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Serratia spp. bacteria evolved in Aotearoa-New Zealand for infection of endemic scarab beetles

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ABSTRACT

The Melolonthinae branch of the beetle family Scarabaeidae has evolved in isolation in Aotearoa, radiating into >100 endemic species, since Aotearoa separated from Gondwanaland 82 million years ago. The group includes important pasture pests, such as the New Zealand grass grub *Costelytra giveni* and the manuka beetle *Pyronota festiva*. These beetles, like other organisms, host their own distinctive microflora including beneficial microbial symbionts and pathogens. A wide range of microbial pathogens infect the Scarabaeidae, but in Aotearoa the bacteria *Serratia entomophila*, *S. proteamaculans* and *S. quinivorans* (Enterobacteriaceae) are frequently found causing natural disease epizootics in *C. giveni*. *S. entomophila* is widespread in Aotearoa pasture soils, with only rare isolations of *S. entomophila* documented in other countries. In contrast *S. proteamaculans* and *S. quinivorans* are globally ubiquitous, and are widely distributed within Aotearoa, with some isolates active against either *C. giveni* or *Pyronota* spp. larvae, or both. Virulence determinants that impart differential host specificity and potency are located on variants of the amber disease associated plasmid (pADAP). The host specificity of the *Serratia*-scarab system and the absence of similar systems in other geographies, suggests that the relationship between *Serratia* spp. and endemic scarabs has evolved in Aotearoa.

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Introduction

The ancestors of the beetle family Scarabaeidae (Coleoptera) can be traced to the Mesozoic era, more than 200 million years ago, and followed an evolutionary radiation across the developing continents (Morón 1984). Scarab beetles, of which there are more than 30,000 species belonging to 1600 genera (Grebennikov et al. 2004), are found in a broad range of ecosystems, from tropical to temperate climates, with many being recognised as destructive grassland pests (Klein et al. 2007). The Gondwanaland fragment which later became Aotearoa separated some 82 million years ago (Rey and Muller 2010) together with the precursors of melolonthine scarabs which

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have radiated into more than 120 endemic species in Aotearoa (Given and Hoy 1952). Despite their wide diversity, scarab beetles have several properties in common. Larvae develop through three larval instars feeding on decaying plant material, dung or roots in the soil. The larval gut is adapted to this diet with a large fermentation chamber for microbial degradation of the ingested material (Zhang and Jackson 2008). The larvae make chambers and pupate in the soil or other concealed locations before emerging as adults, often synchronously in swarms, for feeding on leaves and fruit, mating, and dispersal. The Aotearoa melolonthinae scarabs appear to have evolved in the native grasslands and bush margins with availability of food for both larval and adult stages, but most remain rare in the native habitat. However, some species, such as the New Zealand grass grub (*Costelytra giveni*) (Coca-Abia and Romero-Samper 2016) (formerly *C. zealandica*), known as tūtae ruru by Māori, and the mānuka beetles (*Pyronota* spp.), known as kēkerewai or repowai by Māori, have adapted to feed on introduced pasture species in the modified Aotearoa landscape (LeFort et al. 2014) and have become major agricultural pests (Ferguson et al. 2019). High densities of *C. giveni* larvae decimate the pasture root systems leading to browning of grasses typically observed mid to late March. This severing of the roots enables the 'lifting' of the sward layer when pulled (Figure 1). *C. giveni* is widely dispersed throughout Aotearoa, but has been absent from the West Coast, South Island (Jackson et al. 1991). However, in 2012 a localised population, likely accidently introduced, was identified near the Westport airport (Townsend et al. 2013), and this population has now spread to Cape Foulwind, where it has become established (Hurst et al. 2022).

Mānuka beetle larvae cause similar root damage as *C. giveni* larvae and typically inhabit areas with light soils (Thomson et al. 1979). Although *Pyronota* spp. (*P. festiva, P. laeta*, and *P. setosa*) are smaller in size than *C. giveni*, the larvae of both are similar in appearance (Figure 2). *P. festiva* is widely distributed throughout Aotearoa, while *P. setosa* has a scattered, mainly littoral, distribution in sandy soils in coastal scrubland areas (Brown 1963). Pasture damage attributed to *Pyronota* species has mainly been reported on light soils in proximity to native vegetation or in areas of pasture regrowth, typified by large scale pasture renovation (Thomson et al. 1979; Jackson et al. 2012;

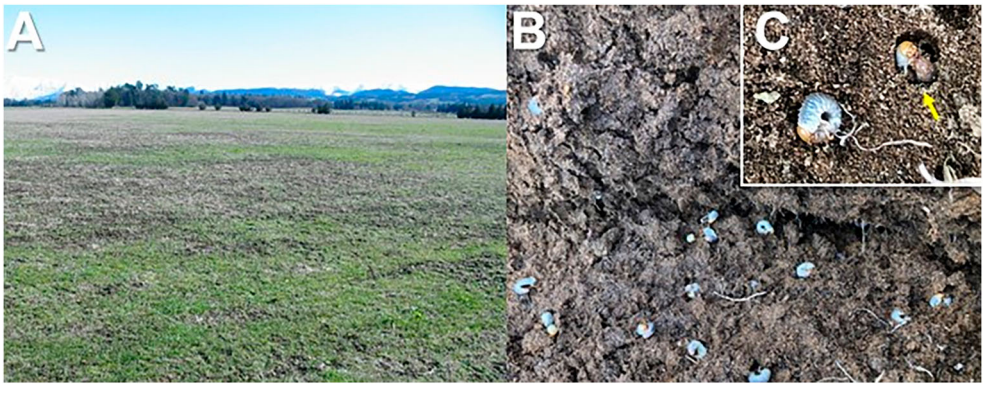

Figure 1. Damage caused by grass grub. **A,** Grass grub damage manifesting as dead patches in pasture (Canterbury). **B,** uplifted sward revealing high *C. giveni* larval densities with larvae forming chambers as depicted in **C**.

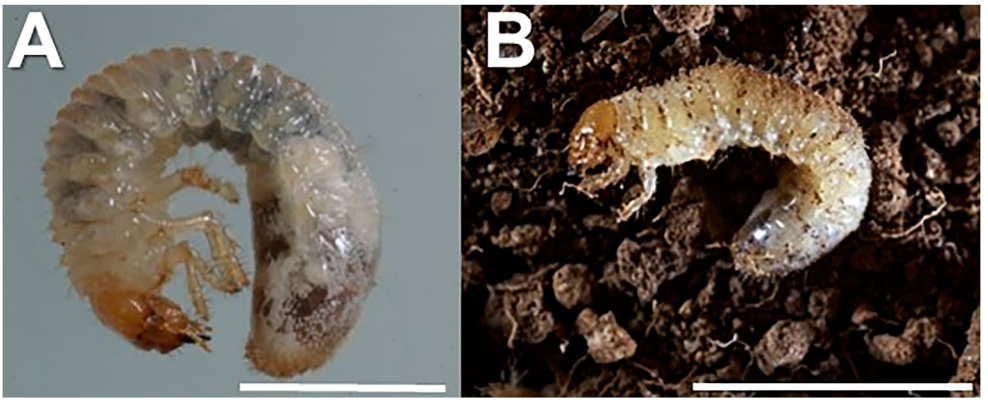

Figure 2. Photographs of grass grub and mānuka beetle larvae. **A,** *C. giveni*. **B**, *P. festiva*. Scale bar 5 mm.

