Molecular epidemiology of antibiotic resistance in the commensal *Escherichia coli* of calves

Catherine M. Yates

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2004
Abstract

In animals, the study of antibiotic resistance in bacteria has been focused on organisms that are pathogenic in human or animal hosts. The development of antibiotic resistance in commensal bacteria is also of concern because they may act as a reservoir of resistance genes. This thesis aimed to determine levels of resistance to veterinary and medical antibiotics in the commensal *Escherichia coli* of calves, to explore the genotypic diversity of isolates, and to study the molecular mechanism and transfer dynamics of resistance to apramycin.

The antibiotic sensitivity testing of calf faecal *E. coli*, obtained by weekly sampling, demonstrated that there was resistance to beta-lactams, cephalosporins, streptomycin, trimethoprim, chloramphenicol, tetracycline and sulphamethoxazole. These resistance phenotypes had not been selected for on antibiotic-containing media, indicating a high prevalence of the corresponding resistance determinants.

Five hundred and forty three isolates were genotyped by pulsed-field gel electrophoresis. Examination of the patterns generated by restriction with *XbaI* and analysed with BioNumerics software revealed a total of 55 different genotypes. Ampicillin resistant isolates were more diverse (24 genotypes) than apramycin or nalidixic acid resistant isolates (5 and 2 genotypes respectively). Apramycin resistance (apr^R^) was conferred by three conjugative plasmids, pUK2001, pUK2002 and pUK2003, of sizes 91, 115 and 181Kb respectively. All apr^R^ plasmids conferred cross-resistance to the medical antibiotics tobramycin and gentamicin. Plasmids pUK2002 and pUK2003 also carried tetracycline and streptomycin resistance. Plasmid pUK2001 demonstrated very high transfer frequencies (4.12x10^3^ during 7 hrs mating), horizontal spread to three different genotypes, and an apparent fitness advantage *in vitro*.

This thesis shows a very high prevalence of antibiotic resistance genes in the commensal faecal flora of food-producing calves. This may have significant implications for the transmission of resistance genes to human clinical bacteria.
Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Catherine M. Yates
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Special thanks also to my wonderful friends and family who have supported, consoled, and encouraged me throughout the many ups and downs.
Publications and Presentations


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>arbitrary fragment length polymorphism</td>
</tr>
<tr>
<td>AMP</td>
<td>ampicillin</td>
</tr>
<tr>
<td>amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ampicillin resistant</td>
</tr>
<tr>
<td>AMX</td>
<td>amoxycillin</td>
</tr>
<tr>
<td>APR</td>
<td>apramycin</td>
</tr>
<tr>
<td>apr&lt;sup&gt;R&lt;/sup&gt;</td>
<td>apramycin resistant</td>
</tr>
<tr>
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<td>azlocillin</td>
</tr>
<tr>
<td>AZT</td>
<td>aztreonam</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cfu</td>
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</tr>
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<td>clavulanic acid</td>
</tr>
<tr>
<td>D</td>
<td>donor</td>
</tr>
<tr>
<td>DM</td>
<td>Davis Mingioli</td>
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</tr>
<tr>
<td>DNE</td>
<td>cephradine</td>
</tr>
<tr>
<td>ESBL</td>
<td>extended spectrum β-lactamase</td>
</tr>
<tr>
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<td>cefoxitin</td>
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<tr>
<td>GNT</td>
<td>gentamicin</td>
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<td>isosensitest</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>LB</td>
<td>Lennox L broth</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MLEE</td>
<td>multilocus enzyme electrophoresis</td>
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</tr>
<tr>
<td>nal&lt;sup&gt;R&lt;/sup&gt;</td>
<td>nalidixic acid resistant</td>
</tr>
<tr>
<td>P</td>
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<td>PCR</td>
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</tr>
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</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>QRDR</td>
<td>quinolone resistance determining region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>R</td>
<td>recipient</td>
</tr>
<tr>
<td>RAPD</td>
<td>random amplified polymorphic DNA</td>
</tr>
<tr>
<td>rep-PCR</td>
<td>repetitive element polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDW</td>
<td>sterile distilled water</td>
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<tr>
<td>SSRL</td>
<td>Scottish Salmonella Reference Laboratory</td>
</tr>
<tr>
<td>STP</td>
<td>streptomycin</td>
</tr>
<tr>
<td>SUL</td>
<td>sulphamethoxazole</td>
</tr>
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</tr>
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</tr>
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</tr>
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<td>ceftizoxime</td>
</tr>
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</tr>
<tr>
<td>URX</td>
<td>cefuroxime</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
# Contents

## Chapter 1. Introduction

1.1 Antibiotic targets of action .......................................................... 1  
1.2 Mechanisms of antibiotic resistance .............................................. 2  
1.3 Mechanisms enabling the dissemination of resistance genes .......... 3  
  1.3a Transduction ........................................................................... 4  
  1.3b Transformation ....................................................................... 4  
  1.3c Conjugation ............................................................................ 5  
  1.3d Transposons and integrons ....................................................... 7  
1.4 Ecology of *E. coli* in farm animals ............................................ 8  
1.5 Agricultural uses of antibiotics ..................................................... 10  
1.6 Effects of antibiotic usage in food animals ................................. 11  
  1.6a Negative effects of antibiotic use in food animals .................. 11  
    i) Selection of resistant bacteria ................................................. 11  
    ii) Antibiotic residues contaminating foodstuffs ...................... 13  
    iii) Selection of antibiotic resistant zoonotic pathogens .......... 14  
    iv) Selection of antibiotic resistant commensals ..................... 15  
  1.6b Advantages of antibiotic use in food animals ......................... 17  
1.7 Conclusions ................................................................................ 18  
1.8 Aims of the thesis ....................................................................... 20

## Chapter 2. Materials and reagents

2.1 Study animals and treatment records ......................................... 21  
2.2 Bacterial isolates ....................................................................... 22  
2.3 Bacterial identification ................................................................ 23  
2.4 Antimicrobial agents ................................................................... 24
### Chapter 3. Antimicrobial susceptibility testing

3.1 Introduction 26
3.2 Materials and Methods 29
   3.2a Bacterial isolates 29
   3.2b Susceptibility testing 30
   3.2c Statistical analyses 31
3.3 Results 33
   3.3a Reproducibility values 33
   3.3b Sensitivity of associations between antibiotics to variation between replicates 34
   3.3c Proportions of resistance 36
   3.3d Between-population comparison of susceptibility proportions 40
3.4 Discussion 44
   3.4a Assessment of levels and sources of variation within the methodology 44
   3.4b Proportions of resistance 48
   3.4c Associations between phenotypes 50
3.5 Concluding remarks 51

### Chapter 4. Genotyping

4.1 Introduction 53
4.2 Materials and Methods 58
   4.2a Bacterial isolates 58
Chapter 4. Analysis of genetic diversity

4.2b Pulsed-field gel electrophoresis  
4.2c Cluster analysis  
4.2d Statistics

4.3 Results

4.3a Population diversity and richness  
4.3b Genotype diversity  
4.3c Within-sample diversity  
4.3d Population diversity as a function of calf age  
4.3e Genotype diversity as a function of sampling date

4.4 Discussion

4.4a Population diversity  
4.4b Genotype diversity  
4.4c Within sample diversity  
4.4d Population diversity as a function of calf age  
4.4e Population diversity as a function of sampling date

4.5 Concluding remarks

Chapter 5. Molecular mechanism of apramycin resistance

5.1 Introduction

5.2 Materials and Methods

5.2a Bacterial isolates

5.2b Antibiotic sensitivity

5.2c Pulsed-field gel electrophoresis

5.2d Conjugation experiments

5.2e Plasmid analysis

5.2f Amplification of the \textit{aac(3)-IV} gene

5.2g Statistical analysis

5.3 Results

5.3a Detection of \textit{apr}^R \textit{E. coli} PFGE types
Chapter 6. The study of plasmid dynamics

6.1 Introduction 110

6.2 Materials and Methods 111
   6.2a Bacterial strains and plasmids 111
   6.2b Conjugations 112
   6.2c Estimates of plasmid transfer frequencies and rates 113
   6.2d Model theory 113
   6.2e Growth curves 115
   6.2f Statistics 115

6.3 Results 116
   6.3a Effect of mating time on estimates of plasmid transfer frequency 116
   6.3b Transfer rates estimated by the end-point method 117
   6.3c Variation in transfer frequency/rate with different methodology 122
   6.3d Modelling plasmid transfer in batch culture 124

6.4 Discussion 132
   6.4a In what manner do plasmid transfer frequency estimates vary as a function of mating time? 133
   6.4b What are the advantages and disadvantages of each technique? 134
   6.4c How do the transfer frequencies/rates measured by each of the methods compare? 136
   6.4d Does the published model of bacterial cell growth and plasmid transfer fit the experimental data of pUK2002 transfer in batch culture? 138
   6.4e If the model does not fit, what biological processes are occurring
which are not taken into account by the model?

6.4f Can the model be used to address questions of plasmid persistence in natural populations?

Chapter 7. Competitive fitness costs of apramycin resistance plasmids

7.1 Introduction 144
7.2 Materials and methods 145
  7.2a Bacterial strains and plasmids 145
  5.2b Five hour growth rate comparisons 145
  7.2c Five hour competition study 146
  7.2d Five day competition experiments 146
  7.2e Reproducibility of viable count method 147
  7.2f Statistical analyses 148
7.3 Results 149
  7.3a Variation introduced by the viable count method 149
  7.3b Five hour growth rate comparisons 150
  7.3c Five hour growth in direct competition 156
  7.3d Five day growth in direct competition 157
7.4 Discussion 159

Chapter 8. The search for extended-spectrum beta-lactamases

8.1 Introduction 164
8.2 Materials and methods 166
  8.2a Bacterial isolates 166
  8.2b Salmonella outbreak 167
  8.2c Antibiotic sensitivity 167
8.2d Determination of the presence of an ESBL 168
8.2e Amplification of blatEM-1 and blasHV-1 168

8.3 Results 169
  8.3a Antibiotic susceptibility 169
  8.3b Determination of the presence of an ESBL 171
  8.3c Amplification of blatEM-1 and blasHV-1 in S. Enteritidis 020003 171

8.4 Discussion 172

Chapter 9. Discussion 175

References 180

Appendices 210

Appendix 1. Results of a sensitivity analysis to determine how different parameters influence the output of the plasmid transfer in batch culture model. 210

Appendix 2. Results of different combinations of the three parameters found to enhance the fit of the model to the experimental data. 216
## List of Tables

1.1 Summary of antibiotic classes and their targets of action. 2
1.2 Examples of mechanisms conferring resistance to specific antibiotics. 3
2.1 Antibiotic treatment records for calves sampled in this study and calves housed with sampled calves. 21
2.2 Characteristics and sources of bacterial strains used in this work. 23
2.3 Antimicrobial agents, solvents and their suppliers. 24
3.1 Isolates investigated in this study, and the populations and numbers of calves from which they were obtained. 30
3.2 Categorisation of kappa values according to Everitt (1989). 31
3.3 Repeat classification of isolates by antibiotic susceptibility at two breakpoint concentrations. 34
3.4 Significance of variation generated by different grouping methods, assessed by Pearson’s $\chi^2$-squared test. 35
3.5 Association in levels of susceptibility between antibiotics of the same family. 41
3.6 Association between resistance to an antibiotic, and resistant population compared to the unselected population. 42
3.7 Association in level of susceptibility between antibiotics to which resistance is commonly carried on plasmids. 43
4.1 Numbers of isolates from each population and calf, typed by PFGE. 59
4.2 Diversity measures of the {\it nal}R, {\it apr}R, {\it amp}R and unselected populations. 69
4.3 Genotypes found within the calf commensal {\it E. coli} for which no genotypic diversity was found following PFGE typing with {\it XbaI}. 71
4.4 Genotypes found within the calf commensal {\it E. coli} for which diversity within a type was found following PFGE typing with {\it XbaI}. 72
4.5 Genotypes and dates of detection of isolates obtained from calf 687. 75
4.6 Genotypes and dates of detection of isolates obtained from calf 693. 76
4.7 Genotypes and dates of detection of isolates obtained from calf 694. 77
4.8 Genotypes and dates of detection of isolates obtained from calf 690. 79
4.9 Presence or absence of genotypes detected in calves 687, 693,694 and 690. 81
5.1 Calves from which apr\(^R\) E. coli were isolated, calf antibiotic treatments and age, dates of apr\(^R\) E. coli detection, and number and genotype of apr\(^R\) isolates. 99

5.2 Plasmid transfer frequencies of pUK2001, pUK2002 and pUK2003, measured on different antibiotic containing selective plates. 102

5.3 MICs of wild type apr\(^R\) E. coli donor isolates, and the transconjugants formed after the conjugation of the apr\(^R\) plasmids into J53. 103

6.1 Mean transfer frequencies (T/D\(_0\)) and number of replicates used in estimates. 117

6.2 Mean transfer rates and confidence intervals for the transfer of pUK2002 from J53 into J62-2 measured after different mating times by the end-point method. 120

6.3 Mean plasmid transfer frequencies as measured by the end-point method and mean plasmid transfer frequencies as measured by T/D\(_0\). 121

6.4 Mean transfer rates of pUK2002 into J62-2 and MG1655. 122

7.1. Descriptive statistics of data from method A and method B. 150

7.2 Relative fitness estimates and selection rate constants of MG1655 harbouring plasmid pUK2001, pUK2002 or pUK2003, in direct competition with MG1655kan. 158

8.1 Susceptibilities of 16 amp\(^R\) isolates selected for further study to \(\beta\)-lactams and cephalosporins. 170

8.2 Comparison of \(bla_{\text{TEM}}\) from S. Enteritidis 020003 and published \(bla_{\text{TEM-1}}\), and \(bla_{\text{TEM-52}}\) sequences. 172
3.1 Comparison of the proportions of resistance of amp$^R$, apr$^R$, nal$^R$ and unselected populations to $\beta$-lactams and quinolones. 37

3.2 Comparison of the prevalence of resistance of amp$^R$, apr$^R$, nal$^R$ and unselected populations to the cephalosporins. 38

3.3 Comparison of the prevalence of resistance of amp$^R$, apr$^R$, nal$^R$ and unselected populations to the aminoglycosides and other classes of antibiotic. 39

3.4 Variation in susceptibility classification due to breakpoint position. 45

4.1a PFGE genotypes in the apramycin resistant population. 63

4.1b PFGE types in the nalidixic acid resistant population. 63

4.1c PFGE types in the ampicillin resistant population. 64

4.1d PFGE types in the unselected population. 65

4.2 Numbers of lineages in the unselected, amp$^R$, and apr$^R$ populations plotted against increasing percentage similarity as calculated by the dice coefficient. 66

4.3 Rank abundance curve of the 543 commensal E. coli genotyped by PFGE in this study. 67

4.4 Rank abundance curves of the unselected, amp$^R$, apr$^R$, and nal$^R$ populations. 68

4.5 Within-sample diversity presented as the number of genotypes detected within a sample from the apr$^R$, amp$^R$, nal$^R$, and unselected populations. 73

4.6 Genotype prevalence with calf age of unselected and selected (amp$^R$, apr$^R$ and nal$^R$) isolates obtained from calf 687. 75

4.7 Genotype prevalence with calf age of unselected and selected (amp$^R$, apr$^R$ and nal$^R$) isolates obtained from calf 693. 76

4.8 Genotype prevalence with calf age of unselected and selected (amp$^R$, apr$^R$ and nal$^R$) isolates obtained from calf 694. 77

4.9 Genotype prevalence with calf age of unselected and selected (amp$^R$, apr$^R$ and nal$^R$) isolates obtained from calf 690. 79

4.10 Genotype prevalence within the unselected population. 83

4.11 Genotype prevalence within the amp$^R$ population. 84

4.12 Genotype prevalence within the nal$^R$ population. 85
5.1 Dates of detection and prevalence of the five different apr$^R$ E. coli genotypes.

5.2 EcoRI plasmid types and the host genotypes from which they were isolated

5.3 Mean transfer frequencies of pUK2001 into 14 different recipients from the unselected E. coli commensal population.

6.1 The effect of mating time on estimates of plasmid transfer frequency as determined by T/Do.

6.2 Eight replicate growth curves of J53 harbouring plasmid pUK2002 in 10ml LB.

6.3 Geometric mean growth curve of J53 harbouring pUK2002 10ml of LB.

6.4 Four replicate experiments showing the effect of mating time on estimates of plasmid transfer rate of pUK2002 from J53 into J62-2, as measured by the end-point method.

6.5 A comparison of four replicate experiments to study plasmid transfer.

6.6 Population dynamics during pUK2002 transfer from J53 to J62-2, using the average plasmid transfer frequency over each of the time points.


6.8 The effect of 0.5, 1 and 1.5hrs lag in growth on the D and R populations.

6.9 The effect of plasmid transfer rate on the R and T populations.

6.10 Best fit of the model to the experimental data, using measured plasmid transfer rate.

7.1 Spread of counts obtained when repeat dilutions of the same bacterial culture was diluted and plated out 30 times, and when 30 repeat aliquots of the same culture were plated out.

7.2 Replicate growth curves of a, MG1655pUK2002; b, J53pUK2002; c, MG1655kan; and d, J53 in 10mL LB.

7.3 Geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 in 10mL LB.

7.4 Replicate growth curves of a, MG1655pUK2002; b, J53pUK2002; c, MG1655kan; and d, J53 in 10mL MM.
7.5 Geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 in 10mL MM.

8.1 MICs of 52 isolates to amoxicillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid.
Chapter 1. Introduction

Since the introduction of antibiotics to human medicine in the 1940s, and later to animal husbandry and veterinary medicine, microbial resistance to these drugs has been on the increase. The massive and often inappropriate use of antimicrobials in human and veterinary medicine, in agriculture and aquaculture, combined with the dramatic reduction in the number of new antimicrobial agents coming onto the market has led to a growing resistance problem. This complex and global phenomenon is threatening the successful management of human infectious disease.

This introductory chapter will take the form of six sections. Firstly, the antibiotic targets of action, the mechanisms by which bacteria may become resistant to different antibiotics, and the processes implementing the dissemination of antibiotic resistance genes will be described. Following this, information available on the ecology of *E. coli* in animals will be reviewed. Finally, the use of antibiotics in food-production animals will be described, before the literature documenting the impact of this usage is reviewed.

1.1 Antibiotic targets of action.

Antibiotics should act on targets present in bacterial cells with minimal host cell damage. The target may not be present in animal or human cells (e.g. a cell wall), or the antibiotic can only enter bacterial cells (e.g. tetracyclines), or the antibiotic binds selectively to the target in bacterial cells but not in mammalian cells (e.g. aminoglycosides). The first antibiotics in general use were the penicillins, which specifically target the synthesis of the bacterial cell wall. The resemblance of the penicillins (and other β-lactam antibiotics) to the substrate of the transpeptidase enzyme, causes the inhibition of this enzyme. This prevents the cross-linking of the peptidoglycan subunits, which are key components of the bacterial cell wall. Other targets of action of antibiotics include the inhibition of protein synthesis, of
tetrahydrofolate synthesis, and of the synthesis of nucleic acids. Representative antibiotics exhibiting these mechanisms of action are listed in Table 1.1.

Table 1.1 Summary of antibiotic classes and their targets of action.

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibiotic class</th>
<th>Antibiotics</th>
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<tr>
<td>Cell wall synthesis</td>
<td>Beta-lactams</td>
<td>Penicillins (ampicillin, amoxycillin)</td>
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<tr>
<td></td>
<td></td>
<td>Cephalosporins (cefoxitin, cefuroxime)</td>
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<tr>
<td></td>
<td></td>
<td>Carbapenems (meropenem)</td>
</tr>
<tr>
<td></td>
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<td>Monobactams (aztreonam)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beta-lactamase inhibitors (clavulanic acid)</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Aminoglycosides</td>
<td>Apramycin</td>
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<td></td>
<td></td>
<td>Streptomycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobramycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamicin</td>
</tr>
<tr>
<td></td>
<td>Macrolides</td>
<td>Erythromycin</td>
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<td></td>
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<td>Tetracyclines</td>
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<tr>
<td></td>
<td>Chloramphenicol</td>
<td>Chloramphenicol</td>
</tr>
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<td>DNA synthesis</td>
<td>Quinolones</td>
<td>Nalidixic acid</td>
</tr>
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<td></td>
<td>Fluoroquinolones</td>
<td>Ciprofloxacin</td>
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<td></td>
<td></td>
<td>Enrofloxacin</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>Rifampicin</td>
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</tr>
<tr>
<td>Tetrahydrofolate synthesis</td>
<td>Sulphonamides</td>
<td>Sulphamethoxazole</td>
</tr>
<tr>
<td></td>
<td>Diaminopyrimidines</td>
<td>Trimethoprim</td>
</tr>
</tbody>
</table>

Adapted from Amyes et al., 1996.

1.2 Mechanisms of antibiotic resistance.

Bacteria may evade the action of an antibiotic by intrinsic or acquired mechanisms. The former is species specific and so determines the spectrum of activity of the drug, whereas the latter is only present in a subpopulation of a species. Specific mechanisms by which bacteria may become resistant to an antibiotic are listed in Table 1.2.
Table 1.2 Examples of mechanisms conferring resistance to specific antibiotics.

<table>
<thead>
<tr>
<th>Resistance mechanism</th>
<th>Antibiotics</th>
<th>Type of resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impermeability</td>
<td>Tetracycline</td>
<td>Transferable</td>
<td>Levy &amp; McMurry, 1974.</td>
</tr>
<tr>
<td>Destruction</td>
<td>Beta-lactams</td>
<td>Transferable or chromosomal</td>
<td>Abraham &amp; Chain, 1940.</td>
</tr>
<tr>
<td>Modification</td>
<td>Aminoglycosides</td>
<td>Transferable or chromosomal</td>
<td>Benveniste &amp; Davies, 1971</td>
</tr>
<tr>
<td>Altered target</td>
<td>Quinolones, streptomycin</td>
<td>Chromosomal</td>
<td>Cox et al., 1964</td>
</tr>
<tr>
<td>Additional target</td>
<td>Trimethoprim</td>
<td>Transferable</td>
<td>Amyes &amp; Smith, 1974</td>
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<tr>
<td>Hyperproduced target</td>
<td>Trimethoprim</td>
<td>Transferable</td>
<td>Young &amp; Amyes, 1985</td>
</tr>
</tbody>
</table>

The resistance mechanisms listed in Table 1.2 are specific to a particular antibiotic, however bacteria may also be intrinsically resistant to a broad spectrum of antibiotics. The reduced number of porins on the outer membrane of *Acinetobacter baumannii* confers reduced susceptibility to a range of antibiotics, by limiting the extent of antibiotic penetration into the cell (Quale et al., 2003). Multiple antibiotic resistant (Mar) *E. coli* mutants have decreased susceptibility to many different classes of antibiotics (including tetracycline, ampicillin, nalidixic acid, rifampicin and chloramphenicol) (Cohen et al., 1993). Phage mediated deletion of genes encoding an efflux pump (*acrAB*) restored the original susceptibility of an *E. coli* Mar mutant, indicating that the AcrAB efflux pump plays a major role in the Mar phenotype (Okusu et al., 1996).

1.3 Mechanisms enabling the dissemination of resistance genes

Although intrinsic resistance to particular antibiotics may limit choices of antibiotics available for treatment, of greater concern is the acquisition of resistance by sensitive bacteria. This may occur by the processes of transduction, transformation and conjugation. These three processes will be described, before the importance of transposable elements (namely transposons and integrons) that make up mobile
resistance elements are discussed in the context of the dissemination of resistance.

1.3a Transduction.

Transduction is mediated by bacteriophages. As these generally have limited host ranges, Davies & Webb (1998) considered transduction less important in the dissemination of resistance genes than conjugation. However, Olsen et al., (1974) have described two phage types capable of infecting any Gram-negative bacterium carrying the resistance plasmid RP1, so could theoretically transfer DNA between unrelated species. During transduction the DNA is carried within the phage particle and so is protected from degradation. This has led to suggestions that in natural habitats transduction as a mechanism of gene transfer may be as important as transformation or conjugation (Zeph et al., 1988).

Jiang & Paul (1998) used a mathematical model to predict the potential for gene transduction in the marine environment. The model was based on one phage-host transduction system studied \textit{in vitro}, and assumed that all marine phages are always infective and that no other factor (such as temperature, pH, non-specific attachment of phage to other particles) influenced transduction rates. The value of a numerical extrapolation of \textit{in vitro} transduction frequencies is unclear because of these assumptions, in addition to the knowledge that transduction rates differ with different phage-host systems (Ichige et al., 1989).

1.3b Transformation

Transformation is the intragenic recombination of chromosomal genes encoding sensitive drug targets, with related genes from other bacteria to form mosaic genes that encode resistant drug targets (Carlson et al., 1983). The selection pressure provided by antibiotic usage has promoted the spread of mosaic genes encoding
penicillin resistant penicillin binding proteins (PBPs) among *Streptococcus* spp. (Dowson *et al.*, 1990) and sulphonamide resistant dihydropterate synthase (dhps) among *Neisseria* spp. (Fermer *et al.*, 1995). Tetracycline resistance, encoded by *tetB*, has also been reported to be disseminated by transformation, between *Haemophilus* spp. and *Moraxella catarhalis* (Roberts *et al.*, 1991).

Although most bacteria can be shown to be competent under certain conditions (Dubnau 1991; Smith *et al.*, 1981), natural transformation has been reported to occur in a limited number of genera (Carlson *et al.*, 1983; Catlin, 1960; Frischer *et al.*, 1990; Goldberg *et al.*, 1966; Juni & Janik, 1969; Lacks *et al.*, 1975; Mathis & Scocca, 1982; Rudin *et al.*, 1974; Wang & Taylor, 1990). Transformation has been considered less important than conjugation as a mechanism for dissemination of resistance genes (Roy 1999). This is because transformation requires the presence of DNA from genetically distinct bacteria in the immediate environment of the bacterium, a DNA uptake system, and a mechanism for the permanent incorporation of recruited DNA into the host chromosome. This is usually mediated by *recA* (Fischer & Haas, 2004; Pearce *et al.*, 1995). Although transformation appears to be rare, antibiotic usage provides an intense selective pressure on the complex microbial populations of the gastrointestinal tract. In this environment gene exchange may involve many steps between unrelated bacteria. The efficiency at which transfer occurs is not important if one considers that only a very small number of resistant bacteria are needed to survive and replicate to create clinical problems.

### 1.3c Conjugation

The transfer of DNA by conjugation requires direct contact between donor and recipient. The genetic elements that are self-transferable by conjugation are plasmids and transposons. Conjugative plasmids can transfer efficiently between different genera of the same Gram-stain group. Although conjugative plasmids can transfer between Gram-positive and Gram-negative bacteria they are often unable to replicate
in the new host because their host range for replication is less broad than that for transfer (Verma et al., 1989). Certain plasmids are however, capable of replication in a bacterium of different Gram-stain but are non-conjugative (Gormley & Davies, 1991; Goze & Ehrlich, 1980).

Transfer of a non-conjugative plasmid may occur by the presence of a co-resident self-transmissible plasmid providing, in trans, a conjugative system to mobilise the co-resident non-conjugative plasmid. Replicative transposons may also enable the transfer of non-conjugative plasmids (Deneer et al., 1982; Shoemaker et al., 1986; Valentine et al., 1988). These two mechanisms of transferring non-mobilisable plasmids may permit plasmid transfer between Gram-negative and Gram-positive bacteria (Doucet-Populaire et al., 1992), and so enhance the fluidity of genetic transfer in mixed bacterial populations, such as the intestinal flora of animals and humans.

Similarities between plasmids as evidence supportive for in vivo transfer have been described on the basis of common resistance patterns (Smith 1975), incompatibility groups (Grant & Pittard, 1974; Levy, 1976) and plasmid molecular weight (Kruse & Sørum, 1994; Nijsten et al., 1995). More convincing evidence for plasmid similarity would be provided by restriction enzyme digests of the plasmid using more than one enzyme, yielding identical banding patterns with each enzyme, because plasmids of apparently identical sizes may prove to be different after restriction analysis.

Plasmids encoding aminoglycoside 3-N-acetyltransferase (conferring apramycin and gentamicin resistance) have been found in isolates from cattle and humans (Chaslus-Dancla et al., 1991). A high degree of genetic homology was detected by preparing plasmid DNA and restricting with three different restriction enzymes, thus providing good evidence for plasmid exchange between bacteria of human and animal origin. Using the same method, identical apramycin resistance plasmids have been found in E. coli and S. typhimurium isolated from calves, where prior to apramycin treatment of the animals, the plasmid had only been found in E. coli. (Hunter et al., 1992).
Conjugative transposons are discrete DNA sequences capable of self-excision and integration into DNA, which are normally integrated into a bacterial genome (Franke & Clewell, 1981). Many multiple-antibiotic resistant Streptococcus and Staphylococcus spp. from clinical isolates have large conjugative transposons (Le Bouguenec et al., 1990). Plasmids have not been identified in these strains suggesting that conjugal transposons were responsible for the spread of multiple resistance observed (Scott, 1992).

Conjugative transposons were first identified in Gram-positive cocci (Franke & Clewell, 1981; Shoemaker et al., 1980), but since then, have also been found in a number of Gram-negative bacteria including Neisseria gonorrhoeae (Swartley et al., 1993), Haemophilus ducreyi (Roberts, 1990), and Bacteroides spp. (Nikolich et al., 1994a). Conjugative transposons are thought to make a considerable contribution to the spread of resistance genes amongst Bacteroides and other bacteria transiently colonising the human intestine (Salyers & Shoemaker, 1996). However this opinion is based on the detection of tetQ, (which is most commonly located on conjugative transposons in Bacteroides spp. (Nikolich et al., 1994b)) as an indicator of the presence of a conjugative transposon in this species (Salyers & Shoemaker, 1996). At present, conjugative transposons have not been found in E. coli.

1.3d Transposons and integrons

The incorporation of DNA, in the form of transposons and integrons, into mobile elements results in the evolution of resistance determinant plasmids (R plasmids) (Collis et al., 1993), which may then be spread via conjugation (Antunes et al., 2004; Heir et al., 2004; Pezzella et al., 2004).

The importance of transposons and integrons is due not only to their ability to enhance the dissemination of resistance genes, but also their ability to integrate into
the bacterial chromosome permitting the persistence of the resistance genes carried. Transposons have been implicated in the spread of tetracycline resistance in *Neisseria* spp. (Swartley et al., 1993) and *Bacteroides* spp. (Nikolich et al., 1994b), the spread of β-lactamase enzymes in *Proteus mirabilis*, *E. coli* (Naas et al., 2003) and amongst *Salmonella* spp. (Orman et al., 2002), and the spread of trimethoprim resistance (linked to streptomycin resistance in 8/12 plasmids examined) in clinical *E. coli* (Towner et al., 1982). Integrons are commonly found on plasmids (Briggs & Fratamico, 1999), but recently a new gene conferring resistance to chloramphenicol and florfenicol has been described that is surrounded by two class I integrons, on the *S. typhimurium* chromosome (Arcangioli et al., 1999). This has been speculated to allow persistence of resistance genes in the absence of antibiotic selection (Threlfall et al., 1994). In a study of sulphonamide resistance in Enterobacteriaceae, stable integration of *sulI* and *sulII* into transposons and plasmids was deduced as the mechanism responsible for the extremely efficient dissemination of these genes in the Gram-negative bacteria studied (Rådström et al., 1991).

1.4 Ecology of *E. coli* in farm animals

*E. coli* typically colonise the lower intestine of new-born mammals within hours. Smith & Crabb (1961) studied the faecal flora of healthy calves, and found large numbers of *E. coli* (approximately $10^5$ cfu/g), *Clostridium welchii* and streptococci after 1 day. Colonising *E. coli* are thought to be acquired from the environment, other animals, directly from faeces, or from contaminated food or water (Maison & Richardson, 1981). After ingestion, *E. coli* may be present in the lumen of the intestine, or may persist in the intestine for longer periods of time by adhering to the intestinal mucosa (Freter et al., 1983) with type 1 somatic pili (Freter, 1981). Certain strains of *E. coli* are found to persist for several weeks or months (Sears & Brownlee 1952; Hinton, 1986) before being replaced by other strains. Caugant et al. (1981), suggest that genetic diversity among *E. coli* strains transiently colonising the gastrointestinal tract is attributable to successive invasion by *E. coli* from
environmental sources, although in this study only 22 faecal samples were obtained over a period of eleven months.

Factors influencing *E. coli* ecology are unclear, partly because of a limited number of studies on the subject, inadequate typing or sampling protocols, and because only a tiny proportion of *E. coli* present in an animal can be studied in any detail. Diet has been found to influence *E. coli* numbers (Diez-Gonzalez *et al.*, 1998; Hovde *et al.*, 1999; Kruse *et al.*, 2003), in addition to stress, disease, composition of other intestinal flora (Hartl & Dykhuizen, 1984; Shedlofsky & Freter, 1974), animal management practices (Hinton *et al.*, 1984) and host genome (Toivanen *et al.*, 2001).

Antibiotic treatment also affects the ecology of *E. coli* in farm animals (Delsol *et al.*, 2003; Dunlop *et al.*, 1998). Resistance to antimicrobial agents is caused by the use of these agents in any dose and over any time period exerting a selective pressure on the microbial population. Those micro-organisms harbouring a mutation(s) in the drug target site, or those that have acquired novel biochemical activities conferring resistance, will survive to produce progeny bearing the same resistance determinants. The selective pressures responsible for the emergence of resistant bacteria in cattle include antibiotic usage for therapy, prophylaxis and growth promotion. There has been considerable debate over the past 40 years on whether antibiotics administered to animals can lead to the selection of resistant bacteria that, on entering the food chain, may result in human infection by these less sensitive strains. In 1969, human health concerns over the widespread use of antimicrobial agents as growth promoters precipitated the appointment of the Swann Committee. Its report (Swann committee 1969), stated that “the administration of antibiotics to farm livestock, particularly at sub-therapeutic levels, poses certain hazards to human and animal health”. More precisely, that this practice had resulted in the emergence of resistant enteric bacteria whose resistance determinants were transmissible to other bacteria, and that these more resistant strains were transferable from animals to man.

A description of the use of antibiotics in agriculture now follows, after which the
potential impact this has on the agricultural industry and on public health is reviewed.

1.5 Agricultural uses of antibiotics

In the UK in 2002, 484 tonnes of active antimicrobial agents were sold for use in food animals (Veterinary Medicines Directorate, 2002). In farm animals, antibiotics are used therapeutically to treat disease (e.g. calf pneumonia and neonatal E. coli infections), prophylactically to prevent disease, and thirdly as growth promoting agents. Ten percent of the antibiotics used in agriculture are given therapeutically, the remainder are used as growth promoters and prophylactic agents (Chadwick & Goode, 1997).

Although the exact mechanisms of action of growth promoters is unknown, the administration of these agents to animals results in an increase in weight gain (between 3 and 5% depending on the species (Select Committee on Science and Technology, 1998). Growth promoters are thought to interact with microbial flora of the gastrointestinal tract, improving digestion, absorption and metabolism, and may suppress infectious disease and metabolic and fermentation disorders (Feighner & Dashkevicz, 1987). Animals fed growth promoters require less food and produce less waste (Gaskins et al., 2002). Growth promoters have allowed savings to be made on space, labour and cost of rearing farm animals, because of the concurrent reduction in the occurrence of disease associated with stress and overcrowding. This has lead to increased intensification of farming practices albeit alongside continuing criticism that growth promoters are being used to substitute good husbandry and hygiene (Select Committee on Science and Technology, 1998).
1.6 Effects of antibiotic usage in food animals

The negative effects of antibiotic usage in animal agriculture include a reduction in the effective life of antibiotics used in animal therapy, foodstuff contamination with antibiotic residues, and the selection of resistant bacteria, both zoonotic and commensal. However, antibiotic use in food animals is required for the treatment of bacterial diseases, resulting in healthier animals and subsequent economic benefits. Reports of the negative effects of antibiotic usage in food production animals will be reviewed first, followed by the advantages of such antibiotic use.

