

# Development and application of polymerase chain reaction-based assays for *Rathayibacter toxicus* and a bacteriophage associated with annual ryegrass (*Lolium rigidum*) toxicity

M. C. Kowalski<sup>A</sup>, D. Cahill<sup>B</sup>, T. J. Doran<sup>A</sup> and S. M. Colegate<sup>A,C</sup>

<sup>A</sup>Australian Animal Health Laboratories, CSIRO Livestock Industries, Geelong, Vic. 3220, Australia.

<sup>B</sup>School of Life and Environmental Sciences, Deakin University, Waurn Ponds Campus, Geelong, Vic. 3220, Australia.

<sup>C</sup>Corresponding author. Email: steve.colegate@csiro.au

**Abstract.** Annual ryegrass toxicity (ARGT) is responsible for significant stock losses in South Australia and Western Australia. The toxicity is caused by corynetoxins produced by the bacterium *Rathayibacter toxicus* (with the possible involvement of a bacteriophage), which infects annual ryegrass (*Lolium rigidum*). Polymerase chain reaction (PCR)-based assays, compatible with an existing enzyme-linked immunosorbent assay for the corynetoxins, have been developed and used to screen *L. rigidum* for both the presence of *R. toxicus* and for the bacteriophage isolate NCPPB 3778. The results from analysing bacterially infected galls from toxic grain screenings showed a positive correlation between the presence of the bacterium and corynetoxins but not with the bacteriophage. Analysis of pasture-derived samples of annual ryegrass showed about a 50% correlation of corynetoxins with bacterial presence and about a 5% correlation of phage with the presence of the bacterium. These observations support the potential application of the PCR-based assays in providing a useful, complementary tool in the assessment of the likelihood of pasture and feed to cause ARGT and to enable a better understanding of the complex aetiology of ARGT.

**Additional keywords:** 16S rRNA, BLAST search, DNA sequencing, nested PCR.

## Introduction

Annual ryegrass toxicity (ARGT) is a potentially fatal disease affecting livestock grazing on pastures or consuming fodder that contains annual ryegrass (*Lolium rigidum*) contaminated with corynetoxins. With an oral median lethal dose of about 1 mg/kg bodyweight for sheep, cattle and pigs (Bourke and Carrigan 1993), corynetoxins are among the more lethal natural toxins. The corynetoxins, a family of tunicaminyluracil glycolipids (Edgar *et al.* 1982), are produced by the bacterium *Rathayibacter toxicus* (Vogel *et al.* 1981) which depends on the nematode *Anguina funesta* as a vector to enable infection of the seedheads of *L. rigidum* (Price *et al.* 1979).

Once carried to the seedhead, the bacterium can outcompete its nematode vector and can fully colonise galls initiated by the nematode. The toxin production within the bacterial gall coincides with the onset of senescence in the annual ryegrass (Stynes and Bird 1983) but the molecular triggers for this production remain undefined. Based upon electron microscopic analysis, it has been speculated (Ophel *et al.* 1993) that a *R. toxicus*-specific bacteriophage, isolated from *R. toxicus*-colonised nematode galls on *L. rigidum*, is involved. This study by Ophel *et al.* (1993) also indicated that *in vitro* infection of *R. toxicus* with the bacteriophage NCPPB 3778 results in an association to form a 'phage-carrier state' and concomitant production of corynetoxins (detected by antibacterial activity) in contrast to the lack of corynetoxin production from cultures

of *R. toxicus* alone. Furthermore, these toxin-producing strains showed unusual morphology with phage particles visible in transmission electron micrographs. Previous examples of enhanced bioavailability or production of specific metabolites by phage-transduced bacteria include the Type A streptococcal exotoxin (McKane and Ferretti 1981; Nida and Ferretti 1982) and the diphtheria toxin released from phage-induced lysogenic *Corynebacterium diphtheriae* (Pappenheimer 1977).

Current methods to confirm a diagnosis of ARGT, or predict the potential for a paddock or feed supplies to cause ARGT, are based on three approaches. The first and most extensively used method relies on the enzyme-linked immunosorbent assay (ELISA)-based detection of water-soluble antigens specific to *R. toxicus* (Masters *et al.* 2006). The second and third approaches indirectly or directly target the corynetoxins rather than the bacterium. A bacterial inhibition assay (Stynes and Vogel 1983; Riley and Ophel 1992) indirectly estimates corynetoxin levels by assessment of the bioactivity of corynetoxins. However, antibacterial activity is not specific to corynetoxins. Direct assays for detection of the corynetoxins include high performance liquid chromatography with peak detection by UV absorption (Cockrum and Edgar 1985) or mass spectrometry (Anderton *et al.* 2004) and a corynetoxins ELISA (Than *et al.* 1998). While any of the current methods, in combination with clinical signs in livestock, can be used to confirm a diagnosis of ARGT, the predictive application relating bacterium presence to

toxicity is problematic (Bucat and Roberts 1994). The cautious approach to predictive capability was highlighted by several presenters at an ARGT workshop (El Cabello Blanco, Western Australia, November 2002) and was related to paddock sampling issues and the highly complex ecosystem that contributes to ARGT.