Townsend et al. 2018). *P. setosa* has caused extensive pasture damage following large scale pasture renovation at Cape Foulwind, West Coast, South Island (Dunbar et al. 2012). *Pyronota* spp. and *C. giveni* often coexist in pastures near bushland or after pasture renovation in hill country.

Both *C. giveni* and *Pyronota* spp. share a similar univoltine seasonal lifestyle, with their eggs typically laid in November–December (late Spring) with feeding damage by the larvae most apparent from autumn (late March–April) to winter (August). The larvae go through three larval instars prior to pupating late August to emerge in November as adults (Thompson et al. 1979). Under adverse climatic conditions such as drought, grass grub larvae can alter their metabolism and enter a two-year life cycle.

In the primary sector these pest species mainly affect, but are not restricted to, pasture comprising ryegrass or clover. Annual pasture losses due to grass grub beetle were estimated at NZ$140–380M for dairy and $75–205M for sheep and beef (Ferguson et al. 2019). This excludes the arable sector comprising an area >160,000 ha, or additional niche cropping markets such vineyards, where adult *C. giveni* feed on foliage (Figure 3A,B). In establishing pastures, *C. giveni* larvae typically reach damaging levels 2–4 years after sowing (Jackson et al. 2017). In some pastures populations of greater than 500 larvae/m^2 are common and over 1000 larvae/m^2 have sometimes been recorded (Jackson et al. 2012). *C. giveni* larval numbers greater than 150/m^2 are considered damaging to pasture (Ferguson et al. 2019). Due to their smaller size, mānuka beetle can exceed larval densities of 2000/m^2 of pasture, although populations greater than 300 larvae/m^2 significantly impact pasture performance (Dunbar et al. 2012).

Pathogens of endemic scarabs

The evolution of scarabs in microbial-rich soil appears to have led to a high degree of resistance to generalist pathogens and the development of a wide range of scarab-specific pathogens including fungi, viruses, rickettsia, bacteria, and protozoa. The scarabs in Aotearoa are hosts to a particular microbiota including both symbionts (Zhang and Jackson 2008) and pathogens (Glare, O'Callaghan, et al. 1993). Scarabaeid

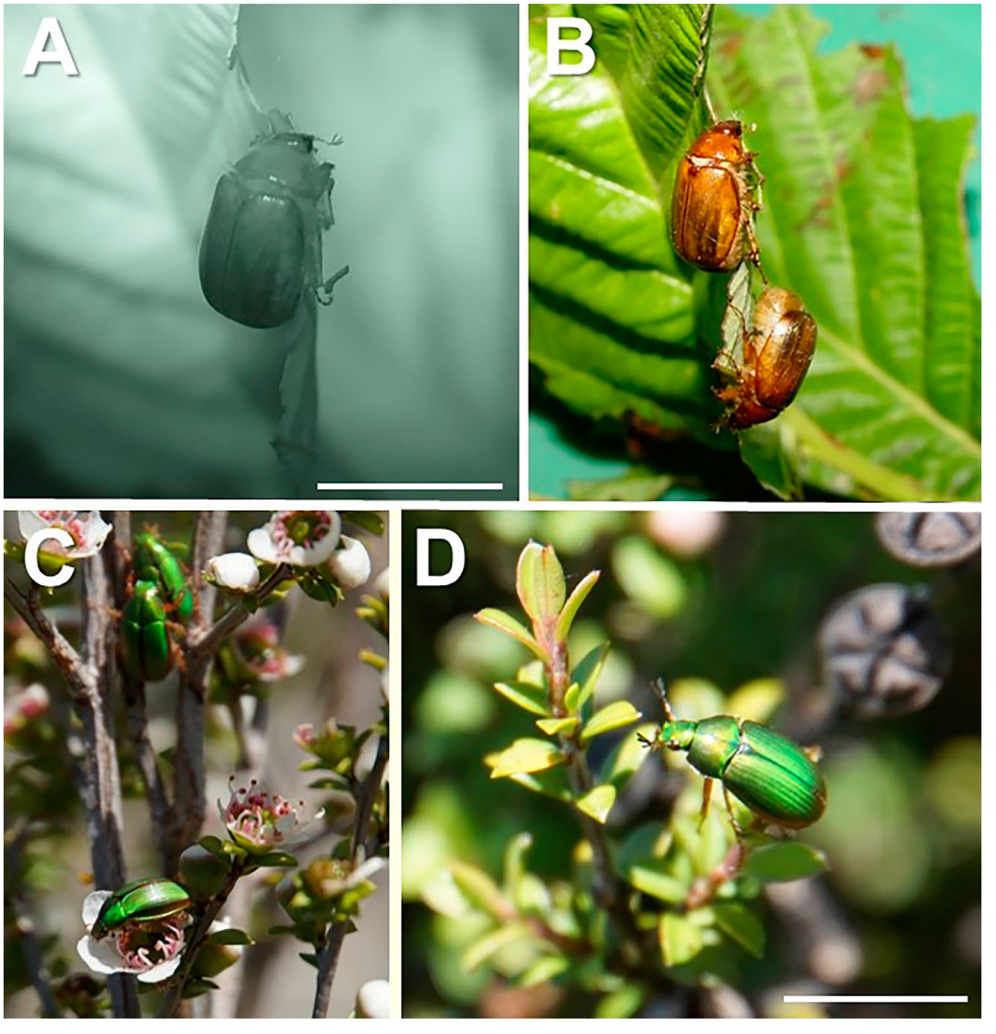

Figure 3. Photographs of adult grass grub and mānuka beetle feeding on foliage. **A,** Nocturnal feeding by *C. giveni* during Spring flights. **B,** *C. giveni* feeding on deciduous leaves during Spring flights; **C-D,** *P. festiva* adult on mānuka (*Leptospermum scoparium*) during mating flights. Scale bar 10 mm.

beetles in general are known to be susceptible to a wide range of pathogens from all classes and some of these microorganisms can play an important role in population regulation. Pathogens are particularly abundant, with more than 30 species of microbial pathogens or entomopathogenic nematodes recorded from *C. giveni* alone (Bourner et al. 1996). The pathogens of Aotearoa Scarabaeidae include obligate pathogens such as the bacterium *Paenibacillus popilliae* (Klein and Jackson 1992) which is ubiquitous, but specific for the Scarabaeidae. Strains of *P. popilliae* are found in scarab populations from all continents, and many are host specific, which suggests that the pathogens arrived and evolved with the founder populations. Species of entomopathogenic fungi attacking Scarabaeidae found in Aotearoa, such as some *Metarhizium* spp., have a wider host range

with susceptible hosts across a range of insect orders (Glare et al. 2021). However, even among the 'broad spectrum' fungi, strain specificity and efficacy can differ (Brunner-Mendoza et al. 2019). *Metarhizium novozealandicum* is the most common fungal pathogen of grass grub in Aotearoa (Villamizar et al. 2021).

Notably the Aotearoa scarabs are also hosts to a range of facultative bacterial pathogens which can live and reproduce on alternative substrates outside the host. These include non-sporeforming bacteria from the Enterobacteriaceae (*Serratia* spp.) which are found to cause epizootics among Aotearoa scarabs. These bacteria can be found in the soil in both pathogenic and non-pathogenic forms (Grimont et al. 1988) and are abundant in long established scarab beetle populations. A less prevalent pathogen is the broad-spectrum entomopathogen *Yersinia entomophaga*, isolated from *C. giveni* larvae, which is highly active towards scarab species, but has yet to be reisolated from a scarab collected from the field (Hurst, Becher, Young, et al. 2011a).

