A STUDY OF THE TYPING OF THE GENUS *ENTEROCOCCUS*

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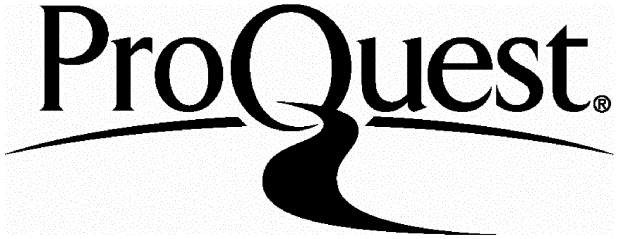

I hereby declare that this thesis has been composed entirely by myself and that all the work described herein was carried out by myself alone, except as otherwise stated in the acknowledgements.

Philippe André Lacoux

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SUMMARY

Restriction fragment length polymorphism (RFLP) of chromosomal DNA for the intra-species characterisation of enterococci is reported. The bacterial DNA was extracted by a rapid method and digested to provide a characteristic DNA banding pattern after agarose gel electrophoresis. Five different restriction endonucleases *Bam* HI, *Sac* I, *Sal* I, *Eco* RI and *Hind* III were assessed for their ability to provide easily readable banding patterns that could be used to differentiate between a representative sample of enterococcal isolates. One hundred and eighty enterococcal isolates from three different sources were then examined using the restriction endonuclease *Sal* I, and this provided 39 distinct DNA banding patterns; this technique provided a discriminatory method of isolate characterisation for *E.faecalis*, *E.faecium* and *E.durans*. Results were compared with biotyping with the API 20 Strep kit, and with antibiogram data using eight antibiotic impregnated discs. The technique of restriction endonuclease analysis with *Sal* I was applied to the temporal analysis of faecal enterococci from an isolated Antarctic community, and the results compared with biotyping and antibiogram data. Southern blots were probed with ribosomal RNA from *Escherichia coli* after digestion of the bacterial DNA with either *Eco* RI or *Hind* III. This revealed some potential as a method for differentiation within the enterococcal species, and also at the inter-species level although *E.faecium* and *E.durans* were difficult to separate.

Recent clinical findings suggest that a typing scheme for *S.bovis* could be important. These developments are discussed, and the methods above applied to three *S.bovis* isolates.

The historical taxonomic changes within the enterococci are reviewed. The clinical importance of the enterococci is discussed in relation to both the changing

pattern of antimicrobial resistance and to the growth in nosocomial infection. Methods of typing the enterococci are reviewed, and the present position of genetic typing methods is discussed.

1. INTRODUCTION

1.1. The genus Enterococcus in historical perspective

The historical taxonomy of the enterococci into a separate genus consisting at the present time of twelve species has closely paralleled the development of new identification techniques in bacteriology. The first use of the term "enterocoque" was by Thiercelin (1899) for a gram-positive diplococcus of intestinal origin. Andrewes and Horder (1906) in their paper giving an overview of the status of the Streptococci at the time entitled "A study of the Streptococci pathogenic for man" found - "a group of streptococci so characteristic of the human intestine that the term "*Streptococcus faecalis*" may be justly applied to it. It is mostly short-chained, rendering broth uniformly turbid, but in disease, at least, forms with long to medium chains occur. It grows readily on gelatin at 20°C with rare exceptions and is distinguished by its great chemical activity."" The species possesses a very great resistance to desiccation, far greater than that of the long-chained throat forms."

Orla-Jenson (1919) suggested the names *S.faecium* and *S.glycerinaceus*, and carefully defined some of the physiological properties of what was to become the enterococci. *S.glycerinaceus* was subsequently confirmed to correspond to the description of *S.faecalis*.

In the subsequent decades there was a great deal of confusion concerning what constituted an enterococcus, and upon the nomenclature which should be used. Sherman (1937) while reviewing the literature felt that many workers had made inadequate attempts to record isolate characteristics. He provided much greater potential for order among the streptococci by suggesting their division into four groups; pyogenic, viridans, lactic and enterococci. The characteristics of the enterococci as suggested by Sherman were growth at 10°C and 45°C, growth in 6.5% sodium chloride and at pH 9.6, also survival at 60°C for 30 minutes, and the

ability to split aesculin. These characteristics have survived remarkably intact, and are still considered among the basic properties of the enterococci (Schleifer, 1984).

Four different species of enterococci were discussed by Sherman (1937) with liquefaction of gelatin (*S.liquefaciens*), and haemolysis in blood agar (*S.zymogenes*) as important tests to differentiate the above from *S.faecalis*. Sherman acknowledged that this sub-division was probably for insufficient reason, and suggested the consideration of varietal status. *S.durans* was more easily classified as a separate species due to its paucity of fermentation reactions. As reflected above much of the earlier work wrestled with the questions about what did and did not constitute an enterococcus, and where species lines were to be drawn.

The serological classification of Lancefield (1933) added the presence of the group antigen D as a characteristic of what are now classified as the enterococci. There was a striking association between the Lancefield serological groupings, and the sources of the cultures. Subsequently, the group D antigen was identified as a teichoic acid and a component, not of the cell wall like those of the Lancefield groups A, B, C, G, and F, but of the cytoplasm (Elliot, 1960; Jones and Shattock, 1960). Elliot also discovered a type specific antigen of carbohydrate situated in the cell wall and which was analagous to the other Lancefield groups.

There was good agreement between the classifications of Lancefield and Sherman for the broad group "streptococci", with minor discrepancies such as the reaction of *S.bovis* with the Lancefield group D antisera.

Further serological work subdivided the enterococci (Sharpe and Shattoch, 1952), but not in a manner that correlated with the physiological characteristics of the emerging enterococcal species. It was not possible to define species in terms of serology, although a broad division was made between *S.faecalis* and its varieties and *S.durans*, *S.bovis* and others.

The position of *S.faecium* and *S.durans* as species or sub-species was unclear for many years. Orla-Jenson (1919) first suggested the species *S.faecium*, this susequently corresponded with tellurite sensitive strains found during the serological study of Sharpe and Shattock (1952), work which was also in support of the species status for *S.faecium*.

Motility was a property which had long been associated with the streptococci, and motile enterococci were observed early in the century as *S.faecium* subsp. mobilis, while yellow motile isolates were called *S.faecium* var. casseliflavus. *S.faecium* had become a repository for many isolates of uncertain classification. *S.malodoratus* was isolated from gouda cheese, and *S.avium* from chickens, the latter possessed both D and Q Lancefield antigens.

Paper chromatography of the hydrolysates of complete cell walls of streptococci (Colman and Williams, 1965), did show 26 patterns from 197 strains, but no overall relationship between taxa or species from the chromatography patterns was evident. Similarly in 1973, Amstein and Hartman used gas chromatography to demonstrate the relative fatty acid composition of 37 enterococci in an attempt to clarify the position between different species. Differences were observed in particular among the motile pigmented strains, however the differences were inadequate for the purpose of species differentiation. The isoprenoid quinones which have a role in electron transport were also examined and although the results did agree in general with other forms of taxonomic information, alone they were insufficient for use as a means of classification (Collins and Jones, 1979).

In 1968 Colman applied computers to the classification of the Streptococci; with three different computer programmes analysing 75 different biochemical and physiological tests. He included 14 enterococci, 12 of which, a mixture of *E.faecalis* and *E.faecium* formed a single cluster, however resolution to the species level was not particularly good. Jones *et al* (1972) examined a larger group of 122

Lancefield group D isolates with 45 different physiological and biochemical tests. They found that the organisms clustered in five main groups consisting of *S.faecalis*, *S.faecium* with *S.durans* (together considered to be one species), *S.avium*, *S.bovis* and *S.equinus*. There were some ungrouped strains, and the previous subdivision of *S.faecalis* isolates into the subspecies liquefaciens and zymogenes was not upheld. Jacob *et al* 1975 showed that the ability to liquefy gelatin (*S.faecalis* subsp. liquefaciens) and to haemolyse red blood cells (*S.faecalis* subsp. zymogenese) are properties controlled by plasmids.

A much reduced group of physiological and biochemical tests was proposed by Gross *et al* (1975) in a survey of over 4000 group D isolates. At this time there was growing concern about the interchangable usage of the terms group D, *S.faecalis* and the enterococci. In particular because of the different antimicrobial treatment of diseases caused by the different group D organisms. Their scheme considered pyruvate fermentation and arginine hydrolysis as useful tests to differentiate the enterococcal species. Using 5-7 initial tests, and 6-11 confirmatory tests they were able to differentiate *S.faecalis*, *S.faecium*, *S.faecium* var. durans, *S.avium* and *S.bovis*.

A numerical taxonomic study of the streptococci by Bridge and Sneath (1983) found 28 different groups (phenons) covering a wide variety of species. The methodology again involved a wide range of biochemical and physiological tests and computer analysis. The enterococci formed four phenons, the system was able to distinguish *E.faecalis* and *E.faecium* isolates, with the *E.faecalis* subsp. liquefaciens and zymogenes forming no distinct separate grouping. *E.durans* isolates were grouped with the *E.faecium* isolates, and a new enterococcal species was proposed "*S.gallinarum*" isolated from the bowel of young chickens. There was also an ungrouped strain "*S.faecalis* subsp. maloderatus". The results largely agreed with those of Jones *et al* (1972).

Interesting results were presented by Plecas and Brandis (1974) who studied enterococcal bacteriophages and found that some were species specific, they were able to identify 370/411 strains as either *S.faecalis*, *S.faecium* or *S.bovis*.

Much of the confusion concerning the taxonomy of the enterococci was clarified by the work of Farrow *et al* (1983) who examined the biochemical, fatty acid, menaquinones and DNA of 50 group D isolates. The main contribution of this work was in defining enterococcal species using DNA homology studies. The varieties of *S.faecalis* had DNA homology values of greater than 70% indicating that they should all be considered the same species. *S.faecalis* and *S.faecium* had homologies of 20-30% confirming the latter as a separate species within the same genus. *S.durans*, previously a sub-species of *S.faecium* was phenotypically similar to *S.faecium* but homology studies suggested that separate species status was appropriate. *S.casseliflavus*, the yellow motile species was confirmed to be a separate species, and the suggested sub-species of *S.faecium* var. mobilis was found to have high homology values (76-97%) with *S.casseliflavus*, and to be unrelated except at the genus level to *S.faecium*. In summary, *S.faecalis*, *S.faecium*, *S.casseliflavus*, *S.durans*, *S.avium* and *S.gallinarum* (Bridge and Sneath, 1982) were different species of the same genus, and *S.bovis* was unrelated at the generic level.

In the last ten years with the use of DNA/rRNA hybridisation studies and comparative analysis of ribonuclease T_1-resistant oligonucleotides of the 16S rRNA (Schleifer and Kilpper-Balz, 1987) a clearer understanding of the streptococci as a whole has emerged; the divisions of Sherman from 1937 continue to be very largely upheld. Schleifer and Kilpper-Balz (1987) with other workers were instrumental in the division of the streptococci into three genera, the Streptococci, Enterococci and Lactococci. This was not fully recognised in the 1984 Bergey's Manual of Bacterial Classification because of overlap in publication dates (Schleifer 1984).

New species of enterococci have emerged, and there are now twelve different enterococcal species (Table 1), with some enterococcal isolates still not identified to this level (Murray, 1990a). Farrow and Collins (1985) described *E.hirae* a species found from pig and chicken isolates. Collins *et al* (1986) described *E.mundtii* a yellow pigmented non-motile strain from plants and soil. *E.pseudoavium*, *E.raffinosus* and *E.solitarius* have been described by Collins *et al* (1989).

Species identification still depends largely upon biochemical testing (Table 2), although DNA homology studies have been the initial basis for the defining of new enterococcal species. Most recently polymorphism among penicillin-binding proteins have emerged as a taxonomic tool. Each of nine different species studied by Williamson *et al* (1986) had a specific pattern of five or more penicillin-binding proteins.

1.2. Present day identification of species

The twelve enterococcal species have been defined principally on genetic DNA/DNA homology criteria. There is some confusion concerning the biochemical differentiation between the enterococcal species. Facklam and Collins (1989) and Rouff *et al* (1990) have both conducted studies to biochemically define the species. There are also many other published biochemical criteria for the distinction of enterococcal species with some disagreements. There are both inconsistent biochemical test results which vary within a species (Table 2) and isolates which fit into no particular species (Facklam and Collins, 1989; Murray, 1990a). It can be argued that it is not possible to confidently distinguish between all of the enterococcal species without some form of examination of the genome.

Table 1. Historical developments within the genus *Enterococcus*

Date	Author(s)	Species recognised
1899	Thiercelin	"enterocoque"
1906	Andrewes and Horder	*S.faecalis*
1919	Orla-Jenson	*S.glycerinaceus*
		S.faecium
1935	Langston	*S.faecium* subsp. mobilis
1937	Sherman	*S.faecalis*
		S.faecalis var. zymogenes
		S.faecalis var. liquefaciens
		S.faecalis var. haemolyticus
1938	Sherman and Wing	*S.durans*
1955	Pette	*S.faecalis* var. maloderatus
1967	Nowlan and Dieble	*S.avium*
1968	Mundt and Graham	*S.faecium* var. casseliflavus
1982	Bridge and Sneath	*S.gallinarum*
1983	Farrow *et al*	*S.faecalis*
		S.faecium
		S.casseliflavus
		S.avium
		S.durans
1984	Schleiffer and	*E.faecium*
	Kilpper-Balz	*E.faecalis*
1984	Collins *et al*	*E.avium*
		E.casseliflavus
		E.durans
		E.malodoratus
		E.gallinarum
1985	Farrow and Collins	*E.hirae*
1986	Collins *et al*	*E.mundtii*
1989	Collins *et al*	*E.raffinosus*
		E.solitarius
		E.pseudoavium

Table 2. Phenotypic characteristics of enterococcal species

Species	Test[a]										
	MAN	SOR	SBS	ARG	ARA	RAF	TEL	MOT	PIG	SUC	PYU
E.avium	+	+	+	-	+	-	-	-	-	+	+
E.raffinosus	+	+	+	-	+	+	-	-	-	+	+
E.malodoratus	+	+	+	-	-	+	-	-	-	+	+
E.pseudoavium	+	+	+	-	-	-	-	-	-	+	+
E.faecalis	+	+	-	+	-	-	+	-	-	+[b]	+
E.solitarius	+	+	-	+	-	-	-	-	-	+[b]	-
E.gallinarum	+	-	-	+	+	+	-	+	-	+	-
E.faecium	+	-[b]	-	+	+	-[b]	-	-	-	+[b]	-
E.casseliflavus	+	-[b]	-	+	-	+	-[b]	+	+	+	-[b]
E.mundtii	+	-[b]	-	+	-	+	-	-	+	+	-
E.durans	-	-	-	+	-	-	-	-	-	-	-
E.hirae	-	-	-	+	-	v	-	-	-	v	-
E.faecalis[c]	-	-	-	+	-	-	+	-	-	-	v

(Facklam and Washington, 1991)

[a] Abbreviations; MAN (mannitol), SOR (sorbitol), SBS (sorbose), ARG (arginine), ARA (arabinose), RAF (raffinose), TEL (0.04% tellurite), MOT (motility), PIG (pigmentation), SUC (sucrose), PYU (pyruvate).

+: >90% positive; -: <10% positive; v: variable 60-90% positive.

[b] Occasional exception (<5% of strains show aberrant reactions).

[c] Variant

The ability of the clinical laboratory to identify enterococci to the species level is important as there are clinically significant differences in antimicrobial susceptibility, for example the greater resistance of *E.faecium* isolates to penicillin/aminoglycoside synergy when compared wih *E.faecalis* isolates (Moellering *et al*, 1979). The work of Mackowiak (1989) has highlighted the differences in the virulence between three enterococcal species (*E.faecalis*, *E.faecium* and *E.avium*) suggesting that great precision is needed in their identification.

A variety of commercial products for the identification of the streptococci have been developed, usually based upon freeze-dried reagents. The names of these products are confusing. In the present study the API 20-Strep has been used, this is manufactured in France by API system, La Balme les Grottes, France, and distributed throughout Europe; in Britain by API-bioMerieux (UK) Ltd., Basingstoke, Hampshire. In the USA the same product is sold under a different name - DMS-Rapid Strep., frequently written as simply the Rapid Strep system. A similar but different product is made by a another company in USA, and is only distributed in the United States. It is not the same product as the API 20-Strep used in this study but has a very similar name - API 20-S, the main difference between the two kits involves the identification of the viridans streptococci. Another product sometimes used for enterococcal studies is the API 50 CH also made by the French company API-bioMerieux. This incorporates 50 carbohydrates, no buffer or media, and can be applied to a wide variety of organisms.

Several studies have compared the various products (Appelbaum *et al*, 1984; Facklam *et al*, 1984; Facklam *et al*, 1985; Fertally and Facklam, 1987). The overall conclusion has been that these products are good at identifying *E.faecalis* and *E.faecium*, but become less accurate for the more recently defined species.

Despite attempts to devise physiological and biochemical means of confidently identifying the enterococci confounding problems continue to emerge. Recently Vincent *et al* (1991) showed that the hitherto relied upon tests of yellow pigment production and of motility, used for the identification of *E.casseliflavus* and *E.gallinarum* can be misleading. They found the penicillin-binding protein profiles and ultimately DNA homology results were required to confidently identify these species. Musher (1988) felt that; "Although DNA homology will almost certainly provide the final word, the clinical laboratory must in the meantime identify streptococci with readily available techniques, and clinicians must utilize these identifications to the best of their ability."

1.3. The clinical importance of the enterococci

Despite their wide distribution in the environment and as part of the normal human bowel, oral cavity, anterior urethra and vaginal flora, the enterococci do not generally cause a significant amount of disease. It has been proposed that this is because they lack the virulence factors found in many other bacteria such as the Staphylococci.

The types of infection that are caused by the enterococci include endocarditis, bacteraemia, urinary tract infection, neonatal sepsis, intra-abdominal sepsis, pelvic sepsis and soft tissue infections. Toala *et al* (1969) noted there was a wide variety of clinical specimens from which enterococci were isolated, with urine being the most common. There is frequently an association with general debility, the extremes of age or the presence of invasive devices such as urinary catheters. Wells *et al* (1990) have shown that *E.faecalis* can translocate across the mouse intestinal tract and be recovered from liver, spleen and lymph nodes when experimental intestinal overgrowth with enterococci is induced. Infections involving the enterococci are also frequently polymicrobial and as a result it is difficult to determine the contribution of each species to the infective problem.

It is becoming clear that the influence of prior antibiotic administration is also significant. The changing pattern of enterococcal infection may be related to the wide use of broad spectrum ß-lactam antibiotics encouraging enterococcal overgrowth (Yu, 1981; Berk *et al* 1983; Morrison and Wenzel, 1986).

1.3.1. Endocarditis

Endocarditis is the most serious manifestation of enterococcal infection. Enterococci are the third most common cause of endocarditis after the viridans streptococci and the staphylococci, and cause approximately 10-20% of cases (Mandell *et al*, 1970). Parker and Ball (1975) found that 7.9% of 223 cases of endocarditis were due to enterococci, and considered that this might be an underestimate as *E.faecalis* is relatively easy to identify and so was less likely to have been sent to the reference laboratory around which the work was based. Enterococcal endocarditis is rare in children, and more common in men of the older age group than in women. The disease frequently runs a subacute rather than acute course, with the genito-urinary tract being the commonest portal of entry (Koenig and Kaye, 1961). It may be related to intravenous drug abuse, when it affects aortic and mitral valves in particular, and is also more common if there is a prior history of valvular heart disease (Koenig and Kaye, 1961). There is one report of human-to-human transmission of enterococcal endocarditis in a couple sharing injection equipment (Hall *et al*, 1976). The mortality rate varies between studies, but is high, Moellering *et al* (1974) 47% of 15 cases, Mandell *et al* (1970) 16% of 36 cases.

There has been an interesting comparison between endocarditis due to enterococci and that due to to *S.bovis*, both being Lancefield group D. In a review of the cases at the Massachusetts General Hospital by Moellering *et al* (1974) the two organisms were equally prevalent, clinical presentation was the same for each, while treatment of *S.bovis* infection can be effective with penicillin alone, and the

mortality of *S.bovis* disease is lower. This illustrates the need to differentiate between these two organisms.

The usual treatment for enterococcal endocarditis is with the synergistic combination of a penicillin or vancomycin with an aminoglycoside. This is under critical review with the advent of resistance in the enterococci to all of these agents. The *ad hoc* group of the committee on Rheumatic fever, Endocarditis and Kawasaki disease from the American Heart Association (Bisno *et al*, 1989) suggest that the species identification of isolates is important as *S.bovis* and *S.mutans* can be treated with penicillin alone. They also suggest where isolates have high level aminoglycoside resistance further *in vitro* susceptibility testing is required, as some of these isolates will be susceptible to streptomycin. Where isolates are ß-lactamase producers or where the patient has a well documented penicillin allergy treatment should be with a combination of vancomycin and gentamicin. However such combinations should be monitored regularly as drug toxicity is an important consideration when therapy may be required for 4-6 weeks.

1.3.2. Bacteraemia

Bacteraemia without endocarditis is a less distinctive but more common clinical problem than endocarditis alone. Parker and Ball (1976) found that 10.6% of 250 bacteraemia isolates were enterococci. Maki and Agger (1988) have shown in a review of cases between 1970-1983 in the University of Wisconsin Hospitals that the incidence of nosocomial enterococcal bacteraemia has increased since 1975. They found a total of 153 enterococcal bacteraemias, with 65 cases involving the simultaneous isolation of other microorganisms, most commonly gram-negative bacilli. The mean age of patients was 50 years, with men twice as common as women. Serious underlying conditions were present in 133 cases, and nosocomial acquisition identified in 118 cases. As with endocarditis, disease of the urinary tract is frequently associated with bacteraemia, as were burns or wounds and the presence

of intravascular catheters (Maki and Agger 1988, Parker and Ball 1975). In a study by Haslett *et al* (1988) on the microbiology of central venous catheters *E.faecalis* was the second most common organism isolated after *Staphylococcus epidermidis*, and was frequently present in combination with another organism. When detected on the vascular catheter tip, *E.faecalis* was frequently also present in peripheral blood and at the insertion site. Musher (1988) found that 23 out of 97 cases of polymicrobial bacteraemia involved enterococci.

Fever may be the only clinical sign when the bacteraemia consists solely of enterococci; this may be due to the lack of endotoxin production by the enterococci. Mortality rates are reported to be between 34% and 46% (Shlaes *et al*, 1981; Malone *et al*, 1986; Maki and Agger, 1988) at least in part due to the serious associated underlying illness. In the study of Maki and Agger (1988) progression of bacteraemia to endocarditis was more common in the solely enterococcal bacteraemias, one suggested reason for this was that in the polymicrobial infections clinical signs were more apparent, and so prolonged bactericidal antimicrobial treatment was more likely to have been instituted. These authors suggest treatment of conditions likely to cause enterococcal bacteraemia because of the high subsequent mortality. Specific therapy of such conditions has been shown to improve survival (Hoge *et al*, 1991).

1.3.3. Urinary tract infection

Urinary tract infections (UTI's) with enterococci are most frequent in men who have had recent instrumentation or have a structural abnormality. Gross *et al* (1976) in a study of elderly men in hospital used antibiograms as the typing method and showed that the mode of spread was endogenous with the individuals own faecal flora as the source of infection, the incidence of enterococcal infection was 21% and transfer by hospital personnel was not involved. In North America enterococci are the third commonest cause of urinary tract infection after

Escherichia coli and *Pseudomonas aeruginosa* causing 14.7% of urinary tract infections (Centre for Disease Control, 1986). In the United Kingdom the incidence was 7.2% in 1980 (Meers *et al*, 1981). Savarino *et al* (1987) found that 11.8% of 1336 streptococcal UTI's were due to enterococci. Morrison and Wenzel (1986) undertook a retrospective survey of nosocomial urinary tract infection due to *Enterococcus sp.* in a single hospital in the United States. They defined nosocomial as an infection that was neither present nor incubating when the patient was admitted to hospital. They demonstrated an increasing incidence of enterococcal UTI, possibly related to cephalosporin use, although no information was obtained pertaining to whether nosocomial infections were due to isolates from the patients own flora or whether there was spread between patients. The same authors also showed that nosocomial UTI's due to the enterococci had increased from 6-16% over a ten year period, and that this closely parallelled the increase in cephalosporin usage over the same time period.

1.3.4. Neonatal infections

Around 10% of neonatal infection is due to enterococci (Murray, 1990a). The setting in which these infections occur has largely involved sick premature infants frequently with invasive devices *in situ*. The source may be a combination of maternal vaginal colonisation and possibly transmission on the hands of staff members (Coudron *et al*, 1984; Luginbuhl *et al*, 1987). Enterococci are probably more important in neonatal infection than has been thought, because of dissmisal in the past of enterococci as bacteriological contaminants. Diagnosis of enterococcal infection, as with many neonatal infections is difficult, with signs and symptoms frequently being non-specific.

1.3.5. Other enterococcal infections

Intra-abdominal and pelvic infections are likely to be endogenous with the enormous reservoir of gut organisms as the obvious source, especially after

abdominal surgery. The pathogenicity of enterococci in abdominal infection is a matter of debate. Some evidence suggests that polymicrobial infections with *Escherichia coli* or *Bacteroides fragilis* are more likely to result in death or abscess formation than infection with enterococci alone (Onderdonk *et al*, 1976). In an animal study Ike *et al* (1984) showed that haemolysis by the strain of enterococcus injected into mice was a determinant for abscess formation.

Soft tissue infections which involve enterococci are usually mixed and affect previously injured tissue such as burns and diabetic or decubitis ulcers rather than previously healthy tissue. Cellulitis does occur, predominantly in a clearly compromised patient. Skin and soft tissue infection is a common source of enterococcal bacteraemia (Musher, 1988).

Pure enterococcal pneumonia was reported by Berk *et al* (1983) in two patients receiving combination cephalosporin and aminoglycoside therapy. In the study of Parker and Ball (1976) enterococci were the cause of 4.3% of 116 cases of purulent disease. Infection of the central nervous system is uncommon, occuring in neonates and older patients. A serious underlying disorder may be present, or as Bayer *et al* (1976) discussed an anatomical cause such as a skull fracture to result in the enterococcal meningitis.

1.3.6. Polymicrobial infection

Onkerdonk (1976) showed that pure enterococcal infection in rats cause little problem, while in combination with gram-negative bacilli death or abscess formation is more likely. One explanation of this is that the presence of *E.faecalis* in a mixed culture has been shown to protect bacteroides strains from the killing effect of a daptomycin and metronidazole combination even at a concentration of 4-8 times the MIC (Nagy *et al* 1990).

1.3.7. Superinfection

In 1981 Yu reported superinfection including two bacteraemias with enterococci associated with a then new broad spectrum antibiotic moxalactam. Several studies on enterococcal bacteraemia have reported data concerning the prior use of broad-spectrum agents ineffective against the enterococci with a range of 42-77% of patients previously exposed (Hoffman and Moellering, 1987). The study of Morrison and Wenzel (1986) on UTI documented the increase in cephalosporin use at the hospital from 64,000-114,000 g/year.

1.4. Nosocomial infection

Nosocomial infections are defined as those acquired while in hospital. The source of such an infection may be from the patient i.e. endogenous, or from some other external source i.e. exogenous. Nosocomial infections may be serious, particularly exogenous ones, as the organism is more likely to be multiply resistant. The disease and increased hospital stay that are encountered result in significant morbidity.

Nosocomial infection caused by the enterococci is of increasing importance. The Centre for Disease Control (Atlanta) in 1986 listed enterococci as the third most common nosocomial pathogen after *E.coli* and *S.aureus*. Indeed the most recent American figures suggest that the enterococci are now a more common nosocomial pathogen than *S.aureus* (Moellering, 1992). Ford-Jones *et al* (1989) found that 4.9% of 4684 hospital acquired infections were due to enterococci, commonly isolated from UTI's and post-operative wound infections. That enterococci are a major cause of nosocomial infection is incontrovertible, the question now is are these endogenous or exogenous infections or a mixture of the two.

1.5. Endogenous versus exogenous

Evidence for an endogenous source of infection was provided by Gross *et al* (1976) in a three month surveillance study on enterococcal urinary tract infection. The workers used antibiograms as their epidemiological marker and found no evidence for exogenous spread of organisms. In a review Kaye (1982) wrote, "All available evidence indicates that in the vast majority of cases, enterococcal infections of all types are acquired from the patients' own flora". During the 1980's a variety of studies using a number of typing techniques challenged this statement.

In 1984 Coudron *et al* reported an outbreak of bacteraemia and meningitis caused by *E.faecium* in a neonatal intensive care unit. Conventional biotyping with 26 different biochemical and physiological tests was found to be inadequate as a means of typing; improved discrimination was obtained by using commercially available rapid identification products. Isolates from infected patients, clustered in the neonatal intensive care unit and had different biotypes from 29 epidemiologically unrelated isolates with which they were compared. Epidemiological information suggested a bowel source for the epidemic strain, and possible transfer on the hands of hospital personnel.

Zervos *et al* (1986; 1987a) showed that nosocomial spread of enterococci does occur. As an isolate marker they used the then uncommon feature of high level gentamicin resistance and also plasmid content to show that environmental surfaces and the hands of hospital personnel were implicated in the spread of enterococci. There was temporal and geographical clustering, and the probability of spread between two hospital sites. In the 1986 study three deaths were associated with enterococcal infection.

In a retrospective study on neonatal enterococcal sepsis Luginbuhl *et al* (1987) used a combination of phenotypic tests and plasmid content as epidemiological markers. They showed that isolates from a peak of enterococcal

sepsis over a six month period were similar to each other and different from the heterogenous endemic nursery strains, supporting the claim of a nosocomial outbreak of infection, and also suggesting exogenous spread.