1.6a Negative effects of antibiotic use in food animals

i) Selection of resistant bacteria

The use of antibiotics in farm animals will select for resistant bacteria (Levy, 1987), in the same way that antibiotic use in humans has selected for resistant bacteria. Antibiotics used prophylactically are usually administered to groups of animals and may occur over prolonged periods of time. Salyers (1999b) described this method of administration as being more likely to lead to the emergence of resistant strains than prophylactic use in human medicine, because of the numbers of animals involved. There appears to be no concrete evidence that this is the case.

The amount of antibiotic used and the time frame of administration are important parameters affecting the frequency at which resistant bacteria arise. Lipsitch & Levin (1997) have used mathematical models to predict the emergence of resistance during antibiotic treatment. Here they show how the probability of a resistant mutant appearing depends on the initial size of the susceptible population, cell division, mutation and net killing rates. If the killing rate of the susceptible bacteria is low compared to the rate of cell division, the likelihood of a resistant mutant appearing is
increased. The model predicts a shorter time span before the appearance of the first mutant if the killing rate is slower, consistent with observations made by others that sub-inhibitory concentrations of antibiotic may be effective in selecting for resistant mutants (Haas et al., 1990; Tenney et al., 1983).

The concentrations of antibiotic used for growth promotion are described as subtherapeutic (Donabedian, et al., 2003a; Johnston, 1998). This term gives the impression that the concentrations used are too low to affect bacterial growth and hence select for resistant populations. Although these concentrations are ineffective for therapy, they may still exert a slight selective pressure on a bacterium, allowing it to grow and acquire new DNA or accumulate mutations increasing its resistance to the drug (Aarestrup et al., 2000; Boerlin et al., 2001; Emborg et al., 2003).

The emergence of resistant bacteria through the use of antibiotics in food production animals may lead to treatment failures and economic problems for the individual farmer and on a wider scale, the agricultural industry. This is exemplified by an outbreak of multi-resistant Salmonella typhimurium that occurred in 1998 on a calf farm in Arizona. The deaths of many calves due to the outbreak resulted in the farm going out of business (Salyers, 1999b).

It has been suggested that if antibiotic selection is continuous, as is the case with the use of growth promoters, bacteria may accumulate compensatory mutations allowing maintenance of their resistance mechanisms without a concurrent reduction in fitness in the absence of the antibiotic (Salyers, 1999b). There are now a few studies reporting an in vitro amelioration of the cost of antibiotic resistance (Dahlberg & Chao, 2003; Lenski et al., 1994; Schrag et al., 1997). Bouma & Lenski (1988) demonstrated that carriage of a plasmid encoding chloramphenicol and tetracycline resistance by E. coli B, reduced bacterial fitness in the absence of chloramphenicol. After 500 generations of culture in the presence of chloramphenicol, the plasmid enhanced the fitness of its host even in the absence of antibiotic. Sequencing and characterisation of the nature of the genetic change would be required to validate
such a conclusion. Additionally, no controls were used to ensure that the genes encoding resistance were being expressed in each generation, as gene repression in the absence of antibiotic may be effective in reducing the cost of resistance.

If fitness costs associated with an antibiotic resistant phenotype are compensated for by adaptive mutations, why are many bacteria still sensitive to drugs introduced some 40 years ago e.g. β-lactams, trimethoprim (Livermore et al., 2000) and tetracyclines (Österblad, 2000)? According to DANMAP (1998), glycopeptide resistant isolates (measured as resistance to avoparcin) from broilers have decreased from 82% in 1995 to 9% in 1998, coinciding with a Danish ban in 1995 on the use of avoparcin as a growth promoter. Similarly, avilamycin has not been used since 1998 and a decline in resistant enterococci from broilers from 79% in 1996 to 29% in 1998 has been demonstrated (DANMAP, 1998). These results are suggestive of a fitness disadvantage associated with the acquisition of a specific antibiotic resistance mechanism.

**ii) Antibiotic residues contaminating foodstuffs**

Antibiotics used in food animal production may remain in the animal’s tissues, and following ingestion of the meat the residues may select for resistant bacteria in the intestine of the consumer (Anadon et al., 1999; McCracken et al., 1976). A major flaw in many of these experiments is that the meat products are usually consumed after cooking, yet raw tissue is often studied (Anadon et al., 1999). Antibiotic residues in foodstuffs are likely to constitute a comparatively minor threat to public health in comparison to the massive use of antibiotics directly administered to humans both in hospitals and in the community (Corpet, 1987; Levy, 1998). Laws governing the number of days an animal should be antibiotic free prior to slaughter are also in place to protect consumers from the risk of antibiotic residues in foodstuffs.
iii) Selection of antibiotic resistant zoonotic pathogens

The potential hazard posed by the selection of zoonotic pathogens (e.g. *Salmonella* spp. *Campylobacter* spp. and *E. coli* O157:H7) follows the contamination of foodstuffs and subsequent human infection where antibiotic therapy is compromised by resistance determinants carried by the pathogen. It has proved difficult to find clear-cut results demonstrating that this kind of scenario is occurring. In addition, as gastroenteritis caused by these pathogens is generally not treated with antibiotics (indeed their use is contraindicated in the case of salmonellosis and *E. coli* O157:H7 infection), this potential hazard has been deemed unimportant (Linton, 1984).

Evidence supporting the transmission of antibiotic resistant *E. coli* from poultry to the human gut, following ingestion of cooked chicken is provided by Linton (1977b). After handling *E. coli* of poultry origin only 1 of 5 volunteers were found to be excreting resistant *E. coli*. The strain from the poultry and that recovered from the faeces of the volunteer were declared identical based on their antibiotic resistance pattern, plasmid profiles and serotyping. A greater number of volunteers and genotyping by PFGE would provide more definitive evidence.

Studies by Endtz et al., (1991) on quinolone resistance in *Campylobacter* have shown an increase in the prevalence of resistant strains coinciding with the extensive use of enrofloxacin in poultry. The conclusions drawn that the resistance observed is mainly due to the use of this drug in the poultry industry, is substantiated by evidence that person to person transmission of *Campylobacter* is rare (Skirrow, 1977).

The spread of chloramphenicol resistant *Salmonella newport* from dairy farms to hamburgers resulting in human illness has been reported by Spika et al. (1987). A risk factor identified by Spika et al. (1987), for illness caused by chloramphenicol-resistant *Salmonella* was the use of penicillin or tetracycline in the month prior to onset. The use of these drugs may provide those bacteria harbouring a multiple
antibiotic resistance plasmid to outgrow other salmonellae ingested at the same time, resulting in an infection by the resistant strains. However there is some evidence that strains of salmonellae carrying multi-resistance plasmids may be more virulent than sensitive ones due to an increased persistence of plasmid bearing host organisms in the intestine (Timoney & Linton, 1982).

Other examples of the transmission of resistant bacteria from animals to humans include indistinguishable glycopeptide-resistant enterococci isolated from turkeys and their farmer in The Netherlands (van den Bogaard et al., 1997), and highly similar ceftriaxone-resistant Salmonella enterica serovar Typhimurium in a child and cattle in Nebraska (Fey et al., 2000). Similar to the study by Spika et al. (1987), in this latter report, the child had received extensive treatment with β-lactam antibiotics (a 10-day course of amoxycillin-clavulanate, prophylaxis with ampicillin-sulbactam before appendectomy, then post operative treatment with amoxycillin-clavulanate) prior to the onset of Salmonellosis. These treatments are likely to have selected for the ceftriaxone-resistant strain. One problem with these studies is that the environmental reservoir of such isolates is not examined, and so the possibility of acquisition of the resistant strain from the environment cannot be excluded.

The literature on the transmission of resistant bacteria from animals to humans is limited. This may be because resistance is not looked for in these illnesses as antibiotics are rarely used, but also because zoonoses are preventable by taking the appropriate care with food preparation.

iv) Selection of antibiotic resistant commensals

The use of antibiotics in agriculture increases resistance levels in animal commensal bacteria (Van den Bogaard & Stobberingh, 2000). The contamination of meat in abattoirs by these commensals is inevitable (Howe et al., 1976b; Linton et al., 1977b) but the extent to which resistant strains are transferred into the human intestine is
unknown.

To date, there appears to be only one report describing how the ingestion of resistant bacteria resulted directly in failed therapy, and this case does not imply contamination from the farm or processing. Ingestion of food contaminated with methicillin resistant *Staphylococcus aureus* (MRSA) resulted in an outbreak of MRSA (Kluymans *et al.*, 1995). However, the index patient was severely immunocompromised and had taken oral ciprofloxacin and antacids (respectively, these two drugs may have selected for the MRSA strain and neutralised gastric acid used as a defence against infection). This kind of scenario is likely to be extremely unusual, but demonstrates food as a vector for resistant organisms.

There are a few reports stating that the antibiotic-resistant organisms causing illness in humans are different strains to those prevalent in animals. Nijsten *et al.* (1996) reported that the resistance of the faecal *E. coli* of pig farmers and their pigs are distinctly different. Similarly, urinary tract infections in women working in poultry processing plants were found to be caused by *E. coli* with different antibiotic resistant patterns to those isolated from the poultry (Parsonnet & Kass, 1987). In contrast to this, studies on vancomycin-resistant enterococci (VRE) have shown that the same ribotypes isolated from poultry were forming part of the normal bowel flora in humans (Bates *et al.*, 1994). The selection of these strains by antibiotic usage in hospitals was suggested as an explanation for the emergence of VRE in hospitals.

Whether or not one particular resistant bacterial strain is directly responsible for human illness is unimportant if one considers that if a bacterium carrying resistance determinants colonises the human gut (even just transiently), there is potential for the spread of these determinants to other intestinal flora. Teuber & Perreten (2000) have reported that the exchange of resistance genes between bacteria from uncooked meat or unpasteurized cheese, and human bowel flora (*Enterococcus* spp.) during passage through the intestine is highly probable. Again, the degree of movement of resistance genes, between bacteria colonising the human bowel, is unknown (Salyers, 1999b).
Evidence that horizontal gene transfer does occur in the intestine is provided by Nickolich et al., (1994b), where tetQ in Prevotella ruminicola from animals had greater than 95% DNA similarity to tetQ from human colonic Bacteroides spp. Based on DNA sequences flanking the gene the authors suggest transfer occurred from humans to animals.

Similarly, almost identical copies (> 99% DNA sequence identity) of the ermG gene, encoding erythromycin resistance, have been found in Bacillus sphaericus and Bacteroides spp. These are found in soil and in the human colon respectively (Cooper et al., 1996). Ingested soil bacteria may have transferred the gene, which is found on a conjugative transposon (Tc Em 7853) to Bacteroides species in the human gut. It is unclear as to in which bacteria the gene is most likely to have arisen. Bacteria that colonise soil may encounter bacteriocins and antibiotics produced by other soil bacteria, but bacteria that normally colonise the human intestine may be dosed with various antibiotics, usually not intended to have an effect on commensal bowel flora such as Bacteroides.

This example illustrates an important flaw in retrospective studies, in that the direction of transfer is difficult to prove with absolute certainty. There are many examples of laboratory experiments demonstrating gene transfer between diverse bacteria (Doucet-Populaire et al., 1992; Chaslus-Dancla et al., 1986; Gordon, 1992; Hunter et al., 1992). These are reviewed by Mazodier & Davies (1991) and are often used to obtain evidence of intergenic transfer of resistance determinants in nature. Intergenic transfer observed in such laboratory experiments is not conclusive evidence that this occurs in nature.

1.6b Advantages of antibiotic use in food animals

Antimicrobial chemotherapy is vital for the treatment and prevention of bacterial diseases in animals (e.g. calf pneumonia, mastitis and neonatal E. coli infections),
which may cause pain and distress or be potentially fatal. For bacterial diseases where vaccines are unavailable, the treatment of animals with antibiotics is necessary for both humane reasons and for economic reasons (preventing further spread of the etiological agent).

Antibiotic usage in food animals may reduce the loads of salmonellae and campylobacter shed by food animals, thereby helping to diminish the risk to humans from these zoonoses (Phillips et al., 2004).

Growth promoters increase rates of bodyweight gain, but may also reduce incidences of acute pneumonia and metabolic and fermentative disorders in cattle, and improve heat tolerance and immune status (Gaskins et al., 2002).

The European ban on the use of several growth promoting agents has resulted in decreases in avoparcin resistant animal enterococci (DANMAP, 2002), virginiamycin resistant enterococci in chickens (DANMAP, 2002), and human vancomycin resistant enterococci (van den Bogaard et al., 2000). However, some therapeutic uses of antibiotics have increased as a direct result of this ban (Casewell et al., 2003). In Denmark increases in tetracycline, penicillins, sulphonamides, macrolides and aminoglycosides have followed the ban on growth promoters (DANMAP, 2002). Similarly, increases in therapeutic use of antibiotics followed the 1986 growth promoter ban in Sweden (Wierup, 2001).

1.7 Conclusions

This review describes the targets of antibiotics and the mechanisms by which bacteria may acquire resistance against antibiotics. Once resistance has evolved in a bacterium, the genes for this resistance mechanism may be spread to other bacteria of the same or of different species, by the processes of transduction, transformation and conjugation.
Antibiotics are used in animal husbandry for the treatment of disease, prophylaxis, and growth promotion. This usage results in the selection of antibiotic resistant zoonotic and commensal bacteria.

*E. coli* is one of the normal microbial constituents of the gastrointestinal tract of mammals, and colonises the mammalian intestine within hours of birth. Although some factors influencing intestinal *E. coli* ecology have been characterised, there is little data available on the ecology of commensal *E. coli* of healthy food animals. The need for surveillance of antimicrobial resistance in both pathogenic and commensal bacteria has been stressed by many governmental and non-governmental bodies, and by independent scientists (Caprioli *et al.*, 2000; Jones, 2000; Masterton, 2000; O’Brien, 1997; Salyers, 1999a; World Health Organisation, 1997). Against a backdrop of debate on the risks to human health posed by antibiotic usage in animal husbandry (Phillips *et al.*, 2004), a better understanding of the ecology of commensal *E. coli* in healthy food animals, the levels of antibiotic resistance present, and the mechanisms of antibiotic resistance harboured by this population is required.
1.8 Aims of the thesis

Although there have been previous studies on antibiotic resistance, these were performed without the benefit of modern molecular techniques. The overall aim of this project is to examine the epidemiology of resistant bacteria in a cohort of animals in close geographic proximity. The more specific aims of the thesis are listed below:

- Determine the antibiotic sensitivities of calf faecal commensal *E. coli* to a range of veterinary and medical antibiotics.

- Determine the genotypic diversity of calf faecal commensal *E. coli*.

- Determine the mechanism of resistance to apramycin in the commensal *E. coli* population.

- Explore the use of a mathematical model in characterising the dynamics of resistance plasmid transfer.

- Determine whether apramycin resistance plasmids confer a competitive fitness disadvantage on a host bacterium.

- Determine whether calf faecal commensal *E. coli* harbour extended-spectrum β-lactamase enzymes.
Chapter 2. Materials and Reagents

2.1 Study animals and treatment records

Eleven beef-suckler calves on a non-organic Scottish farm, born between 14\textsuperscript{th} September and the 1\textsuperscript{st} of October 2001 were studied. The sampled calves were part of a cohort of 49 calves, which were kept separate from all other animals on the farm. The only treatments received by these 49 calves are shown Table 2.1. Four of the eleven calves sampled in this study received antibiotic treatment(s). These calves are highlighted in blue.

Table 2.1 Antibiotic treatment records for calves sampled in this study (blue calf IDs), and calves housed with sampled calves (black calf IDs).

<table>
<thead>
<tr>
<th>Calf ID</th>
<th>Treatment date</th>
<th>Disease</th>
<th>Antibacterial agent</th>
<th>Generic name</th>
<th>Trade name</th>
</tr>
</thead>
<tbody>
<tr>
<td>688</td>
<td>09/10/01</td>
<td>pneumonia</td>
<td>enrofloxacin</td>
<td>Baytril Max</td>
<td></td>
</tr>
<tr>
<td>693</td>
<td>21/11/01</td>
<td>pneumonia</td>
<td>tylosin</td>
<td>Tylan</td>
<td></td>
</tr>
<tr>
<td>23/11/01</td>
<td>pneumonia</td>
<td>tylosin</td>
<td>Nuflor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>05/12/01</td>
<td>pneumonia</td>
<td>florfenicol</td>
<td>Nuflor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>697</td>
<td>01/10/01</td>
<td>navel ill</td>
<td>enrofloxacin</td>
<td>Baytril Max</td>
<td></td>
</tr>
<tr>
<td>09/10/01</td>
<td>navel ill</td>
<td>tylosin</td>
<td>Tylan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12/11/01</td>
<td>navel ill</td>
<td>tylosin</td>
<td>Tylan 200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>698</td>
<td>01/10/01</td>
<td>navel ill</td>
<td>enrofloxacin</td>
<td>Baytril Max</td>
<td></td>
</tr>
<tr>
<td>699</td>
<td>09/10/01</td>
<td>navel ill</td>
<td>tylosin</td>
<td>Tylan</td>
<td></td>
</tr>
<tr>
<td>711</td>
<td>30/10/01</td>
<td>navel ill</td>
<td>enrofloxacin</td>
<td>Baytril Max</td>
<td></td>
</tr>
<tr>
<td>06/11/01</td>
<td>scouring</td>
<td>amoxicillin + clavulanic acid</td>
<td>Synulox bolus</td>
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<td></td>
</tr>
<tr>
<td>09/11/01</td>
<td>navel ill</td>
<td>tylosin</td>
<td>Tylan200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>717</td>
<td>20/12/01</td>
<td>pneumonia</td>
<td>oxytetracycline</td>
<td>Terramycin LA</td>
<td></td>
</tr>
<tr>
<td>719</td>
<td>09/11/01</td>
<td>navel ill</td>
<td>tylosin</td>
<td>Tylan200</td>
<td></td>
</tr>
<tr>
<td>720</td>
<td>09/11/01</td>
<td>navel ill</td>
<td>tylosin</td>
<td>Tylan200</td>
<td></td>
</tr>
<tr>
<td>722</td>
<td>05/12/01</td>
<td>pneumonia</td>
<td>florfenicol</td>
<td>Nuflor</td>
<td></td>
</tr>
<tr>
<td>723</td>
<td>05/12/01</td>
<td>scouring</td>
<td>amoxicillin + clavulanic acid</td>
<td>Synulox bolus</td>
<td></td>
</tr>
<tr>
<td>20/12/01</td>
<td>pneumonia</td>
<td>oxytetracycline</td>
<td>Terramycin LA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2 Bacterial isolates

Rectal faecal samples were taken within 48h of birth. Calves were subsequently sampled approximately weekly until the 14th January 2002 excepting two weeks at Christmas and New Year. Samples were stored at 4°C and processed within 48h of collection. Samples were diluted 1:10 in maximum recovery diluent (Oxoid, Basingstoke, UK). Ten microlitres of each suspension was spread onto Chromocult TBX (tryptone bile X-glucuronide) agar (Merck, Darmstadt, Germany), incorporating 16mg/L ampicillin, or 8mg/L apramycin, or 8mg/L nalidixic acid (Sigma) or without antibiotics. Following overnight incubation at 44°C, glucuronidase positive E. coli were picked from each plate showing growth. The number of picks taken per plate differed depending on the number of colonies present, but in all cases, every discrete colony present on the plate was picked. Colonies were purified by subculturing twice on MacConkey agar (Oxoid, Basingstoke, UK), and then stored at -70°C on cryogenic beads (Mast Diagnostics, Germany). Other bacterial strains used in this study are listed in table 2.2.
Table 2.2 Characteristics and sources of bacterial strains used in this work.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> NCTC10418</td>
<td>Standard strain</td>
<td>R. Paton (Edinburgh University)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> NCTC10662</td>
<td>Standard strain</td>
<td>R. Paton (Edinburgh University)</td>
</tr>
<tr>
<td><em>S. aureus</em> NCTC6571</td>
<td>Standard strain</td>
<td>R. Paton (Edinburgh University)</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC11560</td>
<td>TEM-1 positive strain</td>
<td>R. Paton (Edinburgh University)</td>
</tr>
<tr>
<td><em>E. coli</em> 39R861</td>
<td>Plasmid sizing standard</td>
<td>Scottish Salmonella Reference Laboratory, Glasgow</td>
</tr>
<tr>
<td><em>E. coli</em> V517</td>
<td>Plasmid sizing standard</td>
<td>Scottish Salmonella Reference Laboratory, Glasgow</td>
</tr>
<tr>
<td><em>S. enterica</em> serovar Enteritidis</td>
<td>Outbreak strain</td>
<td>Scottish Salmonella Reference Laboratory, Glasgow</td>
</tr>
<tr>
<td><em>E. coli</em> MG1655</td>
<td>Standard strain</td>
<td>A. Roe, ZAP laboratory (Edinburgh University)</td>
</tr>
<tr>
<td><em>E. coli</em> MG1655kan</td>
<td>Kanamycin resistance in place of lacZY genes</td>
<td>A. Roe, ZAP laboratory (Edinburgh University)</td>
</tr>
<tr>
<td><em>E. coli</em> J53</td>
<td>Pro' Met'</td>
<td>S.G.B.Amyes, MC laboratory (Edinburgh University)</td>
</tr>
<tr>
<td><em>E. coli</em> J62-2</td>
<td>Pro' His' Trp'</td>
<td>S.G.B.Amyes, MC laboratory (Edinburgh University)</td>
</tr>
<tr>
<td></td>
<td>Rifampicin resistant</td>
<td></td>
</tr>
</tbody>
</table>

2.3 Bacterial identification

Glucuronidase positive *E. coli* were identified by the growth of blue colonies on TBX agar following overnight incubation at 44°C.
2.4 Antimicrobial agents

Antimicrobial agents, solvents, and supplier information is listed in table 2.3. Antibiotic stock solutions were prepared on the day of use and diluted with sterile distilled water if necessary.

**Table 2.3** Antimicrobial agents, solvents and their suppliers.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Solvent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxyclovulanic acid</td>
<td>Sterile distilled water (SDW)</td>
<td>Smithkline Beecham</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Apramycin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Ethanol and SDW</td>
<td>Pfizer Ltd</td>
</tr>
<tr>
<td>Azlocillin</td>
<td>NaHCO₃</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cephradine</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>SDW</td>
<td>Glaxo laboratories</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>SDW</td>
<td>Fujisau Pharmaceuticals</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Cephaleothin</td>
<td>SDW</td>
<td>Eli Lilly Pharmaceuticals</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Ethanol and SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>SDW</td>
<td>Bayer plc</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>SDW</td>
<td>Fluka</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Penicillin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Ethanol and SDW</td>
<td>Marion Merrell Ltd.</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>0.1M NaOH and SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>SDW</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>SDW and lactic acid</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
2.5 Media

MacConkey agar, Nutrient agar (NA) and broth (NB), Iso-sensitest agar (IST) and broth were obtained from Oxoid (Basingstoke, UK). Lennox L Broth (LB) was purchased from GibcoBRL Life Technologies Ltd. (Paisley, UK) and Chromocult TBX (tryptone bile X-glucuronide) agar from Merck (Darmstadt, Germany). All media were made up with distilled water according to the manufacturer’s instructions and autoclaved at 121°C and 15psi for 15 minutes prior to use.

2.6 Chemicals, buffers and enzymes

Chemicals were supplied by Sigma-Aldrich (Poole, UK) unless otherwise stated and solutions made up with distilled water. Saline was made with 0.85% (w/v) NaCl. All solutions and buffers were sterilised by autoclaving at 121°C and 15psi for 15 minutes prior to use. Restriction enzymes and PCR reagents were purchased from Promega.
3.1 Introduction

The overall aims of this chapter are three fold: firstly, to identify isolates demonstrating interesting resistance phenotypes for further study (for example, to identify resistance to cephalosporins, which are not used on the animals from which these isolates came, or to detect the emergence of a resistance phenotype). Secondly, to determine the prevalence of resistance amongst \( E. \ coli \) populations selected on ampicillin, apramycin or nalidixic acid containing agar, to a wide range of antimicrobial agents, and to compare these profiles to isolates taken from an unselected population. Thirdly, resistance profiles were compared using statistical tests to determine whether resistance to an antibiotic conferred resistance to other antibiotics of the same family or to other unrelated antibiotics, and to determine whether plasmid-mediated resistance genes were commonly associated with each other.

In research laboratories, \textit{in vitro} antimicrobial susceptibility testing is commonly performed by the determination of the minimum inhibitory concentration (MIC) of an antibiotic to the test organism. This is considered the gold standard of sensitivity tests (Andrews, 2001), and for research purposes is usually carried out with the test antibiotic incorporated into agar plates. A complete range of antibiotic plates containing doubling dilutions of an antibiotic are prepared by adding agar to freshly prepared antibiotic stock solutions. Bacterial cultures, grown overnight in an orbital shaker, are diluted in sterile saline and a multipoint inoculator is used to replicate test cultures onto the plates. Inoculated plates are incubated at 37°C for 16-20 hours and the MIC (the lowest concentration of antibiotic required to inhibit all visible growth) of the antibiotic is read (Andrews, 2001).

Although this method gives the maximum information about the sensitivity of an isolate to an antibiotic, it is time consuming and expensive (Wheat, 2001), so a compromise method is often employed whereby one or two concentrations of an
antibiotic are used. Commonly the antibiotic is incorporated into filter paper discs or agar (Hutchison, 1954). This method is usually referred to as the ‘breakpoint’ technique (Wheat, 2001). Understandably, clinical and routine laboratories testing hundreds of isolates often employ disc sensitivity tests, or agar dilution tests using one breakpoint (Edwards, 2003; Wheat, 2001) to enable an isolate to be classified as sensitive or resistant to a drug. Some researchers also use breakpoint techniques where MIC values would be more informative, even when looking at very low numbers (<40) of isolates (Son et al., 1997).

In this study 410 isolates were to be tested for sensitivity to 25 different antibiotics. To produce MICs for all isolates against all antibiotics would comprise 10,250 tests. Therefore, a breakpoint method of testing was employed whereby a low and high breakpoint concentration of antibiotic was incorporated into agar plates. The use of two breakpoints facilitated the categorisation of isolates into susceptible (S), intermediately resistant (I) and resistant (R) groups. This method had an advantage over using antibiotic discs as it enabled many isolates (n = 33) to be tested on one plate and enabled concentrations of antibiotics relevant to this study to be used.

There is no global standard methodology for MIC tests (Bronzwaer et al., 2002), and many European countries (Germany, The Netherlands, Sweden, Spain, Britain, France and Norway) have issued national guidelines. Methodology may vary in the testing method used (MIC determination by agar dilution or Etest, or disc diffusion), the source of reagents and media, and the interpretive criteria used (Tenover et al., 2001). In Europe, the National Committee for Clinical Laboratory Standards (NCCLS) guidelines are the most frequently followed (Bronzwaer et al., 2002), however The British Society for Antimicrobial Chemotherapy (BSAC) methodology is followed in many British laboratories because it uses MIC ranges and breakpoints most applicable to British clinical isolates. This study had different aims to those of clinical laboratories. One of the aims of this susceptibility profiling was to detect the emergence of reduced susceptibility in E. coli isolated from calf rectal faecal samples, not the presence of clinically relevant levels of resistance with any view to treatment. In this way BSAC methodology was followed but breakpoints specific to
this study were used, as those recommended by the BSAC were not suitable. This has the disadvantage that the results generated cannot be directly compared with other studies using these guidelines. However, it is unlikely that the results would be comparable even with BSAC or NCCLS recommended breakpoints, because the method of selecting out isolates for study would bias any prevalence comparisons with other studies.

Phenotypic susceptibility testing has a number of shortcomings (Andrews, 2001; Bergeron & Ouellette, 1998) and inaccuracies of susceptibility testing in Enterobacteriaceae have been detected for disc diffusion (Block et al., 1998), agar dilution, and automated (e.g. MicroScan and Vitek) testing methodologies (Steward et al., 1999). Variation between repeat tests may arise from the use of different media (Andrews et al., 2002; Koeth et al., 2002) or variation in inoculum density (BSAC, 2004). This may be because of differences between spectrophotometers or human error in visual judgement of density (BSAC, 2004). With disc diffusion assays the antibiotic content of filter paper discs has been found to vary depending on the manufacturer (Darville & Lovering, 2003). The detection of variation within repeat tests of established and routinely used methodologies emphasises the need for validation of the breakpoint method used in this study and the quantification and analysis of any variation detected. Therefore, this chapter will firstly assess the levels and sources of variation within the susceptibility testing technique used. This was achieved by completion of the following:

a) Quantification of variation between replicate tests.
b) Reproducibility values for this method.
c) Assessment of the sensitivity of the comparison of susceptibility profiles between populations to variation between replicates.

After an assessment of the variation present when using this methodology, the original aims of the susceptibility profiling will be addressed. These are summarised as follows:

a) To determine the susceptibility profiles of bovine commensal E. coli, selected from ampicillin, apramycin or nalidixic acid resistant populations.
b) To determine if any associations exist between resistance phenotypes. Resistance genes are commonly found in gene cassettes (Antunes et al., 2004; Heir et al., 2004; Pezzella et al., 2004). These are formed by the action of integrons, which are capable of poaching genes from the environment and incorporating them into bacterial genetic elements (e.g. a plasmid or transposon) (Collis et al., 1993). It was therefore hypothesised that resistance to an antibiotic may be linked to resistance to another unrelated antibiotic as well as to antibiotics of the same family. Association between resistance to antibiotics of the same family, between unrelated antibiotics (i.e. different mechanisms are used to confer resistance), and finally amongst antibiotics to which resistance is commonly plasmid-mediated were examined.

c) To compare the susceptibility profiles of these populations with an unselected population to establish whether certain resistance profiles are specific to a population, and whether isolates from resistant populations more commonly harbour other resistance genes than do isolates from the unselected population.

d) To identify isolates of interest for further study of the molecular mechanisms conferring the resistance phenotype.

3.2 Materials and Methods

3.2a Bacterial isolates

Four hundred and ten bovine commensal E. coli were investigated. The numbers of isolates from the unselected population and from each of the antibiotic resistant populations, selected as described in Chapter 2, are shown in Table 3.1.
Table 3.1 Isolates investigated in this study, and the populations and numbers of calves from which they were obtained.

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of isolates</th>
<th>Number of calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unselected (P)</td>
<td>188</td>
<td>9</td>
</tr>
<tr>
<td>Ampicillin resistant (AmpR)</td>
<td>183</td>
<td>9</td>
</tr>
<tr>
<td>Apramycin resistant (AprR)</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Nalidixic acid resistant (NalR)</td>
<td>27</td>
<td>4</td>
</tr>
</tbody>
</table>

To ensure that each plate contained the correct amount of active antibiotic, three different BSAC recommended standard control strains (*E. coli* NCTC10418, *Pseudomonas aeruginosa* NCTC10662, and *Staphylococcus aureus* NCTC6571) exhibiting different levels of sensitivity to the antibiotics were included on every antibiotic plate. The use of three controls maximised the likelihood of spanning the sensitive, intermediate and resistant categories of the antibiotics under test.

3.2b Susceptibility testing

To categorise isolates as being sensitive (S), resistant (R), or of intermediate level resistance (I) to an antibiotic, a high and low breakpoint were used. The breakpoints were chosen to detect the emergence of resistance and identify isolates of interest for further study. They are listed in Table 3.3.

Molten IsoSensitest (IST) agar, cooled to 55°C was added to freshly prepared antibiotic stock solutions. Bacterial cultures, grown overnight in an orbital shaker (at 37°C and 200 rpm) were diluted five hundred-fold in sterile saline and a multipoint inoculator (Denley, UK) used to replicate test cultures onto the plates giving an inoculum of approximately 10⁶ colony forming units (cfu) per spot. Inoculated plates were dried and incubated at 37°C for 16-20h. Colonies growing on both the
low and high breakpoint plates were classified as R, those growing only on the low breakpoint plate, I, and those growing on neither were classified S. All isolates were inoculated onto agar plates containing no antibiotic to ensure that any inhibition of growth on test plates was due to the presence of the antibiotic.

3.2c Statistical analyses

In order to explore repeatability and determine sources of variation within the breakpoint methodology employed, susceptibility profiling of each isolate was determined twice. The reproducibility of the results obtained from the replicates was assessed in two ways. Firstly, the level of agreement between replicates was described using the kappa statistic. This semi-quantitative approach measures pairwise agreement between the two repeat classifications, and is corrected for chance agreement. Table 3.2 categorises the ranges of kappa according to Everitt (1989). Commonly researchers use 0.6 as a cut off level (Landis & Koch, 1977) with values of 0.6 or greater indicating acceptable levels of variability.

Table 3.2 Categorisation of kappa values according to Everitt (1989).

<table>
<thead>
<tr>
<th>Kappa value</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.81</td>
<td>Almost perfect agreement</td>
</tr>
<tr>
<td>0.61 – 0.8</td>
<td>Substantial agreement</td>
</tr>
<tr>
<td>0.41 – 0.6</td>
<td>Moderate agreement</td>
</tr>
<tr>
<td>0.21 – 0.4</td>
<td>Fair agreement</td>
</tr>
<tr>
<td>0 – 0.2</td>
<td>Slight agreement</td>
</tr>
</tbody>
</table>

Kappa values test the null hypothesis that there is no more agreement than might occur by chance, but because kappa is a semi-quantitative statistic that does not quantify the significance of any differences between repeat experiments, Wilcoxon signed ranks tests were calculated in addition to kappa values.
Susceptibility profiles were used to study any associations between antibiotics, for example whether resistance to one antibiotic conferred resistance to other antibiotics with the same mechanism of action (same antibiotic family). In order to do this, it was important to establish whether associations between antibiotics would be sensitive to variation in replicate results. This was examined using Pearson’s Chi-squared proportions tests, because the associations between antibiotics were to be analysed by comparing proportions of S, I, or R isolates. Two different methods were used to select what data would be used in any subsequent proportions based tests. Firstly, only those results for which replicates were identical would be included in the analysis (method 1). The second approach (method 2) included all results, however where the replicates differed between S and I or I and R, these isolates were allocated into the S or R group respectively. The relative proportions of S, I or R isolates to a particular antibiotic were compared between the two methods. If the relative proportions did not alter between the two methods, method 2 could be used to analyse any associations, thereby allowing the maximum number of isolates to be included in the test giving it more power.

The hypothesis that resistance to an antibiotic was associated with resistance to other antibiotics within the same family, was studied by comparing the proportions of S, I, and R isolates to both the antibiotics being studied, using the G-test of association (Sokal & Rohlf, 1995). The same test was used to determine whether associations existed between plasmid-borne antibiotic resistance mechanisms.

To examine the hypothesis that isolates from resistant populations (i.e. AmpR, AprR or NalR isolates) were more likely to harbour other resistance genes, proportions of isolates resistant to a particular antibiotic were compared between the resistant population and the unselected (P) population with Pearson’s Chi-squared tests. In all statistical analyses, a $P$-value of $< 0.05$ was taken to indicate significance.
3.3 Results

3.3.1 Reproducibility values

The completion of repeat susceptibility profiles for each isolate demonstrated that whilst both repeat results were partially validated by three NCTC control strains there were often discrepancies between the replicates. The reproducibility of the breakpoints used, as assessed semi-quantitatively by the kappa statistic are listed in Table 3.3. According to Everitt’s criteria (Everitt, 1989) 18 of 25 antibiotics (highlighted in green) demonstrated substantial agreement between repeat experiments.

The Wilcoxon signed ranks tests are also listed in Table 3.3. A P-value greater than 0.05 indicates that the replicate means did not differ significantly. Replicate means that did differ significantly have P-values less than 0.05, and these are printed in bold.