Serratia spp. as pathogens of grass grub

In 1981, a honey/amber colouration was recognised as a symptom of ill health in *C. giveni* larvae and found to be caused by a bacterial infection (Trought et al. 1982). The disease, initially termed 'honey disease', was recognised as being widespread and the cause of population decline (Jackson 1984) with disease symptoms caused by two species of soil bacteria (Stucki et al. 1984). Due to concern by beekeepers about the name 'honey disease', the disease was later designated amber disease, based on the distinctive colouration of the infected larvae. Grimont et al. (1988) classified the pathogenic isolates into two species, *Serratia entomophila* sp. nov. and *Serratia proteamaculans*, based on definitive phenotypic and substrate utilisation tests. The newly defined *S. entomophila* was originally represented by nineteen isolates from diseased *C. giveni* larvae collected in Aotearoa and a single isolate designated 222, from the Institute Pasteur, France. *S. entomophila* can be differentiated from other *Serratia* spp. through the ability to utilise itaconate and further differentiated into two biotypes based on further biochemical tests (Grimont et al. 1988).

Since the initial study, *S. entomophila* has been frequently isolated from *C. giveni* larvae and pasture soils in Aotearoa (O'Callaghan et al. 1999) but, to date, despite extensive searches only two further isolates of *S. entomophila* have been reported from outside Aotearoa. These include the Mexican isolate Mor.4.1, reported to control the scarab *Phyllophaga blanchardi* (Nunez-Valdez et al. 2008), and *S. entomophila* strain AB2, isolated from *Heliothis armigera* larvae in India (Chattopadhyay et al. 2011), which has shown pathogenic activity against a range of lepidopteran larvae (Chattopadhyay et al. 2012).

In contrast, *S. proteamaculans* is a ubiquitous bacterium (Grimont and Grimont 2006) which, based on 16S ribosomal RNA phylogeny, is placed within the *Serratia liquefaciens-proteamaculans-grimesii-quinivorans* complex (Begrem et al. 2021). Bacteria of this group have been readily isolated from the phytosphere (Grimont et al. 1981). Subsequently *Serratia proteamaculans* subsp. *quinovora* was reclassified as *S. quinivorans* using DNA–DNA cross hybridisation and substrate utilisation profiling (Ashelford et al. 1983). Using genomic comparisons based on Average Nucleotide Identity (ANI), Williams et al. (2022) recently found that *S. proteamaculans* and *S. quinivorans*

formed separate phylogroups. As detailed below, Aotearoa scarab pathogens have been found belonging to both of these phylogroups.

Using a wide range of historical isolates and genomic approaches, Williams et al. (2022) confirmed the phylogeny of *S. entomophila* which was only found among the Aotearoa isolates and the single French isolate 222. Genomic analysis by these authors defined three lineages within *S. entomophila*; lineage 17 included isolates from the offshore Chatham Islands, and lineage 18 comprised isolates from a single site in Canterbury. The remaining lineage 19 included isolates collected from around Aotearoa and the French isolate 222. Lineages 17 and 18 correspond to Grimont biotype 1 while lineage 19 corresponds to Grimont biotype 2.

In Aotearoa both pathogenic and non-pathogenic isolates of *S. entomophila* and *S. proteamaculans* (some now reclassified as *S. quinivorans*) have been isolated from insects and soil, with the pathogenic forms more likely to be associated with the presence of the host insects (Glare, Corbett, et al. 1993; Dodd et al. 2006). Pathogenicity is defined by bioassay through development of symptoms after ingestion of bacteria (Jackson and Saville 2000). Surprisingly, host range testing found pathogenic *S. entomophila* are specific in causing amber disease in *C. giveni* but had no effect on other scarabs or other insect species (Jackson et al. 1991; Jackson et al. 1998; Jackson 2003). More recently, and as later outlined, isolates of *S. proteamaculans* and *S. quinivorans* have been identified with dual activity towards *Pyronota* spp. and *C. giveni* (Hurst et al. 2018; Hurst et al. 2021).

Amber disease – a chronic infection

An outline of the amber disease process in presented in Figure 4. After ingestion of a pathogenic dose of an amber disease-causing isolate of *S. entomophila* or *S. proteamaculans*, *C. giveni* larvae rapidly cease feeding and the larval gut, which normally contains dark digestive fluid, clears and the larvae take on a characteristic amber colouration (Figure 4F) before entering a long quiescent phase and finally death through septicaemia (Jackson et al. 1993; Jackson et al. 2001). There is no lethal or sub-lethal effect on the larvae from ingestion of non-pathogenic strains, even at very high doses. Ingested bacteria are mostly flushed into the large fermentation chamber prior to cessation of feeding, with some colonisation of cuticular surfaces of the gut and mouthparts (Jackson et al. 2001). Following gut clearance, bacterial growth occurs principally in the foregut, including the oral cavity, and hindgut of the infected larva (Jackson et al. 2001) and reaches more than 1×10^7 bacteria per larva in the chronic phase of infection. Gut clearance is associated with a sharp decrease in levels of the major gut digestive enzymes, trypsin and chymotrypsin (Gatehouse et al. 2009; Jackson 1995), associated with inhibition of exocytosis in the midgut cells (Gatehouse et al. 2008). As the disease progresses the infected larva becomes moribund, fat cells atrophy, and the body develops the clear amber appearance characteristic of the disease (Jackson et al. 1993). In contrast to infections by toxin-bearing *Bacillus thuringiensis* in other insects, there is no destruction or blebbing of the midgut epithelium and the infective bacteria remain within the larval gut until the final stages of infection (Jackson et al. 1993; Jackson et al. 2001). In the infected larva, bacteria can be visualised attached to food particles (Figure 4) or gut membranes but no specific sites of

