# DEVELOPMENT OF A SPECIES-SPECIFIC PROBE FOR DETECTION OF *SERRATIA ENTOMOPHILA* IN SOIL

M.R.H. HURST, S.D. YOUNG and M. O'CALLAGHAN

*AgResearch, Lincoln Research Centre, Private Bag 4749, Christchurch 8140*

*Corresponding author: sandra.young@agresearch.co.nz*

**ABSTRACT**

Strains of the bacteria *Serratia entomophila* and *S. proteamaculans* cause amber disease in the grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae), an important pasture pest in New Zealand. Strains of both *Serratia* species occur naturally in New Zealand pasture soils. *Serratia entomophila* has been developed as a commercial biological control agent, bioshield™ . The persistence of the applied strain in soil is currently measured by enumeration of bacterial colonies on *Serratia*-selective agar and subsequent biochemical tests are carried out to differentiate between the two *Serratia* species, on the basis of their ability to utilise itaconate as a sole carbon source. A species-specific DNA probe has been developed as an alternative to these laborious biochemical tests. Tests against a range of *Serratia* species in colony dot blots showed the probe could be used to differentiate between *S. entomophila* and *S. protemaculans* recovered from treated soil when assessing persistence of bioshield™ inoculum and efficacy of the product in the field.

**Keywords:** *Serratia entomophila, Serratia proteamaculans*, itaconate, DNA probe, grass grub, biocontrol.

## INTRODUCTION

Amber disease of the New Zealand grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae), is caused by some strains of *Serratia entomophila* and *S. proteamaculans* (Enterobacteriaceae) (Jackson et al. 1991). Pathogenicity to grass grub is encoded on a 155 kb plasmid, designated pADAP (Hurst et al. 2003). *Serratia entomophila* is now available to farmers as a commercial product, known as bioshield™ (Ballance Agri-Nutrients, Mt Maunganui, New Zealand). Estimating the environmental persistence of the applied *S. entomophila* is essential to our understanding of the epizootiology of amber disease and also for the assessment of efficacy of the bacterium as a microbial control agent of grass grub populations. Suitable isolation and enumeration techniques are a prerequisite to determining the establishment and persistence of any microbe in soil but the differentiation of species or genera from the multitude of microbial species resident in soil presents a huge challenge. Selective culture media have been used extensively in insect pathology but the selectivity of isolation media is rarely sufficient to allow enumeration of one type of bacterium in the presence of closely related types (Liesack et al. 1997).

The ecology of amber disease-causing *Serratia* spp. has been extensively studied using *Serratia*-selective agar media for isolation and enumeration of the bacteria from soil and grass grub larvae. Most *Serratia* spp. can be isolated on caprylate-thallous agar (CTA), the selectivity of which depends on the uncommon ability of members of the genus *Serratia* to metabolise n-octanoic acid as a sole carbon source and also their ability to tolerate levels of thallium salts toxic to many micro-organisms (Starr et al. 1976). As all *Serratia* spp. (with the exception of *S. fonticola*) can be selectively cultivated from soil on CTA, additional tests are required to differentiate between *Serratia* species. A combination of three test agars has been used to confirm the identity of isolates recovered



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on CTA as *Serratia* spp. and to differentiate *S. entomophila* from *S. proteamaculans*, which is also commonly isolated from New Zealand soils (O'Callaghan & Jackson 1993). Further identification methods, such as API carbohydrate utilisation tests (bioMérieux, Marcy-l'Etoile, France), are needed to differentiate between *S. proteamaculans* and other *Serratia* species.

The development of a species-specific probe allowing differentiation between *S. entomophila* and *S. proteamaculans* strains commonly isolated from field samples would be useful. One of the phenotypic traits currently used to differentiate between the two species is the unique ability of *S. entomophila* amongst the other members of the genus *Serratia* to utilise itaconate as a carbon source (Grimont et al. 1988; O'Callaghan & Jackson 1993). This phenotypic difference suggested that there was a unique gene(s) encoded by *S. entomophila* that facilitates utilisation of itaconate and that this gene(s) could be used for construction of a DNA probe to differentiate *S. entomophila* from other *Serratia* species, in particular *S. proteamaculans*. This paper describes the development and testing of a species-specific probe for *S. entomophila*.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria Bertani (LB) broth or on LB agar (Sambrook et al. 1989) at 37°C for *Escherichia coli* and 30°C for *Serratia entomophila*. Antibiotic concentrations used for *S entomophila* were 100 µg/ml kanamycin and 90 µg/ml chloramphenicol, and for *E. coli* were 50 µg/ml kanamycin, 30 µg/ml chloramphenicol and 100 µg/ml ampicillin.

