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Characterization of Tn5-Induced Mutants of *Xenorhabdus nematophilus* ATCC 19061

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A negative-selection vector, pHX1, was constructed for use in transposon mutagenesis of *Xenorhabdus nematophilus* ATCC 19061. pHX1 contains the *Bacillus subtilis* levansucrase gene which confers sucrose sensitivity. In addition, various Tn5-containing plasmids with different replication origins were transferred by conjugation from *Escherichia coli* into *X. nematophilus* ATCC 19061, and one of these plasmids, pGS9, yields Tn5 insertion mutants of *X. nematophilus* ATCC 19061. By using these two delivery vehicles, more than 250 putative Tn5 insertion mutants of *X. nematophilus* ATCC 19061 were isolated and were then characterized. Mutants that were altered in bromothymol blue adsorption, ability to lyse sheep erythrocytes, production of antibiotics on a variety of media, and virulence for *Galleria mellonella* were found.

Xenorhabdus spp. (*Enterobacteriaceae*) are mutualistically associated with two families of entomogenous nematodes, the Steinernematidae (syn. Neoaplectanidae) and the Heterorhabditidae, that infect more than 250 species of insects (7, 8, 17, 41, 42). The symbiosis is specific in that each nematode species contains its own unique *Xenorhabdus* sp. that lives monoxenically in the gut of the nonfeeding, infective stage of the nematode. The infective-stage nematode invades a host insect, usually through a natural orifice, and then migrates to the hemolymph where it voids its resident *Xenorhabdus* sp. (1, 4, 5, 29, 31, 41, 42). *Xenorhabdus* spp. proliferate in the hemolymph and, in combination with the nematode, usually kill the insect within 48 h (3, 4, 11, 13, 28–30, 42). *Xenorhabdus* spp. produce antibiotics that inhibit the growth of other microorganisms in the insect cadaver, and the bacteria produce nutrients required for optimal nematode development (2, 3, 10, 21, 30, 40). New infective-stage nematodes subsequently leave the carcass in search of new hosts.

Most *Xenorhabdus* spp. share the following characteristics: (i) they exist monoxenically in the gut of specific nematodes, (ii) they have large cells, and (iii) they produce dimorphic or polymorphic clones. *Xenorhabdus luminescens* isolates are catalase positive and luminescent, but other *Xenorhabdus* spp. are catalase negative and nonluminescent. Dimorphism in *Xenorhabdus* spp. involves a type of phase variation between a primary form that is usually isolated from the nematode host and a secondary form that can be isolated from infected insects, from monoxenic in vitro cultures of nematodes and symbiotic bacteria, or from pure cultures of the bacterium (1, 2, 4, 12, 22, 42). The primary forms of most *Xenorhabdus* strains have a distinct colony morphology, produce antibiotics and pili, adsorb certain dyes, support the vigorous development of their host nematode in vivo, and develop large intracellular protein inclusions. The secondary forms have a different colony morphology and do not adsorb the dyes, produce antibiotics or pili, or form protein inclusions efficiently. The *X. luminescens* primary form is usually brightly luminescent, whereas the secondary form is weakly luminescent. Although both forms are pathogenic, the primary forms support superior growth of their host nematodes in vivo better than the secondary forms (1, 10, 22). Secondary forms of *X. nematophilus* revert to primary forms under certain conditions, but the secondary forms of *X. luminescens* have not been reported to revert under any conditions. The switching rate from primary to secondary forms varies between strains (1, 22). Neither the mechanism of switching nor its role in *Xenorhabdus* spp. survival is known.

We report here the development of conjugation and transposon mutagenesis systems for use with *Xenorhabdus* spp. A number of putative Tn5 insertion mutants, including avirulent mutants, were isolated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in modified LB (Luria-Bertani) broth (1% tryptone [Difco Laboratories]–0.5% yeast extract–0.5% NaCl, adjusted to pH 7.5 with NaOH [25]) and maintained on LB agar (LA) and NBT as previously described (22, 45). The accumulation of toxic photoproducts in the media was prevented as previously described (44). Other media were nutrient agar (NA); LA supplemented with bromothymol blue and 2,3,5-triphenyltetrazolium (LBT) at 25 and 30 mg/liter, respectively; blood agar (5% [vol/vol] sheep erythrocytes in Trypticase soy agar); LA plus Congo red (0.01%, wt/vol) (LBCR); Spirit blue agar; milk agar (prepared by mixing equal volumes of sterile 10% [wt/vol] skim milk and double-strength Plate Count Agar after sterilization); egg yolk agar (5% [wt/vol] egg yolk in Trypticase soy agar); Tween 40 and Tween 80 agar (0.2%, vol/vol) (10); and medium X (21). CAS agar for the detection of siderophores was prepared as described by Schwyn and Neilands (33) by using vitamin-free Casamino Acids (grade 0230; Difco). For dipyridyl agar, 2,2′-dipyridyl in 95% ethanol was added to sterile LA before the plates were poured. Except for motility medium, solid media contained 1.5% (wt/vol) Bacto-Agar. All plates were dried for 3 days in the dark at 22°C before use to prevent swarming. Dyes, antibiotics, 2,2′-dipyridyl, Tween 40, and Tween 80 were from Sigma Chemical Co., St. Louis, Mo. Chrome azurol and hexadecyltrimethylammonium bromide were from Fluka Chemical Corporation (Ronkonkoma, N.Y.).