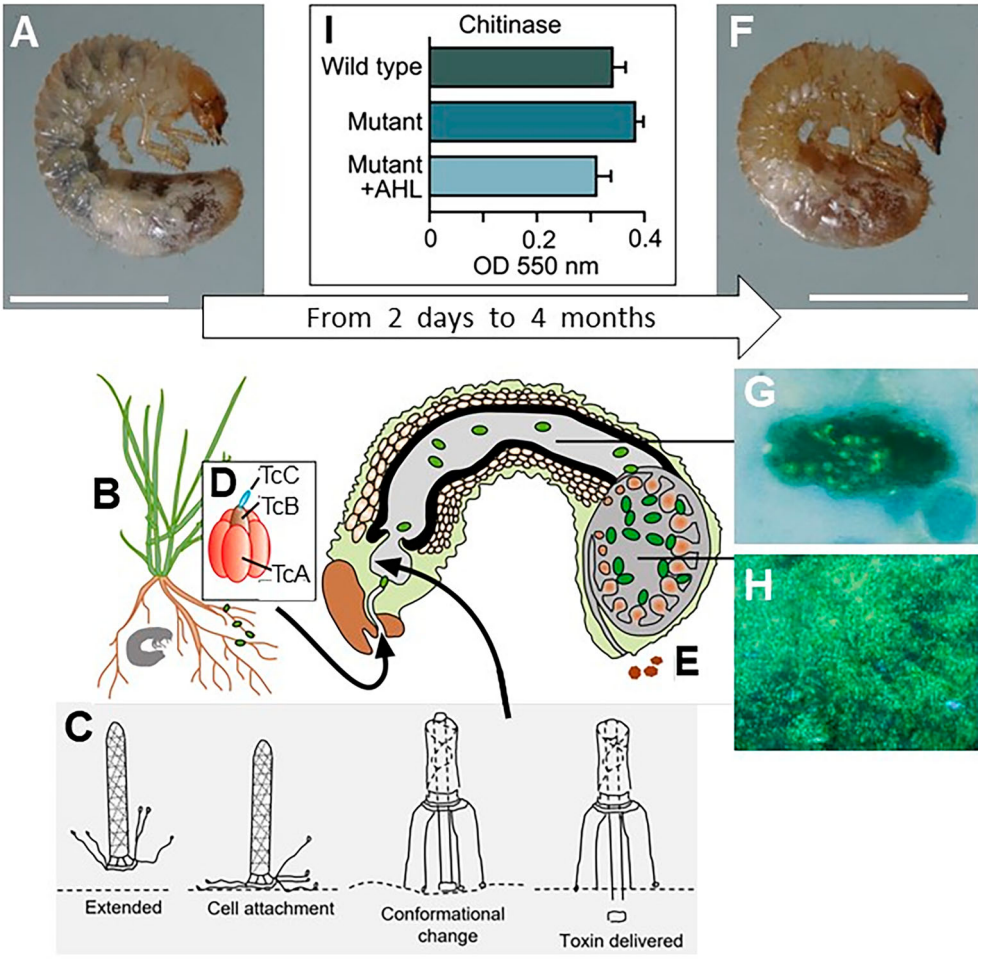

Figure 4. Overview of the *S. entomophila* – *C. giveni* system. **(A),** Healthy *C. giveni* larvae (Scale bar 5 mm) ingest the roots **(B)** and associated *S. entomophila*. Within 2–5 days the larvae cease feeding, a result of as few as 500 anti-feeding prophage (Afp) particles. The proposed model for the action of the Afp is depicted in **(C)**, where the extended virus-like Afp particle latches onto a currently undetermined receptor on cuticular membranes or the intestinal cells using its tail fibres. Once bound (cell attachment), the Afp particle changes conformation to inject the Afp loaded toxin into the eukaryotic target cell. The *S. entomophila* pathogenicity (Sep) Toxin Complex (Tc) **(D)** is released by ingested bacteria. This causes gut clearance, expulsion of gut contents **(E)** and the larvae to become amber in colour **(F)**; scale bar 5 mm. The disease process can last for more than 4 months. There are no apparent sites of *S. entomophila* colonisation on the surface of midgut cells, with bacteria only adhering to particulate matter of the gut and membranous surfaces **(G)**. Within 6 days of ingestion, bacteria multiply within the fermentation chamber of the insect **(H)**. Under conditions of high cell density levels of the *S. entomophila*-derived chitinases are reduced **(I)**, possibly prolonging the time of infection. **I,** measurements of chitinase activity; WT, denotes wildtype *S entomophila*; Mutant, denotes quorum sensing (QS) mutant; +AHL denotes the mutant complemented by addition of an exogenous supply of the QS signal molecule N-Acylhomoserine lactone (AHL). Figure modified from Hurst (2016).

S. entomophila colonisation associated with infection have been identified (Wilson et al. 1992; Jackson et al. 2001; Hurst and Jackson 2002). As time progresses the infected larva

becomes weaker before bacteria eventually invade the haemocoel through the weakened membranes to cause death of the insect by septicaemia and further replication of the bacteria on the cadaver (Jackson et al. 2001). The amber disease process can take more than 4 months from infection to death of larvae and is defined as a chronic infection.

Defining the genetic determinants of amber disease

Ecological studies showed that both *S. entomophila* and *S. proteamaculans* could be isolated from diseased *C. giveni* larvae, but both species occurred in pathogenic and non-pathogenic forms (Grimont et al. 1988, O'Callaghan et al. 1988). This suggested that a transferable genetic element was involved in amber disease. Through plasmid visualisation, Glare, Corbett, et al. (1993) found that amber disease-causing isolates of *S. entomophila* and *S. proteamaculans* both contained a similar 153 kb plasmid that was absent in non-pathogenic isolates. Removal of the plasmid through heat curing resulted in a total loss of virulence. The amber disease encoding plasmid was then designated pADAP for amber disease associated plasmid. Assessment of the DNA restriction enzyme profiles of both the plasmids and genomes of 21 virulent *S. entomophila* isolates showed that they were all quite similar, with only a single *S. entomophila* pADAP variant (p626) identified which differed by a single RE-*Dra*I 4.2-kb band. In contrast *Dra*I plasmid profiles of *S. proteamaculans* strains revealed a wide range of pADAP variants (Dodd et al. 2006).

Variation in pADAP was later examined through the genomic sequencing of plasmids from 51 *S. entomophila* and *S. proteamaculans* isolates derived from diverse geographies and exhibiting diverse disease phenologies (Sitter et al. 2021). Ten sequenced *S. entomophila* pADAP plasmids showed high homology sharing >99% nucleotide identity. In contrast, the 41 sequenced *S. proteamaculans* pADAP plasmids were highly heterogenous in the accessory genes that they encoded despite sharing a common ~63 kb plasmid backbone (Sitter et al. 2021). This study identified and characterised 12 pADAP variants designated A-L. These 12 variants differed only in their accessory regions, several of which encoded different toxin complex (Tc) variants, and these pADAP variants were termed STAMPs for *Serratia* transmissible adaptive mega-plasmids. Two of these STAMP variants reside in non pathogenic *Serratia liquefaciens* isolates 376 and 377 (Sitter et al. 2021).

Using a modified pADAP variant, Grkovic et al. (1995) were able to conjugate pADAP into other members of the Enterobacteriaceae, including strains of *Serratia marcescens, S. liquefaciens, Enterobacter agglomerans,* a *Klebsiella* species and *Escherichia coli.* In all cases, receipt of the plasmid resulted in bacteria that caused amber disease symptoms in the grass grub host. Only pathogenic variants of pADAP have been found in strains of *S. entomophila* and *S. proteamaculans* in the field (Jackson et al. 1991), suggesting that these species encode factors that favour retention of the plasmid.

Through a process of targeted mutagenesis and DNA sequence analysis, the 153 kb pADAP was found to encode two virulence-associated regions. The first region is the *sepABC* (*S. entomophila* pathogenicity) (Hurst et al. 2000) gene cluster encoding a *C. giveni* active Tc (toxin complex) (Figure 4D) (Hurst et al. 2000). Tcs were first identified in the chromosome of the bacterium *Photorhabdus luminescens* (Bowen et al. 1998) and have since been identified in chromosomes of *Pseudomonas* spp. (Rangel et al. 2016), *Xenorhabdus nematophilus* (Morgan et al. 2001), members of *Yersinia* spp. (Fuchs et al.

