The utility of the intergenic transcribed spacer region 1 as a molecular marker for the identification and discrimination of the *Enterobacteriaceae* associated with Acute Oak Decline

James Doonan[1] (bsp017@bangor.ac.uk), Sandra Denman[2], Christoph Gertler[1], Justin A. Pachebat[3], Peter N. Golyshin[1] and James E. McDonald[1]
[1]School of Biological Sciences, Bangor University, Deiniol Road, Bangor, Gwyneed, LL57 2UW, United Kingdom; [2]Forest Research, Alice Holt Lodge, Farnham, GU10 4LH, Surrey, U.K.;
[3]Institute of Biological Environmental Rural Sciences, Aberystwyth University, Penglais, Aberystwyth, Ceredigion, SY23 3DA, United Kingdom .

Abstract

The molecular identification of environmental *Enterobacteriaceae* strains from trees affected by Acute Oak Decline (AOD) is a laborious and costly process, particularly when a large number of isolates are obtained. Currently, strain identification relies on PCR amplification, DNA sequencing and phylogenetic analysis of marker genes such as 16S rRNA or DNA gyrase B. Here, we describe the use of the intergenic spacer region 1 (ITS1) for the rapid, inexpensive and accurate typing of bacterial strains belonging to the *Enterobacteriaceae*. The method was validated using six cultured *Enterobacteriaceae* strains isolated from oak trees. The ITS1 and gyrase B genes of five sub-isolates of each strain (n=30) were amplified using PCR followed by sequencing of ITS1 and *gyrB* amplicons. The number and size of ITS1 amplicons for each sub-isolate was subsequently determined using both 3% agarose gel electrophoresis (figure 1) and polyacrylamide gel electrophoresis (figure 2). Each bacterial species was found to have a unique ITS1 profile (between 3 and 7 amplicons). The validated technique was subsequently used to screen a further 270 sub-isolates derived from 54 *Enterobacteriaceae* strains, providing accurate species specific profiles of ITS1 amplicons. The method was found to have equivalent sensitivity to PCR amplification and sequence analysis of the DNA gyrase B sequencing gene, but with significantly reduced processing time and cost.

Introduction

Acute Oak Decline (AOD) is a syndrome which causes tissue necrosis on the bark of mature oak trees. Previous studies have described the consistent isolation of *Enterobacteriaceae* from necrotic lesions of affected oak trees in sampling sites across England and Wales (Brady *et al*, 2010; Denman *et al*, 2012). It has been suggested that these bacteria have a central role in tissue necrosis.
The rRNA (rrn) operon is a conserved genomic coding region which is commonly used for bacterial genotypic identification (Woese & Fox, 1977). Due to the conserved nature of the operon, resolution of closely related species within certain taxa such as the *Enterobacteriaceae* can be problematic (Janda & Abbott, 2007). The dearth of sequence differences among closely related species contributes to a lack of phylogenetic power and limitations to its abilities of taxonomic resolution. The intergenic or internal transcribed spacer region 1 (ITS1) is part of the rrn operon and is found between the small (16S) and large (23S) subunit rRNA. The ITS1 region is more susceptible to synonymous mutations than other parts of the operon due to its functional role being confined to the coding of tRNAs and processing rRNA (Scheinert *et al*, 1996). This variation makes it an excellent candidate gene for molecular differentiation between both diverse and closely related species. Its use in arboreal microbial analysis has not as yet been tested.

Methods

61 strains of *Enterobacteriaceae* were amplified using ITS1 specific oligonucleotide primers, designed for environmental bacterial communities ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale et al, 2004). PCR amplification products were visualised using 3% agarose gel and polyacrylamide gel. Phylogenetic analysis was performed using the PAST software package (Hammer *et al*, 2001) using a jaccard analysis to evaluate similarities between ITS1 amplicon patterns on acrylamide gels. To validate this approach, the identity of *Enterobacteriaceae* strains exhibiting different ITS1 amplicon sizes and number was confirmed by PCR amplification and sequencing of the *gyrB* and ITS1 genes (figure 3).

Results

Experimental results confirm that ITS1 profiling enabled discrimination between different species. Figure 1 reveals genus level differentiation properties of ITS1 between *Rahnella aquatilis*, *Gibbsiella quercinecans*, *Enterobacter cloacae* and *Brenneria goodwinii*. The polyacrylamide gel shown in figure 2 revels intraspecific amplicon patterns amongst Gibbsiella quercinecans and Brenneria goodwinnii. This strain specificity was further unravelled using the PAST phylogenetic analysis program (figure 4).

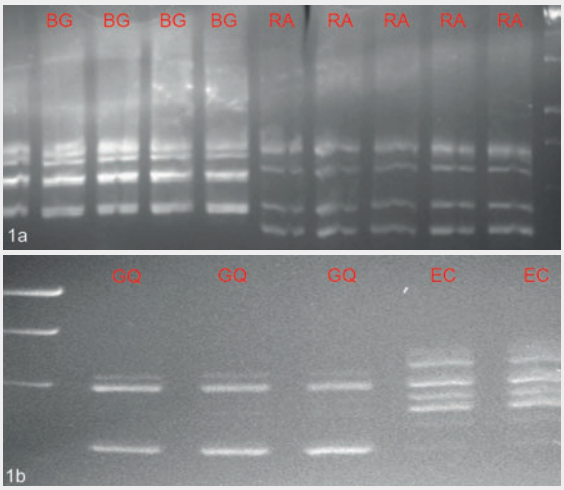

Figure 1a: Agarose gel revealing a distinct ITS1 amplicon pattern between *Brenneria goodwinii* (BG), lanes 1-5 & *Rahnella aquatilis* (RA), lanes 6-10, lane 11 contains a molecular size marker. **Figure 1b**: Agarose gel revealing a contaminant in a *Gibbsiellla quercinecans* (GQ) culture lanes 2-4, and a contaminant bacterium *Enterobacter cloacae* (EC), lanes 5-6. Lane 1 contains a molecular size marker.