Table 3.3 shows that although no replicates were S in one and R in another, a significant level of variation is present amongst replicates for 16 of the 25 antibiotics used.
Table 3.3 Repeat classification of isolates, by antibiotic susceptibility at two breakpoint concentrations. Antibiotics are ordered according to decreasing percentage similarity between repeat classifications. \( P \) values refer to Wilcoxon signed ranks tests. The numbers of isolates that shifted resistance category between repeat experiments are listed in the final two columns.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Breakpoints (mg/L)</th>
<th>S-S</th>
<th>S-I</th>
<th>S-R</th>
<th>I-S</th>
<th>I-R</th>
<th>I-S</th>
<th>R-R</th>
<th>P-value</th>
<th>Kappa</th>
<th>%same</th>
<th>Increased resistance</th>
<th>Decreased resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>1/4</td>
<td>409</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;0.999</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>32/128</td>
<td>194</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>218</td>
<td>0</td>
<td>&gt;0.999</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>1/4</td>
<td>408</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3185</td>
<td>0.6656</td>
<td>99.8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>2/8</td>
<td>393</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0.0835</td>
<td>0.9</td>
<td>99.3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>4/16</td>
<td>344</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>60</td>
<td>0.4148</td>
<td>0.9459</td>
<td>98.5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>1/8</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>349</td>
<td>1</td>
<td>0</td>
<td>0.7062</td>
<td>0.9335</td>
<td>98</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>8/32</td>
<td>355</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>47</td>
<td>0.9</td>
<td>0.9335</td>
<td>98</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2/16</td>
<td>370</td>
<td>0</td>
<td>5</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0.7636</td>
<td>0.8268</td>
<td>97.3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2/8</td>
<td>392</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>0.0005</td>
<td>0.6144</td>
<td>97</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>2/8</td>
<td>323</td>
<td>0</td>
<td>2</td>
<td>34</td>
<td>1</td>
<td>0</td>
<td>13</td>
<td>0.0005</td>
<td>0.8307</td>
<td>96.1</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>4/32</td>
<td>131</td>
<td>11</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>260</td>
<td>0.1338</td>
<td>0.919</td>
<td>96.1</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Cefloxacine</td>
<td>2/16</td>
<td>366</td>
<td>0</td>
<td>16</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.0331</td>
<td>0.1034</td>
<td>94.6</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>4/16</td>
<td>330</td>
<td>0</td>
<td>6</td>
<td>50</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0.0082</td>
<td>0.7595</td>
<td>93.2</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.12/32</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>137</td>
<td>13</td>
<td>0</td>
<td>230</td>
<td>0.0095</td>
<td>0.781</td>
<td>89.5</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>16/64</td>
<td>54</td>
<td>33</td>
<td>9</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.0001</td>
<td>0.6584</td>
<td>87.8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>8/32</td>
<td>312</td>
<td>0</td>
<td>5</td>
<td>35</td>
<td>28</td>
<td>0</td>
<td>1</td>
<td>&lt;0.0001</td>
<td>0.6584</td>
<td>87.8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.5/1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>57</td>
<td>0</td>
<td>3</td>
<td>&lt;0.0001</td>
<td>0.6176</td>
<td>85.4</td>
<td>57</td>
<td>3</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>4/16</td>
<td>35</td>
<td>24</td>
<td>0</td>
<td>24</td>
<td>224</td>
<td>4</td>
<td>0</td>
<td>0.1397</td>
<td>0.6987</td>
<td>83.7</td>
<td>28</td>
<td>39</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>8/32</td>
<td>259</td>
<td>53</td>
<td>0</td>
<td>15</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0.0182</td>
<td>0.919</td>
<td>96.1</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>4/32</td>
<td>135</td>
<td>2</td>
<td>0</td>
<td>25</td>
<td>60</td>
<td>18</td>
<td>0</td>
<td>0.1397</td>
<td>0.6987</td>
<td>83.7</td>
<td>28</td>
<td>39</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1/64</td>
<td>18</td>
<td>7</td>
<td>0</td>
<td>78</td>
<td>104</td>
<td>12</td>
<td>0</td>
<td>0.0091</td>
<td>0.5719</td>
<td>73.2</td>
<td>19</td>
<td>91</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.5/4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>280</td>
<td>101</td>
<td>0</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>0.0538</td>
<td>71</td>
<td>102</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>8/32</td>
<td>122</td>
<td>93</td>
<td>0</td>
<td>36</td>
<td>65</td>
<td>0</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>0.6351</td>
<td>83.4</td>
<td>55</td>
<td>37</td>
</tr>
<tr>
<td>Ampicillin + clavulanic acid</td>
<td>2/8</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>84</td>
<td>13</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>0.3199</td>
<td>64.4</td>
<td>19</td>
<td>127</td>
</tr>
<tr>
<td>Apramycin</td>
<td>4/32</td>
<td>227</td>
<td>9</td>
<td>0</td>
<td>146</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>0.1625</td>
<td>62.2</td>
<td>9</td>
<td>140</td>
</tr>
</tbody>
</table>

S, susceptible; I, intermediate level resistance; R, Resistant; S-S, I-I, and R-R, the same classification on both replicates; S-I, S-R, I-S, I-R, R-S, and R-I, different classification between replicate experiments. Yellow shading indicates identical classifications between replicates, and green shading indicates substantial agreement between replicates. Significant \( P \) values are highlighted in bold.

3.3.b Sensitivity of associations between antibiotics to variation between replicates.

Table 3.3 demonstrates that for some of the antibiotics variation occurred between replicate experiments. On account of this variation, two methods were used to select which data would be used in subsequent statistical tests. Method 1 used only those results for which replicates were identical and method 2 included all results but
grouped any isolates with replicates differing between S and I, or I and R as S or R respectively. The impact of using one method over the other, as assessed by Pearson’s $\chi^2$-squared tests, are shown in Table 3.4

**Table 3.4** Significance of variation generated by different grouping methods, assessed by Pearson’s $\chi^2$-squared test.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>$\chi^2$-squared</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.0004</td>
<td>0.999</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>0.0116</td>
<td>0.9942</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.0489</td>
<td>0.9758</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.0116</td>
<td>0.95</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.1025</td>
<td>0.9244</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.496</td>
<td>0.7804</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1.7562</td>
<td>0.4156</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>1.9158</td>
<td>0.3837</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2.8321</td>
<td>0.2427</td>
</tr>
<tr>
<td>Penicillin</td>
<td>2.9848</td>
<td>0.2248</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3.3239</td>
<td>0.1898</td>
</tr>
<tr>
<td>Apramycin</td>
<td>3.5129</td>
<td>0.1727</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>3.8429</td>
<td>0.1464</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>4.974</td>
<td>0.0832</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>6.5281</td>
<td>0.0382</td>
</tr>
<tr>
<td>Azlocillin</td>
<td>9.3045</td>
<td>0.0095</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>12.7818</td>
<td>0.0017</td>
</tr>
<tr>
<td>Amoxyclovulanic acid</td>
<td>15.8838</td>
<td>0.0004</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>15.6181</td>
<td>0.0004</td>
</tr>
<tr>
<td>Cephradine</td>
<td>18.5139</td>
<td>0.0001</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>83.4351</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>45.3986</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

In the lower part of Table 3.4 are listed eight antibiotics with significant $P$-values. For these antibiotics the selection of data by either method 1 or 2 makes a significant difference to the proportions of S, I and R isolates, and so any further statistical tests
based on relative proportions could only be performed on those data for which repeat experiments yielded the same susceptibility phenotype. Comparing these results with Table 3.3, one can see that all these antibiotics show significant differences between replicate experiments, with the exception of cephalothin. In this case the columns indicating numbers of isolates that shifted resistance category between repeat experiments (columns headed “Increased resistance” and “Decreased resistance”) showed similar numbers (28 and 39). A significant difference between replicate classifications is not recorded by the Wilcoxon signed ranks test, because it measures significant differences in a directional manner. In this way, the two shifts cancel each other out, preventing the detection of a significance difference between the replicates.

The proportions of resistant, intermediately resistant and sensitive isolates did not differ significantly between the two different grouping methods ($P > 0.05$) for 17 of the 25 antibiotics tested. For these drugs subsequent statistical analysis based on proportions were performed on data grouped by method 2, permitting the use of the maximum number of results and thereby giving more power to each test.

3.3. c Proportions of resistance

The four populations of *E. coli* (amp$^R$, apr$^R$, nal$^R$, and unselected *E. coli* populations) were analysed to determine the sensitivities of isolates to 25 antibiotics. Figures 3.1 to 3.3 show the proportions of resistance to these antibiotics within the different populations. In these figures P is the unselected population, A, the amp$^R$, R, the apr$^R$, and X, the nal$^R$ population. Resistance is shaded red, intermediate level resistance, orange, and sensitive, yellow. Sensitivity is shown as a percentage of the total number of isolates. It is important to note that the number of isolates in the population may affect these proportions. The unselected and amp$^K$ populations are much more comparable than the nal$^R$ and apr$^R$ populations as they comprise similar large numbers of isolates (188 and 183 respectively). The nal$^R$ and apr$^R$ populations only represent 27 and 12 isolates respectively.
Figure 3.1 Comparison of the proportions of resistance of $\text{amp}^R$, $\text{apr}^R$, $\text{nal}^R$ and unselected populations to $\beta$-lactams and quinolones.

Figure 3.1 demonstrates that most isolates (408 / 410) were intermediately or fully resistant to penicillin. The $\text{amp}^R$ population had the highest proportion of resistance to amoxyclavulanic acid, azlocillin and aztreonam. The unselected and $\text{apr}^R$ populations had similar proportions of resistance to ampicillin (0.26 and 0.25) and azlocillin (0.24 and 0.25). All $\text{nal}^R$ isolates ($n = 27$) were resistant to penicillin, ampicillin and azlocillin, but demonstrated diversity in their susceptibility to amoxyclavulanic acid and aztreonam.

A high prevalence of nalidixic acid resistance (intermediate or high level) was detected in all populations, but only two isolates were intermediately resistant to the fluoroquinolones (ciprofloxacin and enrofloxacin), and these isolates were picked from unselective plates.
Figure 3.2 Comparison of the prevalence of resistance of $\text{amp}^R$, $\text{apr}^R$, $\text{nal}^R$ and unselected populations to the cephalosporins.

P, unselected; A, $\text{amp}^R$; R, $\text{apr}^R$; X, $\text{nal}^R$; red, resistant; orange, intermediate resistance; blue, sensitive.

The graphs in Figure 3.2 are ordered according to the generation of cephalosporin. The 1$^{\text{st}}$ generation cephalosporins (cephalothin and cephradine) are on the left hand side, the 2$^{\text{nd}}$ generation (cefoxitin and cefuroxime) in the centre, and the 3$^{\text{rd}}$ generation cephalosporins (ceftazidime, cefotaxime and ceftizoxime) are to the right. There is a general trend towards increasing susceptibility with the newer (3$^{\text{rd}}$ generation) cephalosporins. Resistance (red shading) to ceftazidime and ceftizoxime is more prevalent in the unselected population than the $\text{amp}^R$ population.
Figure 3.3 Comparison of the prevalence of resistance of amp\textsuperscript{R}, apr\textsuperscript{R}, nal\textsuperscript{R} and unselected populations to the aminoglycosides and other classes of antibiotic.

The apr\textsuperscript{R} population contained only 12 isolates, however all susceptibility classes were observed when isolates were profiled with streptomycin (Figure 3.3). The vast majority of isolates had decreased susceptibility to rifampicin, because of the barrier function of the gram-negative outer membrane in preventing entry of this antibiotic (Burman et al., 1972).

A greater percentage of amp\textsuperscript{R} isolates also demonstrate reduced susceptibility to streptomycin, trimethoprim, chloramphenicol, tetracycline and sulphamethoxazole, than did the unselected population. Therefore, it is hypothesised that resistance to ampicillin may be linked to resistance to other antibiotics by the carriage of a multi-drug resistance plasmid. Similarly, the apr\textsuperscript{R} population has a higher prevalence of tetracycline and sulphamethoxazole resistance than the unselected population, which

P, unselected; A, amp\textsuperscript{R}, R, apr\textsuperscript{R}, X, nal\textsuperscript{R}; red, resistant; orange, intermediate resistance; blue, sensitive.
may be due to the presence of a plasmid harbouring these resistance determinants within the apr\textsuperscript{R} population. These hypotheses are examined in the following section.

3.3.\textit{d} Between-population comparison of susceptibility proportions

This section aims to address the following three hypotheses:

i) Resistance to an antibiotic confers resistance to other antibiotics of the same family.

ii) Isolates from resistant populations more commonly harbour other resistance genes.

iii) Plasmid mediated resistance genes are commonly associated with each other.

\textit{i) Resistance to an antibiotic confers resistance to other antibiotics of the same family.}

This hypothesis acts as a positive control for the $G$-tests, because resistance to an antibiotic commonly confers resistance to other antibiotics of the same family (Amyes \textit{et al.}, 1996). To examine this hypothesis, the proportions of S, I and R isolates, to one antibiotic were compared to those of another antibiotic from the same antibiotic family (i.e. with the same mechanism of antibacterial action). Representative antibiotics from different families were used in the analysis, the results of which are listed in Table 3.5.
Table 3.5 Association in level of susceptibility between antibiotics of the same family.

<table>
<thead>
<tr>
<th>Antibiotics compared</th>
<th>G value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxyclovulanic acid</td>
<td>95.76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ampicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxyclovulanic acid</td>
<td>65.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Aztreonam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxyclovulanic acid</td>
<td>80.79</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cephalothin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxyclovulanic acid</td>
<td>42.44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>169.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apramycin</td>
<td>45.69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apramycin</td>
<td>6.35</td>
<td>0.1747</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results support the hypothesis that resistance to an antibiotic confers cross-resistance to other antibiotics of the same family. All tests of association between levels of susceptibility to antibiotics of the same family yielded highly significant positive associations ($P < 0.0001$), except apramycin versus streptomycin. This result was expected because the enzyme conferring resistance to apramycin does not confer cross-resistance to streptomycin (Bräu et al., 1984) even though both antibiotics are aminoglycosides. This test was performed on proportions of S, I and R isolates grouped with either method 1 or method 2, depending on the results of section c. As a check, tests were repeated using the more conservative method (method 1), where only those results for which repeat classification yielded the same resistance phenotype. The same significant associations were found.

ii) Isolates from resistant populations more commonly harbour other resistance genes.

Pearson’s $\chi^2$-squared test was used to determine whether isolates from the amp$^R$ and nal$^R$ populations had a significantly greater proportion of resistance to other antibiotics than isolates from the unselected population. The apr$^R$ population was not included in the study as it contained only 12 isolates, which would result in an unreliable test. In this analysis, resistance was classified as either the number of R isolates or the number of non-sensitive (R + I) isolates in the population. Different
definitions of resistance may be used in each comparison as long as the same definition is used within a comparison. This is because the question being addressed is whether there is an association between increases in resistance and the type of population. Representative antibiotics were chosen for this analysis based on their different mechanisms of action, and the results of this analysis are shown in Table 3.6.

Table 3.6 Association between resistance to an antibiotic, and resistant population compared to the unselected population.

<table>
<thead>
<tr>
<th>Population</th>
<th>Antibiotic</th>
<th>R isolates only</th>
<th>R + I isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\chi^2$</td>
<td>$P$-value</td>
</tr>
<tr>
<td>Amp$^R$</td>
<td>Apramycin</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Nalidixic acid</td>
<td>2.37</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>40.98</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>12.45</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>15.58</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Sulphamethoxazole</td>
<td>115.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nal$^R$</td>
<td>Ampicillin</td>
<td>54.41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Apramycin</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>4.724</td>
<td>0.03*</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>149.33</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>0.0451</td>
<td>0.8318</td>
</tr>
<tr>
<td></td>
<td>Sulphamethoxazole</td>
<td>66.03</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Indicates expected frequencies of <5. NA indicates no R isolates were present in one of the populations. R, resistant isolates; I, intermediately resistant isolates.

The ability of the test to detect an increase or decrease in the proportion of resistance between the two populations requires adequate numbers of isolates to be present in the contingency table. This ultimately depends on the ability of the breakpoints used to partition the population, which will differ between antibiotics. The only instance for which the definition of resistance does make a difference to the significance of any association is the presence of tetracycline resistance in the amp$^R$ population. This is because of a large number of isolates in the comparison with intermediate level resistance (41/183 isolates in the amp$^R$ population were intermediately resistant to tetracycline, results not shown). The addition of all I isolates into the R group
dramatically affects the numbers of R isolates used in the test. The results of Table 3.6 indicate that high level tetracycline resistance (MIC $\geq$ 64mg/L) is significantly associated with ampicillin resistance. Ampicillin resistant isolates were also significantly associated with resistance to trimethoprim, chloramphenicol, and sulphamethoxazole (Table 3.6)

Nalidixic acid resistant isolates were significantly associated with sulphamethoxazole and ampicillin resistance. The significance of the association between nalidixic acid resistance and chloramphenicol and trimethoprim resistance should be interpreted with caution because the expected frequencies were fewer than five.

iii) *Plasmid mediated resistance genes are commonly associated with each other.*

To examine this hypothesis, the proportions of S, I and R isolates, to either ampicillin or apramycin were compared to those of other antibiotics to which resistance is commonly plasmid mediated. These results are shown in Table 3.7.

**Table 3.7** Association in level of susceptibility between antibiotics to which resistance is commonly carried on plasmids.

<table>
<thead>
<tr>
<th>Antibiotics compared</th>
<th>$G$ value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.882</td>
<td>0.9271</td>
</tr>
<tr>
<td>Apramycin Chloramphenicol</td>
<td>38.96</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ampicillin Sulphamethoxazole</td>
<td>175.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ampicillin Tetracycline</td>
<td>49.83</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ampicillin Trimethoprim</td>
<td>49.81</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apramycin Chloramphenicol</td>
<td>3.532</td>
<td>0.171</td>
</tr>
<tr>
<td>Apramycin Sulphamethoxazole</td>
<td>2.848</td>
<td>0.2408</td>
</tr>
<tr>
<td>Apramycin Tetracycline</td>
<td>13.36</td>
<td>0.0096</td>
</tr>
<tr>
<td>Apramycin Trimethoprim</td>
<td>3.966</td>
<td>0.1377</td>
</tr>
</tbody>
</table>
Resistance to ampicillin is significantly associated with resistance to chloramphenicol, sulphamethoxazole, tetracycline and trimethoprim (Table 3.7). These conclusions support those of section ii (Table 3.6). AprR isolates were only significantly associated with tetracycline resistance indicating the presence of an apramycin resistance plasmid that also harbours tetracycline resistance. All associations were positive.

3.4 Discussion

This study aimed to determine the susceptibility profiles of 410 isolates to 25 different antibiotics. The number of tests involved meant that the determination of full MICs for each isolate was not practical, and so a breakpoint method of testing was used. By repeating each profile twice, variation arising from this methodology could be measured.

3.4a Assessment of levels and sources of variation within the methodology

Although three control organisms recommended by the BSAC (Andrews, 2001) were included in each experiment (NCTC 10418, NCTC 6571 and NCTC 10662) a significant level of variation was detected with 16 antibiotics, and a less than satisfactory level of reproducibility was observed with seven. Some experimental errors may have remained undetected if the MICs of these standard strains were not close to the breakpoint concentrations chosen.

Discrepancies between replicates can arise from slight variations in inoculum, degradation of antibiotic over time, and variation in reading the plates (BSAC, 2004), but this should be minimal if a single protocol is adhered to and results are recorded by the same operator, as was done in this study. The variation observed is due to the positioning of the breakpoints. The effect of breakpoint positioning on classification is illustrated in Figure 3.4.
Figure 3.4 Variation in susceptibility classification due to breakpoint position.

Figure 3.4 shows a population of bacteria with a range of susceptibilities to an antibiotic, given as MIC values. The population comprises two discrete groups, those that are sensitive, and those that are resistant to the antibiotic. This bimodal distribution is typical of a number of antibiotics used in this study, and is caused by the acquisition of a resistance mechanism resulting in a characteristic increase in MIC. If a breakpoint is positioned at point A, any slight variations in concentration of antibiotic because of degradation of the drug or inaccuracies in making the antibiotic plates will not affect the number of isolates classified as R or S. In contrast, if the breakpoint is positioned at point B, slight variations in antibiotic concentration may result in concentration C being the actual concentration of antibiotic used. This will result in a decrease in the number of isolates that are classed as R, and a corresponding increase in those classed as I. With prior knowledge as to the range of MICs expected within a given population, breakpoint concentrations can be used which, like concentration A, split the population into discrete groups. No information was available on the susceptibilities expected from the isolates examined in this study. To acquire this knowledge would require the completion of full MICs on a very large subset, if not all of the isolates. This is because the isolates are expected to
have extremely varied susceptibility profiles because some were selected on antibiotics prior to isolation and others were not.

The presence of variation between replicates results from the position of the breakpoints used. The extent of the variation observed also differs between the different antibiotics. This is because it depends on the MIC of the majority of the isolates to that antibiotic and the mechanism of resistance to the antibiotic.

According to the BSAC (Andrews, 2001) a variation of plus or minus one two-fold dilution is acceptable when assessing whether a control strain in a sensitivity test has its correct MIC. In this study, if the majority of isolates had an MIC to a particular antibiotic that was at the breakpoint concentration used, repeat experiments could easily result in two different resistance phenotypes being reported because of minor experimental errors. This may explain why many isolates were sensitive to apramycin, azlocillin, cefoxitin, cephradine, or tetracycline in one experiment and demonstrated intermediate level resistance in the repeat experiment.

The isolates in this study varied in their susceptibility to different classes of antibiotic, because of the initial selection of isolates either on media containing an antibiotic or on media without antibiotic supplementation. This range of susceptibilities is a good way of testing the true ability of the breakpoints used to classify isolates into resistance phenotypes. Quality control of susceptibility testing has in the past been conducted with a high proportion of susceptible isolates (Chen et al., 1995) resulting in an unfair test of the methodology.

The mechanism by which bacteria acquire resistance to an antibiotic is also of importance when assessing the effect of variation due to breakpoint position. The categorisation of isolates into the three groups with very few discrepancies between repeats will be obtained where the presence of a resistance mechanism results in large increases in MIC, forming discrete populations, and the breakpoints used have been at positions suitable for distinguishing these populations.
Sulphonamide resistance is mediated by the production of dihydropteroate synthetase with reduced affinity for sulphonamides, but not para-aminobenzoic acid (a precursor in the bacterial metabolic pathway for the synthesis of tetrahydrofolate) (Wise & Abou-Donia, 1975). In this way, the inhibitory effect of the antibiotic is bypassed resulting in a dramatic increase in MIC. The difference in MIC between sensitive and resistant isolates is large enough that no isolates were identified with these breakpoints. Therefore, the breakpoints used must be both present in the trough of the bimodal curve resulting in unambiguous categorisation. This may also be the case with trimethoprim, where there are few (n = 6) discrepancies between replicates.

Trimethoprim resistance occurs by the production of an altered dihydrofolate reductase enzyme (the trimethoprim target) (Amyes & Smith, 1974) resulting in a large increase in MIC.

In contrast, Livermore & Chen (1999) describe the occurrence of a high frequency of resistant *P. aeruginosa* isolates in UK laboratories being reported as sensitive. The high rate of miscategorisation was thought to be due to the unimodal distribution of MIC values. Where a breakpoint cuts the tail of a unimodal distribution, resistance classification by this breakpoint is prone to be erratic, compared to a breakpoint present in between the two discrete populations of a bimodal distribution. This is likely to be the reason for discrepancies in categorisation of susceptibility, and hence the low Kappa values, of the breakpoint tests used in this study to apramycin, cephradine and amoxyclavulanic acid. Here different resistance mechanisms can occur resulting in different MIC values. These include enzymatic modification of apramycin, enzymatic degradation and reduced permeability of the β-lactams and cephalosporins (Moosden, 1997), and efflux pumps capable of removing these antibiotics (Edgar & Bibi, 1997; Rosenberg et al., 2000). This will lead to a greater number of possible MIC values present in the population. Efflux pumps may also play a role in resistance to the quinolones, chloramphenicol and other β-lactams (Sulavik et al., 2001), but the presence of variation between replicates again depends on the position of the breakpoints in relation to the range of MIC values in the population.
The extent of the variation occurring between replicate experiments differed between the antibiotics. This needed to be considered before examining the presence of any associations between resistance phenotypes. Recently, statistics have been applied to the determination of associations between cyclohexane resistance and multiple antibiotic resistance (Randall et al., 2001). Here isolates were classified as S or R based on the results of disc diffusion assays, but the presence of any variation in the categorisation of susceptibility was not examined prior to the determination of any associations between resistance phenotypes. The identification of antibiotics for which the classification was ambiguous would have been a useful addition to the study.

3.4b Proportions of resistance

In the unselected population, resistance amongst the β-lactam antibiotics was detected against penicillin, ampicillin, azlocillin and amoxycylavulanic acid, which indicates that these strains represent a substantial proportion of the calves’ intestinal flora. The sampled calves were not treated with any β-lactams, but penicillin was used to treat two calves reared with the sampled calves. The detection of β-lactam resistance in a high proportion of commensal E. coli may reflect cross-contamination of resistant strains between treated and untreated calves, or a high prevalence of β-lactam resistant strains in the calves’ immediate environment. Environmental sampling and resistance phenotyping would be required to test this hypothesis.

As was expected, many of the isolates selected on ampicillin plates showed cross resistance to other β-lactams, although 120 / 183 of this population were sensitive to aztreonam suggesting that the predominate β-lactamases do not hydrolyse this monobactam.

A low proportion of isolates were resistant to the 3rd generation cephalosporins (eight isolates were ceftazidime resistant and two were ceftizoxime resistant). Surprisingly, resistance to these antibiotics was more common in the unselected population than
the ampR. Resistance to the 3rd generation cephalosporins commonly occurs by mutation of the β-lactam genes blatem-1 or blashv-1 causing an extended spectrum of activity of the corresponding enzyme (Bradford, 2001). Therefore, TEM-1 and SHV-1 enzymes were expected to be more prevalent in the ampR population than those from the unselected population. The mechanism of resistance may be the presence of a cephalosporinase (Alvarez et al, 2004; Zhao et al., 2001) or an efflux pump (Nikaido et al., 1998). This is discussed in more detail in Chapter 8.

Only two isolates demonstrated reduced susceptibility to the fluoroquinolones enrofloxacin and ciprofloxacin. These were picked from unselective plates. Resistance to the quinolones in E. coli commonly occurs by mutation in the gyrA gene (Oram & Fisher, 1991), or reduced levels of quinolone accumulation (Cohen et al., 1988; Hirai et al., 1986a). A single mutation in the quinolone resistance determining region of gyrA confers high level nalidixic acid resistance and low level ciprofloxacin resistance (MIC ≤ 4mg/L) (Vila et al., 1994). This would explain the resistance phenotype observed, but it is surprising that none of the isolates from the nalR population demonstrated this phenotype. Alternatively, the intermediate level resistance to the fluoroquinolones in these two isolates may be due to a reduction in quinolone accumulation. A decrease in the number of OmpF porins (the outer membrane protein channel through which fluoroquinolones are thought to diffuse) has previously been correlated with a 4-fold increase in resistance to both nalidixic acid and ciprofloxacin (Hirai et al., 1986a; Hirai et al., 1986b). Active efflux of quinolones has also been found to contribute to the final MIC of a strain to varying degrees (Everett et al., 1996; Martinez et al., 1998). Sequencing of the QRDR of the gyrA gene, studies of drug accumulation, and outer membrane protein western blots would be required to test these hypotheses.

Although the aprR population comprised only 12 isolates, all levels of streptomycin resistance (S, I, and R) were observed. This indicates variation in the mechanism of streptomycin resistance within the aprR isolates. A number of different streptomycin resistance genes have been identified (Heinzel et al., 1988; Hollingshead & Vapnek, 1985; Scholz et al., 1989) in addition to resistance due to ribosomal alteration (Traub
& Nomura, 1968) and loss of permeability (Zelazna-Kowalska, 1977), all of which may contribute to the variations in resistance profiles observed within this small group.

3.4c Associations between phenotypes

The prevalence graphs comparing resistance phenotypes between the four populations, indicated that the \( \text{nal}^R \) population harboured resistance to ampicillin, sulphamethoxazole, chloramphenicol and trimethoprim, more commonly than the unselected population. When this was examined statistically a significant positive association was found between these four antibiotics and the \( \text{nal}^R \) population \((P < 0.05)\). This association is unlikely to be due to any specific propensity for strains harbouring a chromosomal mutation conferring nalidixic acid resistance to acquire these resistance genes. These results can be explained by the presence of a limited number of genotypes in the \( \text{nal}^R \) population, of which the most prevalent strain also harbours multiple antibiotic resistances. If few other \( \text{nal}^R \) genotypes are present the results of the test will be biased. The extent of clonality within the \( \text{nal}^R \) population is examined by PFGE in chapter 4.

The prevalence graphs also indicated that resistance to trimethoprim, chloramphenicol, tetracycline, and sulphamethoxazole is more common in \( \text{amp}^R \) isolates than isolates from the unselected population. Again, when these potential associations were examined statistically, a positive association was found between each of these antibiotics and the \( \text{amp}^R \) population \((P < 0.05)\). This is likely to be due to the presence of (a) multidrug resistance plasmid(s), which are commonly found in isolates from food-producing animals (Marshall et al., 1990; Mee & Nikoletti, 1983; Oppegaard et al., 2001). The highly significant level of association between these resistance genes and ampicillin resistance, detected by the G-test of association also supports this hypothesis.
The ability of integrons, present in mobile elements, to acquire antibiotic resistance genes from other replicons is well documented (Bass et al., 1999; Collis et al., 1993). This results in the formation of gene cassettes (reviewed by Recchia & Hall, 1995) containing a number of resistance genes, either on conjugative transposons, or transposons within plasmids (Antunes et al., 2004; Heir et al., 2004; Pezzella et al., 2004). To examine whether or not the antibiotic resistances identified are present on one plasmid, transfer frequencies can be compared when the different antibiotics are used as selective agents for transfer. This method is described in more detail in chapter 5, where transfer frequencies were used to determine which resistance genes were present on apr^R plasmids.

The apr^R population had a higher prevalence of tetracycline and sulphamethoxazole resistance than the unselected population, which may be due to the presence of a plasmid harbouring these resistance determinants within the apr^R population. When the significance of any associations between apramycin resistance and other plasmid mediated resistance genes were assessed using the G-test, a significant association was only found between apramycin and tetracycline resistance. This suggests that within the apr^R population is a plasmid carrying apramycin and tetracycline resistance. Because no apramycin (or other aminoglycosides) or tetracycline was used to treat the animals from which these isolates came, the presence of this plasmid is of interest, and is the focus of chapter 5.

3.5 Concluding remarks

The use of two breakpoint concentrations of an antibiotic allowed a large number of isolates (410) to be screened for susceptibility where the usual practice of determining actual MICs by doubling dilution would be unmanageable. Although different degrees of variation were present within replicate experiments, quantification of this variation permitted valid conclusions to be drawn, and enabled the identification of a number of isolates of interest for further study.
These included the detection of intermediate or high level resistance to 3rd generation cephalosporins. These isolates may harbour ESBLs, previously undetected in Scottish food animals, and will be examined in molecular detail in Chapter 8.

The limited diversity of resistance phenotypes within the nal\textsuperscript{R} populations suggested a greater degree of clonality within these isolates compared to the amp\textsuperscript{R}, apr\textsuperscript{R} and unselected populations. The extent of this possible clonality is examined in Chapter 4 using a combination of genotyping by pulsed-field gel electrophoresis, and mathematical estimates of population diversity.

Positive associations between a number of resistance determinants were identified, suggesting the existence of multidrug resistance plasmids. The presence of a plasmid harbouring apramycin and tetracycline is of special interest, as there appears to be no obvious selective pressure maintaining these plasmids. A detailed study on the types of apramycin resistance plasmid, their spread, and the competitive fitness cost of a bacterium harbouring an apr\textsuperscript{R} plasmid is described in Chapters 5 and 7.
Chapter 4. Genotyping

4.1 Introduction

The epidemiology of antibiotic resistance in pathogenic E. coli is well documented (Chaslus-Dancla et al., 1991; Hartman et al., 2003; Oethinger et al., 1998; Kern et al., 2002; Shen et al., 1999), but much less is known about the epidemiology of antibiotic resistance in commensal E. coli. To study this requires a prior understanding of the epidemiology of the commensal E. coli at the sub-species level. This includes the estimation of strain diversity within this population, and information on factors that may affect diversity (for example, calf age, antibiotic treatment and diet). This chapter aims to achieve a better understanding of the diversity of E. coli present within the calf study group. The genotyping results presented in this chapter will also be used in chapter 5 to aid the understanding of the epidemiology of apramycin resistance.

This introduction comprises two parts. Firstly, the different methods of typing bacteria will be discussed, with justification for the method chosen in this study. Secondly, the specific aims of this chapter will be described in detail.

The species E. coli is commonly described as being clonal (Ochman & Selander, 1984; Ørskov et al., 1990; Selander & Levin, 1980). A clone is defined as “any microbial isolate belonging to a set of microbial isolates that have been recovered independently from different sources, in different locations, and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin” (Eisenstein, 1990).

As more advanced typing techniques become available, a greater degree of diversity is being detected within this species, bringing into question this idea of clonality within the E. coli species. Clonality between strains can never be absolute as it depends on the discriminatory power of the techniques used to type the isolates. The more discriminatory the technique, the more certain one will be of any observed
clonality. Therefore to examine strain diversity within the *E. coli* population, and look for clonal relationships, a highly discriminatory method of distinguishing strains is required.

Before the advent of genotypic methods, epidemiology of *E. coli* was examined by serotyping and antibiotic resistance profiling (Eisenstein, 1990). These methods may be useful in studies of the pathogenesis of infections or in patient management (Gemmell, 1999), but are inadequate for exploring *E. coli* population structure. Antibiotic resistance markers, if present on transferable plasmids, may be spread horizontally under selective pressure or may be lost in the absence of selection (Jones *et al.*, 1980), and a substantial amount of chromosomal variation has been detected within some serotypes (Whittam *et al.*, 1993). These factors mean that genotypic approaches are essential for studying *E. coli* population diversity.

A number of genotypic typing methods are now in use in both clinical and research laboratories. Ribotyping is based on restriction fragment length polymorphism of ribosomal RNA genes (Tarkka *et al.*, 1994) and has been applied to the differentiation of *E. coli* O157 isolates from other *E. coli* (Hahm *et al.*, 2003). The differentiation of *E. coli* O157 was successful by this method, which also had the advantage of identifying three isolates that were misclassified as *E. coli* by the cultivation method used. However, ribotyping has been reported to be ineffective at discriminating between isolates within the O157:H7 serotype (Martin *et al.*, 1996). The recent use of ribotyping and multilocus enzyme electrophoresis (MLEE) (where strains are typed by the electrophoretic mobility patterns of multiple metabolic enzymes) (Selander *et al.*, 1986), to determine the clonal structure of avian *E. coli* strains, highlighted MLEE as a better method to discriminate between strains (Silveira *et al.*, 2003). The main disadvantage of MLEE is that typing is based on the structure of between 5 and 15 enzymes, rather than the whole genome.

A number of PCR-based methods are also used in bacterial phylogenetics, including random amplified polymorphic DNA (RAPD) (Power, 1996), arbitrary fragment
length polymorphism (AFLP) (Vos et al., 1995), and repetitive element PCR (rep-PCR) (Versalovic et al., 1991).

RAPD-PCR uses short primers with sequences not directed at any specific genomic sequence, which hybridise at random sites in the DNA to initiate amplification (Welsh & McClelland, 1990). This method was applied to the typing of *E. coli* from beef cattle faeces, carcasses and ground beef (Aslam et al., 2002), leading the authors to conclude that *E. coli* contamination of ground beef originated from cattle faeces. However, RAPD-PCR was found to be less reproducible and less discriminatory than PFGE when these two methods were used to trace fluoroquinolone resistant *E. coli* isolated from cancer patients (Tascini et al., 1999). Other criticisms of RAPD-PCR have been described, including variation in fingerprints due to DNA or primer concentration and staining methodology (Van Belkum et al., 1993), and difficulties in comparison of patterns between gels and between laboratories (Power, 1996).

