Safety of *Serratia entomophila* (Enterobacteriaceae), active organism used in the grass grub biocontrol products Invade™ and Bioshield™

Trevor A. Jackson
AgResearch, PO Box 60, Lincoln, New Zealand. trevor.jackson@agresearch.co.nz

*Serratia entomophila* is a native soil bacterium found in New Zealand. Strains bearing a specific plasmid (pADAP) are able to cause amber disease in the New Zealand grass grub *Costelytra zealandica*. The bacterium has been developed as a biopesticide and has been used for 15 years as a commercial microbial control for grass grub. The bacterium was firstly applied as a liquid product Invade but has recently been developed for application as a solid granule Bioshield. The bacterium has been registered for sale in New Zealand after carrying out required safety testing. Tier 1 testing (high dose challenge) was carried out against small mammals and no detrimental effects were noted following application by oral, intraperitoneal, dermal and ocular routes. Environmental safety was assessed by direct challenge of closely related scarabs and beneficial organisms. Sheep and chickens were tested as representative vertebrates likely to be exposed to the bacterium. No infectivity was demonstrated for any non-target animal tested, indicating high specificity of *S. entomophila* to grass grub. The bacterium has been used in New Zealand for 15 years with no indications of safety problems or unexpected environmental effects from widespread application. Despite widespread testing, no other insect species have been shown to be susceptible to the plasmid bearing strains of *S. entomophila*.

**Toxicity**

Once ingested by the grass grub larva, *S. entomophila* causes a rapid cessation of feeding and gut clearance associated with colonisation by the bacteria. This leads to a prolonged period of chronic infection followed by invasion of the haemocoel and death of the insect by sepsis. Gut clearance is associated with a reduction in gut protease titre. The disease state is permanent once triggered by bacterial ingestion.


**Metabolites**
Grass grub pathogenic strains of *Serratia* produce Sep proteins which are related to the Tc toxins produced by *Photorhabdus* and other bacteria. Sep proteins must be induced, with induction occurring within the grass grub gut. While Tc toxins have shown cytotoxic effects direct toxicity of the Sep proteins is unknown.


**Ecotoxicity**
Plasmid bearing strains of *S. entomophila* occur naturally in New Zealand pastures and are most commonly associated with populations of *C. zealandica* which have been resident in a pasture for some years. Bacterial populations are disrupted by cultivation which leads typically to outbreaks of healthy grass grub in pastures two to three years from sowing. Plasmid bearing strains will become established in high density grass grub populations leading to a disease build up and levels of bacteria of up to $10^6$ bacteria per g of soil. As the population collapses from disease there is little new contribution of plasmid bearing strains to the soil and numbers of pathogens decline to low levels. Commercial application typically takes place in young pastures or where the disease cycle has been disrupted by drought. The objective of application is to mimic the natural process but to provide inoculum and initiate disease in an earlier part of the population cycle before damage has occurred. Levels of applied bacteria never reach higher numbers than occur in natural population events but the timing of the interaction is altered to prevent damage.

As the activity of plasmid bearing *S. entomophila* is mono-specific, it would be surprising if there were non-target effects. Other insects seem unaffected by *S. entomophila* challenge and a wide range of species have been tested. No significant effects on non-target organism have been identified following commercial applications of the bacteria. In total more than 25,000 ha of pasture have been treated with *S. entomophila* with no indications of hazardous effect.


**Alternatives**
*S. entomophila* is the most widely used material for grass grub control in New Zealand aligning with the national priority for “clean-green” agriculture. There is minor use of organophosphates (Diazinon, Chlorpyriphos) and neonicotinoids (imidacloprid) for seed treatment.
**Efficacy**

*S. entomophila* application establishes disease within a grass grub population usually leading to 20% infection within two months of application. Recycling of disease within the population will lead to a 50% reduction in the population in the year of application but most benefit is obtained through continued recycling of bacteria in subsequent generations providing long–term pest suppression.

