Report

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CEH Integrating Project

Title of research project

Interactions between viruses and Lepidopteran larvae in different stress states

Ref: C01547

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CEH Monks Wood:	Dr Daniel Osborn (Co-Project Leader) Mrs Tina Yates (Insect behaviour/pesticide interactions)

Duration of Project: 2000 - 2003

SUMMARY

The aims of this project are to determine the responses of viruses and their lepidopteran hosts to a variety of stresses, including sub-lethal doses of chemical insecticides. The host - pathogen system being used is *Pieris brassicae* (Large white butterfly) and its homologous baculovirus, *P. brassicae* granulovirus (PbGV). To date we have established breeding populations of the insect, thus providing material for use the year round. These insects are routinely maintained on cabbages, but the use of synthetic diet is also being investigated. Preliminary work to monitor tracking of insect larvae with a video/computer-based system was successful, but when virus-infected insects were ready to be tracked, equipment failure thwarted the experiment. Analysis of feeding by insect larvae has shown that sub lethal doses of chemicals inhibit feeding rates significantly. The molecular analysis of virus infection has resulted in the development of a new method for analysing whole virus genomes via their isolation utilising low copy number Bac replicon plasmids in bacteria. We are in a position to be able to dissect a virus population from insects to determine if genetic variation is enhanced by exposure to a chemical stress, e.g. sub-lethal doses of insecticides. Analysis of supposedly uninfected *P. brassicae* populations has also shown the presence of virus at a very low level. We are currently investigating the idea that these insects may harbour a latent baculovirus, which only occasionally manifests itself as an overt infection. This is an unexpected result and could provide additional bonus data to the project. Finally, we have developed further collaborations with scientists at the University of Reading and Pamplona, Spain, which promise to enhance the progress in the project.

ORIGINAL OBJECTIVES

To compare the genetic responses of viruses infecting lepidoptera under a range of larval stresses induced by pesticides, modified secondary plant compound regimes etc

To determine whether, when infected by a virus, larval responses to other stresses are modified

To determine whether individual or multiple stresses are worse for lepidoptera in terms of reproductive success and whether different phenotypes and genotypes survive under different stress regimes.

BACKGROUND

The success of natural insect populations in the environment depends on how they respond and adapt to changing conditions. Individuals in populations are subject to different stressors, some of which determine whether an individual breeds or not. In the previous century, studying such problems was not thought worthwhile by some ecologists. There were a variety of theoretical or conceptual reasons for this. One was that it was felt that provided the population abundance and distribution were maintained then all would be well. This reason is now less accepted, partly because it is recognised that even though a population may be maintained, genetic diversity within it may be so reduced or altered as to raise questions as to whether the population is genetically the same after encountering the stressor as it was before. In the extreme case, it might be found that the species has evolved to counter the stressor (eg all individuals become resistant to a pesticide). Although this in itself might not matter, there could, however, be costs in making the changes that might disadvantage individuals in circumstances where the stressor is removed, or that might reduce fecundity or survival leading to a decline in population numbers or increased population fragility.

Individual organisms do not exist in isolation but in related groupings (eg plant-herbivore, predator-prey, parasite-host). To understand better the ecological role of stressors (eg pesticides, naturally occurring feed stimulants or anti-feedants, starvation, infection with a parasite) studies of impacts on interactions between species are now required. Studies like this have not been done very much in the past, although concerns about indirect effects of pesticides have pointed out how important such effects might be. To be practicable at present, such studies need to use organisms with rapid life cycles, and one such pairing is that between a virus and its lepidopteran host. We propose to determine at the molecular, chemical, individual and population level how a virus responds to differing stress states in its larval host. At the same time much more information will be gathered on how lepidoptera larvae respond to stress.

Baculoviruses are confined to arthropod hosts, with most examples having been isolated from lepidopteran species. They have large (90 - 160 kilobase pairs) circular DNA genomes, which are expressed in a temporal cascade in insect cells. The virus life cycle begins with ingestion of proteinaceous occlusion bodies. These dissolve in the midgut of the insect larva, releasing infectious virus particles, which invade cells lining the gut and initiate virus replication. Rod-shaped virus particles then bud from infected cells, spreading infection throughout the host, which eventually dies at about 10 days post infection. The virus-infected host often liquefies after death to release occlusion bodies, formed in cells throughout the 10-day period. Baculoviruses are classified as either nucleopolyhedroviruses (NPVs) or granuloviruses (GVs) and named after the insect host from which they were isolated. In this study we are working mainly with the *Pieris brassicae* GV, isolated from the Large white butterfly.