Other PCR based methods, although relatively cheap and rapid, are also associated with problems that limit their use for studying the population diversity of *E. coli*. AFLP-PCR uses PCR primers generated by enzymatic digestion of the DNA, which are then used in subsequent PCR of other isolates (Vos et al., 1995). Problems arise if the DNA used is not highly purified and of high quality, leading to artificial amplification (Mueller & Wolfenbarger, 1999). The production of an insufficient number of fragments may also lead to incorrect conclusions of clonal identity (Mueller & Wolfenbarger, 1999).

Rep-PCR is based on the amplification of repetitive intergenic consensus sequences, and was used to type pathogenic and non-pathogenic *E. coli* (Johnson & O’Bryan, 2000). In this study rep-PCR failed to resolve all the major pylogenetic groups previously identified by MLEE, significantly limiting the use of this methodology as a typing tool for *E. coli*. Although here one may argue that the use of a genotypic typing method (i.e. rep-PCR) is a more accurate way to determine genetic relatedness than MLEE, as the latter does not compare the genome directly, but compares the structure of 5'-15 proteins expressed by the genome (Dijkshoorn & Towner, 2001).
In a comparative study, AFLP, rep-PCR, ribotyping and PFGE were applied to the subtyping of foodborne and environmental E. coli isolates (Hahm et al., 2003). All methods were able to differentiate the O157 serotype, but the phylogenetic relationships between the isolates differed depending on the typing method chosen. In this study, PFGE was found to be the most effective method of subtyping E. coli O157. Noller et al. (2003) also report similar findings recommending the use of PFGE typing.

Taking into account the advantages and limitations of the typing methods used previously to study E. coli population structure, PFGE was chosen as the method with which to type the commensal E. coli in this work. The perceived problems associated with this technique are that it is relatively expensive and labour intensive, and where highly diverse patterns are found it cannot be used to determine evolutionary relationships. There are few studies where this method has been used to type commensal E. coli, but it is a highly discriminatory method and the results are extremely reproducible (Osek 2000).

This chapter aims to address four hypotheses, which are outlined below.

1. Commonly, one isolate per faecal sample is examined in epidemiological studies, however it is hypothesised that different genotypes are present in one faecal sample. How commonly this occurs will give an indication of whether or not one isolate per faecal sample is adequate for epidemiological studies.

2. Population diversity can be quantified by statistical estimates. Ecologists and evolutionary biologists have developed statistical approaches to estimating diversity within and between populations (Chao, 1987; Colwell & Coddington, 1994; Martin 2002). To test whether these methods are applicable to the microbial diversity data in this chapter, both Simpson's diversity index, and the Chao1 richness estimator will be used. Once the different methods for quantifying diversity have been explored, they will be used, in addition to non-quantitative graphical methods, to test hypotheses 3.
3. Bacteria may become resistant to ampicillin and apramycin by the acquisition of a plasmid (Chaslus-Dancla et al., 1991; Heffron et al., 1975). In contrast, nalidixic acid resistance is commonly acquired through mutation of the gyrase and topoisomerase genes (Vila et al., 1994). As the mechanisms of resistance acquisition between the resistant populations are different, it is hypothesised that population diversity will also differ between these populations. The unselected population is hypothesised to be the most diverse because it comprises all the other resistant populations in addition to any non-resistant strains.

4. Lastly, because the total numbers of *E. coli* have previously been found to decrease with increasing calf age (Hoyle et al., 2004) it was hypothesised that the population diversity may also change as the calves mature. The intestine of a newborn calf is initially sterile, but is rapidly colonised by different strains from both the environment and from other calves and dams (Hartyl & Dykhuizen, 1984). In ecological terms, Andrews & Hall (1986) consider a given population to comprise two different strategists, those that rely on high rates of reproduction to survive in the community (*r* strategists), and those that rely on optimal utilisation of available resources (*K* strategists). Selection for *r* strategists occurs in un-crowded conditions, but when resources are limited their populations crash. In contrast, *K* strategists depend on specific adaptations to an environment and therefore compete better in crowded and resource limited conditions. The *E. coli* population diversity is hypothesised to decrease with calf age because the early colonisers, most of which will be *r* strategists, are eventually out competed by *K* strategists, which have specific adaptations to the environment and tend to be more stable and permanent members of the community (Atlas & Bartha, 1998). This hypothesis will be tested statistically to determine whether the numbers of genotypes detected change significantly with increasing calf age.
4.2 Materials and Methods

4.2a Bacterial isolates

The isolates used in this study were collected as described in chapter 2. Table 4.1 lists the isolates used in this study, sampling dates, and the calves from which they were obtained. In total 543 commensal *E. coli* were examined by PFGE, comprising 139 unselected isolates, 208 ampR, 45 aprR and 151 nalR isolates.
Table 4.1 Numbers of isolates from each population and calf, typed by PFGE.

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</table>
4.2b Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described by Gautom (1997). Briefly, a single colony or cryopreservative bead was used to inoculate 10 ml LB broth for incubation at 37°C with shaking (150 rpm) for 16 to 18 hours. Cultures were harvested by centrifugation at room temperature at 2,190 x g (3,600 rpm) for 12 min. The cells were washed in SE (75mM NaCl, pH 8.0; 25mM EDTA, pH 8.0) by centrifugation and the optical density adjusted to 1.40 at a wavelength of 610nm. Chromosomal-grade agarose (Bio-Rad Laboratories, Hercules, Calif.) was prepared in TE buffer (10mM Tris; 1mM EDTA, pH 8.0) to a final concentration of 1.5%. Equal volumes of agarose and cell suspension were mixed and dispensed into plug moulds (Bio-Rad Laboratories). Following solidification, plugs were transferred into lysis buffer (50mM Tris, pH 8.0; 50mM EDTA, pH 8.0; 1% sarcosine; 1 mg proteinase K per ml) for 24 h incubation at 55°C. Plugs were then washed eight times with TE for 15 min at 4°C, and then left overnight in TE at 4°C. Unless used that day, plugs were stored in TE at 4°C for up to three months. One third of a plug was then preincubated in 200µl of 1 x XbaI buffer (Promega) for 1 hour at room temperature. The buffer was removed and replaced with a fresh mixture containing 50U of XbaI (Promega) in 1 x restriction buffer and incubated at 37°C for 16 to 20 hours. Following a brief wash in 0.5M Tris-borate-EDTA (TBE) (45mM Tris base, 45mM boric acid, 1mM EDTA, pH 8.0), electrophoresis of the samples was performed on the CHEF-mapper (Bio-Rad Laboratories) system using pulsed-field certified agarose (Bio-Rad Laboratories) with 2 litres of 0.5M TBE running buffer (45mM Tris base, 45mM boric acid, 1mM EDTA, pH 8.0). A lambda ladder PFGE marker (New England Biolabs) was run between every six samples. The following electrophoretic conditions were used: initial switch time, 2.1s; final switch time, 54.2s; run time, 22 hours; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear. After electrophoresis the gels were stained for 30 min in 300ml of sterile distilled water containing 300µl of ethidium bromide (1mg/ml), destained for 1 hour in 1 litre of sterile distilled water, and then photographed with the Gel-Doc 2000 system (Bio-Rad).
4.2c Cluster analysis

TIF images of the PFGE gels were exported into BioNumerics software for further analysis and comparison. PFGE fingerprints were compared using the Dice similarity coefficient and clustered by the unweighted pair group method using arithmetic averages (UPGME). PFGE patterns were interpreted according to criterion suggested by Tenover et al., (1995).

Optimisation and position tolerances, calculated with BioNumerics software, were set at 1%. 629 PFGE patterns (86 of which were repeats to check consistency) from the commensal E. coli isolated in this study, in addition to 150 copies of the lambda ladder PFGE marker were used in the determination of the optimal position tolerance and optimisation settings. Similarity matrices were calculated for all entries in the database with a range of position tolerance values. The optimal value is that which resulted in high similarity scores within groups of the same genotype (or repeat lanes of the lambda ladder PFGE marker), and as low as possible similarity scores between groups of different genotypes. The optimal values were also able to distinguish similar types within a genotype, i.e. subtypes of a genotype, for example, with one band difference. The optimisation settings were calculated in the same way. With the 543 isolates used in this study, a position tolerance and optimisation value of 1% permitted easy and clear identification of different genotypes, but also gave good specificity within a genotype, as isolates with one band difference were clustered within a genotype.

4.2d Statistics

Genotype richness was estimated using the Chao1 richness estimate. This estimator has been found to be useful for microbial data sets (Hughes et al., 2001), which are commonly skewed towards the low abundance classes. Genotype richness was estimated according to the equation;
\[ S_{Chao1} = S_{obs} + \frac{n_1^2}{2n_2} \]

where \( S_{obs} \) is the number of species or genotypes observed, \( n_1 \), the number of genotypes observed once, and \( n_2 \), the number of genotypes observed twice.

Population diversity was quantified by Simpson’s diversity index;

\[ C = \sum n_i \frac{(n_i - 1)}{N(N - 1)} \]

where \( n_i \) is the number of individuals of the \( i \)th species or genotype, and \( N \) is the number of individuals or genotypes in the population. In this chapter, \( D = 1 - C \) is given as the index of diversity because it increases with increasing diversity. This index measures the probability that two isolates taken at random from the population will be of the same genotype.

The effect of calf age, sampling date, and population on genotype richness was analysed with generalised linear models (Crawley, 2002). Population was split into two categories; selected (i.e. antibiotic resistant) or unselected.

4.3 Results

4.3a Population diversity and richness

Population diversity may be expressed as the number of genotypes present (genotype richness), or as the evenness of spread of the genotypes present (diversity). In this chapter the terms richness and diversity will be used to distinguish these two meanings. The genotype richness within the unselected, amp\(^R\), apr\(^R\) and nal\(^R\) populations was studied using all typed isolates irrespective of calf or sampling date.
Figures 4.1a-d show dendrograms of the different genotypes present within each population. Subtypes within six of the genotypes were detected (with \( \leq 2 \) band differences from the majority type of a genotype), and these are also included in the figures. The subtypes within a genotype will be described and discussed in section 4.3b; genotypic diversity.

**Figure 4.1a** PFGE genotypes in the apramycin resistant population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.

**Figure 4.1b** PFGE types in the nalidixic acid resistant population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.
Figure 4.1c PFGE types in the ampicillin resistant population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.
Figure 4.1d PFGE types in the unselected population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.
The highest number of genotypes is found within the unselected population (30 genotypes, two of which contained subtypes) (Figure 4.1d). Of the selected populations, the amp$^R$ population contained the most genotypes (26 genotypes) (Figure 4.1c), and the nal$^R$ population the least (two genotypes) (Figure 4.1b). Five genotypes were found in the apr$^R$ population (Figure 4.1a). In total 55 genotypes were detected within the 543 commensal *E. coli* examined in this study.

Within a given population, diversity may be expressed not only as the number of genotypes detected but also as the relatedness between genotypes. To compare the clustering patterns of each population (excluding the nal$^R$ population as it only contains two genotypes), the numbers of lineages were plotted against increasing percentage similarity (Figure 4.2) as calculated by the dice coefficient. These plots are similar to lineage-per-time plots (Martin, 2002) except that percentage similarity rather than arbitrary time since common ancestry is plotted on the x-axis.

![Figure 4.2](image)

**Figure 4.2** Numbers of lineages in the unselected, amp$^R$, and apr$^R$ populations plotted against increasing percentage similarity as calculated by the dice coefficient. A hypothetical low diversity lineage is plotted in grey dashes as a comparison to these populations.
Figure 4.2 compares the relatedness of the unselected, amp\textsuperscript{R} and apr\textsuperscript{R} lineages. A dendrogram in which there are many closely related lineages would produce a convex curve as shown by the example lineage in Figure 4.2. A concave curve is produced when a large amount of diversity between lineages is present near to the root of the dendrogram. All three populations have concave curves, and there is very little difference between the unselected and the amp\textsuperscript{R} curves. One curve is not obviously more concave than the other. This indicates that the diversity between the different genotypes within these two populations is very similar. In support of this, the maximum percentage difference found between genotypes of the unselected and amp\textsuperscript{R} populations were 52\% and 51\% respectively.

The diversity within the unselected, amp\textsuperscript{R}, apr\textsuperscript{R} and nal\textsuperscript{R} populations were further explored using rank abundance curves. Figure 4.3 shows the total diversity found across all four populations. The curves for each population are shown in Figure 4.4.

![Figure 4.3 Rank abundance curve of the 543 commensal E. coli genotyped by PFGE in this study.](image)

67
Figure 4.4 Rank abundance curves of a; the unselected population, b; ampR, c; aprR, and d; nalR populations.
The rank abundance of each genotype, irrespective of the population from which it was obtained, is shown in Figure 4.3. Although the data may be biased by the use of different numbers of multiple isolates from each faecal sample, the shape of this rank abundance curve is typical of a superdiverse community, as is expected. In Figure 4.3, it can be seen that population contains a few abundant genotypes and many rare genotypes. This produces a long right-hand tail on the rank abundance curve.

Comparing the curve of the unselected population (Figure 4.4a) to that of the ampR population (Figure 4.4b) reveals very little difference between them. In terms of richness, the ampR population has fewer genotypes, but the evenness of spread of genotypes between the ampR and the unselected population are very similar. The diversity of the ampR population is substantially greater than that of the aprR and nalR populations (Figure 4.4c and d).

Finally, the diversity within each of the four populations was compared qualitatively using diversity indices. The two diversity measures used evaluate different aspects of the term diversity. Chao1 is a species richness estimate, whereas Simpson’s diversity index (C) measures the probability of finding two identical genotypes when two isolates are taken at random from a population. Table 4.2 shows the diversity of the four populations according to these diversity measures. 1-Simpson’s index (designated D), is given in Table 4.2, so that D increases with increasing diversity.

**Table 4.2 Diversity measures of the nalR, aprR, ampR and unselected populations.**

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of isolates</th>
<th>No. of genotypes found</th>
<th>Diversity measure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chao1 (richness estimate)</td>
</tr>
<tr>
<td>NalR</td>
<td>151</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>AprR</td>
<td>45</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>AmpR</td>
<td>208</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td>Unselected</td>
<td>139</td>
<td>30</td>
<td>46</td>
</tr>
</tbody>
</table>

The species richness estimate Chao1 predicts that the least number of genotypes will be found in the nalR population (Table 4.2). This is quite reasonable because in *E. coli* resistance to nalidixic acid commonly occurs by mutation in the quinolone
resistance determining region of the \textit{gyrA} gene (Vila \textit{et al.}, 1994). This is not a transferable resistance mechanism and so the number of genotypes harbouring this resistance gene are likely to be less than if it was carried on a transferable element (as are the apramycin and ampicillin resistance determinants). The amp$^R$ population is predicted to have the most number of genotypes \((n = 49)\), but it is unclear whether the differences in diversity or richness of the amp$^R$ and the unselected population are significant (Table 4.2). Similarly, Simpson’s diversity index also predicts the amp$^R$ population to be the most diverse. Qualitatively, the two diversity measures predict the same trends, with the nal$^R$ population being the least diverse and the amp$^R$, the most.

To explore the robustness of the Chao1 richness estimate, one fifth of the isolates from each population were randomly excluded from the analysis. Identical richness estimates were produced with these reduced data sets of the nal$^R$ and apr$^R$ populations, but not with the amp$^R$ or unselected populations. The reduced amp$^R$ population was predicted to contain 35 genotypes (as opposed to 49, see Table 4.2), and the reduced unselected population was estimated to comprise 48 genotypes (rather than 46). Therefore, smaller sample sizes had the greatest effect on the most diverse populations, and much less of an effect on lower diversity populations.

\textit{4.3b Genotype diversity}

Isolates within 24 of the 55 genotypes had identical band patterns following digestion with \textit{XbaI}. These genotypes and the populations from which they were obtained are summarised in Table 4.3.
Table 4.3 Genotypes found within the calf commensal *E. coli* for which no genotypic diversity was found following PFGE typing with *XbaI*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. in genotype</th>
<th>Populations (No. found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>9</td>
<td>apr (8), amp (1)</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>apr</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>plain (4), amp (10)</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>amp</td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>amp</td>
</tr>
<tr>
<td>J</td>
<td>64</td>
<td>amp (46), plain (18)</td>
</tr>
<tr>
<td>K</td>
<td>4</td>
<td>amp</td>
</tr>
<tr>
<td>L</td>
<td>53</td>
<td>amp (8), plain (47)</td>
</tr>
<tr>
<td>M</td>
<td>6</td>
<td>plain</td>
</tr>
<tr>
<td>N</td>
<td>3</td>
<td>amp</td>
</tr>
<tr>
<td>O</td>
<td>18</td>
<td>amp</td>
</tr>
<tr>
<td>P</td>
<td>8</td>
<td>amp</td>
</tr>
<tr>
<td>Q</td>
<td>11</td>
<td>amp (9), plain (2)</td>
</tr>
<tr>
<td>R</td>
<td>22</td>
<td>amp</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
<td>plain</td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>plain</td>
</tr>
<tr>
<td>W</td>
<td>9</td>
<td>plain</td>
</tr>
<tr>
<td>X</td>
<td>2</td>
<td>plain</td>
</tr>
<tr>
<td>Y</td>
<td>2</td>
<td>plain</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
<td>plain</td>
</tr>
<tr>
<td>AA</td>
<td>3</td>
<td>amp</td>
</tr>
<tr>
<td>AB</td>
<td>2</td>
<td>amp</td>
</tr>
<tr>
<td>AC</td>
<td>5</td>
<td>plain</td>
</tr>
<tr>
<td>AD</td>
<td>2</td>
<td>plain</td>
</tr>
<tr>
<td>Uniques</td>
<td>1 per group</td>
<td>apr (2), amp (9), plain (14)</td>
</tr>
</tbody>
</table>

Diversity within a genotype was found in six genotypes. These genotypes were split into subtypes designated by the genotype letter then the number of the subtype. The subtypes are numbered sequentially according to the number of isolates within them. Thus, subtype 1 of a genotype contains the majority of isolates within this genotype. In Table 4.4 these genotypes and subtypes are summarised. The number of isolates within a subtype is given, in addition to the band changes of these isolates compared to the majority band pattern of the genotype.
Table 4.4 Genotypes found within the calf commensal *E. coli* for which diversity within a type was found following PFGE typing with *XbaI*. The populations from which these isolates were obtained are listed in addition to the band changes of these isolates compared to the majority band pattern of the genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. in genotype</th>
<th>Populations (No. of isolates detected)</th>
<th>Subtype</th>
<th>Band differences from majority subtype</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>27</td>
<td>apr (27)</td>
<td>A1</td>
<td>NA</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2</td>
<td>531bp replaced by 563bp</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>158</td>
<td>nal (130), amp (28)</td>
<td>D1</td>
<td>NA</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D2</td>
<td>106bp missing</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D3</td>
<td>94bp missing</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D4</td>
<td>34bp missing</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>32</td>
<td>nal (21), amp (5), plain (6)</td>
<td>E1</td>
<td>NA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E2</td>
<td>151bp missing</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E3</td>
<td>151bp replaced by 159bp</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>20</td>
<td>amp (20)</td>
<td>H1</td>
<td>NA</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H2</td>
<td>139bp missing</td>
<td>1</td>
</tr>
<tr>
<td>S</td>
<td>7</td>
<td>plain</td>
<td>S1</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S2</td>
<td>57bp band extra</td>
<td>1</td>
</tr>
<tr>
<td>U</td>
<td>7</td>
<td>plain</td>
<td>U1</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U2</td>
<td>355bp replaced by 418bp, 172bp band extra</td>
<td>1</td>
</tr>
</tbody>
</table>

NA, these are the majority genotypes against which the subgroup patterns were compared.

Within the six genotypes listed in Table 4.4, all but one subtype differed from the majority subtype by one band difference (i.e. 1 band shifted, missing or extra). The subtype of genotype U (U2) had a two-band difference from the majority subtype U1.

### 4.3c Within-sample diversity

The diversity of genotypes present within one sample was examined with each population. The number of genotypes detected as a function of the number of isolates typed is presented in Figures 4.5a-d.
Figure 4.5 Within-sample diversity presented as the number of genotypes detected within a sample from the a; apr<sup>R</sup>, b; amp<sup>R</sup>, c; nal<sup>R</sup>, and d; unselected populations.
The number of isolates obtained from a sample varied because the criterion for picking colonies was to pick the maximum possible numbers of discrete colonies. In some samples many colonies were present, but in others either low numbers of colonies grew or those that were present were not discrete. Where the same numbers of picks were obtained from more than one sample, the different numbers of genotypes detected in each of these samples are plotted on the graphs in different shades.

When up to 10 colonies were picked from the apramycin plates, a maximum of only three different genotypes were detected (Figure 4.5a). When up to 16 colonies were obtained from a nalidixic acid containing plate, the maximum number of genotypes detected was only two (Figure 4.5c). A maximum of four different genotypes were detected amongst 12 colonies from an ampicillin plate (Figure 4.5b). These results are in contrast to those obtained for the unselective plates where six colonies on one plate contained six different genotypes (Figure 4.5d). The within-sample diversity appeared to level off at a maximum of four genotypes per sample in the ampR population when up to 23 different colonies were obtained from a sample. In the unselected population, within sample diversity was not consistent. Only one genotype was found amongst 23 colonies from one sample, in contrast to the detection of a different genotype in all of six isolates obtained from another sample (Figure 4.5d).

4.3d Population diversity as a function of calf age

To determine if the number and types of commensal E. coli changed with calf age, genotypes from calves 687, 690, 693 and 694 were studied in more detail. These calves were selected for this analysis because they had a good spread of isolates over at least four consecutive sampling dates since birth. None of the calves received any direct antibiotic treatment during the sampling dates used in this analysis (see materials and methods for treatment record details). Figures 4.6, 4.7, 4.8 and 4.9 show the genotypes detected from calves 687, 690, 693 and 694 respectively. The
genotypes detected on each of the weeks after birth are given as a percentage of the total number of isolates obtained on that date. In each graph, the x-axis is split into two groups to present the genotype prevalence from the unselected (U) and selected (S) (comprising amp\(^R\), apr\(^R\) and nal\(^R\) isolates) populations. All unique genotypes are shaded in grey. The other genotypes are indicated with colours that are consistent between the graphs. Below each figure is a table containing a breakdown of which genotypes were present in each of the unselected, amp\(^R\), apr\(^R\) and nal\(^R\) populations, the age of the calf and the corresponding sampling date.

**Figure 4.6 and Table 4.5** Figure 4.6 shows the genotype prevalence with calf age of unselected (U) and selected (amp\(^R\), apr\(^R\) and nal\(^R\)) (S) isolates obtained from calf 687. Table 4.5 lists the dates of detection, exact numbers of each genotype and populations from which they were isolated.
No resistant isolates were detected within 48hrs of birth of calf 687 (week 0, sampling date 17.09.02) (Figure 4.6). After one week, resistant isolates were detected. The \( \text{amp}^R \) genotypes J and F were detected in the selected population for at least two consecutive weeks. These genotypes were both present in the unselected population on more than one occasion, indicating a high prevalence of these strains. The \( \text{nal}^R \) isolate obtained in the first week was different to that obtained in the fourth week (D and E respectively). The waves of different colours across Figure 4.6 are indicative of different genotypes becoming the dominant type and then being replaced by a new dominant type on subsequent dates.

**Figure 4.7 and Table 4.6** Figure 4.7 shows the genotype prevalence with calf age of unselected (U) and selected (amp\(^R\), apr\(^R\) and nal\(^R\)) (S) isolates obtained from calf 693. Table 4.6 lists the dates of detection, exact numbers of each genotype and populations from which they were isolated.
In contrast to calf 687, the most genotypic diversity within the commensal *E. coli* of calf 693 occurred in the first sample (taken within 48h of birth) (Figure 4.7). On this date, isolates were obtained from all four populations (unselected, amp$^R$, apr$^R$ and nal$^R$). However, a week later (01.10.01), when isolates were obtained from the unselected, amp$^R$ and nal$^R$ populations, all isolates were found to be of the same genotype (E). The fact that this genotype accounted for all six of the isolates obtained from an unselective plate indicates that this strain occurred at a high prevalence on this date. This high prevalence of a strain resistant to ampicillin and nalidixic acid does not correlate with any antibiotic treatments of calf 693 (see Chapter 2).

![Genotype prevalence with calf age](image)

**Figure 4.8 and Table 4.7.** Figure 4.8 shows the genotype prevalence with calf age of unselected (U) and selected (amp$^R$, apr$^R$ and nal$^R$) (S) isolates obtained from calf 694. Table 4.7 lists the dates of detection, exact numbers of each genotype, and populations from which they were isolated.
Similar to calf 693, the greatest diversity of genotypes isolated from calf 694, is observed from the sample taken within 48h of birth (26.09.01). In this sample, isolates were obtained from unselective, ampicillin, apramycin and nalidixic acid plates. In contrast to calf 693, the unselected isolates from the week 0 sample of calf 694 were all unique genotypes (genotypes only ever detected once).

The genotype diversity of calf 694 appears to decrease with increasing calf age in both the selected and unselected populations. On the three dates when isolates were obtained from both these populations, the resistant genotypes were never present in the unselected population. This is in contrast to the detection of resistant genotypes in the unselected populations of calves 687 and 693, and indicates that calf 694 has overall a lower prevalence of resistant genotypes than calves 687 and 693.
Figure 4.9 and Table 4.8 Figure 4.9 shows the genotype prevalence with calf age of unselected (U) and selected (\(r{amp}^R\), \(r{apr}^R\) and \(nal^R\)) (S) isolates obtained from calf 690. Table 4.8 lists the dates of detection, exact numbers of each genotype, and populations from which they were isolated.

Examination of the unselected population of calf 690 revealed that the greatest number of different genotypes is found in the first sample (18.09.01). There appears to be a general trend towards decreasing diversity by week four (17.10.01). This trend of decreasing diversity with increasing calf age was observed in samples obtained from calf 693. The \(r{amp}^R\) population also appears to decrease in diversity with increasing age of calf 690.
Similar to calf 687, some genotypes appear on at least two consecutive sampling dates and then disappear below the detection threshold ($\leq 1000\text{cfu/mL}$) (e.g. H, D, O, W and Z). Genotype J is found within the amp$^R$ population on six different sampling dates. J is never detected within the unselected population suggesting that it is present at a lower prevalence in calf 690 than in calf 693, where it was detected in the unselected population. In contrast to calves 693 and 687, resistant genotypes are only found in the unselected population on one occasion (01.10.01).

Overall, three genotypes (F, A and D) were found to persist for at least three consecutive weeks in a particular calf. Eight PFGE types persisted for two weeks (L, J, S, E, W, H, Q, and O). Forty-one genotypes (including unique types) were only found on one sampling date in a given calf. To determine which genotypes were detected in more than one calf, and which were unique to a calf, the presence or absence of each genotype was listed in a table (Table 4.9). Blue shading indicates the presence of a genotype, and no shading, the absence of a genotype. The unique isolates were not included in this table because they were only found on one occasion each, and so are by nature unique to each calf that they were isolated from (at the detection level of the method, $\leq 1000\text{ cfu/mL}$).
Table 4.9 Presence (blue shading) or absence (no shading) of genotypes detected in calves 687, 693, 694 and 690.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of calves</th>
<th>Calf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>687</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AB</td>
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<tr>
<td>AC</td>
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<td>B</td>
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<td>V</td>
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<tr>
<td>Z</td>
<td>1</td>
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</tr>
</tbody>
</table>

Table 4.9 shows that genotypes D, J and L were detected in all four calves, but the majority of genotypes (18/25) were unique to the calf from which they were isolated.

To summarise the data presented in this section of work, the following observations of the data were made:

1. The most genotypic diversity was observed in calves 693, 694 and 690 in the first sample, and appeared to decrease with increasing calf age.
2. Only calf 687 did not harbour resistant isolates on the first sample.
3. Genotypes isolated from antibiotic containing plates were detected in the unselected populations of calves 693 and 687 (and 690 on one occasion) indicating a high prevalence of resistance in these calves.

4. Genotypes isolated from antibiotic containing plates were not detected in the unselected population of calf 694, indicating a lower prevalence of resistance than in calves 693 and 687.

5. The majority of genotypes were unique to the calf from which they were isolated, but genotypes D, J and L were detected in all four calves.

6. Genotypes F, A and D persisted for at least three weeks in at least one calf.

From the data presented in Figures and Tables 4.7 to 4.10, it was hypothesised that genotypic richness (number of types detected) decreases with calf age, and that the level of diversity was specific to a particular calf. When these hypotheses were tested with the data from all 11 calves, genotypic richness was found to decrease with increasing calf age ($F_{1,62} = 9.13, P = 0.004$), but was not associated with specific calves ($F_{10,52} = 1.59, P = 0.14$), sampling dates ($F_{1,61} = 2.63, P = 0.11$), or population (i.e. unselected or selected populations) ($F_{1,61} = 0.024, P = 0.877$). The rate of decrease in genotype richness detected with increasing calf age was dependant on the calf ($F_{8,43} = 3.20, P = 0.007$), but not the sampling date ($F_{1,51} = 0.55, P = 0.463$), or population (unselected versus selected) ($F_{1,42} = 0.46, P = 0.50$).

4.3e Genotype diversity as a function of sampling date

To explore the diversity amongst genotypes as a function of sampling date, genotypes that were detected on more than one sampling date (not necessarily the most abundant genotypes shown in Figure 4.4) were plotted against sampling date for the unselected, amp$^R$ and nal$^R$ populations (Figure 4.11, 4.12 and 4.13 respectively). The apr$^R$ population is excluded from this analysis as it is discussed in detail in Chapter 5.
Figure 4.10 Genotype prevalence within the unselected population. Arrows indicate sampling dates.

The most prevalent genotype (L) on 17.09.01 is not detected the following day (18.09.01), instead AD is the most prevalent genotype (Figure 4.10). The samples obtained from 17.09.01 and 18.09.01 are from different calves, therefore are likely to reflect the differences in genotypes between these calves. Unfortunately, consecutive samples from these two dates from the same calf were not available. After 18.09.01, genotype L shows an overall increase in prevalence over the following 11 days. In contrast, genotypes W and J peak on 26.09.01 and then tend to decrease to undetectable levels on 17.10.01. These dynamics suggest that these more common genotypes prevail for a few days but do not seem to appear and disappear below detection threshold over the sampling period shown. The lack of samples between the dates when samples were obtained means that any trends observed in this graph are difficult to interpret with any certainty.
Figure 4.11 Genotype prevalence's within the amp<sup>R</sup> population. Arrows indicate sampling dates.

The overall impression of the genotypes within the amp<sup>R</sup> population is one of dramatic fluctuations (Figure 4.11). Gradual increases or decreases in genotype prevalence are rarely observed with this sampling protocol. Genotypes that appear to vary between being present and absent on different sampling dates are likely to be present at a prevalence that fluctuates around the detection threshold of the sampling and detection methods.
The most common genotype in the nalR population is D. On one sampling date (19.10.01) it was not detected, and type E is the only nalR genotype found (Figure 4.12). There appears to be a switching of the most prevalent genotype between 29.10.01 and 19.11.01. To prove this hypothesis, further sampling would be required.

4.4 Discussion

The majority of studies of E. coli diversity have focused on pathogenic strains (Hartman et al., 2003; Kern et al., 2002; Noller et al., 2003; Ørskov et al., 1990; Shen et al., 1999; Whittam et al., 1993), but comparatively little is known about the population structure of commensal E. coli. It is essential to determine the background level of population diversity if valid conclusions are to be drawn as to the genetic relatedness of groups of isolates within the E. coli genus (for example outbreak strains, pathogenic strains or antibiotic resistant strains). This chapter aimed to study different aspects of the population diversity of commensal E. coli isolated from calves. Firstly, the genotype diversity within unselected populations and ampR, aprR and nalR subpopulations was compared. Next, the diversity within genotype groups (subtypes of a genotype) and diversity of genotypes within a faecal sample was
examined. Finally, changes in the diversity of genotypes with calf age or with sampling date were explored.

Before each of these sections of work is discussed, it is important to ascertain exactly what is meant by the term diversity. Diversity may be expressed in three different ways; the number of types of an organism (i.e. species richness), the evenness or relative abundance of each type of organism, and the relatedness between groups of organisms. There is no single measure capable of expressing each of these aspects of diversity within a population. In this chapter, a combination of different approaches was used to compare the genotype diversity of the unselected, ampR, aprR and nalR populations.

4.4a Population diversity

Counting the number of genotypes detected within the unselected, ampR, aprR and nalR populations revealed the unselected population to have the most number of genotypes, and the nalR population to have the least. Only four more genotypes were found in the unselected population compared to the ampR population, indicating a high degree of diversity between strains resistant to the β-lactam ampicillin. Although plots of the number of lineages versus percentage similarity demonstrated the genetic relatedness (or dissimilarity) between genotypes within these two populations to be very similar, the spread of genotype abundance within the unselected population was less even, therefore showed less diversity than the ampR population. This emphasises the need to use different measures of diversity in any assessment of diversity between two populations. Each measure on its own does not provide all the information required to assess the three aspects of diversity described at the start of this discussion. In agreement with this, Bohannan & Hughes (2003) also recommend the use of a combination of different approaches to analyse diversity in microbial communities.
Similar to the high level of diversity found within the amp\(^R\) population, Shen et al. (1999) describe a wide genetic diversity amongst clinical isolates of *E. coli* producing extended-spectrum \(\beta\)-lactamases (eight different PFGE patterns were obtained from only 23 isolates). In the comparison of the four populations described in this chapter, the nal\(^R\) population was the least diverse. Similarly, fluoroquinolone resistant *E. coli* from cancer patients were clonal within each patient (Oethinger et al., 1998).

The reason for the different degrees of diversity within the resistant populations studied is likely to be because of the mechanism of resistance harbouried. Resistance to \(\beta\)-lactam antibiotics is commonly plasmid-mediated (Datta & Kontomichalou, 1965; Heritage et al., 1999; Philippon et al., 2002). In addition to the ability to develop resistance individually by mutations in genes controlling AmpC production, resistance may be acquired by the acceptance of a resistance plasmid. A detailed analysis of the diversity of the plasmids conferring amp\(^R\) would confirm this. In contrast, until recently resistance to the quinolones was never considered to be plasmid mediated (Amyes et al., 1996), however evidence of a plasmid vector has been found in isolates from China (Wang et al., 2003). Therefore, the most likely way for a strain to acquire resistance is by mutation of the quinolone resistance determining region of the DNA gyrase and topoisomerase II genes.

Although apramycin resistance is also plasmid-mediated, the genotypic diversity within the apr\(^R\) population is not as great as in the amp\(^R\) population. It is likely that amp\(^R\) plasmids are more common because \(\beta\)-lactams are used more frequently on the farm than aminoglycosides (see Chapter 2). Another factor affecting the spread of plasmid-mediated resistance is plasmid host range. Are the amp\(^R\) plasmids broad host range plasmids and the apr\(^R\) plasmids of a narrow host range? Some results on the acceptance of apr\(^R\) plasmids are presented in Chapter 5.