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TABLE 1. Bacterial strains, plasmids, and viruses

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Escherichia coli		
S17-1	*pro thi hsdR hsdM*$^+$ *recA* Tp^r Sm^r RP4 2-Tc::Mu-Km::Tn*7* Tra$^+$	36
HB101	F$^-$ *hsdR hsdM proA2 leuB6 galK2 lacY1 supE44 thi-1 recA13 rpsL20* Sm^r	25
JEF8	Lambda$^-$ Hfr *thr-31 carB8 relA1 metB1 spoT1*	26
Xenorhabdus nematophilus		
ATCC 19061/1	Amp^r Kan^s	ATCC[a]
ATCC 19061/2	Amp^r Kan^s	45
IM/1	Amp^r Kan^s	This study
X. poinarii	Amp^r Kan^s	45
X. luminescens RH/1	Amp^s Kan^s	22
Plasmids and bacteriophage		
pRS14	ColE1 and RK2 replicons, Kan^r Tet^r	39
pRZ102	ColE1 replicon::Tn*5* Kan^r Mob$^+$	23
pSUP1021	p15A replicon::Tn*5* Kan^r Tet^r Cam^r Mob$^+$	37
pSUP2021	ColE1 replicon::Tn*5* Mob$^+$ Amp^r Cam^r Kan^r	35
pGS9	p15A replicon::Tn*5* Tra$^+$ (N type) Cam^r Mob$^+$ Kan^r	34
pMK409	ColE1 and RK2 replicons, *sacB sacR* Tet^r	This study
pHX1	ColE1 and RK2 replicons::Tn*5* *sacB sacR* Tet^r Kan^r	This study
pUC18	ColE1 replicon, Amp^r	46
Lambda 467	Lambda *B221 rex*::Tn*5* *c*I857 *O*am80 *P*am80	15

[a] ATCC, American Type Culture Collection, Rockville, Md.

Plasmid transformation and mating procedure. Transformation of *X. nematophilus* and *Escherichia coli* was performed as described by Xu et al. (45) and Maniatis et al. (25), respectively. Unless otherwise noted, all cultures of *Xenorhabdus* spp. used in the conjugation and transformation experiments were shaken at 28°C in LB at 200 rpm in a covered rotary water bath shaker. For conjugation, plasmid-containing *E. coli* S17-1 (36) donor cells were grown for approximately 16 h at 37°C without shaking in a 125-ml Erlenmeyer flask containing 5 ml of LB and the appropriate antibiotics. One milliliter of the donor culture was added to 9 ml of antibiotic-free LB in a 250-ml Erlenmeyer flask and grown at 37°C without shaking to a concentration of 1.5×10^8 cells ml^{-1}. *Xenorhabdus* spp. recipients, grown to a cell density of 1.5×10^8 cells ml^{-1}, were washed once in LB to remove antibiotics produced by *Xenorhabdus* spp. and suspended to their original volume in LB. Equal volumes of donor and recipient cells were mixed gently, and 0.2 ml was dropped onto sterile 0.45-μm-pore-size membrane filters (cellulose nitrate; Whatman Inc., Clifton, N.J.) on LA plates. The plates were incubated at 28°C in the dark for 4 h. The cells were resuspended, diluted in LB, and plated on the appropriate medium. The frequency of plasmid transfer is expressed as the number of transconjugants per viable recipient cell.

Biochemical and physiological characterization of mutants. Each isolate was tested at least three times. Tween 80 and Spirit blue plates contained approximately 40 ml of media; all other plates contained approximately 25 ml of media. All plates were patched with 22 test strains and the wild-type primary and secondary strains as controls. All plates, except for the LBCR plates, were incubated for 48 h at 28°C and then at 22°C in the dark for the indicated periods before the results were read. The LBCR plates were incubated for 3 to 4 days at 28°C. Plates that were incubated for longer than 72 h were sealed with Parafilm. The milk and egg yolk plates were treated with $HgCl_2$ (10%, wt/vol) by the method of Frazier (19). Phase-shift stability was determined by monitoring the pattern of bromothymol blue adsorption for 10 to 21 days at 22°C. Unstable mutants developed sectors at the colony edges with the alternative bromothymol blue uptake pattern, whereas stable mutants did not.

Bioassay. *Galleria mellonella* L. (Carolina Biological Supplies, Gladstone, Oreg.) was cultivated in the dark at 32°C by the method of Dutky et al. (16). Virulence assays were performed on the last instar of *G. mellonella*. In preliminary tests, *Xenorhabdus* cells were smeared on the upper rear segments of *G. mellonella* and a sterile stainless steel straight pin was stabbed through the cuticle into the hemolymph. The larvae were incubated in test tubes at 32°C for 48 h. Putative avirulent mutants were tested by injecting 20 μl of culture diluted in Ringer solution (1) into 3 to 10 larvae (in the first intersegmental fold anterior to the prolegs) by using a repeating dispenser (Hamilton Co., Reno, Nev.) fitted with a 0.5-ml disposable syringe and a 27-gauge needle. Three- to 10-fold dilutions, with the lowest concentration ranging between 12 and 50 viable bacteria, were used. Control larvae were inoculated with sterile Ringer solution, avirulent *E. coli* HB101, and wild-type *X. nematophilus* ATCC 19061/1. Putative avirulent mutants were tested three or more times. Numbers of viable cells were determined as described by Poinar and Thomas (31).