Uttley *et al* (1989) when first reporting high level vancomycin resistant enterococci did so from an intensive care unit where the temporal and geographical data suggested the exogenous transfer of bacteria. However two different enterococcal species were involved, *E.faecalis* (two different serotypes) and *E.faecium*. In general the typing information did not allow any firm conclusions about the method of spread of infection between patients. Both plasmid and chromosomal sites for the vancomycin resistance gene were postulated, suggesting the possibility of a transposon.

It has recently been shown that a ß-lactamase-producing, high-level aminoglycoside-resistant *E.faecalis* was rapidly disseminated among patients and staff on an infant-toddler surgical ward (Rhinehart *et al*, 1990). There were no infections attributable to the strain, but patients and staff were colonised. Eradication was not possible despite careful infection control measures and a move to different premises until one of the nursing staff colonised by this strain was removed. A case-control study showed that children who acquired this organism were 12 times more likely to have received care from this nurse. Characterisation of the isolates by restriction enzyme digestion of plamid DNA showed the same pattern from colonised patients and the colonised nurse. It is interesting to note that despite 78 children becoming colonised by this uncommon strain, some of whom had serious underlying illnesses, none developed an associated infection. This information strongly supports the exogenous transfer of enterococci, and contrasts with the findings of Bingen *et al* (1991) who examined 17 vancomycin resistant isolates collected over a one and a half year period from four different wards in one hospital. Using restriction enzyme analysis of chromosomal DNA and ribosomal RNA gene restriction patterns they found no evidence for inter-patient spread; the

only indistinguishable strains were from the same patient. One could argue that this small group of isolates were quite widely dispersed in time and place, and so evidence of spread would be unlikely, however vancomycin resistance is at present an uncommon phenotypic trait, and therefore probably a useful epidemiological marker.

A common pattern can be detected from the above reports. Sick immunocompromised patients such as neonates and renal patients, in environments of high antibiotic usage and frequently with the presence of intravascular devices can succumb to enterococcal infection, but may only be colonised by a new strain. Most of the typing methods mentioned here have relied upon unusual phenotypic characteristics, in particular antibiotic resistances. In order to demonstrate whether exogenous transmission of enterococcal strains which have no readily detectable phenotypic markers is occurring a method of characterisation of these strains is required.

1.6. Antibiotic susceptibilities of the enterococci

The genus enterococcus is already noted for possessing intrinsic resistance to many antimicrobial agents, and in general being less susceptible than the other "streptococci". Those antibiotics which are effective such as penicillin, ampicillin and vancomycin are frequently not bactericidal in action, but can gain bactericidal activity when used in combination with an aminoglycoside. This may not be necessary for an uncomplicated UTI, but is of crucial importance for the more serious infections such as endocarditis.

Toala *et al* (1969) noted a progressive decrease in susceptibility of strains of enterococci to erythromycin, chloramphenicol, and streptomycin, but not to penicillin over the years 1953-1969. In a more recent drug resistance surveillance study Kling *et al* (1989) presented similar data with correlation between local consumption of ampicillin and doxycycline and the prevalence of resistant faecal

organisms. No correlation was observed for cefotaxime or metronidazole, and unfortunately isolates were not identified to the species level.

The range of antimicrobial agents effective in the treatment of severe enterococcal infections has changed in the last 25 years as the organisms have become more resitant. Many of these changes being noted in the last decade. The mechanisms of antimicrobial resistance encountered in the enterococci will be discussed below.

1.6.1. Penicillin resistance

The relative resistance of enterococci to ß-lactam antibiotics is an inherent feature related to the penicillin binding proteins (PBP's). The PBP's are the enzymes involved in the manufacture of the bacterial peptidoglycan cell wall. ß-lactams are analogues of the normal enzyme substrate (D-alanyl-D-alanine) and will interfere with the normal growth process to a certain degree. Cephalosporins and ß-lactams in general have a lower affinity for the PBP's of enterococci than streptococci and so are less active against the enterococci (Fontana *et al*, 1985; Williamson *et al*, 1985). However among the ß-lactams, ampicillin remains a useful treatment for ampicillin sensitive strains. There are also species differences with *E.faecium* having a higher intrinsic resistance to ß-lactams than *E.faecalis* (Mackowiak, 1989; Eliopoulos and Eliopoulos, 1990). Mutations in the structural genes for the PBP's may lead to reduced affinity for the ß-lactams. The affinities of the PBP's for ß-lactams in enterococci have been shown to be directly related to strain sensitivities. Only some of the PBP's are important for determining antibiotic sensitivity (Williamson *et al*, 1985; Chen and Williams, 1987; Fontana *et al*, 1990). PBP 5 in *E.faecium* has a low affinity for penicillin and may be responsible for high level resistance; a novobiocin treated *E.faecium* isolate initially highly resistant to penicillin was found after treatment to have become hypersusceptible to penicillin but was in all other respects identical to the original except for the absence of PBP5.

PBP5 is present in low levels in most enterococci, and is likely to also be the mechanism of the widely acknowledged natural low penicillin susceptibility.

A new development in the resistance of enterococci to the ß-lactams is the synthesis of ß-lactamase, a common feature of Pseudomonas and staphlococcal isolates. This was first reported in *E.faecium* by Murray and Mederski-Samaroj (1983) who also demonstrated that this was present on a transferable plasmid, usually accompanying the genetic information confering high level gentamicin resistance. Such strains have been reported from USA and Argentina, but in general are fairly uncommon (Murray, 1990a; Patterson *et al*, 1988b). The plasmids encoding this information are closely related to those encoding for the ß-lactamase of staphylococci, suggesting direct spread of this information between these genera (Murray *et al*, 1986). Wanger and Murray (1990) showed there was extensive homology between the ß-lactamase determinants from staphylococcal and enterococcal plasmids, but not for other portions of the plasmid suggesting the presence of a transposon. Isolates that produce a ß-lactamase are still treatable with a penicillin/aminoglycoside combination if a ß-lactamase inhibitor such as sulbactam or clavulanic acid are included (Patterson *et al*, 1988b). However detection of these strains can be difficult in the routine laboratory because a small inoculum of the enterococci produces insufficient ß-lactamase to allow growth around a ß-lactam antibiotic disk; this is known as the inoculum effect. Rhinehart *et al* (1990) have demonstrated how quickly and widely ß-lactamase producing isolates can spread.

1.6.2. Aminoglycosides

Enterococci have an intrinsic low level resistance to aminoglycosides. Resistance to the aminoglycosides has altered dramatically over the last thirty years. In 1960 strains in the New York Hospital were usually sensitive to streptomycin (Koenig and Kaye, 1961), however by 1970 40% of enterococci from blood cultures showed high level resistance to streptomycin with previous exposure to

streptomycin a related factor (Mandell *et al* 1970). The mechanisms for this are either ribosomal gene mutation (streptomycin has a single ribosomal binding site as its target) or enzymatic modification. A combination of penicillin and gentamicin was then shown to be an effective treatment.

High level resistance to gentamicin is a more recent development; it was first reported in *E.faecalis* isolates from France by Horodniceanu *et al* (1979) and is now relatively common in many parts of the world. The first report from the USA was by Mederski-Samoraj and Murray (1983). High level gentamicin resistance occurs in 7-10% of enterococci in the UK and 30% of those from blood cultures. Zervos *et al* (1987a) reported an incidence of 55% in a large medical centre in the USA in 1985-6, with figures of 5-45% from other centres. The plasmid characteristics using agarose gel electrophoresis after *Eco* RI digestion from high level gentamicin resistance isolates from ten hospitals in nine geographical areas in the USA were heterogeneous, which suggests that the spread of resistance has been polyclonal (Patterson *et al* 1988a). One gene for high level gentamicin resistance encodes an enzyme with both 6'-acetyltransferase and 2''-phosphotransferase activity and there are other variants (Moellering, 1992). The nucleotide sequences for this gene from *E.faecalis* and *S.aureus* are identical (Feretti *et al*, 1986; Rouch *et al*, 1987). Hodel-Christian and Murray (1990), using plasmid restriction endonuclease analysis and mating experiments, showed that the gentamicin resistance determinant was present on a transposon designated Tn4001. To differentiate the high-level gentamicin resistance from the intrinsic low-level resistance, a single-concentration broth microdilution technique has been proposed (Zervos *et al*, 1987b; Yagupsky *et al*, 1990).

High-level resistance to gentamicin is particularly important because isolates are no longer sensitive to the gentamicin/penicillin synergy, although some of these will still be sensitive to streptomycin (Murray, 1990a). Strains of *E.faecium* with high level gentamicin resistance have also been reported (Hoffman, 1987).

With the documentation of transfer between hospitals (Zervos *et al*, 1987b) emerges the question of screening and infection control measures that are required. Nachamkin *et al* (1988) observed that "neither infection refractory to therapy nor relapse of infection is a common sequelae of gentamicin resistant strains of enterococci in hospitalised patients." At present the optimal treatment for the enterococci resistant to both gentamicin and streptomycin is unknown, some will respond to penicillin, ampicillin or vancomycin. Alternatives are discussed by Eliopoulos and Eliopoulos (1990) and include: a continuous infusion of ampicillin, addition of a ß-lactamase inhibitor if required, the lipopeptide daptomycin, rifampicin or a fluoroquinolone. There are drawbacks to all of these proposals, in particular, they are largely bacteriostatic agents. A combination of ampicillin, vancomycin and azithromycin has been used over an 18 month period in a patient with an *E.faecium* endocarditis resistant to aminoglycosides (unpublished communication, Oxford Public Health Laboratory). In refractory cases of endocarditis early consideration should be given to valve replacement.

1.6.3. Vancomycin

Vancomycin is a glycopeptide. These are cell wall active compounds affecting peptidoglycan synthesis, and like other agents acting at this site are bacteriostatic. The range of minimum inhibitory concentrations (MIC's) for enterococci is around 1-3 ug/ml (Poulson *et al*, 1989). Vancomycin is usually used in combination with an aminoglycoside for serious enterococcal infections or where the patient is allergic to penicillins (Besnier *et al*, 1990).

Vancomycin resistance in enterococci was first reported in Great Britain in seven *E.faecalis* and 48 *E.faecium* isolates from a renal unit (Uttley *et al*, 1988) and was possibly related to prior treatment of sepsis of unknown cause with a combination of vancomycin and ceftazidime. Selection by prior antibiotic administration was probable as 90% of isolates were from patients who had received

ceftazidine, and 50% of isolates were from patients who had had prior vancomycin. MIC's of greater than 4mg/l are generally considered to indicate isolates with resistance. In the above report they were in excess of 64 mg/l. Leclercq *et al* (1988) also reported resistance to high levels of vancomycin and also to teicoplanin in enterococci, and showed that this was plasmid mediated. They compared two resistant *E.faecium* strains, they were of different phenotypes and had different plasmid restriction patterns, but hybridisation experiments demonstrated extensive homology between the vancomycin resistant determinants. There was no cross resistance with the related group of antibiotics the lipopeptides, although isolates resistant to glycopeptides did appear to be prone to a one-step mutation to become lipopeptide resistant. Since 1988 reports of vancomycin resistance with MIC's in the range 64-2000 mg/L have been published from various European countries and the USA (Murray, 1990a). In the UK the Public Health Laboratory Service continues to report vancomycin resistant enterococci; and now from more diverse locations than the original London based reports (CDR Weekly).

Two mechanisms of resistance have been detected, a low level non-transferable chromosomal resistance (MIC 32-64 mg/L) with cross-resistance with teicoplanin after induction by vancomycin, but not inducible by teicoplanin alone; or a high level chromosomal or plasmid-borne resistance with cross resistance to, and inducible by, teicoplanin. One group (Shlaes *et al*, 1989a; Shlaes, 1989b; Al-Obeid *et al*, 1990) have studied the proteins associated with low level and high level vancomycin resistance, and have described associated proteins with molecular weights of 39.5 and 39 KDa respectively. It may be that both proteins function by interference with the pentapeptide target of glycopeptides, although this mechanism has not been confirmed. The protein associated with high level resistance was different in *E.faecium* and *E.faecalis*, which would suggest that dissemination of a plasmid or transposon is not the whole story. However the presence of a transferable gene means that additional therapeutic agents are urgently needed.

A vancomycin resistant *E.gallinarum* strain has been recovered during vancomycin prophylaxis (Kaplan *et al*, 1988), this strain was involved in a polymicrobial infection which eventually resulted in the failure of the femoral haemodialysis graft. Green *et al* (1990) studied vancomycin resistant gram-positive cocci in faeces in a cross-sectional survey in a paediatric ward. They found that 14 isolates from 11 of 48 children were resistant, and 5 of these were enterococci; only one child had previously received vancomycin. These results suggest that vancomycin resistant enterococci may not be uncommon in the gastrointestinal tracts of hospitalised children. Management of patients with vancomycin resistant enterococci includes barrier nursing as with MRSA to control the spread of the organism. It is important to note that there is loss of synergy with gentamicin.

Organisms such as the Leuconostocs, Lactobacilli, Pediococci and Erysipelothrix are inherently resistant to vancomycin, and can be confused with the enterococci under certain circumstances. It is therefore important to ensure that vancomycin resistant enterococci are indeed enterococci (Green *et al*, 1990).

Teicoplanin is also a glycopeptide and initially it was hoped that a once daily regime might be possible with this compound. This has not proved to be the case, and the doses suggested initially have been found to be inadequate (Sullam *et al*, 1985). In general high level vancomycin resistant strains are also resistant to teicoplanin, so the place of teicoplanin in enterococcal infection has, as yet, not been defined.

1.6.4. Quinolones

The fluoroquinolones are broad spectrum bactericidal agents which influence the function of DNA gyrase. This group of antimicrobials have recently become more important. Nalidixic acid, the early example in this group has been used mainly in urinary tract infections (Doulder and Snodgrass, 1989). Ciprofloxacin, one of the newer quinolones, has had wide applications advocated,

including its use in the more severe invasive salmonella infections. Ciprofloxacin in general is not greatly active against the enterococci, but has been useful in the Dulwich outbreak (Uttley *et al*, 1989). A new quinolone WIN 57273 is similar to ciprofloxacin, and has good *in vitro* activity against gram-positive pathogens including MRSA and gentamicin resistant strains of *E.faecalis* (Kaatz *et al*, 1990).

1.6.5. Other antibiotics

Plasmid or transposon-borne resistance has been recorded for chloramphenicol and tetracycline, and also for lincomycin, streptogramin B and erythromycin (Engel *et al*, 1980).

Imipenem is a member of the thienamycins, an antimicrobial group which contains the ß-lactamase ring. It is administered in combination with cilastin sodium an inhibitor of dehydropeptidase I, the enzyme which metabolises imipenem in the normal human kidney. Imipenem appears to be effective against the enterococci at a lower MIC than cefotaxime, cefoperazone or piperacillin (Tutlane *et al*, 1981). Maki and Agger (1988) and others have suggested that *E.faecium* are resistant to imipenem.

Daptomycin, a new lipopeptide, a drug family related to the glycopeptides has good activity against high level gentamicin-resistant strains, ß-lactamase producers and vancomycin-resistant strains, however neurotoxicity has been reported (Stratton *et al* 1987).

1.6.6. Synergy

The phenomenon of increased susceptibility of enterococci to a penicillin and an aminoglycoside in combination when compared to the effects of the compounds separately has been explained as being due to the facilitated entry of aminoglycosides by penicillins damaging the cell wall, and loss of this synergy occurs when the ribosomal site of action of the aminoglycoside becomes modified

(Zimmerman *et al*, 1971). The magnitude of this synergistic effect has been shown to be species specific (Moellering *et al*, 1979).

In summary, the more recent developments concerning antimicrobial agents and the enterococci have been the emergence of high level transferable gentamicin resistance (Horodniceanu *et al*, 1979; Courvalin *et al*, 1980; Patterson *et al*, 1988a), reports of ß-lactamase production (Murray and Mederski-Samoraj 1983) on two or more self transferable plasmids (Patterson *et al*, 1988b), alterations in penicillin binding proteins (Fontana *et al*, 1985; Chen and Williams, 1987) and of resistance to vancomycin (Kaplan *et al*, 1988; Leclercq *et al*, 1988; Uttley *et al*, 1989).

1.7. Plasmids and transposons in the enterococci

Genetic elements which can move between isolates are central to the problem of antibiotic resistance in the enterococci. The first discovery of the association between plasmids and antibiotic resistance in the enterococci was by Courvalin *et al* (1972, in Clewell, 1990). He showed that two different plasmids were associated with the resistances to erythromycin and to tetracycline. Jacob and Hobbs (1974) showed that conjugal transfer of plasmid-borne antibiotic resistance was possible. The conjugal transfer is of two forms, high fequency in broth, and low frequency on solid surfaces. These are also different in that in the former, the transferable plasmids also code for a response to sex pheromones produced by the recipient strain (Engel *et al*, 1980). *E.faecalis* is unique at present in having pheromone-inducible plasmid transfer. Each pheromone will induce a response only in cells carrying a particular plasmid (Dunny, 1990). Once the recipient acquires a copy of the plasmid it shuts down production of only the related sex pheromone, continuing the production of any others so that further pheromone enabled plasmid transfer could occur.

That certain antibiotic resistance determinants on plasmids are widely disseminated among group D streptococci was demonstrated by LeBlanc *et al* (1986) who looked at erythromycin, kanamycin and streptomycin resistance determinants. Using probes for the above determinants, they showed that plasmid-enriched DNA from 70% of 91 isolates from widely dispersed animal and human sources contained these or highly related genes. Detailed analysis of five different sized plasmids showed a 9-11 kilobase-pair region with extensive DNA homology which included the resistance determinants. These results suggest a common origin for the resistance genes studied or wide transfer of this DNA, possibly involving a transposon.

1.8. *Streptococcus bovis*

Interest has been stimulated recently in the non-enterococcal group D streptococci. *S.bovis* and *S.equinus* do possess the Lancefield group D antigen, but do not grow in 6.5% NaCl. *S.equinus* does not appear to be an important cause of human disease, while *S.bovis* has been implicated in disease of veterinary origin (Garvie and Bramley 1979), and also in human disease for example endocarditis. *S.bovis* was first proposed by Orla-Jenson (1919), and subsequently two biotypes have emerged. *S.bovis* biotype I which forms dextran and ferments mannitol, and *S.bovis* biotype II which does not form dextran and usually does not ferment mannitol (Facklam, 1972; Parker and Ball, 1975). *S.bovis* is a normal inhabitant of the human bowel also both are common causes of endocarditis. Whereas enterococci cause a wide range of human disease, *S.bovis* is usually only associated with endocarditis.

The high prevalence of *S.bovis* as a causative agent in endocarditis has been revealed largely through some retrospective surveys of infections thought to have been due to the enterococci. *S.bovis* was found to have been included along with the enterococci (Moellering *et al*, 1974). The phenotypic similarity between

the *Enterococcus sp.* and *S.bovis* resulted in this misidentification. One quarter of cases of endocarditis in patients over the age of 55 years investigated by Parker and Ball (1975) were *S.bovis* biotype I, and it was a more common cause than enterococci. An equal prevalence in endocarditis was suggested by Moellering *et al* (1974). Correct species identification is important in this situation because the antimicrobial treatment is different for these two groups; *S.bovis* is normally sensitive to penicillin, while an enterococcal endocarditis should probably be treated with the synergistic combination of penicillin and the potentially toxic aminoglycosides. Allergy to ß-lactam and failure of previous treatment would be indications for the use of vancomycin-aminoglycoside combinations in *S.bovis* endocarditis (Besnier *et al*, 1990).

An association has been documented between *S.bovis* endocarditis and bacteraemia or septicaemia with colonic malignancy (Klein *et al*, 1977; Klein *et al*, 1979; Klein *et al*, 1987). Over one third of patients with *S.bovis* septicaemia prospectively studied had a colonic abnormality, mostly carcinoma (Klein *et al*, 1979), and such patients should have colonic investigations. In a study of bacteraemia associated with *S.bovis* and *S.salivarius*, Rouff *et al* (1989) showed that the biotype I of *S.bovis* was strongly associated with colonic neoplasia (71%) and bacterial endocarditis (94%), a far greater association than for biotype II of *S.bovis*. This bacteriological marker of serious colonic pathology deserves further investigation, especially because diagnostic delay is the main cause of mortality in colonic malignancy.

1.9. Typing methods

The routine identification of the enterococci has been mainly based upon the criteria of Sherman (1937) and upon the presence of the Lancefield group D antigen, both of which still provide valuable information for the placing of isolates into the genus *Enterococcus*. Individual species can then be assigned using the tests

described in Table 2. In practice this is often unnecessary, and probably only occurs when dealing with life threatening infections. Identification below species level requires more highly discriminatory methods of characterisation, and these are discussed below.

In general phenotypic methods of characterisation of isolates are gradually becoming discredited as it becomes clear that exhibited characteristics relate poorly to the sum total of the genomic information. It is therefore preferable to examine the genome directly. However phenotypic methods are at present commonly used as they are the easiest methods to apply in the routine laboratory. The methods include biotyping, serotyping, bacteriophage typing, antimicrobial susceptibility typing, whole cell protein electrophoresis, multilocus enzyme electrophoresis and the production of bacteriocins. Methods of typing based upon genetic material include plasmid analysis and RFLP either with or without a probe, and the polymerase chain reaction.

1.9.1. Biotyping

Biochemical reactions in general do not show sufficient variation to be useful in the typing of enterococci unless a large number of tests are employed. Several workers have used large batteries of biochemical tests to differentiate isolates. Hall *et al* (1976) when describing human-to-human transmission of enterococci causing endocarditis, used antibiograms and biochemical reactions to compare the strains. Coudron *et al* (1984) used a combination of conventional biotyping and commercially available kits to type *E.faecium* isolates. In a study of neonatal sepsis Luginbuhl *et al* (1987) used a wide range of the more traditional typing methods, with a combination of 35 coventional physiological tests, also 30 tests from the automicrobic Gram-positive identification (GPI) kit, and also plasmid analysis. The authors indicate that the methods employed did distinguish between the epidemic and endemic strains.

1.9.2. Serotyping

Serological typing of the enterococci emerged as a possible method after the group D antigen and also type-specific antigens were detected (Lancefield, 1933; Elliot, 1959). In 1952 Sharpe and Shattock found 24 different serological types in a group of 353 group D strains; 20% of strains were untypable. There did not appear to any direct relationship between serological type and the physiological characteristics of isolates except for a partial correlation with some of the species as then defined. A more recent study using eight typing antisera found only two different serotypes with half of isolates untypable (Smyth *et al*, 1987). Serotyping of the enterococci does not appear to be a useful technique for epidemiological studies.

1.9.3. Antibiograms

Although minor species differences have been observed with respect to antibiotic susceptibilities within the enterococci (Toala *et al*, 1969; Mackowiak, 1989), this has not been found to be useful as a typing method at the sub-species level. However uncommon antimicrobial resistance patterns have been important in some enterococcal epidemiological studies. Gross *et al* (1976) used antibiograms to demonstrate that enterococcal urinary tract infections were derived from the patients own perineal flora. In the study by Zervos *et al* (1987a) into nosocomial infection by *E.faecalis*, the marker of high level resistance to gentamicin was used as a screening test; at the time this was a relatively uncommon marker. In the work of Luginbuhl *et al* (1987) the prime typing method was of ß-lactamase production and aminoglycoside resistance; unusual markers for the hospital where the work was based where screening for them for the previous six years had revealed no previous isolates.

1.9.4. Bacteriophage typing

Bacteriophages are viruses; their use in typing is due to their selective colonisation and either lysis of particular strains of bacteria, or incorporation into the host genome in a state of lysogeny. A set of different bacteriophages is usually selected to increase the sensitivity of the typing, and bacterial strains are then compared by testing them against this set. The main advantage of the technique is that it can be applied to a wide range of bacterial species, particularly ones untypable by other means. The technique can be very sensitive, but is technically demanding, including the need for environmental controls because these can affect the susceptibility of the test bacteria to bacteriophage infection.

The conclusions of the various workers with enterococci in this area have differed. Brock (1964) suggested that bacteriophage typing of the enterococci was unlikely to be useful because of the lack of reproducibility of the results. Smyth *et al* (1987) used a combination of biotyping, serotyping and phage typing to examine a group of 30 isolates including some from haemodialysis in-patients. Surprisingly, only four different combinations of these three properties were obtained, nevertheless the authors presented this as evidence for possible spread of strains. Kuhnen *et al* (1988) used a combination of bacteriophage typing and bacteriocin production to examine 971 group D isolates from two intensive care units. They used bacteriophages that had been used in previous studies, and obtained 26 different groups with 596 (61.4%) isolates being in a single group and 21% of isolates being untypable.

1.9.5. Bacteriocin typing

Bacteriocins (sometimes called enteroccines when produced by the enterococci) are a group of bactericidal antibiotic-like substances produced by bacteria. They have a narrow spectrum of activity, usually acting upon the same species.

Kekessy and Piguet (1971) examined 130 strains of *S.faecalis* by bacteriocin sensitivity typing (six producer strains) and bacteriocin production typing using six indicator strains. They found 13 sensitivity types, and 10 production types. In total there were 25 different combinations of the two. They did not apply the system to any particular epidemiologically defined group. Tagg and Bannister (1978) included 12 group D isolates in a large study on ß-haemolytic streptococci, examining both production and sensitivity to bacteriocins. Both forms of typing were able to subdivide the Lancefield serological groups although incubation conditions were found to be critical, and the system labour intensive. The large study of Kuhnen (1988) has been mentioned above (1.8.4.). With a battery of bacteriocin producers eight groups of isolates were obtained, with 21.5% being untypable. Combining the two methods; bacteriophage and bacteriocin typing, resulted in a method which could type 87% of isolates and gave 187 different combinations when species affiliation was also included.

In general bacteriocin typing is time consuming and the reagents (strains producing the bacteriocins) are difficult to standardise between laboratories, and so the system has not gained general acceptance.

1.9.6. Plasmid analysis

Extra-chromosomal plasmids can be extracted from bacteria and examined directly on agarose gels to determine the size of the plasmid or plasmids present, giving a plasmid profile for that strain. These profiles can then be compared between strains. Plasmids indistinguishable by size can be digested with a restriction enzyme and the resulting fragment sizes compared. This technique is particularly useful for large plasmids because agarose gel electrophoresis is generally not a good technique for differentiating the sizes of large molecules (Farrar, 1983). Many plasmids have been shown to confer resistance to a variety of antibiotics, and are then known as resistance factors or R plasmids. For plasmid analysis to be of use

there must be at least one plasmid present. A good example of total plasmid analysis is that of Schaberg *et al* (1981) where a variety of gram negative bacteria from seven nosocomial outbreaks of infection were studied. The total plasmid patterns were the same for isolates from a given outbreak.

Plasmids in enterococci have been examined without necessarily the intention of using them as a typing method, for example the work of Engel (1980) and that of le Bouguenec and Horodniceanu (1982) when comparing the plasmids of ten *E.faecalis* isolates. Enterococci do not tend to carry a large number of plasmids, and hence the potential of this method as a typing system is limited. As mentioned earlier (in section 1.5), the dissemination of ß-lactamase-producing, aminoglycoside resistant *E.faecalis* among patients and staff on a paediatric surgical ward was assisted by plasmid analysis of some isolates (Luginbuhl *et al* 1987). Plasmids have been used to document cross infection by vancomycin resistant *E.faecium* isolates in a renal unit (Bateman *et al*, 1992). In a study on 66 ampicillin resistant *E.faecium* isolates Donabedian *et al* (1992) found a surprising 51 different groupings when the plasmid DNA was examined by RFLP using the endonuclease *Hind* III.

Some of the difficulties with plasmid analysis include spontaneous plasmid loss during subculture and storage, and difficulties in reproducibly collecting and digesting plasmids (Donabedian *et al*, 1992). Plasmids themselves can be mobile between different strains or even between different species of bacteria. O'Brien *et al* (1980), showed that a single plasmid confering resistance to gentamicin was disseminated into at least six different bacterial species in one hospital. In addition plasmids may contain transposons. Transposons are translocatable DNA sequences which can move between plasmids and between plasmids and genomic DNA. The deletion or addition of this genetic material will alter the size of the plasmid, further complicating plasmid analysis as a typing tool.

Many of the disadvantages of plasmid DNA analysis can be overcome by studying the bacterial chromosome directly.

1.9.7. Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) or "DNA fingerprinting" has been applied to epidemiological studies of a large variety of bacteria (Owen, 1988), fungi (Scherer and Stevens, 1987) and viruses (Buchman *et al*, 1978). In bacteria, the technique involves the extraction of chromosomal or total cellular DNA and then the digestion of this with a restriction endonuclease followed by separation of the fragments by some form of electrophoresis, most commonly using agarose gels. The theoretical advantage of the technique is that a part of the bacterial genome is being directly examined, and then compared between isolates without consideration of the phenotype.

The first microbiological application of RFLP was for the *Herpes simplex* virus by Buchman *et al* (1978) who showed clustering of infection within a hospital and so documented a nosocomial outbreak. An early use of the technique in bacteria was by Collins and de Lisle (1985) who examined 25 different restriction enzymes in their study on *Mycobacterium bovis* and other Mycobacteria and concluded that the method was useful for both the inter- and intra-species classification within the Mycobacteria.

