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Biological Control xxx (2007) xxx–xxx
Biological Control
www.elsevier.com/locate/ybcon

Characterization of baculovirus isolates from *Trichoplusia ni* populations from vegetable greenhouses

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Received 17 October 2006; accepted 26 January 2007

Abstract

A survey of cabbage looper, *Trichoplusia ni*, populations in greenhouse vegetable crops in the Fraser Valley (FV) of British Columbia, Canada led to the isolation of a large number of *Nucleopolyhedrovirus* (NPV) single-infected-larva isolates. These NPVs were identified from cadavers by phase-contrast light microscopy and characterized as either *T. ni* SNPV (TnSNPV) or *Autographa californica* MNPV (AcMNPV) by mulitplex-PCR. Among the 57 NPV isolates collected in 2000, 54 were TnSNPV and three, all from one greenhouse, were AcMNPV. In 2001 over 100 single-infected-cadaver NPV isolates were characterized by PCR and all were TnSNPV. Restriction endonuclease (REN) analysis confirmed the PCR identification of individual isolates. In addition, REN analysis showed that all TnSNPV isolates had identical REN profiles that were similar to but distinct from the reference strain TnSNPV-RJ suggesting that TnSNPV-FV isolates constitute a single unique strain of the virus. In contrast, only a few AcMNPV were isolated and these constitute two strains based on REN profiles that were distinct from other AcMNPV strains. Dose–response bioassays with 2nd and 4th instar *T. ni* indicated there was no significant difference in infectivity of TnSNPV and AcMNPV isolates. However, in 5th instar *T. ni* AcMNPV was as much as 10-fold more infectious than TnSNPV. In addition, AcMNPV appeared to be more virulent as infected 4th instar larvae died approximately 18 h sooner than TnSNPV infected larvae. TnSNPV produced approximately five times more occlusion bodies per cadaver than AcMNPV. Both AcMNPV and TnSNPV appear to have good potential as candidates for biological control agents of *T. ni*.

Keywords: Cabbage looper; *Trichoplusia ni*; *Nucleopolyhedrovirus*; AcMNPV; TnSNPV

1. Introduction

The cabbage looper (*Trichoplusia ni*) (Lepidoptera: Noctuidae) has become a serious pest of greenhouse vegetable (tomato, sweet pepper and cucumber) production in Canada due in part to the development of populations that are resistant to *Bacillus thuringiensis* (*Bt*) based bioinsecticides (Janmaat and Myers, 2003). Much of the Canadian greenhouse vegetable crop is produced without chemical pesticides using IPM systems that rely on biological control agents. Baculoviruses provide an attractive alternative to *Bt* products for cabbage looper control as they are highly infectious and typically host specific and therefore, compatible with IPM systems based on the use of a complex of insect biological control agents. A number of baculoviruses, including *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) Spod-X® LC and *Anagrapha*

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1049-9644/$ - see front matter
doi:10.1016/j.biocontrol.2007.01.011

falcifera multiple nucleopolyhedrovirus (AnfafMNPV), have been registered for greenhouse use in other jurisdictions. The *Baculoviridae* are a diverse family of rod-shaped, occluded viruses that have circular dsDNA genomes ranging in size from 80 to 180 kb and are divided into two genera: the *Nucleopolyhedrovirus* (NPVs) and the *Granulovirus* (GVs). NPVs typically produce large occlusion bodies (OBs) containing numerous virions while GVs produce smaller granular OBs each containing a single virion. A number of baculoviruses have previously been isolated from cabbage looper populations including; *T. ni* granulovirus (TnGV), *T. ni* single nucleopolyhedrovirus (TnSNPV), *T. ni* multiple nucleopolyhedrovirus (TnMNPV) (Jaques, 1970), the later like AnfaMNPV is a strain of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Theilmann et al., 2005).

Baculoviruses, TnSNPV and TnMNPV, have been evaluated as biocontrol agents for *T. ni* in cole crops and deemed to have good potential for development as viral insecticides (Jaques, 1972, 1977; Vail et al., 1999). As indicated above, TnMNPV is one of numerous isolates recognized as being variants of AcMNPV derived from different host species and geographic regions or as plaque purified clones of field isolates (Smith and Summers, 1979; Stiles and Himmerich, 1998; Yanase et al., 2000; Theilmann et al., 2005). Several of these AcMNPV variants have different levels of infectivity for particular host species (Harrison and Bonning, 2003). Similarly, geographic isolates of TnSNPV from various field populations of cabbage loopers have been shown to be genotypically distinct (Bilimoria, 1983; Del Rincón-Castro and Ibarra, 1997) and have significantly different levels of infectivity and virulence for cabbage looper populations. The host range of TnSNPV appears to be much more restricted than AcMNPV (Harper, 1976; Vail et al., 1971; Cory, 2003) and TnSNPV infected only its original host species *T. ni* from among the five species covering five families tested by Del Rincón-Castro and Ibarra (1997).