DNA manipulations. Plasmid DNA isolation, purification, and electrophoresis were performed as described by Maniatis et al. (25). Chromosomal DNA was prepared as described by Ausubel et al. (6). Restriction endonucleases were used as described by the supplier. DNA transfer to GeneScreen Plus, hybridization, and autoradiography with ^{32}P-labeled probes were performed according to the manufacturer's instructions (NEN Research Products, Boston, Mass.). A 3.4-kb internal *Hin*dIII fragment of Tn*5* (15) from pRZ102 was purified from agarose gel by using Gene Clean (Bio 101, Inc., La Jolla, Calif.) according to the manufacturer's instructions, labeled with ^{32}P by using a NEN Random Primer kit, and used as the Tn*5*-specific DNA probe.

Phenotypic nomenclature. When possible, the diagnostic

phenotypes of the wild-type strain of *X. nematophilus* and of the Tn*5* insertion mutants were named according to the conventions of Akhurst (4, 5) and Boemare and Akhurst (10). New symbols and symbol combinations were used for the new diagnostic characterizations employed in this study. We have attempted to use single letters or numbers for all characterizations used in tables and have used three-letter mnemonics to designate phenotypes not previously named. When phenotypic designations for equivalent genes exist in *E. coli*, we have used mnemonics utilized in recent publications. Btb^+ and Btb^- stand for the ability and inability, respectively, to adsorb bromothymol blue, and Suc^r and Suc^s stand for the ability and inability, respectively, to grow on media containing 5% sucrose.

RESULTS

Development of mating conditions between *E. coli* and *X. nematophilus* ATCC 19061/1. The standard conditions for the conjugational transfer of plasmids from *E. coli* S17-1 to *X. nematophilus* ATCC 19061/1 described in Materials and Methods were determined by using the plasmid pRZ102. Under these conditions the frequency of pRZ102 transfer to both the primary and secondary forms of *X. nematophilus* ATCC 19061 was 3.0×10^{-2} to 5.8×10^{-2} and that to *X. poinarii* was 3.5×10^{-7}. No transconjugants were found when *X. luminescens* RH/1 or *X. nematophilus* IM/1 was used as a recipient.

The viable counts of *E. coli* S17-1 fell during mating from a starting ratio of 1:1 to approximately 1:200 within 4 h of mating, and after 20 h of incubation only a rare *E. coli* colony could be detected. The death of *E. coli* during mating made counterselection unnecessary. To determine if *Xenorhabdus* spp. antibiotics were responsible for this killing, *E. coli* S17-1 cells were spread on plates from which filters containing the mating mixtures had been removed after various periods. No inhibition zones developed on plates of matings with *X. nematophilus* ATCC 19061/1 before 20 h. Although *E. coli* S17-1 also died when mated with the non-antibiotic-producing strains *X. nematophilus* ATCC 19061/2 and *X. poinarii*, no inhibition zones were detected even on matings of >20 h. Transconjugants could be measured on selective plates of LA or LBT, but because of their distinct dye uptake patterns both *E. coli* and *X. nematophilus* ATCC 19061/1 could be counted on the nonselective LBT medium. However, the recovery of transconjugants on LBT was 5 to 10% lower than on LA (21a). Since *X. nematophilus* ATCC 19061/1 is resistant to ampicillin (100 $\mu g\ ml^{-1}$), ampicillin was occasionally used to select against the donor.

When the Tn*5*-containing plasmids pSUP1021, pSUP2021, and pGS9 were tested as possible suicide vectors for transposon mutagenesis by conjugation into *X. nematophilus* ATCC 19061/1, the frequency of kanamycin-resistant *X. nematophilus* ATCC 19061/1 colonies was 7.0×10^{-3}, 3.0×10^{-3}, and 5.0×10^{-7}, respectively, and only pGS9 yielded kanamycin-resistant colonies that had lost the plasmid at a useful frequency (15 to 25%).

Because the frequency of recovery of putative Tn*5* transposon mutants of *X. nematophilus* ATCC 19061/1 obtained from matings with pGS9 was low, a negative-selection plasmid, pHX1, was constructed for use as a suicide vector (Fig. 1). The negative-selection characteristic of pHX1 is based on the production of levansucrase by the *sacB* gene of *Bacillus subtilis*. This gene is lethal to many gram-negative bacteria when they are incubated on medium containing sucrose (20, 32). Mutants could be directly isolated by spreading cells transformed with pHX1 onto plates supplemented with kanamycin and 5% sucrose. This procedure usually yielded fewer than 20 colonies per ml. Alternately, mutants could be isolated by first plating the transformants on medium supplemented only with kanamycin and then streaking the colonies to plates supplemented with kanamycin and 5% sucrose. All the colonies from the kanamycin plates yielded Tet^s Suc^r Kan^r isolates. All Tet^s Suc^r Kan^r isolates were assumed to be independent Tn*5* transposon mutants. Putative insertion mutants with a wide variety of combinations of characteristics have been isolated. Many of the putative mutants are distinguished from the wild type only by being kanamycin resistant. In this paper only the mutants with properties that differed in one or more characteristics from the pattern of characteristics assigned to either the primary or secondary wild-type forms (Table 2, columns 2 and 3) are discussed.