Applications of the technique of RFLP to the Streptococci followed. Cleary *et al* (1988) found that DNA fingerprints of *S.pyogenes* were M-type specific, and so correlated with the serological typing, and those not typable serologically did possess a different DNA fingerprint. Denning *et al* (1989) examined a group of 54 Group B Streptococci; these isolates had been epidemiologically carefully characterised, and included two mother-baby pairs and unrelated isolates. Digestion with two different restriction endonucleases for some isolates was employed to increase the specificity. They showed both good discrimination between the isolates

(28 RFLP patterns for the 54 isolates), and also good correlation with the available epidemiological information. Evidence for the widespread dissemination of a limited number of strains was presented. Mogollon *et al* (1990) showed heterogeneity within serotypes of *S.suis* from animal and human infections useful for epidemiological studies. The same authors also found evidence of a clonal relationship between epidemiologically unrelated isolates. Recently Bingen *et al* (1991) examined 16 vancomycin resistant *E.faecium* isolates from four wards in a childrens hospital. RFLP showed that all isolates were different (except for two isolates from the same patient), whilst restriction fragments hybridising to an rRNA gene probe (ribotyping) was less discriminatory.

One problem with RFLP has been the large number of fragments of genomic DNA which may result from digestion using a restriction endonuclease that cuts frequently. This results in a large number of fragments which are closely related in size and hence difficult to separate on an electrophoresis gel. This can be overcome by using an enzyme which cuts less frequently and separating the fragments by pulsed-field gel electrophoresis. This technique was used by Allardet-Servent *et al* (1989) investigating outbreaks of *Acinetobacter calcoaceticus* infection in a urology department, and *Pseudomonas aeruginosa* in an Intensive Care Unit. Murray *et al* (1990b) examined 27 *E.faecalis* isolates by pulsed-field gel electophoresis from three diverse locations and demonstrated both genetic diversity within each location, and possible spread of strains within a location.

1.9.8. Restriction fragment hybridisation to an rRNA gene probe (ribotyping)

As mentioned above there is potential for obtaining an enormous number of bands when total cellular bacterial DNA is digested with a restriction endonuclease, particularly one that cuts frequently, making comparison between banding patterns difficult. The number of bands can be reduced by hybridising with probes for

specific sequences, with the appropriate probe a limited number of bands is obtained making comparison between patterns simpler.

In the latter half of the 1980's a wide range of organisms, including yeasts, mycoplasmas, gram-positive and gram-negative bacteria were studied with ribotyping (Owen, 1989). A wide variety of restriction endonucleases and probes have been used. One particularly useful and commercially available probe is ribosomal RNA from *E.coli*. This has a highly conserved sequence (Woese, 1987) and is present in a multiple but restricted number of copies in all bacteria. Grimont and Grimont (1986) suggested that the resulting patterns were potential taxonomic tools epecially for biochemically relatively inert species. This probe has also been shown to be useful for the intra-species characterisation of bacteria and hence applicable to epidemiological studies. For example Collins *et al* (1990) while examining *Mycobacterium paratuberculosis* from various animal and bird groups demonstrated that there were two distinct groups of strains which were distributed in different host animals, and that ribotyping with a probe specific to a repetitive DNA sequence in *M.paratuberculosis* revealed a larger number of subdivisions than did RFLP. Jordens and Pennington (1991) while examining 94 isolates of *Neisseria meningitidis* by ribotyping found 13 patterns, with outbreak-associated strains all being of the same pattern.

1.9.9. Polymerase chain reaction (PCR)

PCR is a process whereby amplification of a specific DNA sequence results from repeated directed DNA synthesis. Three principal steps are involved: the denaturation of the DNA; binding of specific primers for the 3' and 5' ends of the required sequence; and extension from the primers using the heat stable DNA polymerase from *Thermus aquaticus*. Specific primers are required for the process, and so some knowledge of nucleotide sequence data of the organism is required. At present the predominant clinical use of PCR is to detect pathogens which are

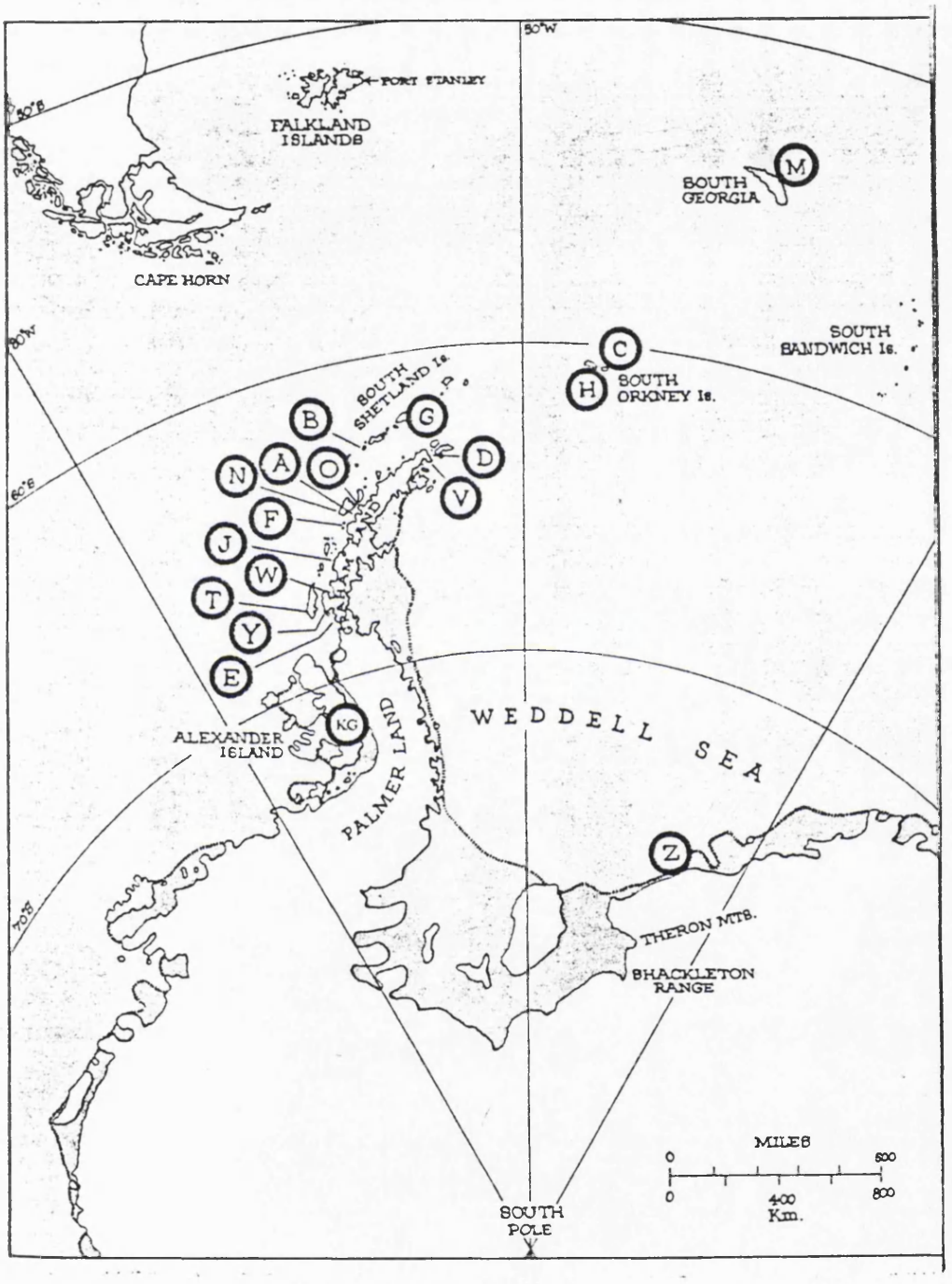
difficult to grow, especially viruses. The applications of PCR in typing is reviewed by Eisenstein (1990), the amplified sequence would then normally be examined by RFLP or by ribotyping.

1.10. The British Antarctic Survey (BAS) and the isolated community

In 1943 a small naval operation code-named Operation Tabarin was launched to prevent the Germans having any influence upon the Southern side of Drakes passage (that area of ocean between Cape Horn and the Antarctic Peninsula) (Figure 1). From this small war-time beginning developed the Falkland Islands Dependencies Survey in 1945, and the British Antarctic Survey in 1959. Since Operation Tabarin, British bases have been permanently staffed in the Antarctic with a swift move towards solely scientific work once World War II had ended.

The British Antarctic Survey operates five permanently staffed stations in Antarctica. Each has a resident wintering group of between three and twenty-five men. The stations are named Bird Island, Signy, Faraday, Rothera and Halley (Figure 1).

Figure 1. Map of British Antarctic Territory[a]



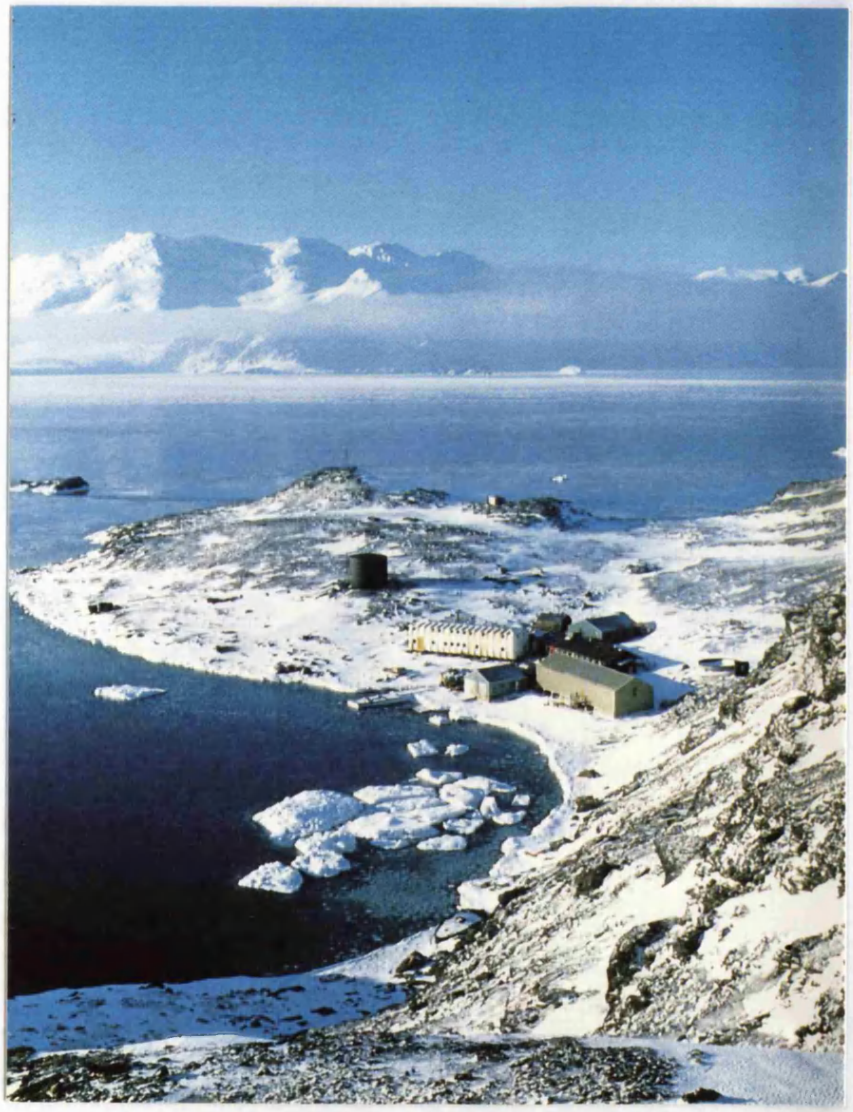
[a]From Of Ice and Men, Sir Vivien Fuchs, 1982, Published by Anthony Nelson. Letters indicate past and present British stations. The five bases in use today are; M, South Georgia (Bird Island); H, Signy Island; F, Argentine Islands (Faraday); T, Adelaide Island (Rothera); Z, Halley Bay.

Most personnel are on two year contracts, with approximately half of the base complement changing at the end of each year. Of these bases, four (H, F, T and Z in Figure 1) are entirely isolated from outside human influence over the Southern winter for a variable amount of time dependent upon the site of the base, the extent of the sea ice and the itinerary of the support ships and aircraft. The base on Signy Island (Figure 2) in the South Orkneys where the Antarctic isolates in this study were collected is principally a biological research base investigating the seal, bird and plant life on the island.

These closed communities on the Antarctic bases provide an ideal environment for the study of the transfer of bacterial strains between individuals in the absence of external sources of human bacteria (Maggs and Pennington, 1989). This will aid the understanding of bacterial spread during nosocomial outbreaks of infection.

Signy station (Figure 2) consists of a relatively unplanned group of partially interconnecting buildings upon the site of what was originally a small whaling station. Signy Island (60°43'S, 45°36'W) is part of the South Orkneys and is approximately 7 kilometers long and 4 kilometres wide and is periodically connected to adjacent islands by sea-ice during the winter period. There is an abundance of wildlife, in particular seals and bird life including penguins, with no indigenous land mammals, although sledge dogs were kept on the base until the early 1960s. Within the base living conditions are comfortable and spacious, although the sleeping accommodation is mostly in the form of shared rooms.

Figure 2. British Antarctic Survey Base Signy, South Orkney Islands



(E. Lemon/BAS)

Materials and personnel were brought to the base over the Southern summer by the BAS supply vessels Royal Research Ship (RRS) Biscoe and RRS Bransfield. Other vessels were also visitors to the base over the summer period. In the winter only vessels of a heavy ice breaking capability would be able to reach the base.

Food, sufficient for the entire year, is supplied in dried, tinned or frozen form. In content it is similar to a conservative British diet with main meals usually consisting of meat, potato and two vegetables with frequent carbohydrate-laden snacks. The living areas within the building area are all well heated throughout the year. The amount of time spent outside the base varies a good deal between individuals, especially in the colder winter months, but work and recreation does continue throughout the whole of the year. Recreation consists of day-trips, or 1-2 week excursions, sleeping in huts or 2 man tents. No specialised thermal equipment is used during the winter; each individual has a personal issue of adequate clothing to last the year. Outside the base the temperature varies between +15°C and -25°C throughout the year.

Four out of the five BAS research bases normally include a Medical Officer in their complement. I was the Medical Officer at Rothera base for a year in 1988-1989. The function of this base is largely to support and coordinate the movement of summer research personnel into the field for a variety of projects. Many doctors have included some research in their workload and several have worked in microbiology. I was collecting gram-negative bacilli from the faeces of humans, husky dogs and seal with a view to conducting a temporal study over the winter, however isolate survival was very poor at less than 10%, possibly due to a break in the cold chain, or alternatively a reflection of the hardiness of the gram-negative bacilli compared with the enterococci. Similar methods were used by Dr C Fenton the Medical Officer on Signy Base for the collection of the Antarctic isolates of enterococci used in this study.

1.11. The aims of this study

The epidemiological investigation of enterococcal infections has been hampered by the lack of a simple typing method which is discriminatory at the intraspecies level (Aber and Mackel, 1981; George and Uttley, 1989; Murray, 1990a). Four of the twelve described enterococcal species are examined in this thesis, including the two most clinically relevant at the present time (*E.faecalis* and *E.faecium*). Representatives of the other genera that have in the past come under the umbrella term streptococcus are included for comparison. These are a type strain of *Lactococcus lactis*, and three isolates of *Streptococcus bovis*, including a type strain.

The aims of this study were to examine the suitability of chromosomal DNA analysis; both restriction fragment length polymorphism (RFLP) and ribotyping, for the intraspecies differentiation of enterococci, and also to compare this data with the characterisation of the same isolates by the more traditional methods of antibiograms and biochemical tests. Warnock (1984) suggested three desirable features that should be worked towards in any typing scheme; that it differentiate a sufficient number of strains, that it should give clear and reproducible results, and that it be reasonably simple to perform. This study examined these criteria when assessing the typing methods employed. A further aim was to apply the typing techniques described above to examine the epidemiology of enterococci in the isolated community. This might aid our understanding of enterococcal transfer in nosocomial outbreaks of infection.

2. MATERIALS AND METHODS

2.1. Isolate collection

2.1.1. Antarctic sample collection

In 1988, the year of this investigation, there were 12 resident base members on the Antarctic base of Signy. Subjects were healthy males aged 20-30 years. Of the 12 resident personnel, 10 volunteered to participate in this study. The degree of this participation did vary amongst the 10 individuals. In 1988 there were no visiting ships to the base over the Southern hemispere winter months between April and October.

Sufficient equipment and materials for the study were brought into the base by the supply ships. Where possible materials were supplied ready for use, but items such as agar plates and incubation broth were prepared by the Medical Officer on site. The medium used for isolation was MacConkeys (Oxoid Ltd.), the broth used for storage was Thioglycollate (Oxoid Ltd.). Media was made up using distilled water from the base supply, and sterilised in a dental autoclave. A methylated spirits lamp was used for loop sterilisation.

2.1.2. Bacterial isolates from Signy Base

Specimen collection began after the last ship had left the base in April 1988 and continued for seven months. Faecal swabs were collected at one monthly intervals, and were plated onto MacConkeys' agar and incubated at 37°C for 24-48 hours. Colonies with appropriate morphology and colour (small magenta colonies) were subcultured onto MacConkeys' agar and incubated overnight for further study. Isolates of presumed enterococci were then confirmed by Gram stain, morphology and a negative catalase reaction and transferred into Thioglycollate broth in 7 ml screw-topped polypropylene bijou bottles for storage. Samples were stored at +4°C while on base and during transport back to the U.K. The maximum length of sample storage was 16 months.

The numbering of the isolates from the Antarctic was designed to give immediate information, and has remained as the Antarctic isolate number throughout this study. The first number refers to the subject from whom the isolate was obtained, the second number to the month in chronological order, and the final number refers to the isolate collected at that time. For example isolate 2105 was from individual 2 in October (month 10) and 5 is the unique identifying number for that isolate. No isolates were collected from subjects 5 or 6.

2.1.3. Control strains

Strains from two other sources were used as controls for the identification and typing of the Antarctic isolates. These were from the National Collection of Type Cultures and the London Hospital.

Seven type strains were obtained from the National Collection of Type Cultures (NCTC). Five were enterococcal type strains: "*Streptococcus faecalis*" NCTC 775, "*Streptococcus faecalis* var. liquefaciens" NCTC 2705, "*Streptococcus faecalis* var. zymogenes" NCTC 10927, "*Streptococcus faecium*" NCTC 7171 and "*Streptococcus durans*" NCTC 8307. Two non-enterococcal but related type strains: "*Streptococcus bovis*" NCTC 8177 and "*Streptococcus lactis*" NCTC 6681 were also included.

The second group of control strains were obtained from faecal specimens from patients attending an outpatient clinic at the London Hospital (17 isolates). These patients were selected because they represented a "normal human population" who had not been on antibiotic treatment. These isolates were numbered London 1-17, abbreviated to LON1-LON17.

2.2. Bacterial identification

2.2.1. Sample revival

All samples from the Antarctic were transported at +4°C to the Department of Medical Microbiology, University of Aberdeen. The thioglycollate broths were sampled with a loop and plated onto blood agar and incubated at 37°C. The plates were examined for growth after 24 and 48 hours and any growth subcultured onto MacConkeys medium. If there was no growth on the original blood agar plate, then approximately 3 ml of nutrient broth (Oxoid Ltd.) was added to each bijou bottle, and the bottle incubated at 37°C for 24-72 hours. These bottles were then sampled for growth by plating onto blood agar and incubating for 24-48 hours. Samples still showing no growth were presumed to have perished during storage and transport.

2.2.2. Species identification

Isolates were subcultured on to blood agar, and API 20 Strep (API-bioMerieux Ltd) was used for species identification, according to the manufacturers instructions. Appropriate additional tests were included as recommended by the API 20-Strep Analytical Profile Index (1989) supplemented by the companies Computer Identification Service (version 4) and with reference to the literature. Any Antarctic isolates which were not identified as enterococci were excluded from further study.

The additional tests used in this study included two of the first described features of enterococci; growth in 6.5% NaCl and growth at 45°C (Sherman, 1937; Schleifer and Kilpper-Balz, 1987). The production of acid from glycerol and from melibiose were used as tests to differentiate *E.faecium* isolates from *E.durans* isolates, with the former positive for both tests and the latter negative for both tests (Farrow *et al*, 1983; Collins *et al*, 1984; Schleifer and Kilpper-Balz, 1987). A yellow colony colour has been considered a basic feature of *E.casseliflavus* isolates (Collins *et al*, 1984), and was used to confirm the identity of the isolate included in this study. These

additional tests were only applied when the API 20 Strep did not give a definite species identification.

Two other tests were included and applied to all isolates. These were for the presence of the Lancefield group D antigen (Lancefield, 1933; Murray, 1990a) as determined by latex agglutination (Oxoid), and susceptibility to vancomycin (Facklam and Collins, 1989; Facklam *et al*, 1989; Murray, 1990a). In general enterococci do possess the group D antigen, and are susceptible to vancomycin, although there are exceptions to both of these generalisations.

The identification of the strains in the two control groups were also confirmed by the methods described above.

2.3. Strain typing

2.3.1. Biotyping

The data collected while identifying the 184 isolates included in this study using the API 20 Strep was applied as a typing scheme. This was done by making the assumption that isolates of the same API 20 Strep profile number were of the same strain. The confirmatory biochemical tests additional to the API 20 Strep were not included here because not all isolates were tested.

2.3.2. Antibiotic susceptibility testing.

Antibiograms were determined for all enterococcal strains by the disc diffusion method. An inoculum of ca. 10^5 cfu/ml in neutralised bacteriological peptone (Oxoid, code L34; 1% wt/vol) pH 7.0, was incubated overnight at 37°C on blood agar (Oxoid). The following antibiotics were used: ampicillin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (30 μg), imipenem (10 μg), piperacillin (75 μg), teicoplanin (30 μg), and vancomycin (30 μg).

Strains were classified as susceptible or resistant, with all intermediate zone sizes being scored as susceptible for ease of interpretation. The zone sizes for imipenem and ciprofloxacin were initially difficult to classify as susceptible or resistant and so all zone sizes for these were measured with a ruler and transillumination. The following zone sizes or smaller (as used for clinical isolates in the Medical Microbiology Department of Aberdeen University) were used to indicate resistance to an antibiotic; ampicillin (11mm), ciprofloxacin (13mm), erythromycin (13mm), gentamicin (12mm), imipenem (13mm), piperacillin (12mm), teicoplanin (10mm) and vancomycin (9mm). The results for most of the antibiotics were clear cut, and with assistance from personnel experienced in reading sensitivity plates a resistant or susceptible label was attached. The Oxford Staphylococcus was used as the control.

Isolates were also tested against an antifolate, using 1.25 μg trimethoprim discs. These results were invalid because enterococci unlike most other bacteria do have the ability to incorporate exogenous folates and so are resistant *in vivo*; *in vitro* testing of enterococci is clinically misleading as it is dependent upon the presence or abscence of exogenous folates or folate precursors in the media (Goodhart, 1984). The possibility of this agent contributing to an antibiogram typing scheme was not pursued, except to note in passing that one enterococcal isolates appeared "resistant" to trimethoprim, as did the four non-enterococcal isolates.

2.3.3. Restriction fragment length polymorphism (RFLP)

2.3.3.1. DNA extraction

Bacterial DNA was extracted by a modification of the method of Pitcher *et al* 1989. Bacteria from 1-2 blood agar plates were harvested with a dry swab into a microfuge tube containing 0.5ml TE buffer (10mM Tris(pH 8.0), 1mM EDTA) and centrifuged (MSE Microcentrifuge) at 6000g for 1 minute. The resulting pellet was resuspended in 100μl of TE buffer containing 500mg/ml lysozyme (Sigma), and incubated at 37°C for 30 minutes. Lysis of bacteria was completed by the addition of

0.5ml GES reagent (containing 5M guanidium thiocyanate, 0.1M EDTA and 0.5% v/v Sarkosyl). The samples were mixed by inversion and then left for 10 minutes at room temperature; 0.25ml 7.5 M ammonium acetate was added at +4°C and the tubes placed on ice for 10 minutes. One half of a millilitre of chloroform:isoamylalcohol (24:1) was then added and the samples mixed thoroughly and then centrifuged at 12,000g for 20 minutes. DNA was recovered from the resulting upper aqueous phase by precipitation with 0.54 volumes of cold isopropanol (-20°C) followed by 30 minutes storage at -20°C and centrifugation at 12,000g for 2.5 minutes. The resulting pellets were resuspended in 0.2ml TE buffer and the DNA re-precipitated by the addition of sodium chloride to a final concentration of 0.1 M. Then 2.5 volumes (0.5ml) of cold absolute alcohol were added followed by 20 minutes storage at -20°C and centrifugation at 6,000g for 2.5 minutes. The resulting DNA pellets were air dried at room temperature and resuspended in 100-200μl of TE buffer, and stored at +4°C.

2.3.3.2. Phenol extraction

Some DNA extracts autodigested in the presence of restriction endonuclease buffer, but in the absence of restriction endonuclease. These DNA extracts were subjected to a phenol extraction to remove this endonuclease activity. This was performed by increasing the volume in the microcentrifuge tube 0.3 ml with TE buffer, and adding an equal volume of phenol/chloroform/isoamylalcohol mixture (25:24:1). This tube was shaken for 5 minutes and then centrifuged at 12000 g for 2 minutes. Using a pipette the upper aqueous layer (approximately 0.2 ml) was transfered to a fresh tube and the DNA collected by precipitation with absolute ethanol in the presence of sodium chloride as described above.

2.3.3.3. Other extraction methods

Several methods of DNA extraction were investigated before consistent yields were obtained. The initial method of bacterial DNA extraction used was that described by Pitcher *et al* (1989) without any of the modifications described above. Some success

was obtained, but there were problems including a poor yield of DNA probably related to inadequate cell lysis. Also the DNA extracts did not dissolve well in the TE buffer.

Mutanolysin has been used for the extraction of DNA from group A Streptococci. An attempt was made to use this lysing agent for DNA extraction in the enterococci. In our hands we were unable to obtain sufficient DNA for restriction endonuclease digestion from enterococci nor indeed from clinical isolates of Group A streptococci causing infections in the Deeside area of Aberdeenshire, and thus the method was abandoned.

2.3.3.4. Evaluation of the purity and yield of DNA.

The purity and yield of DNA was estimated by spectrophotometry (Pye Unicam PU8600 UV/VIS Phillips) at 260 and 280 nm (Maniatis *et al* 1982), where one optical density unit at 260 nm corresponds to 50 ug/ml of double stranded DNA. Quartz cuvettes were used. The integrity of the DNA was determined on 0.8% horizontal agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3).

2.3.3.5. Staining and photographing of gels

Agarose gels were stained with 100 μl of ethidium bromide in 250 ml of tap water for 30 minutes and then destained in plain tap water for a further 30 minutes. Gels were then photographed (Polariod MP-4 Land camera) under ultraviolet transillumination (UVP Cambridge UK model TM-20) using was Polaroid Type 665 positive/negative black and white instant pack film.

2.3.3.6. Restriction endonuclease digestion

The DNA samples (5 μg) were digested overnight with restriction endonucleases (RE's) under conditions recommended by the manufacturer (Boehringer-

Mannheim, Bell Lane, Lewes, East Sussex, UK), with the addition of RNAase to 0.05 mg/ml. The RE's tested included *Bam* HI, *Eco* RI, *Hind* III, *Sac* I and *Sal* I.

After digestion, the loading buffer was added and the DNA fragments were separated by horizontal submerged gel electrophoresis 0.8% agarose gels in TBE buffer at 100 volts on a 15cm x 15cm gel for 4 hours (electrophoresis equipment - Bio-Rad DNA SubCell TM, and electrophoresis power supply - EPS 500/400 Pharmacia). A 1 kb "ladder" (Gibco-BRL) was used as the molecular size marker. A visual migration marker of bromophenol blue was also used. Gels were stained and destained as described above.

2.3.4. Details of the restriction endonucleases

Five different restriction endonucleases were examined in this study. *Bam* HI was isolated from *Bacillus amyloliquefaciens* and recognises the cutting sequence G/GATCC. *Eco* RI was isolated from *Escherichia coli* and recognises the cutting sequence G/AATTC. *Hind* III was isolated from *Haemophilus influenzae* and recognises the cutting sequence A/AGCTT. *Sac* I was isolated from *Streptomyces achromogenes* and recognises the cutting sequence GAGCT/C, there is also an isoschizomer named *Sst* I. *Sal* I was isolated from *Streptomyces albus* and recognises the cutting sequence G/TCGAC.

2.3.5. Ribosomal RNA (rRNA) gene restriction patterns (ribotyping)

2.3.5.1. Probe preparation

Ribosomal 16 + 23s rRNA from *Escherichia coli* (Boehringer) (10 μg) was end labelled with 50 μCi ö^{32}P-ATP (Amersham International, UK) using 10 units of T4 polynucleotide kinase in 50 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine and 0.1 mM EDTA. After 60 minutes incubation at 37°C the reaction was terminated by the addition of 10 μl of 0.2% SDS, 20 mM EDTA, and the probe used without further purification.

2.3.5.2. Southern blotting and DNA hybridization

Restriction endonulease digests of chromosomal DNA using *Eco* RI or *Hind* III were separated on 0.8% agarose gels, and then depurinated, denatured, neutralised and transferred to nylon membranes (Hybond-N, Amersham International) by the method of Southern as modified by Maniatis *et al* (1982). The DNA was fixed to the membrane using a three second exposure to ultraviolet light. These Southern blots were pre-hybridised for 3 hours at 65°C in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 x FPG (1 x FPG is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone 350, 0.02% glycine), 0.5% SDS and 100μg/ml denatured salmon sperm DNA. The probe was added to this and the resulting hybridization mix incubated at 65°C overnight. Hybridized filters were washed (Grimont and Grimont, 1986), and then exposed at minus 70°C to X-ray film for two days using an intensifying screen.

