Microbial characterisation of soils from offal pits

AgResearch

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For:

Summary

A preliminary analysis of the soil bacterial communities in an offal pit used to dispose of GM cattle was carried out. Soil samples were collected from several depths from within the pit, and also from a site adjacent to the pit in May 2004. Samples from various depths in the soil profile were dilution plated to give an estimate of total populations of culturable aerobic bacteria. Numbers of antibiotic resistant bacteria were also estimated and colonies were probed for the presence of puromycin and kanamycin (*npt*II) resistance genes. Bacterial diversity in the various soil samples was compared by 16S rRNA gene-based profiling and denaturing gradient gel electrophoresis (DGGE).

Statistical analysis of the data was not carried out because of the limited number of replicates and lack of comparative samples between the two sites.

Total numbers of culturable aerobic bacteria were generally similar in the offal pit and control soils, although greater numbers were recovered at depth in the offal pit soils.

Kanamycin-resistant bacteria were commonly recovered from offal pit and control soils, in numbers ranging up to almost 10^5 / g soil. When kanamycin-resistant bacteria were probed for the presence of the *npt*II gene, a higher proportion of colonies probed positive, in comparison with those from control soils.

Bacteria tolerant of puromycin (125 µg/ml) were isolated from one offal pit and a comparative control soil, where they constituted less that 2% of the populations of total culturable bacteria. When probed for the presence of the puromycin-resistance gene, between 19 and 88% of the puromycin-resistant colonies probed positive. Other soil samples, from both within and outside the offal pit also yielded bacterial colonies that gave a positive reaction when probed.

DGGE profiles from three of the samples collected from near the soil surface outside the offal pit were very similar, with numerous bands present. Some bands are common to both the control and offal pit soils, but the patterns of soil bacteria recovered from the offal pit samples appear to be more diverse.

Detection of soil bacteria potentially carrying the antibiotic resistance genes for puromycin and kanamycin through colony hybridisation was an exciting result from this study. Further research is needed to confirm the presence of the genes through amplification and sequencing.

Background

Studies on the soil microbiology of offal pits are important in context of disposal of genetically modified animals, which in New Zealand, must be by burial. We could expect that the addition of a large amount of relatively readily decomposable organic resource with a heavy microbial inoculum (in the form of the enteric community of the animal buried) will lead to increased microbial biomass and activity in surrounding soil. However, details of the microbial processes in the decomposition of carcasses of farm animals have not been studied. A preliminary analysis of the bacterial communities in offal pit soil and comparative soil samples from adjacent to the pit was undertaken in May 2004. Samples from various depths in the soil profile were dilution plated to give an estimate of total populations of culturable aerobic bacteria. Numbers of antibiotic resistant bacteria were also estimated and colonies were probed for the presence of puromycin and kanamycin (*npt*II) resistance genes. Bacterial diversity in the various soil samples was compared by 16S rRNA gene-based profiling and denaturing gradient gel electrophoresis (DGGE).

Information collected in this study will be useful in preparation of future ERMA applications, by demonstrating that the method of disposal of GM animal carcasses is safe for the environment. To our knowledge this type of study has not been carried out before and will therefore be of general scientific interest as well.

Methods

Sampling of offal pit

Samples were collected by and transferred to AgResearch for analysis. Because of difficulties with the sampling, fewer samples were collected than initially planned (11 samples from offal pit and 9 from surrounding soil, adjacent to the pit). Again, because of difficulties with sampling, these samples were not always closely matched with respect to depths in profile.

Enumeration of culturable bacterial populations in soil and incidence of antibiotic resistant bacteria

Enumeration of bacteria

Total numbers of aerobic culturable bacteria present in the samples were estimated by plating soil dilutions on 10% Tryptic Soya Agar containing 100mg/ml cycloheximide. Plates were incubated at 20°C for 2 days before colonies were counted.

The incidence of antibiotic resistant bacteria in the soil samples was determined by plating onto TSA containing antibiotics (125 µg/ml puromycin; 50 and 100 mg/ml kanamycin). Because of the high cost of puromycin, only two samples were plated onto this antibiotic-medium to test the feasibility of this approach with this particular antibiotic. Puromycin is active mainly against Gram positive bacteria, so will have little effect on a large proportion of the soil microflora.

Probing for antibiotic resistance genes
Bacterial colonies (1110 in total) isolated on non-selective agar and agar containing kanamycin or puromycin were transferred to fresh media and blotted onto Hybond N^+ nylon membrane (Amersham). The membrane was transferred to blotting paper soaked in 10% SDS for 3 minutes to lyse cells, then to blotting paper soaked in denaturing solution (0.2M NaOH, 0.6M NaCl) for 5 minutes. The blots were neutralised by placing on blotting paper soaked in neutralising solution (0.2M Tris, 0.6M NaCl) for 5 minutes. DNA was fixed to the membrane by exposure to UV light.

The colony blots were probed for the presence of puromycin gene fragment amplified from a plasmid pGL71 containing the puromycin gene (Swiss-Prot accession number P13249) supplied by . Primers used were designed to amplify a 370 bp region of the puromycin resistance gene (puro1: TCACCGAGCTGCAAGAACTC and puro 2: AGCCGCTCGTAGAACGGAA). Conditions for PCR were 1 min. x 94 °C, 1 min x 51°C, 2 min. x 72 °C for 29 cycles, then 72 °C for 2 mins.

Colony blots were also probed for the presence of *npt*II amplified from *Acinetobacter* strain BD413 using NPTII primers (forward: ATGACTGGGCACAACAGACAATC GGCTGCT; reverse CGGGTAGCCAACGCTATGTCCTGATAGCGG). Conditions for PCR were 5 min x 94°C, then 30 sec x 94°C, 30 sec x 55°C, 90 sec x 72°C for 30 cycles, then 7 min x 72°C.

Puromycin and kanamycin gene fragments were labelled with P-33 using the Rediprime II DNA Labelling System (Amersham). The colony blots were hybridised overnight at 65°C in hybridisation solution (2mM EDTA, 0.5M Na_2HPO_4, 7% SDS) to which was added 18µl of denatured, radioactively-labelled probe. The blots were washed three times in 2 x SSC (0.3M NaCl, 0.3M sodium citrate, pH 7.0) at 65°C, then exposed to Kodak XAR for one day before developing.

Molecular analysis of soil bacterial communities

DNA extraction
Total DNA was extracted from a 0.5g soil sample using the FastDNA SPIN Kit for Soil according to the manufacturer's instructions (Qbiogene, Inc.). The method included a cell lysis step using tubes containing ceramic and silica particles shaken in a Biospec mini Bead-beater, and extracted genomic DNA was purified by a GENECLEAN procedure.

PCR Amplification of 16S rDNA
Primers 968-GC; 5'-AACGCGAAGAACCT TAC -3' (Heuer *et al.* 1997) with GC clamp (cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gca cgg ggg g) (Muyzer *et al.* 1993) to the 5' end of the sequence and L1401aR; 5'-CGGTGTGTACAAGGCCC-3' Heuer *et al.* (1997) were used to amplify the bacterial 16S rDNA fragment between positions 968-1401 (*E. coli* numbering) by PCR. DNA was amplified in 30ul volumes using 1ul DNA diluted 1/100, 1x Buffer (ABgene, UK) , 0.2mM dNTPs, 0.2uM each primer, 1.5mM $MgCl_2$, 1ug BSA and 0.775U Taq DNA Polymerase (ABgene, UK). The thermal cycling programme was performed with an initial denaturing step at 94°C

for 5mins then 30 cycles of 94°C for 30 sec, 56 °C for 30 sec and 68 °C for 30 sec, before a final extension step of 68 °C for 7 min.

Denaturing Gradient Gel Electrophoresis

DGGE analysis was carried out using the DCode System DGGE apparatus from Bio-Rad. Inc (Germany). Approximately equal concentrations of PCR products were applied to the wells (up to 20ul) as judged from an ethidium bromide-stained agarose gel 1% (w/v). PCR products were separated in 8% (w/v) polyacrylamide gels [(acrylamide/bisacrylamide (37.5:1)] with a 38-60% denaturing urea gradient [100% denaturant equivalent to 7M urea plus 40%(v/v) formamide]. Gels were run at a constant voltage of 80V for 16hr in 0.5xTAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 60°C, then silver stained (modified method of Sanguinetty *et al* 1994) and dried at 60°C for scanning.