Trichoplusia ni are yearly immigrants to British Columbia; however, *T. ni* can persist in greenhouses and increase early in the spring when new crops are planted. In addition, as the production of vegetables has moved to an almost year round cycle in greenhouses in British Columbia, resident populations of cabbage looper are becoming more persistent. Thus, as a preliminary step in the development of baculoviruses as new microbial control agents for this system, we determined if resident *T. ni* populations harbored baculoviruses and the levels of genetic diversity in these viral populations. This survey was carried out as an extension of a larger survey for *Bt* resistance in the same populations. Furthermore, indigenous baculovirus isolates may be more easily registered as biopesticides in the existing regulatory system and thus, the baculovirus isolates from Fraser Valley greenhouses were evaluated for infectivity and virulence to cabbage lopper in a series of laboratory bioassays.

2. Materials and methods

2.1. Virus isolation

Cabbage looper larvae, 3rd–5th instars, were collected from commercial vegetable greenhouses (nine in 2000 and eight in 2001) in the lower mainland of British Columbia, Canada as part of a larger experimental strategy to determine the level of resistance to *B. thuringiensis* present in these *T. ni* populations (Janmaat and Myers, 2003). In 2001, *T. ni* populations in three broccoli fields in the vicinity of commercial greenhouses were also sampled. Larvae were reared individually on artificial diet until pupation and those dying with baculovirus-like symptoms were collected individually and stored frozen at −20 °C until processed. Cadavers were homogenized in 0.5 ml of sterile ddH_2O in 1.5 ml centrifuge tubes using sterile nylon pestles (Kimble Knotes, Vineland, NJ, USA). Subsequently, 2 μl samples of homogenates were examined microscopically (phase contrast optics) for presence of baculovirus OBs and assessment of AcMNPV or TnSNPV like OB morphology. The OB yield per cadaver ranged between 1.0×10^8 and 9.0×10^9. Cadaver homogenates positive for the presence of NPV OBs were retained for further processing and analysis. The incidence of NPV infection in *T. ni* populations ranged from 0 to 9% for these single time point collections.

2.2. Virus isolates

Previously characterized viruses AcMNPV plaque purified strain AcMNPV-HR3 (Cochran et al., 1982) and a TnSNPV field isolate originally collected in New York state (Jaques, 1970) and referred to here as TnSNPV-RJ (Willis et al., 2005) were used as reference strains for standard comparisons with isolates of the same virus species derived from greenhouse populations of *T. ni*. The NPV virus isolates from Fraser Valley, British Columbia were simply designated in order of isolation from FV#1 to FV#118 for those collected in 2000 and from FV-01#1 to FV-01#108 for those collected in 2001.

2.3. Insects

A long-term colony of *T. ni*, originally obtained from Dr. A. Keddie (University of Alberta, Edmonton, Alberta, Canada), has been maintained at the Saskatoon Research Centre (SRC) since 1996 on a sterile semi-synthetic diet (Keddie and Volkman, 1985) and the larvae reared at 27 °C, 60% relative humidity, and a 20:4 light/dark photoperiod. Embryonated eggs were routinely surface disinfected by a 3 min dip treatment in 0.6% commercial bleach. Two additional colonies of *T. ni* were established (Janmaat and Myers, personal communication) from populations of cabbage looper larvae collected in commercial greenhouses: GIP, a *B. thuringiensis* var. *kurstaki* (Btk) sensitive strain, and BT-R, a population showing significant

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resistance to Btk. These colonies were received from Dr. J. Myers, University of British Columbia, reared in the same manner as the SRC colony and larvae tested in bioassays within three generations of establishment at the Saskatoon Research Centre.

2.4. PCR analysis of field collected cadavers

An AcMNPV-specific primer set was developed based on Ac-*ie0* gene sequence {Primer 174 (forward: 5′ tgtatatgcgtaggagagcc 3′) and Primer 179 (reverse: 5′ ctcgcaact gtttcaagtac 3′)} that produced a 619 bp AcMNPV-specific product. Similarly, a TnSNPV-specific primer set was developed based on Tn-*alkaline exonuclease* gene sequence {Primer 333 (forward: 5′ attcgctgtattgtggtaga 3′) and Primer 334 (reverse: 5′ gcccatcgaaatcaagtgtc 3′)} that produced a 405 bp TnSNPV-specific product (Milks et al., 1997). The specificity of these primers were tested in multiplex against a range of baculovirus DNA samples including, AcMNPV, AnfaNPV, TnSNPV, *Chrysodeixis chalcites* NPV (ChchNPV), *Malacosoma disstria* NPV, *Mamestra configurata* NPVs A & B, *Spodoptera frugiperda* NPV. Positive PCR amplification occurred only with AcMNP-HR3, C6, and AnfaNPV (617 bp product) or TnSNPV-RJ and the closely related baculovirus ChchNPV (405 bp product) (data not shown). As well, BLAST searches of AcMNPV and TnSNPV primer sequence against available baculovirus sequence indicated specificity for AcMNPV variants or TnSNPV and ChchNPV, respectively.