The present study describes the development of polymerase chain reaction (PCR)-based assays to detect the presence of *R. toxicus* and its associated phage. These assays were then used, in combination with the existing corynetoxin ELISA, to examine pasture-derived samples of *L. rigidum* for a correlation between the presence of phage, *R. toxicus* and corynetoxins. It is expected that these assays may be useful in better defining the factors involved in ARGT and perhaps to improving predictability of the disease.

## Materials and methods

### Plant material

A subsample of harvest grain screenings, collected in Western Australia in 1994 and stored at room temperature, was sieved to concentrate the *L. rigidum* seedheads. Using a dissecting microscope to identify the characteristically golden-coloured bacterially colonised galls, 72 bacterially infected galls were selected from this seed concentrate.

Collection of a sample of mature, dry *L. rigidum* from each of 79 paddocks across the wheatbelt of Western Australia was organised by Dr Jeremy Allen (Department of Agriculture, Western Australia) in the summer of 2004–05. These samples, each consisting of a handful of ryegrass from a different paddock, were collected into separate paper bags to avoid cross-contamination.

### Extraction of corynetoxins and DNA from plant material

The 72 isolated galls were immersed separately in 1% (w/v) aqueous methyl- $\beta$ -cyclodextrin solution (about 0.5 mL) and gently shaken at room temperature for 16 h. Sample extracts were stored at 4°C before corynetoxin ELISA and PCR analysis for *R. toxicus* and the bacteriophage within 24 h.

An entire seedhead from each of the 79 pasture samples was manually broken apart (into small pieces, <10 mm in length) into a suitably sized container and covered with 1% (w/v) aqueous methyl- $\beta$ -cyclodextrin solution (about 1 mL), shaken for 20 h at room temperature and stored at 4°C. ELISA and PCR analysis of these stored samples were repeated several times to ensure reproducibility of the observations.

### Bacterium and bacteriophage isolates

*Rathayibacter toxicus* strain CS14 (NCPPB 3552) and *Rathayibacter tritici* CS108 (both provided by Dr Ian Riley, University of Adelaide, South Australia) were grown and maintained on 523M agar plates at 26°C according to the method of Riley and Ophel (1992). The CS14 isolate was shown to be non-toxigenic since it was inactive in the antibacterial assay used to estimate levels of corynetoxins (Riley and Ophel 1992).

The bacteriophage isolate NCPPB 3778 (provided by Dr Ian Riley, University of Adelaide, South Australia) was stored at 4°C in SM buffer (Sambrook *et al.* 1989) in the presence of 0.5% (v/v) chloroform. A routine plaque assay of the phage isolate was used to determine the phage titre in plaque-forming units (pfu).

A *Curtobacterium* sp. (VKM Ac-1376) was a contaminant of some of the *R. toxicus* and *R. tritici* cultures. It was isolated and identified at the genus level by comparison of the 16S rRNA sequence with published sequences (GenBank AB042086).

### Infection of bacterium with phage

A culture broth (523M media, 100 mL) of *R. toxicus* was maintained for 2 days at 25°C resulting in a cellular density of about  $10^8$  cells/mL. At this stage, about  $10^5$  pfu of bacteriophage isolate NCPPB 3778 were added. The resultant cultures were incubated for a further 10–14 days at 25°C following the method of Ophel *et al.* (1993). Visual inspection of the transfected cultures, for signs of cellular debris and a clarification of the media, revealed phage-induced lysis of bacterial cells from 2–3 days of incubation.

### DNA extraction from bacterium and bacteriophage

Bacteriophage DNA was extracted from the phage-lysed *R. toxicus* cultures and purified from the co-extracted *R. toxicus* DNA using the Mini Lambda DNA purification kit (Qiagen, Sydney, NSW) and differential centrifugation as recommended by the manufacturer.

DNA was extracted from non-toxigenic *R. toxicus* CS14 and the closely related bacteria *R. tritici* and *Curtobacterium* sp., all harvested directly from agar culture plates. An inoculation loop-full of the cultured bacterial cells was suspended in TE buffer (870  $\mu$ L) containing lysozyme (7.5 mg/mL, Sigma, South Croydon, Vic.). The mixture was incubated at 37°C for 1–2 h and briefly vortexed every 20–30 min before the addition of 5 mol/L NaCl (20  $\mu$ L), 10% SDS (100  $\mu$ L) and proteinase K (250  $\mu$ g/mL, 10  $\mu$ L, Sigma) and further incubation at 50°C overnight. Co-extracted RNA was degraded by treatment with RNAase (50  $\mu$ g/mL, 5  $\mu$ L, Sigma) for 30 min at room temperature. The resultant mixture was extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) (Sigma) by vortexing the mixture briefly and centrifuging at 13 000g for 5 min. The upper phase (about 200  $\mu$ L) was transferred to an Eppendorf tube (1.5 mL, Eppendorf, North Ryde, NSW) and the DNA precipitated by addition of isopropanol (210  $\mu$ L). After centrifugation (13 000g, 15 min) and removal of the supernatant, the DNA was resuspended in 70% ethanol (1 mL) and centrifuged (13 000g, 5 min). The resultant DNA pellet was air-dried to remove residual ethanol and then dissolved in distilled water (50  $\mu$ L).