Results

Enumeration of total culturable aerobic bacterial populations

Because of the limited number of replicate samples collected from each depth in this preliminary trial, no attempt has been made to statistically analyse the results. Control samples were collected to a maximum depth of 2.4 m, in comparison with offal pit samples which were collected from as deep as 5.5 m. Bacterial counts close to the surface were typical of numbers generally found in agricultural soils, with lower numbers recovered from control soils collected from greater depth. Slightly higher numbers of bacteria were recovered from offal pit soils at all depths.

Table 1: Total numbers of culturable aerobic bacteria /g air dry soil recovered from various depths inside and outside offal pit.

			cfu/g soil	
Depth	**Sample no.**	**Soil moisture**	**Control**	**Offal pit**
0-1 m				
0-2cm	TG1A	24.6%		1.44×10^6
0-15cm	C2B	19.7%	8.02×10^5	
0-20cm	C6A	18.8%	6.65×10^4	
0-30cm	C1B	20.2%	3.14×10^6	
0-1m	TG1B	28.1%		2.19×10^7
80-100cm	C1A	28.4%	1.51×10^6	
1-2 m				
50-150cm	TG2A	31.7%		3.96×10^6
100-125cm	C2A	30.2%	2.04×10^4	
100-120cm	C3B	30.1%	2.40×10^3	
145-160cm	C3A	36.4%	1.19×10^3	
1.5m	TB3B	27.4%		5.30×10^6
170-180cm	C4A	38.1%	ND[1]	
2-3 m				
220-240cm	C5A	38.3%	9.63×10^2	
220-240cm	TG3A	32.7%		1.21×10^4
2-2.4m	TG2B	44.9%		2.34×10^6
> 3 m				
3m	TG4A	40.0%		2.37×10^5
3.5-4m	TB4B	54.2%		5.82×10^5
4m	TG5A	37.8%		1.42×10^4
4.5-5m	TG6A	38.4%		7.57×10^4
5.5m	TG7A	70.2%		4.43×10^6

[1] ND = not detected. Limit of detection is approximately 10^2 bacteria/g soil.

Incidence of antibiotic resistant bacteria

Bacteria resistant to two levels of kanamycin were recovered from both control and offal pit soils (Table 2). In control soils, the proportion of the total bacterial population that was resistant to kanamycin 100 ranged from 0-22%, while the proportion resistant to kanamycin in offal pit soils ranged from 0-13%.

Because of the cost of puromycin, only two samples were plated onto puromycin-containing agar (Table 3) but puromycin-resistant bacteria were recovered from both a control soil and an offal pit soil.

Table 2: Total numbers of kanamycin-resistant bacteria recovered from control and offal pit soil samples.

	Total no. culturable bacteria	Level of kanamycin resistance		% of cfu resistant to Kan 100
		Kana 50	Kana 100	
Control				
C1A	1.51E+06	3.64E+04	1.04E+02	0.0093
C2A	2.04E+04	4.16E+03	4.48E+03	21.9
C3A	1.19E+03	ND	ND	ND
C4A	ND	5.49E+02	5.49E+02	ND
C5A	9.63E+02	ND	ND	ND
C6A	6.65E+04	1.53E+04	1.15E+04	17.3
C1B	3.14E+06	7.78E+04	9.98E+04	3.2
C2B	8.02E+05	9.76E+03	8.96E+03	1.1
C3B	2.40E+03	5.43E+02	2.26E+02	9.4
Offal pit				
TG1A	1.44E+06	9.71E+04	4.44E+04	3.1
TG2A	3.96E+06	1.11E+05	5.35E+04	1.4
TG3A	1.21E+04	5.04E+01	ND	ND
TG4A	2.37E+05	5.59E+02	ND	ND
TG5A	1.42E+04	ND	ND	ND
TG6A	7.57E+04	6.21E+01	6.21E+01	0.08
TG7A	4.43E+06	ND	ND	ND
TG1B	2.19E+07	6.77E+05	1.95E+05	0.90
TG2B	2.34E+06	6.91E+03	8.75E+02	0.04
TB3B	5.30E+06	6.73E+05	6.80E+05	12.8
TB4B	5.82E+05	ND	ND	ND

ND=-none detected

Table 3: Total numbers of puromycin-resistant bacteria recovered from control and offal pit soil samples.

	Total no. culturable bacteria	No. puromycin resistant cfu	% of cfu resistant to puromycin 125
C1A	1.51E+06	8.92E+03	0.6
TG2A	3.96E+06	6.55E+04	1.6

Probing for antibiotic resistant bacteria

Probing for the puromycin resistance and kanamycin *npt*II genes revealed the potential presence of both genes in the general bacterial population. In control soils, 0-88% of colonies probed positive for the puromycin gene segment, while in offal pits, 0-50% probed positive (Table 4). This figure seems extremely high and needs to be confirmed through PCR and sequencing of potential puromycin genes. The puromycin gene segment also contained non-related sequence and this may be the reason for the high incidence of hybridisation. There was some correlation between high incidence of puromcyin-positive colonies and selection of puromycin medium for the control soils, with the 88% of positives being recovered on a puromycin selective plate. For offal pit soils, the highest level occurred on kanamycin selective plates (50%) rather than puromycin (19%).

Probing was also conducted using a segment of the *npt*II gene, as *npt*II has commonly been used as a marker gene in transgenics. For the kanamycin *npt*II gene probe, lower levels of positive colonies were found, especially in the control soils. Surprisingly, on kan100 plates prepared from offal pit soil samples, up to 25.6% of the colonies probed positive for *npt*II. This result needs to be further investigated to confirm that colonies that probed positive contain the kanamycin gene

Table 4: Incidence of bacterial colonies that probed positive for the kanamycin and puromycin resistance genes.

Sample	Depth	Medium on which colonies isolated	No. colonies probed	Puro resistance gene +ve	Kana resistance gene +ve
Control					
C1A	80-100cm	TSA	100	14.0%	0.0%
		TSA, Kana 100	100	2.0%	1.0%
		TSA, Puro 125	100	88.0%	0.0%
C2A	100-125cm	TSA	0	0.0%	0.0%
		TSA, Kana 100	44	0.0%	0.0%
C6A	0-20cm	TSA, Kana 100	46	0.0%	0.0%
C1B	0-30cm	TSA	28	14.3%	0.0%
		TSA, Kana 100	94	27.7%	2.1%
Offal pit					
TG1A	0-2cm	TSA	100	3.0%	2.0%
		TSA, Kana 100	78	50.0%	25.6%
TG2A	50-150cm	TSA	23	8.7%	0.0%
		TSA, Kana 100	83	14.5%	15.7%
		TSA, Puro 125	94	19.1%	13.8%
TG1B	0-1m	TSA	48	27.0%	0.0%
		TSA, Kana 100	79	1.3%	0.0%
TG3B	2-2.4m	TSA	43	18.6%	9.3%
		TSA, Kana 100	100	0.0%	0.0%

Molecular analysis of soil bacterial communities

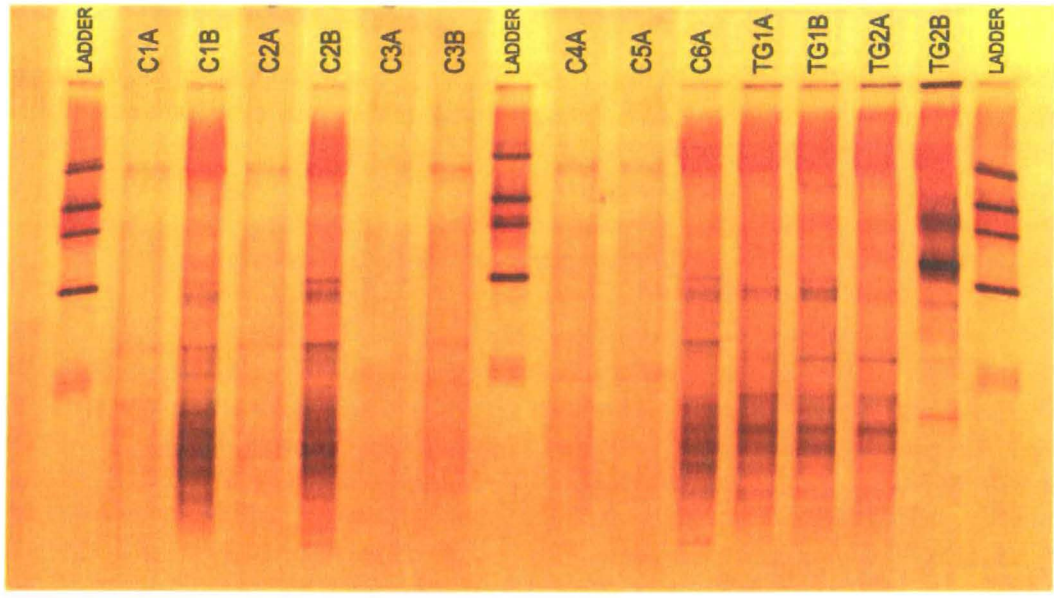
Initially DNA was amplified at different concentrations (concentrated, 1/10, 1/100) to check extraction efficiency. There were problems with amplification of DNA from control soils; C3A (no amplification); C1A, C2A, C3B, C4A and C5A gave weak amplification with concentrated DNA only. Samples C1B, C2B and C6A amplified at 1/10 and 1/100 dilutions only, indicating inhibition of the PCR when DNA was concentrated. Successful amplification was achieved from all transgenic offal pit samples at all concentrations except TG2B, which worked at the lower two dilutions.