**TABLE 1: Bacterial strains and plasmids used in this study.**

| Bacteria/plasmid | Description | Reference |
|---|---|---|
| *Escherichia coli* DH10B | $F^-$*mcr*A △*mrr-hsd*RMS-*mcr*BCϕ80d *lac*Z△M15 △*lacX74 endA1 recA1 deo*R△*ara, leu* 7697 | Lorow & Jessee (1990) |
| *Serratia entomophila* A1MO2 | Contains pADAP, pathogenic to grass grub | Grimont et al. (1988) |
| mini-Tn*5Km*1 | $Kn^R$ | de Lorenzo et al. (1990) |
| pACYC 184 | $Cm^RT^R$ | Chang & Cohen (1978) |
| pUC19 | $Ap^R$ *lac*Zα, multi-cloning site | Yanisch-Perron et al. (1985) |

### Identification of *Serratia* species

Differentiation between *Serratia* spp. was determined by reactions on three test media (DNase agar, adonitol agar and itaconate agar) as described previously (O'Callaghan & Jackson 1993). Identity of selected isolates was further confirmed using API20E and API50CH test strips (API, France).

### Introduction of plasmid DNA into *E. coli* and *S. entomophila*

Plasmids were electroporated into *E. coli* and *S. entomophila* strains using a BioRad Gene Pulser (25 µF, 2.5 kV and 200 ohms) (Dower et al.1988).

### mini-Tn*5Km*1 suicide mutagenesis

To enable selection of the recipient strain, the plasmid pACYC184 was electroporated into *S. entomophila* strain A1MO2 and recipient strains from conjugation events were selected on media supplemented with chloramphenicol, tetracycline and kanamycin. Conjugations for mini-Tn*5Km1* suicide mutagenesis were then performed as previously described (de Lorenso et al. 1990).

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**DNA isolation and manipulation**

Radioactive probes were made using the GE Healthcare Rediprime DNA labelling system (Amersham, Buckinghamshire, UK). Southern blot and colony hybridisations were performed as described in Sambrook et al. (1989). Genomic DNA was prepared using Wizard Genomic DNA Isolation Kit (Promega). Plasmid templates for sequencing were prepared using Concert® (Invitrogen) miniprep kits. The DNA was sequenced by automated sequencing using an Applied Biosystems 377 autosequencer. Databases at the National Centre for Biotechnology Information were searched using BLASTN and BLASTX (Altschul et al. 1997). $P^{32}$-DNA probes were prepared by random *in vitro* labelling following the procedure of the Rediprime DNA labelling system (GE Healthcare) and purified by passage through a Sephadex G-50 column (Sambrook et al. 1989). The sequence determined in this study has been deposited in GenBank under the accession numbers: gi:1100742 (3PM13R) and gi:1100740 (3PM13F).

**Colony blotting and detection**

Bacterial colonies were transferred onto LB agar and grown overnight at 30°C. Plates were incubated for 1 h at 4°C prior to transfer. Hybond $N^+$ nylon membrane (GE Healthcare) was placed over the colonies, which were transferred through capillary action for 5 min. The membranes were then placed colony side up over 10% SDS solution for 5 min, followed by treatment with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min and then twice treated with neutralising solution (0.5 M Tris pH 8, 1.5 M NaCl) for 5 min. Each exposure to the solutions was achieved through Whatman 3 mm filter paper moistened with the appropriate solution and between each step the membranes were dried by placing on dry tissue for 2 min. DNA was fixed to the membrane by 2 min of exposure to UV light on a UVP transilluminator. Control DNA was fixed to the membrane in an asymmetrical pattern to help align the blot's position on the developed film.

All pre-hybridisation, hybridisation and washing steps were carried out in a Biometra (OV3) oven at 65°C. Blots were pre-hybridised in 2 x SSC (0.3 M NaCl, 30 mM sodium citrate) for 15 min, followed by pre-hybridisation buffer (1 mM EDTA, 0.5 M $Na_2PO_4$, 7 % SDS) for 1–3 h. Denatured probe (12 µl; 95°C, 5 min) was then added and the blots were hybridised overnight. After three 15 min washes in 2 x SSC, the blots were exposed to X-ray film (Kodak, X-OMAT AR) overnight.

