A *Xenorhabdus* sp. (Eubacteriales : Enterobacteriaceae) symbiotically associated with *Steinernema kraussei* (Nematoda : Steinernematidae)

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SUMMARY

Examination of *Steinernema kraussei* recently collected from the field revealed that the bacterial symbiont of these nematodes was a *Xenorhabdus* sp. and not the *Flavobacterium* sp. reported by Mráček (1977). Like *Neoaplectana bibionis*, *S. kraussei* infectives carry cells of the bacterium in a specialised vesicle in the foregut.

RÉSUMÉ

Xenorhabdus *sp. (Eubacteriales : Enterobacteriaceae) associé symbiotiquement à* Steinernema kraussei *(Nematoda : Steinernematidae)*

L'examen de spécimens de *Steinernema kraussei*, fraîchement récoltés au champ, a montré que leur symbionte bactérien était un *Xenorhabdus* sp. et non un *Flavobacterium* sp., comme il l'avait été signalé par Mráček (1977). Comme *Neoaplectana bibionis*, les *S. kraussei* infectés transportent les cellules bactériennes dans une vésicule différenciée située à la partie antérieure de l'intestin.

Insect pathogenic nematodes of the families Steinernematidae and Heterorhabditidae are symbiotically associated with specific bacteria. The non-feeding, free-living infective stage nematode carries cells of its bacterial symbiont monoxenically in the intestine. After penetrating an insect host, the nematode migrates to the haemocoel where it releases its symbiont. The bacteria proliferate, killing the insect and providing suitable conditions for growth and reproduction of the nematode (Poinar, 1979).

Mráček (1977) identified nematodes in diseased *Cephalia abietis* L. from Český Rudolec, Czechoslovakia as *Steinernema kraussei* (Steiner) and their bacterial symbiont as a *Flavobacterium* sp. Mráček sent a monoxenic culture of *S. kraussei* and the *Flavobacterium* sp. to this laboratory where it was found that the bacterium was quite different from all other bacteria symbiotically associated with Steinernematidae and Heterorhabditidae (Akhurst, unpubl.). Moreover, although *Xenorhabdus* could be readily seen in smear preparations of and isolated from the intestine of the infective stage of other steinernematid species, the *Flavobacterium* could neither be seen in nor isolated from *S. kraussei* infectives. It was concluded that the *Flavobacterium* sp. may not be the symbiont of *S. kraussei*.

At the author's request, Dr. Mráček kindly collected more of these nematodes from Český Rudolec for further examination. This paper reports the results of this examination.

Materials and methods

The *S. kraussei* used in this study were obtained in 1980 by Dr. Z. Mráček, CSAV, České Budějovice, Czechoslovakia. He collected diseased *C. abietis* from the field, harvested infective stage nematodes produced within them and supplied these infectives to this laboratory.

The intestine of infective stage nematodes was examined for bacteria after extrusion and staining by the method of Poinar (1966).

Bacteria were isolated from surface-sterilised infective stage nematodes by homogenisation (Ak-

hurst, 1980). The homogenate was streaked onto nutrient agar + 0.0025% (w/v) bromothymol blue + 0.004% (w/v) triphenyltetrazolium chloride (NBTA) and incubated at 28°.

Transfer of *Xenorhabdus* by S. *kraussei* from one insect to another was tested. *Galleria mellonella* (L.) larvae were buried in moist sterile sand in 100 ml specimen jars and 100 *S. kraussei* infectives were pipetted onto the surface. After five days at 20° haemolymph from two cadavers was streaked on NA. When infective nematodes were released from the remaining cadavers, they were examined and sampled for bacteria as described above.

Cells of the bacterium harvested from 24 h nutrient broth cultures and diluted with sterile Ringer's solution (Cruickshank, Duguid & Swain, 1970) were injected into the haemocoel of *G. mellonella* larvae to assess pathogenicity. Seven dosages of each species and a Ringer's solution control were injected into 20 larvae/dosage.

Bacteria isolated from infective nematodes, from the haemocoel of infected insect cadavers and from monoxenic cultures established by the method of Bedding (1981) and bacteria isolated from Mráček's original monoxenic cultures were identified from the results of a number of tests conducted at 28°. Mean cell dimensions were estimated by measurement of 50 cells in wet mounts of 24 h culture in yeast-salts broth (YSB) ($NH_4H_2PO_4$, 0.5 g ; K_2HPO_4, 0.5 g ; $MgSO_4.7H_2O$, 0.2 g ; NaCl, 5.0 ; yeast extract, 5.0 g ; water 1 l ; Dye, 1968). Motility was assessed by examination of hanging drops of 24 h YSB cultures and flagella position determined by transmission electron microscopy after negatively staining cells from 24 h YSB cultures. Air dried films of 24 h YSB cultures were stained for the Gram reaction. Colony and cultural characteristics were studied on nutrient agar, NBTA, Simmons citrate agar (Difco) and triple sugar iron agar (Difco).