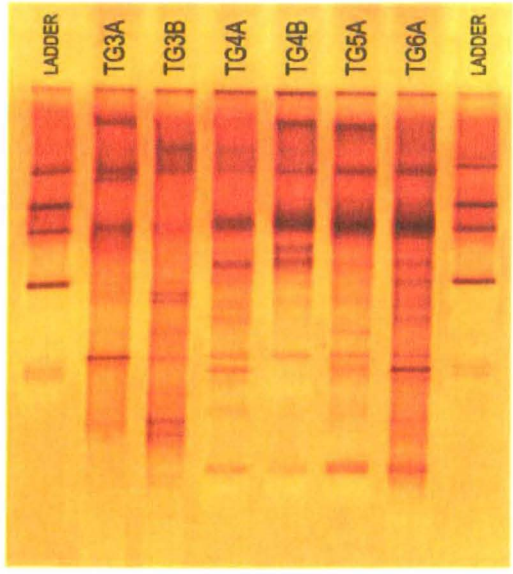
DNA was re-extracted from samples C3A, C3B, C4A and C5A and the bead-beating step was increased by 30sec. DNA from these samples was amplified as above using 2x concentrated, concentrated and 1/10. No PCR products were recovered from C3A and C5A, C3B 1x & 2x and C4A 1/10 weak. A range of different DNA Polymerases (Roche Fast Start with and without GC solution, Amplitaq Gold, Eppendorf Taq Master and ABgene Polymerase) were tried but did not improve results.

PCR was continued with offal pit samples for DGGE, this time using selected dilutions and the addition of T4gene32protein (0.775ug/30ul reaction) instead of BSA (for inhibition in environmental samples) plus a modified PCR programme to improve amplification. The modified thermal cycling programme is as follows with an initial denaturing step at 94°C for 5mins then 15 cycles of 94°C for 30 sec, 56 °C for 30 sec and 68 °C for 30 sec, then 20 cycles of 94°C for 30 sec, 56 °C for 30 sec and T68 °C for 30 sec + 5 sec increase each cycle, before a final extension step of 68 °C for 7 min. The control soil samples C1A, C2A, C3A, C4A and C5A only worked with the Eppendorf MasterTaq Kit with the addition of T4gene32protein (0.775ug in 30ul reaction volume).

No statistical analysis of the patterns has been undertaken because of the limited number of samples. As can be seen in Figure 1, DGGE profiles were achieved for only three of the samples collected from outside the offal pit (C1B, C2B, C6A), and these samples were all from closer to the surface where soil texture was typical of a pasture soil. Visual examination of these three lanes shows that the patterns are quite similar, with numerous bands present. Some bands are common to both the control and offal pit soils, but the patterns of soil bacteria recovered from the offal pit samples appear to be more diverse, with samples taken from close to the surface being more similar to the control samples.

Figure 1: DNA banding patterns of soil bacteria obtained by DGGE analysis of eubacterial-primer based amplicons from soil samples collected from offal pit (TG1A and B, TG2A and B, TG3A and B, TG4A and B, TG5A and B) and control soils from outside the offal pit (C1A and B, C2A and B, C3A and B, C4A, C5A, C6A).





Discussion

The samples provided were very variable in content, with some samples collected from within the pit containing visible animal remains. Control soils collected at depth appeared to contain a high clay content and yielded low numbers of bacteria. These samples also produced few bands in the DGGE analysis, which may have resulted from problems with the DNA extraction technique. Further work would be necessary to overcome these technical difficulties, but viable counts also indicate that low numbers of culturable bacteria are present at depth in the soil profile. This is in contrast with the offal pit soils, where high numbers of bacteria were still recovered at 5 m.

Statistical analysis of the data was not possible because of the limited replication, but there does not appear to be a significant difference in the numbers of antibiotic-resistant bacteria from control and offal pit soils. Kanamycin-resistant bacteria were recovered frequently, which is consistent with other studies which have found that a considerable fraction of the soil bacterial populations were found to be resistant to kanamycin (Recorbet et al 1992; van Elsas et al. 1991).

Probing results suggest that bacteria containing the puromycin resistance gene are present in New Zealand soils, but there was no evidence of increased levels of puromycin-resistant bacteria in offal pit samples. Indeed, the highest incidence of colonies probing positive for the puromycin resistance gene was found in control soil selected on puromycin medium, at 88% of all colonies. Because the segment of the gene used as a probe may contain some unrelated DNA or point mutations, further research is required to determine if the colonies which probed positive definitely contain the puromycin resistance gene.

Similarly, probing for the *npt*II gene found a number of positive colonies, which is interesting as we have never before detected *npt*II in New Zealand bacteria. This result needs to be further investigated. In previous work, we have not detected the *npt*II gene in New Zealand soil bacteria and it is interesting that numbers of *npt*II gene-positive colonies were higher in the offal pit than in control soil samples. In a previous study on the prevalence of *npt*II gene in kanmycin-resistant bacteria from various environments, colonies reacting positively to a 925 bp *npt*II specific probe were primarily obtained from sewage samples, with fewer obtained from pig manure slurry and river water. Bacteria containing the *npt*II gene were not found in soil samples (Smalla et al. 1993). *npt*II was detected in some soils via PCR of environmental DNA extracts. Resistance to kanamycin can be achieved by several different mechanisms. Phosphorylation of kanamycin has been identified as the main modification method and many different phosphorylating enzymes, including the enzyme encoded by *npt*II, have been identified. It appears that most soil bacterium utilise mechanisms other than the enzyme encoded by *npt*II, neomycin phosphotransferase-II) in kanamycin resistance.

DGGE profiles from control samples where PCR could be successfully carried out tended to less variable than those of the offal pit samples. Given that there was still a large amount of animal matter still present in the samples, it is not surprising that there were differences in the bacterial community profiles produced by DGGE analysis. This analysis was carried out using general bacterial primers and further

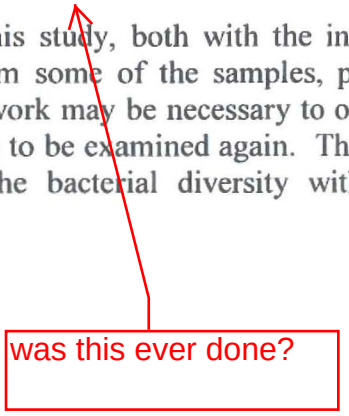
differences may be detected using taxon-specific primers, for example to detect enteric bacteria.

Suggestions for further work

An interesting result in this work was the number of colonies which probed positive for *nptII* and the puromycin resistance gene. We have not found *npt*II in New Zealand soil bacteria previously, so PCR amplification and sequencing from probe positive colonies would greatly increase our knowledge of the background level of antibiotic gene presence in New Zealand soils. Puromycin, in particular, was detected through colony hybridisation at high levels. As far as we are aware, no previous study has examined soil bacteria for the presence of this gene. Identification of the bacteria may allow us to determine whether colonies carrying the *npt*II gene are typical soil bacteria or of animal origin.