### Assessment of DNA quality

Aliquots (3  $\mu$ L) of all DNA preparations were subjected to electrophoresis on 1% (w/v) agarose gels in 1  $\times$  TAE buffer. After staining with ethidium bromide (0.5  $\mu$ g/mL), DNA bands were photographed and observed for band-excision on a UV transilluminator. The integrity and concentrations of the DNA solutions were estimated spectrophotometrically at 260–280 nm (ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE).

### Cloning a phage DNA fragment for DNA sequence analysis

Phage DNA (50 ng) was digested with *Bam*HI restriction enzyme (Promega, Madison, WI). An electrophoresis-separated and excised 2.6-kb fragment from the digest was ligated into the

*Bam*HI site of the vector pUC19 (Progen, Toowong, Qld) and transformed into *Escherichia coli* Top10F' (Invitrogen, Mt Waverley, Vic.) using standard procedures (Sambrook *et al.* 1989). A successful recombinant plasmid was sequenced by Newcastle DNA Express Sequencing (Newcastle, NSW) using the universal M13 primer.

#### PCR amplification

Forward (F) (TD260) and reverse (R) (TD261) phage DNA primers (Table 1) were designed based on the DNA sequence obtained for the cloned 2.6-kb fragment derived from *Bam*HI restriction enzyme digestion of the phage DNA. The F (TD264) and R (TD265) primers for *R. toxicus* genomic DNA were designed based on the partial sequence of the *R. toxicus* 16S rRNA gene (GenBank accession number: D84127). All primers were synthesised by Sigma.

The PCRs were performed using 96-well plates (Eppendorf). Thus, forward and reverse primers (0.1 µL of 100 µmol/L solutions), Taq (0.1 µL of 5.5 units/µL, BIOTAQ, Bioline, Alexandria, NSW), 10 × buffer (5 µL, Bioline), MgCl<sub>2</sub> (4 µL of 25 mmol/L, Bioline), dNTPs (0.4 µL of 25 mmol/L, Bioline) and dH<sub>2</sub>O (40.60 µL) were added to the template (5 µL of the aqueous solutions of phage or *R. toxicus* DNA, or the aqueous cyclodextrin extracts of the gall or plant material) in each well. The PCR conditions were 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (extension).

Positive control samples (*R. toxicus* and phage DNA) and negative controls (1% aqueous methyl-β-cyclodextrin) were included with every assay in the application of the PCR assay to the plant sample extracts. DNA from *R. tritici* CS108 and *Curtobacterium* sp. VKM Ac-1376 were included as additional controls to monitor the specificity of the *R. toxicus* primers. PCR products were examined for size against a standard DNA ladder (MBI Fermentas, Hanover, MD) by electrophoresis on 1% (w/v) agarose gels and stained with ethidium bromide (0.5 µg/mL) and viewed under UV light.

#### PCR inhibition investigation

Samples of *L. rigidum*, free of *R. toxicus* and the phage, were extracted as above and the extracts spiked with various amounts of bacterial and phage DNA. These DNA-spiked extracts were PCR amplified along with control samples of the DNA in water. In addition, extracts of those samples that were shown to be phage-negative were serially diluted (10<sup>-1</sup>–10<sup>-8</sup>) and

spiked with 75 ng of phage DNA and reanalysed using PCR amplification.

#### Nested PCR

Nested PCR primers (internal primers) were designed (F: TD262 and R: TD263) based on the internal sequence of the phage PCR product obtained using the Phage (F) (TD260) and Phage (R) (TD261) primers (external primers) (Table 1).

Thus, in two successive PCRs, each conducted under the same conditions as previously for the phage and *R. toxicus* PCRs, 0.5 µL of the product from the first amplification (using primers TD260 and TD261) was used as the template in the second PCR amplification using the internal primers TD262 and TD263. The nested PCR product was analysed using agarose gel electrophoresis as previously described.

#### Detection of corynetoxins

An ELISA was used to quantitatively analyse all gall and plant material for corynetoxins according to the method described by Than *et al.* (1998). Briefly, aliquots of the 1% (w/v) aqueous methyl-β-cyclodextrin extracts of the samples were analysed using an indirect ELISA in which the sample analytes in solution competed with a toxin-derived antigen, immobilised on the ELISA plate, for polyclonal corynetoxin antibodies present in diluted serum obtained from a sheep vaccinated with a corynetoxins-derived hapten conjugated to fetal calf serum. After the reaction and washing phases, the samples were mixed with donkey anti-sheep IgG coupled to peroxidase for 2 h at room temperature. Subsequent washing and addition of 3,3',5,5'-tetramethylbenzidine (Sigma) produced a colour in inverse proportion to the amount of corynetoxins in the sample. The concentrations of corynetoxins were subsequently calculated from the optical densities of the samples against a standard curve, measured at 450 nm using an ELISA plate reader (Than *et al.* 1998).