## RESULTS

A total of 1200 mutants was screened for inability to grow on itaconate media, by patching onto LB agar and LB agar plates supplemented with kanamycin and itaconate. Two colonies were incapable of growth on media containing itaconate, indicating that the mini-Tn*5K*m1transposon had inserted into a gene involved in metabolism of itaconate. To define the mini-Tn*5K*m1 insertion points in each of the mutants, genomic DNA from the two mutants was isolated and digested in separate reactions with restriction enzymes that did not interrupt the kanamycin encoding sequence of the transposon mini-Tn*5K*m1 (*Eco*RI, *Kpn*I and *Pst*I, Fig. 1) and ligated into the analogous sites of the vector pUC19. Ligation mixes were used to transform *E. coli* DHB101 and this was plated onto LB agar supplemented with ampicillin and kanamycin. Two transformants were identified and designated p1R1 and p3Pst1. The insertion points of each of the clones was defined using the universal M13F and M13R primers and a mini-Tn*5Km*1 specific primer (5'ACTTGTGTATAAGAGTCAG3').

**Probe development**

Sequence data from the mini-Tn*5Km*1 clones p1R1 and p3Pst1 showed that the DNA of p1R1 was conserved to other species showing significant DNA similarity to *Escherichia coli* and *S. marcescens* suggesting that such similarity would also encompass other species of *Serratia* and work with this clone was not continued. However, although the translated sequence generated off the p3Pst1 clone had significant translated similarity to a LysR type transcriptional activator, its DNA showed no similarity to DNA sequences in the current data-base. The 1.7 kb *Eco*RI – *Pst*I fragment was eluted and used to generate a species-specific probe. The probe was used to probe a Southern blot containing *Hin*dIII digested genomic DNA of a selection of *S. entomophila* and *S. proteamaculans* strains (Table 2, Fig. 2). The probe hybridised only to strains of *S. entomophila*.

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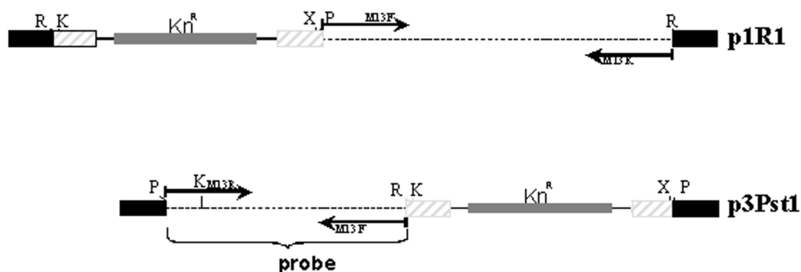


**FIGURE 1: Restriction enzyme maps of clones p1RI and p3PstI. These were obtained from mutagenesis with the mini-Tn*5K*m1 transposon (hatched DNA sequence) and failed to grow on media containing itaconate. The insert was subsequently cloned into the pUC19 vector (solid black). The Kn$^R$ kanamycin resistance encoding gene is denoted by the grey DNA sequence. Arrows denote direction of nucleotide sequence using either the M13 forward (M13F) or M13 reverse (M13R) primers as indicated. Restriction enzymes are abbreviated as follows: R=*Eco*RI; K=*Kpn*I; P=*Pst*I; and X=*Xba*I. Location of the 1.7 kb *Kpn*I – *Eco*RI fragment that was used as the species-specific probe is indicated.**

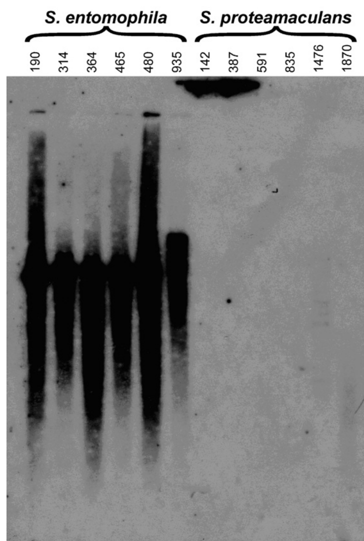


**FIGURE 2: Southern blot hybridisation of *Hin*dIII digested genomic DNA of *Serratia entomophila* and *S. proteamaculans* strains.**

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To assess the probe's utility as a standard method for identifying *S. entomophila* colonies, the probe was used to screen colony blots containing a range of *Serratia* species: *S. entomophila*, *S. proteamaculans*, *S. liquefaciens*, *S. marcescens*, *S. rubidaea* and *S. grimesii*. Only *S. entomophila* colonies probed positive for this fragment (Table 2; Fig. 3).