A well known problem is that only a small percentage (estimates range between 0.1-10%) of bacteria are accessible through cultivation or viable plating. Therefore studies based on cultivation of bacteria tell us little about the presence of antibiotic-resistance genes in the majority of environmental bacteria. To investigate the prevalence of antibiotic resistance genes in the total bacterial community present in the offal pit soil (including non-culturables), PCR-mediated amplification of resistance genes could be applied to total community extracts obtained from offal pit soils (e.g. Smalla and van Elsas 1995; Aminov et al 2001; Riesenfeld et al 2004). This preliminary study has shown that the bacterial community is different from that found in the surrounding soil and that the prevalence of the *npt*II gene also appears to differ between the soils. Further investigation of the range of antibiotic resistance genes present in the offal pit soil bacterial community is warranted.

Difficulties were encountered in this study, both with the initial sampling and the subsequent extraction of DNA from some of the samples, particularly those from deeper in the soil profile. Further work may be necessary to overcome difficulties in DNA extraction if deep samples are to be examined again. The use of taxon-specific primers would further elucidate the bacterial diversity within the offal pit soil bacterial community.



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Offal pit summary for 2005

Puromycin

Probing control was unlabelled DNA – 10ul on blot, undiluted and 1:10

520 colonies were probed
Of these 176 colonies left *some* impression on the autoradiograph film – however faint.

56 of the "positive" colonies were attempted to be amplified with puromycin fragment
- should be ~ 400bp

4 had a band at around 800-900 bp and one had a band at ~ 600bp.

These bands were faint and inconsistent when repeated.
- have not been sequenced, due to inability to obtain enough DNA

nptII

Control was *Acinetobacter* BD314 transferred onto square plates with offal pit colonies.

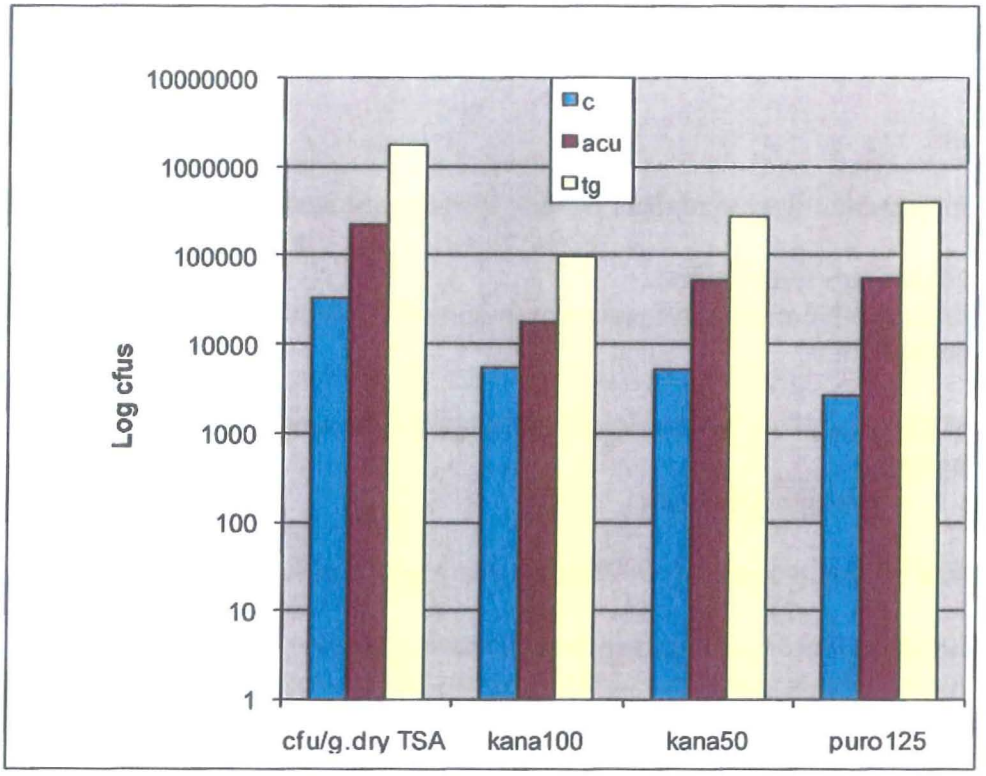
1130 colonies probed (there were two levels of antibiotic plate – kana 50 and kana 100).

Of these 27 left some mark on autoradiograph – MUCH fainter than any of the puromycin colonies

24 were attempted to be amplified with nptII (and compared with BD413) and were all negative.

nptII hybridisation looks like isotope "sticking" to proteins on blots – only one colony even approaches the same opacity as the controls and it was negative when pcr'd

puromycin hydridisation turned up a significant number (~100) of strongly positive colonies when compared to the control. But none of the colonies tested had the 400bp puromycin fragment.
- problem with DNA used for probe?
- puromycin DNA was also used for the PCR and band ran at correct place 400bp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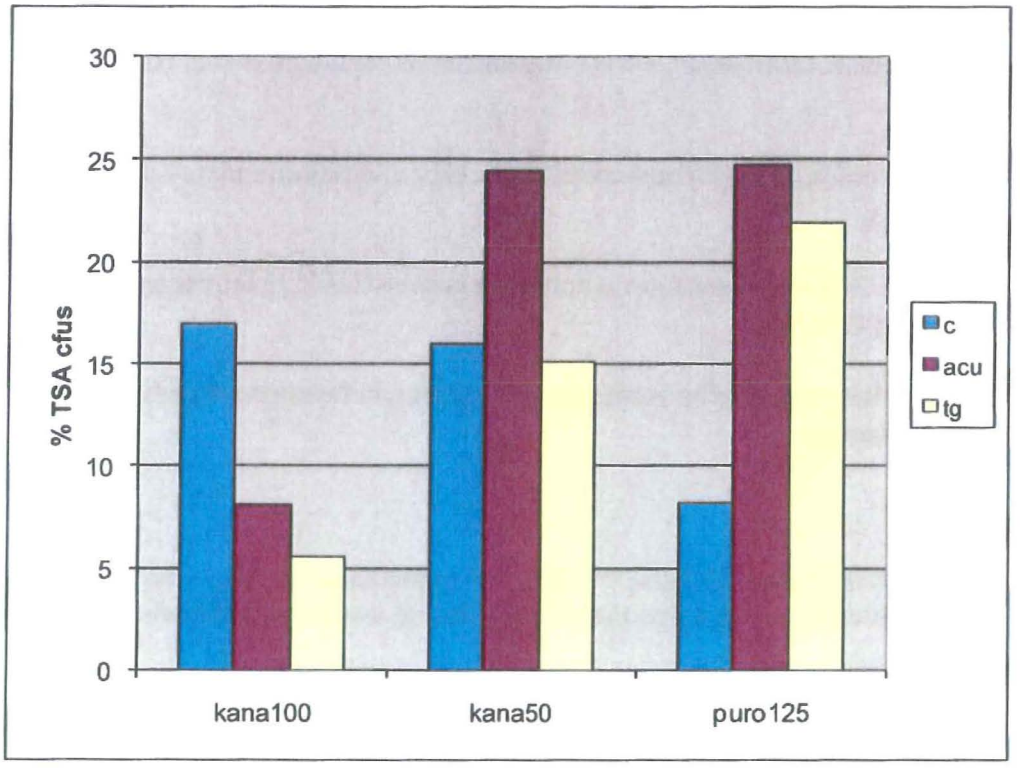
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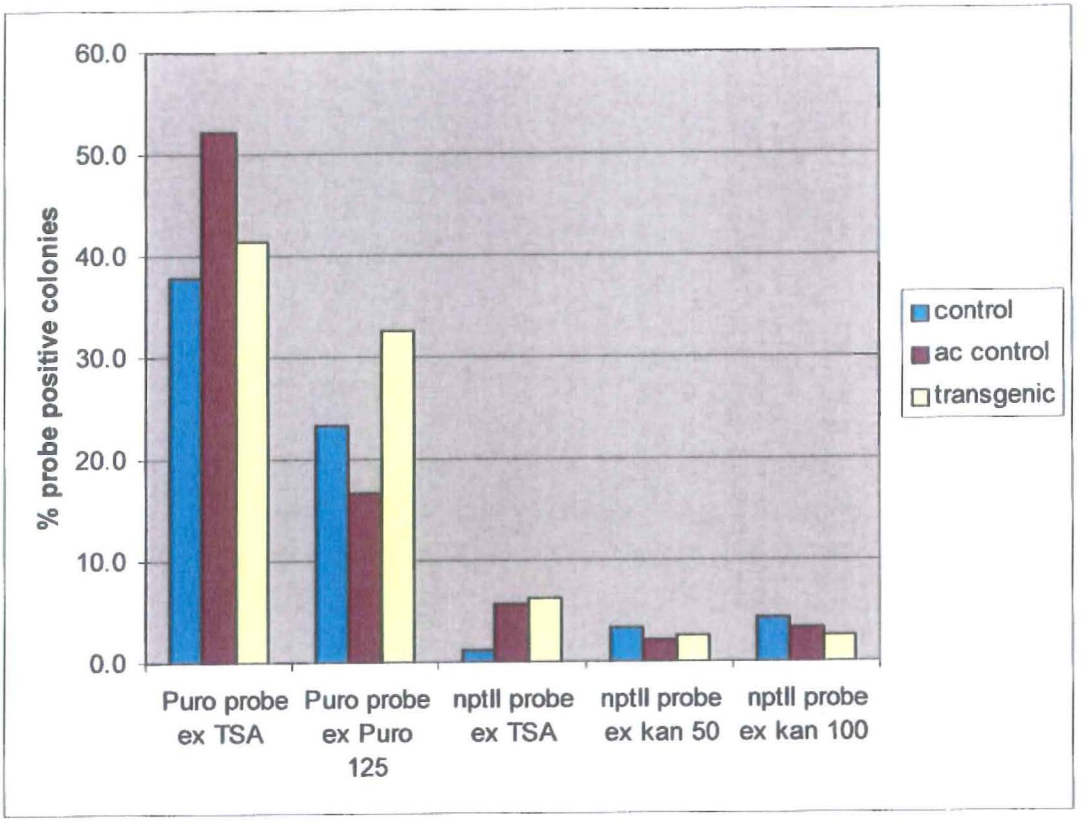
60.0
50.0
40.0
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20.0
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0.0
% probe positive colonies
Puro probe ex TSA
Puro probe ex Puro 125
nptII probe ex TSA
nptII probe ex kan 50
nptII probe ex kan 100
control
ac control
transgenic