To determine if *T. ni* cadavers were infected with AcMNPV- or TnSNPV-like viruses, 2.0×10^7 OBs (~1–5 μl of cadaver homogenate) suspended in 100 μl of TE, pH 7.5, were incubated with 100 μl of OB dissolution buffer (0.1 M Na_2CO_3, 0.17 M NaCl, 0.001 M Na–EDTA, pH 10.8) for 15 min. Released occlusion-derived virions (ODV) were pelleted by centrifugation (20 min at 60 K in Beckmann TLA 120.2 rotor) and resuspended in 25 μl of TE. Ten microliter of ODV was incubated in 90 μl of PCR detergent buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3, 0.1 mg/ml gelatin, 0.45% Nonidet P 40 (NP-40), 0.45% Tween 20) with 6 μg of Proteinase K at 60 °C for 60 min, and then heated to 95 °C for 15 min. Standard PCRs were run using 10 μl of ODV DNA template in a 25 μl reaction volume containing 0.5 U of *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada), 0.4 mM dNPTs, 4 mM $MgCl_2$, and 0.8 μM of each primer. PCRs were carried out in a Strategene Robocycler thermocycler (Stratagene, LaJolla, CA, USA) with an initial denaturation cycle at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; followed by a final extension cycle at 72 °C for 10 min. These same conditions worked well for multiplex PCR with all four primers together. PCR products were electrophoresed through 1.0% agarose gels containing 0.5 μg/ml ethidium bromide and using 1× TAE running buffer.

2.5. Occlusion-derived virion purification, DNA extraction and restriction endonuclease analysis of NPV isolates

In most cases virus OB yield per field-collected larva was sufficient that OB suspension derived from 1/2 an individual could be used to purify DNA for restriction endonuclease (REN) analysis. In instances where insufficient virus was collected directly from individual field-collected larva the virus isolate was amplified by infecting 5–4th instar *T. ni* larvae with 1.0×10^3 OB applied to a canola leaf disc as described below. Virus DNA was purified from ODV isolated from 1 to 5×10^8 OBs essentially as previously described (Erlandson, 1990). Briefly, OB suspensions were incubated with an equal volume of OB dissolution buffer (pH 10.8) for 10–15 min, the suspension neutralized by addition of 50 μl of 1.0 M Tris–HCl, pH 7.2, and undissolved OBs and cell debris pelleted by low speed centrifugation. Finally the supernatant containing ODV was layered onto 100 μl of 20% sucrose cushion and centrifuged for 20 min in a Beckmann Benchtop Ultra-Centrifuge using the TLA-100.3 fixed angle rotor at 60 K rpm (150,000*g*) to pellet the virions.

Occlusion-derived virions were resuspended in 300 μl TE, pH 7.5, adjusted to 1% SDS, 50 μg/ml proteinase K and incubated at 37 °C for 2 h. The preparations were then extracted twice with an equal volume of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1). Finally the aqueous phase was dialyzed against TE, pH 7.5, at 4 °C for 48 h.

Approximately 500 ng of purified virus DNA was digested with *Eco*RI, *Hin*dIII, *Pst*I or *Xho*I (Invitrogen, Burlington, ON) in manufacturer supplied REN buffer at 37 °C for 2 h. The DNA restriction fragments were separated by electrophoresis on 0.7% agarose gels in Tris–borate, EDTA buffer at 55 V (35 mA) for approximately 18 h. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed on a UV transilluminator.

2.6. Bioassays

Bioassays were conducted using a leaf disc feeding assay to inoculate 2nd, 4th or 5th instar larvae. In each case 4 mm diameter canola leaf discs (12.5 mm^2) were placed on 1.0 ml of 1.0% agar in 24-well Nu-Trend trays (Clear Lam Packaging Inc., Elk Grove Village, IL). Occlusion body suspensions were diluted in 0.05% Triton X-100 to specific concentrations and 2 μl samples applied to the leaf discs. After 1 h drying period, a single larva was placed in each well and the trays sealed with a ''breathable'' mylar cover (Clear Lam Packaging Inc., Elk Grove Village, IL). A minimum of five doses were tested for each isolate in each bioassay as follows: 2nd instar larvae—2-fold dilution series ranging from 2 to 32 OB/larva; 4th instar larvae—4-fold dilution series ranging from 5 to 320 OB larva; and 5th instar larvae—5-fold dilution series ranging from 10 to 6250 OB/larva. Five replicate assays were run with different

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cohorts of larvae and for each assay 24 larvae were treated per dose. Mortality was recorded daily until control larvae pupated or 10 days post-treatment. All virus-related mortality was confirmed by phase-contrast light microscopy of cadaver wet mounts. Dose–response mortality data were analyzed using SAS v.8.2—Probit procedure for multiple experiment bioassays using the normal distribution model to give estimates of LD_{50} values.