2008, Hurst et al. 2011a), and *B. thuringiensis* (Blackburn et al. 2011), and other bacteria (Song et al. 2021). Typically, Tcs are chromosomally encoded to produce three proteins, designated TcA, TcB, and TcC (Figure 4D), which combine to form the final insect-active complex (ffrench-Constant and Waterfield 2005). The TcA component enables the delivery of the toxin effector and imparts host range specificity (Sergeant et al. 2006, Meusch et al. 2014). The TcC component is a two-domain protein comprising a distinct *C*-terminal effector domain (Hurst et al. 2000) and a conserved Rhs *N*-terminal domain which, together with the TcB component, envelopes the TcC *C*-terminal effector (Busby et al. 2013), allowing its docking to the TcA delivery component. Once in the midgut the Sep protein causes flushing of the gut and prevents the secretion of digestive enzymes such as trypsin and chymotrypsin by interfering with exocytosis leading to symptoms of amber disease (Gatehouse et al. 2009). Of interest *C. giveni* larvae challenged with low amounts of purified Sep proteins cleared the gut and turned amber within 5 days but later reverted to the healthy phenotype (Hurst, Jones, et al. 2007). This reversion did not occur after antibiotic elimination of the bacteria (Jackson et al. 2001).

The second pADAP virulence determinant is a pyocin-like particle called the anti-feeding prophage (Afp) (Hurst et al. 2004; Desfosses et al. 2019), variants of which have been identified in a diverse range of microbes (Sarris et al. 2014; Chen et al. 2019) and have been collectively termed extracellular contractile injection machines (eCIS) (Böck et al. 2017). Following ingestion by *C. giveni* larvae, as depicted in Figure 4C, the Afp is proposed to bind via its tail fibres to a yet-to-be-defined target cell, from where it then contracts to deliver its protein effector payload to the cell cytosol, resulting in cessation of feeding activity (Hurst et al. 2004; Hurst, Beard, et al. 2007). The Afp induces an effect independently of the bacterial cell; Rybakova et al. (2013) found that as few as 500 Afp particles were required to elicit cessation of feeding activity in 50% of the larval population three days post *per os* challenge. Higher concentrations of Afp caused a rupture of the gut membranes inducing a brown bacterial septicaemia and death within days of ingestion (Hurst et al. 2004). In addition to these findings, unlike other *Serratia* species, a targeted mutation of the *S. entomophila* cell density-dependent quorum sensing (QS) gene, exhibited increased chitinase production under *in vitro* conditions (Figure 4I; Hurst 2016), a finding that may have implications for amber disease progression as discussed further below.

The disease process in *C. giveni* caused by *S. entomophila* shows some similarities to that of the obligate Gram-positive pathogen *Paenibacillus popilliae* (Bacilliae) (Klein and Jackson 1992). Both pathogens grow within their hosts without triggering any apparent host responses, such as melanisation or recruitment of haemocytes. *P. popilliae* invades midgut cells and penetrates the haemocoel where it multiplies in the haemolymph without reaction. In contrast, *S. entomophila* colonises and multiplies in the insect intestine. In both systems events are triggered that lead to cessation of feeding and the depletion of the larval fat bodies, leading to starvation and death of the host. In both scenarios it appears that the purpose of pathogenicity is to provide an environment suitable for replication of the pathogen.

Atypical disease-causing *Serratia* isolates

While both *S. entomophila* and *S. proteamaculans* pathogenic isolates generally produce similar symptoms of amber disease in *C. giveni* larvae, several *Serratia* isolates have been

identified that cause atypical disease symptoms. These include isolate *S. proteamaculans* 143 (now *S. quinivorans* 143) which caused only 60–70% of challenged *C. giveni* larvae to exhibit an amber colour (Glare, Corbett, et al. 1993; Hurst, Becher, O'Callaghan 2011b) regardless of the dose of bacteria administered. Recently, several *S. proteamaculans* and *S. quinivorans* isolates recovered from either diseased *C. giveni* or *Pyronota* spp. larvae, sourced from a range of geographies, were found to cause heightened virulence and/or affected the larvae of both *C. giveni* and *Pyronota* spp. Unlike the stark cessation of feeding activity caused by *S. entomophila,* many of the larvae challenged with these *S. proteamaculans* or *S. quinivorans* isolates continued to feed but at a reduced capacity. The diseased larvae cleared their guts, transitioning to an amber phenotype and then turned brown prior to their death within 5–12 days of pathogen ingestion. In some cases, these isolates did not cause symptoms in all challenged larvae, with only 80–90% of the challenged larvae symptomatic and the remaining larvae appeared healthy over the duration of the 12-day bioassay (Hurst et al. 2021).

Bioinformatic analysis of the genomes of the atypical *S. proteamaculans/S. quinivorans* isolates and *S. entomophila* revealed the presence of seven distinct *C. giveni* or *Pyronota* spp. active Tcs and an Afp variant (Figure 5). These included four distinct *tcA* (*tcA1–tcA4*) and *tcC* (*tcC1–tcC4*) variants with three of the *tc* clusters encoding two different TcC proteins. Several of the *S. proteamaculans* and *S. quinivorans* Tc-encoding isolates had activity towards both *Pyronota* spp. and *C. giveni*, with other isolates being specific to either *Pyronota* spp. or *C. giveni* (Figure 5). Two *S. quinivorans* isolates, C and Man4, were found to cause an incomplete disease state, with the challenged larvae reverting from diseased to healthy (Hurst et al. 2021), a reversion effect like that previously observed with *C. giveni* larvae challenged with low levels of Tc Sep proteins. Differences in both host range (isolate Cfb affects both *C. giveni* and *Pyronota* spp.; Figure 5) and virulence capacity (e.g. *C. giveni*-specific isolate 143; Figure 5) of isolates encoding the same virulence determinants were also noted. Other isolates, such as *S. proteamaculans* 28F and *S. quinivorans* MH5, exhibited variable pathogenicity over different bioassays, being effective against *C. giveni* larvae one season but not the next. Although yet to be proven, this seasonal variability may reflect genetic differences in the larvae sourced from different geographic locations used for bioassay assessments, as noted by Richards et al. (1997). The *S. entomophila sep* Tc-encoding region is phylogenetically distinct to the more closely related *S. proteamaculans* and *S. quinivorans* Tc orthologues (Hurst et al. 2021). When the highly variable TcC effector domain was excluded from the analysis, the divergence in these Tc clusters was more pronounced across the various *Serratia* TcA components which, based on studies of *P. luminescens* Tcs (Gatsogiannis et al. 2013; Meusch et al. 2014), impart host specificity.