Monitoring offal pits for puromycin resistance bacteria and the presence of genes encoding resistance to puromycin

Prepared by:

13 June 2007

Report For:

Background

In previous studies on the microbial communities in an offal pit used for the disposal of transgenic cattle at Ruakura, it was found that significant numbers of puromycin and kanamycin resistant bacteria were present in both control and offal pit soils, and that many colonies probed positive for the gene constructs used in the transgenic cattle project, but PCR failed to amplify a segment of the puromycin gene from any of the bacteria. Baseline community diversity and bacterial density data was collected to allow future comparisons of microbial community change in offal pit and surrounding soil.

This report covers ongoing monitoring of the offal pits for antibiotic resistant bacteria in December 2006.

Methods

Soil sampling

Soils were supplied by and team , sampled from offal pit directly and control soil nearby, sampled in December 2006. Due to the difficulty of sampling at depth in control pastures and the results of the last study which showed little pattern in the depth at which resistant bacteria were recovered, sampling will be restricted to core or spade squares down to approximately 20-30cm.

Bacterial isolations

Soil was diluted: 20 g of soil in 180 ml of 0.1% peptone (1g bacteriological peptone in 1l water). Ten-fold dilutions in 100 mM phosphate buffer were plated on selective media.

Bacteria were isolated on TSA + cycloheximide (100 µg/ml) or TSA + puromycin (125 µg/ml). Colony counts were also conducted using TSA + kanamycin (50 and 100 µg/ml) but no isolates were kept for further analysis, as kanamycin was not used in selection of transgenic cattle.

Randomly selected single colonies were transferred to appropriate (10% TSA or 10% TSA + puromycin) agar stabs in 2ml microcentrifuge tubes and stored at 4°C until probing.

Probing

Bacteria were transferred directly from agar stabs (10% TSA and 50% TSA with appropriate antibiotics) to 10% TSA square plates for colony blotting. All colonies grew after 48 hours incubation at 20°C.

Plates were chilled at 4°C and the colonies then blotted onto Hybond N^+ nylon membrane. The membranes were transferred (colony side up) to filter paper soaked in 10% SDS for five minutes to lyse the colonies. Membranes were then transferred to filter paper soaked in denaturing solution (0.2M NaOH, 0.6M NaCl) for five minutes. This was followed by two x five minutes on filter paper soaked in neutralising solution (0.2M Tris, 0.6M NaCl). Finally the membrane was air dried and fixed by UV crosslinking on a transilluminator for three minutes.

To probe the colony blots a 370 bp region of the puromycin resistance gene was amplified from plasmid pGL71 using puro1 (TCACCGAGCTGCAAGAACTC) and puro2 (AGCCGCTCGTAGAACGGAA) primers under the following PCR conditions: 1 min. x 94°C, 1 min x 51°C, 2 min. x 72°C for 29 cycles, then 72°C for 2 mins. Product was cleaned up using the Roche High Pure PCR purification kit and diluted in sterile distilled H_2O at a ratio of 2µl of product in 45µl total volume.

The puromycin gene fragment was labelled with P-33 dCTP using the Rediprime II Labelling system (GE Healthcare). Hybridisation of colony blots was carried out overnight at 65°C in hybridisation buffer (2mM EDTA, 0.5M Na_2HPO_4, 7% SDS) to which 20µl of denatured probe was added. Blots were washed three times in 2 x SSC (0.3M NaCl, 0.3M sodium citrate, pH 7.0) at 65°C and then exposed to Kodak XAR film for three days before developing.

A strip of membrane to which denatured, diluted PCR product was fixed was included with each hybridisation run and film cassette as a positive control.

Colonies having a signal equal or greater than the control DNA were recorded:

Amplification of putative puromycin resistance encoding genes

These colonies were streaked onto 10% TSA and whole cells resustpended in 1 ml of distilled water for use directly in PCR. Two ul of this suspension was used in PCR with the puro1 and puro2 primers under the same conditions as the previous PCR.

Species determination for colonies containing homologous genes

Any isolates found to have genes for puromycin after sequencing will be subjected to 16s gene sequencing and API identification to determine genus and species.

Results

Bacterial isolations from soil

Colony numbers of the various antibiotic containing media are shown in Table 1. The numbers vary between samples, but there was no increase in puromycin resistance bacteria from offal pit samples (ACU) compared to control paddock soil (CP). Higher numbers of colonies were recovered from soils using cycloheximide and puromycin as the selective agent (Tale 1). Kanamycin resistance bacteria were recovered in similar numbers from both, but kanamycin was not used as a selective agent in the transgenic cattle project and is simply used as a control antibiotic for comparison of bacterial resistance.

Table 1: Colony forming units of bacteria per g of soil (dry weight equivalent) cultured on four media (mean of 2 plates)

Sample ID[1]	10% TSA + Puromycin medium		10% TSA + Kanamycin (100) medium		10% TSA + Kanamycin (50) medium		10% TSA + Cycloheximide medium	
	Bacteria g^{-1} soil	Log_{10} g^{-1} soil	Bacteria g^{-1} soil	Log_{10} g^{-1} soil	Bacteria g^{-1} soil	Log_{10} g^{-1} soil	Bacteria g^{-1} soil	Log_{10} g^{-1} soil
CP1	3961375	6.60	9232*	3.97	73856*	4.87	30465600	7.48
CP2	3979005	6.60	NG		92125*	4.96	11976250	7.08
CP3	12013950	7.08	74028*	4.87	259098	5.41	37939350	7.58
CP4	1.4E+08†	8.14	240279	5.38	2957280	6.47	39738450	7.60
CP5	6600000	6.82	27728*	4.44	NG		38818500	7.59
AVERAGE	**33310866**	**7.05**	**70253**	**3.73**	**676471.8**	**4.34**	**31787630**	**7.47**
ACU1	75940200†	7.88	NG		55764*	4.75	23235000	7.37
ACU2	5186160	6.71	18522*	4.27	2315250	6.36	21300300	7.33
ACU3	25081650	7.40	27765*	4.44	55530*	4.74	34243500	7.53
ACU4	6676200	6.82	NG		92725*	4.97	20399500	7.31
ACU5	1207635	6.08	46448*	4.67	371580	5.57	46447500	7.67
AVERAGE	**22818369**	**6.98**	**18547**	**2.67**	**578169.8**	**5.28**	**29125160**	**7.44**

[1] CP: Control paddock (5 replicate samples); ACU: Animal control unit (5 replicate samples);

NG: No growth on any of the plates

† Colony count from the CP4 and ACU1 samples on puromycin medium was unusually high.