Time-to-mortality bioassays were undertaken in 4th instar larvae with selected AcMNPV and TnSNPV isolates at an approximately LD_{99} dose, 320 OB/larva, as described above. Mortality assessments were done twice daily from days 4 to 8 post-inoculation. Four replicate assays (24 larvae/isolate/replicate) were conducted with different cohorts of larvae, and time-to-mortality data were pooled and analyzed using the SAS v.8.2—Life Test procedure to estimate survival time 50% (ST_{50}) for each isolate.

In order to determine OB yield for AcMNPV-HR3 and TnSNPV-RJ, four replicate bioassays were conducted with 25-5th instar larvae infected with an LD_{99} dose, 500 OB/larva for AcMNPV-HR3 and 6250 OB/larva for TnSNPV-RJ, and infected larvae harvested individually into 1.5 ml centrifuge tubes as they became moribund (5–7 days post-inoculation). Individual larva were homogenized using a Teflon™ pestle in 1.0 ml of sterile ddH_2O and the number of OBs/ml estimated microscopically using a Neubauer hemocytometer to count a 1:100 or 1:1000 dilution of each homogenate. The data (OBs/larva) for each isolate were analyzed by a one-way ANOVA (Statistix v.8, Analytical Software, Tallahassee, FL, USA).

3. Results and discussion

3.1. Characterization of NPV field isolates

The multiplex PCR assay developed for the detection and identification of TnSNPV and AcMNPV proved useful in characterizing NPV infections in cabbage looper cadavers (Fig. 1a and b). As predicted by the DNA sequences of AcMNPV (GenBank Accession No. L22858) and TnSNPV (GenBank Accession No. DQ017380), the species-specific primers produced a PCR product of 619 bp for AcMNPV and 405 bp for TnSNPV, and both PCR products were present when the two virus DNA templates were mixed together in the PCR (Fig. 1a).

Based on light microscopy analysis of tissue smears, cadavers positive for NPV OBs were isolated from cabbage looper populations in seven of the nine greenhouses surveyed in the Fraser Valley region of British Columbia in 2000 and from six of eight greenhouses surveyed in 2001, as well as from a single broccoli field. Fifty-seven single-cadaver NPV isolates were characterized by PCR from among samples of cabbage looper larvae collected greenhouse in 2000; 54 were TnSNPV isolates and three, all from one greenhouse, were AcMNPV isolates. Only nine NPV infected cadavers were collected from the greenhouse complex which yielded the AcMNPV isolates and of these four

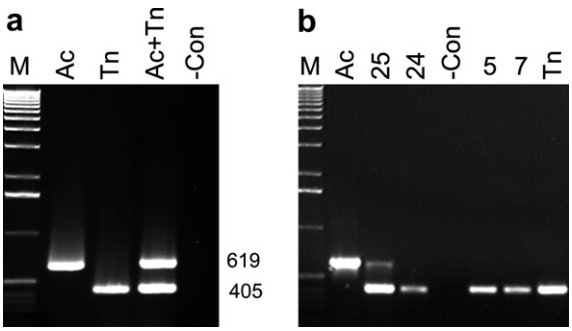

Fig. 1. Species-specific multiplex PCR assay for AcMNPV and TnSNPV. (a) Purified genomic DNA from AcMNPV-HR3 (Ac), TnSNPV-RJ (Tn) or mixed DNA (Ac+Tn) samples were subjected to multiplex PCR, as outlined in Section 2, and electrophoresed on a 1.0% agarose gel (1× TAE). PCR product sizes are indicated between panels, AcMNPV-609 bp and TnSNVP-405 bp. (b) Occluded virions purified from 2.0×10^7 OB harvested from single cadaver isolates from Fraser Valley populations of cabbage looper larvae were subjected to multiplex PCR as in (a). Note that FV#25 isolate is a mixed population of AcMNPV and TnSNPV viruses.

contained mixed AcMNPV+TnSNPV infections as detected by PCR (for example, see isolate FV#25, Fig. 1b), two displayed pure TnSNPV infections and three cadavers had pure AcMNPV infections. In 2001 over 100 single-cadaver NPV isolates, including 12 from broccoli fields, were characterized by PCR and all were TnSNPV. In all cases cadavers that were positive for baculovirus OB by microscopic analysis were demonstrated to be either AcMNPV or TnSNPV by PCR.