2.3.6. Analysis of banding patterns

Both the RFLP patterns and the ribotyping patterns were compared visually. Isolates with similar patterns but on different gels were then run on the same gel for further comparison.

3. RESULTS

3.1. Survival of Antarctic isolates.

Of nearly 700 presumptive enterococcal isolates collected in Antarctica 172 were found to be viable when subcultured after reaching the UK. The overall survival of the isolates was therefore relatively poor with only 25% of the samples collected surviving. Of these 172 isolates, 160 were confirmed as enterococci. The 172 isolates had been collected up to 16 months prior to their laboratory examination in Scotland, selected in the Antarctic on the criteria of being gram positive cocci, catalase negative and on colonial morphology when grown on MacConkeys medium. Since 160/172 of the surviving isolates were enterococci, the identification criteria used in the Antarctic were therefore appropriate.

The results of the identification of the 172 Antarctic isolates by API 20 Strep was as follows; *Enterococcus faecium* (112), *Enterococcus faecalis* (12), *Enterococcus durans* (34), genus *Enterococcus* (2), *Streptococcus viridans* (10), *Lactococcus lactis* (1) and *Streptococcus salivarius* (1). The Antarctic *S.salivarius*, *S.viridans* and *L.lactis* isolates were excluded from all further study, with concentration upon the enterococcal species (160 isolates, 93% of the surviving isolates), and their comparison with enterococci from the two other sources described.

3.2. Species identification

The 160 isolates from the Antarctic, together with the 7 NCTC strains and 17 London Hospital strains, making 184 isolates in total were all identified to the species level (with the exception of 2 isolates from Antarctica, identified only to the level of the genus *Enterococcus*) using the biochemical tests (API 20 Strep) with supplementary tests as described in the methods. All isolates except for the type strain of *L.lactis* possessed the Lancefield group D antigen.

3.2.1. API results

Twenty-four different API profiles were obtained for the 184 isolates; 20 different API profiles if the four non-enterococcal strains are excluded (Table 3). Additional confirmatory tests for API profiles of low discrimination between species were required for 102 (55%) isolates.

3.2.2. Confirmatory testing

Details of the additional confirmatory tests used are given in Table 3. Tests not specifically requested by the API system are recorded in parenthesis.

The API profiles 7157411 (40 isolates) and 7357411 (19 isolates) were listed as "low discrimination", with *E.durans* and *E.faecium* to be differentiated. The biochemical tests used to differentiate between these two species were the aerobic production of acid from glycerol, and also from melibiose. Both of these sugar fermentation tests were positive for all the 7357411's (19/19), and most of the 7157411's (38/40); these organisms were therefore classified as *E.faecium*. The two remaining 7157411 isolates fermented melibiose, but not glycerol and could not be identified to the species level using this system, although they were confirmed to be *Enterococcus* sp. They were ultimately identified to species on the basis of their DNA fingerprint as discussed below. The physiological tests of growth in 6.5% NaCl and at 45°C were positive for all of these 59 isolates.

Table 3. API 20 Strep profiles and confirmatory tests

API profile	Isolates[a] Tot	Ant.	Species	Confirmatory tests[b]
1240443	1	0	*S.bovis*	
5043411	1	0	*L.lactis*	
5113411	5	5	*E.durans*	6.5%+ (45°C+ AM+)
5157511	4	4	*E.faecium*	
5240463	1	0	*S.bovis*	
5650452	1	0	*S.bovis*	
7113411	29	29	*E.durans*	6.5%+ (45°C+ AM+)
7143711	6	0	*E.faecalis*	
7153311	1	1	*E.faecalis*	
7157411	40	40	38 *E.faecium*	6.5%+ AG+ 45°C+ (AM+)
			2 *Enterococcus* sp.	6.5%+ AG- 45°C+ (AM+)
7157510	1	1	*E.faecium*	
7157511	24	22	*E.faecium*	
7157551	1	0	*E.faecium*	
7173311	7	7	*E.faecalis*	
7173711	7	4	*E.faecalis*	
7353400	1	0	*E.durans*	
7353411	1	0	*E.durans*	
7357411	19	19	*E.faecium*	AG+ (6.5%+ 45°C+ AM+)
7357510	9	9	*E.faecium*	
7357511	13	13	*E.faecium*	
7357541	1	1	*E.faecium*	
7357551	8	3	*E.faecium*	Y-
7377510	2	2	*E.faecium*	
7357571	1	0	*E.casseliflavus*	Y+
Tot 24	184	160		

All API profiles except 5043411 represent isolates which were positive for the Lancefield group D antigen.

[a]Tot., total number of isolates. Ant., number of isolates from the Antarctic.

[b]Confirmatory tests: 6.5%, growth in 6.5% NaCl; 45°C, growth at 45°C; AG, the production of acid from glycerol; AM, the production of acid from melibiose; Y, colony colour yellow.

+, positive for a confirmatory test; -, negative for a confirmatory test.

Parenthesis indicate those confirmatory tests not suggested by the API database version 4.

The other main area of confirmatory testing was for isolates with the API profile 5113411 (5 isolates) and isolates with the API profile 7113411 (29 isolates) which were poorly discriminatory between the species *E.durans* and *L.lactis*. All 34 isolates grew at 45°C, and also in 6.5% NaCl which are not characteristics of *L.lactis*, and so they were confirmed to be *E.durans*. These isolates as mentioned earlier also possessed the Lancefield group D antigen.

The single isolate with the API profile 7357571 was identified as one of *E.gallinarum*, *E.faecium*, or *E.casseliflavus*. The latter normally has yellow pigmented colonies as did this isolate, and hence this isolate was classified as *E.casseliflavus*.

The eight isolates with the API profile 7357551, were listed as a "doubtful profile" on version 4 of the API computer database. This doubtful profile does not distinguish between *E.gallinarum* and *E.faecium*; they did not produce yellow colonies, and so were not *E.casseliflavus*. The July 1982 API 20 Strep catalogue, and the version 3 of the database both list this API profile as *E.faecium*, the isolates with this profile were therefore classified as *E.faecium*. This will be discussed later.

The summary of species identified in this study by biochemical tests with the API 20 Strep was as follows, 120 *E.faecium*, 36 *E.durans*, 21 *E.faecalis*, 1 *E.casseliflavus*, 2 isolates of the genus *Enterococcus* but not initially allocated to species, 3 *S.bovis* and 1 *L.lactis*. The source and species distribution of the 184 isolates is given in Table 4.

Table 4. Source and species distribution of isolates

Species	NCTC*	Outpatient	Antarctic	Total
E.faecalis	3	6	12	21
E.faecium	1	7	112	120
E.durans	1	1	34	36
E.casseliflavus	0	1	0	1
S.bovis	1	2	0	3
L.lactis	1	0	0	1
genus *Enterococcus*	0	0	2	2
Total	7	17	160	184

Assignment to species is based upon the combined results of API 20 Strep profile and confirmatory tests.

*National Collection of Type Cultures, London, U.K.

3.3. Typing of the enterococci

3.3.1. Biotyping

The API 20 Strep is designed with the aim of differentiating between different species within the streptococci and related taxa. It is not designed as a tool for differentiating between strains. However the large number of different tests incorporated into the system can be used to give an indication of the amount of biological variation between isolates of the same species. Isolates are discussed below with assignment to species as described earlier, and also with the two isolates of the genus *Enterococcus* included within the species they were assigned to by RFLP in section 3.3.4.

3.3.1.1. *E.faecalis*

The 21 *E.faecalis* were represented by 4 different API profiles (Table 3).

The following tests were positive for all *E.faecalis* isolates; acetoin production from pyruvate (Voges-Proskauer), hydrolysis of hippurate, bile-esculin reaction, pyrrolidonylarylamidase, leucine arylamidase, arginine dihydrolase, acid from ribose, mannitol, sorbitol, trehalose and starch.

The following tests were negative for all *E.faecalis* isolates; α-galactosidase, ß-glucuronidase, acid from L-arabinose, inulin, raffinose and glycogen, and for ß-haemolysis.

Three different biochemical tests gave variable results for the 21 *E.faecalis* isolates, and these are detailed in Table 5.

3.3.1.2. *E.faecium*

This larger group of 121 isolates was represented by 11 different API profiles.

The following tests were positive for all *E.faecium* isolates; acetoin production from pyruvate (Voges-Proskauer), bile-aesculin reaction, pyrrolidonylaryamidase, ß-galactosidase, leucine arylamidase, arginine dihydrolase, acid from L-arabinose and lactose.

The following tests were negative for all *E.faecium* isolates; ß-glucuronidase, acid from sorbitol, inulin and starch and for ß-haemolysis.

Eight biochemical tests out of 21 were variable for the 121 *E.faecium* isolates, and these results are detailed in Table 6.

3.3.1.3. *E.durans*

Most of the 37 *E.durans* isolates appeared to be biochemically similar with five different API profiles including the isolate identified by DNA fingerprinting.

The following tests were positive for all *E.durans* isolates; acetoin production from pyruvate (Voges-Proskauer), bile-aesculin reaction, pyrrolidonylarylamidase, ß-galactosidase, arginine dihydrolase, acid from ribose and lactose.

The following tests were negative for all *E.durans* isolates; ßglucuronidase, alkaline phosphatase, acidification of manitol, sorbitol, inulin, raffinose and starch, and for ß-haemolysis

Six different biochemical tests gave variable results within the 37 *E.durans* isolates examined, these results are detailed in Table 7.

Table 5. *E.faecalis* biotyping results

No. of isolates	API 20-Strep	Test[a]			*Sal* I pattern
		ßGAL	AP	LAC	
6	7143711	-	-	+	6U(a-f)
1	7153311	+	-	-	1J
7	7173311	+	+	-	7J
7	7173711	+	+	+	3U(g-i), 4J

Total 21

[a]ßGAL, ß-galactosidase; AP, Alkaline phosphatase; LAC, acid from lactose.

Table 6. *E.faecium* biotyping results

No. of isolates	API 20-Strep	Biochemical test[a]								*Sal* I[b]
		HIP	αGAL	AP	RIB	MAN	TRE	RAF	AMD	
39[c]	7157411	+	-	-	+	-	+	-	-	4
4	5157511	-	-	+	-	+	-	+	+	1
1	7157510	+	-	-	+	+	+	-	-	1
24	7157511	+	-	-	+	+	+	-	+	8
1	7157551	+	-	-	+	+	+	+	+	1
19	7357411	+	+	-	+	-	+	-	+	2
9	7357510	+	+	-	+	+	+	-	-	2
13	7357511	+	+	-	+	+	+	-	+	7
1	7357541	+	+	-	+	+	-	+	+	1
8	7357551	+	+	-	+	+	+	+	+	6
2	7377510	+	+	+	+	+	+	-	-	1

Tot 121

[a]HIP, hydrolysis of hippurate; αGAL, α-galactosidase; AP, alkaline phosphatase; acid from RIB, ribose; MAN, mannitol; RAF, raffinose; AMD, starch.

[b]Number of RFLP patterns obtained for each API 20 Strep profile. Details in Table 16.

[c]Includes one isolate assigned to species by DNA fingerprint pattern.

Table 7. *E.durans* biotyping results

No. of isolates	API	Test[a]						*Sal* I
		HIP	αGAL	LAP	ARA	TRE	AMD	
5	5113411	-	-	-	-	+	+	5K
29	7113411	+	-	-	-	+	+	28K, 1UD1
1	7353400	+	+	+	-	-	-	1UD2
1	7353411	+	+	+	+	+	+	1UD3
1[b]	7157411	+	-	+	+	+	+	1K
Total 37								

[a]HIP, hydrolysis of hippurate; αGAL, αgalactosidase; LAP, leucine arylamidase; acid from ARA, arabinose; TRE, trehalose; AMD, starch.

[b]Isolate assigned to species by RFLP pattern.

3.3.1.4. *S.bovis*

Each of the three *S.bovis* isolates gave a different API profile.

The following tests were positive for the three *S.bovis* isolates; acetoin production from pyruvate (Voges-Proskauer), α-galactosidase, leucine arylamidase, acid from lactose, raffinose and starch.

The following tests were negative for the three *S.bovis* isolates; hydrolysis of hippurate, pyrrolidonylarylamidase, alkaline phosphatase, arginine dihydrolase, acid from ribose, arabinose, mannitol, sorbitol, and for ß-haemolysis. The six different biochemical characteristics which were variable are shown in Table 8.

A summary of the constant and variable biochemical results for the four species presented above is given in Table 9.

3.3.2. Antibiotic susceptibility testing

An antibiotic resistance pattern (antibiogram) was determined for all 184 isolates examined in the study. Five different combinations of antibiotic resistances were encountered, these 'Antibiograms' are detailed in Table 10. All 184 isolates were resistant to gentamicin, and were susceptible to ampicillin, piperacillin, vancomycin and teicoplanin. Out of the 184 isolates in this study, 23 were resistant to more than gentamicin alone, they are detailed in the Table 11. The distribution of antibiograms for each bacterial species is given in Table 12.

Table 8. *S.bovis* biotyping results

No. of isolates	API	Test[a]						*Sal* I
		ESC	ßGUR	ßGAL	TRE	INU	AMD	
1	1240443	-	-	-	-	-	+	UB3
1	5240463	+	-	-	-	+	+	UB2
1	5650452	+	+	+	+	-	-	UB1
Total 3								

[a]ESC, bile aesculin reaction; ßGUR, ß-glucuronidase; ßGAL, ß-galactosidase; acid from TRE, trehalose; INU, inulin; AMD, starch.

Table 9. Individual API 20 Strep test variability for each enterococcal species

API test[a]	*E.faecalis* (21)[b]	*E.faecium* (121)	*E.durans* (37)	*S.bovis* (3)
VP	+	+	+	+
HIP	+	v	+	-
ESC	+	+	+	v
PYRA	+	+	+	-
αGAL	-	v	v	+
ßGUR	-	-	-	v
ßGAL	v	+	+	v
PAL	v	v	-	-
LAP	+	+	v	+
ADH	+	+	+	-
RIB	+	v	+	-
ARA	-	+	v	-
MAN	+	v	+	-
SOR	+	-	-	-
LAC	v	+	+	+
TRE	+	+	v	v
INU	-	-	-	v
RAF	-	v	-	+
AMD	+	-	-	v
GLYG	-	+	+	+
HEM	-	-	-	-

+ positive; - negative; v variable

[a]VP, acetoin production from pyruvate (Voges-Proskauer); HIP, hydrolysis of hippurate; ESC, bile-esculin reaction; PYRA, pyrrolidonyarylamidase; αGAL, α-galactosidase; ß-glucuronidase; ßGAL, ß-galactosidase; PAL, alkaline phosphatase; LAP, leucine arylamidase; ADH; arginine dihydrolase; acid from RIB, ribose; ARA, arabinose; MAN, manitol; SOR, sorbitol; LAC, lactose; TRE, trehalose; INU, inulin; RAF, raffinose; AMD, starch; GLYG, glycogen; HEM, ß-haemolysis.

[b]Number of isolates in parentheses

Table 10. Antibiograms

Antibiogram	No. of isolates[a]	Resistant to:
A	161 (144)	Gentamicin
B	15 (10)	Gentamicin, Ciprofloxacin
C	6 (4)	Gentamicin, Erythromycin
D	1 (1)	Gentamicin, Imipenem
E	1 (1)	Gentamicin, Erythromycin, Imipenem
	Total 184 (160)	

[a]Number of Antarctic isolates in parenthesis

Table 11. Isolates resistant to more than gentamicin alone.

Antibiogram Ident No[a]	API Profile	Species	Source	Antibiotic resistance[b]	*Sal* I
B 173	7157511	*E.faecium*	Antarctic	Cip.	D
B 255	7357411	*E.faecium*	Antarctic	Cip.	C
B 486	7157511	*E.faecium*	Antarctic	Cip.	F
B 754	7157411	*E.durans*	Antarctic	Cip.	K
B 7171	7157511	*E.faecium*	NCTC	Cip.	U10
B LON6	7357571	*E.casseliflavus*	London	Cip.	UCa
B LON8	7157551	*E.faecium*	London	Cip.	U15
B 351	5113411	*E.durans*	Antarctic	Cip.	K
B 356	7113411	*E.durans*	Antarctic	Cip.	K
B 377	7113411	*E.durans*	Antarctic	Cip.	UD1
B 458	7113411	*E.durans*	Antarctic	Cip.	K
B 4710	7113411	*E.durans*	Antarctic	Cip.	K
B 1153	7113411	*E.durans*	Antarctic	Cip.	K
B LON17	1240443	*S.bovis*	London	Cip.	UB3
B 6681	5043411	*L.lactis*	NCTC	Cip.	ULa
C 71011	7357511	*E.faecium*	Antarctic	Ery.	H
C 1054	7357551	*E.faecium*	Antarctic	Ery.	I
C 10619	7357551	*E.faecium*	Antarctic	Ery.	U9
C 10714	7357411	*E.faecium*	Antarctic	Ery.	Nil
C LON9	7143711	*E.faecalis*	London	Ery.	Ua
C LON16	5650452	*S.bovis*	London	Ery.	UB1
D 256	7357411	*E.faecium*	Antarctic	Imip.	C
E 799	7357511	*E.faecium*	Antarctic	Imip., Ery.	H

Total 23

[a]Refers to either the Antarctic identification number as described in section 2.1.2. or the London (LON) number or the National Collection of Type Cultures (NCTC) number.

[b]Cip., ciprofloxacin; Ery., erythromycin; Imip., imipenem.

Table 12. Distribution of antibiograms by species

Species	Antibiogram[a]	Number of isolates	
E.faecium	A	110	
	B	5	
	C	4	
	D	1	
	E	1	(not A 9.1%)
E.faecalis	A	20	
	C	1	(not A 4.8%)
E.durans	A	30	
	B	7	(not A 18.9%)
E.casseliflavus	B	1	
S.bovis	A	1	
	B	1	
	C	1	
L.lactis	B	1	
		Total 184	

[a]Abbreviations as in Table 10

In general the methodology used here is too imprecise for any detailed examination of the antibiotic susceptibilities of these 180 enterococcal isolates, although it equates with the routine work up for many clinical isolates. The main intention was an assessment of the degree of variability of these phenotypic characteristics within this group of isolates and subsequently the applicability of this as a typing scheme. Over 85% of the isolates in this study had the same antibiogram (Table 10).

3.3.3. DNA finger-printing

3.3.3.1. DNA extraction

An effective method of extraction of total cellular DNA from enterococci was developed during the study. DNA of sufficient quantity and purity to obtain fingerprints using at least the restriction enzyme *Sal* I was obtained from a high percentage of isolates 180/184 (98.5%) using this rapid small scale extraction method. A total of 343 individual DNA extractions were performed.

It was not possible to obtain DNA of sufficient quantity and purity for DNA fingerprinting from four isolates. These included two *E.faecium* isolates obtained from the Antarctic (isolates 791 and 10714), and two of the London controls also both *E.faecium* isolates (LON2 and LON4). There were no particular indicators to suggest which isolates it would be difficult to extract DNA from, and the fact that the four isolates were all *E.faecium* may only reflect their preponderance in this study.

Two heavily inoculated overnight blood agar plates were found necessary for the DNA extraction in order to obtain a reasonable DNA yield. In general a final phenol extraction did improve the quality of the fingerprints by reducing the

frequency of overdigestion and increasing the clarity of banding pattern, but there was also an associated reduction in the yield of DNA.

The extraction method yielded 0.02-0.4 mg of intact DNA suitable for RFLP study from 98.5% of isolates.

3.3.3.2. Choice of restriction endonuclease

A pilot study was conducted to determine which of five restriction enzymes was most appropriate for the subsequent examination of all isolates. A broadly representative sample of 17 isolates (Table 13) was selected for this pilot study, and the same isolates later examined by ribotyping. The results of digesting these isolates with the restriction endonucleases *Bam* HI, *Sac* I, *Sal* I, *Eco* RI, and *Hind* III are presented in Figures 3-7.

As shown in Figures 6 and 7, *EcoR* I and *Hind* III resulted in a large number of fragments. By careful scrutiny of the banding patterns it is possible to suspect that the two *E.faecalis* isolates in tracks 2 and 3 are indistinguishable as might be the three *E.durans* isolates in tracks 7, 8 and 9. However the considerable overlap and merging of the large number of bands makes comparison between adjacent tracks very difficult, and the comparison of tracks distant to each other but on the same gel almost impossible.

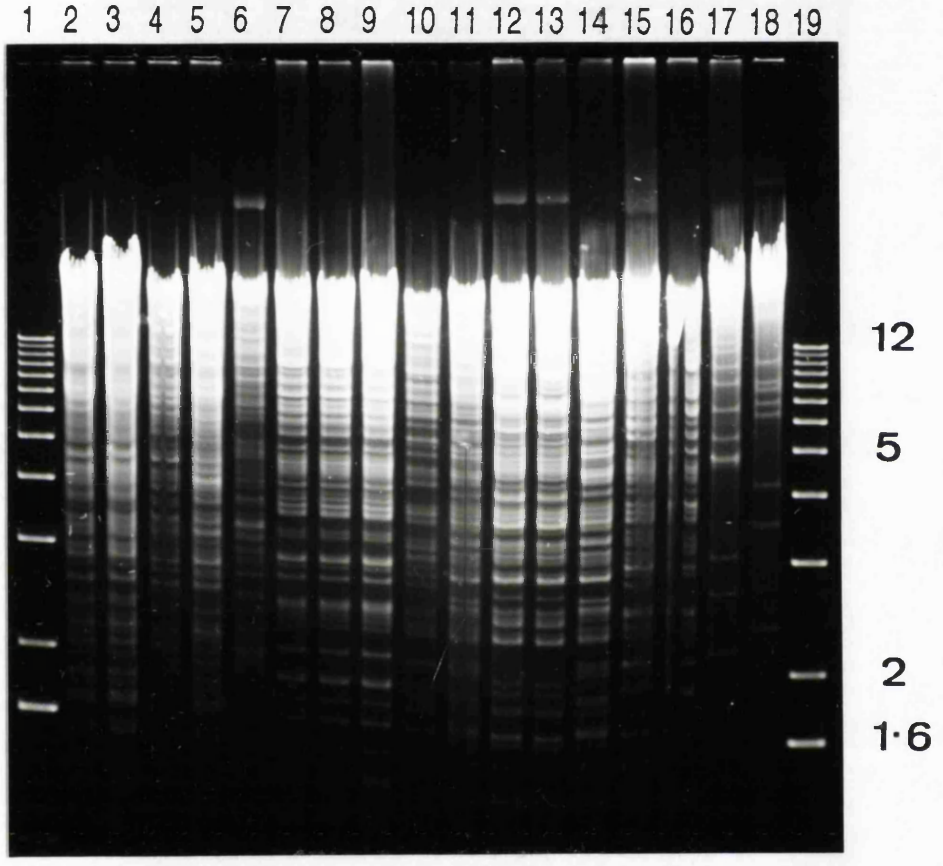
Table 13. Details of the pilot study isolates in Figures 3-7, 11 and 12

Track	Isolate number[a]	Source	*Sal* I pattern[b]	Species
1	1kb marker			
2	872	Antarctic	J	*E.faecalis*
3	8108	Antarctic	J	*E.faecalis*
4	775	NCTC	Ug	*E.faecalis*
5	2705	NCTC	Uh	*E.faecalis*
6	8307	NCTC	UD2	*E.durans*
7	4813	Antarctic	K	*E.durans*
8	377	Antarctic	UD1	*E.durans*
9	1155	Antarctic	K	*E.durans*
10	7171	NCTC	U10	*E.faecium*
11	1068	Antarctic	C	*E.faecium*
12	8725	Antarctic	B	*E.faecium*
13	8812	Antarctic	U5	*E.faecium*
14	1071	Antarctic	C	*E.faecium*
15	178	Antarctic	D	*E.faecium*
16	154	Antarctic	E	*E.faecium*
17	8177	NCTC	UB2	*S.bovis*
18	6681	NCTC	ULa	*L.lactis*
19	1kb marker			

[a]Either the National Collection of Type Cultures (NCTC) number or the Antarctic isolate number as explained in section 2.1.2.

[b]DNA fingerprint pattern using the Restriction endonuclease *Sal* I. All unique patterns are prefixed by the letter U.

Figure 3: Digestion of the pilot study isolates with the restriction enzyme *Bam* HI.

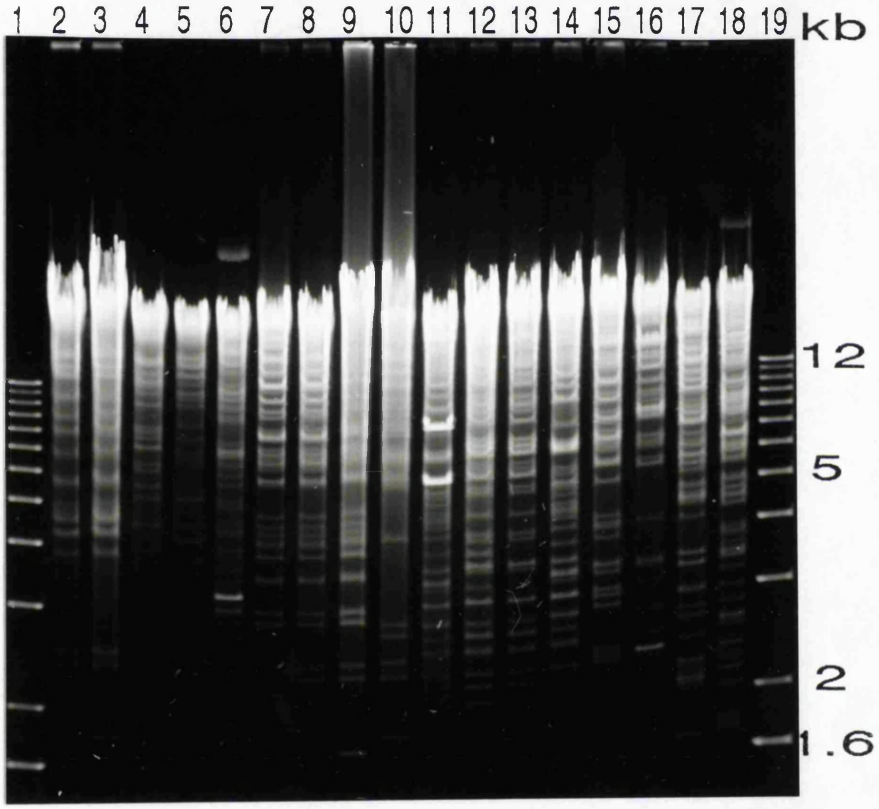


The RFLP patterns in tracks 2-5 are from *E.faecalis* isolates; tracks 6-9 *E.durans* isolates; tracks 10-16 *E.faecium* isolates; track 17 *S.bovis* and track 18 *L.lactis* (see Table 13). Tracks 1 and 19 contain the kilobase (kb) marker.

There is interference from an artefact in track 16.

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, AND NCTC 6681. Further details in the Summary Table.

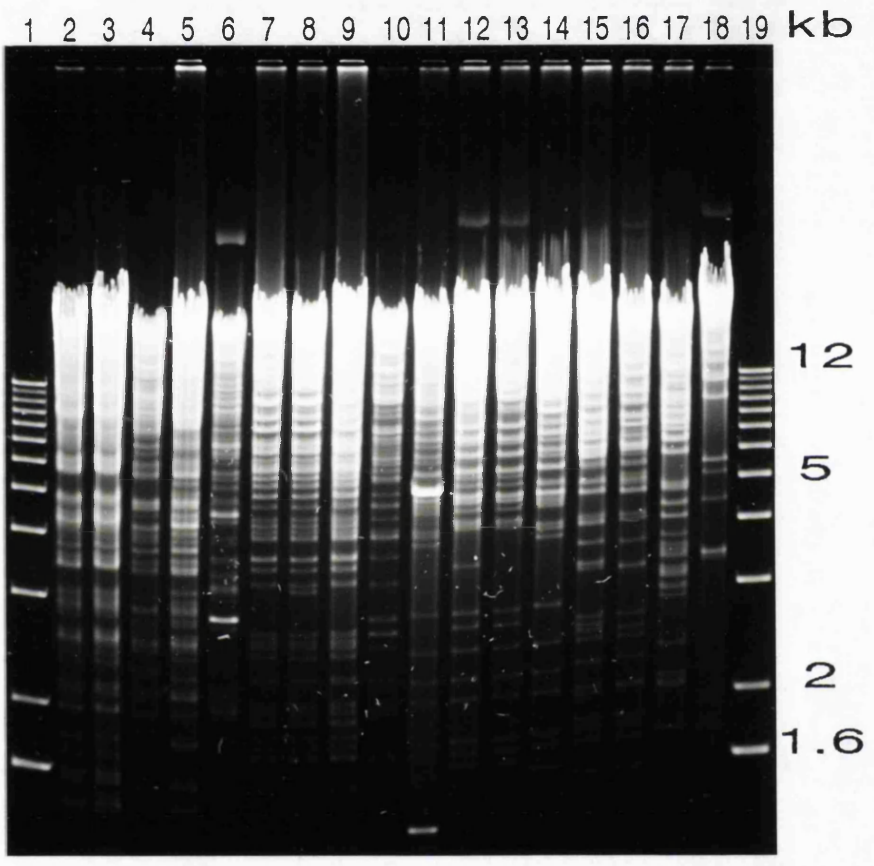
Figure 4: Digestion of the pilot study isolates with the restriction enzyme *Sac* I.



The RFLP patterns in tracks 2-5 are from *E.faecalis* isolates; tracks 6-9 *E.durans* isolates; tracks 10-16 *E.faecium* isolates; track 17 *S.bovis* and track 18 *L.lactis* (see Table 13). Tracks 1 and 19 contain the kilobase (kb) marker.

