Biological Control 52 (2010) 77–83

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Mixed-genotype infections of *Trichoplusia ni* larvae with *Autographa californica* multicapsid nucleopolyhedrovirus: Speed of action and persistence of a recombinant in serial passage

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ARTICLE INFO

Article history:
Received 16 June 2009
Accepted 1 October 2009
Available online 8 October 2009

Keywords:
Autographa californica multicapsid nucleopolyhedrovirus
Trichoplusia ni
egt gene
Genetically modified organism
Bioassay
Time to death
Fitness
Competition
Real-time PCR

ABSTRACT

Fast-acting recombinant baculoviruses have potential for improved insect pest suppression. However, the ecological impact of using such viruses must be given careful consideration. One strategy for mitigating risks might be simultaneous release of a wild-type baculovirus, so as to facilitate rapid displacement of the recombinant baculovirus by a wild-type. However, at what ratio must the two baculoviruses be released? An optimum release ratio must ensure both fast action, and the eventual competitive displacement of the recombinant virus and fixation of the wild-type baculovirus in the insect population. Here we challenged *Trichoplusia ni* larvae with different ratios of wild-type *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and a derived recombinant, vEGTDEL, which has the endogenous *egt* gene (coding for ecdysteroid UDP-glucosyltransferase) deleted. Time to death increased with the proportion wild-type virus in the inoculum mixture, although a 1:10 ratio (wild-type: recombinant) resulted in equally rapid insecticidal action as vEGTDEL alone. Five serial passages of three different occlusion body (OB) mixtures of the two viruses were also performed. OBs from 10 larval cadavers were pooled and used to initiate the following passage. Although the wild-type baculovirus was maintained over five passages, it did not go to fixation in most replicates of the serial passage experiment (SPE), and there was no good evidence for selection against the recombinant. Long-term maintenance of a recombinant in serial passage suggests an ecosystem safety risk. We conclude that for assessing ecological impact of recombinant viruses, SPEs in single and multiple larvae are relevant because of potential modulating effects at the between-host level.

1. Introduction

Baculoviruses have shown potential as agents for biological control of pest insect species (Moscardi, 1999). These viruses are highly virulent (Bianchi et al., 2000b; Cory and Myers, 2003) and have a restricted host range (Federici, 1997). A major drawback of baculoviruses in insect pest control is their slow speed of action (Moscardi, 1999). Crop damage after application of a baculovirus spray can therefore be substantial, even if mortality in target insects is eventually high (Bianchi et al., 2000a). With the advent of recombinant DNA techniques, it has become possible to engineer baculoviruses with faster speeds of action (Stewart et al., 1991; Inceoglu et al., 2006). These fast-acting baculoviruses can provide improved protection of crops in comparison with wild-type baculoviruses (Cory et al., 1994; Sun et al., 2004b). However, the ecological impact of the release of fast-acting recombinant baculoviruses is not fully understood and deserves further consideration to avoid unintended impacts on non-target organisms and other environmental ramifications.

Many studies have addressed different aspects of recombinant baculovirus fitness. The trend in these studies is that fitness of recombinant viruses is either not distinguishable from that of the parental wild-type virus (Bianchi et al., 2000b; Sun et al., 2004a) or is reduced (Cory et al., 1994, 2004; Sun et al., 2005; Zhou et al., 2005). Lower virus yield associated with shorter survival time of infected insects (Cory et al., 2004) suggests that recombinant baculoviruses may be less fit than wild-type viruses at the

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doi:10.1016/j.biocontrol.2009.10.002

between-host level. These patterns in speed of kill and virus yield do not necessarily extend to all susceptible species, however (Hernández-Crespo et al., 2001). In some instances insect behavior is also altered, which removes the virus-killed insect from plant surfaces (Hoover et al., 1995) and thereby reduces secondary transmission (Hails et al., 2002).

Two studies have addressed the within-host fitness of fast-acting recombinant baculoviruses derived from *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). Milks et al. (2001) found that a recombinant AcMNPV expressing the scorpion-derived, invertebrate-specific toxin AaIT had unaltered within-host fitness, when in direct competition with its parental wild-type virus. Zwart et al. (2009b) studied the behavior of vEGTDEL, an AcMNPV-derived recombinant lacking the endogenous *egt* gene, and found decreased within-host fitness compared to its parental wild-type virus. The *egt* gene encodes the ecdysteroid UDP-glucosyltransferase enzyme, which inactivates ecdysteroids and thereby modulates host development in a manner that results in a higher occlusion body (OB) yield (O'Reilly and Miller, 1989; Cory et al., 2004; Zwart et al., 2009b). In some hosts deletion of *egt* leads not only to a reduction in OB yield, but also to reduced cadaver weight and shorter time to host death (O'Reilly and Miller, 1989; Cory et al., 2004).

The available evidence therefore suggests that fast-acting baculoviruses lacking the *egt* gene may be ecologically impaired at both the within-host and between-host levels, and could be displaced by wild-type baculoviruses after release. Therefore, to hasten the competitive displacement of the fast-acting recombinant, a wild-type virus strain could be added to the recombinant virus inoculum. However, can an application of both recombinant and wild-type baculovirus retain the improved speed of action of the recombinant virus and, at the same time, result in the displacement of the recombinant virus in agro-ecosystems?