These results were similar to survey data from field populations of *T. ni* larvae collected on cole crops in Ontario and the Maritimes which showed that a "small polyhedrosis" (based on the size of the occlusion body) virus (presumably TnSNPV) was the most abundant virus in these populations while a "large polyhedrosis virus" (presumably AcMNPV or TnMNPV) occurred more rarely (Jaques, 1970). As well, mixed infections of "large" and "small" NPVs, presumably AcMNPV and TnSNPV, respectively, have commonly been reported from *T. ni* in the past (Heimpel and Adams, 1966; Vail et al., 1999). Although no thorough study of mixed infections has been conducted in field populations, Milks et al. (2001) showed that TnSNPV and AcMNPV were equally fit in intra-host competition studies.

In order to confirm PCR identity and to check the potential degree of genetic diversity among the NPV isolates derived from the various cabbage looper populations, REN analysis of purified virus DNA was undertaken. Fig. 2 shows a *Hin*dIII digest of a few of the TnSNPV and AcMNPV isolates collected during 2000. The REN analysis of Fraser Valley (FV) NPV isolate DNAs confirmed the PCR identity as either TnSNPV or AcMNPV related strains. Additional REN analysis (*Hin*dIII, *Eco*RI and *Pst*I digests of all FV isolates, data not shown) demonstrated that all of the TnSNPV-FV isolates had identical REN profiles and were similar to but distinct from the TnSNPV-RJ isolate used as a TnSNPV reference strain.

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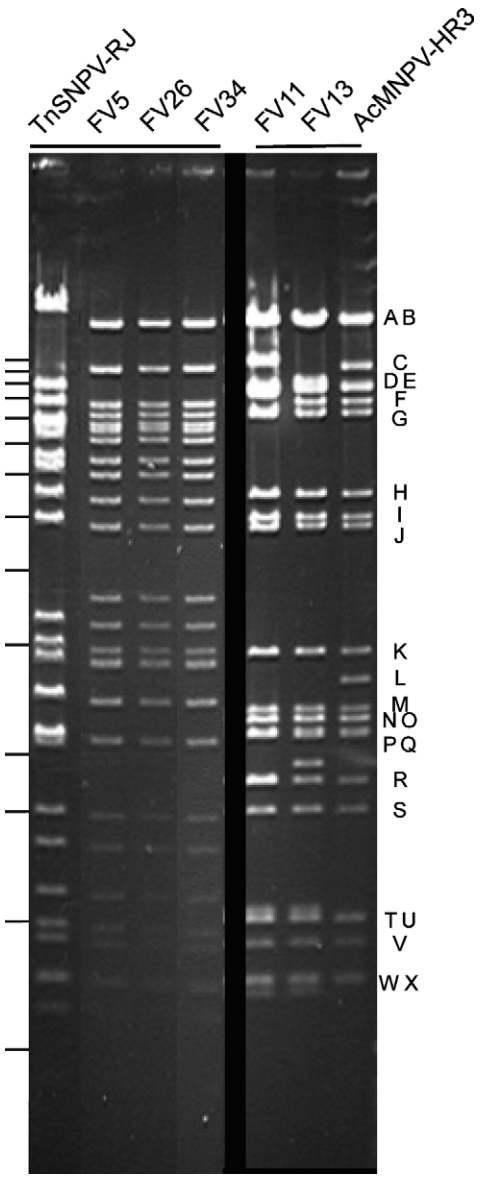

Fig. 2. Restriction endonuclease analysis of Fraser Valley (FV) Nucleopolyhedrovirus (NPV) isolate DNA. Genomic DNA was purified from OBs isolated from single cadaver isolates as outlined in Section 2, digested with *Hin*dIII and electrophoresed on a 0.7% agarose gel (1× TBE) for 18 h, stained with ethidium bromide and photographed. AcMNPV-HR3 and TnSNPV-RJ DNAs were included as references for each virus species. The FV isolates are grouped as TnSNPV and AcMNPV isolates. The tick marks on the left of the panel indicate the migration of marker DNA (1 kb ladder—12.2 kbp to 517 bp, Invitrogen). The letters are right side of the panel designate the AcMNPV-HR3 *Hin*dIII fragments, largest to smallest alphabetically. Note that each of the AcMNPV-FV isolates have unique *Hin*dIII patterns while all of the TnSNPV-FV isolates have an identical *Hin*dIII profile.

The difference in the *Hin*dIII patterns between TnSNPV-RJ (Willis et al., 2005) and the TnSNPV-FV isolates result from additional *Hin*dIII sites internal within the genomic region represented by *Hin*dIII A and B fragments in TnSNPV-RJ (Fig. 2). The position of the additional *Hin*dIII sites in FV isolates were confirmed by DNA sequence analysis of cloned fragments of TnSNPV-FV#5 and #34 (~50 kbp) and these appeared to result from single base pair differences in the two strains (Willis et al., 2005; Erlandson and Theilmann, unpublished TnSNPV-FV sequence data). Apart from these differences, the *Hin*dIII fragment profiles of the two TnSNPV strains appear to be very similar and sequence data indicated ~99% identity (Erlandson and Theilmann, unpublished data). The REN profiles of the TnSNPV-FV isolates were also unique when compared to previously published *Hin*dIII profiles of TnSNPV isolates from Alabama and Arkansas (Bilimoria, 1983) and from Mexico and China (Del Rincón-Castro and Ibarra, 1997). This suggests that TnSNPV-FV isolates are a genetically distinct strain of TnSNPV.