An important discovery in recent years was that baculoviruses are genetically homogeneous. This is apparent when some virus isolates are examined using restriction enzyme digestion of genomic DNA. When the products are fractionated in an agarose gel, submolar bands are apparent, in addition to the major fragments. The role of this variation is unclear. It may represent a mechanism for adaptation to particular hosts or environmental conditions experienced by those insects. This idea is supported by laboratory studies with a number of baculoviruses, which show that different genotypes may be selected in alternative insect species.

Similar genetic variation has been observed in GVs isolated from *Pieris* butterflies, which use various species of the plant family Cruciferae as food plants for their larvae. Crooke (1986) showed thirteen isolates of GVs from *P. rapae* and *P. brassicae* could be placed in three subtypes according to similarities and differences in DNA profiles generated after restriction enzyme digestion. Within isolate variation was also evident by the appearance of sub-molar DNA bands in agarose gel analysis of restriction enzyme-digested DNA.

Studies of the impacts of organophosphorous chemicals (dimethoate, fenitrothion) on *Pieris* species over the past 2 years have identified a number of effects ranging from the molecular and sub-cellular to the behavioural. The aim of this work is to proceed to determine the ecological significance of these changes by growing larvae on after sub-lethal dosing with the chemicals. This work (done in association with Prof Nicholson's group at Imperial College) has established a range of general methods that can be used to look at a wide variety of stressors.

It is proposed to transfer these methods to examine the ways pesticides, naturally occurring toxic substances, food availability and palatability, and virus infection affects the "fitness" of individual larvae. A further objective is to see whether when lepidoptera under different stress states are infected with a virus this results in the selection of a particular subset of genotypes within the virus population. If this proves to be the case, it will suggest that a particular profile of virus gene expression is better suited to replication in a given set of environmental conditions.

RESULTS

Establishing breeding populations of Pieris brassicae

To provide a regular supply of larvae for experimentation, a continuous cycle of breeding *P.brassicae* needed to be established. This species is normally bi-voltine in the UK, but only two broods a year under natural day length and temperature conditions would severely limit the number and timing of experiments. A light/ temperature regime was quickly established that gave continuous broods of the butterfly, with a subsequent regular supply of experimental larvae. However, initially cabbages for the larval food had to be purchased. Although care was taken only to buy organic cabbages, which should have been pesticide free, one batch of cabbages virtually wiped out the colony. Subsequently, any cabbages that were bought in were sourced directly from an organic grower, where it was possible to check that not even permitted pesticides such as *Baccilus thuringensis* had been used. A regular succession of cabbages are now also being grown in Monks Wood greenhouses, and although one succession was virtually wiped out by an invasion of *P. rapi*, this is proving to be the best method of feeding the stock larvae.

In terms of continuous breeding of the large white, a temperature of 20 ^{o}C, with lighting on an 18 hours on/ 6 hours off regime, produces a complete life cycle of 42-45 days. Nine complete generations of the butterfly were successfully reared under this regime. Unfortunately the population went through two genetic bottlenecks in that time, partly due to the problems with cabbage supplies describe above, and the final generation produced males that did not respond to the females. Inbreeding is the logical explanation, but more stock has fortunately been obtained from the same source.

A more efficient way of rearing the larvae would be to use artificial diet, and this has been used at CEH Oxford for the larvae dosed with virus. Experiments with this artificial diet showed that it did not sustain the larvae sufficiently, with individuals growing more slowly and having a higher mortality than those reared on cabbage. The formulation was changed, after collaboration with staff from the Dept. of Horticulture and Landscape at Reading University, and work continues in assessing the performance of artificial diet. The new formulation sustains the larvae through normal growth to the pupal stage, but subsequent emergence from the pupae was poor, and the adults that did emerge failed to breed. Collaboration with the University of Reading continues, and biochemical analysis of larvae fed on different diets is planned, alongside that of larvae subject to sub-lethal doses of pesticide.