* indicate that the results for some samples are not reliable (less than 5 colonies per plate) on kanamycin media.

26.9% (43+19+8+6)/(106 +97+44+36)

Probing

A total of 687 individual bacterial isolated randomly selected from TSA isolation plates, both containing puromycin and without, were probed using a fragment of the puromycin resistance encoding gene from pGL71. In the control soils, 16 of the bacteria probed positive, while in offal pit soil 8.9% probed positive (Table 2). Using antibiotics in the medium, 27.9% of colonies probed positive when isolated on purmycin containing medium compared to 2.2% on cycloheximide containing medium. In total, 85 colonies probed positive (12.4%).

where is this data in table 2?

Table 2: Probing colonies recovered from control (CP) and offal pit (ACU) soils using fragment of the puromycin resistance encoding gene.

Treatment	Antibiotic used	Strength of medium	Total colonies probed	Positive	% Positive
CP	puromycin	10% TSA	106	43	40.6
ACU	puromycin	10% TSA	97	19	19.6
CP	puromycin	50% TSA	44	8	18.2
ACU	puromycin	50% TSA	36	6	16.7
CP	cyclo	10% TSA	144	1	0.7
ACU	cyclo	10% TSA	165	5	3.0
CP	cyclo	50% TSA	44	2	4.5
ACU	cyclo	50% TSA	51	1	2.0

Total probed 687, total positive 85.

Four randomly selected colonies that had a autoradiograph signal, but yielded no PCR product were identified by partial sequencing of the 16SrRNA region. All four were identified by sequencing and colony morphology as *Pseudomonas* sp. It is common with colony blots for *Pseudomonas* spp. to probe positive to any DNA probe.

PCR amplification of putative puromycin genes

A 700 bp product was amplified from 13 of the 85 colonies which probed positive using the puromycin gene specific primers.

The 700 bp product that 13 of the colonies yielded was at too low a concentration to send for sequencing. Instead this product from three colonies was used as a template for a repeat of the PCR with puro1 and puro2 primers.

This secondary PCR yielded products at ~ 700 bp and ~100-150 bp, while the predicted product for the puromycin gene would have been 370 bp. These products were cloned into DH10B using the pGEM T Easy vector system (Promega) and sequenced. Few successful sequences were obtained, but for four products the amplified product was the vector DNA, pGEM.

Conclusions

Use of a puromycin resistance encoding gene in the production of the transgenic cattle grazed at Ruakura, Hamilton, led to some concern as to whether antibiotic resistance encoding genes from carcasses buried in soil could be uptaken by bacteria. Monitoring of the soil bacterial populations for increase in puromycin resistance in bacterial population in the offal pits have been monitored for several years.

Samples taken in December 2006 were analysed for i) puromycin resistance in bacterial populations and ii) presence of homologous puromycin resistance encoding genes in isolated bacteria. No increase in puromycin resistance in bacteria recovered from offal pits compared to control paddock soils was found. Over 600 individual bacterial colonies were probed for homology to a portion of the puromycin gene originally used in the cattle. Eight-five colonies probed positive, however subsequent examination show most were *Pseudomonas* spp., which often show non-specific binding of DNA probes in colony blots. Thirteen of 85 probe positive colonies had weak bands after PCR using specific puromycin gene primers, but sequencing showed only vector sequence.

In conclusion, there was no evidence of any puromycin resistance gene similar to the one used in the transgenic cattle in the offal or control soil bacteria, and no increase in puromycin resistant bacteria in offal pits compared to control soil.

extremely poor experimental procedure. the sequencing results could be explained by PCR inhibitors. why wasn't the positive control sequence not mixed with the environmental samples to determine if conditions were creating a false negative?

Monitoring offal pits for the presence of puromycin resistant bacteria and the presence of genes encoding resistance to puromycin

Prepared by:

30 January 2008

Report For:

Background

In previous years, microbial community studies of soils from offal pit used for disposing the carcasses of transgenic cattle at Ruakura, showed that significant populations of puromycin and kanamycin resistant bacteria were present in offal pit soils as well as nearby soils which have not been exposed to transgenic cattle (controls). Many of these colonies probed have shown to be positive for the gene constructs used in the transgenic cattle project, but PCR failed to amplify a segment of the puromycin gene from any of the bacteria. Baseline community diversity and bacterial density data was collected to allow future comparisons of microbial community change in offal pit and surrounding soil.

This report covers ongoing monitoring of the offal pits for antibiotic resistant bacteria in December 2007.

Methods

Soil sampling

Soils were supplied by and team , sampled from offal pit directly and control soil nearby, sampled in December 2006. Due to the difficulty of sampling at depth in control pastures and the results of the last study which showed little pattern in the depth at which resistant bacteria were recovered, sampling were restricted to a random number of cores collected to a depth of approximately 20-30cm.

Bacterial isolations

Soil dilutions were prepared in 0.1% peptone (1g bacteriological peptone in 1l water) Twenty gram quantity (oven dried equivalent) of soil was placed in 180 ml of peptone solution and subjected to ultrasonic treatment at maximum power for 3 min to disrupt the soil aggregates (Kerry's Ultrasonics model KG 100). This was placed on a wrist-action shaker for 10 min to further homogenise the soil suspension and 10-fold dilutions were made in 100 mM phosphate buffer before spread-plating on selective media.

Bacteria were isolated on 10% tryptose soy agar (TSA) containing cycloheximide (100 µg/ml) or 10% TSA + puromycin (125 µg/ml). The presence of cycloheximide inhibits the growth of most fungi while the puromycin will inhibit the growth of all microbes sensitive to this antibiotic.

Randomly selected single colonies from both media were transferred to appropriate (10% TSA or 10% TSA + puromycin) agar stabs in 2ml microcentrifuge tubes and stored at 4°C until probing.

Probing

Bacterial isolates collected from samples of offal pit soil were screened for the presence of a 370bp fragment of a puromycin resistance gene.

The colonies initially isolated on TSA + cycloheximide and TSA + puromycin were transferred to $10cm^2$ 10% TSA plates for colony blotting. Plates were incubated for 48 hours at 20°C, chilled to 4°C and the colonies blotted onto Hybond N^+ (GE Healthcare) nylon membranes. Membranes were transferred (colony side up) to filter paper soaked in 10% sodium dodecyl sulfate (SDS) for five minutes to lyse the colonies. The membranes were then transferred to filter paper soaked in denaturing solution (0.2M NaOH in 0.6M NaCl) for five minutes. This was followed by two transfers to filter paper soaked in neutralising solution (0.2M Tris in 0.6M NaCl). Finally the membrane was air dried and fixed by UV crosslinking on a transilluminator for three minutes.

To probe the colony blots a 370 bp region of the puromycin resistance gene was amplified from plasmid pGL71 using puro1 (TCACCGAGCTGCAAGAACTC) and puro2 (AGCCGCTCGTAGAACGGAA) primers under the following PCR conditions: 1 min. × 94 °C, 1 min × 51oC, 2 min. × 72 °C for 29 cycles, then 72 °C for 2 min. The product was cleaned up using Roche's High Pure PCR purification kit and diluted in sterile distilled water at a ratio of 2µl of product in 45µl total volume.

The puromycin gene fragment was labelled with P-33 dCTP using the Rediprime II Labelling system (GE Healthcare). Hybridisation of colony blots was carried out overnight at 65°C in hybridisation buffer (2mM EDTA, 0.5M Na2HPO4, 7% SDS) to which 20µl of denatured probe was added. Blots were washed three times in 2 x SSC (0.3M NaCl, 0.3M sodium citrate, pH 7.0) at 65°C and then exposed to Kodak XAR film for 48 hours before developing.

Amplification of putative puromycin resistance encoding genes

The PCR amplification was carried out under identical conditions as above. The template used was 1-3 well isolated colonies suspended in 500µl of sterile distilled water (4µl per well). Control DNA from the pGL71 plasmid was also run with each batch.

