Virulence of *Serratia* Strains against *Costelytra zealandica*

Binglin Tan, Trevor A. Jackson,* and Mark R. H. Hurst

Biocontrol Technologies, AgResearch, Canterbury Agricultural and Science Centre, P.O. Box 60, Lincoln, New Zealand

Received 3 March 2006/Accepted 11 July 2006

Strains of *Serratia* spp. showed a high level of virulence when injected into the hemocoel of larvae *Costelytra zealandica*, with *Serratia entomophila*, *S. plymuthica*, and *S. marcescens* showing significantly higher virulence than *S. proteamaculans*. Toxicity was independent of the amber disease-causing plasmid pADAP, suggesting a generalized *Serratia* toxin.

The genus *Serratia* (*Enterobacteriaceae*) contains insect pathogenic strains (5) which are usually considered to be opportunistic or facultative pathogens, as they are often avirulent to insects when present in the digestive tract but are lethal upon entering insect hemocoel following injury or stress (3). In contrast, strains of *Serratia entomophila* and *S. proteamaculans* containing the pADAP plasmid consistently produce amber disease after ingestion by the New Zealand grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae) (7, 9). Amber disease is a gut-colonizing disease caused by a cluster of three genes termed *sep* (*S. entomophila* pathogenicity) genes on the pADAP plasmid, with the bacteria invading the hemocoel only after a long period of chronic infection (4, 7, 10). The *sep* proteins show significant similarity to the toxin complexes produced by bacterial entomopathogens *Photorhabdus luminescens* and *Xenorhabdus nematophila*, which are usually nematode vectored, leading to death of the nematode by septicemia, but can also be orally active (1, 13, 14). Thus, while oral activity of amber disease-causing strains has been well characterized (9, 10), the effects of coelomic delivery were not known. In this study, we examined virulence and pathology of pADAP-bearing and pADAP-free *Serratia* strains against *Costelytra zealandica* larvae, estimated the intracoelomic lethal doses of the bacteria, and examined the resultant pathology.

The bacterial strains bioassayed in this study are listed in Table 1. Bacteria were produced in an overnight shake culture in Luria-Bertani (LB) broth at 37°C for *Escherichia coli* and 30°C for *Serratia* (7). Cell densities of bacteria were estimated from CFU after dilution plating with phosphate-buffered saline (PBS) onto LB agar plates.

pADAP-bearing strains *S. entomophila* 154+, *S. proteamaculans* 2742, and *S. proteamaculans* 142 and pADAP-free strains *S. plymuthica* 590, *S. marcescens* 363, and *E. coli* DH10B (11) were injected into healthy grass grub larvae. Two microliters of diluted suspension of bacterial culture was injected into the hemolymph through the larval head capsule by using a 1-ml Hamilton syringe with a 30.5-gauge needle (8) to achieve doses ranging from $10^0$ to $10^6$ cells per insect. Larvae for uninfected controls were injected with equal volumes of PBS. The symptoms of toxicity were recorded and the mortalities monitored over time postinjection. The experiment was repeated on three occasions in a completely randomized design. The experimental unit was a group of eight larvae. Inoculated larvae were individually placed in wells of 24-well tissue culture plates, held in the dark in an incubator at 15°C, and observed until 14 days postinjection. Data were analyzed using Genstat 7.1 software (Genstat UK) with treatment mortalities corrected for control mortalities and median lethal doses estimated by probit analysis.

To assess the ability of bacteria to survive and grow within the insect hemocoel, we compared the *S. entomophila* A1MO2 (pADK6) *E. coli* DH10B and labeled by green fluorescent protein using plasmid pGFPSum (6). Bacteria were delivered as described above, with doses of approximately $10^2$ to $10^5$ cells larva$^{-1}$ for *E. coli* DH10B and approximately $10^1$ to $10^3$ cells larva$^{-1}$ for the *S. entomophila* strain containing pADK6. Larvae were bled up to 48 h postinjection when 20 μl of hemolymph was removed from each larva and dilution plated with PBS onto LB agar plates containing the appropriate antibiotics (6). Resultant CFU were assessed by visualization of green fluorescent protein-based fluorescence after exposure on a UV transilluminator. The experiment was carried out with six larvae for each dose rate of each strain, and three larvae assessed at each time point for all dose rates of each strain. Pathology was monitored by examination of hemolymph extracts by phase-contrast microscopy and examination of thin sections of embedded tissue fixed from moribund larvae.