Physical characterization of Tn*5* insertion. To characterize the nature of the transpositions, *Eco*RI fragments of chromosomal DNA from putative insertion mutants were separated on agarose gels and were analyzed by hybridization with a Tn*5*-specific DNA probe. *X. nematophilus* ATCC 19061/1 mutants isolated on medium containing 20 μg of kanamycin ml^{-1} produced multiple bands (data not presented), whereas DNA from the majority of mutants that were isolated by using 10 μg of kanamycin ml^{-1} produced single-hybridization bands (Fig. 2). These data suggested that Tn*5* was inserting at various sites, because multiple copies of the resistance gene were required in order to produce resistance to the higher concentration of kanamycin used. Bands of different sizes were also observed, suggesting that the insertion was random (Fig. 2, lanes a through f and h). At 10 μg of kanamycin ml^{-1}, spontaneous resistant mutants appeared on the selective plates after 48 h of incubation; however, these resistant mutant colonies were distinguished from those containing the transposon by their smaller sizes and their delayed growth. The lack of hybridization of the probe with the DNA isolated from one of the colonies (Fig. 2, lane g), suggests that it was a spontaneous kanamycin-resistant mutant.

Tn*5* also contains a Str^r gene that is expressed in some bacteria. When putative Tn*5* insertion mutants of *X. nematophilus* ATCC 19061/1 were streaked onto LA containing 100 μg of streptomycin ml^{-1}, only a few colonies developed. Hybridizational analysis of the genomic DNA of several of these Str^r mutants that originated from strains carrying only a single copy of the Tn*5* showed that all contained multiple bands that hybridized with the Tn*5* probe and that the banding pattern in each was unique (44a). These data support the suggestion that a single copy of chromosome-inserted Tn*5* provides only low-level resistance to the antibiotics.

Avirulent mutants. The protein profiles of the avirulent *X. nematophilus* ATCC 19061/1 mutants, AV1, 7A-2/1G, and 7A-2/1SA (Table 2), are shown in Fig. 3. Although the protein patterns of the wild-type and avirulent mutants are similar, there are some differences. All of the avirulent strains are missing or are deficient in a number of high-M_r protein bands and in particular, a major band with an apparent molecular mass of 32.5 kDa (Fig. 3). The significance of these missing proteins in virulence remains to be determined.

All three avirulent mutants were Btb^+ (Table 2) and produced the same fragment pattern when their genomic DNA was digested with restriction enzymes (data not presented). Strain 7A-2/1SA was unusual in its sensitivity to

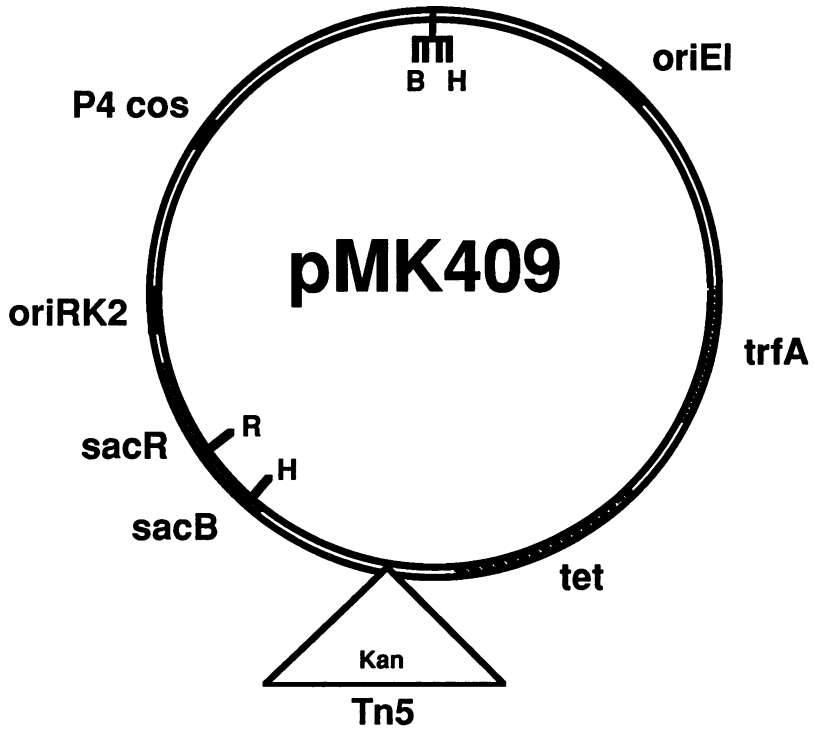

FIG. 1. Partial restriction map of the plasmid pMK409 and its Tn*5* derivative, pHX1. pRS14, which carries both the RK2 and ColE1 origins of replication and the Tet^r and Kan^r genes (39), was digested with *Bam*HI and *Hin*dIII and ligated to similarly digested pUC18 to replace part of the Kan^r gene in pRS14 with part of the linker region of the pUC18 plasmid. One of the resulting Tet^r Kan^s Amp^s plasmids was then digested with *Bgl*II and ligated to the *Bam*HI *sacB sacR* cartridge from pUM24 (32). Because the inhibition of growth of *E. coli* strains that contain the levansucrase genes was greater on LA containing 5% (wt/vol) sucrose at 30 than at 37°C, selection for sucrose resistance and screening for sucrose sensitivity were done at 30°C. Tet^r Kan^r transformants that contained the insertion of the sucrose-kanamycin cartridge were then partially digested with *Pst*I to remove Kan^r, and Tet^r Kan^s Suc^s transformants were isolated. The structure of one of these isolates (pMK409) was confirmed by restriction digests. *E. coli* JEF8(pMK409) was infected with lambda::Tn*5* 467 as described by de Bruijn and Lupski (15). A plasmid pool from cultures grown in LB containing kanamycin and tetracycline at 50 and 12.5 μg ml^{-1}, respectively, was used to transform *E. coli* HB101. pHX1, isolated from an *E. coli* HB101 colony that was Tet^r Kan^r Suc^s, was transformed into *X. nematophilus* ATCC 19061/1, and a single Tet^r Kan^r Suc^s *X. nematophilus* ATCC 19061/1(pHX1) isolate was used to obtain pHX1 for subsequent transposon mutagenesis experiments. Filled areas represent significant genes. Restriction sites: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. The multiple sites at the top represent the linker fragment of pUC19 bounded by the *Bam*HI and *Hin*dIII sites.