Various diversity estimates have been used previously to compare bacterial diversity between different environments (Dunbar et al., 1999; McCaig et al., 1999; Sigler & Zeyer, 2002). Simpson’s diversity index measures the probability that two
individuals from a population will be the same if the isolates are drawn at random. This was considered an appropriate measure because it expresses the dominance of one or two of the commonest species (or genotypes). The sampling protocol used in this work was suitable for determining the commonest genotypes. In contrast, the commonly used Shannon-Weaver diversity index is strongly affected by the abundance of the genotypes present in the middle of a range of genotype abundances. Although this measure has been applied to microbial populations (Dunbar et al., 1999; McCaig et al., 1999; Sigler & Zeyer, 2002) it was considered inappropriate for the bacterial populations described in this chapter because they contained a few common genotypes and many rare ones (as demonstrated by the rank abundance curves). The Shannon-Weaver diversity index assumes that the random sample taken from the population represents all of the species in the population. This assumption could not be even partly satisfied with this data set because of the extreme diversity of the commensal E. coli population (see Figure 4.3) and the sampling protocol adopted in this study.

Simpson’s diversity index (D) ranges from 0 to 1. Values around 0 reflect the dominance of a few genotypes, and values nearer 1 reflect a population containing many different genotypes. The low D observed with the nalR resistant population (D = 0.241) demonstrates the high dominance of a few genotypes. In contrast the diversity of the ampR population was high (D = 0.899). This level of diversity is comparable to that found amongst E. coli strains in piglets within the first week of birth (mean D = 0.848) (Katouli et al., 1995).

Qualitatively, diversity as estimated by Simpson’s was similar to the diversity (defined as species richness) estimated by Chao1. Of 139 unselected E. coli comprising 30 different genotypes, total genotype richness was estimated at 46 genotypes. This is substantially lower than the number of E. coli predicted (predicted n = 128) with the same richness estimate amongst 280 E. coli from a bovine feedlot (Yang et al., 2004). However, the number of types detected will depend on the typing method, the definition of a type, and the sampling effort. In the study of bovine feedlot E. coli, PCR-based typing was performed and operational taxonomic
units (OTUs) were defined with a cut-off of 85% similarity. Differences in methodology and analysis mean it is difficult to compare diversity between studies.

Interestingly, Seurinck et al. (2003) conclude that generally 32 isolates per faecal source were sufficient to characterise the richness of the *E. coli* within that source. In the present study, a maximum of 24 isolates were obtained from any one faecal sample, and so this hypothesis could not be challenged. However, bacterial population dynamics are based on the ability to persist at low levels and then increase when the selective environment favours the particular strain. It is possible that there could be a dominant strain that is 100-fold more abundant than any of the others, particularly if the environment favoured it. The other strains at lower concentrations would remain undetected by Seurinck et al. s criteria.

There are two important problems associated with the use of statistical approaches to estimate bacterial diversity. Firstly, they rely on estimates of relative abundances of OTUs, which may be inaccurate because of sampling biases. In the present study, the different numbers of isolates analysed from each sample may have introduced some bias into the data. Bias may also occur because of differences in survival during storage at 4°C between *E. coli* genotypes, prior to analysis. Secondly, the statistical methods used in this chapter rely only on the information from the genotypes observed and therefore cannot account for very rare genotypes. Although this will result in an underestimate of the actual number of genotypes present within a sample, they do provide a lower boundary of genotype diversity.

### 4.4b Genotype diversity

Subtypes within a genotype were identified in six of the 55 genotypes detected in the commensal *E. coli*. The presence of subtypes within a genotype depends on the interpretive criteria used to define a genotype. Commonly, criteria outlined by Tenover et al. (1995) are used in defining PFGE types. The usefulness of these definitions depend on the type of bacteria studied (more genotypic diversity may be
expected between *Streptococcus milleri* group (Bartie et al., 2000) than between enterococci isolates (Perlada et al., 1997), the size of geographical area from which isolates were obtained (isolates from different countries are likely to be less similar than isolates from the same town unless they are related) and the diversity expected between the isolates (diversity between *E. coli* O157 is less than between commensal *E. coli* isolates comprising many serogroups (Vali et al., 2004)).

In this chapter, a high degree of diversity was expected between the commensal isolates because there was no (known) selection for specific serogroups. The genotypes detected were highly diverse, with an uncountable number of band differences between genotypes. Because the isolates were found to be so diverse, the presence of types that differed from a genotype by up to two bands were considered related to that genotype. Only by taking into account the background level of diversity within the commensal population could this judgment be made. Similarly, Foxman et al. (2002) established a high degree of diversity between *E. coli* from pairs of heterosexual partners, which meant that finding the same genotype within a couple was of significance. In this chapter, the criteria devised by Tenover et al. adequately distinguished between different genotypes, which were highly dissimilar, and subtypes within a genotype. The same interpretive criteria have been used to characterise extended-spectrum β-lactamase producing clinical *E. coli* (Shen et al., 1999) and enterohaemorrhagic *E. coli* (Rios et al., 1999).

### 4.4c Within sample diversity

Increasing the numbers of colonies taken from a sample did not necessarily increase the number of genotypes detected. In the ampR population increases in genotype numbers appeared to level off at 12 picks (where a maximum of four genotypes were detected in samples from which 12 to 23 isolates were obtained). The maximum genotype richness in the unselected population was found when six isolates were obtained, and increasing the number of isolates from a sample did not increase the richness detected (up to 23 isolates per sample). Based on the shape of the rank
abundance curve in Figure 4.4a it is hypothesised that a far greater number of rarer genotypes would be detected were log fold increases in isolates examined. This hypothesis is supported by the fact that when selection is applied to the commensal population (i.e. plating onto ampicillin plates) almost as many different genotypes are observed as in the unselected population (Figure 4.1c and d).

There appears to be no published data describing genotypic diversity of commensal organisms within samples (faecal or other), although phenotypic diversity has been described with 12 E. coli isolates from piglet faeces (Katouli et al., 1995). The detection of more than one genotype in over half of the samples from the unselected population (11/21) and in 19 of the 63 samples from the selected population has important implications for the design of experiments intended to study genotypic diversity in either antibiotic resistant or unselected bacterial populations.

4.4d Population diversity as a function of calf age

The genotypic diversity (number of types) was found to decrease significantly with calf age (irrespective of whether the selected or unselected population was studied). This result contrasts with that of Katouli et al. (1995), where the mean number of faecal E. coli biochemical phenotypes found in piglets increased as the animals aged. Similar to Katouli et al., most types were found to be transient and were replaced by new types in the following samples. Genotypes F, A and D were found to persist for at least three weeks. These genotypes may have been better able to colonise and maintain their populations in the calf intestine than the majority of genotypes (n = 44), which were not detected on consecutive sampling dates. Colicin resistance has been found in established strains of E. coli (Ofek et al., 1977), which was thought to aid persistence in the lower intestine.

Alternatively, genotypes F, A and D, may simply have been the first to colonise the calves. Freter et al. (1983) describe difficulties in implanting E. coli in the mouse intestine when established intestinal flora are present. In contrast, when the E. coli
are inoculated first, then the caecal flora, stable \textit{E. coli} populations persist indefinitely.

4.4e \textit{Population diversity as a function of sampling date}

The changes in PFGE types (detected on more than one occasion) did not show any general trends during the sampling period. The sampling protocol of approximately weekly sampling was not frequent enough to determine how genotype prevalences changed with time. A general impression of dramatic fluctuations of genotype prevalence was observed with both the unselected and amp\textsuperscript{R} populations. Similarly successive waves of phenotypically different \textit{E. coli} have been found to colonise the intestine of piglets (Katouli \textit{et al.}, 1995), although the sampling protocol of this study was less frequent than that described in this chapter.

The most prevalent \textit{nal}\textsuperscript{R} genotype (D) appeared to be succeeded by a different genotype (E), but because 14 days had elapsed between the sampling dates when D was detected at a high prevalence, and the sampling date when a high prevalence of E was found, the dynamics of this switching of types are not clear.
4.5 Concluding remarks

Problems arose in analysing the diversity based on genotype prevalence, because the numbers of isolates obtained from each sample ranged from one to 24. A better protocol would be to spread diluted samples evenly over each agar plate, and to pick equal numbers of colonies from each plate showing growth. In addition, weekly sampling was found to be inadequate for studying the fluctuations of different genotypes with time. More frequent sampling (at least daily) is suggested for future analyses.

In this chapter a high degree of diversity amongst calf faecal commensal *E. coli*, in terms of the total number of different genotypes detected, the evenness of spread of types, and the genetic relatedness of genotypes was detected. Referring back to the original aims of the chapter, this work demonstrated the following:

1. Different genotypes were hypothesised to be present within one faecal sample. This was shown to be true in many but not all samples (11/21 unselected samples, 19/63 selected samples).

2. The statistical predictions of diversity produced qualitatively reasonable estimates, although the predicted numbers of genotypes are likely to be underestimates of the actual numbers present because they rely only on the number of genotypes observed. In this way, very rare classes are ignored because they are present below the detection threshold of the method. Rare classes may only be “rare” at the time of sampling because genotypes were found to fluctuate dramatically with time. On another sampling date, or following a change in feed, environment, housing or treatment, rarer types may be selected into becoming dominant types.

3. It was hypothesised that population diversity would differ between the resistant populations because the mechanisms by which bacteria may acquire resistance is different. The diversity observed differed between the four
populations, but the diversity between the unselected and amp\textsuperscript{R} populations were surprisingly similar. This was thought to be because ampicillin resistance is commonly plasmid mediated. The greater number of amp\textsuperscript{R} genotypes compared to the apr\textsuperscript{R} genotypes may reflect differences in the number and host range of these two types of resistance plasmid. In addition, the number of resistance genes that confer ampicillin resistance are numerous (Amyes et al., 1996), in contrast to the single gene found to confer apramycin resistance (Shaw et al., 1993).

4. The \textit{E. coli} population diversity was hypothesised to decrease with calf age because the first colonisers, which did not have to compete for space and nutrients, would be out competed by genotypes better able to adapt to a more crowded and resource limited environment. Diversity (number of genotypes) was found to decrease significantly with increasing calf age, but was not significantly associated with sampling date.
Chapter 5. Molecular mechanism of apramycin resistance

5.1 Introduction

In 1978 the aminoglycoside apramycin was licensed for veterinary use in the United Kingdom, and has since been used extensively in animal husbandry (Hunter et al., 1992). Although this antibiotic has not been used in human medicine, apramycin resistance has been detected in human isolates of *Salmonella typhimurium* (Chaslus-Dancla et al., 1989), *Klebsiella pneumoniae* (Johnson et al., 1995) and *E. coli* (Johnson et al., 1995; Hunter et al., 1993). Resistance to apramycin is conferred by the production of the aminoglycoside-modifying enzyme, 3-N-aminoglycoside acetyltransferase type IV (AAC(3)IV) (Davies & O'Connor, 1978) which has been found on diverse conjugative plasmids (Platt & Smith, 1991; Pohl et al., 1993). A high degree of genetic homology has been detected between plasmids harbouring AAC(3)IV isolated from bacteria of human and animal origin (Chaslus-Dancla et al., 1991). In addition to the acetylation of apramycin, AAC(3)IV also acetylates tobramycin and gentamicin, which are used to treat serious infections in humans (Amyes et al., 1996). Recently, 7% of *E. coli* isolated from calf enteritidis faecal samples in Scotland were found to be resistant to apramycin (Gunn & Low, 2003). The aim of this study was firstly to determine whether calves that had not been treated with aminoglycosides harboured apramycin resistant (aprR) commensal *E. coli*, and secondly to study the molecular epidemiology of the resistance genes to this antibiotic.

5.2 Materials and Methods

5.2a Bacterial isolates

Apramycin resistant *E. coli* isolates (n = 45) obtained as described in chapter 2 were examined in this study.
5.2b Antibiotic sensitivity

Minimum inhibitory concentrations (MICs) of ampicillin, apramycin, gentamicin, streptomycin, sulphamethoxazole, tetracycline (Sigma, Poole, UK) and tobramycin (Faulding Pharmaceuticals, Warwickshire, UK) were determined using a multipoint inoculator to replicate test cultures onto antibiotic-containing Isosensitest agar plates (Oxoid CM471) at $10^4$-$10^5$ cfu/spot, following BSAC guidelines (Andrews, 2001). The MIC was defined as the lowest concentration of antibiotic to give complete inhibition of bacterial growth after incubation at 37°C for 18 hours.

Control organisms (NCTC10418, NCTC10662 and NCTC6571) were included to detect any errors in the preparation of antibiotics or plates, and all isolates were inoculated onto agar plates containing no antibiotic to ensure that any inhibition of growth on test plates was due to the presence of the antibiotic.

5.2c Pulsed-field gel electrophoresis

PFGE typing as described in Chapter 4 was used in this study.

5.2d Conjugation experiments

Apramycin resistance plasmids were transferred by conjugation into a rifampicin resistant *E. coli* J62-2 (pro' his' trp') (Bachmann, 1972) by incubating a 1:100 ratio of donor to recipient, in nutrient broth (Oxoid) at 37°C for 7 hours (method adapted from Provence & Curtiss, 1994). Transconjugants were isolated and purified on MacConkey plates (Oxoid) incorporating 20mg/L rifampicin and 16mg/L apramycin.

Transfer frequencies, completed in triplicate, were defined as the proportion of transconjugants over total number of donors (D) at the start of mating (Provence &
Curtiss, 1994) and were measured between J62-2 (D) and the related auxotroph J53 (pro’ met’) (Bachmann, 1972). Overnight nutrient broth (NB) (Oxoid) cultures of donor and recipient were incubated in NB at 37°C in a 1:100 ratio respectively, for 7 hours (Provence & Curtiss, 1994). Transconjugants were selected on Davis Mingioli plates (DM) supplemented with 16mg/L apramycin, 50mg/L proline and 50mg/L methionine. Average transfer frequencies were compared to those of the well-characterised plasmids RP4 (Datta et al., 1971) and R46 (Brown & Willetts, 1981) using the same mating protocol.

The recipient ability of 28 different genotypes of commensal E. coli from the unselected population was measured by repeat conjugation experiments with J62-2 harbouring plasmid pUK2001. The same 7 hr mating protocol was used as that described above. Transconjugants were selected on DM plates containing no amino acids and 16mg/L apramycin.

5.2e Plasmid analysis

Plasmid DNA was extracted from J53 transconjugants using a Maxiprep kit (Qiagen) and restricted with EcoRI and ApaI (Promega) according to the manufacturer’s instructions. Gels were stained with ethidium bromide as described earlier. Plasmid size estimates were determined by comparison to the standard plasmids harbour by E. coli 39R861 and E. coli V517.

5.2f Amplification of the aac(3)IV gene

Primers 5'-GTCGTCCAATACGAATGGCG-3' and 5'-CAGCAATCAGCGCGACCTTG-3' were used to amplify the region between base pairs 241 to 1076 of the published aac(3)IV gene (GenBank X01385) (Bräu et al, 1984) from purified plasmid DNA. DNA was denatured for 5 min at 94°C, followed by 30 cycles of 1 min denaturation (94°C), 1 min anneal (55°C), and 1 min extension
(72°C). Sequencing was performed by chain termination (Sanger et al., 1977) in both directions and compared to the published sequence (Bräu et al., 1984).

5.2g Statistical analysis

Statistical analysis was performed with S-Plus (Insightful, Seattle). Analyses of the carriage of apramycin resistant E. coli (presence/absence) by the 11 calves was performed using generalised linear mixed effects models, with binomial errors. Calf identity was entered as a random effect to account for both inherent differences between calves and the lack of independence between samples. Time and calf age were entered into separate models as fixed effects.

5.3 Results

5.3a Detection of apr\textsuperscript{R} E. coli PFGE types

Although no aminoglycoside antibiotics were used on the 11 calves sampled in this study, six calves harboured apr\textsuperscript{R} E. coli (≥1000 cfu/mL) during the four months sampling period. Detection dates, calf identities, antibiotic treatment records and genotypes of apr\textsuperscript{R} E. coli are listed in Table 5.1.
Table 5.1: Calves from which aprR E. coli were isolated, calf antibiotic treatments and age, dates of aprR E. coli detection, and number and genotype of aprR isolates.

<table>
<thead>
<tr>
<th>Calf</th>
<th>Treatment (date)</th>
<th>Sampling dates of aprR E. coli detection</th>
<th>AprR E. coli isolates</th>
<th>PFGE types (number of each type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>690</td>
<td>None</td>
<td>01/10/2001</td>
<td>2</td>
<td>A (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12/11/2001</td>
<td>1</td>
<td>B (1)</td>
</tr>
<tr>
<td>687</td>
<td>None</td>
<td>17/10/2001</td>
<td>10</td>
<td>A (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24/10/2001</td>
<td>10</td>
<td>A (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12/11/2001</td>
<td>3</td>
<td>A (3)</td>
</tr>
<tr>
<td>694</td>
<td>None</td>
<td>26/09/2001</td>
<td>8</td>
<td>C (8)</td>
</tr>
<tr>
<td>696</td>
<td>None</td>
<td>12/11/2001</td>
<td>6</td>
<td>A (1), B (4), D (1)</td>
</tr>
<tr>
<td>698</td>
<td>enrofloxacin (01/10/2001)</td>
<td>10/12/2001</td>
<td>1</td>
<td>A (1)</td>
</tr>
</tbody>
</table>

From four calves (693, 694, 696 and 698), aprR E. coli were found on only one occasion (Table 5.1). The analysis of carriage of aprR E. coli by the 11 calves revealed that the detection of aprR E. coli was not related to calf age ($F_{1,139} = 1.26, P = 0.26$), or sample date ($F_{1,139} = 1.22, P = 0.27$). To ensure that those calves for which aprR E. coli were never detected were not biasing the analysis, models were repeated with only calves that were found to harbour aprR E. coli during the sampling period. These repeat tests yielded similar $F$ and $P$ values for the effect of age ($F_{1,73} = 0.74, P = 0.39$), or sample date ($F_{1,73} = 1.33, P = 0.25$) on the presence of aprR E. coli.

Forty-five aprR E. coli were isolated. Genotyping of the isolates by PFGE revealed that the aprR population was not clonal, and comprised five distinct genotypes (Chapter 4, Figure 4.1a).

The majority (7/9) of samples contained only one genotype (Table 5.1). Two calves (690 and 687) were found to harbour aprR E. coli on more than one occasion. Calf 690 carried different genotypes on the two dates where aprR E. coli were detected, whereas calf 687 harboured the same genotype on all dates of detection. PFGE type
A was the most common genotype and was found in four of the calves. Genotypes C, D and E were only found on one sampling date each. To determine if genotypes were associated with specific sampling dates, the prevalence of each genotype was plotted against the sampling date (Figure 5.1).

![Genotype prevalence graph]

**Figure 5.1** Dates of detection and prevalence’s of the five different apr$^R$ *E. coli* genotypes.

On four of the six dates on which apr$^R$ *E. coli* were detected, the only genotype present was PFGE type A. All isolates of this genotype ($n = 27$) were found to have the same resistance phenotype (resistant to tetracycline, sulphamethoxazole, apramycin, tobramycin and gentamicin). On two sampling dates (26/09/01 and 12/11/01) more genotypic diversity (three different genotypes) was present. These are the only two dates on which apr$^R$ *E. coli* were isolated from more than one calf.

### 5.3b Plasmid analysis

Plasmid profiling with *EcoRI* revealed three different plasmids, designated pUK2001, pUK2002, and pUK2003, of size 91Kb, 115Kb and 181Kb respectively.
The EcoRI restriction profiles of these plasmids are shown in Figure 5.2. Plasmid types were confirmed with Apal restriction (results not shown). pUK2002 and pUK2003 were found in only one genotype each, but pUK2001 was found in three different genotypes indicating horizontal spread. It was not possible to restrict plasmid pUK2003 despite repeated attempts, but the different sizes and transfer frequencies (described below) indicate that pUK2003 and pUK2002 are different plasmids.

![Plasmid restriction profiles](image)

**Table 5.2**

<table>
<thead>
<tr>
<th>Plasmid restriction</th>
<th>Calf</th>
<th>Plasmid</th>
<th>Host genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEO1.0690</td>
<td>pUK2001</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>LEO1.0693</td>
<td>pUK2001</td>
<td>Unique</td>
<td></td>
</tr>
<tr>
<td>LEO1.0694</td>
<td>pUK2001</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>LEO1.0696</td>
<td>pUK2002</td>
<td>Unique</td>
<td></td>
</tr>
<tr>
<td>LEO1.0690</td>
<td>pUK2003</td>
<td>B</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.2** EcoRI plasmid types and the host genotypes from which they were isolated.

5.3c *Transfer frequencies*

All three apramycin resistance plasmids were conjugative. Transfer frequencies were measured between the *E. coli* K12 derivatives J62-2 and J53, using the different auxotrophisms of these strains as a selective mechanism. The transfer frequencies of each plasmid, and the selective plate used for transconjugant enumeration are listed in Table 5.2.
Table 5.2 Plasmid transfer frequencies of pUK2001, pUK2002 and pUK2003, measured on different antibiotic containing selective plates.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Selective plate</th>
<th>Transfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUK2001</td>
<td>16mg/L apramycin</td>
<td>4.19 x 10^2</td>
</tr>
<tr>
<td>pUK2002</td>
<td>16mg/L apramycin</td>
<td>1.73 x 10^3</td>
</tr>
<tr>
<td></td>
<td>10mg/L tetracycline</td>
<td>1.57 x 10^3</td>
</tr>
<tr>
<td></td>
<td>16mg/L apramycin + 10mg/L tetracycline</td>
<td>1.37 x 10^3</td>
</tr>
<tr>
<td></td>
<td>8mg/L streptomycin</td>
<td>1.87 x 10^3</td>
</tr>
<tr>
<td></td>
<td>16mg/L apramycin + 8mg/L streptomycin</td>
<td>2.02 x 10^3</td>
</tr>
<tr>
<td>pUK2003</td>
<td>16mg/L apramycin</td>
<td>4.64 x 10^6</td>
</tr>
<tr>
<td></td>
<td>10mg/L tetracycline</td>
<td>4.27 x 10^6</td>
</tr>
<tr>
<td></td>
<td>16mg/L apramycin + 10mg/L tetracycline</td>
<td>5.18 x 10^6</td>
</tr>
<tr>
<td></td>
<td>8mg/L streptomycin</td>
<td>4.41 x 10^6</td>
</tr>
<tr>
<td></td>
<td>16mg/L apramycin + 8mg/L streptomycin</td>
<td>4.27 x 10^6</td>
</tr>
<tr>
<td>RP4</td>
<td>10mg/L tetracycline</td>
<td>1.0 x 10^4</td>
</tr>
<tr>
<td>R46</td>
<td>10mg/L tetracycline</td>
<td>2.1 x 10^5</td>
</tr>
</tbody>
</table>

Plasmid pUK2001 transferred at the highest frequency (4.19 x 10^2), which was substantially greater than that of the standard plasmids RP4 and R46 (Table 5.2). Plasmid pUK2002 also transferred at a frequency faster than the standard plasmids (Table 5.2). The similar transfer frequencies observed when pUK2002 or pUK2003 were selected on tetracycline, streptomycin or these antibiotics in combination with apramycin, indicate that tetracycline and streptomycin resistance determinants are also carried by these two plasmids. This was supported by measuring the MICs of the donor strains, recipient J53, and transconjugants.

Table 5.3 shows the MICs of the recipient strain J53, the original donor strains (WF006555Y17 to WF007225Y01), and the transconjugants. The plasmid harboured by each of the donors is listed in the second column.
Table 5.3 MICs of wild type apr\textsuperscript{R} E. coli donor isolates and the transconjugants formed after the conjugation of the apr\textsuperscript{R} plasmids into J53.

<table>
<thead>
<tr>
<th>Isolate / strain</th>
<th>Plasmid</th>
<th>APR</th>
<th>TOB</th>
<th>GNT</th>
<th>STP</th>
<th>AMP</th>
<th>TET</th>
<th>SUL</th>
</tr>
</thead>
<tbody>
<tr>
<td>J53</td>
<td>pUK2001</td>
<td>2</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>WF006555 Y17</td>
<td>pUK2001</td>
<td>&gt;128</td>
<td>64</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>WF006562 Y14</td>
<td>pUK2001</td>
<td>&gt;128</td>
<td>32</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>WF006716 Y17</td>
<td>pUK2001</td>
<td>&gt;128</td>
<td>32</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>WF007204 Y15</td>
<td>pUK2002</td>
<td>&gt;128</td>
<td>16</td>
<td>8</td>
<td>32</td>
<td>4</td>
<td>&gt;128</td>
<td>8</td>
</tr>
<tr>
<td>WF007225 Y01</td>
<td>pUK2003</td>
<td>&gt;128</td>
<td>16</td>
<td>8</td>
<td>32</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>pUK2001 in J53</td>
<td>pUK2001</td>
<td>&gt;128</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>pUK2002 in J53</td>
<td>pUK2002</td>
<td>&gt;128</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>&gt;128</td>
<td>8</td>
</tr>
<tr>
<td>pUK2003 in J53</td>
<td>pUK2003</td>
<td>&gt;128</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>&gt;128</td>
<td>8</td>
</tr>
</tbody>
</table>

APR, apramycin; TOB, tobramycin; GNT, gentamicin; STP, streptomycin; AMP, ampicillin; TET, tetracycline; SUL, sulphamethoxazole.

In addition to apramycin resistance (MIC >128 mg/L) all three plasmids conferred resistance to tobramycin and gentamicin (MICs of 16 and 8 mg/L respectively). Plasmids pUK2002 and pUK2003 also conferred resistance to streptomycin (MIC of 16 mg/L) and tetracycline (MIC >128 mg/L).

The ability of other commensal E. coli from the unselected population to accept plasmid pUK2001 was studied in 28 different genotypes. Of these, 14 were able to receive pUK2001. The frequencies at which this plasmid was transferred to these 14 isolates are shown in Figure 5.3.
Figure 5.3 Mean transfer frequencies of pUK2001 into 14 different recipients from the unselected E. coli commensal population. Error bars show ± 2x the standard deviation.

The transfer frequencies of pUK2001 into different commensal E. coli from the unselected population ranged from 2.03 x 10³ to 3.43 x 10⁸ (Figure 5.3). Fourteen strains were unable to accept the plasmid. Of the 14 strains able to accept pUK2001 \textit{in vitro}, seven were isolated from samples that also contained genotypes carrying pUK2001. Two genotypes (6947-1 and 6947-3) were isolated from a sample from which genotypes presumed to carry pUK2001 were present. This presumption is based on the fact that the presumptive apr\textsuperscript{R} carriers had identical PFGE patterns to isolates from which pUK2001 was actually extracted, although a plasmid extraction was not carried out on an apr\textsuperscript{R} isolate from this sample.

5.3d Amplification of the aac(3)\textit{IV} gene

PCR with primers based on the published \textit{aac(3)IV} sequence (Bräu et al., 1984) yielded a product of the expected size (836bp). A comparison of the sequenced
products with the published \textit{aac(3)IV} gene revealed that all three plasmids harboured this gene. The sequence of each product was identical to that of the published nucleotide sequence.

5.4 Discussion

Although the detection of \textit{aprR} bacteria isolated from food-producing animals and humans has been reported previously (Chaslus-Dancla \textit{et al.}, 1989; Hunter \textit{et al.}, 1992; 1993; 1994; Johnson \textit{et al.}, 1994; 1995) these reports have concentrated on organisms that are pathogenic either to humans or animals. Little is known about the epidemiology of resistant commensal bacteria, which constitute a huge reservoir of resistance genes (Schwarz \textit{et al.}, 2001). In this study, PFGE and detailed molecular typing of the resistance mechanism were combined to give a better understanding of the epidemiology of apramycin resistance.

A cohort of calves that had not been treated with any aminoglycosides was selected for this work, to avoid any direct selection of \textit{aprR} \textit{E. coli} during the study. During the four months sampling period, six of the 11 calves sampled were found to carry \textit{aprR} \textit{E. coli} on one or more sampling dates. The presence of \textit{aprR} \textit{E. coli} was found to be unrelated to calf age or sampling date. This is in contrast to the detection of ampicillin resistant \textit{E. coli}, which are significantly more prevalent in new-born and young calves (Hoyle \textit{et al.}, 2004). Similarly, other workers report a higher incidence of antibiotic resistance amongst bacteria isolated from calves than from older animals (Hinton \textit{et al.}, 1985; Linton, 1977a).

The farm, from which the isolates in this study came, had not used apramycin since at least July 2000 (the date from which records of drug usage on the farm were kept). During this time only one calf (not sampled in this study) was treated with Penstrep (see Chapter 2), a combination drug containing penicillin and streptomycin. Although streptomycin resistance is conferred by a different resistance mechanism (enzymatic phosphorylation or adenylation of the antibiotic) to that conferring
apramycin resistance (antibiotic acetylation) (Shaw et al., 1993), plasmids pUK2002 and pUK2003 also carry streptomycin resistance, which may indicate cross-contamination of either strains or antibiotics between calves.

The presence of apr\textsuperscript{R} plasmids in the calves in this study demonstrates the persistence of apramycin resistance without recent selection by apramycin. A similar result has been reported in E. coli from disease outbreaks in pigs (Hunter et al., 1994). Antibiotic usage in cattle is dramatically less than the quantities used in pigs (Veterinary Medicines Directorate, 2001), and so the detection of APR resistance in the commensal flora of calves is of significance.

A total of 45 apr\textsuperscript{R} E. coli were obtained from the six calves. The genotypic diversity amongst these isolates was examined using PFGE, which has previously only been applied to pathogenic apr\textsuperscript{R} organisms. Amongst the 45 isolates, five distinct genotypes were found. Because APR resistance is commonly found to be mediated by transferable plasmids (Hunter et al., 1992; 1994; Johnson et al., 1994; 1995) (although the putative presence of the \textit{aac(3)}\textsubscript{IV} gene on the chromosome has been reported by Johnson et al., 1994) genotypic diversity amongst apr\textsuperscript{R} E. coli was expected to be high (compared to a non plasmid-mediated resistance mechanism such as quinolone resistance). Johnson et al. (1994) reported different serogroups amongst clinical isolates of apr\textsuperscript{R} E. coli, which is consistent with our findings of genotypic diversity within the apr\textsuperscript{R} E. coli population. Similarly, aminoglycoside resistance amongst different salmonella serotypes (Wray et al., 1986) and genotypically diverse enterococci isolates has been described (Donabedian et al., 2003b; Papaparaskevas et al., 2000). Further PFGE studies on a greater number of isolates would be required to determine the extent to which apramycin resistance plasmids have spread to different E. coli genotypes.

The most prevalent apramycin resistant genotype (type A) was detected on five of the six dates where apr\textsuperscript{R} E. coli were recovered. This genotype was also resistant to tetracycline and sulphonmethoxazole in addition to the aminoglycosides tested. The high prevalence of this strain in calves that have never been treated with tetracycline,
sulphamethoxazole or aminoglycosides shows the signature of antibiotics used previously or the cross-contamination of resistant strains within the farm.

The sampling dates where more genotypic diversity was detected were those in which apr\textsuperscript{R} \textit{E. coli} were recovered from different calves. This would suggest that the intestinal flora of each calf differs in the prevalence and range of genotypes present. This hypothesis is supported by Howe et al. (1976a; 1976b), where a total of 103 different O-antigen serotypes of commensal \textit{E. coli} were found colonising the intestines of calves. Different selective forces may be exerted on each ecosystem, such as the use of antibiotics, disinfectants, initial colonisation of the gut by different micro-organisms, and the production of bacteriocins by other flora colonising the intestine. Any apr\textsuperscript{R} \textit{E. coli} present will be subject to these selective pressures and are therefore likely to differ between calves. The study of the predominate genotypes in cattle, and any associations between genotype and animal, would require the genotyping of apr\textsuperscript{R} \textit{E. coli} from a larger cohort of animals. Work is currently underway to address this issue.

Restriction analysis of the apramycin resistance plasmids from the five apr\textsuperscript{R} genotypes demonstrated that the gene conferring resistance was carried on diverse plasmids. Similar findings have been reported in pathogenic bacteria, based on incompatibility groups (Wray et al., 1986) and restriction endonuclease fragmentation pattern (Platt & Smith, 1991; Pohl et al., 1993). Sequencing analysis of the \textit{aac(3)\textsubscript{IV}} gene revealed identical genes in all apr\textsuperscript{R} plasmids. This degree of plasmid diversity and gene homology suggests that the \textit{aac(3)\textsubscript{IV}} gene is carried on a transposable element. Previously, the \textit{aac(3)\textsubscript{IV}} gene has been found to be associated with insertion sequences (Bräu et al., 1984; Salauze et al., 1990) which is compatible with this hypothesis. This would greatly increase the potential for dissemination of the gene to different replicons.

The apr\textsuperscript{R} plasmids were all conjugative. The frequency at which the transfer of apr\textsuperscript{R} plasmids occurs is rarely measured, but one study has measured transfer frequencies of apr\textsuperscript{R} plasmids isolated from \textit{E. coli} and salmonellae isolated from disease
outbreaks of cattle, pigs, sheep and poultry (Wray et al., 1986). Two Apr\textsuperscript{R} E. coli isolates from diseased cattle were found to transfer apramycin resistance at frequencies of $<10^{-8}$ and $<10^{-10}$ transconjugants per donor overnight. Higher transfer frequencies were detected in three apr\textsuperscript{R} E. coli isolated from pigs, where frequencies of $10^{-3}$ and $10^{-2}$ per donor were detected when the plasmids were transferred to secondary recipients during a one hour mating (Wray et al., 1986). A seven-hour mating was required to give the similar transfer frequencies detected for plasmids pUK2001 and pUK2002 in this work. In the following chapter, mating times of one hour were studied, which are directly comparable with those described by Wray et al. (1986). In addition to these high transfer frequencies, the use of PFGE to genotype each isolate allowed the detection of one plasmid (pUK2001) in three different genotypes. To the best of my knowledge, this is the first report of horizontal spread of apramycin resistance in commensal organisms.

The ability of wild type isolates to accept apr\textsuperscript{R} plasmids has not been studied previously, and there are very few publications in which the recipient ability of wild-type strains is assessed. Of 28 different genotypes of commensal E. coli from the unselected population, 14 were able to accept pUK2001. Of interest is the fact that nine of these recipients were isolated from samples in which isolates containing (or presumed to contain for two isolates) pUK2001 were present. The donor and recipient genotypes are likely to be at similar cell densities within the calves because they were isolated from the same diluted sample. These results indicate that there are barriers to in vivo conjugation that may not be present in vitro. Resource limitation and conditions of stress have been found to decrease the rate of plasmid transfer (Arana et al., 1997; Curtiss et al., 1969; Muela et al., 1994), and the use of rich media has been shown to increase the probability of mating pair formation compared to minimal media (Curtiss et al., 1969). Some kind of selective pressure may be required in order for the donor bacterium to expend energy and resources in transferring its plasmid. This may have been the use of apramycin many years ago, or resistance to disinfectants or feed supplements, resulting in the spread of pUK2001 into three different genotypes. The calf may have acquired other genotypes capable of accepting the plasmid at a later date, but without any selective pressure
the plasmid may just remain in these three genotypes only. Alternatively, the trigger for transfer may be related to growth phase, and other workers have shown that donor ability is greatest during exponential phase growth (Curtiss et al., 1969). The dynamics of plasmid transfer are discussed in more detail in Chapter 6.

The selective pressures maintaining these large conjugative resistance plasmids in the commensal flora are not clear. The calves sampled were not treated with aminoglycosides or tetracyclines. The presence of pUK2002 and pUK2003 suggests that the spread of plasmids or resistant strains resulted from previous tetracycline or streptomycin use. pUK2001 only carried apramycin resistance. The selective force driving the spread of this plasmid may be previous farm usage of apramycin, or the employment of disinfectants or feed supplements on the farm. Sequencing of the plasmids, which is currently underway, may reveal the presence of such resistance determinants. Unfortunately, it was not possible to determine when in the evolutionary history of the strains acquisition of pUK2001 occurred.

The presence and spread of apr\textsuperscript{R} plasmids in commensal \textit{E. coli}, under unknown selective pressures, poses important implications for the transmission of this resistance determinant into clinical bacteria.
Chapter 6. The study of plasmid dynamics

6.1 Introduction

In the previous chapter, the transfer frequencies of each apramycin resistance plasmid found in the calf faecal commensal E. coli population were defined as the number of transconjugants divided by the number of donors at the start of mating (Provence & Curtiss, 1994). The initial density of the donors and recipients, and the time permitted for mating, were kept constant to allow an accurate comparison of transfer frequencies between the aprR plasmids, and with the well-characterised plasmids RP4 and R46.