In contrast to the homogeneous nature of the numerous TnSNPV single-infected-larva isolates derived from Fraser Valley cabbage looper populations, only three AcMNPV single-infected-larva isolates were detected and these appeared to constitute two strains based on *Hin*dIII (Fig. 2) and *Eco*RI (data not shown) REN profiles. The *Hin*dIII profile of AcMNPV-FV#11 and AcMNPV-FV#15 were identical (data not shown) and were similar to AcMNPV-HR3 except that *Hin*dIII-C had higher molecular weight, *Hin*dIII-F was absent or its mobility altered, and the *Hin*dIII-L fragment was absent but additional fragments of approximately 1.2 and 0.7 kbp were apparent (Fig. 2). The *Hin*dIII profile AcMNPV-FV#13 was also similar to AcMNPV-HR3 except that *Hin*dIII-C was absent or its mobility altered as there appeared to be multiple co-migrating fragments at approximately 9.5–10.0 kbp. The *Hin*dIII-L fragment was also absent in FV#13 and additional *Hin*dIII fragments of 1.8, 1.2 and 0.7 kbp were apparent (Fig. 2). In addition to the AcMNPV-FV isolates being distinct from AcMNPV-HR3 (Cochran et al., 1982) (Fig. 2), they were also distinct from other AcMNPV strains such as E2 and L1 (data not shown), and other AcMNPV field isolates (Smith and Summers, 1979; Stiles and Himmerich, 1998; Yanase et al., 2000). Although the REN profiles of AcMNPV-FV isolate appear to be unique, the variations relative to AcMNPV-HR3 occur in regions of the AcMNPV genome that commonly differ between strains, namely *Hin*dIII C, F and L (Stiles and Himmerich, 1998; Miller and Dawes, 1978).

3.2. Biological characterization of field isolates

One of the concerns with characterizing infectivity and virulence of insect pathogens using long standing laboratory cultures of insects is the possibility that these cultures do not reflect the susceptibility of naturally occurring populations of the insect pest in question. To this end we conducted simultaneous dose–response bioassays in 2nd instar larvae of three populations of *T. ni* including two recently established colonies from field collected *T. ni* (Table 1). There were no significant differences in LD_{50} estimates, based on exclusivity of 95% confidence intervals, among the different *T. ni* populations for either the AcMNPV-HR3 isolate (LD_{50} estimates—5.4–9.3 OBs/larva) or the

Table 1
Dose mortality responses day 7 post-infection, lethal dose 50% (LD_{50}) OBs/larva, for 2nd instar *Trichoplusia ni* larvae from colony and field sources infected with AcMNPV and TnSNPV isolates in leaf disc feeding exposure assays

T. ni source	Virus strain	LD_{50} (95% CI)	*b*	X^2/df
SRC	AcMNPV-HR3	5.4 (3.7–7.4)	1.3	4.43/3
GIP	AcMNPV-HR3	9.3 (6.9–12.9)	1.3	4.67/3
BT-r	AcMNPV-HR3	6.9 (5.1–9.2)	1.4	3.87/3
SRC	TnSNPV-RJ	2.4 (1.2–3.6)	1.2	2.03/3
GIP	TnSNPV-RJ	3.0 (2.2–3.7)	1.4	1.67/3
BT-r	TnSNPV-RJ	1.8 (1.0–2.6)	1.6	1.67/3

TnSNPV-RJ isolate (LD_{50} estimates—1.8–3.0 OBs/larva). These results indicate that bioassay data generated with our laboratory strain of *T. ni* will have relevance to expected results with field populations of *T. ni*. As well, in this series of bioassays TnSNPV-RJ appeared to be two to three times more infectious than AcMNPV-HR3. Preliminary droplet-feeding bioassays with AcMNPV-HR3 and TnSNP-RJ in neonate *T. ni* larvae also indicated higher infectivity (lower LD_{50}) for TnSNPV-RJ compared to AcMNPV-HR3 (data not shown).