ampicillin, resistance to streptomycin (100 μg ml^{-1}), and slow growth. Strain 7A-2/1SA also produced two colony morphologies on LA after incubation for 48 h at 28°C: a large colony of 1.0 to 1.3 mm in diameter and a small colony of <0.5 mm in diameter. Both of these forms generate the alternative form, and repeated attempts to purify them from clonally isolated colonies have failed. Strain 7A-2/1G appears to be defective in iron uptake, since it is unable to grow in the presence of the iron-chelating agents 2,2′-dipyridyl or Chrome azurol S (Table 2).

Biochemical mutants. The biochemical and physiological characteristics of several putative Tn*5* insertion mutants of *X. nematophilus* ATCC 19061/1 were compared with each other and the wild-type forms (Table 2). No stable, primary-form bromothymol blue-adsorbing mutants were isolated. Since all the Btb$^+$ mutants also produced Btb$^-$ forms. However, the degree of instability among the Btb$^+$ adsorption mutants varied from mutants in which only occasional red sectors appeared through strains that produced approximately equal proportions of red and blue sectors or that were maroon to mutants whose colonies were predominantly Btb$^-$ with rare blue sectors. The intensity of the bromothymol blue adsorption varied from deep blue through pale blue to green (Table 2). Further, the rate of switching between Btb$^+$ and Btb$^-$ was influenced by the conditions of the testing, with more sectors appearing in strains grown on LBT at 28°C than those grown on NBT at 22°C. Several stable Btb$^-$ mutants had different intensities of red pigmentation (Table 2).

Among mutants modified in antibiotic synthesis were several whose production was influenced by the medium used (Table 2). Several antibiotic-producing mutants had the bromothymol blue adsorption pattern of secondary forms (Table 2, strains XuH1, 141, 200, and 209). The ability to lyse erythrocytes is often considered a virulence factor (18). Several mutants that had primary-form characteristics were nonhemolytic (Table 2, strains AV1, 113, 210, 7A-2/1SA, and 7A-2/1G), whereas others that had some secondary-form characteristics were hemolytic (Table 2, strains 141, 200, and 209). Furthermore, some mutants produced lytic zones consistently larger or smaller than the wild type, and others produced cloudy lytic zones, the size of which varied among mutants.

Because Congo red adsorption is related to virulence in some gram-negative bacteria (27), we examined the pattern of its adsorption by *X. nematophilus*. Three characteristics of Congo red adsorption were genetically variable: (i) the development of a red ring immediately around the colony,

TABLE 2. Characteristics[a] of Tn*5* insertion derivatives of *X. nematophilus* ATCC 19061/1

Phenotypic characteristics	Result with strain:															
	19061/1	19061/2	AV1	7A-2/1SA	7A-2/1G	XuH1	113	114	R28A	R28B	200	223	209	210	141	Xu9
Btb adsorption[b] (°C)																
NBT (22)	B	M	B	w	w	R	M	B	Gw	Gw	R	B	R	B	R	M
NBT (28)	B	R	M	w	w	R	M	M	Gw	Gw	R	B	R	B	R	M
LBT (22)	B	M	B	B	B	R	M	M	R	M	R	M	R	B	R	M
LBT (28)	B	R	B	B	B	R	M	M	R	B	R	B	R	B	R	M
Btb stability	U	U	U	U	U	S	U	U	S	U	S	U	S	U	S	U
Congo red[c]	++W	−−R	++W	++W	++W	−+R	w+W	++W	−−R	++W	−−R	++W	−−R	++W	−−R	−−R
Hemolysis[d]	+	w	−	−	−	c	c	+	−	+	+	+	+	−	+	c
Resistance and susceptibility																
Kan[e]	s	s	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Amp[e]	r	r	r	s	r	r	r	r	r	r	r	r	r	r	r	r
Str[e]	s	s	h	r	s	h	h	h	h	h	h	h	h	h	h	h
Virulence[f]	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+
Antibiotic[g]																
LA	+	−	−	+	+	+	+	w	−	+	+	−	+	+	+	w
NA	+	w	w	w	w	+	+	w	−	w	+	+	+	+	s	w
X	+	−	+	w	w	+	w	−	−	w	+	−	+	+	+	−
BHI	+	−	w	−	w	+	−	w	−	w	+	−	+	+	w	−
YS	+	−	+	+	+	s	+	+	−	+	+	−	+	+	+	+
Lecithinase[h]	+	−	+	+	+	+	+	−	−	−	−	+	−	+	−	+
Milk[i]	−	+	−	−	s	−	−	s	+	+	−	−	+	−	+	−
Spirit blue[j]	−B±−	−BS−	−WRw	−BS+	−B±−	−BSw	−BR+	−BS+	+LS−	−TS−	−WR−	+WR−	−WR−	+WS−	+TR−	−BR+
Tween 80[k]	+	−	+	−	−	+	−	−	−	+	+	+	−	+	+	+
CAS[l]	+	+	+	+	0	ND	+	+	+	+	ND	ND	ND	ND	+	ND
2,2′-Dipyridyl[m]	+	+	+	+	0	ND	+	+	+	+	ND	ND	ND	ND	+	ND
Motility[n]	+	w	−	−	−	ND	−	+	−	−	ND	ND	ND	ND	−	ND