## Results

#### PCR amplification products

The forward and reverse primers specifically designed for the phage and *R. toxicus* PCRs (Table 1) resulted in amplified products of about 1 kb and 200 bp, respectively (Fig. 1). The same bands were observed with PCR amplification of the methyl-β-cyclodextrin extracts of the isolated bacterial galls but not with cyclodextrin solution alone (Fig. 1). No amplified products were obtained with either primer set when tested on their non-target DNA i.e. phage (for the *R. toxicus*-derived primers), *R. toxicus* (for the phage-derived primers), and *R. tritici* or *Curtobacterium* sp. Furthermore, sequence analysis of the 1-kb and 200-bp PCR products revealed DNA sequences with exact homology to the 2.6-kb phage DNA fragment and the 16S rRNA sequence of *R. toxicus* respectively.

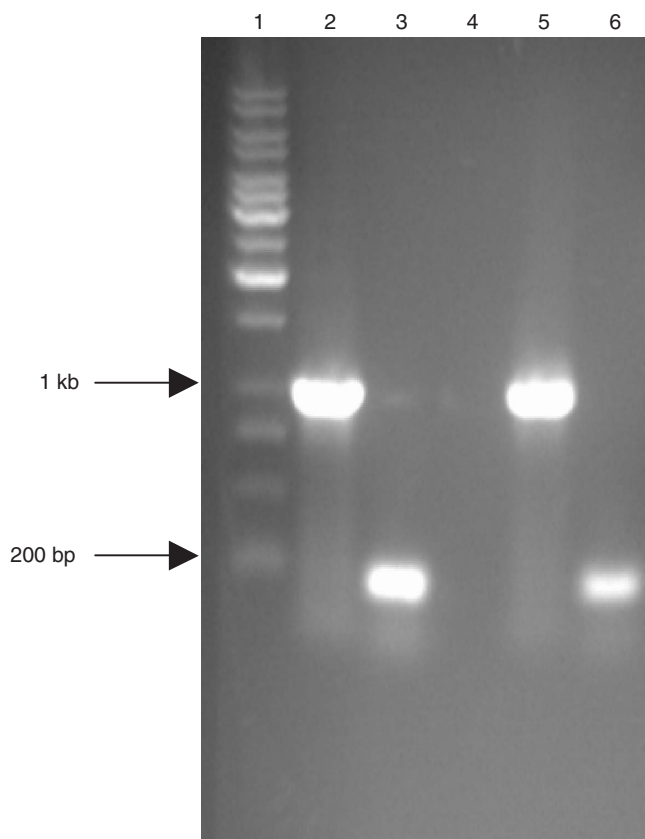
#### Analysis of samples derived from annual ryegrass

The 72 bacterially infected galls selected from concentrated grain screenings were all strongly positive (in excess of the ELISA corynetoxins standard at 6400 pg/0.1 mL extract) for corynetoxins by ELISA. All of these samples were also PCR-positive for the presence of *R. toxicus*. However, only 49 of the 72 were PCR-positive for the bacteriophage. Five phage-positive

**Table 1. Oligonucleotide sequences of the forward (F) and reverse (R) PCR primers used to amplify bacteriophage NCPPB 3778 and *Rathayibacter toxicus* DNA**

The primers for the nested phage PCR are also shown

Primer	5'–3' nucleotide sequence
TD260 – Phage (F)	ACCGCACATCTGCTCTATCC
TD261 – Phage (R)	CGTACAGCAGGAGGGTACTC
TD262 – Nested phage (F)	TACCACTCCGACAACATCAC
TD263 – Nested phage (R)	CACCTTATTCAAAACCCGCA
TD264 – <i>Rathayibacter toxicus</i> (F)	AGCGAATCCCAAAAAGCC
TD265 – <i>Rathayibacter toxicus</i> (R)	ACTTAGTCCTAATCACCGATC



**Fig. 1.** PCR-amplified products resulting from testing gall material. Lane 1, 1 kb Plus DNA ladder; lane 2, positive control: 1 kb amplified product from phage genomic DNA; lane 3, positive control: 200 bp amplified product from *R. toxicus* genomic DNA; lane 4, negative control: cyclodextrin solvent; lane 5, 1 kb phage amplified product from positive gall; and lane 6, 200 bp *R. toxicus* amplified product from positive gall.

and five phage-negative samples were diluted and reanalysed for corynetoxins. The dilution steps (up to 1 : 1000) aligned the corynetoxin concentration in the sample to the linear portion

of the calibration curve (100–6400 pg/100  $\mu$ L) allowing a more accurate estimation of content. Given that each gall was extracted with 200  $\mu$ L of solvent, the ELISA concentrations (pg/100  $\mu$ L) of corynetoxins in the phage-positive samples were calculated to represent 10.5, 7.1, 3.7, 3.2 and 0.5  $\mu$ g/gall and 5.0, 3.2, 2.6, 2.1 and 0.6  $\mu$ g/gall in the phage-negative samples.