A preliminary single dose (50 OB/2nd instar larva) time-to-mortality bioassay with Fraser Valley NPV isolates, including randomly selected TnSNPV isolates and all AcMNPV-like isolates, showed variation in time-to-mortality between some isolates (data not shown). Thus to more thoroughly examine the infectivity of the FV NPVs, several isolates representing a full range of time-to-mortality responses were chosen for dose-mortality bioassays in 2nd instar larvae. The bioassay results indicated that one isolate of each virus species, FV#11 (AcMNPV) and FV#26 (TnSNPV), was significantly less infectious, LD_{50} estimates of 10.9 and 13.6 OB/larva, respectively, than the other isolates for each virus species (Table 2). Further investigation will be required to determine if variations in the *Hin*dIII profiles of the AcMNPV isolates are related to the differences in their infectivity. With the exception of these two isolates there were no significant differences between the LD_{50} estimates, 2.1–6.1 OB/larva, for the other isolates (Table 2).

The most infectious FV isolates of AcMNPV and TnSNPV were selected for further dose–response bioassay assessments in 4th and 5th instars. In 4th instar bioassays no significant differences were detected in LD_{50} estimates for any of the strains, LD_{50} ranged from 14.0 to 34.8 OB/larva (Table 3). However, there was a trend to lower LD_{50} estimates for the AcMNPV isolates which was much more pronounced in 5th instar bioassays in which AcMNPV-HR3 and FV#13 had significantly lower LD_{50} estimates, 38.3 and 47.5 OB/larva, respectively, than TnSNPV-RJ, FV#5 and FV#34 (205–425 OB/larva) (Table 4). There were no significant differences in LD_{50} estimates for isolates within virus species for 5th instar *T. ni*; however, the TnSNPV isolate FV#34 had a 2-fold lower LD_{50} than the other two TnSNPV isolates.

Previous comparative bioassay studies with TnSNPV and AcMNPV in cabbage looper larvae have yielded somewhat variable results. In some cases, TnSNPV was more infectious for neonate *T. ni* larvae than was AcMNPV (Granados et al., 1994; Davis and Wood, 1996; Del Rincón-Castro and Ibarra, 1997). Our preliminary neonate bioassays and comparative assays in the three *T. ni* colonies also indicated higher infectivity for TnSNPV-RJ compared to AcMNPV-HR3. In contrast, Vail et al. (1971) found that an AcMNPV isolate was four to five times more infectious than a TnSNPV isolate for 1st instar *T. ni* larvae. While the bioassays in 2nd and 4th instar *T. ni* in the current study showed was no significant difference in the LD_{50} estimates for AcMNPV and TnSNPV isolates, AcMNPV isolates were significantly more infectious than TnSNPV in 5th instars. The variability in these results may relate to differences in fundamental susceptibility of the *T. ni* populations used in the assays, the specific strains of each virus used or the techniques used for infection in the bioassays. Our *T. ni* neonate assays were conducted using a droplet feeding assay (Hughes and Wood, 1981) in a similar fashion to assays conducted by Davis and Wood (1996). In

Table 2
Dose mortality responses day 7 post-infection, lethal dose 50% (LD_{50}) OBs/larva, for 2nd instar *Trichoplusia ni* larvae in leaf disc feeding exposure assays

Virus strain	LD_{50} (95% CI)	*b*	X^2/df
AcMNPV-HR3	6.1 (4.3–8.4)	1.6	5.10/3
FV#11 (AcMNPV)	10.9 (8.0–15.9)	1.3	6.30/3
FV#13 (AcMNPV)	3.4 (1.7–5.0)	1.5	1.29/3
TnSNPV-RJ	5.6 (2.8–9.0)	1.3	4.87/3
FV#5 (TnSNPV)	5.4 (2.7–8.8)	1.5	2.72/3
FV#26 (TnSNPV)	13.6 (9.5–23.0)	1.1	2.20/3
FV#34 (TnSNPV)	2.1 (0.8–3.8)	1.5	0.74/3

Table 3
Dose mortality responses day 7 post-infection, lethal dose 50% (LD_{50}) OBs/larva, for 4th instar *Trichoplusia ni* larvae in leaf disc feeding exposure assays

Virus strain	LD_{50} (95% CI)	*b*	X^2/df
AcMNPV-HR3	17.6 (10.4–26.8)	1.1	1.07/3
FV#13 (AcMNPV)	14.0 (7.5–22.3)	1.1	2.56/3
TnSNPV-RJ	21.1(13.1–31.5)	1.2	0.86/3
FV#5 (TnSNPV)	34.8 (22.9–68)	1.2	0.94/3
FV#34 (TnSNPV)	20.1 (3.6–37.6)	1.1	0.61/3

Table 4
Dose mortality responses, lethal dose 50% (LD_{50}) OBs/larva, for 5th instar *Trichoplusia ni* larvae in leaf disc feeding exposure assays

Virus strain	LD_{50} (95% CI)	*b*	X^2/df
AcMNPV-HR3	38.3 (29–50)	1.9	0.74/2
FV#13 (AcMNPV)	47.5 (30–77)	1.7	0.86/2
TnSNPV-RJ	425 (265–665)	1.6	0.15/2
FV#5 (TnSNPV)	442 (279–685)	1.9	0.15/2
FV#34 (TnSNPV)	205 (118–351)	1.6	1.82/2