[a] +, Positive reaction, equivalent to wild-type control; −, negative reaction; w, positive reaction or growth, but significantly less so than the wild-type control; s, positive reaction significantly stronger than wild-type control; ND, not done; 0, no growth; ±, mixed characteristics, i.e., colonies with approximately equal numbers of sectors with two different characteristics. For some of the phenotypic charactertistics, more-specific information about these symbols is given in the corresponding footnotes.

[b] Results on LBT were recorded after 48 to 72 h at 28°C and after 96 to 120 h at 22°C. Results on NBT were recorded after 72 to 96 h at 28°C and after 96 to 144 h at 22°C. B, Dark blue, with rare red sectors if unstable; R, red, with rare blue sectors if unstable; M, maroon or approximately equal mixture of red and blue sectors; G, green; w, weak growth, dye adsorption indeterminate; S, stable, does not produce alternative bromothymol blue adsorption form; U, unstable, produces alternative bromothymol blue adsorption form.

[c] Results were recorded after 72 to 96 h at 28°C. First symbol, red-to-purple ring immediately around colony; second symbol, clearing of dye around colony, outside of the ring when ring is present; third symbol, colony color by reflected light. W, White; R, red. See footnote *a* for additional symbol definitions.

[d] Hemolysis was read after 48 h of incubation at 28°C. +, Clear lysis zone surrounding colony; −, no clearing; c, cloudy lysis. See footnote *a* for additional symbol definitions.

[e] s, Susceptibility, no or rare growth of clonally plated cells on LB (pH 7.5) containing 10 μg of kanamycin ml^{-1}, 100 μg of ampicillin ml^{-1}, or 100 μg of streptomycin ml^{-1}; r, resistance, growth of clonally plated cells was on the media described above; h, high frequency of resistant mutants to 100 μg of streptomycin ml^{-1}.

[f] *G. mellonella* larvae died within 48 h of being injected with approximately 20 to 200 bacteria. See footnote *a* for symbol definitions.

[g] All plates were incubated at 28°C. Antibiotic production was scored by flooding the plates with 0.1 ml of an overnight culture of *Bacillus sphaericus* 1593 (grown overnight with shaking at 28°C in LB) diluted to 1.0 ml in LB. The size of the inhibition zones relative to the diameter of the colonies was measured after 8 to 26 h at 37°C. Indicator bacteria were added to LA, brain heart infusion (BHI), and YS plates after 24 h of incubation, to NA plates after 36 h of incubation, and to medium X plates after 48 h of incubation. See footnote *a* for symbol definitions.

[h] +, Formation of white halo immediately surrounding colony after 5 to 7 days at 28°C. See footnote *a* for other symbol definitions.

[i] Results were read after 5 to 7 days at 28°C. +, Clear zone under colony following treatment with $HgCl_2$. See footnote *a* for other symbol definitions.

[j] Results were read after growth at 22°C for approximately 50 days. Characteristics are as follows: first symbol, presence of blue ring around colony; second symbol, colony color (W, white; B, brown); third symbol, colony surface (R, rough; S, smooth); fourth symbol, papilla formation after approximately 30 to 50 days. See footnote *a* for other symbol definitions.

[k] +, Formation of crystals around and under colony after 5 to 7 days of incubation. See footnote *a* for other symbol definitions.

[l] +, Ability to grow in CAS medium and to produce yellow halo around colony, indicating siderophore formation. See footnote *a* for other symbol definitions.

[m] +, Growth on LB (pH 7.5) containing 300 μM 2,2′-dipyridyl. See footnote *a* for other symbol definitions.

[n] LB medium was prepared with 1.2% Bacto-Agar and stored at 4°C shortly after solidification. Colonies were examined under a dissecting microscope after 24 and 48 h for the formation of flares extending out from the colony edge. Symbols: +, formation of flares;−, no flares; w, rare flare appearing after 24 h of incubation. See footnote *a* for other symbol definitions.