Quantifying feeding and movement

The original project plan proposed to measure the effects on larval movement of exposure to virus and / or pesticides. The intention was to measure this by video tracking individual larvae in an arena. The protocol for video tracking larvae was established after some experimentation.

The video tracking system has to be “trained” when larvae are first introduced to the camera, so that pixels that represent larvae can be differentiated from those caused by the arena walls or light reflectance. Background shadows can be minimised by strong overhead lighting, but as the larvae have to be confined by a clear surface to the arenas,

the trade-off is increased reflectance, which can also confuse the imaging system. The larger the larvae, the more clear the contrast between the animal and its background. However, smaller larvae are more susceptible to virus and pesticide dosing, so training the system to recognise larvae at the smallest possible size was needed. Consistent results were obtained with larvae as small as 0.03g, which was ideal for both virus infection and pesticide dosing. Unfortunately, we were unable to perform tracking with the first batch of experimental larvae infected with PbGV. Just prior to the tracking experiments the system underwent electrical safety testing as part of the annual CEH Monks Wood monitoring programme, and was subsequently discovered to be unusable. Trials are now underway to see if it is possible to collect data on larval movement by other methods, namely, timing the movement of experimental and control animals as they search for food.

Despite these difficulties in monitoring insect movement, progress has been made in elucidating the effect of stressors on insect feeding rates. Data originally collected by a student as a preliminary to this project has been analysed to compare feeding rates after exposure to insecticides. The results show that sub-lethal dosing with dimethoate inhibits feeding rates to a statistically significant degree. (This work in the process of being written up, and has also been submitted as the subject of an oral paper at the BES Winter Meeting in Birmingham, December 2001).

When the feeding data are subject to Dunett simultaneous tests, to compare the rate of feeding of dosed larvae, with that of the controls, there is a statistical significance in the results above a dose level of 0.077 µg/insect (see Table 1). For reference, scaling up from the work of Sinha *et al*. (1990) the expected LD_{50} for larvae of the size used would be around 250 µg/insect.

Table 1. Results of Dunett simultaneous tests.

Dose (µg/insect)	Difference of means	SE of Difference	T-value	Adjusted P-value
0.039	-0.161	0.2264	-0.711	0.9258
0.077	-0.815	0.2264	-3.598	0.0063
0.154	-1.423	0.2264	-6.287	0.0000
0.308	-2.021	0.2264	-8.927	0.0000
0.616	-2.056	0.2264	-9.080	0.0000

Molecular analysis of virus infection

Before embarking on experiments to determine how viruses might adapt to altered conditions within an insect hosts, for example, sub-lethal doses of chemical insecticides, we developed a method to examine genetic variation in virus populations. In earlier work, described by other authors (Smith and Crook, 1988; 1993) limit dilution cloning (LDC) techniques were used to isolate variants from baculovirus populations. Although very successful, the method is time consuming and labour intensive. Essentially, wild type baculoviruses are diluted in water, fed to insect larvae and the virus progeny from

individuals examined to determine if it differs genetically from the original inoculum. Multiple rounds of LDC are usually required to ensure that the virus stock is genetically homogeneous. The method was also unsuitable for our purposes for another reason. We plan to propagate baculoviruses in insects subjected to various stressors, e.g. sub-lethal doses of insecticides. To examine the effect on virus genotype selection, we need to isolate those viruses that have been selected in preference to others. The problem with using LDC is that the insects used for the method will not be subjected to the chemical stressor. Even if they were, we would be selecting the baculoviruses in an environment where continued stress was present. The virus progeny eventually selected would not reflect those present in the experimental cohort. Clearly, an improved method for isolating baculovirus genotypes is required.

We have developed the technique of whole genome cloning (WGC) to isolate intact baculovirus genomes from an experimental population. The method permits us to take a snapshot of the virus population derived from individual insects or pools of infected individuals. It is based on the use of Bac replicons, which can be propagated in *Escherichia coli*. These plasmids replicate in bacterial cells but only at very low copy number (<10 per cell). In consequence, they will accept and stably maintain very large DNA inserts. When they were used to clone human chromosomal DNA, up to 300 Kbp was faithfully amplified in the host. Since the PbGV genome is about 110 kbp, the insertion of these DNA molecules into the vectors was not anticipated to present any problems.