A novel *S. proteamaculans* isolate AGR96X was found to consistently kill larvae of both *C. giveni* and *Pyronota* spp. faster than other isolates, across multiple bioassays over different seasons. AGR96X challenged *C. giveni* and *Pyronota* spp. larvae died within 5–12 days of ingestion. Unlike *S. entomophila*, AGR96X was found to invade the *C. giveni* larval haemocoelic cavity within days where it rapidly multiplied to reach 8.2×10^9 bacteria per larva, compared to 2.7×10^7 of *S. entomophila* while confined to the gut at 6 days post challenge (Hurst et al. 2018). The main virulence determinant of AGR96X is a variant of the Afp designated AfpX (Figure 5) which, unlike the Afp, contains two tail-length termination proteins (AfpX16a and AfpX16b), two predicted toxins

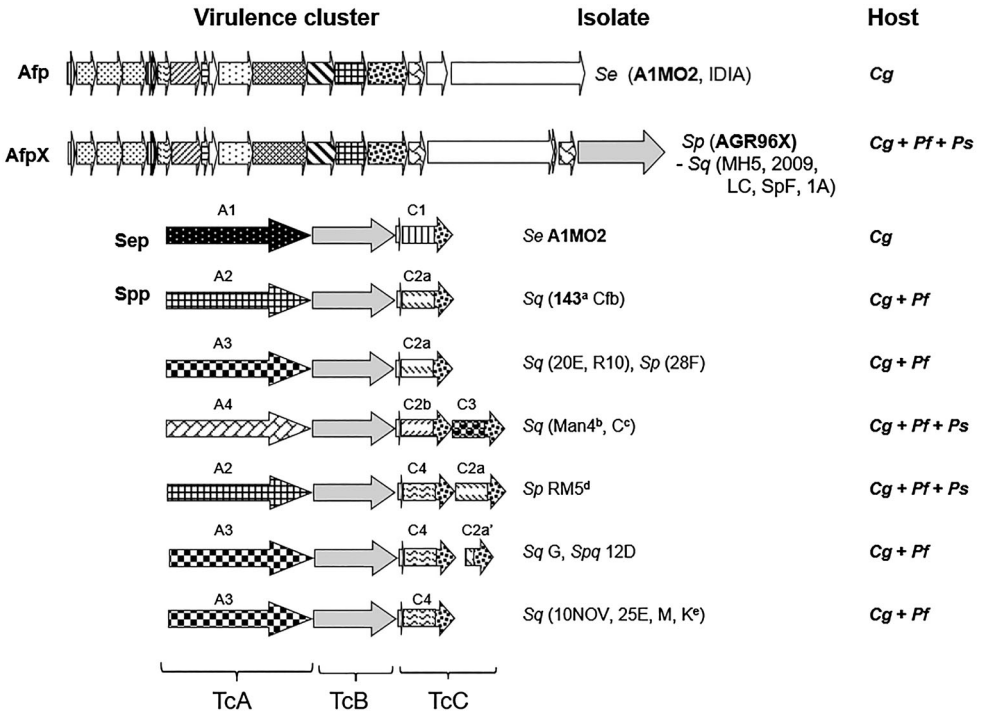

Figure 5. Schematic of the *S. entomophila* (Se), *S. proteamaculans* (Sp) and *S. quinivorans* (Sq) Afp and Tc variants and their associated *C. giveni* (*Cg*), *P. festiva* (*Pf*), and *P. setosa* (*Ps*) host. Patterned arrows de note the Tc (TcA1-A4, TcC1 – C4) variants. Relevant isolate identifiers are listed where bold text denotes the previously documented *S. entomophila* Sep and Afp and the *S. quinivorans* isolate 143, and AGR96X AfpX virulence-associated region. Isolate names are listed where superscript letters indicate: [a]no activity against *Pyronota* species, [b]transient activity against *C. giveni*, [c]no activity against *C. giveni*, transient activity against *Pyronota* species, [d]transient activity against *Pyronota* spp., [e] *Pyronota*-active. Figure modified from Hurst et al. (2021).

(AfpX17 and AfpX18) and differs in its Afp13 receptor binding tail fibres. Like AGR96X, the purified AfpX is also active towards *C. giveni* and *Pyronota* spp. larvae, causing a similar disease phenology (Hurst et al. 2018). The broader host range, with consistent increased speed of kill, suggests that AGR96X could be useful in biopesticide formulations in pasture systems.

Ecology and epidemiology of the host specific pathogen *Serratia entomophila*

Serratia spp. are a common component of the soil microflora which can contain up to 10^9 bacteria/g soil. The study of the ecology of *Serratia* species is aided by their fortuitous ability to grow on otherwise toxic caprylate thallous agar (Starr et al. 1976). This enables *Serratia* spp. to be selectively isolated and quantified from complex microbial communities including clinical samples, dead insects and soil. Once putative *Serratia* colonies have been isolated on the semi selective caprylate thallous agar, identity of genera and species of interest can be made by sequential plating on DNAse agar, a

positive test to confirm *Serratia* spp., then itaconate agar, selective for *S. entomophila*; and finally, adonitol agar which is able to differentiate *S. entomophila* from *S. proteamaculans* (O'Callaghan and Jackson 1993a). Virulence of isolates is subsequently confirmed by bioassay (Jackson and Saville 2000). Using this selection regime, *Serratia* species populations in the soil can be quantified and compared to populations and life stages of *C. giveni*.

Serratia populations, particularly *S. entomophila,* are generally absent from new pastures but are common where *C. giveni* is established (O'Callaghan et al. 1999). *Serratia* populations in *C. giveni* colonised pasture soils averaged 5×10^4 bacteria/g soil but the distribution within pastures was uneven and populations as high as 10^7 bacteria/g soil were recorded in locations with high *C. giveni* density. High density patches of *C. giveni* favour transmission between larvae. Natural infections with >50% amber disease have been recorded (Jackson et al. 1991) and a single larva can contribute > 3×10^7 *S. entomophila* cells (Jackson et al. 2001; Hurst et al. 2018) to the soil on decay of the cadaver. Accordingly, after a cycle of disease the numbers of pathogenic *Serratia* in soil increase during June-July and amber disease in *C. giveni* is more prevalent in high density *C. giveni* populations, where host proximity favours transmission (O'Callaghan et al. 1999). To establish new foci of infection, it appears that pathogenic *S. entomophila* is dispersed from infected sites by adult beetles during the flight season (O'Callaghan and Jackson 1993b). This scenario would account for the dispersal of *Serratia* during the beetle flight season and colonisation of areas where the pathogen is absent (Zydenbos et al. 2016).

The field density of amber diseased *C. giveni* larvae will, to a large extent, determine the number of *Serratia* available to cause disease in the subsequent season (Godfray et al. 1999). Grass grub populations typically increase over a 5-year period before undergoing a natural decline as the incidence of disease increases in the grub population, concurrent with increased *S. entomophila* numbers in soil (O'Callaghan et al. 1999). Following collapse of the host population, pathogen levels decline allowing the host population to recover in a delayed, density-dependent oscillation until the density is such that transmission is favoured, and a further epizootic occurs (Jackson et al. 2017). In the absence of a host, amber disease-causing isolates of *Serratia* decline resulting in the majority of *Serratia* isolates recovered from soil being non-pathogenic (O'Callaghan et al. 1999).

These pathogen–host dynamics may be affected by external influences such as climate. Under laboratory conditions, *S. entomophila* populations rapidly declined in dry soils with the decline accentuated when the soil temperature increased from 10°C to 20°C (O'Callaghan et al. 2001). The impact of desiccation stress on *Serratia* populations and other grass grub pathogens in soil can also be inferred from field observations where grass grub outbreaks in pasture commonly occur after dry summers (Barlow and Jackson 1998).

The seasonal increase in soil populations and the maintenance of *Serratia* populations across years may also result from saprophytic growth. Non-pathogenic modes of reproduction have been reported in other entomopathogens and are common for many disease-causing bacteria in mammals (e.g. *E. coli*). *Serratia* spp. are capable of rapid growth in soil in the absence of competition with soil microorganisms (for example in sterile soil) and in response to addition of nutrients to soil (O'Callaghan et al. 1988).