Results

Bacterial isolations from soil

Colony forming units (CFUs) observed on the two media used are shown in Table 1. The numbers vary between samples, but there was no increase in puromycin resistance bacteria from offal pit samples (ACU) compared to control paddock soil (CP). Higher numbers of colonies were recovered from soils in the absence of puromycin (Table 1)

Table 1: Colony forming units of bacteria per g (log_{10}) of soil (dry weight equivalent) cultured on two media (mean of 2-4 plates)

Sample ID[1]	10% TSA + Puromycin medium	10% TSA + Cycloheximide medium
CP1	4.81	6.48
CP2	5.25	6.66
CP3	5.37	6.57
CP4	5.74	6.35
CP5	6.16	6.49
Average	**5.70**	**6.52**
ACU1	5.92	OG
ACU2	OG	OG
ACU3	4.98	6.89
ACU4	5.29	7.00
ACU5	4.41	6.89
Average	**5.36**	**6.71**

[1] CP: Control paddock (5 replicate samples); ACU: Animal control unit (5 replicate samples);

OG: Colonies were overgrown to get an accurate count.

Probing

Detection of the puromycin gene fragment

All isolates that produced a signal on the film (no matter how faint) were used as a template for PCR with the puro1 and puro2 primers to check for the presence of the 370bp fragment.

Table 2: Probing colonies recovered from control (CP) and offal pit (ACU) soils using fragment of the puromycin resistance encoding gene.

Treatment	Antibiotic used	Strength of medium	Total colonies probed	Positive	% Positive
CP	puromycin	10% TSA	124	11	9%
ACU	puromycin	10% TSA	125	17	14%
CP	cycloheximide	10% TSA	125	20	16%
ACU	cycloheximide	10% TSA	125	14	11%

Total probed 499, total positive 62.

PCR amplification of putative puromycin genes

None of the colonies tested showed any DNA amplification to the puro primers

this is again poor experimental procedure. there are many reasons why amplification was not successful.

Conclusions

There is some concern regarding the possibility of horizontal transfer of puromycin resistance encoding gene from transgenic cattle carcasses buried at Ruakura to soil

bacteria Monitoring of the soil bacterial populations for increase in puromycin resistance in bacterial population in the offal pits have been monitored for a number of years.

Samples taken in December 2007 were analysed for puromycin resistance in the soil bacterial community and the presence of homologous puromycin resistance encoding genes in the isolated bacteria. No increase in puromycin resistance in bacteria recovered from offal pits compared to control paddock soils was found. Five hundred individual bacterial colonies were probed for homology to a portion of the puromycin gene originally used in the cattle. Sixty two colonies from all the sources probed positive. Although these colonies were not specifically identified, experience from previous years indicates that colonies belonging to the genus *Pseudomonas* may generate weak positive results from the radioactive probe adhereing to cellular debris on the blotting membrane. It is also possible the puromycin fragment is hybridising to weakly homologous sequences in the DNA from naturally occurring puromycin resistance genes.

There was no evidence of any puromycin resistance gene similar to the one used in the transgenic cattle in the offal or control soil bacteria, and no increase in puromycin resistant bacteria in offal pits compared to control soil.

agresearch Farming, Food and Health. First

Monitoring offal pits for the presence of puromycin resistant bacteria and the presence of genes encoding resistance to puromycin

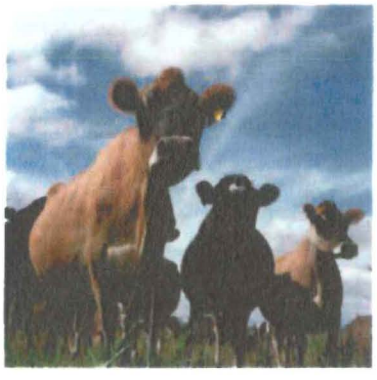
April 2009

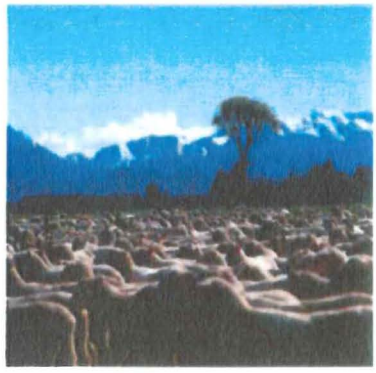
Report for:

Client report number: SC110/2009/32

2020 SCIENCE

New Zealand's science. New Zealand's future.





CONTENTS

1. EXECUTIVE SUMMARY

Transgenic cattle carcasses are disposed into offal pits at Ruakura campus, AgResearch. In the past, significant populations of puromycin and kanamucin resistant bacteria have been detected in offal pit soils as well as nearby soils which have not been exposed to transgenic cattle. A number of puromycin resistant colonies showed some homology to the gene constructs when screened using DNA-DNA hybridisation but no region of the puromycin gene construct was detected in DNA from these colonies by PCR methods. Therefore, there is no evidence to suggest the presence of any puromycin resistance gene similar to the one used in the transgenic cattle in the offal pit soil bacterial community, and no significant increase in puromycin resistant bacteria in offal pit soil compared to control soil.

This report present the results of the 2009 study examining the possible horizontal gene transfer from transgenic cattle to soil bacterial community.

2. BACKGROUND

In previous years, microbial community studies of soils from offal pit used for disposing the carcasses of transgenic cattle at Ruakura, showed that significant populations of puromycin and kanamycin resistant bacteria were present in offal pit soils as well as nearby soils which have not been exposed to transgenic cattle (controls). Many of these colonies probed have shown to be positive for the gene constructs used in the transgenic cattle project, but PCR failed to amplify a segment of the puromycin gene from any of the bacteria. Baseline community diversity and bacterial density data was collected to allow future comparisons of microbial community change in offal pit and surrounding soil.

This report covers ongoing monitoring of the offal pits for antibiotic resistant bacteria in December 2008.

3. METHODS

3.1 Soil sampling

Soils were supplied by and team , sampled from offal pit directly and control soil nearby, sampled in December 2008. Due to the difficulty of sampling at depth in control pastures and the results of the last study which showed little pattern in the depth at which resistant bacteria were recovered, sampling were restricted to a random number of cores collected to a depth of approximately 20-30cm.

3.2 Bacterial isolations

Soil dilutions were prepared in 0.1% peptone (1g bacteriological peptone in 1l water) Twenty gram quantity (oven dried equivalent) of soil was placed in 180 ml of peptone solution and subjected to ultrasonic treatment at maximum power for 3 min to disrupt the soil aggregates (Kerry's Ultrasonics model KG 100). This was placed on a wrist-action shaker for 10 min to further homogenise the soil suspension and 10-fold dilutions were made in 100 mM phosphate buffer before spread-plating on selective media.

Bacteria were isolated on 10% tryptose soy agar (TSA) containing cycloheximide (100 µg/ml) or 10% TSA + puromycin (125 µg/ml). The presence of cycloheximide inhibits the growth of most fungi while the puromycin will inhibit the growth of all microbes sensitive to this antibiotic.

Randomly selected single colonies from both media were transferred to appropriate (10% TSA or 10% TSA + puromycin) agar stabs in 2ml microcentrifuge tubes and stored at 4°C until probing.

3.3 DNA-DNA hybridisation

The colonies initially isolated on 10% TSA + cyclohexamide and 10% TSA + puromycin were transferred to 10 cm^2 10% TSA plates for colony blotting. The plates were incubated for 48 hours at 20°C, chilled to 4°C and the colonies blotted onto Hybond N+ (GE Healthcare) nylon membranes. The membranes were transferred (colony side up) to filter paper soaked in 10% sodium dodecyl sulfate (SDS) for five minutes to lyse the colonies. The membranes were then transferred to filter paper soaked in denaturing solution (0.2 M NaOH in 0.6 M NaCl) for five minutes. This was followed by two transfers to filter paper soaked in neutralising solution (0.2 M Tris in 0.6 M NaCl). Finally the membrane was air dried and DNA fixed by baking at 80°C for 2 hours.

To probe the colony blots a 370 bp region of the puromycin resistance gene was amplified from plasmid pGL71 using puro1 (TCACCGAGCTGCAAGAACTC) and puro2 (AGCCGCTCGTAGAACGGAA) primers under the following PCR conditions: 1 min x 94°C, 1 min x 51°C, 2 min x 72°C for 29 cycles, then 72°C for 2 min. The PCR product was purified using Roche's High Pure PCR purification kit and diluted in sterile distilled water at a ratio of 2 µl of product in 45 µl total volume.