Prior to inserting PbGV genomes into the Bac replicons, we conducted a preliminary study to identify restriction endonucleases that only cleaved the virus DNA once. Two were identified: *Avr*II and *San*DI. A Bac replicon, pBace3.6, was then modified by inserting cloning sites for these two enzymes to create pBace3.6-*Avr*II and pBace3.6-*San*DI. These modified vectors were then digested with the appropriate enzyme (*Avr*II or SanDI) before ligating with linear PbGV DNA digested with either enzyme. The ligation mixture comprising pBace3.6-*Avr*II and PbGV digested with *San*DI was used to transform *E. coli* DH10B electrocompetent cells, which were then spread onto agar plates containing chloramphenicol as a selectable marker. After incubation at 37°C for 24 hours, many colonies were identified on the plates. 120 were isolated, amplified in liquid medium containing chloramphenicol and processed to yield plasmid DNA. This was digested with *Hin*dIII and individual clones analysed using a 1% agarose gel, before staining with ethidium bromide (data not shown). These results demonstrated that intact virus genomes had been inserted into the original plasmid. However, since DNA yields from the bacteria were low, consistent with a low copy number replicon, we used a more sensitive technique for analysing the cloned DNA. pBacPbGV genomes were digested with Sal1 and SanDI and radiolabelled with ^{32}P, prior to separation by gel electrophoresis. The gel was then dried and exposed to X-ray form to detect the DNA bands. Figure 1 shows 5 representative clones, which exhibit evidence for genetic variability. The DNA profiles for the virus genomes are all very similar, particularly the PbGV wild stock, used in the original cloning experiment, and ArGV1 obtained from Professor Chris Payne (Reading). The 5 Bac clones have an additional band, indicated by arrow 4 in Fig. 1, of about 2kbp, which represents part of the pBace3.6-SanDI vector. The remainder of this vector is about 6.2 kbp and migrates just below a virus specific band of 6.5 kbp (arrow 2, Fig. 1). Arrow 1 (Fig. 1) indicates where a large DNA band is absent from clone E. This is thought to be an artefact of the DNA extraction procedure where material has been sheared by physical forces. Clone C has

many other sub molar bans, which be a consequence of complete or partial digestion. Band 3 (Fig. 1) indicates a region of the PbGV genome where size variation is apparent.

We then returned to our original PbGV isolate to determine if we could identify evidence for genetic variation in this virus population. Virus DNA was digested with a range of restriction enzymes, radiolabelled and fractionated using a 1% agarose gel (Figure 2). Genetic variation should be evident by the presence of sub molar DNA bands between the ones representing the majority profile of the virus genome. None were present in a range of restriction enzyme digest products.

Latent baculovirus infections in P. brassicae populations

Some intriguing results were obtained, which are relevant to the possibility that *P. brassicae* harbour latent baculovirus infections. In the routine exchange of material between Monks Wood and Oxford, the *P. brassicae* eggs are occasionally surface sterilised with formalin to reduce the risk of virus or bacterial infection of the neonates. On one occasion, however, after sterilisation of the eggs and emergence of the larvae, the neonates died within a few days of what appeared to a baculovirus infection. The insects liquefied after death. Since the virus deaths occurred in small, 1st instar larvae, the individuals were pooled and processed for virus purification using our normal method. DNA was extracted from the purified virus and analysed using PCR, with primers specific for the granulin gene region (Figure 3). Lane 4 (Fig. 3) shows a band of about 420 bp, which comigrates with a positive control from PbGV DNA (lane 11; Fig. 4). This indicates that the deaths in the insect population could be due to infection with PbGV or a related virus.

We also obtained some dead *P. brassicae* larvae from Prof. Chris Payne, of the Dept. of Horticulture and Landscape at Reading University. These had been maintained on artificial diet and never been deliberately exposed to baculoviruses. Virus DNA was purified from two cadavers, amplified in a PCR with granulin-specific primers and the products analysed using an agarose gel. A faint band is evident for both infected insects, which comigrates with the positive control. These samples will be subjected to further detailed analysis to identify the nature of the virus.