ENVIRONMENTAL SAFETY OF INUNDATIVE APPLICATION OF A NATURALLY OCCURRING BIOCONTROL AGENT, *Serratia entomophila*

1. ORIGIN AND DEVELOPMENT OF *SERRATIA ENTOMOPHILA* AS A BIOPESTICIDE

The grass grub, *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae), has been one of New Zealand’s most troublesome grassland pests. It is one of a small number of endemic insects that have been able to flourish in the modified grassland habitats of New Zealand developed by the European settlers. Grass grub has been recorded as a pest since the origins of pasture development for grazing animals. The insect is widespread throughout the country and has been well known as a chronic problem, reducing persistence and yield of sown pasture, and sometimes occurring in outbreaks that cause total pasture loss.

There was a brief respite from grass grub in the 1950s with the introduction of DDT which was widely used for pasture pest control. By the late 1960s, however, DDT was losing its effect as grass grub developed resistance. The chemical was finally withdrawn from use in 1968 for environmental reasons, in particular its accumulation in animal fat. Thus in the 1970s, grass grubs were back with a vengeance and research was concentrated on this pest. While occurrence of the pests was predictable in young pastures, populations under long term monitoring would frequently decline to low levels and the causal factor became the target of investigations.

In 1982, unusual looking larvae were discovered; they were non-feeding and had clear alimentary tracts resulting in an amber colouration. This condition was eventually attributed to a new disease, later designated amber disease of the grass grub. The disease was bacterial in origin and found to be caused by strains of the naturally occurring soil bacteria *Serratia entomophila* Grimont et al. (Grimont et al. 1988) and *Serratia proteamaculans* (Paine and Stansfield). Both bacteria occur in pathogenic and non-pathogenic forms and strains of *S. entomophila* have been selected and developed as the commercial biocontrol product, Invade®, for grass grub (Jackson et al. 1992). Pathogenicity is encoded by genes carried on a 140kb plasmid (pADAP) which is carried by pathogenic strains (Glare et al. 1993).

Disease causing bacteria must be ingested by larval grass grub and will colonise the gut of the host insect and cause cessation of feeding within 24-48 hours from ingestion. The infected insect will then characteristically clear the midgut of food and digestive enzymes, which produces the resultant amber colouration. A long, chronic disease period follows, eventually resulting in death of the infected host (Jackson et al. 1993a, Jackson et al. 2001).

Amber disease is often found in the field and is commonly associated with “old” grass grub populations, those that have been in a particular pasture for more than 5 years. In young pastures, less than 3 years from sowing, the disease is rare. The disease appears to build up and affect the grass grub population in a delayed density-dependent manner causing population crashes after high populations of the host insect have become established. Indeed, the level of disease in a population can be used as an indicator of incipient population collapse (Jackson 1984, Jackson et al. 1999). *S. entomophila* can be used effectively as a biopesticide by introducing the bacterium into healthy populations, thereby promoting early epizootics of disease and preventing pasture damage. In order to use the bacterium as a biopesticide, a production method was developed which resulted in a high concentrate fermentation broth which was effective when applied at the rate of 1 litre (4 x 10^{13} viable bacteria)/ha. The manufactured strains of *S. entomophila* were safety-tested and registered as New Zealand’s first indigenous insect microbial control agent (Invade®) and the first in the world to be based on a member
of the Enterobacteriaceae (Jackson et al. 1992). Since 1992, approximately 15,000 ha of pasture have been treated with Invade®.

2. CHARACTERISTICS OF S. ENTOMOPHILA

As outlined by Glare and O’Callaghan (Chapter 7) the potential for non-target impacts will depend on the specificity, mode of action, persistence and transmission of the bacterium. These factors are examined in detail below.

2.1. Specificity
Specificity of an organism can be determined by host range studies. In determining the host range, most attention is logically applied to closely related species but tests should also be made against other species representative of the local fauna and especially beneficial species. The stage of insect development will also be important. To test for the possible effects of amber disease causing Serratia spp. we have used maximum challenge tests in which high doses (10⁷ to 10⁸ cells of bacteria) were applied to the food of the target species. As the bacterium appears to act through the gut, it is important to ensure that the food is ingested in order to be able to evaluate the result. Laboratory tests have been carried out against a wide range of feeding scarab larvae and other insects (Table 1). There are no cases where the bacterium has caused cessation of feeding and disease as occurs in C. zealandica. To date, we have been unable to find another insect species that is susceptible to the disease.