All *Serratia* strains caused mortality after injection into the larvae. Larvae inoculated with doses of $\geq 10^7$ cells larva$^{-1}$ of *Serratia* strains began to die within 24 h postinjection, while larvae inoculated with doses of $\leq 10^5$ cells larva$^{-1}$ tended to die after 48 h postinjection. After six days, *S. entomophila* 154+ and 154−, *S. plymuthica* 590, and *S. marcescens* 363 produced the highest mortality, with estimated 50% lethal doses (LD$_{50}$) that were significantly ($P<0.05$) lower than those of *S. proteamaculans* 2746 and 142, which were in turn significantly ($P<0.05$) lower than those of controls, including *E. coli* DH10B (Table 1). Disease symptoms included lethargy and a darkening of the larvae, but gut clearance, a primary symptom of amber disease, was not a symptom here. Following intracoelomic inoculation of the pathogenic *S. entomophila* strain containing pADK6, the bacteria grew rapidly within the hemolymph, with the CFUs reaching $10^9$ cells per ml of hemolymph in 48 h. In contrast, after injection of *E. coli* DH10B, the bacteria tended to die.
coli DH10B, no CFUs were recovered, indicating that cells were rapidly cleared from the hemolymph shortly after inoculation. Microscopic visualization of hemolymph revealed strong bacterial growth accompanied by vacuolization of the hemocytes, leading to cell lysis. Infection was accompanied by a severe disruption of the midgut epithelium in moribund larvae.

These experiments show that the *Serratia* strains tested had a high virulence against larvae of the New Zealand grass grub, *C. zealandica*, when injected directly into the hemocoel and can be considered potential pathogens by the definition of Buchar (2). The effects of *Serratia* sp. inoculation were clearly distinct from those produced by *E. coli* DH10B, which did not cause mortality even when applied at a concentration of 10⁵ cells larva⁻¹. *Serratia*-induced mortality could be produced with inoculation of very low numbers of cells, and in this regard, our results coincide with those of Lysenko (12) for intracoelomic inoculation of *S. marcescens* into caterpillars. Microscopic examination of larvae through the disease process indicates that *Serratia* virulence is related to the ability of the bacteria to avoid or overcome the insect host’s defenses and proliferate in the hemocoel producing cytotoxic effects against both hemocytes and gut epithelial tissue.

This rapid pathology accompanied by cytotoxic effects is clearly distinct from the chronic ameboid disease produced by strains of *S. entomophila* and *S. proteamaculans* (9, 10). No relationship was found between the presence of the ameboid disease-causing plasmid pADAP and virulence in these experiments. Indeed, the matched strains *S. entomophila* 154+ and 154− showed similar virulences, while the plasmid-bearing *S. proteamaculans* strains showed the lowest virulence of the *Serratia* strains tested. Furthermore, the characteristic ameboid disease symptoms of cessation of feeding and gut clearance (9, 10) were not observed in these experiments. Thus, the pathogenicities of these strains of *Serratia* to grass grub larvae following coelomic injection appear to be related to a generalized *Serratia* toxin rather than caused by the toxin complex/Sept-type gene complex found on the pADAP plasmid.

We are grateful to the members of the Biocontrol Technologies Team, AgResearch, for their excellent technical assistance and David Saville for data analysis. This research was funded by grant C10X0313 of the New Economy Research Fund, administered by the New Zealand Foundation for Research, Science and Technology.

### REFERENCES