DISCUSSION

The work to establish breeding population of *P. brassicae* has gone well. In our original proposal, we intended to maintain other *Pieris* species, but constraints to the budget for the project as awarded to us do not render this feasible, so we are concentrating on one species for most of our work. The *P. brassicae* appear to be most readily maintained in continuous culture using cabbages, providing that these are free of pesticides. Artificial diet offers an alternative, but some problems have been encountered in completing the butterfly life cycle. For short-term maintenance of insect larvae for experimental purposes and virus dosing, artificial diet was very useful. It remains to be seen whether the switch from a natural to a semi-synthetic diet will significantly affect results.

Our attempts to monitor the movement of insect larvae after dosing with virus and/or chemicals were frustrated by equipment failure. It is not instructive to dwell on these technical problems here, suffice to say that this is a serious hold up to the project, which everyone is working hard to resolve. We have been able to utilise some earlier data on feeding responses to pesticides to show that dimethoate, given in sub-lethal doses to insects, inhibits feeding rates. It will be interesting to combine both chemical and virus treatments and monitor the effect(s) on feeding and movement.

The delay in developing the tracking studies on *P. brassicae* infected with the GV has had an unexpected bonus. At CEH Oxford, we have been able to devote more time to the molecular analysis of virus infection. This has resulted in the development of a method for cloning intact baculovirus genomes, via the use of low copy number Bac plasmid replicons from bacteria. Preliminary analysis of 120 virus clones has revealed at least 5 with significant genetic variation. This low number of clones with genome variation was surprising, since earlier work had shown that PbGV contained a number of genotypes (Smith and Crook, 1988; 1993). We are, however, working with a different virus stock, albeit of the same species, which may account for the different results. The WGC method allows us for the first time to examine the individual genomes in virus populations, without the need to separate viruses by time consuming limit dilution with insect larvae. The application of the method is in its early stages, but we hope to use it to examine virus populations that have amplified in the presence of sub-lethal doses of pesticides and other stressors.

The method also has the potential to recover viable virus after transfection (via injection) of insects with Bac clones of PbGV DNA. At present, we can only examine the genotype of the variants, but if we can succeed in establishing a normal virus infection it will be possible to compare the phenotype of the clone with the original PbGV isolate. Experiments with other baculoviruses have shown that is possible to inject their DNA genomes into the haemocoel of susceptible larvae, in the presence of a liposome reagent, and obtain productive infection of the host. Mature, occluded virus can then be recovered for further analysis.

In a another spin-off from the project, we have established a collaboration with Dr Primitivo Caballero (Pamplona, Spain), who is very interested in applying the WGC method to the analysis of *Spodoptera exigua* NPV, which is used as a biocontrol agent of *S. exigua* in Europe. He is going to supply suitable virus DNA to us for the generation of genome libraries, which will then be characterised by his staff in Spain.

We are currently seeking funding to enable exchange of personnel between laboratories. Another collaboration established is with Professor Chris Payne and Dr Melanie Hunt at Reading University, who have been very helpful in establishing *P. brassicae* on artificial diet and supplying virus isolates. This collaboration has also been useful in our studies on latent baculoviruses, since dead insects supplied by the Reading group have proven to contain virus DNA sequences. Although it is too early to be too confident in our results, these data coupled with our own experiences with larvae supplied by CEH Monks Wood are suggestive of the fact that *P. brassicae* harbour latent or persistent virus infections. If we can conform that this host has a latent baculovirus infection, it will support results published some years ago by our laboratory that lepidopteran species can maintain these viruses as sub-lethal infections (Hughes *et al.*, 1993).