2.2. Mode of action
The specificity of pADAP bearing strains of Serratia is unusual. An acute oral dose of as few as 3 x 10⁴ cells constitutes the IC₅₀ (concentration necessary for 50% infection) (Jackson et al. 2001) with most of these cells floating freely in the gut lumen (Hurst and Jackson 2002). Ingestion of these cells triggers a reaction starting with cessation of feeding and leading to gut clearance and the switching off of digestive enzyme production in the midgut epithelial cells (Jackson 1995). Pathogenicity is determined by a plasmid-borne set of genes encoding proteins similar to those in the tc family of toxins produced by Photorhabdus and Xenorhabdus bacteria (Hurst et al. 2000). However the mode of action of pADAP containing Serratia spp. is quite distinct (Jackson et al. 2002). No cellular damage has been observed in the midgut epithelial cells in spite of ingestion of high concentrations of bacteria. Through the chronic stages of disease, bacteria are confined to the insect gut where they multiply and are only released into the soil on the death of the insect host. The chronic phase of disease, (non-feeding and with a clear gut), cannot be reversed by elimination of bacteria through the administration

<table>
<thead>
<tr>
<th>Order; Family; Subfamily</th>
<th>Species</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptera; Scarabaeidae; Melolonthinae</td>
<td>Costelytra zealandica (White)*</td>
<td>✓</td>
</tr>
<tr>
<td>C.; S.; Melolonthinae</td>
<td>Odontria striata Broun*</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Melolonthinae</td>
<td>Odontria smithii Broun*</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Melolonthinae</td>
<td>Pyronota festiva (F.)*</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Melolonthinae</td>
<td>Stethaspis sp.*</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Melolonthinae</td>
<td>Melolontha melolontha (L.)</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Dynastinae</td>
<td>Pericoptus truncatus (F.)*</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Dynastinae</td>
<td>Adoryphorus couloni, (Burn.)</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Dynastinae</td>
<td>Heteronychus arator (F.)</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Rutelinae</td>
<td>Popillia japonica Newman</td>
<td>X</td>
</tr>
<tr>
<td>C.; S.; Aphodidae</td>
<td>Acrossidius tasmaniae (Hope)</td>
<td>X</td>
</tr>
<tr>
<td>C.; Lucidae</td>
<td>Paralissoetes sp.*</td>
<td>X</td>
</tr>
<tr>
<td>Lepidoptera;Pyralidae</td>
<td>Galleria mellonella (L.)</td>
<td>X</td>
</tr>
<tr>
<td>L.;Tortricidae</td>
<td>Epiphias postvittana (Walker)</td>
<td>X</td>
</tr>
<tr>
<td>L.; Hepialidae</td>
<td>Wiseana cervinata (Walker)</td>
<td>X</td>
</tr>
<tr>
<td>Hymenoptera; Apidae</td>
<td>Apis mellifera L.</td>
<td>X</td>
</tr>
<tr>
<td>Haplotaxida; Lumbricidae</td>
<td>Aporrectodea calliginosa (Savigny)</td>
<td>X</td>
</tr>
</tbody>
</table>

* New Zealand endemic species

Table 1. Invertebrate species tested for susceptibility to pADAP bearing strains of S. entomophila. ✓, Susceptible; X, No effect.
of antibiotics (Jackson et al. 2001). Thus the mode of action of pADAP bearing strains appears to be unique but the exact mechanisms have yet to be elucidated.

2.3. Persistence
Persistence can be a negative property of pest control agents especially if there are unwanted side effects, as has been seen with the residues of organochlorine pesticides. Persistence, however, is an essential factor in the success of biocontrol of soil insects (Jackson 1999). After application to the soil, pathogenic *Serratia* cells stabilise at a level of about $10^5$ cells/g in the presence of grass grub. The level is maintained by multiplication in infected insects and subsequent release into the soil on death of the host. If the host grass grub population declines to low levels, the applied pathogenic strain will die out.