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, AND NCTC 6681. Further details in the Summary Table.

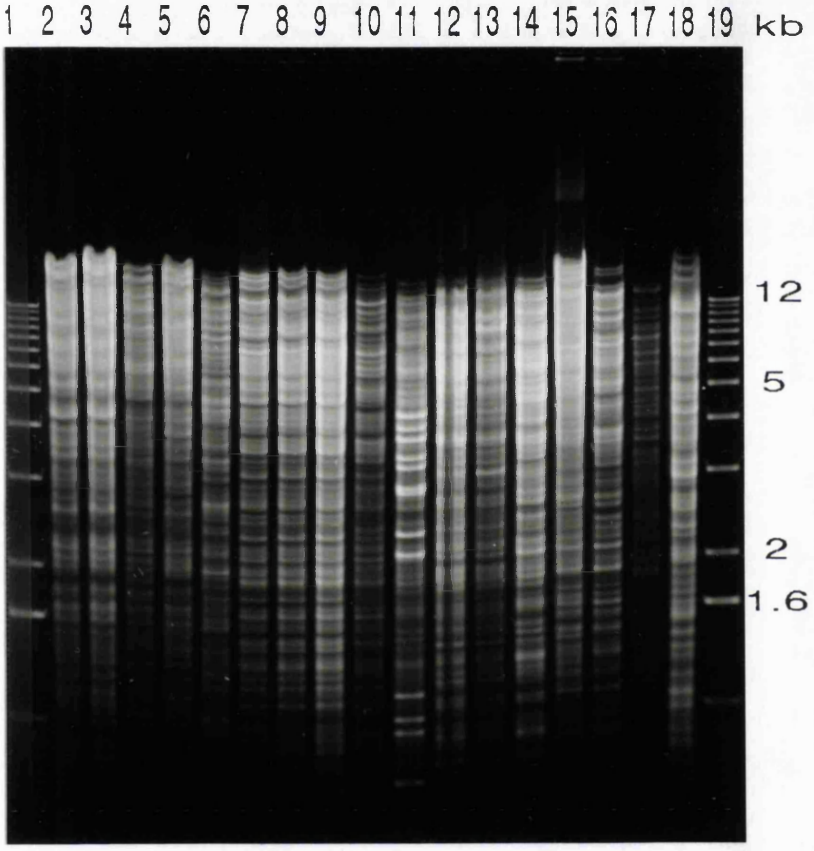
Figure 5: Digestion of the pilot study isolates with the restriction enzyme *Sal* I.



The RFLP patterns in tracks 2-5 are from *E.faecalis* isolates; tracks 6-9 *E.durans* isolates; tracks 10-16 *E.faecium* isolates; track 17 *S.bovis* and track 18 *L.lactis* (see Table 13). Tracks 1 and 19 contain the kilobase (kb) marker.

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, AND NCTC 6681. Further details in the Summary Table.

Figure 6: Digestion of the pilot study isolates with the restriction enzyme *Eco RI.*

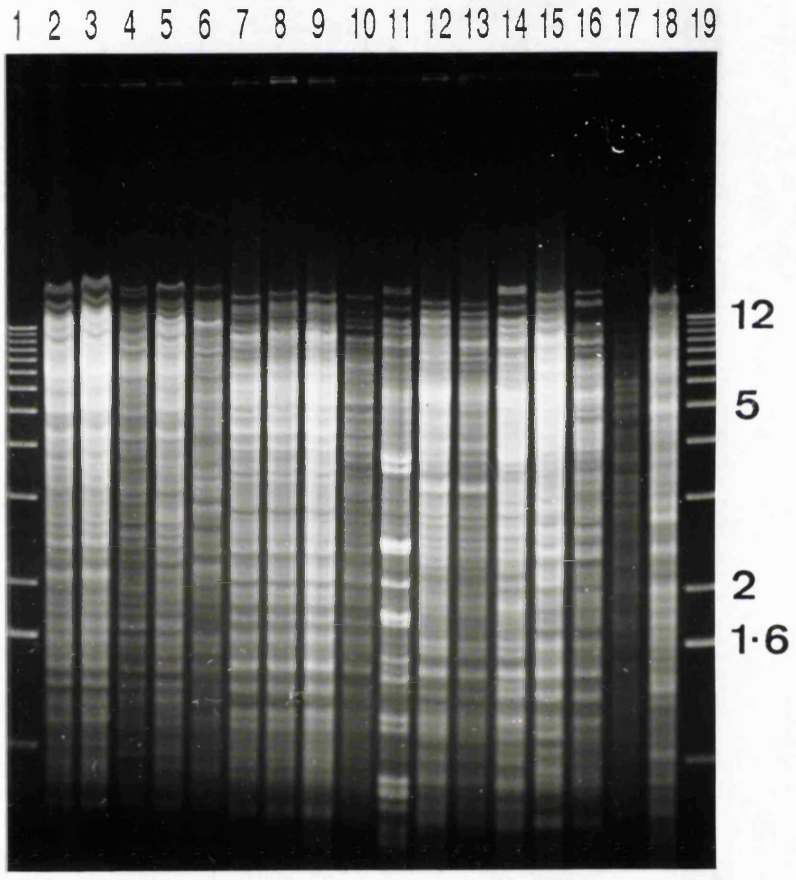


The RFLP patterns in tracks 2-5 are from *E.faecalis* isolates; tracks 6-9 *E.durans* isolates; tracks 10-16 *E.faecium* isolates; track 17 *S.bovis* and track 18 *L.lactis* (see Table 13). Tracks 1 and 19 contain the kilobase (kb) marker.

There is interference from an artefact in track 12.

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, AND NCTC 6681. Further details in the Summary Table.

Figure 7: Digestion of the pilot study isolates with the restriction enzyme *Hind III*.



The RFLP patterns in tracks 2-5 are from *E.faecalis* isolates; tracks 6-9 *E.durans* isolates; tracks 10-16 *E.faecium* isolates; track 17 *S.bovis* and track 18 *L.lactis* (see Table 13). Tracks 1 and 19 contain the kilobase (kb) marker.

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, and NCTC 6681. Further details in the Summary Table.

Digestion with the restriction enzymes *Bam* HI, *Sac* I and *Sal* I resulted in fewer bands (Figures 3, 4 and 5). It was generally possible to compare distant tracks on the same gel, and determine whether the banding patterns were indistinguishable or not. This was only easily done over a limited molecular size range, because the larger molecular size bands were closely packed. The range 1.6-5.0 kb was chosen. Results with these three restriction endonucleases did not reveal any overlapping patterns between the five bacterial species in this pilot study. The initial speciation was upon biochemical grounds, and the REA patterns did not conflict with this.

This study was directed towards the discrimination between bacterial isolates within a species. Table 14 shows the number of banding patterns which resulted when three different RE's were used to digest the pilot study isolates. More patterns arise from the use of the enzyme *Sal* I (14 patterns) than for either *Bam* HI (12 patterns) or *Sac* I (13 patterns). All three are equally effective at distinguishing between the four *E.faecalis* isolates, but for the four *E.durans* and seven *E.faecium* isolates, the enzyme *Sal* I was the most discriminatory at the intraspecies level (Table 14), however only a small selection of each species is examined here. Bright bands are evident in track 11 (isolate 1068) of most of these pilot digests (Figures 3-7), suggesting the presence and digestion of a plasmid present in a large number of copies.

Sal I was selected for the digestion of all 184 study isolates because it gave readily distinguishable patterns with 10-20 discrete bands in the 1.6-5.0 kb range. The results are summarised in Table 15.

Table 14. Pilot study RFLP pattern results for *Bam* HI, *Sac* I and *Sal* I[a]

Track	Species	Banding pattern[b] *Bam* HI	*Sac* I	*Sal* I
2	*E.faecalis*	1	1	1
3	*E.faecalis*	1	1	1
4	*E.faecalis*	2	2	2
5	*E.faecalis*	3	3	3
6	*E.durans*	4	4	4
7	*E.durans*	5	5	5
8	*E.durans*	5	5	6
9	*E.durans*	5	5	5
10	*E.faecium*	6	6	7
11	*E.faecium*	7	7	8
12	*E.faecium*	8	8	9
13	*E.faecium*	8	9	10
14	*E.faecium*	7	7	8
15	*E.faecium*	9	10	11
16	*E.faecium*	10	11	12
17	*S.bovis*	11	12	13
18	*L.lactis*	12	13	14

Tracks 1 and 19 contain the kilobase marker

[a]See Figures 3-5

[b]Numbers refer to each new banding pattern

Table 15. Summary of the RFLP patterns with *Sal* I

Species	Fingerprint[a]	No of isolates	Source[b]
E.faecium	A	*10	Antarctic
	B	20	Antarctic
	C	22	Antarctic
	D	7	Antarctic
	E	29	Antarctic
	F	8	Antarctic
	G	2	Antarctic
	H	2	Antarctic
	I	2	Antarctic
	U1-15	15	Antarctic (9) NCTC (1) London (5)
	Nil	4	Antarctic (2) London (2)
		SubTotal 121	
E.faecalis	J	12	Antarctic
	Ua-i	9	London (6) NCTC (3)
		SubTotal 21	
E.durans	K	*34	Antarctic
	UD1-D3	3	Antarctic (1) NCTC (1) London (1)
		SubTotal 37	
E.casseliflavus	UCa	1	London
S.bovis	UB1-B3	3	NCTC (1) London (2)
L.lactis	ULa	1	NCTC
		Total 184	

[a]All unique patterns are prefixed by the letter U. These are numbered 1-15 for *E.faecium*, a-i for *E.faecalis*, D1-D3 for *E.durans*, Ca for *E.casseliflavus*, B1-B3 for *S.bovis* and La for *L.lactis*.

[b]Number of isolates from the different sources is indicated in parenthesis.

*These fingerprint patterns each contained one enterococcal isolate that it was not possible to assign to species by the biochemical tests described.

3.3.3.3. *E.faecium* digestion with *Sal* I

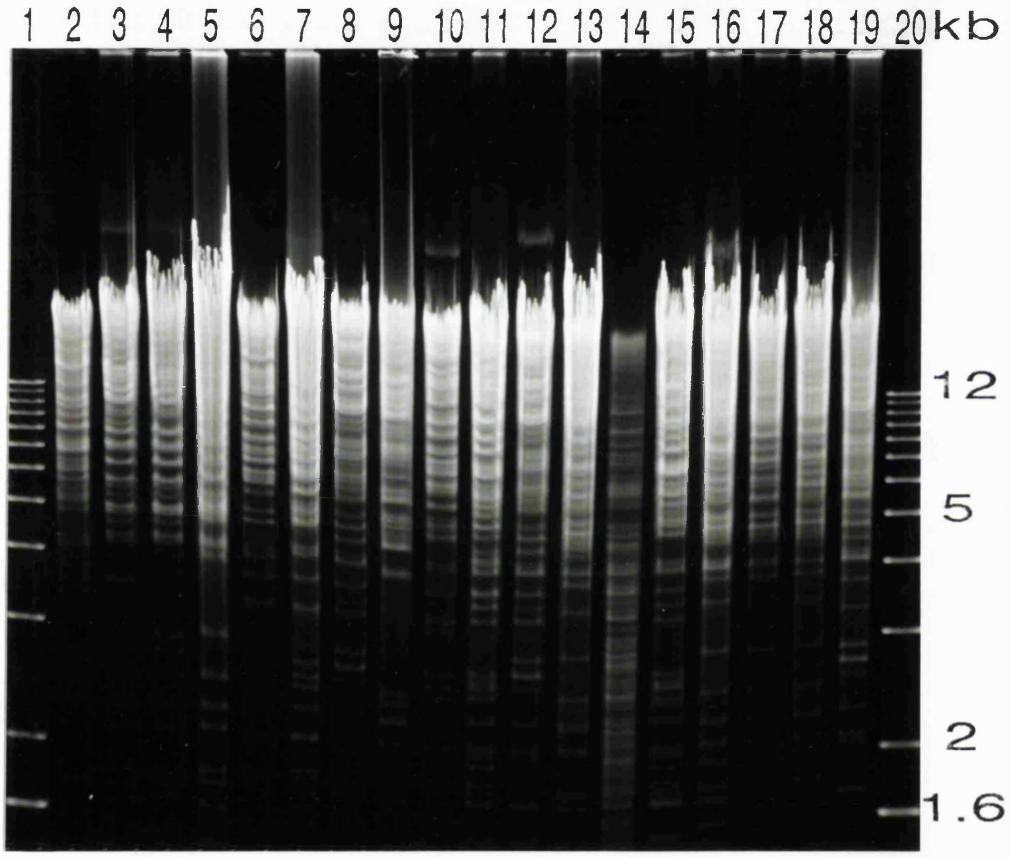
The 117 typable isolates of *E.faecium* resulted in 24 different DNA fingerprint patterns, representing between 1 and 29 isolates; 15 of these patterns were unique. The largest group of isolates with the same pattern consisted of 29 isolates all from the Antarctic, with pattern designated E. They were obtained from 5 subjects. The 111 typable Antarctic isolates resulted in 18 different DNA fingerprint patterns, and half of these patterns were from single unique isolates. Most of the *E.faecium* DNA fingerprint patterns from the Antarctic isolates are presented in Figure 8 and it can be seen that the patterns are easy to distinguish. The type strain NCTC 7171 and the 5 typable isolates from the outpatient department gave unique patterns. Four *E.faecium* isolates from a total of 121 isolates were untypable.

In Table 16 a display of the *Sal* I patterns obtained from each API 20 Strep profile is presented. For example all four isolates of the API profile 5157511 were of the *Sal* I pattern D, while the 13 isolates of the API 20 Strep profile 7357511 are of the *Sal* I patterns B, C, G, H, I, U4, and U8. The relationship between these two typing systems is not simple. It will reflect the disparity between a phenotypic typing scheme and one based upon the genome.

3.3.3.4. *E.durans* digestion with *Sal* I

The 37 *E.durans* isolates gave 4 different DNA fingerprint patterns. The 35 Antarctic isolates resulted in only two patterns, with 34/35 isolates being indistinguishable. They were obtained from 6 subjects. The type strain (NCTC 8307) and the outpatient isolate both gave unique patterns. Only the pattern from the outpatient isolate is absent from the illustration in Figure 9.

Figure 8: Restriction endonuclease analysis of Antarctic *E.faecium* isolates.



All patterns are from *E.faecium* isolates except pattern K (track 11) which was from the isolate identified as *E.durans* by RFLP. There are 16 different Antarctic RFLP patterns of *E.faecium* isolates collected from 7 subjects.
Tracks 5, 13, 14, 17 and 18 all contain RFLP patterns of isolates from subject 10, with those in tracks 5 and 17 being indistinguishable (RFLP pattern C). Tracks 2, 3 and 4 contain three different RFLP patterns from subject 8.
Tracks 1 and 20 contain the kilobase (kb) marker.

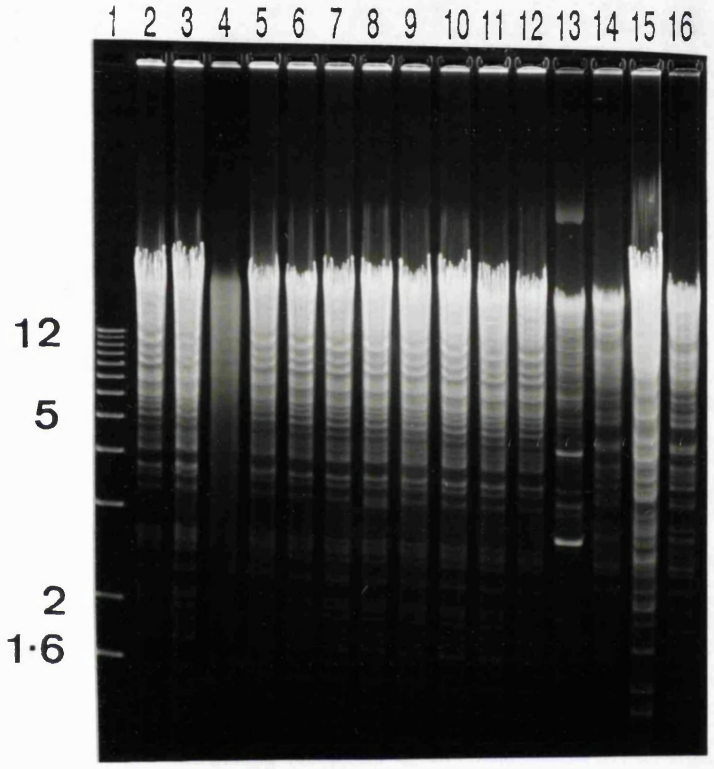
The isolate numbers are: 854, 887, 8812, 1055, 1716, 2612, 4718, 799, 459, 754, 973, 1054, 10617, 455, 7103, 1051, 10619, and NCTC 7171.

Table 16. *E.faecium* API profile and *Sal* I patterns

API profile	Sal I pattern											Total
	A	B	C	D	E	F	G	H	I	U[a]	Nil	
7157411	10				26					2	1	39
5157511				4								4
7157510		1										1
7157511		6		3	3	8				4		24
7157551										1		1
7357411			17							1	1	19
7357510		8								1		9
7357511		3	3				2	2	1	2		13
7357541			1									1
7357551			1						1	4	2	8
7377510		2										2
Totals	10	20	22	7	29	8	2	2	2	15	4	121

[a]U refers to the number of unique *Sal* I patterns.

Figure 9: Restriction endonuclease analysis of Antarctic *E.durans* isolates.



Tracks 2-12 contain Antarctic *E.durans* isolates; all are of pattern K except track 8 (pattern UD1). Tracks 3-12 were collected from a single individual over a three month period.
There is overdigestion of track 4. Track 13 is the *E.durans* type strain, and tracks 14-16 are the *E.faecalis* type strains.
Track 1 contains the kilobase (kb) marker.

The isolate numbers are: 151, 351, 353, 354, 355, 356, 377, 378, 379, 3711, 3713, NCTC 8307, NCTC 775, NCTC 2705, NCTC 10927.

3.3.3.5. *E.faecalis* digestion with *Sal* I

The 21 *E.faecalis* isolates gave 10 different RFLP patterns. The 12 Antarctic isolates were all of the same pattern, whilst the 3 type strains (NCTC 775, 2705 and 10927) and the 6 outpatient department isolates all had unique patterns. The RFLP patterns from the three *E.faecalis* type strains are illustrated in Figure 9, the outpatient isolates are illustrated in Figure 10, and two of the Antarctic isolates are shown in Figure 5.

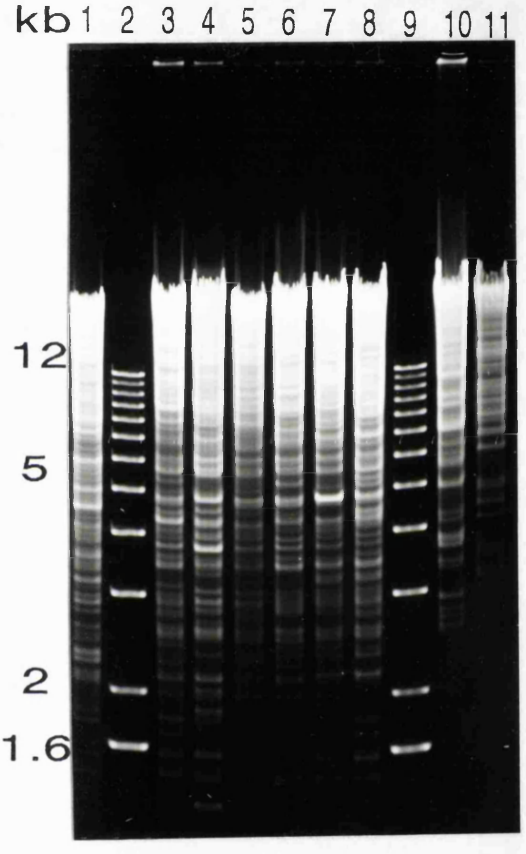
3.3.3.6. Digestion of other species with *Sal* I

The three *S.bovis* isolates each gave different and unique DNA fingerprint patterns, two of which are illustrated in Figure 10. The single *E.casseliflavus* (Figure 10) and *L.lactis* (Figure 5) isolates resulted in unique RFLP patterns.

3.3.3.7. Summary of results with *Sal* I

Table 15 has summarised the results of restriction endonuclease analysis with *Sal* I. A total of 43 RFLP patterns were obtained from the 178 typable isolates. Thirty-two of these patterns represented single isolates and so were unique. There were between 1 and 34 isolates with the same RFLP pattern; a mean of 4 isolates per pattern. Each of the 7 type strains, and also each of the 15 typable outpatient isolates gave a unique RFLP pattern. There was no crossover of the RFLP patterns obtained with Sal I between the 6 species included in this study; these included 4 *Enterococcus sp.*, and 2 non-enterococcal species. Multiples of the same RFLP pattern were only obtained from the Antarctic group of isolates.

Figure 10: Restriction endonuclease analysis of London *E.casseliflavus*, *E.faecalis* and *S.bovis* isolates.



Track 1 contains the single *E.casseliflavus* isolate. Tracks 3-8 contain six London *E.faecalis* isolates. Tracks 10 and 11 contain two *S.bovis* isolates. All patterns were unique.
Tracks 2 and 9 contain the kilobase (kb) marker.

The isolate numbers are: LON6, (kb), LON13, LON14, LON12, LON10, LON11, LON9, (kb), LON17, and NCTC 8177. Further details in the Summary Table.

3.3.4. Species identification by RFLP

Concerning the two isolates identified in section 2.2.2. as far as the genus *Enterococcus*; it would have been possible to apply further phenotypic tests (Table 2) with the intention of deciding upon their identification. However in this study after the planned tests had not indicated the species their RFLP patterns with *Sal* I were compared with those of the other enterococcal isolates. An identical RFLP pattern was considered to indicate the same species and identification thereby obtained.

The two isolates were numbers 754 and 1064, both of the API profile 7157411 (low descrimination between *E.durans*, *E.faecium* and *L.lactis*). These were not *L.lactis*, as they were both of the Lancefield group D, they both grow at 45°C and in 6.5% NaCl. Each had a different RFLP pattern. Isolate 754 had the RFLP pattern K, and so was identified as *E.durans*. Thirty-three other isolates identified as *E.durans* possessed the same RFLP pattern. Isolate 1064 was of the RFLP pattern A, and was identified as *E.faecium*. Nine other isolates which had been confidently identified as *E.faecium* were of the same RFLP pattern (Table 15).

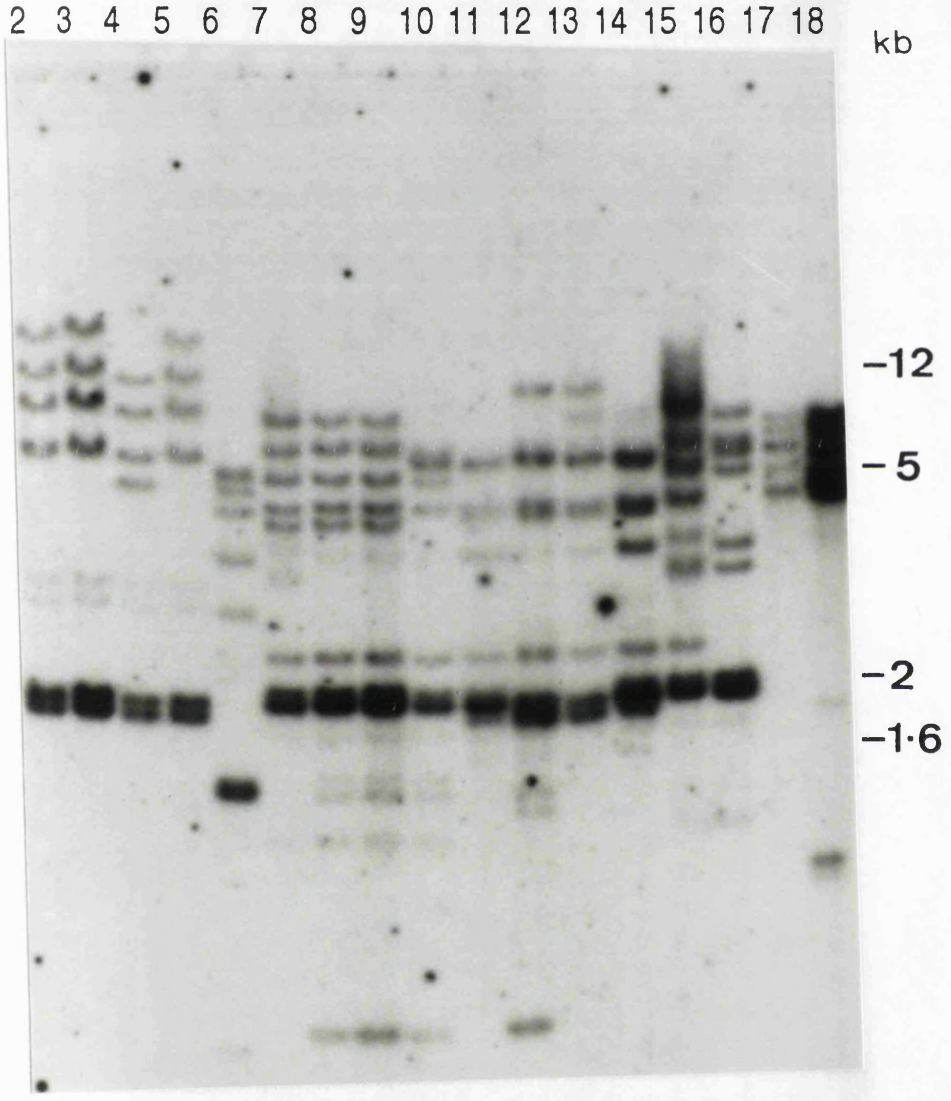
As explained earlier in section 3.2.2. eight isolates were assigned to the *E.faecium* group with what could be interpreted as equivocal evidence. These were of the API profile 7357551, a profile which did not distinguish between *E.faecium* and *E.gallinarum* isolates. The *Sal* I patterns for these eight isolates were one each of C and I, four unique patterns (U9, U12, U13 and U14), and there were two isolates that were untypable. RFLP patterns C and I were both patterns displayed by other *E.faecium* isolates (Tables 15 and 16). This evidence supports the assignment of two of the isolates of the API 20 Strep profile 7357551 to the species *E.faecium*, although a question must remain concerning the identity of the remaining six; those two isolates not fingerprinted and also the four unique isolates.

3.3.5. Ribosomal RNA gene restriction patterns (ribotyping)

Ribosomal RNA gene restriction patterns obtained after digestion of DNA from the pilot study isolates listed in Table 13 with *Eco* RI were more discriminatory than those with *Hind* III, as shown in Figures 11 and 12 and Table 17. Twelve different banding patterns were obtained using *Eco* RI, and 9 different banding patterns were obtained with *Hind* III. Ten ribotype patterns were obtained using *Eco* RI for the 15 enterococcal isolates included in the pilot study, with indistinguishable patterns only occurring within a species (Figure 11, Table 17). This was not as discriminatory as RFLP with *Sal* I where 12 patterns were obtained for the same 15 pilot study isolates digested (Table 14). After digestion with *Eco* RI ribotyping differences were evident both between and within the three enterococcal species (*E.faecalis*, *E.faecium* and *E.durans*).

The most dramatic difference between the results with ribotyping and those with RFLP was that ribotyping with *Hind* III resulted in an overlap of patterns between two different species of enterococci. The ribotype pattern 4 was common to both *E.faecium* and *E.durans* isolates (Figure 12, Table 17).

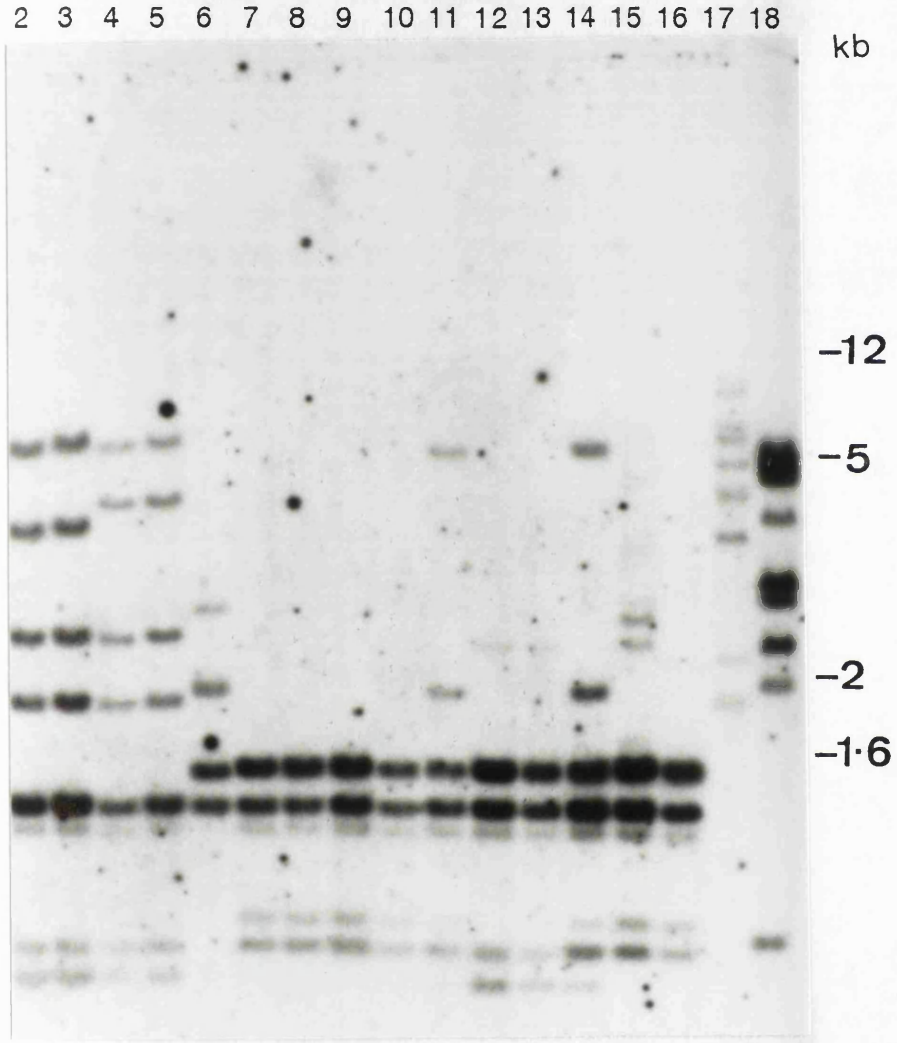
Figure 11: Southern blot of the pilot study isolates digested with *Eco* RI.