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contrast our assays with 2nd, 4th and 5th instars were conducted using a leaf disc assay (12.5 mm^2 canola leaf disc) and those larvae completely consuming the disc within a 1 h period were included in the assay. It is possible that the different delivery substrate, plant versus aqueous suspension, could impact virus stability or initial infection of the midgut. The bioassays conducted by Vail et al. (1971) and Del Rincón-Castro and Ibarra (1997) used inoculation of the surface of artificial diet as means of infecting larvae and LC_{50} rather that LD_{50} estimates were derived. In general, our LD_{50} estimates for the AcMNPV-HR3 reference virus are in agreement with previously published data for AcMNPV in 2nd–4th instar *T. ni* larvae.

In the current study significant differences were noted for LD_{50} estimates in 5th instar *T. ni* with AcMNPV isolates being 5- to 10-fold more infectious than TnSNPV isolates. Developmental resistance has been noted in many host-NPV interactions in which significantly higher doses are required to kill late instar larvae (Evans, 1981; Rovesti et al., 2000) with increases in LD_{50} estimates often being positively correlated with increases in larval weight in later instars (Evans, 1981). However, developmental resistance is not as marked in all insect species; Rovesti et al. (2000) found that resistance to MbMNPV isolates increased by a factor of 40,000 from L1 to L4 in *Mamestra brassicae* larvae but only by 1300 in *Heliothis armigera*. Engelhard and Volkman (1995) showed that, within an instar, developmental resistance is not only related to increasing body weight but also to increasing rates of midgut cell sloughing as larvae age. It may also be possible that the level of developmental resistance within a single host species may vary depending on the virus species used to challenge the insects. Thus the differences in increases of LD_{50} from L2 to L5 *T. ni* larvae for AcMNPV and TnSNPV warrants further investigation to determine if there is a fundamental difference in the infection process for the two viruses in last instar *T. ni*.

A separate series of experiments was performed to estimate the speed with which AcMNPV and TnSNPV isolates killed 4th instar *T. ni*. The survival time for 50% of larvae (ST_{50}) infected at an LD_{99} dose (320 OB/larva) were significantly lower for AcMNPV-HR3 and AcMNPV-FV#13, 140.5 ± 2.5 and 138.0 ± 3.1 h post-infection, respectively, than for TnSNPV-RJ, and TnSNPV-FV#5, 157.0 ± 2.1, and 156.3 ± 2.1 h post-infection, respectively (Wilcoxon—$X^2 = 35.8$, 3 *df*, $p < 0.001$). It was routinely observed that in the dose–response assays the AcMNPV-infected larvae died 12–24 h before TnSNPV-infected larvae when comparing biologically equivalent doses. These results are in agreement with the observation of Harper (1976) that SNPV isolates took longer to kill their host than did MNPV isolates.

Another key feature of baculovirus biology that impacts the ecological interaction with their host insects is the amount of infectious virus produced in each infected host. The amount of AcMNPV or TnSNPV OBs produced per infected *T. ni* larva was examined in infected 5th instar cabbage looper larvae. Approximately fivefold more OBs were produced in TnSNPV-RJ infected larvae ($2.55 \pm 0.47 \times 10^9$ OB/larva) compared to AcMNPV-HR3 infected larvae ($5.31 \pm 1.01 \times 10^8$ OB/larva). Thus, although TnSNPV kills the host more slowly, this results in higher production of OBs which could be advantageous to its transmission efficiency.

In evaluating the potential of NPVs as microbial control agents a number of characteristics can be considered. The higher infectivity of AcMNPV isolates than TnSNPV isolates in 5th instar cabbage loopers and the fact that AcMNPV appears to take less time to kill *T. ni* larvae than does TnSNPV suggest that AcMNPV is the more effective pathogen in *T. ni*. On the other hand, TnSNPV-infected larvae produce approximately five times the OBs of AcMNPV-infected *T. ni* larvae although each of these OBs may contain fewer virions. The observations here for cabbage loopers collected from tomato, sweet pepper, cucumber and broccoli, and those by Jaques (1970) on loopers from field cole crops show that TnSNPV is by far the more frequently recovered NPV species. The higher prevalence of TnSNPV and higher yield of TnSNPV OBs in *T. ni*, which may increase the efficiency of *in vivo* production, have positive implications for the development of TnSNPV as viral insecticide. Overall the results from this study suggest that indigenous isolates of either TnSNPV or AcMNPV have the infectivity and virulence to warrant consideration for investigation as viral insecticides for the control of cabbage looper larvae in greenhouse vegetable crops in Canada.

Acknowledgments

The financial support of the Natural Sciences and Engineering Research Council of Canada (NSERC) under the Biocontrol Research Network is gratefully acknowledged. We thank Alison Paton for technical assistance in rearing *T. ni* larvae.

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