2.4. Transmission
Potential negative environmental effects of microbes can result from transmission of the pathogen to new, unintended hosts. Applied *Serratia* strains can be transmitted to new sites by soil movement or passively by the movement of contaminated adult beetles (O’Callaghan and Jackson 1993a). However as the host range is so limited and the target insect is a major pest, transmission of the bacteria between hosts will produce a positive effect and is unlikely to cause any non-target effects.

3. ENVIRONMENTAL IMPACTS

3.1. Microbial population dynamics
In applying a biopesticide to the soil, it is important to consider the quantitative aspects of application. Application of Invade® to the soil at the commercial rate of $4 \times 10^{13}$ cells/ha results in a population of *S. entomophila* of approximately $4 \times 10^{4}$ cells/g soil. These levels can be validated using a combination of selective media and traditional and molecular identification methods (O’Callaghan and Jackson 1993b) which indicate that there is a >70% efficiency of establishment of the applied cultures. Grass grubs are estimated to be potentially damaging on one million hectares of New Zealand grassland, where surveys indicate an average density of grass grub pathogenic *Serratia* spp. of approximately $2 \times 10^5$ cells/g soil (O’Callaghan et al. 1999). This figure is, of course, highly variable and at the height of a natural epizootic of amber disease, the density of natural pathogenic strains can exceed $10^6$ /g soil while in recently infested areas often no pathogenic isolates are recovered. On the basis of these figures, the total number of naturally occurring, grass grub pathogenic *Serratia* spp. in New Zealand soils can be estimated as $10^{17}$ bacteria. In the past decade 15,000 ha of New Zealand pasture have been treated with Invade which means that $6 \times 10^{16}$ bacteria have been cultured and applied to the pasture. This is a massive number and almost equivalent to the estimated total number of pathogenic bacteria in New Zealand soils (Table 2).
Table 2. Quantitative aspects of natural and applied grass grub pathogenic Serratia spp. in New Zealand soils.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average pathogenic <em>Serratia</em> /g soil in grass grub infested areas</td>
<td>2 x 10^2</td>
<td>O’Callaghan et al. 1999</td>
</tr>
<tr>
<td>Pathogenic <em>Serratia</em> /hectare</td>
<td>2 x 10^11</td>
<td></td>
</tr>
<tr>
<td>Pasture area infested with grass grub</td>
<td>1,000,000 hectares</td>
<td>MAF Survey</td>
</tr>
<tr>
<td>Estimated total number of grass grub pathogenic <em>Serratia</em> in New Zealand soils</td>
<td>2 x 10^17</td>
<td></td>
</tr>
<tr>
<td>Commercial application rate of bacteria/ha</td>
<td>4 x 10^13</td>
<td>Jackson et al. 1992</td>
</tr>
<tr>
<td>Area treated with Invade</td>
<td>15,000</td>
<td>Commercial records</td>
</tr>
<tr>
<td>Total number of bacteria applied</td>
<td>6 x 10^16</td>
<td></td>
</tr>
</tbody>
</table>

While these figures suggest that there has been a doubling of grass grub pathogenic *Serratia* spp. in the environment, in fact, this is not the case. The figure for natural bacterial abundance reflects a carrying capacity of pathogenic bacteria which is, on average, a stable figure. On the other hand, applied populations of bacteria decline in numbers until they establish infections in the target insect population. Pathogenic bacteria will then recycle through the target population maintaining stable levels until the insect numbers have declined to low densities. At this point the pathogenic strains appear to be outcompeted by non-pathogenic strains and decline to undetectable levels in the pastures. After 10 years of application, the commercially applied strain *S. entomophila* 154 remains uncommon in New Zealand soils and is only rarely found outside the sites of application.