In this chapter, a second method of quantifying plasmid transfers originally described by Simonsen et al. (1990), was used. The method, designated the end-point method, is described by the authors as one which “overcomes the shortcomings of previous techniques because it is not affected by cell density, donor: recipient ratio and mating time”.

There are three reasons for calculating the plasmid transfer frequencies by two different methods. Firstly, this allows a comparison of the methods to determine the advantages and disadvantages of each. Secondly, different researchers have used a variety of methods to calculate plasmid transfer frequencies, and so the use of two methods allows a comparison of transfer frequencies with a greater number of published results than if only one method was used. Lastly, the results from the end-point calculation may be incorporated into a mathematical model, originally presented by Levin et al. (1979). The model describes bacterial cell growth and plasmid transfer in batch culture, and may be used to explore the dynamics of plasmid transfer by altering input variables and observing their effects.
This chapter aims to address the following questions:

1. In what manner do plasmid transfer frequency estimates vary as a function of mating time?
2. What are the advantages and disadvantages of each technique?
3. How do the transfer frequencies/rates measured by each of the methods compare?
4. Does the published model of bacterial cell growth and plasmid transfer fit the experimental data of pUK2002 transfer in batch culture?
5. If the model does not fit, what biological processes are occurring which are not taken into account by the model?
6. Can the model be used to address questions of plasmid persistence in natural populations?

6.2 Materials and Methods

6.2a Bacterial strains and plasmids

The plasmids used in this comparative study were the apr\textsuperscript{R} plasmids identified in Chapter 5 (pUK2001, pUK2002 and pUK2003). The plasmid pUK2002 was chosen for the more detailed study of variation within and between the two methods of calculating plasmid transfer frequencies. This plasmid was chosen because it had an intermediate transfer frequency in relation to the other apr\textsuperscript{R} plasmids, and the transfer frequency measured in Chapter 5 indicated that this plasmid was unlikely to be derepressed for transfer. To determine plasmid transfer rates by the end-point method, plasmids were maintained in \textit{E. coli} K12 strain J53 (\textit{pro}^-' \textit{met}') (Bachmann, 1972). The \textit{E. coli} K12 strains J62-2 (\textit{lac}-, \textit{pro}^-' \textit{trp} ' \textit{hisf}') (Bachmann, 1972) and MG1655\textit{kan} (\Delta \textit{lac}, \textit{Km}\textsuperscript{R}) were used as recipients.
6.2b Conjugations

To explore the effect of mating time on the estimates of plasmid transfer frequency, transfer frequencies were determined as described in Chapter 5, but over mating times of 1, 7, 10 and 16 hours. To determine rates of plasmid transfer as estimated by the end-point method, matings were carried out in 50 ml Erlenmeyer flasks containing 10 ml LB (Lennox L broth) (Invitrogen). Overnight cultures of donor and recipient were diluted 1:10 in saline and used to inoculate the flasks, to give an initial density of approximately $1 \times 10^6$ cfu/ml and a 1:1 ratio of donors to recipients. The cultures were incubated at 37°C with shaking at 150 rpm, and sampled at 30 min intervals after initial inoculation ($t = 0$) for six hours. Donor, recipient and transconjugant cell densities were estimated by serial dilution in saline and selective plating. In matings between J53 and J62-2, donors were selected on Davis Mingioli (DM) plates supplemented with 50mg/L proline and 50mg/L methionine. Recipients were selected on DM plates with 50mg/L proline, 50mg/L tryptophan and 50mg/L histadine, and transconjugants selected on MacConkey plates containing 16mg/L apramycin and 20mg/L rifampicin. When plasmids were transferred between J53 and MG1655kan, donors and recipients were distinguished by colony colour on the same plate. Here S-gal plates (Sigma) were used, which contain a chromogenic β-galactosidase substrate. Cleavage of this substrate results in the black coloration of colonies. Recipient cells MG1655kan have a deletion of the lacZ gene, resulting in the presence of white colonies. Transconjugants were selected on S-gal plates containing 40mg/L apramycin and 32mg/L kanamycin. Growth rate estimates were based on the average of three replicates. The exponential phase population growth rate was assumed to be the average of the individual growth rates of the donor and recipient, again based on three replicate experiments.
6.2c Estimates of plasmid transfer frequencies and rates

Plasmid transfer rates were estimated as a function of the cell densities of donors \((D)\), recipients \((R)\) and transconjugants \((T)\), during exponential growth, in accordance with the following equation (Simonsen et al., 1990):

\[ \gamma = \psi \left( \frac{1}{N - N_0} \right) \cdot \ln \left( 1 + \frac{T \cdot N}{R \cdot D} \right) \]

Equation 1.

Where \(\psi\) is the average growth rate per hour of the two strains \((D \text{ and } R)\) involved in the mating during exponential growth, \(N\), the total cell density at the time of sampling, and \(N_0\), the total cell density at the start of mating.

Plasmid transfer frequencies were also determined as the number of transconjugants to donors at the start of mating, as described in chapter 5. Although this method is often quoted as the T/D ratio, mathematically this is not strictly a ratio because \(T\) and \(D\) are measured at different time points during the experiment (\(T\) is measured at the end of the mating and \(D\), at the start). To make clear this distinction, the transfer frequency as estimated by this method will be referred to as \(T/D_0\).

6.2d Model theory

The model used to explore this system was that presented by Levin et al. (1979) to describe cell growth and plasmid transfer in a batch mating culture. Here, changes in the densities of donors, recipients and transconjugants due to cell growth and plasmid transfer are considered with respect to time, according to the following equations:

\[ \frac{dD}{dt} = \psi(C) \cdot D \]

Equation 2.
Where \( D, R \) and \( T \) are the densities (cfu/ml) of the donors, recipients and transconjugants respectively. \( \psi(C) \) is the growth rate per hour, \( \psi \), at a given concentration, \( C \), of resource (mg/L), and \( e \) (\( \mu \)g) the amount of resource required to make a new cell. The exact concentration of resource in 10ml LB could not be calculated, so an estimated concentration of 500 \( \mu \)g/mL was used in the model, based on trial and error against experimental results. The plasmid transfer rate constant, \( \gamma \), (ml per cell h\(^{-1}\)) is calculated as described above. Growth rates and plasmid transfer rates are assumed to be Monod functions (Monod, 1949) of resource concentration \( C \). Growth and conjugation cease when resources are exhausted, according to the following equations:

\[
\frac{dR}{dt} = \psi(C) \cdot R - \gamma(C) \cdot R \cdot (D + T)
\]

Equation 3.

\[
\frac{dT}{dt} = \psi(C) \cdot T + \gamma(C) \cdot R \cdot (D + T)
\]

Equation 4.

\[
\frac{dC}{dt} = -\psi(C) \cdot (R + D + T) \cdot e
\]

Equation 5.

\[
\psi(C) = \psi_{\text{max}} \cdot \frac{C}{(Q+C)}
\]

Equation 6.

\[
\gamma(C) = \gamma_{\text{max}} \cdot \frac{C}{(Q+C)}
\]

Equation 7.

Here, \( \psi_{\text{max}} \) and \( \gamma_{\text{max}} \) are the maximum growth and plasmid transfer rates respectively, and \( Q \), the half saturation constant (mg L\(^{-1}\)).

There are four basic assumptions of the model, which are summarised as follows:
1. Mating occurs randomly with a frequency that is jointly proportional to the donor and recipient densities, and independent of bacterial growth rates.

2. Plasmids are not lost by segregation.

3. Transconjugants are immediately able to transfer the plasmid at the same rate as donor cells.

4. All clones grow at the same rate.

Model simulations were produced with Berkley Madonna™ differential equation solving package (www.berkleymadonna.com).

6.2e Growth curves

Growth curves were measured in 50 ml Erlenmeyer flasks containing 10 ml LB (Lennox L broth) (Invitrogen). Overnight cultures of plasmid-harbouring J53 (J53pUK2002) were diluted 1:10 in saline and used to inoculate the flasks, to give an initial density of approximately $1 \times 10^6$ cfu/ml. Cultures were incubated at 37°C with shaking at 150 rpm, and sampled at 30 min intervals since initial inoculation ($t = 0$) for 5.5 hours. Cell densities were estimated by serial dilution in saline and selective plating onto MacConkey plates supplemented with 16mg/L apramycin.

6.2f Statistics

To analyse the variation in plasmid transfer frequencies as a function of time and plasmid, analysis of variance and multiple linear regressions were performed. In all tests, the number of degrees of freedom is written as a subscript of the test statistic and a significant result was recorded if $P < 0.05$. Linear mixed effects models were used to test whether log-transformed transfer rates differed significantly with mating time. As the temporal data were non-independent, each replicate was entered as a random effect.
6.3 Results

6.3a Effect of mating time on estimates of plasmid transfer frequency

Mean transfer frequencies, determined as $T/D_0$, were calculated from replicate matings of 1, 7, 10 and 16 hours. Transfer frequencies of pUK2003 could not be determined within a one-hour mating period, as this plasmid required longer than one hour for its transfer. The transfer frequencies of each of the plasmids during the four different mating times are shown in Figure 6.1. Table 6.1 shows the mean transfer frequency and number of replicates used in the estimates.

![Graph showing transfer frequency vs mating time]

Figure 6.1 The effect of mating time on estimates of plasmid transfer frequency as determined by the number of transconjugants to donors at the start of mating.
Table 6.1 Mean transfer frequencies (T/D₀) and number of replicates used in estimates.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>1</th>
<th>7</th>
<th>10</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUK2001</td>
<td>1.14 x 10⁻⁵ (8)</td>
<td>4.19 x 10⁻⁵ (3)</td>
<td>1.84 x 10⁻² (2)</td>
<td>3.07 x 10⁻¹ (2)</td>
</tr>
<tr>
<td>pUK2002</td>
<td>9.46 x 10⁸ (35)</td>
<td>1.73 x 10⁻³ (5)</td>
<td>3.41 x 10⁻³ (6)</td>
<td>3.03 x 10⁻² (3)</td>
</tr>
<tr>
<td>pUK2003</td>
<td>ND</td>
<td>4.65 x 10⁶ (5)</td>
<td>4.84 x 10⁶ (5)</td>
<td>4.43 x 10⁶ (3)</td>
</tr>
</tbody>
</table>

As can be seen in Figure 6.1, the estimate of plasmid transfer frequency varies according to the time allowed for mating. Transfer frequencies of pUK2003 appeared least affected by mating time, whilst pUK2002 and pUK2001 showed general trends towards increased frequencies during longer mating times. The analysis of variance performed on this data confirmed these trends; a significant increase in transfer frequencies was present with increasing length of mating time (F₁,7₀ = 255.3, P < 0.001). The plasmid transfer frequencies were significantly different (F₂,₇₀ = 214.6, P < 0.001), and the relationship between time and transfer frequency was found to be plasmid dependant (F₂,₇₀ = 25.47, P < 0.001), with pUK2001 and pUK2002 transfer frequency estimates increasing with mating time (t₁₃ = 9.9, P < 0.001; and t₄₆ = 17.85, P < 0.001 respectively). pUK2003 transfer frequencies did not change significantly with mating time (t₁₁ = -0.23, P = 0.82) (Figure 6.1).

6.3b Transfer rates estimated by the end-point method

The end-point method has been described as being independent of mating time because transfer rates and growth rates during exponential growth are assumed to be related in accordance with equation 1 (Simonsen et al., 1990). To determine when during the course of the experiment, the exponential growth phase is reached, growth curves of the donor strain (J53) harbouring pUK2002 were plotted. The growth curves were determined in eight replicate experiments carried out in the same type and volume of broth, and under the same experimental conditions, as would be used
for determining plasmid transfer rates. The individual growth curves of the replicate experiments are shown in Figures 6.2, and the geometric mean growth curve of all replicates is shown in Figure 6.3.

**Figure 6.2** Eight replicate growth curves of J53 harbouring plasmid pUK2002 in 10ml LB.

**Figure 6.3.** Geometric mean growth curve of J53 harbouring pUK2002 10ml of LB.
Figure 6.2 shows that a lag phase occurs during approximately the first hour of incubation. This is followed by an approximately exponential growth phase spanning from one to approximately four hours, after which the growth rate of this strain declines. Figure 6.3 shows the geometric mean of these growth curves. In this graph, the start of the exponential phase is distinct, but the end of this phase is less well defined. To determine if plasmid transfer rates as measured by the end-point method were independent of time of sampling, transfer rates of pUK2002 were determined on the proportion of donors, recipients and transconjugants at each time point. For each transfer rate estimate, the growth rate specific to that time point was used. Here, average growth rates for each of the time points were calculated by performing linear regression of the slope between pairs of consecutive time points for each replicate growth curve, and taking the mean of these rates. Transfer rates at 0-0.5 and 0.5-1 hours were excluded from the analysis because although the mean growth rates of the eight replicate growth curves were very low, some replicates were not growing. Additionally, in two of four replicate mating experiments, the transconjugant population was below the detection threshold of the method (<100 cfu/mL).

The results of this analysis are presented in Table 6.2, where each transfer rate (with the exception of $t = 3.5$ and $t = 4$, where the number of replicates $\leq 2$) has been calculated from the geometric means of the cell densities ($D$, $R$ and $T$) of replicate experiments. Figure 6.4 shows the transfer rate estimates of all replicates. The numerical values at each time point are listed in Table 6.2.
Table 6.2 Mean transfer rates and confidence intervals for the transfer of pUK2002 from J53 into J62-2 measured after different mating times by the end-point method.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>No. of replicates</th>
<th>Transfer rate (mL per cell h⁻¹)</th>
<th>Mean transfer rate (mL per cell h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate 1</td>
<td>Replicate 2</td>
<td>Replicate 3</td>
</tr>
<tr>
<td>1.5</td>
<td>3</td>
<td>2.91 x 10⁻¹¹</td>
<td>3.23 x 10⁻¹¹</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1.32 x 10⁻¹⁰</td>
<td>5.36 x 10⁻¹¹</td>
</tr>
<tr>
<td>2.5</td>
<td>4</td>
<td>5.78 x 10⁻¹¹</td>
<td>2.32 x 10⁻¹¹</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1.51 x 10⁻¹¹</td>
<td>5.21 x 10⁻¹²</td>
</tr>
<tr>
<td>3.5</td>
<td>1</td>
<td>ND</td>
<td>2.10 x 10⁻¹²</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>ND</td>
<td>9.24 x 10⁻¹³</td>
</tr>
<tr>
<td>4.5</td>
<td>3</td>
<td>ND</td>
<td>1.71 x 10⁻¹³</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>ND</td>
<td>4.73 x 10⁻¹⁴</td>
</tr>
</tbody>
</table>

Figure 6.4 Four replicate experiments showing the effect of mating time on estimates of plasmid transfer rate of pUK2002 from J53 into J62-2, as measured by the end-point method.
As can be seen in Figure 6.4, estimates of transfer rate tend to be lower when calculated from cell densities of samples taken later in the mating time. The decrease in transfer rate with mating time was significant ($t_{19} = -5.08, P < 0.001$).

The transfer rate appeared to be fastest during the lag phase and slowest after the exponential growth phase when the growth rate is also decreasing. To ensure that the plasmid transfer rate estimates for the three apr\textsuperscript{R} plasmids were as comparable as possible, and would not be affected by variations in the length of lag phase, they were estimated from cell densities of samples taken at approximately the middle of the exponential growth phase. This will be a conservative estimate, as plasmid transfer rates are thought to be fastest during the lag phase (Simonsen et al., 1990). The plasmid transfer frequencies were then calculated from the cell densities of a sample removed after 2.5 hours of mating. Growth rates were calculated as the rate of growth during exponential phase (1-4 hours). These results are listed in Table 6.3.

Table 6.3 Mean plasmid transfer rates as measured by the end-point method, and mean transfer frequencies as measured by $T/D_0$.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid transfer rate (ml per cell h\textsuperscript{-1})</th>
<th>Plasmid transfer frequency ($T/D_0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUK2001</td>
<td>$2.83 \times 10^{-9}$</td>
<td>$4.19 \times 10^{-2}$</td>
</tr>
<tr>
<td>pUK2002</td>
<td>$3.74 \times 10^{-11}$</td>
<td>$1.73 \times 10^{-3}$</td>
</tr>
<tr>
<td>pUK2003</td>
<td>$1.15 \times 10^{-15}$</td>
<td>$4.65 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

The transfer rates listed in Table 6.3 demonstrate that plasmid pUK2001 has the fastest rate of transfer, and pUK2003 has the slowest. Qualitatively, these results correlate well with the transfer frequencies obtained by calculating $T/D_0$.

In Table 6.3, different $E.\ coli$ K12 recipient strains were used for the end point matings (MG1655kan) compared to the $T/D_0$ matings (J62-2). This was to minimise pipetting errors when determining D and R densities with the end-point method. To determine whether the choice of recipient strain used in the mating experiment affected the plasmid transfer frequency obtained, pUK2002 was mated with
MG1655kan and with J62-2, and the transfer rates compared. Conjugations were repeated four times with each recipient. Cell densities were estimated from samples removed after 2.5 hours of mating. The growth rates used in each calculation were the average growth rates of each mating pair during exponential growth (1-4 hours). The results of this analysis are shown in Table 6.4.

### Table 6.4 Mean transfer rates of pUK2002 into J62-2 and MG1655kan.

<table>
<thead>
<tr>
<th>Mating pair</th>
<th>Average transfer rate (ml per cell h⁻¹)</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>J53 + pUK2002 into J62-2</td>
<td>3.74 x 10⁻¹¹</td>
<td>7.62 x 10⁻¹²</td>
<td>6.72 x 10⁻¹¹</td>
</tr>
<tr>
<td>J53 + pUK2002 into MG1655</td>
<td>3.53 x 10⁻¹¹</td>
<td>9.18 x 10⁻¹²</td>
<td>6.14 x 10⁻¹¹</td>
</tr>
</tbody>
</table>

The mean transfer frequencies of pUK2002 into J62-2 and MG1655kan were very similar, and the upper and lower 95% confidence intervals indicate similar variation with the two recipients. A standard two sample t-test revealed that the two transfer systems (into J62-2 or into MG1655kan) did not give significantly different estimates of transfer frequency (t₅ = 2.57, P = 0.87).

### 6.3c Variation in transfer frequency/rate with different methodology

To explore the variation over a range of mating times, when the plasmid transfer frequency or rate was measured by each of the two different methods, the results of the mating experiment described in Figure 6.4 were plotted with the T/D₀ frequencies of the same data. The data for each of the four replicates is shown in Figure 6.5.
Figure 6.5 A comparison of four replicate experiments to study plasmid transfer. EP1-4; transfer rates measured by the end-point method (mL per cell h⁻¹), T/D 1-4; transfer frequencies measured as the density of transconjugants over donors at the start of mating (T/D₀).

Figure 6.5 shows that neither measure of plasmid transfer is independent of mating time. The frequency of transfer as measured by T/D₀ increases with mating time, whereas the transfer rate, measured by the end-point method, decreases as mating time increases. A significant decrease in transfer rate with mating time (t₁₉ = -5.08, P < 0.001) was described earlier for the end-point method (Figure 6.4). This analysis was repeated for the T/D₀ data, and revealed a significant increase in
transfer frequency with mating time ($t_{33} = 4.82, P < 0.001$). The replicate points of the end-point method appear more closely clustered than those of the T/D0 measure (Figure 5.5), indicating less variation within this method.

6.3d Modelling plasmid transfer in batch culture

A model describing bacterial cell growth and plasmid transfer in batch cultures (see section 6.2d in materials and methods) was used to describe mathematically the experimental results obtained during the calculations of plasmid transfer frequencies by the end-point method, and to predict the effect of altering various parameters where this could not be achieved practically. The plasmid transfer results selected for this section of work were those of plasmid pUK2002. To ensure the model accurately describes the experimental data, the cell densities, plasmid transfer rate, and average growth rates from the mean results of four replicate pUK2002 conjugation experiments were incorporated into the model. The model was then run using Berkley-Madonna™ software and the output compared to the experimental results.

When the model was run using the maximal cell growth rates for each population it did not represent the experimental data. The T, D and R populations were overestimated throughout the five hours mating time (results not shown). The model predicted resource limitation at 2.5 hours, but this was not observed in the experimental data. The results shown in Figure 6.2 demonstrate that growth rates vary during the experiment, and so the model was repeated with the average growth rates over the five-hour experiment for each population. This gave a better representation of the experimental data than when the maximal growth rates were used, but the transconjugant population was still greatly overestimated by the model (results not shown). Table 6.2 and Figure 6.4 indicate that the plasmid transfer rate varies during different growth phases. It was hypothesised that the model would fit the real data better if the average transfer rate during the five-hour experiment was used. This hypothesis was tested and the comparison of real and model data is presented in Figure 6.6.
Figure 6.6 Population dynamics during pUK2002 transfer from J53 to J62-2, using the average plasmid transfer frequency over each of the time points.

Figure 6.6 shows that the model-generated data follows the general trend of each of the populations, but it does not capture the sigmoid shape of the D and R experimental curves. Although the slope of the modelled T population is similar to that of the real data, the main discrepancy between the model and real data is that it overestimates the T population throughout the five hours. In an attempt to enhance the fit of the model to the real data, two further models were run in which cell densities, growth rates, and plasmid transfer rates from the lag phase and secondly from the post-exponential phase were used. The use of data from these specific growth phases only, resulted in very inaccurate modelling of all populations, even during the particular growth phases from which the data came (results not shown).
The model shown in Figure 6.6 was also repeated with a range of transconjugant growth rates (vT) from 0 to 2.5, to determine whether the overestimation of T density was because of transconjugant growth. The numerical solutions and the resulting graph for all these vT values were almost identical to those for Figure 6.6 (results not shown), indicating that the transconjugant population plotted in Figure 6.6 is a direct result of conjugation events and that transconjugant growth has an undetectable effect on population density.

A sensitivity analysis was performed to determine how each parameter influences the output of the model. The graphs of this analysis may be found in appendix 1. The key findings of this analysis are as follows:

1. Reducing the donor or recipient growth rates (vD and vR respectively) enhanced the fit of the model to the T population, but resulted in very inaccurate modelling of the D and R populations.

2. When vD > vR, donors constituted a greater proportion of the total population at resource limitation. Conversely, when vR > vD, recipients occupied a greater proportion of the broth than D. The reduction in D as a function of increasing vR, was less than the reduction in R due to increasing vD. This is likely to be because D only acts in competition with the other strains present, whereas R is affected by conversion to T, in addition to competition with both D and T.

3. Lowering the initial densities of D or R to $1 \times 10^5$ lowered the T curve to fit the experimental T data much better, but this also caused a lowering of the D and R curves, which was a very poor representation of the experimental data for these populations.

4. There was a trade off between an increase in resource concentration increasing the fit of the D and R populations, but resulting in a reduced fit to the T population.
5. The parameter changes that did enhance the fit of the model to the experimental data were the introduction of a lag in conjugation, increased resources, a lag in growth of all D and R, and a reduction in plasmid transfer frequency. A more detailed discussion of these parameter changes now follows.

A lag in plasmid transfer was introduced by offsetting the model-generated results of the transconjugant population from the corresponding time points by 30, 60 and 90 minutes. The results of this analysis are shown in Figure 6.7.

![Figure 6.7](Image)

**Figure 6.7** The effect of a lag in conjugation on the dynamics of pUK2002 transfer between *E. coli* J53 and J62-2.
The model more accurately represents the experimental data when a 30 minute (0.5 hours) lag in transfer is introduced (Figure 6.7), but after 3 hours the increase in T density is less than is predicted by the model. A lag in growth of approximately one hour also needs to be introduced before D and R growth begins. This is illustrated in Figure 6.8.

**Figure 6.8** The effect of 0.5, 1 and 1.5hrs lag in growth on the D and R populations.
To compensate for the one hour lag before cell growth, the growth rates of the D and R populations needs to be increased. A growth rate of $2.4h^{-1}$ was found to be most suitable.

Next, the effect of varying transfer rate ($\gamma$) was explored. This analysis is shown in Figure 6.9, where for simplicity, only the effects on the R and T populations are shown. The D population is only affected by direct competition between the other strains and so is expected to grow according to its growth rate until resource limitation is reached.

Figure 6.9 The effect of plasmid transfer rate on the R and T populations.
Figure 6.9 shows that a plasmid transfer rate of between $1 \times 10^{-10}$ and $1 \times 10^{-15}$ does not affect the R population (shown as just a turquoise dashed line; the other colours cannot be seen, as they are directly under this line). At $\gamma \geq 1 \times 10^{-9}$, the R population is reduced, and dies out when $\gamma \geq 1 \times 10^{-7}$ because all the R’s are converted into T’s during the course of the experiment. From this figure it appears that the best plasmid transfer rates to fit the experimental data is between $1 \times 10^{-12}$ mL per cell h$^{-1}$ and $1 \times 10^{-11}$ mL per cell h$^{-1}$.

Different combinations of the three parameters found to enhance the fit of the model (lag in transfer, lag in D and R growth, and increased resource concentration) were studied in combination. The results of this analysis may be found in Appendix 2, in graphs 1 to 27.

Figure 6.10 shows the results of the best fit of the model (selected by eye), which includes a 30min lag before plasmid transfer, a 1.5 hour lag before D and R cell growth, D and R growth rates of 2.4h$^{-1}$, a plasmid transfer rate of $1 \times 10^{-11}$ mL per cell h$^{-1}$ and a resource concentration of 500μg/mL. Here it is important to note that the concentrations of resource input into the model are arbitrary. LB contains both tryptone peptide and yeast extract, both of which may be used as a C source. This meant that it was not possible to calculate exactly how much of each different carbon source was initially present and subsequently used up during the experiment.
Figure 6.10 Best fit of the model to the experimental data, using measured plasmid transfer rate ($1.98 \times 10^{-11}$ mL per cell h$^{-1}$).

In Figure 6.10, the density of transconjugants throughout the experiment are overestimated. With a resource concentration of $500\mu$g/mL, the culture reaches resource limitation at 4 hours. Experimentally, resource limitation is not reached until at least 5 hours. However, increasing resources further also affects the T population (see Figure A1 in appendix 1) by increasing the number of transconjugant cells formed. To reduce this, a lower plasmid transfer rate may be used, but this is not logical because the transfer rate is measured during the experiment based on the densities and growth rates of the cells involved. Therefore, either the measurement of transfer rates with the end-point method is not accurate, or the use of the transfer rate in the model requires a further variable to reduce the transfer rate with increasing
mating time. Biologically, the reason for this is likely to be because of a reduction in donor ability during exponential phase growth compared to lag phase.

The final section of this chapter intended to explore which parameters are influential in allowing persistence of the plasmid in a population. The use of an accurate model is critical for this analysis, as it allows the alteration of different parameters in a thorough and stepwise manner, which is not possible experimentally. Although only a crude exploration of parameter space was completed due to time limitations, it is clear from the results above (Figures 6.6 to 6.10) that the model is not able to capture all of the behaviour of the experimental data. The model needs to allow the input of transfer rates that change throughout the experiment, depending on the growth phase of the donor and recipient strains. The application of a mathematical model to the phenomenon of conjugative plasmid transfer has however highlighted processes and dynamics of apr$^R$ plasmid transfer that would not have been observed by simply measuring transfer frequencies or rates.

6.4 Discussion

This chapter is concerned with gaining a better understanding of the biology of the apr$^R$ plasmids described in chapter 5. Two different methods of determining plasmid transfer frequencies were examined to allow a comparison of these two published methods, in addition to a comparison of plasmid transfer frequencies with other worker's results. The use of the end-point method enabled experimental data to be compared with data generated using a mathematical model describing cell growth and plasmid transfer in batch culture. Testing the model against experimental data highlighted limitations of the model and identified biological processes likely to be involved in plasmid transfer. The discussion of the results described earlier will be organised according to the original aims set out in the introduction.
In what manner do plasmid transfer frequency estimates vary as a function of mating time?

When plasmid transfer frequencies were measured as $T/D_0$ (where $T = \text{cfu/ml of transconjugants}$, and $D_0 = \text{cfu/ml of donors at the start of mating}$), overall there was a significant increase in transfer frequency estimate with increasing mating time. This is because transconjugants are formed by plasmid transfer to a recipient (horizontal spread) as well as by clonal replication (vertical spread) of a transconjugant cell. The number of transconjugants formed by both modes of plasmid transmission will increase as the mating time is increased. The longer the mating time the greater the effect of transconjugant growth on the number $T/D_0$, because of the logarithmic nature of bacterial cell growth.

In contrast, the transfer rates measured by the end point method decreased with mating time. The fastest transfer rates occurred during the early exponential growth phase, and a significant decrease in the rate of plasmid transfer occurred during 2.5 to 5 hours of mating. Similarly, Smets et al. (1993) found that the highest rates of plasmid transfer were observed when donor cultures were in the exponential growth phase. The decrease in plasmid transfer between 2.5 and 5 hours is because with increasing mating times $\ln(1+T/R \cdot N/D)$ increases, but at a slower rate than the decrease in $1/(N-N_0)$. This indicates that in order to compare results between different labs, the same growth phase should be used in the estimate of transfer rate. This result is in contrast to the results of Simonsen et al. (1990) where constant transfer rates were found throughout the exponential, post-exponential and stationary phases of growth.

The correlation of maximum transfer rates with early exponential growth phase contrasts strongly with the results of other workers, where minimum transfer frequencies (measured as the frequency of $T/D_0$) were measured during the donor exponential growth phase (Muela et al., 1994).
6.4b What are the advantages and disadvantages of each technique?

The measure T/D₀ does not take into account the growth of the transconjugant population. This, as outlined above, may lead to inaccurate transfer frequency estimates with longer mating times. In the determination of transfer frequencies of the three apr⁸ plasmids identified in chapter 5, a mating time of one hour would have been less affected by transconjugant growth than the seven hours used. However, the minimum mating time that could be used was seven hours, as pUK2003 transfer could only be detected after this time.

Additionally, transfer frequencies obtained by T/D₀ are likely to vary if different donor: recipient ratios are used. A lower initial density of donors to recipients is likely to have more of an effect than a reduction in the initial density of recipients. This is because the expression of genes required for the transfer of most conjugative plasmids occurs in only a small proportion of the cells that harbour them (Hardy, 1984), and so the rate-limiting step would be donor ability. In Enterobacteriaceae, the conjugative ability of F-like R plasmids is only expressed in approximately 0.1% of cells containing the plasmid (Hardy, 1984). Walmsley (1976) describes the limiting step in the formation of mating pairs under normal physiological conditions, as the production of F pili by donor cells. Considering both these observations, one would expect that a reduction in donor numbers would be likely to reduce transfer frequencies more dramatically than a reduction in the number of recipients. To test whether this hypothesis is true for the strains, plasmids and media used in these conjugation experiments would require repeat mating experiments with a variety of donor: recipient ratios.

Fernandez-Astorga et al. (1992) report constant transfer frequencies in tryptone soya broth, as measured by T/D₀, when the density of recipient cells is higher than that of donor cells. With ratios of 30:1 (D: R) or higher donor densities, a significant decrease in transfer frequency was detected. A ratio of 1:10 (D: R) was used in the measurements of transfer frequencies by T/D₀ in this and the previous chapter,
therefore any variation in inocula (introduced by pipetting errors) were expected to have a minimal effect of the transfer frequency obtained.

In summary, this method of calculating plasmid transfer frequencies allowed an accurate comparison between the three aprR plasmids and the two reference plasmids (RP4 and R46). In addition, a comparison of the transfer frequencies obtained following the detection of transconjugants on different selective plates, enabled inferences to be made as to whether other resistance genes were present on the same plasmid or different but mobilisable plasmids. The main disadvantage of the T/D0 measure is that comparisons with other researchers are not possible if slightly different methodologies have been used (for example, initial donor and recipient inoculums and ratios, different mating times and different culture conditions).

The rate of plasmid transfer was next measured by the end-point method. According to Simonsen et al. (1990), plasmid transfer and cell growth are thought to remain proportional to each other and to vary little during exponential growth. The period during which exponential growth occurred for the cells and culture conditions used was determined by plotting growth curves. This is an important first step, as the different phases of bacterial cell growth vary in both their presence and duration (Monod, 1949), depending on the media, strain, amount of aeration, and starting temperature.

Transfer rates could not be determined until after one hour of mating, because some replicates were not growing at all, and two of the four replicates did not yield transconjugants at that time. To give an average transfer rate of these replicates would not be an accurate representation of the data. At this early stage in the experiment, the formation of some transconjugants were expected, but their numbers may have been below the detection threshold of the method (<100 cfu/mL), giving the appearance of an absence of plasmid transfer. According to Simonsen et al. (1990), plasmid transfer rates are at their maximum during the lag phase, so we would expect the highest rates to occur during the first hour of mating. In agreement
with this statement, Muela et al. (1994) demonstrate high transfer frequencies during donor cell lag phase compared to the exponential growth phase.

To sum up, the end-point method is more complicated and labour intensive than the T/D₀ measure, but is less affected by mating time, and demonstrated less variation in replicate experiments. A disadvantage of the end-point method is that the calculation of transfer rates during lag phase may be inaccurate or impossible because of very low or negative growth rates.

6.4c How do the transfer frequencies/rates measured by each of the methods compare?

Two different recipients, J62-2 and MG1655kan were used in the measurement of transfer frequencies/rates by the two methods. The use of a second recipient in the end-point method was to minimise pipetting errors introduced by plating samples onto different agar plates. Before the transfer frequencies/rates could be compared, it was necessary to ensure that the use of different recipients would not affect the results.

The ability of different E. coli strains to receive plasmids is likely to vary if the recipient strain has a different restriction modification system to that of the donor cell (Murray, 2002). Gordon (1992) found the variation in transfer rate between E. coli isolated from a variety of natural populations to be mainly due to recipient effects. Both the genetic relatedness of the two strains involved, and the native plasmid burden of the isolate influenced its ability to receive plasmid R1 (Gordon, 1992). Mutations affecting the inner core of lipopolysaccharide (LPS) and the porin OmpA have also been found to result in poor recipient ability in broth matings, and are thought to reduce mating pair stabilisation (Klimke & Frost, 1998).

It was expected that MG1655kan would have a very similar if not identical ability to receive plasmids from J53. The main reason for this hypothesis is that it is also a K12
derivative strain, and so is genetically related to both J53 and J62-2. Additionally, MG1655kan does not contain any other plasmids. Transfer rates obtained with the same plasmid and each recipient were not significantly different, and so the frequencies of T/D₀ and the plasmid transfer rates measured by the end-point method could be compared.

Qualitatively, the transfer rates of plasmids pUK2001, pUK2002 and pUK2003 estimated by the end-point method, were found to correlate well with those obtained by measuring T/D₀. Plasmid pUK2001 transferred at the fastest rate (2.83 x 10⁻⁹ ml per cell h⁻¹). Plasmids pUK2002 and pUK2003 transferred at slower rates (3.74 x 10⁻¹¹ and 1.15 x 10⁻¹⁵ ml per cell h⁻¹ respectively). A study of the transfer rates of R1 between different E. coli strains from natural populations revealed that the fastest rate of transfer for this plasmid was 5.2 x 10⁻¹¹ ml per cell h⁻¹ (Gordon, 1992). Transfer rates varied according to the donor and recipient strains used, and averaged at 1.3 x 10⁻¹⁵ ml per cell h⁻¹. This rate is several log-fold slower than the rate of pUK2001 transfer.

However, the rates estimated in this chapter are likely to be faster than those obtained by mating experiments with the natural host strain. This is because the E. coli K12 strains used in this work are well-adapted to culture conditions in the laboratory, and perhaps more importantly, they are isogenic and so there is likely to be no restriction on the transfer of plasmids between them (Murray, 2002). In support of this hypothesis, Gordon (1992) found that the transfer of R1 into the lab strain CSH50 was on average 100 times faster than when wild-type strains served as both donors and recipients.