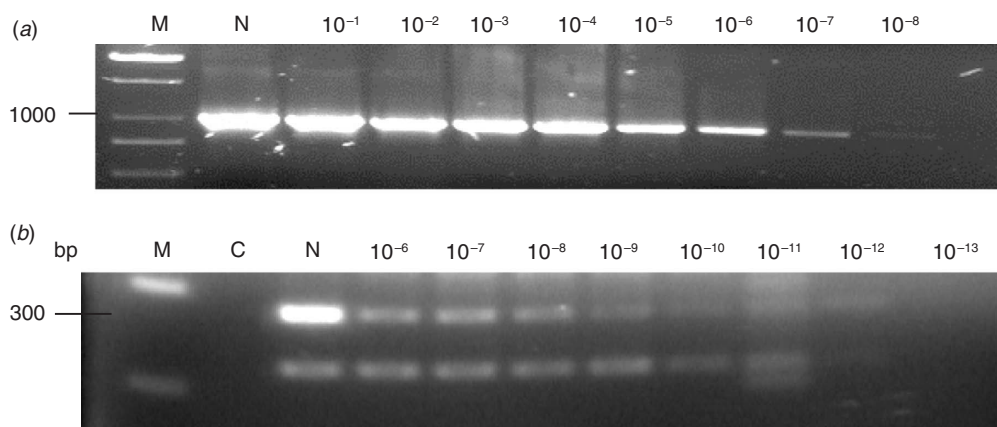
Out of the seedheads from the 79 pasture samples of *L. rigidum*, 68 were positive for *R. toxicus* and 32 of these were also positive for corynetoxins, 30 of which returned levels in excess of the maximum calibration standard used in the ELISA, i.e. 6400 pg/0.1 mL. Only 4 of the 79 pasture seedhead samples were positive for phage. The four phage-positive samples were also positive for *R. toxicus* and for corynetoxins. Nine of the 32 corynetoxin-positive samples, including the four phage-positive but none of the corynetoxins-negative samples, displayed the yellow bacterial slime associated with ARG. The repeat analysis of the extracts of the seedheads from the entire 79 pasture samples provided the same observations.

#### PCR inhibition and nested PCR

The phage DNA-spiked samples all resulted in the expected phage amplification product. There was no evidence of matrix-related inhibition of the PCR amplification of phage DNA. The nested PCR phage DNA amplification product was about 300 bp and enabled the detection of phage-related DNA at dilutions down to  $10^{-12}$  of the primary phage PCR template solutions (Fig. 2). This product was observed with the PCR phage-positive samples but not with any of the phage-negative samples tested. A lower band observed in the nested PCR was considered to be non-specific, perhaps even a primer dimer, as it did not titrate out as did the upper band with increasing dilution of the sample.

#### Discussion

In order to achieve PCR amplification of *R. toxicus* and phage DNA, specific sequence primers were successfully developed. In the case of the phage, the specific sequence was obtained by first digesting the phage DNA and selecting a 2.6-kb fragment for insertion into a plasmid. The nucleotide sequence of the cloned plasmid was compared to those existing in GenBank by performing a BLAST search (Altschul *et al.* 1990). The



**Fig. 2.** Phage PCR and nested phage PCR sensitivity. 10-fold dilutions of phage DNA (75 ng/ $\mu$ L) from  $10^{-1}$  to  $10^{-8}$  were used. Undiluted phage DNA (N), a blank control (C) and molecular mass marker 1 kb plus ladder (M) were also included. (a) Phage PCR: product was detected up to  $10^{-7}$  dilution, which corresponds to about 100 phage particles. (b) Nested phage PCR: product was detected up to the dilution  $10^{-12}$ , which corresponds to <1 phage particle.

lack of any existing homology supported the uniqueness of the 2.6-kb fragment to this phage isolate and increased confidence that the PCR primers designed from the derived sequence would be specific for the phage.

For PCR detection of bacteria, the primers are often derived from 16S rRNA gene sequences (Deng and Hiruki 1991; Lee *et al.* 1993; Namba *et al.* 1993). This gene is highly conserved between species; however, it does contain some variable regions that can be used to develop bacterial species-specific primers. Consequently, analysis of 16S rRNA has proven to be very useful and reliable in the classification of microorganisms and has allowed for rapid differentiation of phytopathogenic coryneform bacteria for diagnostic application (Drennan *et al.* 1993; Pallen *et al.* 1994; Lee *et al.* 1997). The coryneform *R. toxicus* has previously been distinguished from other species in the same genus on the basis of 16S rRNA gene typing (Sasaki *et al.* 1998). Therefore, primers for the PCR amplification of *R. toxicus* were designed from the reported partial sequence of the 16S rRNA gene (GenBank accession number D84127) to specifically amplify *R. toxicus* DNA and differentiate it from *R. tritici*. Specifically, the reverse primer falls within the variable region 9 (V9) domain of 16S rRNA (Coenye and Vandamme 2003).