The ribotype patterns in tracks 2-5 are from *E.faecalis* isolates, tracks 6-9 *E.durans* isolates, tracks 10-16 from *E.faecium* isolates, track 17 *S.bovis* and track 18 *L.lactis* isolates (Table 13).
There are indistinguishable patterns in tracks 1, 2 and 4, tracks 7-9, and tracks 11 and 14 (Table 17).

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, AND NCTC 6681. Further details in the Summary Table.

Figure 12: Southern blot of the pilot study isolates digested with *Hind* III.



The ribotyping patterns in tracks 2-5 are from *E.faecalis* isolates, tracks 6-9 *E.durans* isolates, tracks 10-16 from *E.faecium* isolates, track 17 *S.bovis* track 18 an *L.lactis* isolates (see Table 13).
There are indistinguishable patterns in tracks 2 and 3, tracks 4 and 5, tracks 7-10 and 16, tracks 11 and 14, and in tracks 12 and 13 (Table 17).

The isolate numbers are: 872, 8108, NCTC 775, NCTC 2705, NCTC 8307, 4813, 377, 1155, NCTC 7171, 1068, 8725, 8812, 1071, 178, 154, NCTC 8177, AND NCTC 6681. Further details in the Summary Table.

Table 17. Ribotype patterns after digestion with *Eco* RI[a] and *Hind* III[a]

Track	Species	Banding pattern *Hind* III[b]	*Eco* RI[b]
2	*E.faecalis*	1	1
3	*E.faecalis*	1	1
4	*E.faecalis*	2	2
5	*E.faecalis*	2	1
6	*E.durans*	3	3
7	*E.durans*	4	4
8	*E.durans*	4	4
9	*E.durans*	4	4
10	*E.faecium*	4	5
11	*E.faecium*	5	6
12	*E.faecium*	6	7
13	*E.faecium*	6	8
14	*E.faecium*	5	6
15	*E.faecium*	7	9
16	*E.faecium*	4	10
17	*S.bovis*	8	11
18	*L.lactis*	9	12

[a]See Figures 11 and 12

[b]Numbers refer to each new banding pattern

Another anomaly of the ribotyping results concerned the different sources of isolates within each species. After digestion with *Eco* RI one of the type strains of *E.faecalis* (NCTC 2705, track 5, Figure 11) was indistinguishable from those of two of the Antarctic *E.faecalis* isolates (tracks 2 and 3). After digestion with *Hind* III (Figure 12) the NCTC 2705 isolate in track 5 was indistinguishable from another Antarctic isolate, present in track 4 (Table 17). A major feature of the RFLP results with *Sal* I was that there was no overlap of patterns between the three sources of isolates (NCTC, Antarctic and London), a feature not demonstrated by the ribotyping results.

3.4. Epidemiology in Antarctica

The 160 isolates from the isolated Antarctic community can be examined in a temporal manner with the three different typing methods used in this study: API biotyping, antibiograms and RFLP. The main limitation placed upon the interpretation of this data is the poor survival (25%) of the isolates collected in the Antarctic. The actual number of isolates examined by genomic molecular techniques here is larger than in the majority of previously reported studies, and the largest to examine enterococci (Allardet-Servent *et al*, 1989; Denning *et al*, 1989; Mogollon *et al*, 1990; Murray *et al*, 1990b; Bingen *et al*, 1991; Jordens and Pennington, 1991; Donabedian *et al*, 1992; Hall *et al*, 1992)

The temporal results obtained with each of the typing techniques for the large *E.faecium* group are shown in Figures 13, 14 and 15. Composite figures contain the results from the three methods of typing for *E.durans* (Figure 16) and *E.faecalis* (Figure 17).The blank spaces on the temporal figures indicate that enterococcal isolates were collected and did survive, but were not of the species to which the figure refers.

Figure 13. Temporal analysis of the Antarctic *E.faecium* RFLP results using *Sal* I

	Month							
Subject	4	5	6	7	8	9	10	Total
1	+	EEE	+	DDD DDDD	+	+	+	10
2	+	CCC CCCC	EEE EEEE	+	+	EE EEE	EEE	22
3	+		-	B	+	-	-	1
4	+	AAAA E U_1U_2	-	FF	FF	+	-	11
7	+	A	E	EEEEE F G	-	E F H Nil	E G H U_3	17
8	B	AAAA	+	BBB BBB	BBBBB BBBBBB U_4U_5	+	B	25
9	+	-	-	U_6	-	-	-	1
10	+	CCCC I	CCCCC A I $U_7U_8U_9$	CCCCC Nil	+	+	C	22
12	+	+	+	FF EE	-	-	-	4
Total	1	27	18	34	15	9	9	113

\+ Isolates collected and died
\- No isolates collected
Nil No fingerprint obtained
Each letter refers to the RFLP pattern of a single isolate.

Figure 14. Temporal analysis of the Antarctic *E.faecium* API 20 Strep biotyping[a] results

	Month							
Subject	4	5	6	7	8	9	10	Total
1	+	22 4	+	1111 444	+	+	+	10
2	+	555 5555	4 222 222	+	+	22 222	222	22
3	+		-	4	+	-	-	1
4	+ +	222 2222	-	44	44	+	-	11
7		2	2	22222 4 7		22 4 7	44 77	17
8	7	2222	+	444 66 10	3 4 6666666 777 10	+	4	25
9	+	-	-	4	-	-	-	1
10	+	8 55 7 9	2 5555 7777 9	555555	+	+ +	9	22
12	+	+	+	22 44	-	-	-	4
Total	1	27	18	34	15	9	9	113

Footnote to Figure 14.

+ Isolates collected and died
- No isolates collected

Each number in Figure 14 refers to the biotype code of a single isolate.

[a]Biotype code	API profile	No. of isolates
1	5157511	4
2	7157411	39
3	7157510	1
4	7157511	22
5	7357411	19
6	7357510	9
7	7357511	13
8	7357541	1
9	7357551	3
10	7377510	2
	Total	113

Figure 15. Temporal analysis of the Antarctic *E.faecium* antibiogram[a] results

Subject	Month 4	5	6	7	8	9	10	Total
1	+	AAA	+	AAAAAA B	+	+	+	10
2	+	AAAAA B D	AAA AAAA	+	+	AAAAA	AAA	22
3	+		-	A	+	-	-	1
4	+	AAA AAAA	-	AA	A B	+	-	11
7	+	A	A	AAA AAAA	-	AAA E	AAA C	17
8	A	AAAA	+	AAA AAA	AAAA AAAA AAAAA	+	A	25
9	+	-	-	A	-	-	-	1
10	+	AAAA C	AAAA AAAAA C	AA AAA C	+	+	A	22
12	+	+	+	AAAA	-	-	-	4
Total	1	27	18	34	15	9	9	113

\+ Isolates collected and died

\- No isolates collected

[a] Antibiograms listed in Table 10

Each letter refers to the antibiogram code of a single isolate.

Figure 16. Temporal analysis of the Antarctic *E.durans* isolates by three typing methods

	Month							
Subject	4	5	6	7	8	9	10	Total
1	+	1[a] (A)[b] K[c]	+		+	+	+	1
3	+	1 2222 (AAA) (BB) KKKKK	-	222 2222 (AAAAAA) (B) KKKKKK UD1	+	-	-	12
4	+	22 (A) (B) KK	-	222 (AA) (B) KKK	2222 (AAAA) KKKK	+	-	9
7	+	2222 3[d] (AAAA) (B) KKKKK			-			5
8		222 (AAA) KKK		2 (A) K		+		4
11	+	111 2 (AAA) (B) KKKK	+	-	-	-	-	4
Total0	20	0	11	4	0	0	35	

\+ Isolates collected and died - No isolates collected

[a]Biotype code	API profile	No. isolates
1	5113411	5
2	7113411	29
3	7157411	1

[b] Antibiograms in parenthesis (Table 10)
[c] RFLP pattern (Table 15)
[d] Isolate identified to species by RFLP pattern
Each entry under a, b or c refers to a single isolate.

Figure 17. Temporal analysis of the Antarctic *E.faecalis* isolates by three typing methods

Subject	Month 4	5	6	7	8	9	10	Total
1	+		+	3[a] (A)[b] J[c]	+	+	+	1
8				1 222222 33 (AAAA AAAAA) JJJJ JJJJJ		+	2 3 (AA) JJ	11
Total0	0	0	10	0	0	2	12	

\+ Isolates collected and died

[a]Biotype code	API profile	No. isolates
1	7153311	1
2	7173311	7
3	7173711	4

[b] Antibiograms in parenthesis (Table 10)
[c] RFLP pattern (Table 15)
Each entry under either a, b or c refers to a single isolate.

3.4.1. RFLP

In comparison with the biotyping or antibiogram data RFLPA with *Sal* I was more discriminatory.

Repeated isolation of organisms with the same RFLP pattern over a number of months from a single individual probably reflects colonisation of the individual by a particular strain. The results suggest that one strain (i.e. organisms with the same RFLP pattern) can be shared between individuals and may persist for several months in more than one individual. An example of this is with the *E.faecium* RFLP pattern E which was present in subjects 1, 2, 4, 5 and 9, and was shown to colonise subject 2 and 5 (Figure 13). Colonisation simultaneously by more than one strain and by more than one enterococcal species was also detected, for example four different strains of *E.faecium* (RFLP patterns E, F, G and H) colonised subject 7 (Figure 13). Three different enterococcal species colonised subject 8; *E.faecium* (pattern B, Figure 13), *E.durans* (pattern K, Figure 16) and *E.faecalis* (pattern J, Figure 17).

The 113 *E.faecium* isolates (111 of which were typed by RFLP) from the Antarctic community were distributed between 9/10 (90%) of the participating subjects with colonisation demonstrated in 5 of these for periods of up to seven months (Figure 13).

The 35 *E.durans* isolates from the Antarctic community (34/35 of which had indistinguishable RFLP patterns) were distributed between 6/10 (60%) of subjects with colonisation demonstrated in half of these for periods of up to four months (Figure 16).

The 12 *E.faecalis* isolates from the Antarctic community all possessed indistinguishable RFLP patterns and were obtained from 2/10 (20%) of subjects with colonisation demonstrated in one of these for a period of 4 months (Figure 17).

3.4.2. API biotyping

Whether or not API biotyping can be taken to reflect differences between strains requires some discussion. However there was evidence of a widespread community presence of certain biotypes, for example the *E.faecium* biotype code 2; this was present in seven subjects (1, 2, 4, 7, 8, 10, and 12) (Figure 14). There was also evidence of persistence of a single biotype in one or more subjects over time, as demonstrated in subjects 7 and 8 with biotype code 7 (Figure 14). The persistence of more than one biotype in an individual was evident, as in subject 7 and the biotypes 2, 4 and 7. In Figure 16 of the *E.durans* results the biotype code 2 can be seen to persist in subjects 3, 4 and 8. There were no examples of more than one *E.durans* biotype persisting in the same subject. Persistence of biotypes 2 and 3 of *E.faecalis* in subject 8 is illustrated in Figure 17.

3.4.3. Antibiograms

Meaningful information is probably not available from such a poorly discriminatory method as the antibiograms are for the typing of these 160 Antarctic isolates. Over 90% of the Antarctic isolates are of antibiogram A (Table 10). For comparison the data is presented in a temporal format in Figures 15, 16 and 17; the uniformity is the main significant feature.

Possibly interesting typing information is presented by the isolates of antibiogram C (resistant to gentamicin and erythromycin). Three of the four Antarctic *E.faecium* isolates of this antibiogram were from the same subject 10 and collected over three consequtive months (Figure 14). The association between *E.durans* and ciprofloxacin resistance (antibiogram B) was noted earlier, four individuals were involved, two of these (subjects 3 and 4) over a three month period (Figure 16).

3.4.4. Combined Grouping

Combined typing systems have been used by several workers to increase isolate discrimination. Figure 18 represents the effect of combining the results of RFLP, antibiograms and biotyping for the Antarctic *E.faecium* isolates. The significance of the results is discussed in section 4.8.

3.5. Summary Table

Table 18 contains all of the information collected on each individual isolate, except for the ribotyping data. The table can be used in conjunction with Figures 13-18 and other tables to compare features of particular isolates.

Table 18. Summary table

NUM	PE	MO	SA	SPECIES	1	2	3	4	5	6	7	AM	6.5	45	AG	G	P	C	CIPm	E	I	IMIPm	V	A	TE	ANTI	LA	SAL	OTHERNAME
1	1	5	2	E.faecium	7	1	5	7	5	1	1	2	2	2	0	R	S	S	16	S	S	26	S	S	S	A	D	E	
2	1	5	3	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	28	S	S	S	A	D	E	
3	1	5	4	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	22	S	S	S	A	D	E	
4	1	7	1	E.faecium	5	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	24	S	S	S	A	D	D	
5	1	7	3	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	R	12	S	S	22	S	S	S	B	D	D	
6	1	7	5	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	26	S	S	S	A	D	D	
7	1	7	6	E.faecium	5	1	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	26	S	S	S	A	D	D	
8	1	7	8	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	26	S	S	S	A	D	D	
9	1	7	9	E.faecium	5	1	5	7	5	1	1	0	0	0	0	R	S	S	14	S	S	24	S	S	S	A	D	D	
10	1	7	16	E.faecium	5	1	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	26	S	S	S	A	D	D	
11	2	5	1	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	28	S	S	S	A	D	C	
12	2	5	2	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	20	S	S	S	A	D	C	
13	2	5	3	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	18	S	S	S	A	D	C	
14	2	5	4	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	16	S	S	S	A	D	C	
15	2	5	5	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	R	12	S	S	14	S	S	S	B	D	C	
16	2	5	6	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	14	S	R	12	S	S	S	D	D	C	
17	2	5	7	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	18	S	S	S	A	D	C	
18	2	6	3	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	E	
19	2	6	4	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	E	
20	2	6	5	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	E	
21	2	6	6	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	30	S	S	S	A	D	E	
22	2	6	12	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	22	S	S	S	A	D	E	
23	2	6	13	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	24	S	S	S	A	D	E	
24	2	6	14	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	30	S	S	S	A	D	E	
25	2	9	2	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	E	
26	2	9	3	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	18	S	S	S	A	D	E	
27	2	9	4	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	28	S	S	S	A	D	E	
28	2	9	9	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	E	
29	2	9	18	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	28	S	S	S	A	D	E	
30	2	10	5	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	28	S	S	S	A	D	E	
31	2	10	15	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	30	S	S	S	A	D	E	
32	2	10	16	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	28	S	S	S	A	D	E	
33	3	7	4	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
34	4	5	1	E.faecium	7	1	5	7	4	1	1	0	0	0	2	R	S	S	16	S	S	24	S	S	S	A	D	A	
35	4	5	3	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	28	S	S	S	A	D	E	
36	4	5	4	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	22	S	S	24	S	S	S	A	D	A	
37	4	5	5	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	26	S	S	S	A	D	U1	
38	4	5	6	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	24	S	S	S	A	D	A	
39	4	5	9	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	U2	
40	4	5	10	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	14	S	S	24	S	S	S	A	D	A	
41	4	7	14	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	14	S	S	20	S	S	S	A	D	F	
42	4	7	18	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	22	S	S	S	A	D	F	
43	4	8	6	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	R	12	S	S	20	S	S	S	B	D	F	
44	4	8	7	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	14	S	S	18	S	S	S	A	D	F	
45	7	5	3	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	22	S	S	S	A	D	A	
46	7	5	4	E.durans	7	1	5	7	4	1	1	2	2	2	1	R	S	R	12	S	S	24	S	S	S	B	D	K	
47	7	6	2	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	26	S	S	S	A	D	E	
48	7	7	1	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	18	S	S	S	A	D	G	
49	7	7	4	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	28	S	S	S	A	D	E	
50	7	7	7	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	30	S	S	S	A	D	E	
51	7	7	9	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	30	S	S	S	A	D	E	
52	7	7	10	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	30	S	S	S	A	D	E	
53	7	7	12	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	28	S	S	S	A	D	E	
54	7	7	16	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	14	S	S	20	S	S	S	A	D	F	
55	7	9	1	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	18	S	S	S	A	D	Nil	
56	7	9	2	E.faecium	7	1	5	7	4	1	1	0	0	0	2	R	S	S	18	S	S	28	S	S	S	A	D	E	
57	7	9	5	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	14	S	S	20	S	S	S	A	D	F	
58	7	9	9	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	18	R	R	12	S	S	S	E	D	H	
59	7	10	3	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	18	S	S	S	A	D	U3	

NUM	PE	MO	SA	SPECIES	1	2	3	4	5	6	7	AM	6.5	45	AG	G	P	C	CIPm	E	I	IMIPm	V	A	TE	ANTI	LA	SAL	OTHERNAME
60	7	10	6	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	18	S	S	S	A	D	E	
61	7	10	7	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	28	S	S	S	A	D	G	
62	7	10	11	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	22	R	S	19	S	S	S	C	D	H	
63	8	4	1	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
64	8	5	1	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	14	S	S	24	S	S	S	A	D	A	
65	8	5	3	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	22	S	S	26	S	S	S	A	D	A	
66	8	5	4	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	28	S	S	S	A	D	A	
67	8	5	5	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	26	S	S	S	A	D	A	
68	8	7	14	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	16	S	S	S	A	D	B	
69	8	7	17	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	18	S	S	S	A	D	B	
70	8	7	20	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	20	S	S	S	A	D	B	
71	8	7	21	E.faecium	7	3	7	7	5	1	0	0	0	0	0	R	S	S	18	S	S	18	S	S	S	A	D	B	
72	8	7	25	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	22	S	S	18	S	S	S	A	D	B	
73	8	7	26	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	20	S	S	22	S	S	S	A	D	B	
74	8	8	2	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	18	S	S	20	S	S	S	A	D	B	
75	8	8	3	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	18	S	S	S	A	D	B	
76	8	8	5	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
77	8	8	6	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
78	8	8	7	E.faecium	7	3	7	7	5	1	0	0	0	0	0	R	S	S	18	S	S	14	S	S	S	A	D	B	
79	8	8	8	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
80	8	8	10	E.faecium	7	1	5	7	5	1	0	0	0	0	0	R	S	S	16	S	S	16	S	S	S	A	D	B	
81	8	8	11	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	28	S	S	S	A	D	U4	
82	8	8	12	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	16	S	S	18	S	S	S	A	D	U5	
83	8	8	13	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	20	S	S	16	S	S	S	A	D	B	
84	8	8	14	E.faecium	7	3	5	7	5	1	0	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
85	8	8	18	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
86	8	8	19	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	20	S	S	S	A	D	B	
87	8	10	28	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	B	
88	9	7	3	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	14	S	S	20	S	S	S	A	D	U6	
89	10	5	1	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	18	S	S	S	A	D	C	
90	10	5	2	E.faecium	7	3	5	7	5	4	1	0	0	0	0	R	S	S	18	S	S	18	S	S	S	A	D	C	
91	10	5	3	E.faecium	7	3	5	7	4	1	1	0	0	0	2	R	S	S	18	S	S	18	S	S	S	A	D	C	
92	10	5	4	E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	18	R	S	22	S	S	S	C	D	I	
93	10	5	5	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	23	S	S	S	A	D	C	
94	10	6	4	E.faecium	7	1	5	7	4	1	1	2	2	2	1	R	S	S	16	S	S	30	S	S	S	A	D	A	
95	10	6	5	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	20	S	S	S	A	D	C	
96	10	6	7	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	20	S	S	S	A	D	I	
97	10	6	8	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	18	S	S	S	A	D	C	
98	10	6	9	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	20	S	S	18	S	S	S	A	D	C	
99	10	6	14	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	14	S	S	S	A	D	U7	
100	10	6	15	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	16	S	S	S	A	D	C	
101	10	6	17	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	18	S	S	S	A	D	U8	
102	10	6	18	E.faecium	7	3	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	20	S	S	S	A	D	C	
103	10	6	19	E.faecium	7	3	5	7	5	5	1	2	2	2	0	R	S	S	20	R	S	18	S	S	S	C	D	U9	
104	10	7	1	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	16	S	S	S	A	D	C	
105	10	7	3	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	16	S	S	S	A	D	C	
106	10	7	5	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	22	S	S	S	A	D	C	
107	10	7	7	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	16	S	S	S	A	D	C	
108	10	7	9	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	18	S	S	18	S	S	S	A	D	C	
109	10	7	14	E.faecium	7	3	5	7	4	1	1	2	2	2	2	R	S	S	14	R	S	18	S	S	S	C	D	Nil	
110	10	10	8	E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	20	S	S	30	S	S	S	A	D	C	
111	12	7	6	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	16	S	S	18	S	S	S	A	D	F	
112	12	7	9	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	24	S	S	S	A	D	E	
113	12	7	16	E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	18	S	S	30	S	S	S	A	D	F	
114	12	7	17	E.faecium	7	1	5	7	4	1	1	2	2	2	2	R	S	S	16	S	S	30	S	S	S	A	D	E	
115				E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	R	8	S	S	20	S	S	S	B	D	U10	NCTC 7171
116				E.faecium	7	1	5	7	5	1	1	0	0	0	0	R	S	S	20	S	S	22	S	S	S	A	D	U11	LON1
117				E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	18	S	S	24	S	S	S	A	D	Nil	LON2
118				E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	18	S	S	20	S	S	S	A	D	U12	LON3
119				E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	14	S	S	20	S	S	S	A	D	Nil	LON4
120				E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	14	S	S	22	S	S	S	A	D	U13	LON5
121				E.casseli.	7	3	5	7	5	7	1	0	0	0	0	R	S	R	10	S	S	28	S	S	S	B	D	UCa	LON6

NUM	PE	MO	SA	SPECIES	1	2	3	4	5	6	7	AM	6.5	45	AG	G	P	C	CIPm	E	I	IMIPm	V	A	TE	ANTI	LA	SAL	OTHERNAME
122				E.faecium	7	3	5	7	5	5	1	0	0	0	0	R	S	S	20	S	S	28	S	S	S	A	D	U14	LON7
123				E.faecium	7	1	5	7	5	5	1	0	0	0	0	R	S	R	12	S	S	20	S	S	S	B	D	U15	LOM8
124				E.faecalis	7	1	4	3	7	1	1	0	0	0	0	R	S	S	14	R	S	24	S	S	S	C	D	Ua	LON9
125				E.faecalis	7	1	4	3	7	1	1	0	0	0	0	R	S	S	14	S	S	18	S	S	S	A	D	Ub	LON10
126				E.faecalis	7	1	4	3	7	1	1	0	0	0	0	R	S	S	18	S	S	26	S	S	S	A	D	Uc	LON11
127				E.faecalis	7	1	4	3	7	1	1	0	0	0	0	R	S	S	18	S	S	24	S	S	S	A	D	Ud	LON12
128				E.faecalis	7	1	4	3	7	1	1	0	0	0	0	R	S	S	16	S	S	24	S	S	S	A	D	Ue	LON13
129				E.faecalis	7	1	4	3	7	1	1	0	0	0	0	R	S	S	22	S	S	24	S	S	S	A	D	Uf	LON14
130				E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	16	S	S	22	S	S	S	A	D	Ug	NCTC 775
131				E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	16	S	S	24	S	S	S	A	D	Uh	NCTC 2705
132				E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	14	S	S	24	S	S	S	A	D	Ui	NCTC 10927
133	1	7	0	E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	16	S	S	24	S	S	S	A	D	J	
134	8	7	2	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	16	S	S	22	S	S	S	A	D	J	
135	8	7	4	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	20	S	S	24	S	S	S	A	D	J	
136	8	7	6	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	16	S	S	20	S	S	S	A	D	J	
137	8	7	11	E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	16	S	S	22	S	S	S	A	D	J	
138	8	7	13	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	16	S	S	20	S	S	S	A	D	J	
139	8	7	15	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	16	S	S	22	S	S	S	A	D	J	
140	8	7	23	E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	16	S	S	22	S	S	S	A	D	J	
141	8	7	24	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	18	S	S	26	S	S	S	A	D	J	
142	8	7	27	E.faecalis	7	1	5	3	3	1	1	0	0	0	0	R	S	S	16	S	S	24	S	S	S	A	D	J	
143	8	10	1	E.faecalis	7	1	7	3	7	1	1	0	0	0	0	R	S	S	20	S	S	22	S	S	S	A	D	J	
144	8	10	8	E.faecalis	7	1	7	3	3	1	1	0	0	0	0	R	S	S	18	S	S	22	S	S	S	A	D	J	
145	1	5	1	E.durans	5	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	22	S	S	S	A	D	K	
146	3	5	1	E.durans	5	1	1	3	4	1	1	2	2	2	0	R	S	R	12	S	S	20	S	S	S	B	D	K	
147	3	5	3	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	24	S	S	S	A	D	K	
148	3	5	4	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	26	S	S	S	A	D	K	
149	3	5	5	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	22	S	S	S	A	D	K	
150	3	5	6	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	R	10	S	S	22	S	S	S	B	D	K	
151	3	7	7	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	R	12	S	S	24	S	S	S	B	D	UD1	
152	3	7	8	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	26	S	S	S	A	D	K	
153	3	7	9	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	24	S	S	S	A	D	K	
154	3	7	11	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	24	S	S	S	A	D	K	
155	3	7	13	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	26	S	S	S	A	D	K	
156	3	7	18	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	22	S	S	S	A	D	K	
157	3	7	19	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	22	S	S	S	A	D	K	
158	4	5	2	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	22	S	S	S	A	D	K	
159	4	5	8	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	R	12	S	S	26	S	S	S	B	D	K	
160	4	7	10	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	R	12	S	S	22	S	S	S	B	D	K	
161	4	7	11	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	24	S	S	S	A	D	K	
162	4	7	17	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	28	S	S	S	A	D	K	
163	4	8	3	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	22	S	S	S	A	D	K	
164	4	8	4	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	22	S	S	S	A	D	K	
165	4	8	8	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	28	S	S	S	A	D	K	
166	4	8	13	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	22	S	S	S	A	D	K	
167	7	5	1	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	22	S	S	S	A	D	K	
168	7	5	2	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	24	S	S	S	A	D	K	
169	7	5	5	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	26	S	S	S	A	D	K	
170	7	5	6	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	24	S	S	S	A	D	K	
171	8	5	2	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	26	S	S	S	A	D	K	
172	8	5	6	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	26	S	S	S	A	D	K	
173	8	5	7	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	24	S	S	S	A	D	K	
174	8	7	34	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	30	S	S	S	A	D	K	
175	11	5	1	E.durans	5	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	24	S	S	S	A	D	K	
176	11	5	2	E.durans	5	1	1	3	4	1	1	2	2	2	0	R	S	S	14	S	S	24	S	S	S	A	D	K	
177	11	5	3	E.durans	7	1	1	3	4	1	1	2	2	2	0	R	S	R	10	S	S	24	S	S	S	B	D	K	
178	11	5	5	E.durans	5	1	1	3	4	1	1	2	2	2	0	R	S	S	16	S	S	28	S	S	S	A	D	K	
179				E.durans	7	3	5	3	4	0	0	0	0	0	0	R	S	S	20	S	S	18	S	S	S	A	D	UD2	NCTC 8307
180				E.durans	7	3	5	3	4	1	1	0	0	0	0	R	S	S	22	S	S	26	S	S	S	A	D	UD3	LON15
181				S.bovis	5	6	5	0	4	5	2	0	0	0	0	R	S	S	18	R	S	30	S	S	S	C	D	UB1	LON16
182				S.bovis	5	2	4	0	4	6	3	0	0	0	0	R	S	S	18	S	S	30	S	S	S	A	D	UB2	NCTC 8177
183				S.bovis	1	2	4	0	4	4	3	0	0	0	0	R	S	R	12	S	S	30	S	S	S	B	D	UB3	LON17

NUM	PE	MO	SA	SPECIES	1	2	3	4	5	6	7	AM	6.5	45	AG	G	P	C	CIPm	E	I	IMIPm	V	A	TE	ANTI	LA	SAL	OTHERNAME
184				L.lactis	5	0	4	3	4	1	1	0	0	0	0	R	S	R	12	S	S	28	S	S	S	B	NT	ULa	NCTC6681

Table 18: Footnote to Summary table

NUM	numerical order
PE	subject number
MO	month number
SA	sample number
	PE, MO and SA combine to form the Antarctic isolate number as described in section 2.1.2.
1-7	digits of the API 20 Strep profile
AM	acid from melibiose
6.5	growth in 6.5% NaCl
45	growth at 45°C
AG	acid from glycerol
	0, test not done; 1, test negative; 2, test positive
G	gentamicin
P	piperacillin
C	ciprofloxacin
CIPm	ciprofloxacin zone size in mm
E	erythromycin
I	imipenem
IMIPm	imipenem zone size in mm
V	vancomycin
A	ampicillin
TE	teicoplanin
	S, susceptible; R, resistant
ANTI	antibiogram (Table 10)
LA	Lancefield serological group
SAL	RFLP pattern with *Sal* I
OTHERNAME	name for non Antarctic isolates

Figure 18. Temporal analysis of the Antarctic *E.faecium* results using Combined Groupings

	Month							
Subject	4	5	6	7	8	9	10	Total
1	+	bbf	+	eeu cccc	+	+	+	10
2	+	huu hhhh	bbb bbbf	+	+	bb bbb	bbb	22
3	+		-	d	+	-	-	1
4	+	aaaa b U_1U_2	-	gg	gu	+	-	11
7	+	a	b	bbbbb g l	-	f g u Nil	b l u U_3	17
8	j	aaaa	+ +	ddd iil	ddjmu iiiiii U_4U_5	+	d	25
9	+	-	-	U_6	-	-	-	1
10	+	hhuu u	hhhkk a u $U_7U_8U_9$	hhhhh Nil	+	+	u	22
12	+	+	+	gg bb	-	-	-	4
Total	1	27	18	34	15	9	9	113

U_X Unique by RFLP (and thus also for Combined Grouping)
u Unique Combined Grouping
Each letter refers to the Combined Grouping of a single isolate.
(also refer to Figure 13)

4. DISCUSSION

4.1. Species identification

One of the central problems that surrounds the present day study of the enterococci is the lack of an easy to use gold standard for the identification of the individual species. This is partly because many of the species have been quite recently described and so a reliable body of information concerning them is yet to accumulate. A second reason is the concern about the reliability of individual phenotypic tests for species identification. This objection is partly countered by the use of a large battery of tests such as the API 20 Strep, where single phenotypic characteristics have a lesser influence upon the result. In practise however even with the API 20 Strep, identification frequently depends upon a small number of tests, as illustrated by the large proportion of isolates in this study identified by confirmatory testing (Table 3).