The transfer rates and frequencies obtained in this and the previous chapter are likely to be the maximum possible for each plasmid. Nutrient rich media was used, the broths were well aerated and constantly mixed, and no other competing bacteria were present. These conditions do not represent the conditions likely to occur in the intestine of a calf. The use of a rich medium has been shown to increase the mean number and length of donor pili and increase the probability of mating pair formation.
(Curtiss et al., 1969) compared to minimal media. Starvation prior to mating, or conditions of stress have also been found to inhibit plasmid transfer (Muela et al., 1994; Curtiss et al., 1969; Arana et al., 1997).

6.4d Does the published model of bacterial cell growth and plasmid transfer fit the experimental data of pUK2002 transfer in batch culture?

The transfer rates determined by the end-point method, may be readily incorporated into a mathematical model (Levin et al., 1979) describing bacterial cell growth and plasmid transfer in batch culture. The reason for wishing to use a model in this work was to enable an exploration of the dynamics of plasmid transfer by altering input variables which were not achievable experimentally, and subsequently observing their effects. The model was found to fit the growth curves of the donor and recipient population much better than it did the transconjugant population, although it did not capture all of the behaviour of the data. The rate of plasmid transfer was found to vary depending on the growth phase, a result that has been reported by others (Smets et al., 1993; Muela et al., 1994; Curtiss et al., 1969) as well as demonstrated in this work. Although repeat runs of the model with the average transfer rate over the whole experiment fitted the data better, it was still overestimating the number of transconjugants throughout the mating time. The introduction of a 30 minute lag before plasmid transfer did appear to improve the fit to the T population. This illustrates that the dynamics of transfer of plasmid pUK2002 between J53 and J62-2 are not as simple as predicted by the model, and highlights the need to identify biological processes occurring during the transfer of pUK2002 that are not considered by the model.
6.4e If the model does not fit, what biological processes are occurring which are not taken into account by the model?

Firstly, a lag in growth was detected (Figure 6.2) at the start of the experiment despite the fact that the LB broth was warmed to 37°C prior to inoculation. The presence of a lag phase in bacterial growth has been found to depend on the specific conditions and the properties of the organism (Monod, 1949). Additionally, an apparent lag may be caused if the inoculate contains a large proportion of non-viable cells (Monod, 1949). In the end-point mating experiments described in this chapter, an overnight culture was diluted in total 1 in 1000 into the LB. Although it is not clear what proportion of the total cells present in this inoculum were viable, all of the viable cells would be in stationary phase (after 16-18hrs incubation in 5mls LB) at the moment of inoculation. Here, there will be a delay in growth (the duration of which will depend on the bacteria and the media) while the necessary genes for growth are transcribed and translated into protein products. The model requires a function to allow for this lag in growth, the duration of which in vivo is unknown.

Secondly, plasmid transfer has been found to be more frequent during the lag and early stationary phase of donor cells than during the exponential phase (Muela et al., 1994). The results of this chapter suggest that the plasmid transfer rate varies as a function of growth phase, not necessarily growth rate. The model does not take into account any variation in ability to donate or receive plasmids by the strains involved, which has also been found to vary with growth phase (Smets et al., 1993; Fernandez-Astorga et al., 1992).

6.4f Can the model be used to address questions of plasmid persistence in natural populations?

Models simplify observed phenomenon into mathematical terms to determine whether the data can be explained in terms of apparently few parameters and variables, and by doing so have to make assumptions. By studying the assumptions
made, one can determine whether the model is likely to be a realistic representation of plasmid transfer \textit{in vivo}.

The model assumes that the frequency of matings increases at a rate proportional to the increase in donor and recipient populations. A consequence of this assumption is that the mating culture must be subjected to continuous mixing, which increases the likelihood that donor cells will encounter recipient cells. \textit{In vivo} studies have indicated that the majority of plasmid transfers occur in the mucus layer of the intestine (Poulsen \textit{et al.}, 1994). Here the bacteria are thought to retain fixed spatial positions despite peristalsis and the fluidity of the mucus (Licht \textit{et al.}, 1999). These conditions permit little mixing of the population, which is thought to account for the observation of a brief period of rapid conjugation after inoculation in mouse colonisation experiments, followed by no further plasmid transfer (Licht \textit{et al.}, 1999). Additionally, the density of cells able to receive the plasmid is likely to be far lower \textit{in vivo} than in these broth matings, because of differences in restriction modification systems present in other bacteria and even within different \textit{E. coli} strains (Murray, 2002).

A second assumption of the model is that mating frequency is independent of bacterial growth phase. The results described in this chapter demonstrate that the rate of plasmid transfer is affected by the growth phase of the donor and recipient population, because significantly different transfer rates were measured at different mating times, and different growth rates were demonstrated at each of these mating times. Here the fastest rate of transfer occurred during exponential growth of the donors and recipients, and the slowest rates were measured in the lag and post-exponential growth phases. Although other workers have found different relationships between growth rates and plasmid transfer frequencies (Smets \textit{et al.}, 1993; Muela \textit{et al.}, 1994; Curtiss \textit{et al.}, 1969), these studies support the hypothesis that plasmid transfer is not independent of bacterial growth phase.

The model also assumes that transconjugants are immediately able to transfer the plasmid at the same rate as donor cells. Cullum \textit{et al.} (1978) report that most newly
formed transconjugants are only able to mate after approximately 90 minutes. Similarly, Andrup & Anderson (1999) report a “recovery period” of about 40-80 minutes, following acceptance of the F plasmid in E. coli or plasmid pCF10 in Enterococcus faecalis before tranconjugants mature into proficient donors. These reports indicate that this assumption may introduce inaccuracy into the model.

The are a number of studies which report that the growth rates of plasmid carrying cells are slower than those of plasmid free cells (Gillespie, 2001; Goodwin & Slater, 1979; Helling et al., 1981; Inselberg, 1978; Kayama & Yara, 1975). In contrast, other workers have identified plasmids for which carriage does not impart a fitness cost on the host bacterium (Bouma & Lenski, 1988; Lenski et al., 1994; Enne et al., 2004b). The model used in this chapter assumes that the growth rate of all clones is identical. This is tested in the next chapter, where plasmid-carrying and plasmid-free isogenic strains are grown in competition with each other.

The growth rates of all strains involved is likely to be much lower in vivo than in a nutrient rich broth like LB. Generation times of 20mins have been observed in broth cultures compared to a mean generation time of 12h in the alimentary canal (Hardy, 1984). Cell growth and plasmid transfer are energy requiring processes, and so are both likely to be slower when nutrients are more limited than in LB. A comparative study on the transfer rates of the derepressed plasmid R1drd19 in batch culture demonstrated transfer rates in mouse caecal contents or mouse caecal mucus were 4- and 50-fold lower than those obtained in L-broth (Licht et al., 1999). In vivo, plasmid transfer was only detected during a brief period following inoculation, after which no further transfer occurred, even though rapid division of donors and recipients was observed (Licht et al., 1999). Similarly, Thomas et al. (2001) report a reduced plasmid transfer frequency between Bacillus spp. in mosquito larvae compared to broth culture. These results support the hypothesis that the transfer rates obtained in the broth mating experiments described in this chapter, are likely to be over-estimates of the rates achievable in vivo.
Finally, the model predicts that plasmids with faster transfer rates will transfer to a greater number of recipients, resulting in a greater prevalence than plasmids with lower transfer rates. Of the three apr\textsuperscript{R} plasmids identified in the calf faecal \textit{E. coli} population, pUK2003 transferred at the slowest rate (Chapter 5), yet it is the second most prevalent plasmid. This demonstrates that the factors responsible for plasmid persistence \textit{in vivo} are more complicated than can be explained simply in terms of growth rates and plasmid transfer rates of the two strains involved in a mating. Other factors that may influence plasmid persistence include the presence of other genes on the plasmids (e.g. bacterial attachment pili, metabolic genes), which may not be expressed \textit{in vitro}, as yet unknown selective pressures (e.g. bacteriocin production by other gut flora), and costs associated with high transmission rates that may be undetectable in the closed \textit{in vitro} system used. More information about the immediate environment in which these apr\textsuperscript{R} plasmids reside is required before questions about plasmid persistence \textit{in vivo} can begin to be addressed.

In summary, two methods were used to measure the transfer rate or frequency of the apr\textsuperscript{R} plasmids described in Chapter 5. The measurement of T/D\textsubscript{0} allowed an accurate comparison of transfer frequencies within a study, but was more affected by mating time and showed a greater degree of variation than the end-point method. The end-point method is likely to be more comparable with other workers results because it takes into account the densities of all cells involved in a mating, but problems may arise when attempting to measure transfer rates during lag phase.

Secondly, a model was applied to the experimental data. It proved difficult to get a very close fit of the model with the experimental data, but exploring the effects of altering the parameters enabled the identification of biological processes occurring during the transfer of the apr\textsuperscript{R} plasmid pUK2002 that may not otherwise have been questioned. For example, the presence of a lag phase prior to conjugation, and the non-linear (sigmoid) shape of transconjugant density demonstrating the different rates of plasmid transfer with growth phase. The study of the underlying assumptions of the model prompts many questions about the process of conjugation both in batch
cultures and in vivo. It is this prompting of questions, more than any attempts to use the model in addressing hypotheses on plasmid persistence, which has been useful in the study of the transfer of pUK2002.
Chapter 7. Competitive fitness costs of apr\(^R\) plasmids

7.1 Introduction

The three apr\(^R\) plasmids identified in chapter 5 were all of high molecular size (>90kb). The carriage of large conjugative resistance plasmids is generally thought to engender a competitive fitness cost on the host bacterium in the absence of selection for the resistance phenotype (Gillespie, 2001; Goodwin & Slater, 1979; Helling \textit{et al.}, 1981). Lower growth rates of plasmid-containing cells compared with plasmid-free cells have been observed in both closed-culture experiments (Inselburg, 1978; Kayama & Yara, 1975) and chemostat systems (Goodwin & Slater, 1979; Noack \textit{et al.}, 1981). Plasmid-carrying strains are thought to display decreased growth rates because of the energy expended and the carbon sources needed for both maintenance and replication of the plasmid, and its functions (Goodwin & Slater, 1979).

Previous studies of the competitive fitness cost engendered by plasmid carriage have focused on common laboratory plasmids or cloning vectors (Dahlberg & Chao, 2003; Bjorkman & Andersson, 2000). There appears to be no studies to date that have determined fitness costs associated with plasmids from veterinary isolates.

The aim of this chapter is to determine whether the carriage of plasmids pUK2001, pUK2002 and pUK2003 confer detectable competitive fitness costs on a host bacterium, either in single culture experiments (5hr growth rate comparisons) or during direct competition (for 5hr and for 5 days).
Materials and Methods

Bacterial strains and plasmids

The competitive fitness costs of the apr\textsuperscript{R} plasmids pUK2001, pUK2002 and pUK2003, identified in Chapter 5 were studied. Plasmid pUK2002 was selected for a more detailed analysis of growth rates of plasmid-carrying and plasmid-free strains. This plasmid was chosen because its size and transfer frequency was intermediate among the three plasmids isolated. Plasmids were maintained in \textit{E. coli} MG1655 and \textit{E. coli} J53. The \textit{E. coli} K12 strains J62-2 (lac\textsuperscript{+}, pro\textsuperscript{-} trp\textsuperscript{-} his\textsuperscript{-}) and MG1655kan (\Delta lac, Km) were used as isogenic competitors in J53 competitions and in MG1655 competitions, respectively.

Five hour growth rate comparisons

Cultures of each strain (plasmid-harbouring J53 or MG1655 and plasmid-free J53 or MG1655) were grown in 5ml Lennox L broth (LB) (Invitrogen) or Davis Mingioli broth supplemented with 0.4% glucose (designated MM for minimal media), for 16-18h at 37\textdegree C with shaking at 150 rpm. Starter cultures were diluted 1:10 in saline and used to inoculate 10mL LB or MM to give an initial density of approximately \(1 \times 10^6\) cfu/mL. During incubation at 37\textdegree C with shaking at 150 rpm, samples were taken at 30 min intervals since initial inoculation for five hours. Cell densities were estimated by serial dilution in saline and selective plating. To determine whether plasmid segregation was occurring during the course of the experiment, viable counts of plasmid-harbouring strains were plated onto MacConkey plates, with or without 16mg/L apramycin.
7.2c Five hour competition study

The same protocol as that described for the five hour growth rate comparison was used to study plasmid-carrying and plasmid-free strains in direct competition, except that MG1655kan and J62-2 were used as the plasmid-free competitors. This was to enable the identification of plasmid-carrying, plasmid-free and transconjugant strains during the experiment. Starter monocultures were diluted 1:10 in saline and used to inoculate 10ml LB or MM at a ratio of 1:1 and an initial density of $1 \times 10^6$ cfu/mL of each strain. The formation of any transconjugants was measured by serial dilution and plating onto selective MacConkey plates. Supplementation with 40µg/mL apramycin and 32µg/mL kanamycin was used for MG1655 competitions, and 32µg/mL apramycin and 32µg/mL rifampicin for J53 and J62-2 competitions.

7.2d Five day competition experiments

Five-day competition experiments between plasmid-carrying MG1655 and plasmid-free MG1655kan were performed in MM to limit the frequency of plasmid transfer. Overnight cultures of the competing strains were grown up in separate bijoux containing 5mL MM. The two cultures were mixed in a 1:1 ratio (mixO) and used to inoculate 250µL of broth in each of six wells of a microtitre plate, to give an initial inoculum of approximately $1 \times 10^6$ cfu/mL. Initial counts of plasmid-carrying MG1655 and plasmid-free MG1655kan were determined by serial dilution and plating onto S-gal plates (Sigma). The presence of any transconjugants was detected by plating 100µL mixO onto MacConkey plates supplemented with 40µg/mL apramycin and 32µg/mL kanamycin. Microtitre plates were incubated at 37°C with gentle shaking (150 rpm.). After 24 hours of growth, cultures were diluted 1:100 into fresh media. This dilution step was repeated daily for a total of five transfers. The densities of plasmid-free, plasmid-harbou ring and transconjugant cells were estimated every 24 hours by serial dilution and selective plating. S-gal was used for the estimates of plasmid-carrying (black) and plasmid-free cells (white), and
MacConkey plates supplemented with 40mg/L apramycin and 32mg/L kanamycin for the quantification of transconjugants.

Relative fitness ($S$) was estimated as described by Lenski et al (1991)

$$S_y = \left\{ \frac{\ln[N_x(1)/N_x(0)] - \ln(2)}{\ln[N_y(1)/N_y(0)] - \ln(2)} \right\} - 1$$

Where $N_x(0)$ and $N_y(0)$ are the initial densities of the tested clone and the common competitor (MG1655kan) respectively, and $N_x(1)$ and $N_y(1)$ are their corresponding final densities.

Fitness was estimated after 24 hours of direct competition and also after five days of direct competition (with a 1:100 dilution of each culture into fresh broth after each 24 hour period). Some workers have calculated fitness only after 24 hours (Dahlberg & Chao, 2003). The reason for estimating fitness after 24 hours and after five days of competition was to determine if the estimates differed with longer competition times. In all assays, the relative fitness was calculated as the growth of MG1655 relative to MG1655kan. In this way a positive relative fitness calculated as described by Lenski et al. (1991) demonstrates a growth advantage of MG1655 compared to MG1655kan.

The selection rate constant, $r$ (day$^{-1}$) was calculated by regressing the natural log-transformed ratio of plasmid-bearing to plasmid-free cell densities against time (Lenski et al., 1994), over the five day time period.

7.2e Reproducibility of viable count method

It was hypothesised that the differences between growth rates of plasmid-carrying and plasmid-free strains may be slight, if detectable. It was important therefore, to determine the level of variation introduced by the viable count method used to quantify the numbers of bacteria present. Four colonies of E. coli 10418 were
inoculated into 5mL LB and grown for 8 hours at 37°C with shaking at 150 rpm. The culture was then diluted 100,000 fold in saline. To study the variation with repeat dilutions, thirty 100μL aliquots were diluted 10-fold in saline and plated onto MacConkey plates for quantification (method A). To study variation within one dilution (method B), a 1mL aliquot was diluted 10-fold in 9mL saline, and 30 aliquots of this dilution were subsequently plated out onto MacConkeys plates.

7.2f Statistical analyses

The effects of plasmid, host, media (LB or MM) and detection plate (MacConkeys or MacConkeys supplemented with 16mg/L apramycin) on the number of cfu/mL during the five-hour growth experiments and five-day competition assays were analysed with generalised linear mixed effects models. Data that were not normally distributed were log-transformed before the analysis was performed. Different flasks or wells were entered as a random effect to account for both the variation between replicate experiments and the lack of independence between samples. Type of detection plate was included in the model as a fixed effect to determine whether plasmid segregation was occurring in any of the experiments.

A second linear mixed effects model was run to study the effect of plasmid carriage during the five-hour growth experiments. Non-significant interactions were removed from the previous models, and time, host (J53 or MG1655), media (LB or MM) and plasmid carriage were entered as fixed effects. Different flasks were again entered as a random effect.
7.3 Results

7.3a Variation introduced by the viable count method

Thirty replicate dilutions of *E. coli* 10418 (method A) and 30 replicate aliquots of the same diluted *E. coli* 10418 culture (method B) were plated onto MacConkeys plates for quantification by the viable count method. Figure 7.1 shows the spread of counts obtained with each method. The mean count for each method is highlighted on the graph.

![Figure 7.1](image)

**Figure 7.1** Spread of counts obtained when repeat dilutions of the same bacterial culture was diluted and plated out 30 times (blue bars; method A), and when 30 repeat aliquots of the same culture were plated out (pink bars; method B).

Figure 7.1 shows that although all replicate counts were performed on dilutions of the same 8-hour bacterial culture, a variety of counts are obtained. The counts were not significantly different between the two methods (*t*<sub>58</sub> = 0.54, *P* = 0.591), but ranged from 21 to 81. If the colony counts of two samples differed by less than 60 colonies, it would be difficult to state whether the two samples contained significantly different cfu/mL. This is because the variation observed may be a result
of variation introduced by the methodology. Table 7.1 lists the descriptive statistics of the data sets from each method.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Method</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of replicates</td>
<td></td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>46.6</td>
<td>45</td>
</tr>
<tr>
<td>Standard Error</td>
<td></td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>12</td>
<td>10.4</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td></td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>60 (21-81)</td>
<td>48(22-70)</td>
</tr>
</tbody>
</table>

**Table 7.1** Descriptive statistics of data from method A and method B.

The range of counts, standard error and standard deviation indicate that method A (multiple dilutions of the same culture) generates slightly more diversity in the colony counts than multiple aliquots of a diluted culture (method B). The variation between the two methods was insignificant ($F = 1.55$, $P = 0.248$). From the results described in Figure and Table 7.1, it was decided that a difference of at least 60 colonies should be present between identical dilutions of two given cultures, to be certain that they had different cfu/mL of bacteria.

**7.3b Five-hour growth rate comparisons**

Firstly, the growth curves of each strain (MG1655pUK2002, MG1655kan, J53pUK2002 and J53) in LB and MM was plotted (at least five replicates) to determine the phases of growth exhibited under these conditions. The growth curves of replicate cultures in LB are shown in Figure 7.2, and the geometric means of each of these, in Figure 7.3. Similarly, the growth curves of replicate cultures in MM and the geometric means of these results are shown in Figures 7.4 and 7.5 respectively.

The missing data points on the graph occur when the number of cfu on a plate was of an uncountable number (>300cfu). The exclusion of these data points does not affect
the ability of the linear mixed effects model to determine the significance of any observed trends.

Figure 7.2 Replicate growth curves of a, MG1655pUK2002; b, J53pUK2002; c, MG1655kan; and d, J53 in 10mL LB.

In Figure 7.2 it can be seen that the replicate experiments show similar trends overall, but there are times during the experiment where more variation is present. During the first hour, more variation between the five replicates of MG1655kan is observed than at 1.5 and 2 hours. This may result in inaccurate estimates of growth rates if they are measured from the moment of initial inoculation ($t = 0$), as the slopes
of each replicate of MG1655kan during the first hour are quite different. In the graphs of J53pUK2002 and J53 (b and d respectively) replicate cultures, the slopes of each replicate appear very similar except at 4 and 5 hours. Here, the viable counts of some replicates continue to increase where in other replicates they either decrease or reach a stationary phase with no overall increases in viable cell numbers. Overall, it appears that variation between replicate growth curves is most evident during the first hour and after the fourth hour of growth.

**Figure 7.3** Geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 in 10mL LB.

In the comparison of the geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 (Figure 7.3), all four cultures demonstrate a one
hour period of reduced growth in comparison to the exponential phase occurring between one and four hours. The decreased growth rate is more evident with cultures of MG1655pUK2002 and J53. After four hours, the cultures enter a post-exponential growth phase, where J53pUK2002 and J53 cultures show a more dramatic decrease in growth rate than MG1655pUK2002 and MG1655kan. In this way, after four hours the growth curve varied in a manner dependent on the host strain (J53 or MG1655).

With respect to determining differences in growth rates between plasmid carrying and plasmid free strains, it is important to define periods of growth that are most comparable. From Figure 7.2, it is clear that less variation between replicate growth curves is present during one and four hours. Between the four different strains, obvious variation was present during the lag and post-exponential growth phases. In the post-exponential phase, the pattern of growth appeared dependent on host strain. Therefore, only data from the exponential phase (between one and four hours) was used to compare growth rates between plasmid-free and plasmid-carrying strains.

The increase in cfu/mL over time was host specific, with MG1655 cultures (irrespective of plasmid carriage) containing significantly less cfu/mL with time (slower growth) than J53 strains, $t_{50} = -4.17, P = 0.0001$. Plasmid segregation was discounted as changes in cell numbers over time revealed no significant difference in the recovery of plasmid-carrying cells on MacConkey plates or on MacConkey plates supplemented with 16mg/L apramycin, $t_{50} = 0.59, P = 0.56$.

In MM more variation between replicate experiments was apparent (Figure 7.4) than between replicates in LB (Figure 7.2). This is likely to be because of variation introduced when making the MM, as each flask was made up separately from double concentrate DM, distilled water and glucose. Slower growth was predicted in MM than in LB, and this is what is observed, with the presence of <18 Ln cfu/mL after five hours compared to around 21 Ln cfu/mL in LB. The J53pUK2002 starter culture may have contained more cells than the other starter cultures, because the cultures at time 0hr contain more cfu/mL than the other three growth curve experiments.
The geometric mean growth curves in MM (Figure 7.5) show less well-defined growth phases compared to those in LB (Figure 7.3). The growth curves of MG1655kan and J53 have much more distinct lag phases than those of the plasmid-carrying strains (MG1655pUK2002 and J53pUK2002). None of these curves reach a post-exponential phase, which was observed in LB.

To keep the two data sets (LB growth and MM growth) as comparable as possible, again only data between one and four hours were used in the statistical analysis.

Figure 7.4 Replicate growth curves of a, MG1655pUK2002; b, J53pUK2002; c, MG1655kan; and d, J53 in 10mL MM.
Figure 7.5 Geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 in 10mL MM.

In MM, cultures of J53 (with or without plasmid) contained significantly more cfu/mL overall, during the course of the experiment than cultures of MG1655 (with or without plasmid) $t_{57} = -2.41, P = 0.039$. The increase in cfu/mL with time was host specific, with J53 cultures growing significantly faster than MG1655 cultures, $t_{61} = -2.16, P = 0.035$. Plasmid segregation was again discounted as changes in cell numbers over time revealed no significant difference in the recovery of plasmid-carrying cells on MacConkey plates or on MacConkey plates supplemented with 16mg/L apramycin, $t_{57} = -92, P = 0.36$. 
In the final model the growth of J53, J53pUK2002, MG1655kan and MG1655pUK2002 were compared. The number of cfu/mL in each culture of plasmid-carrying strains (J53pUK2002 and MG1655pUK2002) throughout the course of the experiment did not differ significantly from those of plasmid-free cultures, $t_{31} = 1.35, P = 0.19$. Plasmid carriage did not affect the changes in cfu/mL over time (i.e. cell growth), $t_{171} = 0.363, P = 0.7172$. The increase in cfu/mL over time was host-dependant, with MG1655 cultures growing significantly slower than J53 cultures, $t_{176}, P < 0.0001$.

7.3c Five-hour growth in direct competition

When plasmid-carrying (J53pUK2002 and MG1655pUK2002) and plasmid-free (J53 and MG1655kan) strains were grown in direct competition, the mean colony counts of competing strains did not differ by > 60 colonies. This suggests that plasmid carriage did not affect growth because this level of variation could occur on account of the methodology. When tested statistically, overall fewer cfu/mL were present in MG1655 cultures (either plasmid-carrying or plasmid-free MG1655) than J53 cultures, $t_{12} = -3.86, P = 0.002$, but the growth rates of MG1655 strains and J53 strains were not significantly different, $t_{245} = 0.94, P = 0.35$.

Plasmid carriage did not affect the numbers of cfu/mL over the course of the experiments, $t_{243} = -0.14, P = 0.89$, and did not affect the growth rates of the host strain (increase in cfu/mL with time), $t_{243} = 1.06, P = 0.29$. Plasmid segregation was discounted in both LB and MM, as changes in cfu/mL with time were not significantly different when measured on MacConkey plates and MacConkey plates supplemented with 16mg/L apramycin, $t_{46} = -0.2, P = 0.85$ (LB data), and $t_{70} = -1.19, P = 0.24$ (MM data).
7.3d Five-day growth in direct competition

Relative fitness estimates and selection rate constants of plasmid-carrying MG1655 in direct competition with MG1655kan are listed in Table 7.2. The control for this 5-day competition assay was the direct competition of MG1655 and MG1655kan. Relative fitness estimates of $S_y = 0.005 \pm 0.005$ after 24 hours revealed that the insertion of the kanamycin resistance gene into MG1655 marginally reduced the competitive fitness of the strain (Table 7.2). The relative fitness cost of the kanamycin resistance gene is more evident after five days of competition.

If there is no difference in the growth rate of two competing cultures but one has a higher inoculum, the difference in competing cell densities may be amplified with each repeat passage. To ensure that variation in the number of bacteria present in the initial cultures did not influence the outcome of the competition, control experiments were repeated with an initial ratio of 1:2 and of 2:1 MG1655 to MG1655kan. The selection rate constants of six replicate competitions did not differ significantly when initial ratios of 1:2 or 2:1 were used ($t_6 = -0.386$, $P = 0.712$).
Table 7.2 Relative fitness estimates and selection rate constants of MG1655 harboring plasmid pUK2001, pUK2002 or pUK2003, in direct competition with MG1655kan.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Replicate</th>
<th>Selection rate constant (r) (day(^{-1}))(^{a})</th>
<th>Relative fitness (S_j^{b}) 24h</th>
<th>Relative fitness (S_j^{b}) 5 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUK2001</td>
<td>1</td>
<td>0.253</td>
<td>0.145</td>
<td>0.290</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.219</td>
<td>0.149</td>
<td>0.236</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.293</td>
<td>0.295</td>
<td>0.405</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.330</td>
<td>0.188</td>
<td>0.593</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.107</td>
<td>0.144</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.136</td>
<td>0.211</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>0.223 ± 0.088</td>
<td>0.189 ± 0.024</td>
<td>0.351 ± 0.054</td>
</tr>
<tr>
<td>pUK2002</td>
<td>1</td>
<td>0.358</td>
<td>0.140</td>
<td>0.638</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.378</td>
<td>0.182</td>
<td>0.361</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>0.350</td>
<td>0.332</td>
</tr>
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<td></td>
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<td>0.143</td>
<td>0.301</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.351</td>
<td>0.199</td>
<td>0.405</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.511</td>
<td>0.124</td>
<td>0.437</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>0.334 ± 0.117</td>
<td>0.190 ± 0.034</td>
<td>0.412 ± 0.049</td>
</tr>
<tr>
<td>pUK2003</td>
<td>1</td>
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<td>0.003</td>
<td>-0.071</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>0.015</td>
<td>-0.043</td>
</tr>
<tr>
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<td>3</td>
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<td>0.029</td>
<td>-0.072</td>
</tr>
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<td>0.016</td>
<td>-0.056</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.0065</td>
<td>ND</td>
<td>-0.041</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-0.0601</td>
<td>ND</td>
<td>-0.057</td>
</tr>
<tr>
<td></td>
<td>mean</td>
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<td>0.016 ± 0.01</td>
<td>-0.057 ± 0.01</td>
</tr>
<tr>
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<td>0.077</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>0.008</td>
<td>0.069</td>
</tr>
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<td>ND</td>
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<td>6</td>
<td>0.0051</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>0.0391 ± 0.0302</td>
<td>0.005 ± 0.005</td>
<td>0.043 ± 0.012</td>
</tr>
</tbody>
</table>

\(^{a}\) Standard deviation is given for each mean estimate, \(^{b}\) standard error is given for each mean estimate.

Table 7.2 shows that pUK2001 and pUK2002 confer fitness advantages on MG1655 that are far greater than the advantage of this strain over its kanamycin resistant counterpart. The fitness advantages conferred by carriage of these plasmids are more evident after five days of competition. The mean colony counts of the raw data showed a difference of > 60 colonies after day three and day one for pUK2001 and pUK2002 respectively. The selection rate constants for MG1655 carrying these plasmids were significantly different from the control (pUK2001; \(t_{10} = -4.87, P = \)
Plasmid segregation of pUK2001 or pUK2002 was discounted, as changes in cfu/mL with time did not differ significantly when measured on S-gal plates or MacConkey plates supplemented with 16mg/L apramycin ($t_{15} = 0.96, P = 0.342, t_{112} = 1.18, P = 0.24$ respectively). Over the five days of competition, cfu/mL of MG1655pUK2001 differed significantly from those of MG1655kan ($t_{51} = -1.24, P < 0.0001$). Cfu/mL of MG1655pUK2001 increased significantly with time ($t_{23} = 4.67, P = 0.0001$).

The increase over time of cfu/mL of MG1655pUK2002, compared to MG1655kan was significant, $t_{19} = -11.133, P < 0.0001$. When separate models of the MG1655pUK2002 or the MG1655kan data were run, the cfu/mL of MG1655kan decreased significantly over time, $t_{57} = -12.89, P < 0.0001$.

In contrast, pUK2003 appeared to confer a fitness cost which was detectable after five days of competition (Table 7.2). After only 24 hours of competition, the difference in relative fitness was not significant ($t_9 = -1.533, P = 0.176$). Fewer cfu/mL of MG1655pUK2003 compared to cfu/mL of MG1655kan were detected during the course of the assay, but the difference was not significant ($t_{33} = -0.92, P = 0.36$). Cfu/mL of MG1655pUK2003 decreased with time, but this decrease was not significant ($t_{14} = -0.72, P = 0.48$). Consistent with these statistical findings, the colony counts of competing strains throughout the five-day competition did not differ by > 60 colonies.

### 7.4 Discussion

Resistance plasmids are threatening the successful management of both human and animal bacterial infections. Traditionally, the carriage of resistance plasmids is thought to engender a competitive fitness disadvantage on the host bacterium, in the absence of selective pressure by antibiotics (Goodwin & Slater, 1979; Lee & Edlin, 1985; Noack et al., 1981; Zund & Lebeck, 1980). In this way, the presence of plasmid-mediated resistance may be limited by restricting the use of the selective...
antibiotics, allowing sensitive strains to outcompete the resistant bacteria. A few reports have documented the amelioration of a fitness disadvantage conferred by the presence of chromosomal mutations (Bjorkman et al., 1998; Reynolds, 2000), transposable elements (Hartl et al., 1983; Modi et al., 1992) conjugative and non-conjugative plasmids (Bouma & Lenski, 1988; Enne et al., 2004b; Negri et al., 2000). Very few of these reports have studied naturally occurring plasmids, focussing mainly on cloning vectors and common laboratory plasmids (Bjorkman & Andersson, 2000). There appears to be no studies of fitness costs associated with plasmids isolated from veterinary isolates. The apr^R plasmids identified in the commensal E. coli of the calves sampled in this study were large (> 90kb), and so the presence of a fitness disadvantage associated with the carriage of these plasmids was expected.

Previously, the biological cost of resistance has been measured experimentally by estimating growth rates of sensitive and resistant bacteria in monocultures (Bjorkman et al., 1998), or by pairwise competition studies in batch cultures (Dahlberg & Chao, 2003; Enne et al., 2004b; Lenski et al., 1994), chemostats (Helling et al., 1981, Modi et al., 1992) or experimental animals (Bjorkman et al., 1998; Enne et al., 2004a; Negri et al., 2000). In this chapter growth rate comparisons of monocultures and competing cultures were compared, in addition to pairwise competition studies in batch culture.

The growth of isogenic strains with and without pUK2002, in single culture experiments and during direct competition, did not reveal any significant differences in growth rates. Similarly, Enne et al. (2004b) did not find significantly shorter generation times of plasmid-carrying strains compared to their isogenic plasmid-free counterparts. The use of linear mixed effects models to analyse the data, enabled more information to be gleaned than by comparing growth rates by paired t-tests, in that interactions between factors such as media and host strain could be analysed. This meant it was possible to account for any differences for example, between the host strain used, when studying the effect of plasmid carriage. The growth of isogenic plasmid-carrying and plasmid-free strains for five hours, either in direct
competition or in single cultures may be too short a period for any slight differences in growth rate to be detected. A five-day pairwise competition was performed to test this hypothesis.

Different fitness cost estimates have been used to quantify the difference in competitive fitness between two strains (Lenski et al., 1991). These include estimates of relative fitness (Helling et al., 1981; Reynolds, 2000), per generation fitness impact (Enne et al., 2004a, 2004b), generation times (Bjorkman et al., 1998) and selection rates or coefficients (Dahlberg & Chao, 2003; Lenski et al., 1994; Modi et al., 1992; Negri et al., 2000). The lack of a standard estimate to quantify fitness costs leads to difficulties in comparing results between studies. In this chapter, both the selection coefficient and the relative fitness (as described by Lenski et al., 1991) were used to allow the results to be compared with those of similar published studies.

During five days of pairwise competition, the average selection coefficient of pUK2002 was found to be \( r = 0.334 \) day\(^{-1} \pm 0.117 \). Similar selection coefficients \( (r = 0.216 \) day\(^{-1} \pm 0.043) \) have been described by Lenski et al. (1994) for a 2.9kb plasmid conferring tetracycline resistance. The authors suggest that the tetracycline resistance gene (tetC) was the factor responsible for the enhanced fitness with plasmid carriage in the co-evolved host. Interestingly, plasmids pUK2002 and pUK2003 also carry a tetracycline resistance determinant (tetB). Plasmid pUK2003 did not confer a fitness advantage, and demonstrated a potential fitness cost \( (r = -0.069 \pm 0.044 \) day\(^{-1} \) although more replicate experiments and longer competition times would be required to improve the significance of this result, so this gene may be unrelated to the fitness advantage of pUK2002.

The competitive fitness advantage of pUK2001 and pUK2002 was observed in hosts that had no evolutionary history of association. This is a surprising result because the competitive fitness advantage described by Lenski et al. (1994) was only present when the plasmids and hosts had coevolved for 500 generations. In contrast, Enne et al. (2004b) report a fitness advantage in both naïve and adapted hosts, of a 6.2kb plasmid conferring sulphamethoxazole and streptomycin.
The relative fitness of pUK2001 and pUK2002 ($S = 0.189 \pm 0.024$ and $0.190 \pm 0.034$ respectively) after 24 hours of competition are within the range of mean fitness estimates of R1- or RP4-bearing J53 after 24h following approximately 1100 generations of growth in an antibiotic-free environment (range of 0.16 to 0.35) (Dahlberg & Chao, 2003).