Godfray et al. (1999) constructed a model that incorporated saprophytic growth of the bacteria and illustrated the benefit of a saprophytic phase in *Serratia* – *C. giveni* population dynamics.

Use of *S. entomophila* for management of *Costelytra giveni*

The natural impact of amber disease on populations of *C. giveni* suggested that the causative bacterium, *S. entomophila*, could be used as an applied agent for control of the pest (Jackson 1984). The bacterium was cultured by fermentation and delivered in an aqueous drench to field populations of *C. giveni* resulting in 30–59% infection within three months and residual activity (Jackson et al. 1986). Cessation of larval feeding allowed a 30% increase in pasture production over the test period. However, the large volume of liquid inoculant and the sensitivity of bacteria to UV light on the soil surface limited the practicality of the approach. These problems were overcome with development of a high bacterial density aqueous formulation which was injected into the soil through a modified seed drill (O'Callaghan et al. 1987). After further field efficacy and safety testing the bacterial product was registered and marketed as 'Invade' (Jackson et al. 1992). The limitations of this approach (the need for specialist application equipment and refrigerated storage) were overcome with development of an ambient temperature stable granular formulation of the bacteria which could be applied through standard drills which was marketed as 'Bioshield' (Johnson et al. 2001; Jackson 2007). When compared with a chemical insecticide (diazinon) for long term management of *C. giveni* (Zydenbos et al. 2016) application of *S. entomophila* established a cycle of disease in the larval population and maintained the population at low levels for several seasons. In contrast, the insecticide treatment caused a rapid decline in larval numbers shortly after application, but after two years the insect population resurged to high, damaging levels.

Successful use of *S. entomophila* in control of *C. giveni* requires consideration of the characteristics of the pest and the bacterium. Healthy populations of the beetles multiply rapidly in new pastures resulting in a damage peak frequently observed 2–3 years post pasture establishment. *S. entomophila* requires a basal population of insects sufficient for multiplication and transmission. If applied to a rising population a level of 20% infection within six weeks of application is usually sufficient to establish a recycling epizootic of amber disease-causing bacteria which will hold the pest population to low levels (Jackson et al. 2017). As a biological control agent, *S. entomophila* is limited by its specificity to a single pest (Jackson et al. 1991) and its need for accurate application timing; these are characteristics borne out of close evolution with its host.

Epidemiology of non-amber disease *Serratia* pathotypes

Although the epidemiology of *S. entomophila* and *C. giveni* are reasonably well understood, the same is not true of atypical disease causing *Serratia* pathotypes. It is likely that faster acting pathogens such as *S. proteamaculans* AGR96X may be associated with localised disease outbreaks characterised by a 'boom to bust' life cycle or, alternately, suited to cooler climes where reduced larval metabolism and movement limits spread of the pathogen (Read 1994; Paez and Fleming-Davies 2020). Unlike *S. entomophila,*

AGR96X invades the haemoceol cavity early in the infection and can reach cell numbers of greater than 8×10^9 cells per larva. This, combined with mortality and decay within 12 days of infection, will increase the opportunity for recycling of AGR96X through infection of heathy larvae within the season. The ability of some *S. proteamaculans* or *S. quinivorans* isolates to cause disease in 60–90% of the challenged larvae (Hurst et al. 2021) may reflect differences in virulence regulation and/or larval genetics and physiology. In this scenario the unaffected larvae may ensure the long-term persistence of the pathogen by maintaining availability of the host in subsequent years.

The diversity of *S. proteamaculans* and *S. quinivorans* isolates with activity against both *C. giveni* and *P. festiva* likely reflects the overlapping distribution of these insect species. Amber disease-encoding isolates along with other *Serratia* Tc-encoding variants have been mainly isolated from low-lying regions of modified pasture (typically comprising clover and ryegrass) inhabited by *C. giveni* and/or *Pyronota* species (Jackson et al. 1991; Dodd et al. 2006; Hurst et al. 2021), although only limited sampling has been conducted to date. The most virulent *Serratia* isolates (e.g. strains encoding AfpX) were recovered from modified pastures in hill country areas of Aotearoa. In addition, both the AfpX encoding AGR96X and the broad host-range entomopathogen *Y. entomophaga* (Hurst, Becher, Young, et al. 2011a) were isolated from the same field site (Hurst et al. 2021), indicative of a multi-entomopathogen complex at this location. This site was at elevation and in proximity to the native estate wherein potential pathogen spill-over from areas of endemic flora comprising tussock and other plant species may have occurred. Although yet to be defined, aside from the likely temperature differences (low land versus sub alpine -possibly affecting larval metabolism), the different plant species present in the native estate or modified pasture (clover and ryegrass) may impart positive/negative selective pressures for *S. entomophila* and the various species of *S. proteamaculans* or *S. quinivorans*. It is plausible that, as previously noted with the endophytic nature of *S. proteamaculans* isolate 568 (Taghavi et al. 2009), some Aotearoa *Serratia* isolates may be plant associated.

Origins of pathogenicity of *Serratia* spp. to scarabs within Aotearoa

In pre-human Aotearoa, *C. giveni* and *Pyronota* spp. probably lived in tussock patches on the edge of the native bush or in areas temporarily cleared and revegetated after avalanche or flood (much as is seen in the high country today). In these situations, a balance develops between pathogen and host. With human intervention through agriculture, soil disturbance during cultivation can virtually eliminate pests and with them their natural pathogens, explaining the healthy state of scarab beetles colonising new pastures. In the depauperate new pasture environment *C. giveni* populations increase without constraint. The absence of pathogens leads to spectacular pest outbreaks in pasture, especially after dramatic land use change (Jackson et al. 2012). *C. giveni* and *Pyronota* spp. are usually univoltine and females can have an egg load of up to 60. For both *C. giveni* and *P. setosa* most eggs are laid close to where the female emerges leading to high density, pasture damaging, patches of larvae.

The bacterial pathogens of Aotearoa scarabs have evolved from the Gammaproteabacteria widespread on Earth nearly 2 billion years ago. Bacteria evolved together with other life forms and formed associations with invertebrates, some of which were pathogenic.

Evidence of ancient relationships is provided by *P. popilliae* and *P. lentimorbus* which are only known to infect members of the coleopteran family Scarabaeidae, including those in Aotearoa. The precursors of the current scarab beetle species are known from the fossil record in the Mesozoic period, before the breakup of the Pangaea supercontinent about 175 million years ago (Morón 1984; Ahrens et al. 2014). Scarab beetles can be found on all habitable continents and, with them, infections of *Paenibacillus* spp. which appear to show host specificity (Klein and Jackson 1992), suggesting that they co-evolved with the beetles and emerged from ancient origins before continental break-up. The suggestion of ancient origins is supported by molecular evidence from Zeigler (2013). Similarly, the unique associations of *Photorhabdus* spp. bacteria with *Heterorhabditis* spp. nematodes and *Xenorhabdus* spp. bacteria with *Steinernema* spp. nematodes from all continents suggests coevolution before the Pangaea separation (Poinar 2011; Poinar and Grewal 2012). The nematode/*Photorhabdus* association is of interest as this suggests that Tc genes, or their precursors, were widespread before the breakup of Pangea with origins in the Mesozoic and have evolved into the insect pathogens of the *Xenorhabdus* (Sergeant et al. 2006), *Photorhabdus* (Duchaud et al. 2003), *Yersinia* (Fuchs et al. 2008) and *Serratia* genera of the Enterobacteriaceae (Jackson et al. 2017).