The only widely available and rapid method of enterococcal species identification namely biotyping is still evolving (Facklam and Collins, 1989; Ruoff *et al*, 1990). It is possible that phenotypic identification will always be insufficiently discerning for some isolates. This may not be important for routine clinical use, but is relevant to more detailed epidemiological work. DNA/DNA homology studies (Facklam and Collins, 1989) or perhaps the penicillin binding proteins (Williamson *et al*, 1986) are at present the best means of species identification, but are not practical either for a study of the size presented here nor for most routine clinical work.

The Lancefield serological groupings were a dramatic advance in the classification of the Streptococci. Probably too great a reliance was placed upon this system over 30-40 years, so inhibiting the further development of streptococcal classification (Schleifer and Kilpper-Balz, 1987). The vast majority of enterococci

do possess the Lancefield group D antigen (Murray, 1990a). In one study, which included many enterococcal isolates of unusual phenotype, only 77% of 188 isolates possessed this antigen (Facklam and Collins, 1989). All of the 180 enterococcal isolates presented in this study did possess the group D antigen.

The API 20 Strep was selected for the initial speciation of isolates. Several authors have shown that *E.faecalis*, *E.faecium* and *E.durans* are reliably identified by this system (Tillotson, 1982; Appelbaum *et al*, 1984; Colman and Ball, 1984; Facklam *et al*, 1984; Facklam *et al*, 1985). Colman noted in 1984 that the API 20 Strep does not easily descriminate between *L.lactis* and *E.faecium* isolates, but was able to identify the *S.bovis* biotypes, which is in agreement with this study. There has been much less work with reference to the more recently described species. The API 20 Strep system as with some other biochemical bacterial identification systems functions on a data base of the probabilities of a particular biochemical test result being positive for the different species. The changes within the genus *Enterococcus* have resulted in the updating of the API 20 Strep data base, but there is inevitably a delay before this is performed. As noted by previous workers we found that confirmatory tests were frequently necessary, and were applied to 56% of the 180 enterococcal isolates studied here. It is likely that the API 20 Strep will require not only an update of the data base, but also the introduction of further biochemical tests if it is intended to identify all of the newer enterococcal species.

The tests I have used for the separation of *E.faecium* and *E.durans* do not agree with those of Facklam and Collins (1989) or Ruoff *et al* (1990). These authors suggest that neither species will produce acid from glycerol, and that the majority of both will produce acid from melibiose. To an extent disagreement is inevitable because the two enterococcal species *E.durans* and *E.faecium* have proven difficult to distinguish phenotypically in previous work. Jones, Sackin and Sneath (1972) in their computor analysis of 122 group D Streptococci felt that *S.durans* should be classified as the same species as *S.faecium*; however pictorially

at least the two dimensional representation of the clusters they published does show considerable spread within their single species. It was not until Farrow *et al* (1983) examined the DNA/DNA homologies of 16 *E.faecium* and *E.durans* isolates that separate species status was confirmed to be appropriate. In the present study there was no crossover of the *E.faecium* and *E.durans* RFLP patterns on digestion with *Sal* I, although the three other typing methods; biotyping, antibiograms and ribotyping all confused these two species on at least a single occasion. In the future more extensive DNA/DNA homology studies between the *E.faecium* and *E.durans*, examining a larger group of isolates than the 16 examined by Farrow *et al* (1983) might further clarify their position as distinct species.

Two isolates both possessed the API profile 7157411 and produced acid from melibiose but not from glycerol, and so could not be placed within a species grouping, they were not further differentiated biochemically. Interestingly they were different species when their RFLP patterns were compared with the isolates of known species identity. One being *E.durans* (pattern K), and one *E.faecium* (pattern A). This demonstrates that where a bank of banding patterns from known species is available there is a place for RFLP in the identification of isolates to the species level. At present the availability of such a bank of banding patterns is uncommon. This situation may change with greater automation and standardisation of the RFLP technique. Within the enterococci this development would be encouraged by the difficulties encountered in phenotypic species identification.

Eight isolates of the API 20 Strep profile 7357551 were assigned to the *E.faecium* species group. This API 20 Strep profile did not distinguish between *E.faecium* and *E.gallinarum*. If Table 16 is examined it can be seen that two of these eight isolates possessed RFLP patterns indistinguishable from those of isolates confidently identified as *E.faecium*, in the case of pattern C a large number of *E.faecium* isolates. This supports the view that two of these isolates are *E.faecium*.

However from these results it is still possible that the other six isolates could be another enterococcal species.

The single isolate of *E.casseliflavus* had a unique RFLP banding pattern. It is not possible to be confident of the species identification of this isolate because of the report by Vincent *et al* (1991) suggesting that the yellow colour of colonies of this species are not a reliable characteristic. This monothetic approach to species identification is considered inherently less than satisfactory by systematists, and in the case of *E.casseliflavus* this has now been confirmed.

Most studies have found that in human faeces *E.faecalis* is more commonly found and is in greater numbers than *E.faecium* (Murray, 1990a). However Mead (1978) found the reverse applied. It may be that the disparate results from a range of studies on the prevalence of species of enterococci in human faeces is a reflection of a normally dynamic system where no one species consistently dominates. The results from the Antarctic isolates in this study tentatively suggest that *E.faecium* and *E.durans* isolates were more common than *E.faecalis* isolates by multiples of 10 and 3 to 1. It is possible that either the Antarctic strain survival in storage was entirely random or that it was dependent upon the species. If strain survival was random then the findings of more *E.faecium* than *E.durans*, and more *E.durans* than *E.faecalis* is interesting, as most studies suggest the reverse should occur. If true this could have been influenced by the communities isolation or diet. If however strain survival was a function of species then these results suggest *E.faecium* is a hardier species than *E.faecalis* under these conditions. RFLP analysis suggests that single clonal groups of *E.durans* and *E.faecalis* were collected and have survived, rather than the genetic diversity displayed by the larger *E.faecium* group. This might be because these clones had become the predominant clone in the isolated population, or this may reflect the hardiness of the surviving clone in comparison with other clones of the same species. These hypothesese could be partially tested with storage survival studies on a predetermined collection of

isolates, but further work from the isolated community with improved isolate survival is also required. Six out of the 10 participating Antarctic base members (60%) carried *E.durans*, this is a higher figure than Noble (1978) reported. It is also interesting that no isolates of *S.bovis* were obtained from the Antarctic; *S.bovis* are reported to be present in 5% of adults (Noble, 1978). Their absence in the Antarctic collection may be due to their poor ability to survive in storage, their absence in this small group of men, or simply missing this species during the collection. From the point of view of Antarctic work, bacterial collection with the inevitably long time lag between collection and the laboratory has now been aided by the introduction of freeze drying facilities on the Antarctic bases.

As might be expected considering their well documented phenotypic distinctions there was no difficulty in differentiating the RFLP or ribotyping patterns obtained for any of the *Enterococcus* sp. from those of the *S.bovis* or *L.lactis* isolates. In the pilot study, differences were observed with each of the five restriction endonucleases used for RFLP (Figures 3-7), and after probing *Eco* RI or *Hind* III digests (Figures 11 and 12).

4.2. Clonality

The concept of clonality is at the core of studies in microbial epidemiology. The comparison of isolates of the same species and the search for those which are of the same clone assumes great importance when an outbreak of infection is suspected. Orskov and Orskov (1983) suggest that members of a clone are those recovered "independently from different sources, in different locations, and perhaps at different times, but showing so many identical phenotypical and genetic traits that the most likely explanation for this identity is a common origin." Selander *et al* (1987b) used the term clone in a more general genetic sense "to refer to a group of genetically identical or nearly identical cells that owe their similarity to (recent) descent from a common ancestral cell, in the absence of chromosomal

recombination". The operational definition of a clone will depend upon the technique used to examine the isolates.

Studies using multilocus enzyme electrophoresis have provided compelling evidence that many bacterial populations are clonal. This technique involves the separation of bacterial cell extracts by gel electrophoresis, with subequent band staining using specific enzyme substrates, allowing an estimation of each enzyme's electrophoretic mobility (Selander *et al*, 1986). Approximately 50% of amino acid substitutions are detectable by this method (Selander *et al*, 1987b). Applying this method to the *Enterobacteriaceae*, *Neisseria* species and *Haemophilus influenzae* (Selander *et al*, 1986), substantial genetic variation has been found within each bacterial species. Many different electromorphs were seen for most of the enzymes studied, so that theoretically, many hundreds of combinations of electromorphs were possible. However in practise, only a limited number of patterns were found, a phenomenon referred to as "linkage disequilibrium" (Selander *et al*, 1987a) and convincing evidence of bacterial clonality.

Tibayrenc *et al* (1991) have suggested some genetic criteria including linkage disequilibrium to point to clonal groups within a population. Two of their criteria; correlation between independent sets of genetic markers, and the overrepresentation of a widespread identical genotype are observable in this study. The RFLP patterns of the 35 Antarctic *E.durans* isolates were all essentially the same, and so could be argued to be overrepresented. The widespread presence of single RFLP patterns over time and between individuals could be classed as correlation between independent sets of genetic markers, and is shown in Figure 13, for example pattern with E. These suggestions of clonality are perhaps weakened by the absence of any crossover in RFLP patterns between the Antarctic, London and NCTC collections, and also by the artificial and closed nature of the Antarctic community, and the problems with isolate survival. Perhaps a greater insight into this area would have been obtained in the present work if a scoring method had

been used to numerically compare the RFLP patterns of dissimilar isolates because the RFLP patterns were considered either the same or different with no middle ground.

When the isolated community is considered alone, diversity is evident only within the *E.faecium* group, while *E.faecalis* and *E.durans* isolates are largely indistinguishable. This might reflect clonal selection within the isolated group, however the data is far from complete. If selection of clones of *E.durans* and *E.faecalis* during the period of isolation had occurred, an expected result would have included a greater diversity of RFLP patterns during the earlier part of the winter for these two species. Another possibility is that the *E.durans* and *E.faecalis* clones were selected prior to arrival upon the Antarctic base, perhaps during the months spent on board the BAS ships where living and eating were communal. There is a stark contrast betwen the diversity of RFLP patterns in the *E.faecium* group, and the lack of diversity in the *E.durans* and *E.faecalis* groups. This may be related to isolate storage and survival - perhaps a majority of the *E.faecium* groups survived but only single clones of the two other species. This is conjecture, positive conclusions with respect to species distribution and clonal spread would only be possible with higher survival rates for the collected isolates. Clonal groupings in the Antarctic community are demonstrated in this work, but clonal spread is not. One recent study has provided strong evidence for the clonal spread of a ß-lactamase producing *E.faecalis* in the USA (Murray *et al*, 1992).

The present study suggests that while the traditional methods of biotyping and antibiograms are probably still useful in a routine diagnostic setting, they are tools too crude to give any useful information regarding the clonal structure of a bacterial population. As described in section 1.9.1. biotyping has been used to to document person to person spread of enterococci, on occasions nosocomial. The work of Gross *et al* (1976) used biotyping to refute the suggestion of an exogenous source of enterococci causing UTI, suggesting that the infections were from the

patients own flora. The main difficulties with biotyping are; that variation is to a degree proportional to the number of tests performed, the test conditions can be critical to the result obtained, and that biotype is a phenotypic picture and so only indirectly an examination of the genome. Antibiograms are a form of biotyping and so the same critisisms apply, and in addition there is very limited variation in antimicrobial susceptibility within the enterococci. The work of Mackowiak (1989) on enterococci suggested there was minor species differences in antibiotic susceptibilities, however not eneough to separate species, and certainly insufficient for subdivision at the intra-species level. Both the results of RFLP and any form of biotyping can be influenced by plasmid movement. Figures 13-17 compare the typing results using biotyping, antibiograms and RFLP for the Antarctic isolates. RFLP patterns are one way of sampling the genome, and ribotyping is another. Pulsed field electrophoresis has the potential to compare the complete genomes of isolates. Perhaps in the future extensive sequencing data will be available to compare large numbers of isolates in detail.

4.3. Restriction fragment length polymorphism (RFLP)

The rapid small scale DNA extraction method described by Pitcher *et al* (1989) has here been successfully applied to a large group of enterococci. The average yield was 0.08 mg of DNA, sufficient for 16 separate digestions when digesting 5 μg of DNA. DNA extraction took 4 hours using 1-2 overnight blood agar plates. Some DNA samples gave apparent digestion in the absence of restriction enzyme, these were subjected to a single phenol extraction followed by precipitation with sodium chloride and ethanol in order to remove what was presumed to be endogenous endonuclease activity. A further DNA extraction was required in 15% of isolates to obtain DNA of digestable quality, and also to provide sufficient material to cope with the extensive cross checking of related RFLP patterns. The lysing agent used in this study was lysozyme, and has been used by

others (Murray *et al*, 1990; Hall *et al*, 1992). One variation in lysing agent that has recently been employed is a combination of lysozyme and mutanolysin (Goering and Winters, 1992). Of the six different bacterial species examined here by RFLP, this was the first to study *E.durans* and *S.bovis*.

The choice of restriction enzyme was decided by the requirement to obtain a limited number of bands over a practically useful kilobase range. A large number of overlapping bands such as occurred using *Eco* RI (Figure 6) would be difficult to interpret. *Sal* I resulted in a limited number of bands in the 1.6-5.0 kb range enabling a simple visual comparison to be made between isolates. A kilobase range within which to compare isolates is necessary with this technique because the heavier bands are too tightly packed to allow visual comparison. After the RFLP patterns with *Sal* I had been assigned the photographs of the gels were reviewed and no conflicting banding differences present outside the chosen range were noted.

The number of bands obtained is related to the cutting sequence recognised by the restriction enzyme chosen, and the G+C content of the DNA. The G+C contents of *E.faecalis*, *E.faecium* and *E.durans* are similar at 37-40%, with that of *E.casseliflavus* higher at 41-45% (Schleifer and Kilpper-Balz, 1987). The three isolates of *S.bovis* (G+C ratio 36-38%) were the least frequently cut by *Sal* I (Figure 10).

Any difference in the RFLP banding pattern with *Sal* I has been taken to define a separate strain. The question arises as to how different the banding patterns need to be; for example patterns K (tracks 7 and 9) and UD1 (track 8) in Figure 5 diverge by only a single band difference and so are clearly related. Such a band difference could be due to the acquisition or loss of a plasmid which might happen to have appropriately sized *Sal* I digestion fragments. It could also be caused by the movement of a transposon. In Figure 5 tracks 11 and 14 are indistinguishable except that two heavy bands were present in track 11 from what was presumed to be a

plasmid. In this study heavy banding likely to be due to the digestion of a plasmid was not usual, and noted in only two isolates both represented in Figure 5 (tracks 6 and 11). There was no evidence of any alteration in banding pattern over time, such as after second or third DNA extractions and subsequently in the many different digestions. This is evidence against there being any significant influence on the RFLP patterns by movable genetic elements. Another possible cause for a change in the banding pattern of an isolate with time would be episodes of partial digestion by the restriction endonuclease. As all banding patterns remained consistent there was also no evidence that this had occurred.

The technique described here examines only part of the genome; the small fragments generated after restriction enzyme digestion. Pulse field electrophoresis has the potential for examination of the complete genomic DNA especially if an infrequently cutting restriction enzyme is used to limit the band numbers. The patterns can be clear and epidemiologically useful (Murray *et al*, 1990; Donabedian *et al*, 1992). There have not been further reports using this technique on enterococcal isolates, perhaps because of its' greater complexity in comparison with RFLP of smaller fragments.

One minor anomally was the banding pattern in track 14 (Figure 8); one of the unique *E.faecium* patterns, this is very different in appearance from all other *E.faecium* patterns; in retrospect it would have been interesting to repeat the identification and DNA extraction of this isolate.

Considering the enterococcal species individually; *E.faecium* was the largest and most diverse group with 121 isolates, 11 API profiles and 24 RFLP patterns. The genetic diversity observed here within the species *E.faecium* has been noted before (Kilpper-Balz *et al*, 1982). The *E.durans* isolates were a more homogenous group, with 37 isolates, 5 API profiles and 4 RFLP patterns. DNA from 34/35 isolates from the Antarctic community were indistinguishable when

digested with *Sal* I, and as discussed above the single different Antarctic isolate had only a single extra band at the 3 kb level (Figure 5, track 8). The type strain and the out-patient strain of *E.durans* were unique. The *E.faecalis* group consisted of 21 isolates, 4 API profiles and 10 RFLP patterns. The 12 isolates from the Antarctic community were indistinguishable, while the 3 type strains and 6 out-patient isolates were all unique. Genetic heterogeneity was demonstrated within all three enterococcal species.

The superior discriminatory power of RFLP over biotyping is reinforced when the patterns from the 7 type strains and 13 outpatient isolates are considered alone. For example the three *E.faecalis* type strains were all of the same API profile, but each gave a separate RFLP pattern (Figure 9). The six outpatient strains of *E.faecalis* were of the same API profile and again each possessed a unique RFLP pattern. Conversly the 12 indistinguishable Antarctic *E.faecalis* isolates by RFLP were represented by three different API profiles; one explanation for this is the variable expression of phenotypic characteristics.

RFLP with the endonuclease chosen here was more discriminatory than biotyping with API 20 Strep. However the fundamental advantage of RFLP is that the genomes are being compared and not the characteristics which the genomes control.

4.4. Biotyping

Phenotypic characteristics have frequently been considered for the role of strain differentiation. However difficulties can arise because many markers of phenotype are not stable characteristics. Specific examples are the variable expression of proteolytic activity on blood agar in the streptococci (Deible *et al*, 1963), and the alteration in surface proteins dependent upon growth conditions in *E.faecalis* (Lambert *et al*, 1990). There is also limited variation (normally either

positive or negative) in many phenotypic characteristics, and so the discrimination can be poor.

Multiple tests on each isolate can be time consuming, and have problems with reproducibility. There is also the difficulty in deciding when an adequate number of tests have been performed; Colman (1968) used 75 different physiological and biochemical tests in his computer analysis of the Streptococci. Coudron *et al* (1984) found the use of commercial kits more discriminatory than conventional biotyping with 26 tests.

In Table 9 a number of the API 20 Strep tests were found to be variable within the different species. Some of these would be appropriate tests to utilise when contemplating enterococcal strain differentiation by biochemical means. However, as explained above, there would remain great uncertainty as to the reliance that could be placed upon such a typing scheme.

There appeared to be little or no correlation between the isolate characterisation using biochemical tests, and that resulting from RFLP with *Sal* I; this is illustrated by Table 16. For example all ten of the isolates of *Sal* I pattern A were of the same API biotype (7157411), whilst the twenty isolates of the *Sal* I pattern B were represented by five different API biotypes. Conversely not all of the API biotype 7157411 had the *Sal* I pattern A, two had unique patterns and 26 were of pattern E. Similar results were displayed by *E.faecalis* and *E.durans* isolates in Tables 5 and 7. Twenty different API 20 Strep profiles were obtained for the 180 enterococcal isolates, as compared with twice this number (39) of different RFLP patterns.

4.5. Antibiograms

Antibiograms of the 184 isolates with eight antimicrobials resulted in poor isolate discrimination, with 161/184 isolates (87.5%) with the same antibiogram;

there were only 5 different susceptibility patterns (Table 10). The range of antibiograms obtained in this study were an expected result. There was no evidence for a direct relationship between antibiogram and species (Tables 11 and 12). The *E.faecalis* isolates appeared to have the least variation in antibiograms, with only 1/21 that was not of antibiogram A. Of the 23 isolates resistant to more than just gentamicin (Table 11), 16 (70%) were from the Antarctic, 5 (22%) from the outpatients department, and 2 (8%) were type strains. Considering these as proportions of the total from each source, then 16/160 (10%) were from the Antarctic, 5/17 (29%) were from the outpatients department and 2/7 (28%) were type strains. For isolates resistant to more than gentamicin alone it was three times more likely that they were from the outpatient department than from the Antarctic.

Ampicillin susceptibility occurred in all 184 isolates. Ciprofloxacin resistance (antibiogram B) was noted in 15 isolates, and was with gentamicin alone. Cipofloxacin resistance appeared more commonly among the *E.durans* isolates (Table 12), however four of the five other species also have representatives with the antibiogram B, and it was far from being a unique *E.durans* marker as it was present in only 7/37 isolates. George and Uttley (1989) stated that among the quinolones ciprofloxacin was the most active against enterococci, but that the MIC's were close to the upper break point for susceptibility. Erythromycin resistance is found in enterococci, this may be plasmid mediated (Engel *et al*, 1980). Only seven out of the 184 isolates in this study were resistant, six with gentamicin alone, and one in combination with imipenem. Five of the erythromycin resistant isolates were from the Antarctic, including the isolate also resistant to imipenem (Table 11). Only two isolates were resistant to imipenem, one in combination with gentamicin alone, and one with gentamicin and erythromycin (Table 11). Both resistant isolates were *E.faecium*, and this has been noted before (George and Uttley, 1989). Maki and Aggar (1988) have suggested that imipenem resistance is a feature of *E.faecium*, but this was not confirmed here. Low level gentamicin resistance was noted in 100% of

isolates. No information was obtained concerning high level resistance where a 200 μg disc would be required (Sahm and Torres, 1988). Resistance to a 30 μg disc does not reflect any intracellular ribosomal influence, as the antibiotic is unable to enter the cell in the absence of a cell wall active compound such as a penicillin. This is not a useful dosage of gentamicin to include when testing for resistance in the enterococci. Piperacillin susceptibility was noted in 100% of isolates using a 75μg disc. This is in keeping with the knowledge that the broad spectrum penicillins have good activity against enterococci excluding those that produce ß-lactamase in which case activity is reduced (George and Uttley 1989). Normally enterococci are susceptible to the glycopeptides vancomycin and teicoplanin, and this was confirmed in our study. Resistance does occur, however at present this is unusual. Synergy between gentamicin and either penicillin, ampicillin or vancomycin was frequently observed on the susceptibility testing plates, but was not recorded. Loss of synergy would be expected only if high level gentamicin or vancomycin resistance had been revealed, or if isolates had been ß-lactamase producers.

The antibiogram data could be interpreted as agreeing with the findings of Mackowiak (1989) that *E.faecium* has a broader pattern of antibiotic resistances than does *E.faecalis*. However this is rather a small sample of isolates weighted in numbers towards *E.faecium*. 11/121 (9%) *E.faecium* isolates were resistant to more than gentamicin alone, while 1/21 (5%) *E.faecalis* isolates were resistant to more than gentamicin alone.

Antibiograms alone did not appear to represent a useful typing scheme for these isolates, which is in agreement with the body of previous work. In specialised circumstances antibiotic resistances for example high level gentamicin resistance have proven epidemiologically useful (Zervos *et al*, 1987a). Gross *et al* (1976) used antibiograms as their typing technique in a study on enterococcal urinary tract infections and concluded that urinary tract infections were endogenously acquired.

The poor discriminatory power of the technique does question the validity of their conclusions.

The results in the work presented here must partly reflect the choice of antimicrobials and also their doses, in particular that of gentamicin, but in essence probably simply demonstrates that any inter- or intra-species differences that might exist (Mackowiak, 1989) will not be revealed using this methodology.

4.6. Ribotyping

One major advantage of ribotyping over RFLP is the normally limited number of bands generated. Another potential advantage of ribotyping over RFLP is that the influence of unstable genetic elements to falsely inflate banding differences is reduced. Common problems with the technique include the considerable amount of background interference that is typically present. These features are illustrated in Figure 11, the minor smudges in tracks 7-9 confusing what are otherwise indistinguishable patterns. The significance of minor smudges when interpreting the pictures is a topical problem at present in the field of forensic medicine. Further examples are presented in the paper of Hall *et al* (1992). A disadvantage of ribotyping as used here is the inconvenience of radioactive probes; this could be avoided using one of the alternate systems such as biotin labelling (Pitcher *et al*, 1987). Another important consideration is the greater time and technical expertise required by ribotyping as compared with RFLP.

In this study ribotyping of the pilot study isolates did not prove as discriminatory at the intraspecies level as RFLP using *Sal* I. This may be a function of the chosen probe; perhaps rRNA is too highly conserved for this purpose. Hall *et al* (1992) found that ribotyping after digestion with *Bsc* I was less discriminatory than RFLP with *Sst* I, and a similar finding was demonstrated in the study of Bingen *et al* (1991) on vancomycin resistant *E.faecium* isolates where RFLP was the more discriminatory of the two techniques. Interestingly I obtained indistinguishable

ribotype banding patterns from two isolates which were biochemically different species, the same two species which have historically been difficult to distinguish biochemically - *E.faecium* and *E.durans*. Apart from this significant anomally species separation with ribotyping was good.

Only the 17 pilot study isolates were examined by ribotyping, and they were a mixture of different species and so it is difficult to form an opinion of the method with any confidence, however the general trend of the present work on enterococci including those of Bingen *et al* (1991) and Hall *et al* (1992) is that the intra-species discriminatory power of ribotyping by the methods used so far is less good than that obtained by RFLP. Future work should examine the results using a probe other than rRNA, and so avoid the critisism that rRNA is too highly conserved whilst continueing to benefit from the advantages of the technique.

4.7. *S.bovis*

Three isolates of *S.bovis*, including a type strain have been included in the present study to examine the possibility of their characterisation by RFLP, and to compare the results obtained for enterococci with another group D streptococcus. *S.bovis* bacteraemia and endocarditis has been strongly associated with colonic malignancy (Klein *et al*, 1987). A variety of hypotheses have been put forward to account for this finding, with the possibility of *S.bovis* being a carcinogen, or of this being a purely physical association related to an increased *S.bovis* carriage rate in a disordered bowel. Ruoff *et al* (1989) have demonstrated an interesting 100% association between bacteraemia with the *S.bovis* biotype I and colonic neoplasm (if thoroughly examined by colonoscopy, laparotomy or autopsy), or a 71% overall association. It would be interesting to question whether the patients with *S.bovis* endocarditis did carry the same strain in their bowel as this is the presumed source. It would also be possible to examine the strains causing endocarditis and bacteraemia, might there be a more virulent strain. These questions could be

investigated using the techniques described here. The three *S.bovis* biotype II isolates that I examined were from diverse sources and each had a unique RFLP pattern.

4.8. Epidemiology

There are practical problems involved in working within an isolated community over several months which include the motivation of both the participants and the investigator. Two wintering base members out of the twelve at Signy base in 1988 declined any involvement in the study, and the involvement of some others was limited.

Several factors have combined to potentially skew the population of Antarctic isolates collected. Enterococci will grow on MacConkeys medium, however from faecal swabs they are outnumbered several fold by gram-negative organisms, so there may be competition for both nutrients and space. Many workers would now prefer to use a media which was more enterococcal selective. The long storage time of the isolates between their collection and laboratory examination was unaviodable in this setting, however it introduces a risk of loss of samples. One cause of this is related to the logistics required to maintain the cold chain (isolates were stored at +4°C) between the Antarctic and Aberdeen. This is a formidable problem although the British Antarctic Survey are well used to such obstacles.