3.2. Monitoring of field application sites

Field application sites have been monitored after application of Invade®, principally for effect on grass grub populations and persistence of bacteria. This has provided the opportunity for multiple visits to treated areas and no obvious negative effects on non-target species have been observed. Detailed population estimates of effects on non-target fauna were carried out on three experimental sites. Populations of surface fauna were estimated by vacuum sampling from defined areas of turf in treated and untreated plots and no differences have been recorded in numbers of the ubiquitous Argentine stem weevil (*Listronotus bonariensis* (Kuschel)), spiders, surface dwelling beetles and collembola. The result is not altogether surprising as the bacteria do not survive in the vegetation on the pasture surface. Invertebrate populations in the soil were also assessed and no differences were recorded in numbers of earthworms or soil dwelling lepidopteran larvae from the treated and control plots. Thus the field experimental results are in accordance with the experimental data from maximum challenge laboratory tests.

4. GENE TRANSFER AND GENETIC STABILITY

What of the genetic stability of the bacterium? We know that the most likely form of instability is plasmid loss which removes the insect-killing properties of the bacterium and converts it back to a common soil bacterium. Plasmid transfer to other *Serratia* species can be achieved in the laboratory (Glare et al. 1993) but *S. entomophila* and *S. proteamaculans* are the only species known to carry the plasmid in field soils. We also know that the genetic basis of pathogenicity is similar to that of the more broadly active bacteria containing tc toxins. While there has been no indication of genetic
instability and mutation to other forms, it is important to maintain strict quality control of starter cultures and product and to be on the lookout for unwanted effects.

5. REGISTRATION OF INVADE®

Invade® has been registered as a biological pesticide in New Zealand according to guidelines set by the New Zealand Pesticides Board. The New Zealand regulations are modelled on those developed by the EPA and European agencies and involve three levels of testing. At the first level, the principle concern is mammalian safety. In New Zealand, microbes must pass Tier I testing before they can be used in field trials under a “Not for Sale” permit for biopesticide evaluation. On the basis of satisfactory efficacy and non-target organism testing, a limited sales permit is issued which can be upgraded to full registration given favourable results from monitored commercial applications. Invade® was the first endemic microbe to be registered as a microbial pesticide in New Zealand (Jackson et al. 1993b) and has passed all mammalian, avian and environmental assessments without problem.

6. CONCLUSIONS

All tests and observations have failed to indicate that there is any environmental hazard from the use of \(S.\ entomophila\). The bacterium occurs naturally at high levels in New Zealand pastures and there have been no indications of ill effects on livestock or wildlife. Thus, to have found that the bacterium was hazardous would have been surprising. Does this mean, therefore, that the effort put into safety testing was a waste of time? The answer is probably no. While an organism does occur naturally, when it is applied as a biopesticide we are changing the timing and scale of interaction with its environment. In the case of Invade, we are providing an inoculum at levels which would take several years to build up naturally. Operators are exposed to highly concentrated bacteria with numbers that can be found in a hectare of soil concentrated into a litre of product. Hence operator safety is a primary concern. \(Serratia\ entomophila\) in its product form, Invade®, has to all intents and purposes been proved safe through detailed testing followed by widescale use and observations in the field. It appears that the plasmid-bearing forms have a competitive advantage in a narrow niche, the grass grub gut, but do not have the ability to succeed in other environments. While there will always be the potential of unanticipated and/or unrecognised side effects from the use of bacterial biopesticides, the likelihood of these occurring will be diminished with a good understanding of the science behind their use. Our assurance in the safety of \(S.\ entomophila\) as a biopesticide has been supported by our ability to track the organism in the environment and our understanding of the molecular genetics of disease. Without a sound understanding of the biology and ecology of the microbe in use, environmental safety cannot be guaranteed.

7. ACKNOWLEDGEMENTS

I wish to acknowledge the positive contribution of the whole team of the Microbial Control Group, Biocontrol and Biosecurity, AgResearch, Lincoln in the development and evaluation of \(Serratia\ entomophila\) as a microbial pesticide.

8. REFERENCES


15. AUTHOR AFFILIATIONS

AgResearch, PO Box 60, Lincoln, New Zealand
email: trevor.jackson@agresearch.co.nz