The phage PCR did not amplify the bacterial DNA used in the present study, nor did the *R. toxicus* PCR amplify phage DNA or the DNA extracted from the non-target bacteria used in the present study. This observation increased the confidence in the presumed specificity of the primers designed for PCR amplification of both the phage and *R. toxicus* DNA. The sensitivities of the PCRs were determined by spiking and serially diluting 50 ng of phage or *R. toxicus* genomic DNA into an extract of *L. rigidum* already shown to be negative for both PCRs. The *R. toxicus* PCR detected 5 fg ( $10^{-7}$  dilution) of DNA, which correlates to about two *R. toxicus* cells assuming that the total genome is 2500 kb (Agarkova *et al.* 2006). The phage PCR also readily detected 5 fg ( $10^{-7}$  dilution) correlating to about 100 phage particles given that the phage genome is about 45 kb (Ophel *et al.* 1993).

For application of the new PCR-based assays, the samples were extracted with a 1% aqueous  $\beta$ -cyclodextrin solution that provided an extract that was compatible with the PCR assay and the corynetoxins ELISA. Thus, each sample could be analysed for the presence of *R. toxicus*, bacteriophage NCPPB 3778 and the corynetoxins without the sampling issues associated with preparing different samples for different analyses.

The PCR assays and the corynetoxins ELISA were initially applied to extracts of 72 bacterially colonised galls collected from annual ryegrass-contaminated grain screenings obtained at harvest in Western Australia. As expected, the selected galls were each positive for the presence of the bacterium and the corynetoxins. However, the observation that 23 of these bacterium- and corynetoxin-positive samples were negative for the phage was inconsistent with a tripartite relationship between the bacterium, bacteriophage and corynetoxins. Five randomly selected phage-positive extracts and five randomly selected phage-negative extracts were re-analysed to establish whether phage presence impacted upon the concentration of corynetoxins in the bacterially infected gall. This re-analysis made optimum use of the quantitative capability of the corynetoxin ELISA (Than *et al.* 1998) by applying sufficient dilution of the strongly

corynetoxin-positive extracts to ensure that the final optical density was within the linear portion of the calibration standard curve included on each ELISA plate. As expected, since the size of the galls varied, the results indicated a wide range of corynetoxin content (0.5–10  $\mu$ g corynetoxin/gall). However, there was no difference between corynetoxin content in phage-positive or phage-negative galls.

The analytical results from the 79 pasture samples of *L. rigidum* clearly indicated two things. Firstly, the presence of bacterium, in the present study of single seedheads selected from pasture samples of annual ryegrass, did not imply the presence of corynetoxins, even though the samples were collected at a time of plant senescence when the bacterium is expected to produce the toxins (Stynes and Bird 1983). Secondly, the presence of corynetoxins and/or *R. toxicus* did not imply the presence of phage in the present study. The lack of a positive correlation between the presence of *R. toxicus* and corynetoxins supports the observation that, on average, only 1 in 5.75 bacterially infected paddocks are toxic to sheep (D. Roberts, ARGT Workshop, El Cabello Blanco, Western Australia, November 2002, pers. comm.). However, this support would need to be considered in the context of the complex interactions that constitute overt toxicity. Thus, lack of ARGT on bacterially infected pasture may also be due to factors such as the dose of toxin ingested by sheep and the period of exposure, the condition of the sheep, overall pasture composition and vagaries such as local weather conditions. Additionally, given the sensitivity of the PCR assay, very low levels of toxigenic bacterium could well be detected at a stage before the bacteria have reached a population size that would initiate toxin production via quorum sensing for example (for an overview of quorum sensing the reader is referred to <http://www.nottingham.ac.uk/quorum/> and references therein, verified 10 October 2006). It could be envisaged that once the *R. toxicus* population had reached a sustainable population, then, faced with decreasing nutrients derived from a senescing plant, small-molecule signalling (quorum sensing) could initiate a concerted expression of antibiotic toxin by the *R. toxicus* population to inhibit competitors.

These observations with the isolated galls and the pasture samples could indicate that phage is not required for the production of corynetoxins by *R. toxicus*. The absence of corynetoxins in about 50% of those pasture samples that were positive for *R. toxicus* may indicate an as yet unknown or undefined stimulus required to initiate toxin production. In the absence of any PCR amplification product from *R. tritici* or the *Curtobacterium* sp. it is unlikely that the PCR product in the corynetoxin-negative samples is the result of non-specific amplification. However, differential sensitivities of the assays leading to false negatives could not be totally discounted on this data.