ACKNOWLEDGEMENTS

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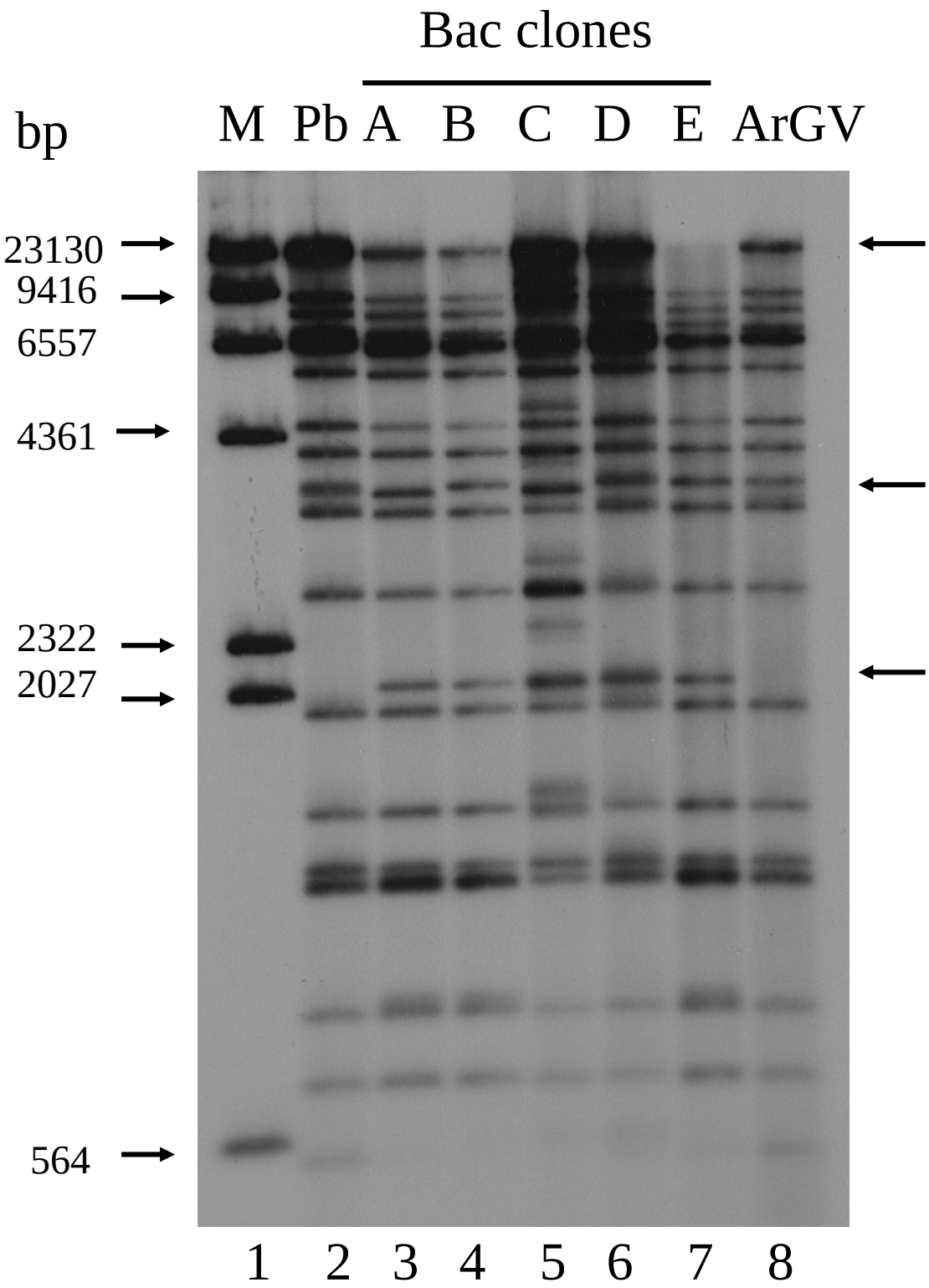

Figure 1. Restriction enzyme profiles of DNA recovered from PbGV Bac clones propagated in bacteria as low copy number plasmids. DNA was digested with *Sal*I and *San*DI, end-radiolabelled with ^{32}P and fractionated in a 1% agarose gel. The gel was dried, then exposed to X-ray film for 8 hours. Pb, original wild type virus used for cloning of virus genomes; A-E, Bac clones isolated from bacteria. ArGV, an *Artogeia (Pieris) rapae* GV provided by Prof. Chris Payne, Reading University. The arrows on the right side of the gel indicate region s of interest, which are described in the text. M, lambda bacteriophage DNA digested with *Hin*dIII and radiolabelled to provide molecular size standards.