The five-day pairwise competition study described in this chapter has an advantage over the five-hour competition study in that more components of bacterial growth are compared. For example, differences in initial lag phase, speed of growth in exponential phase, and efficiency of resource utilisation. Over 24 hours, all available resources are used up and the cells enter stationary phase. Upon subculture into fresh broth, this complete cycle of growth is repeated. A substantial amount of variation occurred between the replicate competition experiments described in this chapter, demonstrating the necessity for repeat estimates of fitness.

The results of this chapter indicate that pUK2001 and pUK2002 confer a competitive fitness advantage on the naïve host strain MG1655, under the conditions tested (minimal media, incubation at 37°C with shaking at 150 rpm.). At present the nature of this advantage is unknown. The plasmids may carry a metabolic function aiding growth of the host strain under the conditions tested. To determine whether a gene present on the plasmid is responsible for conferring a competitive advantage will require sequencing of the plasmids.

It is also unclear at this stage whether the competitive advantage observed in vitro will also occur in vivo. Although an attempt was made to stress the cells by growing them under nutrient limited conditions, this is unlikely to be as stressful as conditions in vivo. Intestinal E. coli must compete for nutrients against many other intestinal micro-organisms (bacteria; Bacteroides spp, Klebsiella, Proteus, Streptococcus faecalis; the yeasts Candida and Torulopsis; and protozoa; Entamoeba) (Stanier et al., 1983), some of which may produce bacteriocins (Avelar et al., 1999). Gut bacteria are likely to be exposed to cycles of nutrient starvation and abundance in
vivo (Hardy, 1984). The carriage and maintenance of a large accessory genetic element is likely to require more of the cells available energy, than to not carry it. During periods of starvation, when the cell must reduce its metabolic activity in order to survive, strains without the plasmid are likely to overgrow those with a plasmid unless there is direct selection for its presence. Other conditions may arise when it is more advantageous to carry such a plasmid. However, on consideration of the occurrence of these apr$^R$ plasmids (which required selective isolation for their detection), they are rare in the commensal population studied, and so it is likely that a selective pressure not yet identified is responsible for their presence. This may be co-selection by tetracycline or streptomycin farm usage for pUK2002, or the use of disinfectants or feed supplements, as is discussed in Chapter 5.

Although the fitness advantage described in this chapter should be interpreted with caution until in vivo studies have been performed, this work demonstrates an in vitro competitive fitness advantage associated with the carriage of pUK2001 and pUK2002 that is comparable to other published studies.
Chapter 8. The search for extended-spectrum beta-lactamases

8.1 Introduction

In 1965 the first plasmid-mediated β-lactamase in a gram-negative organism, was described (Datta & Kontomichalou, 1965). This enzyme was designated TEM-1 after the Greek patient, Temoniera, from whom it was isolated. TEM-1 efficiently hydrolyses amoxycillin, and is commonly found on transposons (Yamamoto et al., 1982) and plasmids (Gulay et al., 2000; Simpson et al., 1980). Because of the mobility of these genetic elements, the enzyme TEM-1 has been able to spread to other species of bacteria, such as Klebsiella (Jeong et al., 2004a), Proteus (Chanal et al., 2000), Salmonella (Rivera et al., 1991), Pseudomonas (Calderwood et al., 1982) and Acinetobacter (Vila et al., 1993). TEM-1 has since spread worldwide, and is currently the most common plasmid-mediated β-lactamase (Amyes et al., 1996). A sulphydryl variable of this enzyme (SHV-1), originally isolated from Klebsiella pneumoniae is also commonly found amongst Enterobacteriaceae (Heritage et al., 1999). This enzyme is usually plasmid mediated in E. coli isolates (Bradford, 2001).

Extended-spectrum β-lactams were developed to combat organisms harbouring β-lactamases. These are based on the original β-lactam structure but with additional side-chains intended to prevent their hydrolysis. However, each new extended-spectrum β-lactam used therapeutically has been met with the discovery of a new β-lactamase (designated extended-spectrum β-lactamases or ESBLs) capable of its hydrolysis. Most ESBLs are derived from TEM or SHV enzymes, by the presence of between one and five point mutations enabling the extended-spectrum β-lactams antibiotics to fit into the active site of the enzyme for subsequent hydrolytic inactivation (Bush et al., 1995). Other classes of ESBL include those that are based on CTX-M and OXA enzymes. The latter is commonly found in Pseudomonas isolates (Danel et al., 1999; Hall et al., 1993; Mugiier et al., 1998), but CTX-M enzymes have been isolated mainly from salmonella (Edelstein et al., 2004; Tzouvelekis et al., 2000) and E. coli strains (Briñas et al., 2003b; Jeong et al.,
CTX-M enzymes preferentially hydrolyse cefotaxime and are becoming a huge problem (Pitout et al., 2004; Pournaras et al., 2004). ESBLs are commonly encoded by self-transmissible or mobilisable plasmids, which further exacerbate the therapeutic challenges posed by these enzymes.

The presence of ESBLs in organisms causing infections is now a global problem. The prevalence of ESBLs amongst clinical isolates varies between countries and even within a country (Winkur et al., 2001). In France 3.2% of 2506 Enterobacteriaceae isolated in 1998 produced an ESBL (De Champs et al., 2000). In contrast, a recent survey in Japan estimated that less than 0.1% of E. coli and 0.3% of K. pneumoniae strains isolated possessed an ESBL (Yagi et al., 2000). The type of ESBL most commonly identified is also often country or region specific. In the United States TEM-10 producing organisms have caused several unrelated outbreaks (Bradford et al., 1994; Schiappa et al., 1996; Wiener et al., 1999), but in Europe this enzyme is much less common (De Champs et al., 2000; Perilli et al., 2002). In Chicago, a common plasmid harbouring TEM-10 was found in 17 isolates from 20 hospitals or nursing homes (Wiener et al., 1999). PFGE demonstrated that seven different Klebsiella pneumoniae and E. coli strains were carrying the plasmid, leading the authors to presume that the plasmid was present in the commensal flora of the patients. This interesting and alarming speculation is substantiated by other studies on β-lactam resistance in commensal organisms (Thomson et al., 1993). In Turkey, a high incidence of ampicillin resistance has been found in commensal faecal E. coli from healthy volunteers (Gulay et al., 2000). In South Africa, 94.3% of ampicillin resistant commensal faecal gram-negatives from healthy volunteers were found to harbour TEM-1 (Shanahan et al., 1995).

Although extended-spectrum β-lactams are used in animal husbandry, there have been very few studies on the characterisation of β-lactamases from healthy food-producing animals (Brinas et al., 2002; 2003b; Zhao et al., 2001). ESBLs have been identified in pathogenic organisms from veterinary isolates (Brinas et al., 2003a) but not yet in commensal isolates. This may be because therapeutic failures have not occurred or not been detected, and so the presence of ESBLs has not been sought.
Alternatively, the use of cephalosporins in animal husbandry may have not yet lead to the selection and development of resistant isolates. The aim of this chapter was to determine whether any of the commensal E. coli collected from the calf cohort, demonstrating reduced susceptibility to 3rd generation cephalosporins, harboured an ESBL. In addition, a Salmonella enterica serotype Enteritidis isolate (S. Enteritidis), identified by the Scottish Salmonella Reference Laboratory (SSRL) in Glasgow was included in the study because it was implicated in a Salmonellosis outbreak during which therapeutic failures with 3rd generation cephalosporins were reported. Because antibiotic resistance in relatively uncommon in S. Enteritidis (Vahaboglu et al., 2001), the mechanism of resistance was of interest.

The two aims of this chapter are described as follows:

1. To determine if the cephalosporin resistant commensal E. coli isolated from the calves in this study harbour ESBLs.
2. To establish the mechanism of resistance of the S. Enteritidis isolate to 3rd generation cephalosporins.

8.2 Materials and Methods

8.2a Bacterial isolates

This study was carried out on 52 isolates of the ampR population identified by work described in Chapter 3 as having reduced susceptibility to 3rd generation cephalosporins (cefotaxime MIC ≥ 2mg/L, ceftazidime MIC ≥ 4mg/L).

The S. Enteritidis isolate was collected from an outbreak of salmonellosis in a general hospital in Glasgow, Scotland. The epidemiology of this outbreak and the work completed by the Scottish Salmonella Reference Laboratory (SSRL) is outlined below.
8.2b Salmonella outbreak

The Salmonella outbreak occurred from the 20th December 2001 to the 21st January 2002, during which nine isolates were obtained from five patients with salmonella gastroenteritis, and two asymptomatic members of staff. The SSRL identified the isolates as *Salmonella enterica* serovar Enteritidis PT21, by agglutination to polyvalent antisera and phage typing. Isolates of *S. Enteritidis* from two patients and one member of staff were sensitive to fourteen common antibiotics. The isolates from the remaining patients and one member of staff were resistant to ampicillin and cefotaxime. Plasmid profiling and PFGE were used by the SSRL to demonstrate that these isolates were clonal and carried a plasmid of 95 kb. One isolate (020003) was used to determine the mechanism of resistance of the isolates.

8.2c Antibiotic sensitivity

Initial antibiotic susceptibility testing of 183 ampR isolates was performed with a panel of 25 commonly used antibiotics (Chapter 3). The susceptibility of *S. Enteritidis* 020003 to ampicillin and cefotaxime was determined by the SSRL by disc diffusion.

MICs of the ampR *E. coli* to amoxycillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid (in a ratio of 2:1 antibiotic to β-lactamase inhibitor), were determined following BSAC guidelines (Andrews, 2001). NCTC strains *E. coli* 10418, *S. aureus* 6571 and *E. coli* 11560 were used as controls. Susceptibility testing of *S. Enteritidis* 020003 to ampicillin, cefaclor, cefotaxime and ceftazidime was performed using Etest strips (AB Biodisc, Sweden) following the manufacturer’s instructions.
8.2d Determination of the presence of an ESBL

The presence or absence of an ESBL was determined by Etest strips specific for the detection of ESBLs, containing cefotaxime or ceftazidime, with and without clavulanic acid, and by double disc synergy tests (Livermore & Brown, 2001). Discs containing 10μg cefpodoxime, 30μg ceftazidime or 30μg cefotaxime with and without clavulanic acid (1μg with cefpodoxime and 10μg with ceftazidime or cefotaxime) (Oxoid, Basingstoke, UK) were placed on IST agar plates (Oxoid) inoculated with 0.5 McFarland standard of test organism. Plates were incubated for 12-18 hours at 37°C. A positive result for the presence of an ESBL was recorded if the inhibition zone around the disc containing cephalosporin and clavulanic acid was ≥5mm larger than that around the disc containing only the cephalosporin.

NCTC organisms E. coli 11560 (TEM-1 +ve) and E. coli 10418 (ESBL –ve) were used as positive and negative controls respectively, for both the double disc synergy tests and Etest strips.

8.2e Amplification of blaTEM-1 and blaSHV-1

The β-lactamase genes blaTEM-1 and blaSHV-1 were sought by PCR using a Taq polymerase kit (Promega) and the following oligonucleotide primer pairs: blaTEM-1, 5’-ATGAGTATTCACATTTCCG-3’, 5’-CCCATGCTTATCAGTGAGG-3’; and blaSHV-1, 5’-GCCCGGGTTATTCTTATTTGTCGC-3’, 5’-CTTTCCGATGCGCCGCACTGCA-3’. DNA was initially denatured for 5 min at 95°C, followed by 30 cycles of amplification, and a final 10 min extension at 72°C. The amplification of blaTEM-1 comprised a 30 sec denaturation at 95°C, 1 min of annealing at 53.3°C, and 1 min extension at 70°C. For blaSHV-1 cycles of 30 sec denaturation at 95°C, 30 sec of annealing at 68°C, and 50 sec extension at 72°C, were used. Sequencing was performed by the chain termination method (Sanger et al., 1977) in forward and reverse directions, and the sequences were compared with sequences in public databases.
8.3 Results

8.3a Antibiotic susceptibility

Initial breakpoint susceptibility testing revealed that 52 of 183 amp\textsuperscript{R} commensal *E. coli* tested, demonstrated reduced susceptibility to the 3\textsuperscript{rd} generation cephalosporins cefotaxime (≥ 2mg/L) or ceftazidime (≥ 4mg/L). The MIC values of these isolates to amoxycillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid are shown in Figure 8.1. To display the MICs of the 52 isolates in a graph, isolates were sorted in ascending order according to firstly their amoxycillin MIC, then cefotaxime MIC, and final by their ceftazidime MIC. This was to aid the identification of isolates for further study. The isolates selected (n = 16) are highlighted on Figure 8.1 by black squares. These isolates were chosen to give a range of isolates representative of a group with similar sensitivity profiles, or because they were outliers in the general trend of the graph.

![Figure 8.1](image)

**Figure 8.1** MICs of 52 isolates to amoxycillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid. Sixteen isolates were selected for further study. These are highlighted with black squares.
The susceptibilities of these 16 isolates to a range of cephalosporins and β-lactams are listed in Table 8.1. The breakpoint susceptibilities were determined as described in Chapter 3. The actual MIC values of amoxicillin, ceftazidime, cefotaxime, and these antibiotics in combination with clavulanic acid, are given in brackets next to the phenotype.

Table 8.1 Susceptibilities of 16 amp\textsuperscript{R} isolates selected for further study to β-lactams and cephalosporins.

<table>
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<th>PEN</th>
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<th>URX</th>
<th>FOX</th>
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<th>AMX</th>
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<td>I</td>
<td>S</td>
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<td>R</td>
<td>I</td>
<td>R</td>
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<td>I</td>
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<td>R</td>
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<td>R</td>
<td>I</td>
<td>(&gt;256)</td>
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<td>I</td>
<td>R</td>
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<td>R</td>
<td>S</td>
<td>R</td>
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<td>I</td>
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</tr>
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<td>R</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>(&gt;256)</td>
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<td>I(2)</td>
<td>R(16)</td>
<td>I(16)</td>
</tr>
<tr>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>(&gt;256)</td>
<td>R(32)</td>
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<td>I(2)</td>
<td>R(16)</td>
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<td>WF006959-1</td>
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<td>R</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>(&gt;256)</td>
<td>R(32)</td>
<td>I(2)</td>
<td>I(2)</td>
<td>R(16)</td>
<td>I(16)</td>
</tr>
<tr>
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<td>R</td>
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<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>(&gt;128)</td>
<td>R(128)</td>
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<td>I(2)</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>(&gt;128)</td>
<td>R(128)</td>
<td>I(2)</td>
<td>I(2)</td>
<td>R(16)</td>
<td>I(16)</td>
</tr>
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<td>WF007558-1</td>
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<td>R</td>
<td>R</td>
<td>(&gt;128)</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>(&gt;128)</td>
<td>R(128)</td>
<td>I(2)</td>
<td>I(2)</td>
<td>R(16)</td>
<td>I(16)</td>
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</table>


Table 8.1 shows that all 16 isolates are resistant to the 1\textsuperscript{st} generation cephalosporins; cephalothin and cephradine. More variation in resistance phenotype is present with the 2\textsuperscript{nd} generation cephalosporins; cefuroxime (URX) and cefoxitin (FOX). No resistance to the 3\textsuperscript{rd} generation cephalosporin ceftizoxime (TIZ) is observed, in contrast to ceftazidime (TAZ) to which 10/16 are resistant. The presence of an ESBL
is indicated, but not proven by an eight-fold difference in the MIC of a 3rd generation cephalosporin and the MIC obtained when this antibiotic is used in combination with a β-lactamase inhibitor (e.g. clavulanic acid). According to these criteria, none of the isolates were thought to possess an ESBL.

Screening for \textit{bla}_{\text{TEM-1}} revealed that the following isolates (isolate number is given after the sample number separated by a dash) (WF00-) 6720-3, 6720-4, 6831-5, 6831-6, 6996-3, 7104-4, 7104-9, 7490-3, 7490-7 and 7558-1 harboured this gene. These are highlighted in yellow in Table 8.1. The resistance profiles of isolates (WF00-) 6986-4, 7204-4, 7013-1, 6959-1, and 7766-1 are suggestive of AmpC-type β-lactamases. These enzymes are usually resistance to ampicillin, amoxycillin, ceftazidime (MIC > 4mg/L), cefotaxime (MIC > 1mg/L), cefoxitin (MIC > 16mg/L), and often (but not always) resistant to monobactams such as aztreonam. AmpC-type β-lactamase MICs are usually higher for ceftazidime than for cefotaxime, a result that is observed for all 16 isolates (Table 8.1).

The salmonella isolate \textit{S. Enteritidis} 020003 was resistant to ampicillin (>128mg/L), cefaclor (>128mg/L), cefotaxime (8mg/L) and ceftazidime (>32mg/L).

\textit{8.3b Determination of the presence of an ESBL}

E-test strips and double disc diffusion tests confirmed that none of the 16 commensal \textit{E. coli} isolates harboured an ESBL. \textit{S. Enteritidis} 020003 did have an ESBL according to these tests.

\textit{8.3c Amplification of \textit{bla}_{\text{TEM-1}} and \textit{bla}_{\text{SHV-1}} in \textit{S. Enteritidis} 020003}

PCR with \textit{bla}_{\text{SHV-1}} primers failed to detect a gene encoding an SHV-1 derivative in \textit{S. Enteritidis} 020003. Amplification of \textit{bla}_{\text{TEM-1}} in this isolate yielded a product of the expected size (858bp). The comparison of the DNA sequence of this product with
bla$_{\text{TEM-1}}$ (Lefon-Guilbout et al., 2000) and bla$_{\text{TEM-52}}$ (Lee et al., 2003; Poyart et al., 1998) showed that this gene encoded bla$_{\text{TEM-52}}$. Bla$_{\text{TEM-52}}$ differs from bla$_{\text{TEM-1}}$ by amino acid substitutions at positions Glu-104→Lys (GAG→AAG), Met-182→Thr (ATG→ACG) and Gly-238→Ser (GGT→AGT). The changes identified in addition to a silent mutation identified at codon 134 are listed in Table 8.2.

**Table 8.2** Comparison of bla$_{\text{TEM}}$ from *S. Enteritidis* 020003 and published bla$_{\text{TEM-1}}$, and bla$_{\text{TEM-52}}$ sequences

<table>
<thead>
<tr>
<th>β-lactamase</th>
<th>Amino acid (codon) at position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>104   Glu (GAG) Ala (GCG) Met (ATG) Gly (GGT)</td>
<td>Lefon-Guilbout <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>TEM-52 (<em>S. Enteritidis</em> 020003)</td>
<td>134   Lys (AAG) Ala (GCG) Thr (ACG) Ser (AGT)</td>
<td>This work</td>
</tr>
<tr>
<td>TEM-52 (<em>S. Saint Paul</em>)</td>
<td>182   Lys (AAG) Ala (GCT) Thr (ACG) Ser (AGT)</td>
<td>Lee <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>TEM-52 (<em>K. pneumoniae</em>)</td>
<td>238   Lys (AAG) Ala (GCG) Thr (ACG) Ser (AGT)</td>
<td>Poyart <em>et al.</em>, 1998</td>
</tr>
</tbody>
</table>

8.4 Discussion

Although the 52 isolates identified in this work had reduced susceptibility to 3rd generation cephalosporins, they did not harbour any ESBLs. Similarly, Brinas *et al.* (2002) did not find any ESBLs in ampicillin resistant *E. coli* from food, humans and healthy animals. Based on susceptibility profiles, the isolates were thought to harbour either bla$_{\text{TEM-1}}$ (conferring high level resistance to amoxycillin), an AmpC-type β-lactamase, or both. In *E. coli*, the chromosomal ampC gene is normally expressed at low levels, but production of this enzyme can be increased by gene duplication or by mutation in the ampC promoter or attenuator (Caroff *et al.*, 2000; Nelson & Elisha, 1999). More recently, plasmid-mediated AmpC-type β-lactamases have also been found in *E. coli* from food-producing animals (Zhao *et al.*, 2001) and *Salmonella spp.* (Allen & Poppe, 2002).
The primary aim of this section of the chapter was to determine if the calf faecal *E. coli* harboured any ESBLs. Reduced susceptibility to the cephalosporins was demonstrated in 16 isolates, and *bla*_{TEM-1} was detected in ten of these. Unfortunately due to time restrictions, it was not possible to determine the exact mechanism of this reduced susceptibility in the other six isolates. The hypothesis that some of these isolates harboured AmpC β-lactamases, may be tested by isoelectric focusing and PCR to amplify and sequence the *bla*AmpC gene implicated by the pI value (Allen & Poppe, 2002; Brinas *et al*., 2003b; Zhao *et al*., 2001). It would also be of interest to determine if the resistance gene was transferable by conjugation, as has been found for some AmpC-type β-lactamases (Alvarez *et al*., 2004; Knox, 1995; Zhao *et al*., 2001).

The *S.* Enteritidis isolate was of interest because antibiotic resistance is not common in this serovar (Vahaboglu *et al*., 2001). The isolate was implicated in a Salmonellosis outbreak during which therapeutic failures with 3rd generation cephalosporins were reported. In the treatment of extraintestinal salmonella infection the antibiotics of choice are extended-spectrum cephalosporins and fluoroquinolones. Recently, *Salmonella* isolates harbouring extended-spectrum β-lactamases (ESBLs) capable of hydrolysing third-generation cephalosporins have been reported (Lee *et al*., 2003; Vahaboglu *et al*., 2001). This is of particular concern for the treatment of salmonellosis in children, because fluoroquinolones cannot be used in this age group.

The resistance detected to the 3rd generation cephalosporins ceftazidime and cefotaxime was mediated by *bla* _{TEM-52} (mutations at codons 104, 182 and 238, compared to *bla*_{TEM-1}).

A change in residue 104 from either glutamic or aspartic acid to lysine, is the second most common substitution of the TEM-type ESBLs (Knox, 1995). Here, a negatively charged residue is replaced by a positively charged residue with a long basic side chain, which may interact with the carboxylic acid group in ceftazidime, ceftibuten or aztreonam, increasing initial binding (Knox, 1995). In TEM-type ESBLs, the substitution from methionine to threonine at position 182 adds an extra hydrogen.
bond between the hydroxyl group of threonine and the carbonyl group of the glutamic acid in position 64. This may increase the stability of the protein where multiple mutations are present (Poyart et al., 1998). The substitution Gly-238→ Ser, occurs in 27 TEM variants and in all SHV mutants. Position 238 is in a key beta-strand of the catalytic site of class A beta-lactamases, and this mutation has been found to be critical in increasing resistance to cefotaxime (Blazquez et al., 1995). A mutation at position 238 has been hypothesised to slightly expand the lower portion of the β-lactam binding site, allowing larger cephalosporins such as cefotaxime to form hydrogen bonds with residue 237 in the binding site (Huletsky et al., 1993). A silent mutation was present in the blaTEM-52 allele of isolate 020003 at position Ala-134 (GCT→ GCG) that is not present in other published salmonella TEM-52 sequences (GenBank no. AF126444, AY220520), but which has been reported in a Klebsiella pneumoniae TEM-52 sequence (GenBank no. Y13612).

In conclusion, reduced susceptibility to cephalosporins was found in 52/183 ampR commensal E. coli, but no ESBLs were detected amongst a subset (n = 16) of these isolates. The outbreak strain S. Enteritidis 020003 was found to harbour an ESBL, and because antibiotic resistance is not common in this serovar the detection of an ESBL is significant. The enzyme was identified as TEM-52, which has not previously been reported in Salmonellae in the UK. The TEM-52 gene described here differed from all published salmonella TEM-52 sequences by one silent mutation.
Chapter 9. Discussion

Resistance to antibiotics arises from the use of these agents in human and animal medicine, and in animal husbandry. The advent of antibiotics into animal husbandry for treatment, but especially for prophylaxis and growth promotion, has precipitated concern that this usage poses risks to human health (Swann Committee, 1969; Salyers, 1999b; Khachatourians, 1998; van den Bogaard & Stobberingh, 2000). The debate over whether or not human health is being compromised (or will be in the future) by antibiotic use in animal husbandry is ongoing, with several working groups and independent scientists stressing the need for more surveillance of antibiotic resistance not only in pathogens but also in commensal organisms (Caprioli et al., 2000; Jones et al., 2000; Masterton, 2000; O’Brien, 1997). It is within this context that the key findings from this thesis are most relevant.

Chapters 3 to 8 contain detailed discussions of the specific results therein, and so this final chapter aims to address some of the wider issues emerging from these results.

To date there are many reports documenting antibiotic resistance in both human and animal pathogens, but comparatively little is known about levels of resistance in commensal organisms in food animals (Salisbury et al., 2002). Notwithstanding this imbalance in the data available, the hypothesis that antibiotic uses in animals pose a risk to human health is supported by claims that commensal organisms constitute a huge reservoir of resistance genes (Salyers, 1999a; Schwarz et al., 2001). On inspection of the results presented in Chapter 3, it is clear that the commensal E. coli of calves do harbour resistance genes to a range of antibiotics. In the unselected population these include ampicillin, amoxyclyclavulanic acid, cephalosporins (1st, 2nd and 3rd generation), tetracycline, streptomycin, chloramphenicol, trimethoprim and sulphamethoxazole.

The fact that isolates harbouring resistance mechanisms to these antibiotics were identified without selection to amplify their numbers indicates that they constitute a significant proportion of the population (Chapter 3). However, the relative
abundance of a particular resistance mechanism is not as important as the fact that it is present in the population. When the bacterial population is exposed to sufficient concentrations of an antibiotic, susceptible strains are suppressed and any resistant strains are amplified (Amyes et al., 1996). Reduced susceptibility within the commensal E. coli was also found to nalidixic acid, apramycin, gentamicin and tobramycin. With the exception of apramycin, all of the antibiotics to which reduced susceptibility was demonstrated are used in human medicine. From this, the commensal E. coli of calves is concluded to constitute a substantial reservoir of resistance genes.

After the demonstration of numerous resistance phenotypes, the genotypes of members of the unselected and the three selected (antibiotic resistant) populations were examined. Knowledge of the background level of population diversity is essential if studies on the genetic relatedness of pathogenic or antibiotic resistant E. coli are to be put into context. In chapter 4, genotyping by PFGE was found to be highly discriminatory, enabling the distinction of 55 different genotypes within the commensal population, in addition to subtypes within six of these genotypes. Fewer antibiotic resistant genotypes and less diversity of genotypes correlated with increasing calf age. In the context of the current debate, these results suggest that meat from older animals is less likely to contain antibiotic resistant E. coli than that from calves, and so poses less of a risk to human health.

So far, the results from this research have demonstrated that the commensal E. coli of calves constitute a large reservoir of resistant genes, and that resistant commensal E. coli comprise many diverse genotypes. For human health to be compromised by the presence of these resistant commensals, meat or other foodstuff contaminated with these organisms must be ingested, or the resistance genes transferred to organisms pathogenic to humans (Piddock, 1996).

In Chapter 5, the horizontal conjugative transfer of a high frequency apramycin resistance plasmid (pUK2001) into three different genotypes was demonstrated. This transfer may have occurred in vivo, or in the environment before the strains colonised
the calves. Two other apramycin resistance plasmids were identified, both of which had lower transfer frequencies and were not found in multiple genotypes. Interestingly, the ability of pUK2001 to transfer into other commensal *E. coli* genotypes, isolated from the same sample as that from which pUK2001 was isolated, was demonstrated *in vitro*. Therefore, pUK2001 was capable of transfer into these genotypes, which were present in similar abundances, but had not transferred into these strains *in vivo*. This suggests that there are barriers to the spread of resistance plasmids that are present *in vivo*, but which may not be evident in *in vitro* experiments.

The nature of these barriers include the presence of restriction modification systems (Murray, 2002), the presence of other plasmids of different incompatibility groups in a potential recipient (Gordon, 1992), and spatial and temporal barriers such as the *in vivo* proximity of donors and recipients (Licht *et al.*, 1999). The application of a mathematical model to the phenomenon of plasmid transfer within a population (Chapter 6) revealed a further inhibition to the spread of plasmids. This is the growth phase of the mating pair. Experimental data of the population dynamics of plasmid transfer (chapter 6) did not fit the outcome predicted by the model. The model overestimated the numbers of transconjugants formed in the later stages of the mating experiment (from around 2.5 hours) because plasmid transfer varied with growth phase, not growth rate. In this way, the growth phase of possible donors and recipients is concluded to be a potential barrier to plasmid spread in a mixed population.

The results of chapter 6 also demonstrate that the dynamics of plasmid transfer cannot be simply explained in terms of cell densities, growth rates and plasmid transfer rates. The model predicts that plasmids with high transfer rates will be present at a greater prevalence than plasmids with lower transfer rates. These comparative prevalences are not observed *in vivo*, demonstrating that many more factors influence the dynamics of plasmid transfer than may be incorporated into an *in vitro* experiment (see chapter 6 for details).
A fitness advantage was observed in *E. coli* hosts harbouring pUK2001 or pUK2002, compared to their isogenic counterparts. This is the first demonstration of an *in vitro* fitness advantage of plasmids obtained from commensal isolates. The fitness advantages measured were comparable to fitness advantages described by other studies of cloning vectors and common laboratory plasmids. However, the fact that these plasmids required selection in order to be isolated from the commensal *E. coli* population shows that within this population they are rare, and so the *in vivo* advantage must be slight, if present at all.

The results from chapters 6 and 7 are very interesting in themselves, but their full interpretation requires a comparison with the situation *in vivo*. This comparison phase has been omitted in some earlier studies (Dahlberg & Chao, 2003; Lenski *et al.*, 1994; Modi *et al.*, 1992). The results of such *in vitro* experiments have been used to support the hypothesis that resistant bacteria in food animals are potential risks to human health (McGeer, 1998; Salyers & Amabile-Cuevas, 1997). Chapters 6 and 7 demonstrate that although apramycin resistance plasmids may transfer horizontally to other *E. coli*, and may not confer a competitive disadvantage on the host bacterium, *in vivo*, there are barriers to the spread of these plasmids. The low prevalences of these plasmids suggest that the cost of carriage is also likely to be greater *in vivo*.

As mentioned earlier in this discussion, one potential hazard posed by the presence of resistant bacteria in the flora of food animals is the contamination of foodstuffs for human consumption. This is most easily tracked with zoonotic organisms such as *Salmonella* spp. *Salmonella* is a common resident of the calf intestinal flora, and causes infections and outbreaks in humans following ingestion (Threlfall, 2002). One such outbreak is described in chapter 8. Here the mechanism of resistance was found to be an ESBL. To the best of my knowledge ESBLs have not yet been found within the commensal organisms of calves, and were not harboured by the *E. coli* isolates characterised in this thesis. Based on this evidence, and the fact that cephalosporins are commonly used in the treatment of extraintestinal salmonella infections and other serious infections (Amyes *et al.*, 1996), the most likely way in which the salmonella
strain causing the outbreak acquired its plasmid-mediated resistance was from human usage of cephalosporins.

There are a few reports where resistance mechanisms present in human isolates are thought to have been acquired from animal isolates (Fey et al., 2000; Spika et al., 1987; van den Bogaard et al., 1997), but the majority of resistance in human isolates does not appear to have arisen from the use of antibiotics in animals, but from their use in human medicine (Cook, 1997; Phillips et al., 2004). Although antibiotic resistant bacteria are present within the flora of food animals, the way to reduce the potential hazard this poses is to minimise the spread of organisms between animals and humans. This can be achieved by better food hygiene practices, such as reducing cross contamination of raw animal products and cooked foods, washing fruit and vegetables (as manure is often used in their cultivation), and adequate cooking of meat based products.

In conclusion, the work presented in this thesis demonstrates that commensal E. coli of calves do represent a substantial reservoir of resistance genes, of which resistance to apramycin has been demonstrated to have spread horizontally. For resistant organisms to pose a threat to human health, they must either contaminate foodstuffs or transfer their resistance mechanisms to pathogenic organisms. Although the transfer of apramycin resistance plasmids has been demonstrated, in vivo barriers exist that are capable of preventing extensive spread throughout a population. The risk posed by the contamination of foodstuffs can be minimised by good food hygiene practices. From the evidence provided by this thesis, it is suggested that the use of antibiotics in animal husbandry poses far less of a risk to human health than the use of these agents in human medicine. All antibiotic usage provides a selective pressure for the emergence of resistance, and efforts now need to be concentrated on reducing antibiotic usage and keeping the reservoirs of animal and human resistant organisms separate.
References cited


DANMAP (1998). Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Laboratory, Copenhagen, Denmark. ISSN 1397-1409.

DANMAP (2002). Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Laboratory, Copenhagen, Denmark. ISSN 1600-2032.


Salyers, A.A (1999a). Beware the commensals! Their role in antibiotic resistance spread may be greater than we think. *ASM News* **65**, 459-469.


Appendix 1. Results of a sensitivity analysis to determine how different parameters influence the output of the plasmid transfer in batch culture model.

Figure A1. The effect of donor growth rate on the transconjugant population during the transfer of pUK2002 between J53 and J62-2.

Figure A1 demonstrates that the best fit for the T population is a low donor growth rate (vD) (vD of 0.5 cfu/mL), but this results in very inaccurate modelling of the D population. Additionally, resource limitation is reached at too low a cell density compared to the experimental data, indicating that the model should be run with a greater amount of resource than that currently used.
Figure A2. The effect of donor growth rate on the recipient population during the transfer of pUK2002 between J53 and J62-2.

The effect of donor growth rate (vD) on R (Figure A2) is one of direct competition. When vD > vR, D constitutes a greater proportion of the total population at resource limitation than R, and vice versa.
Figure A3. The effect of recipient growth rate on the transconjugant population during the transfer of pUK2002 between J53 and J62-2.

Figure A3 is almost identical to that of the effect of vD on T (Figure A1), with the best fit of the experimental T population being achieved with the lowest recipient growth rate (vR), 0.5. The use of a low vR results in inaccurate modelling of R.
Figure A4. The effect of recipient growth rate on the donor population during the transfer of pUK2002 between J53 and J62-2.

In a similar way to the effect of vD on R (Figure A1), if vR > vD, R will occupy a greater proportion of the broth than D (Figure A4). The reduction in D as a function of increasing vR is less than the reduction in R due to increasing vD because D only acts in competition with the other strains in the broth, whereas R is affected by conversion to T at a rate determined by \( \gamma \), in addition to the effect of competition with D and T.
Figure A5. The effect of different initial D densities on the population dynamics of pUK2002 transfer.

When the initial density of D (iD) is greater than initial density of R (iR) the density of R is not affected throughout the experiment (Figure A5). When iD > iR, D reaches resource limitation before R, therefore R occupied a smaller proportion of the broth than D throughout the experiment. When the model was run with different initial densities of R, similar results were obtained in that when iR < iD, the density of D is not affected but when iR > iD, R occupies a greater proportion of broth than D (results not shown). Lowering iD or iR produces the same T population curve, but at lower cell densities.
Figure A6. The effect of resource limitation on the population dynamics of pUK2002 plasmid transfer. The resource concentrations used are listed in the key as D or T and the concentration of glucose added (200-1000).

There is a trade off between an increase in resource concentration, enhancing the fit of the donor (and recipient) populations, but resulting in a reduced fit to the transconjugant population. This graph also makes it clear that the model is unable to take into account an initial lag in growth of the donors (and recipients). The sigmoid curve of these populations is not reproduced by the model, which assumes bacterial growth is constant and exponential from the moment of initial inoculation.
Appendix 2. Results of different combinations of the three parameters (resource concentration, lag in D and R growth, and lag in transfer) found to enhance the fit of the model to the experimental data.

Three different values of each of the three parameters were tested. In each of the following graphs (1-27) the value of each parameter is listed in the title according to the following key:

R200; 200µg/mL of resource.
R500; 500µg/mL of resource.
R1000; 1000µg/mL of resource.

L0.5; 0.5h lag before D and R growth.
L1; 1h lag before D and R growth.
L1.5; 1.5h lag before D and R growth.

T0; 0h lag before plasmid transfer.
T0.5; 0.5h lag before plasmid transfer.
T1; 1h lag before plasmid transfer.