Examination for bioluminescence was made in total darkness with 48 h nutrient agar cultures. Moeller's (1955) method was used to test for arginine dihydrolase and lysine, ornithine and glutamic acid decarboxylases. Cytochrome oxidase was tested by Schaeffer's (1961) method, phosphatase as described by Cowan and Steel (1974) and phenylalanine deaminase by the method of the Difco Supplement (1962). Gelatin hydrolysis was tested in Oxoid nutrient gelatin and casein hydrolysis on Dye's (1968) OY agar containing 10% (w/v) skim milk. Methods described by Dye (1968) were used for the remaining tests.

Antibiotic production was tested by the method of Poinar, Hess and Thomas (1980) with twenty species of bacteria.

Dr. R. Bedding, CSIRO, examined adult nematodes and confirmed that these were of the same species as those originally sent in monoxenic culture with the *Flavobacterium* sp. by Dr. Mráček.

Results

S. kraussei infectives contained a vesicle within the foregut (Fig. 1A) similar to that described for *Neoplectana bibionis* by Bovien (1937) (Fig. 1 B). Bacteria were seen in the vesicle in 15/16 infectives from *C. abietis* parasitised in the field, 20/20 from *G. mellonella* parasitised by such infectives and 19/20 from monoxenic cultures with the *Xenorhabdus* sp. Bacteria could not be seen in any of over 100 infectives from monoxenic culture with the *Flavobacterium* sp. or isolated by homogenisation of surface sterilised infectives (100 from each of three such cultures).

The bacterium isolated from infectives sent by Dr. Mráček was found to be the same as that which overwhelmingly dominated the flora of the *G. mellonella* cadavers and as that recovered from the infectives produced from these cadavers.

Characteristics of the bacterium isolated from the intestine of infective stage *S. kraussei* and of the *Flavobacterium* sp. isolated from Mráček's monoxenic cultures are listed in Tab. 1. On nutrient agar, the *Flavobacterium* sp. formed two colony types ; one was convex, circular with a smooth margin, and 1 mm diameter at four days while the other was similar but surrounded by a flat, irregular apron. Colonies formed by the bacterium isolated from infective stage *S. kraussei* were convex, circular with slightly irregular margin, 1.5-2 mm diameter at 4d and slightly granular. Two colony types were obtained from monoxenic cultures of *S. kraussei* and the bacterium isolated from the infective stage ; one colony type was as described for the bacterium isolated from the infective stage while the other was flatter, wider (2.5-3.5 mm at four days) and less intensely pigmented on NA. The latter type did not absorb bromothymol blue from NBTA.

While intrahaemocoelic injection with 100 cells of the bacterium isolated from infective stage *S. kraussei* was inevitably fatal for *G. mellonella* larvae (LD50 = 16.5), injection of 25 000 cells of the *Flavobacterium* sp. did not kill any *G. mellonella* larvae.

Discussion

The bacterium isolated from the intestine of infective stage *S. kraussei* conforms substantially to the description of the genus *Xenorhabdus* given by Thomas and Poinar (1979). It also occurs in two

Table 1

Comparison of bacteria isolated from intestinal lumen of infective stage *S. kraussei* and of a *Flavobacterium* sp. previously reported as being symbiotically associated with *S. kraussei*

Characteristic	*Bacterium isolated from intestinal lumen of infective stage* S. kraussei	Flavobacterium *sp. isolated from Mrácek's monoxenic cultures*
No. isolates	6	2
Cell morphology	Rod	Rod
Mean cell length (range)	4.8 (1.6-12.1) µm	2.7 (1.6-3.2) µm
Mean cell width (range)	1.1 (0.8- 1.6) µm	0.5 (0.3-0.5) µm
Motility	+	−
Flagella	Peritrichous	none
Pigment (nutrient agar)	yellow	yellow
Gram stain	−	−
Anaerobic growth	+	−
Catalase	−	+
Oxidase	−	+
Cytochrome oxidase	−	+
Hugh & Leifson-open	+	+
Hugh & Leifson-closed	+	−
Nitrate reduction	−	−
Indole	−	−
Methyl red	−	−
Acetoin	−	−
KCN, inhibition by	+	−
Simmons citrate	−	−
TSI (slope/butt/H_2S)	alkaline/acid/-	nc (²)/nc/-
Arginine dihydrolase	−	−
Lysine decarboxylase	−	−
Ornithine decarboxylase	−	−
Glutamic acid decarboxylase	−	−
Phenylalanine deaminase	−	+
Gelatine liquefaction	+	−
Bioluminescence	−	−
Absorbtion of BTB	+ (¹)	−
Urease	−	+
Phosphatase	−	+
Casein hydrolysis	+	−
Aesculin hydrolysis	+	−
Antibiotic production	+ (¹)	−
Carried within intestine of Infective stage *S. kraussei*	+	−

(¹) By one colony only.
(²) No change.