**TABLE 2: *Serratia* species probed with 1.7 kb DNA fragment from itaconate negative mutant. Eight colonies of each strain were probed.**

| Species | Strain Number[1] | Hybridisation to itaconate probe |
|---|---|---|
| *S. entomophila* | 190, 220, 314, 364, 465, 480, 798, 935, 956[2], 964 | Yes |
| *S. proteamaculans* | 142, 387, 461, 591, 835, 1476, 1870 | No |
| *S. ficaria* | 458 | No |
| *S. fonticola* | 799 | No |
| *S. grimesii* | 348 | No |
| *S. liquefaciens* | 447 | No |
| *S. marcescens* | 363, 505 | No |
| *S. rubidaea* | 242 | No |

[1]AgResearch entomopathogenic bacteria culture collection.
[2]One colony did not grow.

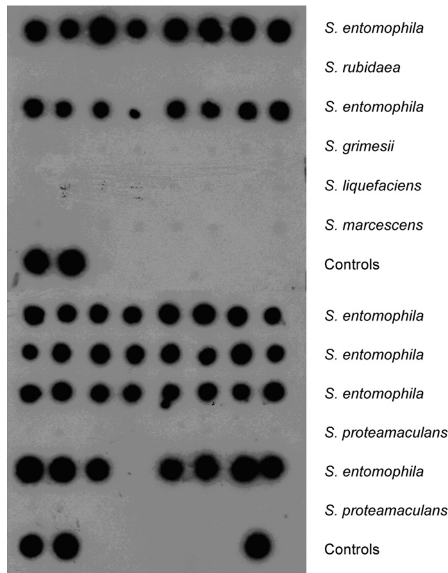


**FIGURE 3: Colony blot hybridisation of *S. entomophila*, *S. proteamaculans*, *S. liquefaciens*, *S. marcescens*, *S. rubidaea* and *S. grimesii* using the 1.7 kb p3Pst1 derived probe. Positive controls are p3Pst1 DNA.**

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## DISCUSSION

Measurement of the establishment and persistence of *S. entomophila* applied to pasture soil for grass grub control is an essential step in optimising the efficacy of this microbial control agent. Assessments have been made by soil dilution plating onto *Serratia*-selective agar, CTA, and then confirming species identity by transferring a representative sample of 25 isolates growing on CTA to three differential agars. This process takes approximately 10 days to complete and is very laborious, prompting the search for alternative identification methods. Through transposon mutagenesis screening, we have identified unique regions of *Serratia* DNA involved in the utilisation of itaconate and the probe developed from these regions enables clear-cut differentiation of colonies of *S. entomophila* from five closely related *Serratia* species. Colonies growing on *Serratia* isolation plates can be transferred for blotting with a p3Pst1 derived PCR-generated probe for *S. entomophila*, reducing the time taken to confirm the presence of *S. entomophila* by approximately 4 days. The method also allows rapid screening of much greater numbers of *Serratia* colonies present on a CTA plate than can be carried out using the differential test agars, thereby improving the statistical reliability of estimates of *S. entomophila* populations. In addition, the colony blots could be used in conjunction with other probes such as the 2.2-kb pADAP pathogenicity probe (Jackson et al. 1997), which is now used routinely as an alternative to bioassays that determine if *Serratia* isolates are pathogenic. In addition, an alternate methodology that combines rapid PCR screening using a combination of primer sets derived from the 1.7 kb *Eco*RI – *Pst*I p3Pst1 species probe and 2.2 kb *Eco*RI-derived pathogenicity probe is also feasible. However, for rapid screening of large numbers of colonies, as is required for studies on the ecology and efficacy of the bacterium, the use of colony blots and the DNA probes remains the most efficient and economic methodology at present.

The translated sequence from the clone p3Pst1 had significant similarity to a regulator and a gene responsible for the degradation of citrate, suggesting the putative genes are responsible for the regulation and degradation of itaconate, respectively. Further investigation into the role of these genes in the ecology of *S. entomophila* is warranted.

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