The puromycin gene fragment was labelled with P-32 dCTP using the Rediprime II Labelling system (GE Healthcare). Hybridisation of colony blots was carried out overnight at 65°C in hybridisation buffer (2 mM EDTA, 0.5 M Na2HPO4, 7% SDS) to which 20 µl of denatured probe was added. Blots were washed three times in 2 x SSC (0.3 M NaCl, 0.3 M sodium citrate, pH 7.0) at 65°C and then exposed to Kodak XAR film for 24 hours before developing.

3.4 Amplification of putative puromycin resistance encoding genes

The PCR amplification was carried out under identical conditions as above. The template used was 1 to 3 well isolated colonies suspended in 500µl of sterile distilled water (4 µl per well). Five µl of each reaction was run on a 0.8% x 0.5 TBE gel at 80V for 1 hour with 100 bp ladder. The gel was stained with EtBr for 15 minutes and destained for 5 minutes in tap water before visualisation on BioRad Gel Doc.

4. RESULTS AND DISCUSSION

4.1 Bacterial isolations from soil

Colony forming units (CFUs) observed on the two media used are shown in Table 1. The numbers vary between samples. There was a trend in ACU samples showing increased numbers of CFU growing in puromycin plates compared to cycloheximide plates but these differences and the differences in CFUs in control soils were not statistically significant (Table 1).

Table 1: Colony forming units of bacteria per g (log_{10}) of soil (dry weight equivalent) cultured on two media (mean of 5-6 plates)

Sample ID	10 % TSA+ Puromycin	10 % TSA + Cyclohexamide
CP1	5.22	7.11
CP2	6.00	6.54
CP3	5.22	5.99
CP4	5.47	6.37
CP5	5.34	6.18
Average	**5.45**	**6.43**
ACU1	5.48	6.23
ACU2	7.63	6.76
ACU3	7.34	6.26
ACU4	7.74	6.31
ACU5	7.76	6.37
Average	**7.19**	**6.38**

CP: Control paddock; ACU: Animal control unit (5 replicate samples from each)
*in control samples, CFUs were significantly ($P<0.01$) lower in puromycin medium but in ACU samples, differences were not significant.

4.2 Probing

A total of 502 colonies were probed using the 370 bp fragment of the puromycin resistance gene. Of these colonies 83 had some homology to the probe (Table 1). These colonies were used as templates for PCR with the puro1 and puro2 primers (as used for amplification of the 370 bp sequence form the puromycin insert). Of

these reactions, two colonies produced PCR products at >1500 bp and ~ 800 bp respectively. Both of these colonies were isolated on agar containing 125 mg/ml puromycin. One colony was from the control block, the other from the ACU block. (Table 2).

Table 2: Probing colonies recovered from control (CP) and offal pit (ACU) soils using fragment of the puromycin resistance encoding gene.

Treatment	Antibiotic used	Total colonies probed	Colony Hybridisation	% Hybridised	PCR product from 370 bp
CP	puromycin	73	37	5	
ACU	puromycin	68	16	2	
CP	cyclohexamide	178	14	2	
ACU	cyclohexamide	183	12	8%	0

why is it assumed that the primers are identifying the same kind of construct? only cloning and sequencing the bands seen by hybridization would prove the negative

No evidence of the puromycin 370 bp fragment was found in the colonies tested. As expected there is some homology between the fragment and naturally occurring puromycin resistance; 38% of colonies isolated on puromycin containing agar hybridised to the probe, compared with only 7% of those isolated on agar without puromycin. No 370 bp or similar sized PCR products were amplified from any of the colonies.

4.3 Cloning and sequencing PCR products

Colonies PACU2-6 and PCP2-14 were used as template in a PCR reaction with puro1 and puro2 primers as described above. The PCR products at 1500 bp and 800 bp respectively were purified using Roche's High Pure PCR Product Purification kit. The DNA was ligated into pGEM using the pGEM T-easy kit. (2x buffer, 5 µl; T4 1 µl; pGEM 1µl and DNA 3 µl). Ligation was incubated overnight at 4°C. Ligations were transformed into *E. coli* DH10B and plated onto Lauria Bertani agar containing 100 mg/L ampicillin and 100 mg/L x-gal.

Five clones were randomly selected from each transformation. Clonal plasmid DNA was isolated and digested with *Eco*R1 at 37°C for 2 hours, then run on a 0.8% TBE gel for one hour at 100 V. Two clone variations were found from each

transformation. Plasmid isolations from each of these variants were sent for sequencing with M13F and M13R primers at AWGCS at Massey University, Palmerston North.

None of the sequences returned had significant matches to either the puro primers or to the 370 bp fragment. The sequences' closest matches were to plasmid vectors or to fragments of bacterial DNA sequences.

were they detected by the probe too? or are these unrelated to what the probe was detecting?

5. Conclusions

There is some concern regarding the possibility of horizontal transfer of puremycin resistance encoding gene from transgenic cattle carcasses buried at Ruakura to soil bacteria Monitoring of the soil bacterial populations for increase in puromycin resistance in bacterial population in the offal pits have been monitored for a number of years.

Samples taken in December 2008 were analysed for puromycin resistance in the soil bacterial community and the presence of homologous puromycin resistance encoding genes in the isolated bacteria. No increase in puromycin resistance in bacteria recovered from offal pits compared to control paddock soils was found. Five hundred and two individual bacterial colonies were probed for homology to a portion of the puromycin gene originally used in the cattle. Eighty three colonies from all the sources had some homology to the probe. Although not specifically tested, experience from previous years indicate that these belong to the genus *Pseudomonas*. It is also likely that the puromycin fragment is hybridising to weakly homologous sequences in the DNA (possibly from naturally occuring puromycin resistance genes).

There was no evidence of any puromycin resistance gene similar to the one used in the transgenic cattle in the offal or control soil bacteria, and no significant increase in puromycin resistant bacteria in offal pit soil compared to control soil.

Interim Report

Monitoring offal pits for puromycin resistance bacteria and the presence of genes encoding resistance to puromycin

Prepared by:

14 December 2009

Report For:

Background

In previous studies on the microbial communities in an offal pit used for the disposal of transgenic cattle remains at Ruakura, it was found that significant numbers of puromycin resistant bacteria were present in both control and offal pit soils, and that many colonies probed positive for the gene constructs used in the transgenic cattle project, but PCR failed to amplify a segment of the puromycin gene from any of the bacteria. Baseline community diversity and bacterial density data was collected to allow future comparisons of microbial community change in offal pit and surrounding soil.

This report covers ongoing monitoring of the offal pits for antibiotic resistant bacteria in December 2009.

Methods

Soil sampling

Soils were supplied by and team , sampled from offal pit directly and control soil nearby, sampled 30 November 2009. Samples analysed in the present study were collected using a soil corer (25 mm diameter) down to a depth of 15 cm.

Bacterial isolations

Soil dilutions were prepared by suspending 20g (dry weight equivalent) in 180 ml of 0.1% peptone (1g bacteriological peptone in 1l water). Ten-fold dilutions were made in 100 mM phosphate buffer were plated on selective media.

Bacteria were isolated on TSA + cycloheximide (100 µg/ml) or TSA + puromycin (125 µg/ml).

Randomly selected single colonies were transferred to appropriate (10% TSA or 10% TSA + puromycin) agar stabs in 2ml microcentrifuge tubes and stored at 4°C until probing.

Results

Bacterial isolations from soil

Colony forming units (CFUs) observed on the two media used are shown in Table 1.

Table 1: Colony forming units of bacteria per g (log_{10}) of soil (dry weight equivalent) cultured on two media (mean of 5-10 plates)

Sample ID[1]	10% TSA + Puromycin medium	10% TSA + Cycloheximide medium
CP1	6.27	6.71
CP2	5.66	6.35
CP3	5.71	6.63
CP4	7.27	7.32
CP5	6.16	7.04
Average	**6.66**	**6.94**
ACU1	7.39	6.92
ACU2	6.53	6.70
ACU3	6.25	6.99
ACU4	6.33	7.11
ACU5	6.19	6.84
Average	**6.82**	**6.94**

[1] CP: Control paddock (5 replicate samples); ACU: Animal control unit (5 replicate samples);

Summary

There were significant population of bacteria in both groups of soil samples (CP and ACU) that can grow on selective media containing puromycin.