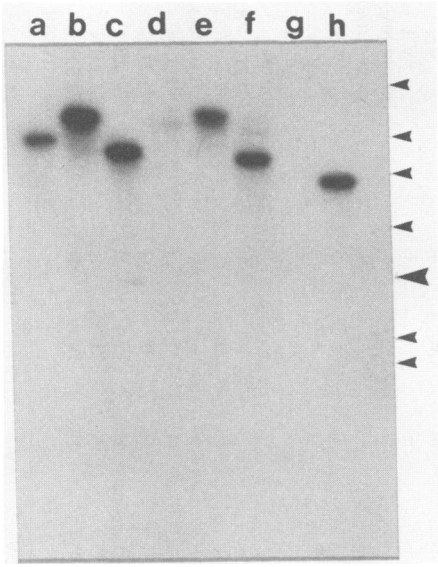

FIG. 2. Demonstration of Tn*5*-specific sequences in genomic DNA of putative Tn*5* insertion mutants of *X. nematophilus* ATCC 19061/1 by Southern hybridization. *Eco*RI digests of chromosomal DNA were separated by electrophoresis in 0.7% agarose buffered with Tris-borate and hybridized with a ^{32}P-labeled Tn*5*-specific probe. Lanes: a, 3-2/1; b, 3-6/1; c, 6-2/1; d, 5A-1/1; e, 5A-2/1; f, 3A-3/1; g, 8-1/1; h, 7A-2/1. The small arrows indicate the migration of lambda *Hin*dIII size standards determined by ethidium bromide staining of the gel (from top to bottom, in kilobases): 23.7, 9.46, 6.67, 4.26, 2.25, and 1.96. The large arrow indicates the position of the 3.5-kb Tn*5* probe.

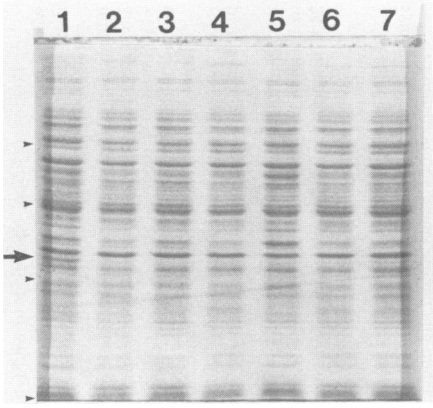

FIG. 3. Comparison of the total cellular protein-banding pattern of *X. nematophilus* ATCC 19061/1 wild type with avirulent Tn*5* mutants. Cells were grown for 20 to 24 h at 28°C with shaking in 1.5 ml of LB; pelleted in a microcentrifuge for 5 min; suspended in the original volume of sterile, deionized water; and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as previously described (24). The proteins (in 20-μl volumes) were separated on a 1.5-mm-thick 12% acrylamide gel and stained with Coomassie brilliant blue R. Lane 1, *X. nematophilus* ATCC 19061/1; lane 2, AV1; lane 3, AV1S (derivative of AV1 resistant to 100 μg of streptomycin ml^{-1}); lane 4, 7A-2/1SA; lane 5, 7A-2/1G; lane 6, 7A-2/1SAL (large colony from clonal plating of 7A-2/1SA); lane 7, 7A-2/1SAS (small colony from clonal plating of 7A-2/1SA). The small arrows indicate the position of the molecular mass protein standards (from top to bottom, in kilodaltons): BSA, 66; ovalbumin, 45; carbonic anhydrase, 29; lysozyme, 14.3. The large arrow indicates the position of the prominent 32.5-kDa protein that is missing in the avirulent strains.

(ii) the clearing of dye from the medium to form a colorless zone outside the red ring, and (iii) the color of the colony by reflected light. The primary-form colony on LBCR was opaque white, and it developed a prominent red ring, surrounded by a clear zone, whereas the secondary form colony was red by reflected light and produced no rings. A number of mutants with mixtures of these characteristics were isolated (Table 2, strains XuH1 and 113).

Since *Xenorhabdus* spp. grow in insects that are rich in fats, the ability to digest lipids may contribute to their pathogenicity. Therefore, the phenotype of *X. nematophilus* ATCC 19061/1 on the Spirit blue lipase indicator medium was examined. Differentiation on Spirit blue medium required incubation periods of up to 50 days (Table 2). Phenotypic differences could be discerned on this medium in colony color (colorless, brown, light blue, or dark blue), colony appearance (rough or smooth), and the development, after 20 to 40 days, of prominent, raised, yellow-brown papillae. Sectors of the various colony morphologies in a variety of combinations were present in many colonies, but other colonies were homogeneous in given characteristics.

Mutants in lecithinase production, Tween 80 hydrolysis, motility, and the hydrolysis of milk casein were also characterized (Table 2). Although only the response of mutants on milk in a plate count agar base is reported here, we also tested some strains on milk in an LA base and observed a different phenotype for some mutants on the two media (data not presented). The adsorption of neutral red from MacConkey agar (1) was too weak for consistent differentiation between *X. nematophilus* ATCC 19061/1 strains. None of the strains tested possessed urease or catalase activity and none utilized citrate, whereas all tested positive for Tween 40 hydrolysis.

DISCUSSION

The results presented here are the first demonstration of the use of Tn*5* mutagenesis for the induction of mutations in *Xenorhabdus* spp. and of conjugative plasmid transfer from *E. coli* to *X. nematophilus*. The frequencies of successful transfer of the various plasmids conjugatively transferred into *X. nematophilus* varied by several orders of magnitude. pRZ102 was transferred at the highest frequency of any of the plasmids tested, whereas pGS9 was infrequently recovered in the recipient cells. The low frequency of transfer observed for pGS9 could be due to inefficient mobilization by the N transfer system used by this plasmid (34) or to some effect linked to its larger size, such as a higher susceptibility to restriction. We have previously reported evidence that restriction barriers exist between *Xenorhabdus* spp. and *E. coli*, since plasmids grown in *E. coli* are transformed at a much lower frequency than plasmids obtained from *Xenorhabdus* spp. (45). However, the instability of the Kan^r exconjugates obtained with pGS9 suggests that the problem might also be due to inefficient maintenance of the plasmid. The low frequency of transference of pRZ102 into *X. poinarii* and its failure to transfer conjugatively into *X. nematophilus* IM/1 and *X. luminescens* RH/1 show that this means of transfer is strain specific. We have not investigated the possibility of conjugation between the various *Xenorhabdus* spp. or between *Xenorhabdus* spp. and other bacterial species.