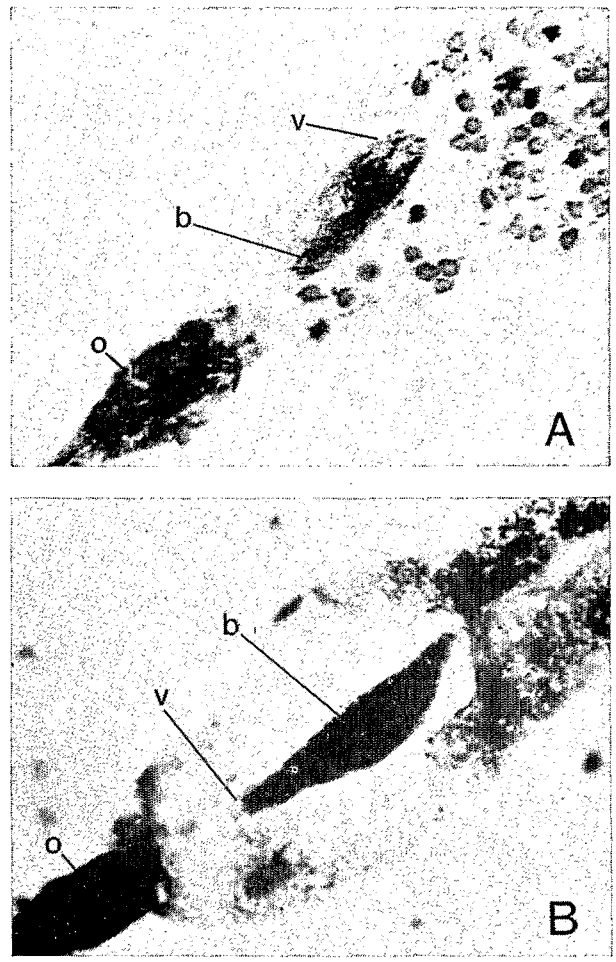

Fig. 1. Ventricular portion of the intestine of the infective stage after extrusion by the method of Poinar (1966) and staining with safranin (× 100). A : *Steinernema kraussei* ; B : *Neoaplectana bibionis* ; b : bacteria ; o : basal bulb of oesophagus ; v : vesicle.

forms as described by Akhurst (1980) for *Xenorhabdus* spp. and the primary form, like the primary form of *Xenorhabdus* isolated from infectives of seven species of steinernematid and heterorhabditid nematodes (Akhurst, unpubl.), produces an antibiotic factor. This bacterium is clearly a *Xenorhabdus* sp. and very different from the *Flavobacterium* sp. originally thought by Mráček (1977) to be the symbiont of *S. kraussei* (Tab. 1). The discovery of a *Xenorhabdus* sp. symbiotic with *S. kraussei* means that infectives of each species of nematode of the families Steinernematidae and Heterorhabditidae so far examined (Thomas & Poinar, 1979 ; Akhurst, unpubl.) carry a bacterium of this genus monoxenically within their intestines.

The *Flavobacterium* sp. originally considered symbiotic with *S. kraussei* does not have the same relationship with the nématode as does the *Xenorhabdus*. This latter bacterium is transported from cadaver to fresh host in a specialised organ of the infective's intestine ; the *Flavobacterium* sp. cannot be so carried. The ability of the nematode to grow and reproduce in monoxenic culture with the *Flavobacterium* sp. is of little significance since in such cultures fecundity is much lower than in cultures with the *Xenorhabdus* sp. and several *Neoaplectana* spp. have also been cultured monoxenically with this *Flavobacterium* sp. (Akhurst, unpubl.). Moreover, the *Xenorhabdus* sp. was highly pathogenic for insects and able to inhibit the growth of other microorganisms while the *Flavobacterium* sp. was not.

The bacterial symbiont was obviously lost from the *S. kraussei* described by Mráček (1977). This is not the only instance of an unwitting separation of a steinernematid nematode and its bacterial symbiont ; the symbiont of *Neoaplectana glaseri* and of an undescribed *Neoaplectana* sp. (Akhurst, unpubl.) have also been accidentally lost. The potential of these nematodes as control agents cannot be adequately tested in the absence of their symbionts. Consequently it is most important that the symbiont be isolated as soon as possible after detection of a new steinernematid population. Since the infective stage of steinernematids contains only the symbiotic bacterium and other stages may also contain a number of bacterial species from the insect cadaver (Poinar, 1966 ; Akhurst, unpubl.), the symbiont should be isolated from surface sterilised infectives.

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