Although the corynetoxin ELISA can detect as little as 50 pg/100  $\mu$ L, the *R. toxicus* PCR is estimated to detect as few as two bacteria. Therefore, it is conceivable that trace amounts of *R. toxicus* DNA may be amplified and detected without a corresponding detection of corynetoxins. This could be addressed by developing a quantitative approach to the PCR assay (real time PCR) and correlating corynetoxins to *R. toxicus* in isolated bacteria-infected galls. The derived correlation could then be applied to field samples of pasture where *R. toxicus* was

detected to produce an indication of the level of corynetoxins that should be expected.

The potential for false negatives in the phage PCR assay was addressed in two ways: (i) the elucidation of any inhibitory matrix effects in the PCR assay; and (ii) by enhancing the specific sensitivity of the phage assay via a nested PCR approach. Thus, seven of the isolated bacterially infected gall samples that failed to amplify phage DNA were serially diluted (to lessen any matrix-related inhibitory effect) and spiked with phage DNA. All spiked samples were phage-positive in subsequent PCR reactions indicating that nothing inhibitory was present within the sample to prevent the phage PCR. Application of the nested PCR approach enabled an enhancement of about 10 000-fold in the detection of phage, ensuring detection of a single phage particle. Nested PCR analysis of eight gall samples, which included both PCR phage-positive and phage-negative samples, only produced an amplified product in those samples that were PCR phage-positive, thereby increasing the confidence that the negative phage results were real.

In this preliminary application of the PCR assays in combination with the corynetoxin ELISA, neither the geographic location of sample collection, presence of the yellow, bacterial slime nor the level of corynetoxins could differentiate the samples that were both corynetoxin- and phage-positive from those that were both corynetoxin-positive and phage-negative.

As the proposed involvement of the phage in corynetoxin production in the field is speculative, it is not known how much phage would be required and whether the current PCR assay is sensitive enough to detect a minor involvement. It is also possible that unknown matrix effects might hinder the extraction and or PCR amplification of phage DNA from annual ryegrass samples. However, several experiments in which methyl- $\beta$ -cyclodextrin extracts of annual ryegrass were spiked with phage DNA did not identify problems with sample matrix effects (data not shown). Additionally, the extraction and PCR amplification of *R. toxicus* DNA was not apparently hindered and it would require a significant difference in the biology and or ecology of the bacterium and its putative associated phage if detection of one and not the other was so severely affected. Another possibility is that more than one species or strain of phage, that was not identified in earlier research (Ophel *et al.* 1993) nor detected by the specific primers designed for phage NCPPB 3778, might be involved in the production of the corynetoxins.

Combined with potential development of a PCR-based method of detection for the nematode vector (J. Allen, pers. comm.) and the existing ELISA for corynetoxins, the PCR-based assays for *R. toxicus* and the bacteriophage NCPPB 3778 may provide a method for assessing pasture and feed for all the known, or in the case of the phage, suspected, factors contributing to ARGT. Such a rapid and comprehensive detection system could be regularly used before and during the ARGT season to provide for more timely and effective control and management of ARGT.

## Acknowledgements

Dr Jeremy Allen (Department of Agriculture, Western Australia) and Dr Ian Riley (University of Adelaide, South Australia) are gratefully acknowledged for providing material for this study. Neil Anderton and Yu Cao (Plant Toxins Research Group, Geelong) are thanked for their assistance with the gall selections and corynetoxins ELISA respectively.

Dr Vicky Stevens and Dr Volker Haring (Australian Animal Health Laboratory, Geelong) are thanked for reviewing this document as part of the CSIRO Livestock Industries internal review process. The project was funded by Deakin University and the CSIRO. The authors sincerely acknowledge the contribution of the anonymous referees in this final version of this report.