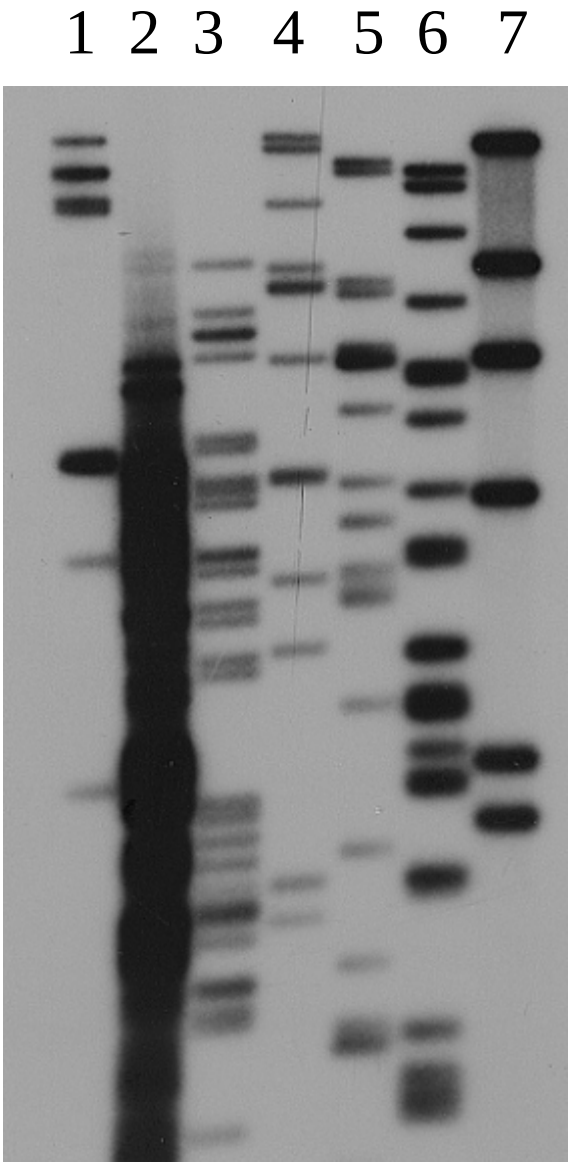

Figure 2. Restriction enzyme profiles of PbGV DNA from the wild type virus. Virus DNA was digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), *Cla*I (lane 3), *Asp*718 (lane 4), *Sal*I (lane 5) or *Mlu*I (lane 6), radiolabelled and fractionated in a 1% agarose gel prior to autoradiography. Lane 6, lambda DNA digested with *Hin*dIII.