Other strategies for mitigating the persistence of recombinant baculoviruses have been previously suggested. For example, Hamblin et al. (1990) suggested the use of 'co-occluded' OBs containing both a wild-type virus and the AcMNPV recombinant Ac-E10 that lacks the *polyhedrin* gene and is therefore incapable of generating OBs autonomously. Wood et al. (1993) and Hughes and Wood (1996) suggest the use of only Ac-E10 virions (i.e., preoccluded virions) for biological control, because (i) they are highly infectious, ensuring effective biological control, and (ii) they remain infectious only for short periods of time, and therefore the virus will be quickly lost from the environment. The approach we study here – using a mixture of OBs – would be an attractive alternative because it is relatively simple, requiring only quantification of the different OBs. Moreover, because recombination between wild-type and recombinant viruses will occur in the field, understanding the competitive fitness of recombinant occluded baculoviruses (i.e., those retaining the *polyhedrin* gene) is also relevant to evaluating the approaches suggested by Hamblin et al. (1990) and Hughes and Wood (1996).

Zwart et al. (2009b) found that when *Trichoplusia ni* (Hübner) larvae were challenged with an LD_{80} (a dose that produces 80% mortality) of AcMNPV at a wild-type to recombinant ratio of 1:100, most larvae were solely infected by the recombinant virus. It is plausible that at this dose a simple sampling effect is responsible for the absence of the wild-type virus in the host; i.e., the founder number for the infection is well below 100, and only 1 in 100 founders is of the wild-type virus (see Zwart et al., 2009a). On the other hand, with the same dose but at a wild-type to recombinant ratio of 1:10 all larvae contained both the wild-type and recombinant viruses at death, indicating a founder number well above 10 virions per larva (Zwart et al., 2009b). Thus, at an LD_{80} dose and a wild-type to recombinant ratio of 1:10, the wild-type virus will be present in the majority of infected insects. This inoculum fulfills one of the requirements for a recombinant virus application strategy based on co-packaging of a wild-type virus in a biological control product, but we also need to know: (i) What is the speed of action at this dose and ratio? (ii) Will the wild-type virus go to fixation and displace the faster-acting recombinant virus?

We first determined time to death for different ratios of wild-type to recombinant viruses in bioassays at LD_{80}. As a wild-type virus, we used the parental virus of vEGTDEL, AcMNPV Wt L1 (Lee and Miller, 1978). An LD_{80} is suitable for initial evaluation of recombinant baculoviruses because medium to high OB doses will be required for effective biological control (Cory et al., 1994; Bianchi et al., 2002). A serial passage experiment (SPE) was performed starting with different ratios of the wild-type and recombinant viruses, to test whether the wild-type virus would competitively displace the recombinant completely and go to fixation. Alternatively, the recombinant might competitively displace the wild-type virus, or both viruses might be maintained in the population. Sustained maintenance of two genotypes over many generations would indicate that competition is neutral (no stronger competitor), or it could also indicate that both virus genotypes occupy somewhat different niches within the host, such that their co-occurrence represents a stable equilibrium (e.g., Gotelli, 2001). Note that by 'maintenance' we mean that a genotype remains present over time in the virus population, irrespective of its frequency. By 'fixation' we mean that only a single genotype is represented in the population, and other genotypes have been competitively displaced.

In previously reported SPEs with recombinant baculoviruses, each replicate was performed in a single larva (Milks et al., 2001; Zwart et al., 2009b). This experimental design focuses on the competitive process within a single host. In the field, however, a larva may ingest OBs originating from multiple larval cadavers. This is especially likely to happen when, during an epizootic, infectious larval cadavers occur at high densities. In our study we therefore chose to perform a SPE with an inoculum preparation from 10 larval cadavers. This experimental design focuses on the competitive process at the between-host level. The two situations – passaging in a single larva or passaging in 10 larvae – probably represent extremes compared to what may happen in the field. This approach complements previously reported studies, as the ensemble of previous results and those reported here bracket the relevant range of the number of OB sources for insect larvae.

2. Materials and methods

2.1. Insects and viruses

T. ni larvae were reared as previously reported (Zwart et al., 2009b; see also Smits et al., 1986). Larvae were reared communally on artificial diet based on wheat germ, in plastic boxes with a paper towel as a lid. Diet composition was identical to that described by Smits et al. (1986) except that cornmeal was replaced with wheat germ. Larvae were allowed to spin cocoons and pupate on the paper towel lids. Moths were kept in cages with paper towels placed along the sides for egg-laying. Paper towels with eggs were collected daily and surface-sterilized as described by Smits et al. (1986). Eggs were occasionally kept at 4° or 16 °C, for 1 or 2 days, in order to keep the insects in a regular rearing scheme. Larvae, pupae and moths were kept with a 16-h photoperiod and at 27 °C.

AcMNPV Wt L1 and vEGTDEL (O'Reilly and Miller, 1991) were amplified in 30 fourth-instar *T. ni* larvae (L4) inoculated at a high dose (approx. $100 \times LD_{99}$). Larval cadavers were collected upon death, stored at −20 °C, and OBs were subsequently purified as described by Zwart et al. (2008). Briefly, larval cadavers were