The pADAP encoded *sep* and *afp* genes together with the high specificity of isolates causing amber disease of *C. giveni* suggest that this is a more recently evolved host–pathogen relationship. Bacterial evolution is aided by fluid genomes adapting by mutation and homologous recombination with the potential for plasmid transfer of genetic material between cells. For the toxin-bearing *B. thuringiensis*, strain evolution has probably been driven quite rapidly with exchange of toxin-coding plasmids (Bizzarri and Bishop 2008) such that there are a huge range of strains of *B. thuringiensis* in the environment containing functionally different toxins (Cry, Cyt, VIP) governing pathogenicity to different insect groups (Jurat-Fuentes and Jackson 2012). The diversity of Tc-encoding clusters across Proteobacteria signifies that the Tc proteins play a key role in the ecology of these bacteria (Song et al. 2021) and alludes to their potential mobility. It may also reflect an enhanced evolutionary capacity for these Tc encoding regions to acquire or interchange different *tcC* components, as originally proposed by Hill et al. (1994). The diversity of Tc encoding clusters parallels the diversity of three-domain Cry toxins in *B. thuringiensis*, where the various composite domains are proposed to have evolved at their own rates (Bravo et al. 2013), with greater variability in the host-targeting and effector regions (Wu et al. 2007). In this instance the varying TcA, Afp13 cell targeting, and TcC effector regions (Hurst et al. 2000; Hurst et al. 2004; Hurst et al. 2021) have likely evolved to target and/or have greater affinity for certain host species. This selective process, in turn, may be driven by a pathogen–host arms race as has been proposed in other effector systems (Galan 2009; Białas et al. 2018). Given the variability within Tc orthologs and associated TcC effectors, it remains unclear as to why there are only two main Afp variants (Afp and AfpX).

Sitter et al. (2021) and Williams et al. (2022) considered that pADAP-type plasmids are restricted to genus *Serratia*. To date the pathogenicity encoding genes for the Aotearoa Scarabaeidae have been defined as plasmid located (Sitter et al. 2021). It is therefore tempting to speculate that the various STAMPs and their associated toxins likely reflect the ecology of the pathogen and the host. None of the non amber disease encoding strains of *S. proteamaculans* or *S. quinivorans* encoded both a *tc* and an *afp*

variant. This may reflect that rapid onset of disease imparted by these isolates overrides the requirement for a second virulence determinant. Alternately, the cessation of feeding activity imparted by the Afp may necessitate the requirement for Sep proteins to clear the gut. In turn the voided gut may make intestinal conditions more favourable to pathogen establishment.

The host specificity of the various *S. proteamaculans, S. quinivorans* and *S. entomophila* strains, combined with the absence of non-Aotearoa STAMP variants in the current public bioinformatic databases, supports the notion that the assembly of STAMPs has been essential for success of the disease since the separation of Aotearoa from Gondwanaland. Bioinformatic analysis of the various STAMP variants revealed that the key virulence determinants have been independently acquired in a distinct region of the STAMP backbone (Hurst et al. 2011b; Sitter et al. 2021). The association of STAMPs within *Serratia* spp., wherein *S. entomophila* is more geographically localised (Aotearoa), is indicative of more recent evolution. The isolation of an avirulent *S. proteamaculans* pADAP variant pPuna18 from Aotearoa, encoding a nitrogen fixation cluster, further suggests that the pADAP linage is diverse within Aotearoa and may be confined to *S. entomophila*, *S. quinivorans, S. proteamaculans*, and *S. liquefaciens* (Sitter et al. 2021). It is likely that the precursors of the Sep and Afp toxins were present among the Enterobacteriacae and acquired in *Serratia* spp. to form a primitive STAMP. Further to this the altered virulence of *S. proteamaculans* isolate 3041 transconjugant containing the plasmid p145, was less virulent than the *S. proteamaculans* 145, plasmid p145 donor. This finding suggests that the chromosomal background affects disease regulation and that these plasmids are likely confined to an isolate lineage (Sitter et al. 2021).

Relative to other genera, the pan genome of *Serratia* is considered restrained and metabolically diverse, indicative of long-term niche adaptation within evolutionary time scales (Williams et al. 2022). The *S. entomophila* chromosome has been defined as 'closed', with reduced signatures of horizontal gene transfer, pointing to speciation at the time that pADAP was acquired (Vaughan et al. 2022; Williams et al. 2022). Of interest, *S. entomophila* encodes the species-specific itaconate encoding region. An itaconate-deleted mutant resulted in a delay in the onset of amber disease indicating a role of this metabolic pathway in the initial disease process and perhaps a tenuous link to speciation (Vaughan et al. 2022).

The pathogenesis of amber disease caused by *S. entomophila* with these toxins (Sep and Afp) in Aotearoa scarabs is unusual (Jackson et al. 1993; Jackson et al. 2001) in that there is no observable tissue damage in the early stages of infection but there are physiological responses (cessation of feeding and gut clearance) caused by combination of expression of the Sep and Afp toxins. As previously noted, a continuous supply of Sep proteins is required to maintain gut clearance, while an oversupply of Afp causes larval mortality. This, combined with elevated chitinase levels in a *S. entomophila* QS mutant (Hurst 2016), suggests that gene regulation within this bacterium is tightly regulated to prolong the amber disease process. In this scenario, under conditions of high cell density such as can occur in the *C. giveni* hindgut, chitinase production is likely decreased, reducing the ability of *S. entomophila* to degrade the peritrophic membrane or other chitinaceous material. This, in turn, may increase extend the time required before the bacteria can invade the haemocoelic cavity, thereby potentially extending the persistence of the pathogen by protecting it from environmental stresses in the soil (Hurst 2016).

Recombinations and/or genetic acquisitions of the *S. proteamaculans/S. quinivorans* plasmid by *S. entomophila* probably led to the variant with the capability and evolutionary advantages of infection and growth in scarab larvae. Chance association and plasmid transfer would have conferred this advantage on *S. entomophila*, which is a relatively rare bacterium in the non-pathogenic form, both internationally and in Aotearoa pasture soils. With European colonisation and wide scale pasture development in Aotearoa, the pasture scarabs and their *Serratia* pathogens have proliferated to become common features of the local environment. This is most evident with the host specific *S. entomophila – C. giveni* relationship and the chronic nature of amber disease where the prolonged infection is suggestive of a dedicated pathogen with a lifestyle somewhat dependent on its *C. giveni* host.

Conclusion

The isolation of *Serratia* strains encoding different Tc and Afp clusters offers the potential for development of biopesticides that are more efficacious in specific environments. Further, combining isolates with different virulence properties, such as *S. entomophila* and the more rapid-killing *S. proteamaculans* isolates may afford greater control of the insect host (Paez and Fleming-Davies 2020). This, combined with the identification of *Serratia* pathogens that target *C. giveni* larvae with differing effects, suggests genetic heterogeneity within *C. giveni* across different geographies. This may in part explain the presence of several different Tc encoding clusters. To validate these scenarios detailed genetic analysis of the *C. giveni* larvae sourced from different locations needs to be undertaken. Such studies will be aided through advances in metagenomic and target specific quantitative assays which will enable the differentiation of pathogen and host type across both the natural and modified Aotearoa landscapes.

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