1 2 3 4 5 6 7 8 9 10 1112 13

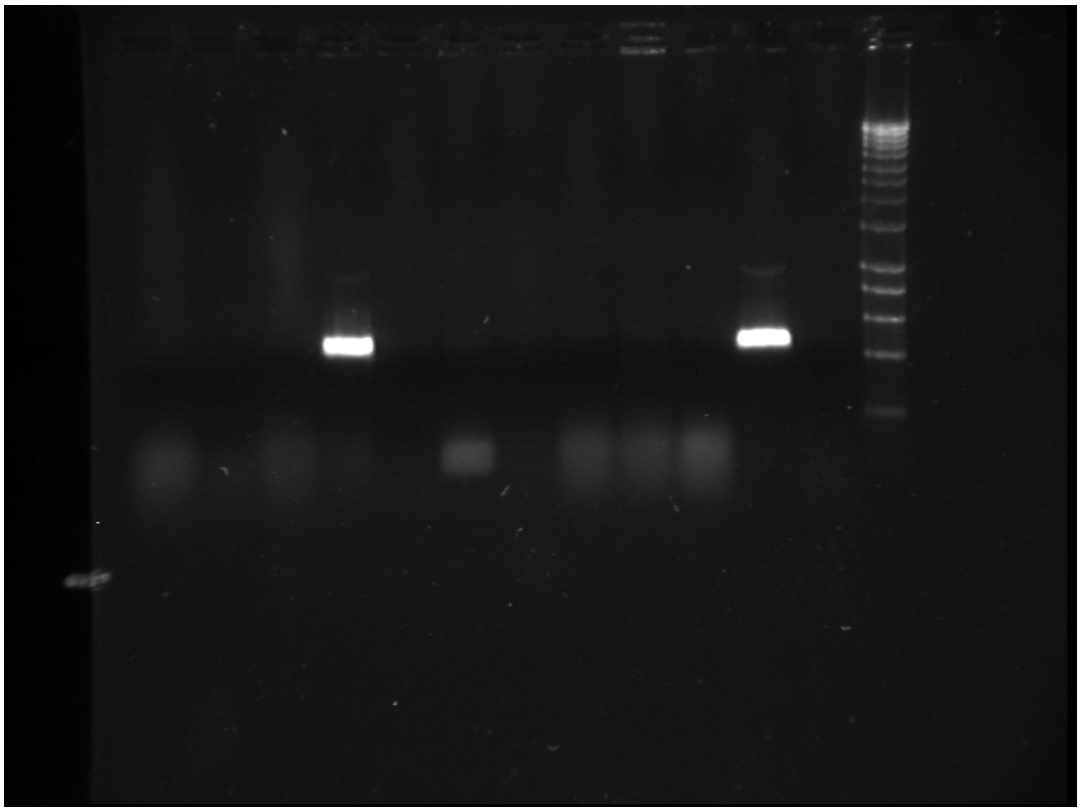

Figure 3. Polymerase chain reaction (PCR) analysis of virus DNA extracted from neonate *P. brassicae* larvae. Primers specific to each end of the PbGV granulin gene were used to amplify virus DNA sequences in material purified from insect larvae that had emerged from eggs treated with formalin and subsequently died with GV symptoms (lane 4). A positive control utilising PbGV DNA in the PCR is shown in lane 11, with a negative control shown in lane 12. The remaining lanes contained samples from another experiment. Lane contains molecular size markers.

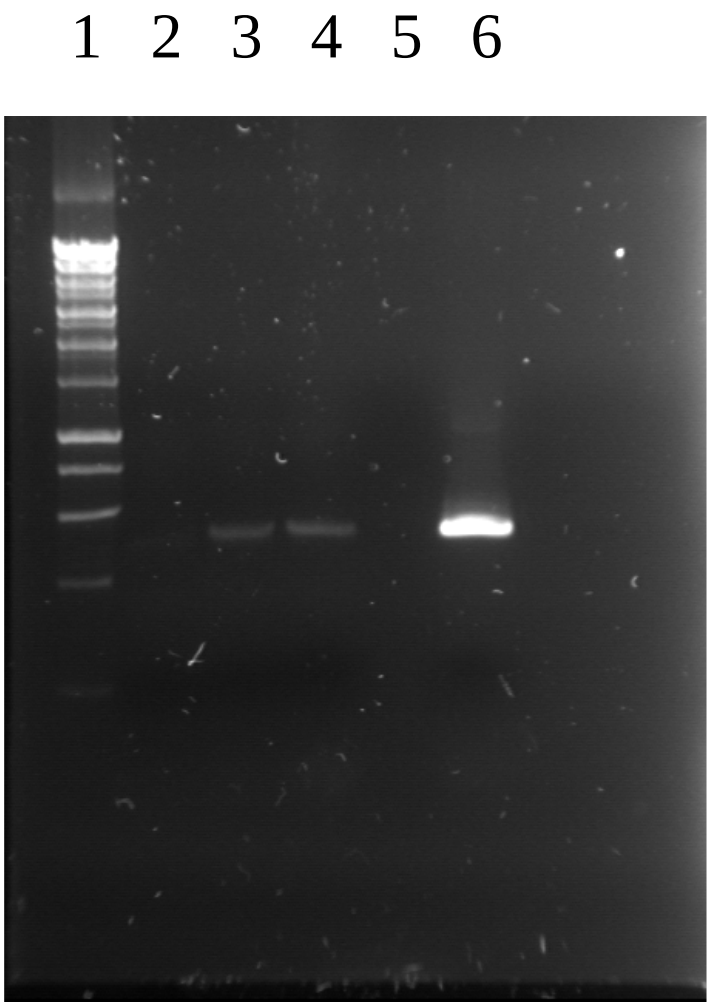

Figure 4. PCR analysis of virus DNA extracted from *P. brassicae* larval cadavers. Lane 2, negative control; lanes 3 and 4, DNA from larvae that might have died from virus infection; lane 5, blank' lane 6, positive PbGV control.