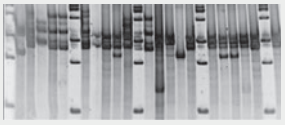

Figure 2: Polyacylamide gel with ITS1 profiles for *G. quercinecans*, lanes 2-6; 8-12; 14-15 and *B. goodwinii*, lanes 16-18; 20-24. Lanes 1,7,13,19, 25 contain a molecular size marker.

Isolate (sub-isolate)	Sequence ID (gyrB)	Number of ITS1 bands & amplicon size (bp) S	equence ID (ITS1)
Gibbsiella quercinecans FRB98 (I8A) *E*	*nterobacter cloacae*	3 (230, 400, 420) 3	bands sequenced, all ITS1
Gibbsiella quercinecans FRB98 (I8B) *E*	*nterobacter sp.*	3 (230, 400, 420) 3	bands sequenced, all ITS1
Gibbsiella quercinecans FRB98 (I8C) *E*	*nterobacter cloacae*	3 (230, 400, 420) 3	bands sequenced, all ITS1
Gibbsiella quercinecans FRB98 (I8D) *G*	*ibbsiella quercinecans* FRB98	6 (340, 360, 380, 390, 400, 450) 4	bands sequenced, all ITS1
Gibbsiella quercinecans FRB98 (I8E) *G*	*ibbsiella quercinecans* FRB98	6 (340, 360, 380, 390, 400, 450) 5	bands sequenced, all ITS1
Brenneria goodwinii R43477 (I67A) *B*	*renneria goodwinii R43477*	6 (340, 360, 370, 400, 430, 520) 3	bands sequenced, all ITS1
Brenneria goodwinii R43477 (I67B) *B*	*renneria goodwinii R43477*	6 (340, 360, 370, 400, 430, 520)	2 bands sequenced, all ITS1
Brenneria goodwinii R43477 (I67C) *B*	*renneria goodwinii R43477*	6 (340, 360, 370, 400, 430, 520) 3	bands sequenced, all ITS1
Brenneria goodwinii R43477 (I67D) *B*	*renneria goodwinii R43477*	6 (340, 360, 370, 400, 430, 520) 3	bands sequenced, all ITS1
Brenneria goodwinii R43477 (I67E) *B*	*renneria goodwinii R43477*	6 (340, 360, 370, 400, 430, 520) 1	band sequenced, ITS1

Figure 3: Identification of bacterial isolates and confirmation of purity. The bacterial identity is shown in column 1 and the strain validation method is in columns 2-4, using DNA gyrase B, ITS1 amplicon pattern and ITS1 sequencing.

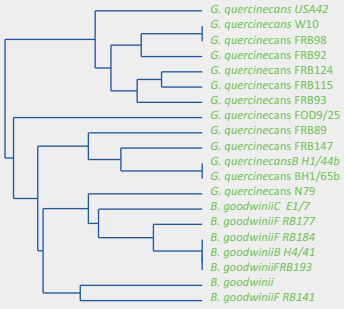

Figure 4: Preliminary data from a phylogenetic analysis of AOD isolates using the ITS1 molecular marker.

Conclusions

This is a simple, accurate, inexpensive method for rapid screening of *Enterobacteriaceae* isolates and provides robust species specific profiles. The method can be applied without specialised laboratory equipment and does not require technical expertise. This allows environmental screening of a large number of isolates providing species identification and differentiation data or further analysis to ensure purity of isolates.

References
Brady C, Denman S, Kirk S, Venter S, Rodriguez-Palenzuela P, Coutinho T (2010) Description of *Gibbsiella quercinecans* gen. nov., sp. nov, associated with acute oak decline. *Syst Appl Microbiol*; **33**, 444-450.
Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol*, **70**, 6147-6156.
Denman S, Brady C, Kirk S, Cleenwerck I, Venter S, Coutinho T, De Vos P (2012) *Brenneria goodwinii* sp.nov., associated with acute oak decline in the UK. Int J *Syst Evol Microbiol*; **62**, 2451-5.
Gurtler V & Stanisich (1996) New approaches to typing and identification of bacteria using the 16-23S rDNA spacer region. Microbiology; 142, 3-16.
Hammer O, Harper DAT, Ryan PD (2001) PAST: Paleontological Statistics software package for education and data analysis. *Palaeontol Electron* **4**(1), 9.
Janda MJ & Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils and pitfalls. *J Clin Microbiol*; **45**(9), 2761.
Scheinert P, Krausse R, Ullmann U, Soller R, Krupp G (1996) Molecular differentiation of bacteria by PCR amplification of the 16-23S rRNA spacer. *J Microbiol Meth*; **26**, 103-117.
Woese CR & Fox GE (1977) Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proc Natl Acad Sci USA*; **74**, 5088–5090.

Acknowledgements
We thank the Forestry Commission, Woodland Heritage and the Rufford Foundation for funding this study, and Andy Peace for the statistical analyses.