## References

- Agarkova IV, Vidaver AK, Postnikova EN, Riley IT, Schaad NW (2006) Genetic characterization and diversity of *Rathayibacter toxicus*. *Phytopathology*, in press. doi: 10.1094/PHTO-96-1270
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410. doi: 10.1006/jmbi.1990.9999
- Anderton N, Beales KA, Cao Y, Colegate SM, Edgar JA, Michalewicz A, Riley IT, Stewart PL, Than KA (2004) The identification of corynetoxin-like tunicaminyluracil-glycolipids from nematode galls in *Festuca nigrescens* from North America and New Zealand. In 'Poisonous plants and related toxins'. (Eds T Acamovic, CS Stewart, TW Pennycott) pp. 204–209. (CABI: Wallingford, UK)
- Bourke CA, Carrigan MJ (1993) Experimental tunicamycin toxicity in cattle, sheep and pigs. *Australian Veterinary Journal* **70**, 188–189.
- Bucat J, Roberts WD (1994) Prevalence of the causative agent of annual ryegrass toxicity in areas where the disease is endemic or sporadic. In 'Plant-associated toxins: agricultural, phytochemical and ecological aspects'. (Eds SM Colegate, PR Dorling) pp. 57–61. (CAB International: Wallingford, UK)
- Cockrum PA, Edgar JA (1985) Rapid estimation of corynetoxins in bacterial galls from ryegrass (*Lolium rigidum* Gaud.) by high-performance liquid chromatography. *Australian Journal of Agricultural Research* **36**, 35–41. doi: 10.1071/AR9850035
- Coenye T, Vandamme P (2003) Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiology Letters* **228**, 45–49. doi: 10.1016/S0378-1097(03)00717-1
- Deng S, Hiruki C (1991) Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods* **14**, 53–61. doi: 10.1016/0167-7012(91)90007-D
- Drennan JL, Westra AAG, Slack SA, Delsere LM, Collmer A (1993) Comparison of DNA hybridization probe and ELISA for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. *Plant Disease* **77**, 1243–1247.
- Edgar JA, Frahn JL, Cockrum PA, Anderton N, Jago MV, Culvenor CCJ, Jones AJ, Murray K, Shaw KJ (1982) Corynetoxins, causative agents of annual ryegrass toxicity; their identification as tunicamycin group antibiotics. *Journal of the Chemical Society. Chemical Communications* **4**, 221–224.
- Lee IM, Hammond RW, Davies RE, Gunderson DE (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma like organisms. *Phytopathology* **83**, 834–842.
- Lee IM, Klopmeyer M, Bartoszyk IM, Gunderson-Rindal DE, Chou TS, Thomson KL, Eisenreich R (1997) Phytoplasma induced free-branching in commercial poinsettia cultivars. *Nature Biotechnology* **15**, 178–182. doi: 10.1038/nbt0297-178
- Masters AM, Gregory AR, Evans RJ, Speijers JE, Sutherland SS (2006) An enzyme-linked immunosorbent assay for the detection of *Rathayibacter toxicus*, the bacterium involved in annual ryegrass toxicity, in hay. *Australian Journal of Agricultural Research* **57**, 731–742.
- McKane L, Ferretti J (1981) Phage-host interactions and the production of Type A streptococcal exotoxin in group A streptococci. *Infection and Immunity* **34**, 915–919.
- Namba S, Oyaizu H, Kato S, Iwanami S, Tsuchizaki T (1993) Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. *International Journal of Systematic Bacteriology* **43**, 461–467.

- Nida S, Ferretti J (1982) Phage influence on the synthesis of extracellular toxins in group A streptococci. *Infection and Immunity* **36**, 745–750.
- Ophel KM, Bird AF, Kerr A (1993) Association of bacteriophage particles with toxin production by *Clavibacter toxicus*, the causal agent of annual ryegrass toxicity. *Molecular Plant Pathology* **83**, 676–681.
- Pallen MJ, Hay AJ, Puckey LH, Efstratiou A (1994) Polymerase chain reaction for screening clinical isolates of corynebacteria for the production of diphtheria toxin. *Journal of Clinical Pathology* **47**, 353–356.
- Pappenheimer A (1977) Diphtheria toxin. *Annual Review of Biochemistry* **46**, 69–94. doi: 10.1146/annurev.bi.46.070177.000441
- Price PC, Fisher JM, Kerr A (1979) On *Anguina funesta* n. sp. and its association with *Corynebacterium* sp., in infecting *Lolium rigidum*. *Nematologica* **25**, 76–85.
- Riley IT, Ophel KM (1992) Rapid detection of corynetoxins produced by *Clavibacter toxicus*. *Letters in Applied Microbiology* **14**, 96–99.
- Sambrook J, Fritsch EF, Maniatis TA (1989) 'Molecular cloning: a laboratory manual.' 2nd edn. (Cold Spring Harbor Laboratory: New York)
- Sasaki J, Chijimatsu M, Suzuki K (1998) Taxonomic significance of 2,4-diaminobutyric acid isomers in the cell wall peptidoglycan of actinomycetes and reclassification of *Clavibacter toxicus* as *Rathayibacter toxicus* comb. nov. *International Journal of Systematic Bacteriology* **48**, 403–410.
- Stynes BA, Bird AF (1983) Development of annual ryegrass toxicity. *Australian Journal of Agricultural Research* **34**, 653–660. doi: 10.1071/AR9830653
- Stynes BA, Vogel P (1983) A bacterial inhibition assay for corynetoxins from parasitised annual ryegrass. *Australian Journal of Agricultural Research* **34**, 483–489. doi: 10.1071/AR9830483
- Than KA, Cao Y, Michalewicz A, Edgar JA (1998) Development of an immunoassay for corynetoxins. In 'Toxic plants and other natural toxicants'. (Eds T Garland, AC Barr) pp. 49–54. (CAB International: Wallingford, UK)
- Vogel P, Petterson DS, Berry PH, Frahn JL, Anderton N *et al.* (1981) Isolation of a group of glycolipid toxins from seedheads of annual ryegrass (*Lolium rigidum* Gaud.) infected by *Corynebacterium rathayi*. *The Australian Journal of Experimental Biology and Medical Science* **59**, 445–467.

Manuscript received 17 June 2005, accepted 19 June 2006