species. However, *X. bovienii* represents another *Xenorhabdus* species associated to several host species. In this case, different 16S rDNA genotypes more or less specific to a *Steinernema* species have been identified. Phenotypic data also reflect the heterogeneity of this bacterial species (Fig. 2). Molecular studies of host ribosomal genes indicate that the different associated nematodes are not phylogenetically closely related [22]. This particular symbiosis needs more data to elucidate the relation between the diversity of the symbiont and those of their host.

More comprehensive data are available to interpret the association of *X. poinarii* with two nematode species. When *S. cubanum* was described, it was con-

sidered as a related species of *S. glaseri* because some morphological characters were closely related [6]. These morphological similarities make it difficult to distinguish the two species [26]. The restriction analysis of the intergenic transcribed spacer of the ribosomal genes revealed high molecular similarities between *S. cubanum* and *S. glaseri* [26] and the construction of a dendrogram evidenced their close phylogenetic relatedness [22]. A 'recent' common ancestor could explain high morphological and molecular similarities between these two species. The geographical isolation of the fauna from Cuba could have led to their speciation, which may be relatively recent. However, the species concepts for the two types of organisms are different and a relatively larger degree of evolutionary change on the DNA level may be required to allow the creation of a bacterial species compared to a nematode species. Although the bacterial symbionts of the two nematodes at present belong to the same species, the phenomenon of co-speciation, usually observed between *Steinernema* and *Xenorhabdus* species, may be in course.

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