The observed death of donor *E. coli* cells during conjuga-

tion with *Xenorhabdus* spp. is similar to that reported for donor cells conjugated with *Zymomonas mobilis* (38) and *Pseudomonas fluorescens* (43) strains. Killing of *E. coli* donor cells by *X. nematophilus* ATCC 19061/1 was more rapid than that reported for *Z. mobilis*. The sensitivity of the donor cells during matings with both *Z. mobilis* and *P. fluorescens* was attributed to antibacterial compounds produced by these bacteria (38, 43). *E. coli* S17-1 showed zones of inhibition when tested against *X. nematophilus* ATCC 19061/1 but not when tested against *X. nematophilus* ATCC 19061/2 and *X. poinarii*, yet donor cells were also killed when mated with the last two strains. It is possible that the last two strains produce antibiotics of a kind or quantity that are lethal to donor cells in intimate contact with the strains but which cannot be detected by the production of an inhibition zone. *X. nematophilus* ATCC 19061/2 occasionally produces a narrow zone of inhibition on NA when *B. sphaericus* 1593 is used as an indicator (Table 2).

pSUP1021, pSUP2021, and pGS9 have all been used as suicide vectors for Tn*5* mutagenesis in gram-negative organisms that do not support their replication (34, 35), but the stability of pSUP1021 and pSUP2021 in *X. nematophilus* ATCC 19061/1 precludes their use for this purpose in this organism. However, putative Tn*5* insertion mutants were isolated from matings with pGS9 by screening for Kan^r Cam^s transconjugates.

pHX1 was an efficient negative-selection vector for use with *X. nematophilus* ATCC 19061/1 and is likely to serve the same function in the other *Xenorhabdus* spp. as well as in other gram-negative bacteria. We have transformed pHX1 into *X. luminescens* RH/1 and have isolated a number of Kan^r putative Tn*5* insertion mutants. A mobilizable version of pHX1 has been constructed (unpublished data).

Hybridization analysis of a number of putative Tn*5* insertion mutants and the isolation of a wide range of Kan^r mutants indicated that Tn*5* insertion in *X. nematophilus* is sufficiently random to be suitable for transposon mutagenesis. The observation of multiple Tn*5* inserts at higher kanamycin concentrations suggests that several copies of the kanamycin resistance gene were required for growth at these concentrations. The reason for this is not known, but it may be that the kanamycin resistance gene is inefficiently expressed in *X. nematophilus*. Alternatively, the Tn*5* inserts, which have been shown to be unstable in some cases (9), may produce multiple inserts in a given isolate over time. Exposing the single-insertion Tn*5* mutants to a high concentration of streptomycin to select for multiple insertions may be useful in increasing the chance of obtaining a particular mutation, although it would then be necessary to sort through the insertions to identify the mutation of interest.

We have extended the observations of Boemare and Akhurst (10) that *Xenorhabdus* spp. characteristics (e.g., bromothymol blue adsorption and antibiotic production) are influenced by the medium used and the temperature of incubation (Table 2). We have introduced new media for the characterization of *X. nematophilus* mutants, although the biochemical explanations for the differences are unknown. The pattern of Congo red adsorption, while useful in distinguishing avirulent from virulent strains of some gram-negative bacteria (14, 27), did not relate to the virulence of *X. nematophilus* ATCC 19061/1. Since we have not identified a pattern of phenotypic characteristics that identifies avirulent mutants, the in vivo virulence assay remains the only means of detecting avirulent mutants of *X. nematophilus*.

The general variability and phase variation of *X. nematophilus* (1, 10) makes unambiguous interpretation of some of the diagnostic data difficult. Akhurst (1) has reported that a variety of bromothymol blue adsorption strains exists among isolates of *X. nematophilus*. Therefore, the phenotypic changes in dye adsorption tentatively ascribed here to Tn*5* insertions may, in some strains, be due to the isolation of spontaneous dye adsorption variants. Final proof of the effect of each Tn*5* insertion must await correlation of the phenotypic change with transfer and incorporation of the affected gene into the chromosome of the wild-type cell as well as complementation studies. However, in 3 years of work with *X. nematophilus* ATCC 19061/1 we have not seen spontaneous variations of the types present in the putative Tn*5* mutants shown in Table 2.

The procedures described in this report provide powerful tools for probing the unique characteristics of the *Xenorhabdus* spp. These procedures will make it possible to identify and isolate the genes responsible for *Xenorhabdus* spp. virulence, as well as for a variety of other abilities of the *Xenorhabdus* spp., such as the genes responsible for the switching from primary to secondary form, for the specificity of the *Xenorhabdus* sp.-nematode host relationship, and for antibiotic production. The isolation of three avirulent mutants from the first 250 putative Tn*5* insertion mutants acquired shows that this technique yields avirulent mutants at a reasonable frequency. The nature of the mutations leading to loss of virulence is currently under investigation.

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