In some studies on enterococcal typing workers have combined more than one technique to increase discrimination. Examples include the biotyping, serotyping and phage typing of *E.faecalis* by Smythe *et al* (1987), and the use of bacteriocin and phage typing by Kuhnen *et al* (1988). In Table 16 all of the *E.faecium* biotypes and RFLP patterns with *Sal* I are presented; there are 34 different combinations of these features. Since most isolates were of the antibiogram A, little increased discrimination would be gained by also combining the antibiogram data. Only the 17 pilot study isolates were examined by ribotyping, and

so this information is not available for all isolates. The validity of the combination of such diverse typing methods is an important question. Biotyping and antibiograms are measuring expressed characteristics, while the genome is examined by RFLP. RFLP should probably not be used in combination with theoretically unreliable typing systems based upon phenotypic characteristics which are likely to introduce misleading information. If however the biotyping and RFLP data were combined, is there any correlation between the two. Considering the Antarctic *E.faecium* isolates then Table 16 would suggest not. This Table is effectively a scattergram with no aparent correlation; there is no single API 20 Strep profile being represented by a single *Sal* I pattern. Figure 18 uses lower case letters which indicate combinations of the results from RFLP with *Sal* I, the antibiograms and the biotype with API 20 Strep profiles of the Antarctic *E.faecium* isolates. This letter is referred to as the Combined Grouping. For comparison and ease of interpretation examine Figure 18 in conjunction with Figure 13. The results obtained from the Combined Grouping are very similar to those from *Sal* I alone, except that the number of unique isolates increases from nine with *Sal* I to 23 for the Combined Grouping. The 121 Antarctic *E.faecium* isolates which formed 18 patterns with *Sal* I, form 34 Combined Groupings. Some observations of the Combined Grouping approach can be made. Most of the RFLP pattern E's are of the Combined Grouping b, except for a scattering of f's in subjects 1, 2 and 7. In subject 7 this has the effect of reducing the recorded colonisation period from five to four months if the Combined Groupings are believed. *Sal* I patterns H and I cease to reveal evidence of persistence in subjects 7 and 10, and so become sporadic unique isolates (all recorded as u in Figure 18). The powerful evidence for long term (7 months) colonisation of subject 8 by *Sal* I pattern B becomes considerably subdivided, and colonisation by Combined Grouping d (for 4 months), i (for 2 months), j (for 5 months), and m (for 2 months) is revealed. Figures 16 and 17 contain the equivalent information for *E.durans* and *E.faecalis*, although Combined Grouping letters have

not been assigned. Combined Grouping letters for the NCTC and London isolates are irrelevant as these were all unique by RFLP.

I am skeptical about the Combined Groupings approach in providing any useful information. The RFLP patterns with *Sal* I were assigned after considerable cross checking of isolate patterns ultimately upon the same gel. The antibiogram and biotyping information was only obtained upon one occasion, and so has not been equivalently validated. More importantly, the phenotypic approaches to typing suffer from the inability to quantify the the influence of the differential expression of genes, while, as mentioned before, methods which directly examine the genome avoid this problem. Combining this data introduces greater uncertainty, and also dilutes the power of the RFLP data.

Comparison of the diversity of banding patterns within the different sources of isolates in this study lends weight to the applicability of RFLP as an epidemiological typing tool. The seven type strains all had unique RFLP patterns with *Sal* I, as did the 13 typable isolates (out of 15) from the outpatients department, while for the 158 typable isolates (out of 160) from the geographically isolated Antarctic community, only 22 patterns emerged; a mean of 8 isolates per RFLP pattern with a range of 1-34. Thus in a situation where collection of multiple isolates of the same strain would be expected they were obtained. The RFLP patterns obtained from the outpatients and Antarctic community did not overlap for any isolates, and nor were they indistinguishable from any of the type strains, suggesting that enterococcal strains in general are genetically diverse which is in agreement with Farrow *et al*, 1983.

In this study, carriage of multiple strains of a single enterococcal species by individuals has been demonstrated (Figures 13, 16 and 17). By examination of Figure 13 it can be seen that in general the more isolates that were collected the more strains were detected, this suggests that carriage of multiple strains by a single

individual is probably the norm. This is of some significance for future studies on enterococcal carriage and spread where large collections of isolates are probably necessary to genuinely reflect the diversity.

Aber and Mackel (1981) suggest that the ideal typing scheme should be standardised, reproducible, sensitive, stable, inexpensive, available, widely applicable and field tested. The Warnock (1984) criteria (section 1.11) include the same basic features. The application of RFLP to the temporal analysis of the Antarctic isolates has tested many of these features. The technique is certainly reproducible, sensitive, stable, reasonably inexpensive and available. Standardisation is possible at present with only some parameters, such as the restriction endonuclease used, and is to an extent irrelevant as the important comparisons are generally between isolates from the same "outbreak" and these can normally be compared on the same gel. In the future automated means of reading gels may eliminate some of the problems of standardisation so that results can be compared between laboratories. RFLP is certainly a widely applicable technique as demonstrated by the broad range of situations in which it has been applied (Buchman *et al*, 1978; Collins and de Lisle, 1985; Scherer and Stevens, 1987; Owen, 1988; Cleary *et al*, 1988; Denning *et al*, 1989; Mogollon *et al*, 1990; Jordens and Pennington, 1991; Bingen *et al*, 1991; Hall *et al*, 1992). This study has field tested the technique of RFLP in the enterococci, as have Bingen *et al* (1991) and Hall *et al* (1992) each with interesting and relevant results. It would also be intrigueing to apply the technique to an unequivocal outbreak situation such as the vancomycin resistant isolates reported by Uttley *et al* (1989).

The present study has succeeded in applying RFLP to the enterococci and showing that a suitable diversity of patterns is obtained. It has not succeded in the aim of documenting the enterococcal distribution and spread in the isolated community because of poor isolate survival. A more satisfactory study could be arranged if several different problems were addressed. Isolate identification by

phenotypic means requires reassessing because the API 20 Strep has been inadequate. One possibility would be to use the identification schemes of Facklam and Collins (1989b) or Ruoff *et al* (1990b). The biochemical differentiation between *E.faecium* and *E.durans* requires particular attention. Should antibiograms be used then their range and dosage require review, in particular the introduction of a 200 μg gentamicin disc. The Antarctic isolate collection could be improved by making greater effort to collect from each individual on a regular basis, and by using a more enterococcal specific collection medium. Improved survival should result from either freeze drying, which is the current practise, or by using deep frozen bacterial impregnated beads. An alternative approach would be to provide the appropriate laboratory facilities in the Antarctic. The timing of sampling could be extended to include a period before departure from the UK, again whilst on board the Royal Research Ships, also whilst on the research base during the period of isolation, and finally when back in the UK. This would provide better information about the normal enterococcal gut flora, and the effects of isolation, but would be organisationally difficult. The comparison between RFLP and ribotyping could be more fully explored if either all of the collected isolates were examined by both techniques, or more realistically a larger group of one particular species examined by both techniques. A probe other than ribosomal RNA should be considered. The core of this study - typing of enterococci by RFLP has been successfully examined, but the application of this to epidemiology in the isolated community requires further work.

4.9. Conclusions

This was a complex investigation involving biochemical profiles, antibiograms and the molecular technique of restriction fragment length polymorphism (RFLP) to characterise a large group of mainly enterococcal isolates. A subset of isolates were also examined by ribotyping. Questions are raised concerning the identification of enterococcal species, the validity of phenotypic

typing, and the place of molecular methods in the epidemiological study of enterococci. Further work is required, particularly in the area of species identification by phenotypic means. The technique of RFLP of small fragments should be validated by the application of it to a group of enterococcal isolates which have been epidemiologically defined by another means.

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Characterization of enterococcal isolates by restriction enzyme analysis of genomic DNA

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SUMMARY

A restriction enzyme analysis (REA) of chromosomal DNA for the intra-species characterization of enterococci is reported. The DNA was extracted by a rapid method and digested with the restriction enzyme *Sal* I to provide a characteristic 'fingerprint' consisting of 10–20 bands in the 1·6–5·0 kb range. One hundred and eighty enterococcal isolates were examined; 5 were type strains, 15 from an out-patient clinic and 160 from a geographically isolated British Antarctic Survey Base. The epidemiologically unrelated out-patient clinic isolates gave readily distinguishable patterns, whereas isolates from the geographically isolated community showed evidence of colonization. This technique provided a highly discriminatory method of isolate characterization for *Enterococcus faecalis*, *E. faecium* and *E. durans* suitable for epidemiological studies.

A sample of isolates were probed with 16 + 23 S ribosomal RNA from *Escherichia coli*. Discrimination between isolates was poorer than with REA, although good correlation was observed between the results of the two techniques.

INTRODUCTION

Enterococci cause a variety of infections, with the critically ill host particularly at risk [1]. An endogenous source has frequently been implicated [2], but nosocomial infection appears to be on the increase [3, 4]. This increase in hospital-acquired infection may be related to the wide use of broad spectrum β-lactam antibiotics encouraging enterococcal overgrowth [5]. Also of concern is the rise in antibiotic resistance in the enterococci; the genus is already noted for possessing intrinsic resistance to many antimicrobial agents. High level transferable gentamicin resistance [6], transferable β-lactamase production [7], alterations in penicillin binding proteins [8] and resistance to vancomycin [1, 9] in the enterococci are all likely to exert an increasing influence on future clinical prescribing practice.

The establishment of the genus Enterococcus is a relatively recent development

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in the taxonomy of streptococci [10]. Recent work indicates that the genus is made up of a heterogeneous group of organisms. In serious infections the practice of identifying isolates solely to the genus level must be questioned [11].

Epidemiological investigation of enterococcal infection has been hampered by the lack of a simple typing method [12]. Restriction enzyme analysis (REA) of chromosomal DNA and ribosomal RNA gene restriction patterns have been advocated as epidemiological typing methods for a wide range of bacterial pathogens [13, 14]. The aim of this study was to examine the suitability of chromosomal DNA analysis by REA and ribosomal RNA gene restriction analysis for the intraspecies differentiation of enterococcal isolates.

MATERIALS AND METHODS

Isolates

Strains were obtained from three sources. Seven type strains were obtained from the National Collection of Type Cultures (NCTC) (Public Health Laboratory Service, Colindale Avenue, London, UK), of which five were enterococcal type strains: *Streptococcus faecalis* NCTC 775, *Streptococcus faecalis* var. *liquefaciens* NCTC 2705, *Streptococcus faecalis* var. *zymogenes* NCTC 10927, *Streptococcus faecium* NCTC 7171 and *Streptococcus durans* NCTC 8307. Two non-enterococcal type strains, *Streptococcus bovis* NCTC 8177, and *Streptococcus lactis* NCTC 6681 were also included.

The other isolates, all enterococci, came from two human sources: (*a*) faecal specimens from an out-patient clinic (representing people living in the community who had not been on antibiotic treatment) at the Royal London Hospital, London (15 isolates) and (*b*) faecal swabs from 10 members of an isolated group of 12 healthy men living on the British Antarctic Survey (BAS) research base Signy, situated on the South Orkney Islands, Antarctica (160 isolates). Whilst in the Antarctic presumptive enterococci were selected by colonial morphology on MacConkey's medium (Oxoid) and stored in thioglycollate broth (Oxoid) at +4 °C before transfer to the UK for detailed identification. A mean of 5 isolates were collected (range 1–13) from the culture of each swab. Swabs were taken at up to 1 month intervals from subjects in the study.

Biochemical identification

Isolates were subcultured on 10% blood agar (Oxoid) at 35 °C in room air and identified to species level with API 20 STREP (API-bioMérieux Ltd), plus additional tests as indicated below based upon the recommendations of the API 20 STREP Analytical Profile Index (1989) supplemented by the company's Computer Identification Service.

The supplementary tests used to differentiate between *E. faecium* and *E. durans* were the production of acid from melibiose and the production of acid from glycerol [10, 15]. *E. faecium* isolates were positive for both tests, and *E. durans* isolates negative for both tests. To differentiate *E. durans* and *Lactococcus lactis*, the presence of the Lancefield group D antigen, determined by latex agglutination (Oxoid) and growth in 6·5% sodium chloride were used, with *E. durans* giving positive results for both tests and *L. lactis* giving negative results for both tests.

Antimicrobial susceptibility testing.

The susceptibilities of all isolates to seven clinically relevant antibiotics were determined by the Kirkby–Bauer disk diffusion method after overnight incubation on unlysed blood agar at 37 °C. The following antibiotics were used: ampicillin (10 μg), ciprofloxacin (5 μg) erythromycin (15 μg), imipenem (10 μg), piperacillin (75 μg), vancomycin (30 μg) and teicoplanin (30 μg). To simplify the interpretation of results all intermediate zone sizes were recorded as sensitive. The testing of sensitivity to vancomycin also reduced the chance of the inclusion of lactobacilli, *Leuconostoc* sp. and pediococci because they are resistant whilst enterococci are usually susceptible [16].

DNA extraction

Bacterial DNA was extracted by a modification of the method of Pitcher and colleagues [17]. Bacteria from two blood agar plates were harvested into a microfuge tube containing 0·5 ml 10 mM Tris (pH 8·0), 1 mM-EDTA (TE buffer) and centrifuged 6000 g for 1 min. The resulting pellet was resuspended in 100 μl of TE buffer containing 500 mg/ml lysozyme, and incubated at 37 °C for 30 min. Lysis of bacteria was completed by the addition of 0·5 ml GES reagent (containing 5 M guanidium thiocyanate, 0·1 M-EDTA and 0·5% v/v Sarkosyl), for 10 min at room temperature. 0·25 ml 7·5 M ammonium acetate (+4 °C) was added and the tubes placed on ice for 10 min. 0·5 ml chloroform:isoamylalcohol (24:1) was added, the samples mixed thoroughly and centrifuged at 12000 g for 20 min. DNA was recovered from the resulting aqueous phase by precipitation with 0·54 volumes of cold (−20 °C) isopropanol followed by 30 min at −20 °C and centrifuging at 12000 g for 2·5 min. The resulting pellets were resuspended in 0·2 ml TE buffer and the DNA reprecipitated by the addition of sodium chloride to a final concentration of 0·1 M and 2·5 volumes of cold (−20 °C) absolute alcohol followed by 20 min at −20 °C and centrifugation at 6000 g for 2·5 min. Pellets were air dried at room temperature and resuspended in 100–200 μl of TE buffer, and stored at +4 °C. The purity and yield of DNA were estimated by spectrophotometry at 260 and 280 nm [18]. The integrity of the DNA was determined on 0·8% horizontal agarose gels in TBE buffer (89 mM-Tris, 89 mM boric acid, 2 mM-EDTA, pH 8·3).

Restriction endonuclease analysis

The DNA samples (5 μg) were digested overnight with restriction endonucleases (REs) under conditions recommended by the manufacturer (Boehringer-Mannheim, Bell Lane, Lewes, East Sussex, UK), with the addition of RNAase to 0·05 mg/ml. The REs tested included *Bam*H I, *Eco*R I, *Hin*d III, *Sac* I and *Sal* I. The resulting fragments were separated by horizontal electrophoresis on 0·8% agarose gels in TBE buffer at 100 volts on a 15 × 15 cm gel for 4 h. A 1 kb 'ladder' (Gibco-BRL) was used as the molecular size marker. Banding patterns were compared by eye on the same gel, and the groupings obtained confirmed by running the isolates together on a subsequent gel. Any difference in banding pattern was considered to be a separate DNA fingerprint.

RNA probe

Ribosomal RNA (16+23 S) from *E. coli* (Boehringer-Mannheim) (10 μg) was end-labelled with 50 μCi [γ-^{32}P]ATP (Amersham International, UK) using 10 units of T4 polynucleotide kinase in 50 mM-Tris-Cl (pH 7·6), 10 mM-$MgCl_2$, 5 mM dithiothreitol, 0·1 mM spermidine and 0·1 mM-EDTA. After 60 min incubation at 37 °C the reaction was terminated by the addition of 10 μl of 0·2% SDS, 20 mM-EDTA, and the probe used without further purification.

Southern blotting and DNA hybridization

RE digests of total cellular DNA from 17 isolates using *Eco*R I or Hind III were separated on 0·8% agarose gels, and then depurinated, denatured and transferred to nylon membranes (Hybond-N, Amersham International) by the method of Southern as modified by Maniatis and colleagues [18]. The Southern blots were pre-hybridized for 3 h at 65 °C in 2 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M sodium citrate), 5 × FPG (1 × FPG is 0·02% Ficoll 400, 0·02% polyvinylpyrrolidone 350, 0·02% glycine), 0·5% SDS and 100 μg/ml denatured salmon sperm DNA. The probe was then added to this and the hybridization mix incubated at 65 °C overnight. Hybridized filters were washed [6], and then exposed to X-ray film (Kodak X-OMAT AR) for 2 days at −70 °C using an intensifying screen.

RESULTS

Biochemical identification

The origin and species distribution of isolates used in this study are listed in Table 1.

All isolates except *L. lactis* NCTC 6681 were Lancefield group D. One hundred and eighty out of the 182 isolates could be identified to species by the API 20 STREP and supplementary tests. Twenty-two different API profiles were obtained for the 182 isolates and 96 (53%) isolates required supplementary biochemical tests to confirm the species identification. Two enterococcal isolates were not speciated by these methods; but were included in the study and subsequently identified to species level on the basis of their DNA fingerprint.

Restriction endonuclease analysis

The extraction method yielded 0·02–0·4 mg of high molecular weight DNA suitable for digestion with restriction enzymes from 178/182 (98%) isolates.

After a pilot study looking at five different restriction endonucleases, *Sal* I was selected as it gave readily distinguishable patterns with 10–20 discrete bands in the 1·6–5·0 kb range (Fig. 1). For the 15 enterococcal isolates (Table 2) digested with *Sal* I in Fig. 1, 12 different banding patterns were obtained with indistinguishable patterns only occurring within a species. *Sal* I was used for the characterization of all 178 isolates from which DNA had been obtained, and this resulted in 41 different banding patterns (Table 3).

The two isolates not identified below genus level by biochemical means were identified to species level by REA with *Sal* I as they had patterns indistinguishable from isolates which had been confidently identified to species level by the biochemical tests. One isolate was identified as *E. faecium*, the other as *E. durans*.

Table 1. *Origin and species distribution of isolates*

	NCTC*	London out-patients	Antarctic	Total
E. faecalis	3	6	12	21
E. faecium	1	7	112	120
E. durans	1	1	34	36
E. casseliflavus	0	1	0	1
S. bovis	1	0	0	1
L. lactis	1	0	0	1
genus Enterococcus	0	0	2	2
Totals	7	15	160	182

Assignment to species is based upon the biochemical tests (API 20 STREP and supplementary tests) as described in the methods.

* National Collection of Type Cultures, London, UK.

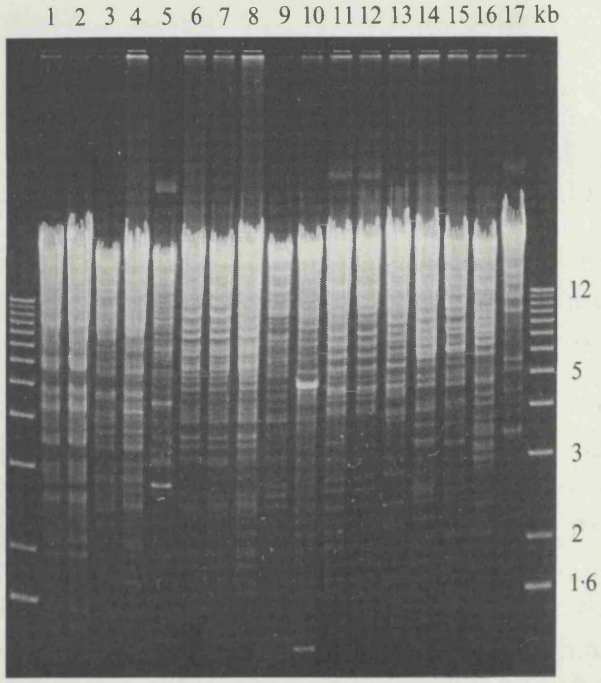

Fig. 1. DNA fingerprint patterns of the isolates described in Table 2, using the restriction enzyme *Sal* I. Isolates 1 and 2; and 6 and 8, are indistinguishable, isolates 10 and 13 are indistinguishable except that two bright bands, from a presumed plasmid, are present in isolate 10. M = 1 kb ladder as the molecular size marker.

Considering the DNA fingerprint patterns of the enterococcal species individually (Table 3), *E. faecium* was the largest and most diverse group with 117 typable isolates which were represented by 11 API profiles and 24 DNA fingerprints. No correlation between API profile and DNA fingerprint pattern was observed.

The *E. durans* isolates were a more homogeneous group, with 37 isolates, 5 API profiles and 4 DNA fingerprint patterns. DNA from 34 out of 35 isolates from the Antarctic community were indistinguishable when digested with *Sal* I and the single different Antarctic isolate differed from these only in having a single extra band (Fig. 1, lane 7). The type strain and out-patient strain were unique. The

Table 2. *Details of isolates illustrated in Figures 1 and 2*

Track	Source	Fingerprint* DNA	Fingerprint* rRNA	Species
1	Antarctic	J	a	*E. faecalis*
2	Antarctic	J	a	*E. faecalis*
3	NCTC 775	Unique	Unique	*E. faecalis*
4	NCTC 2705	Unique	a	*E. faecalis*
5	NCTC 8307	Unique	Unique	*E. durans*
6	Antarctic	K	b	*E. durans*
7	Antarctic	Unique	b	*E. durans*
8	Antarctic	K	b	*E. durans*
9	NCTC 7171	Unique	Unique	*E. faecium*
10	Antarctic	C	c	*E. faecium*
11	Antarctic	B	Unique	*E. faecium*
12	Antarctic	Unique	Unique	*E. faecium*
13	Antarctic	C	c	*E. faecium*
14	Antarctic	D	Unique	*E. faecium*
15	Antarctic	E	Unique	*E. faecium*
16	NCTC 8177	Unique	Unique	*S. bovis*
17	NCTC 6681	Unique	Unique	*L. lactis*

* Letters assigned to indicate whether isolates had banding patterns which were distinguishable or not from those of other isolates when the same method of typing was used.

E. faecalis group consisted of 21 isolates, 4 API profiles and 10 DNA finger-prints. The 12 isolates from the Antarctic community had indistinguishable fingerprints, whilst the 3 type strains and 6 out-patient isolates were all unique.

Ribosomal RNA gene restriction patterns

DNA from a sample of 17 isolates (Table 2) was probed with rRNA after digestion with either *Hind* III or *Eco*R I. The hybridization patterns resulting from digestion with *Eco*R I were more discriminatory than those with *Hind* III. At the inter-species level, *E. faecium* and *E. durans* had closely related patterns which were difficult to distinguish from each other, while *E. faecalis*, *L. lactis* and *S. bovis* were different from each other, and from the *E. faecium*/*E. durans* group. At the intra-species level minor differences were evident within the three enterococcal species (*E. faecalis*, *E. faecium* and *E. durans*). For the 15 enterococcal isolates in Fig. 2, 10 rRNA gene restriction patterns were obtained with indistinguishable patterns only occurring within a species; however, the significance of some faint smudges was difficult to interpret.

With two exceptions strains with indistinguishable rRNA gene restriction patterns also gave indistinguishable patterns with REA (Figs. 1 and 2). The major discrepancy is one of the type strains of *E. faecalis* NCTC 2705 (lane 4) which was indistinguishable from two other *E. faecalis* isolates, those in lanes 1 and 2 by rRNA gene restriction patterns. The isolate in lane 7, which had been unique on REA was very similar to isolates 6 and 8 by rRNA gene restriction pattern. These three isolates had closely related patterns on REA with *Sal* I and were indistinguishable when the restriction enzymes *Bam*H I or *Sac* I were used for the analysis. The type strain of *E. durans* (NCTC 8307) in lane 5 had a significantly different rRNA gene restriction pattern from the 14 other enterococci examined.

Table 3. *Summary of DNA fingerprint pattern results with Sal I*

Species	Pattern and no. of isolates		Source
E. faecium	A	*10	Antarctic
	B	20	Antarctic
	C	*22	Antarctic
	D	7	Antarctic
	E	29	Antarctic
	F	8	Antarctic
	G	2	Antarctic
	H	2	Antarctic
	I	2	Antarctic
	Unique	15	Antarctic (9)
			NCTC (1)
			London (5)
	Nil	4	Antarctic (2)
			London (2)
	total 121		
E. faecalis	J	12	Antarctic
	Unique	9	London (6)
			NCTC (3)
	total 21		
E. durans	K	*34	Antarctic
	Unique	3	Antarctic (1)
			NCTC (1)
			London (1)
	total 37		
E. casseliflavus	Unique	1	London
S. bovis	Unique	1	NCTC
L. lactis	Unique	1	NCTC
	Total 182 isolates		

* These fingerprint patterns contained one enterococcal isolate that it was not possible to assign to species by the biochemical tests described.

Biochemical identification

Between the different DNA fingerprint patterns the biochemical test results displayed relatively few differences. Considering the largest species group *E. faecium*, there was variation in up to six biochemical test results (Table 4). Similar results but with less variation were obtained for *E. faecalis* and *E. durans*.

Antibiograms

All isolates were sensitive to ampicillin, piperacillin, vancomycin and teicoplanin. As a method of typing enterococci these antibiograms were of little value; only five different combinations were detected and 87% of the isolates had the same pattern of resistance.

Temporal analysis

The distribution of enterococcal isolates over time from the subjects in the isolated community is shown in Table 5. Repeated isolation of organisms with the same DNA fingerprint pattern over a number of months from a single individual probably reflects colonization. The results suggest that one strain (i.e. organisms

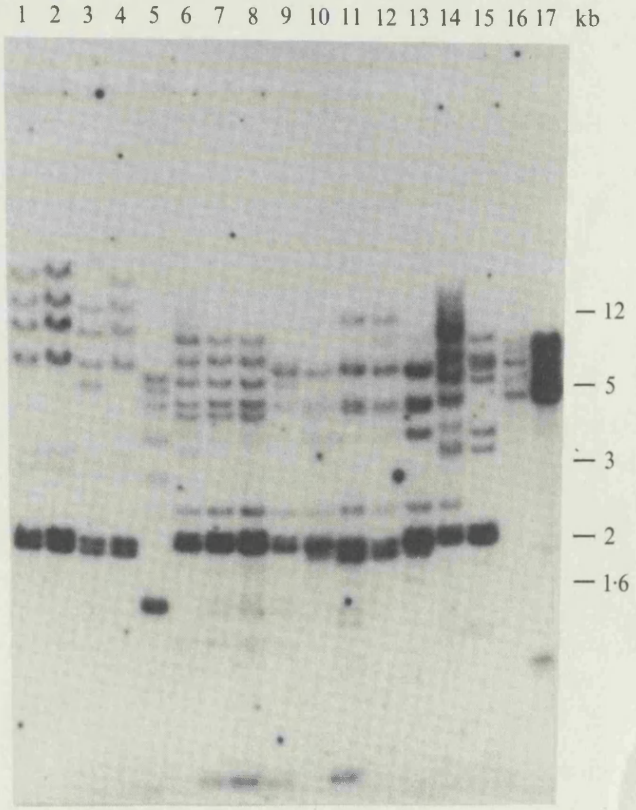

Fig. 2. Ribosomal RNA gene restriction patterns of the isolates described in Table 2, after digestion of bacterial DNA with *Eco*R I. Isolates with indistinguishable patterns are: 1, 2 and 4; 6, 7 and 8 (see text), and 10 and 13.

Table 4. *Variable* E. faecium *biotyping results**

	Sal I DNA fingerprint pattern										
	A, U_1, U_2	B	C	D	E	F, U_3, U_6, U_{10}, U_{11}	G, H, I, U_4, U_8	U_5	U_7	U_9, U_{12}, U_{13}, U_{14}	U_{15}
Hippurate	+	+	+	v	+	+	+	+	+	+	+
α-Galactosidase	−	v	+	−	−	−	+	+	+	+	−
Alkaline phosphatase	−	v	−	−	−	−	−	−	−	−	−
Mannitol	−	+	v	+	v	+	+	+	−	+	+
Raffinose	−	−	v	−	−	−	−	−	−	+	+
Starch hydrolysis	+	v	+	+	+	+	+	−	+	+	+

* (+) positive; (−) negative; (v) variable.
† Unique isolates indicated by letter U.

with one DNA fingerprint pattern) can be shared and so may persist for several months in more than one individual. An example of this is with the DNA finger print pattern E which was present in subjects I, II, III, V and IX, and is shown to colonize subject II and V. Colonization simultaneously by more than one strain and by more than one enterococcal species was also detected, for example four different strains of *E. faecium* (DNA finger print patterns E, F, G and H) colonized subject V, and three different enterococcal species colonized subject VI, *E. faecium* (pattern B), *E. faecalis* (pattern J) and *E. durans* (pattern K).

Table 5. *Temporal analysis of enterococcal isolates from an isolated Antarctic community*

Subject	Month 4	5	6	7	8	9	10
I	+*	*E, K*	+	*D, J*	+	+	+
			E----------	----------	----------	*E*----------	*E*
II	+	*C*		+	+		
		K----------	----------	*K*			
III	+		−†		+	−	−
				B, U_D			
		A, E		*F*----------	*F*		
IV	+	U_1, U_2	−			+	−
		K----------	----------	*K*----------	*K*		
			E	*E*----------	----------	*E*----------	*E*
V		*A, K*		*F*----------	*F*		U_3
				G----------	----------	----------	*G*
	+				−	*H*----------	*H*
	B----------	----------	*B*----------	----------	*B*----------	----------	*B*
		J----------	----------	----------	----------	----------	*J*
VI		*K*----------	----------	----------	*K*		
		A			U_4, U_5	+	
VII	+	−	−	U_6	−	−	−
		C----------	*C*----------	*C*----------	----------	----------	*C*
VIII		*I*	*I*				
	+		A, U_7, U_8, U_9		+	+	
IX	+	+	+	*E, F*	−	−	−
X	+	*K*	+	−	−	−	−

* +, Samples died during transport to UK. † −, No sample taken.

Letters indicate DNA fingerprint patterns (as in Table 3). Unique patterns indicated by the letter U. *E. faecium* isolates are labelled A–I and U_1–U_9; *E. faecalis* isolates are labelled J; *E. durans* isolates are labelled K and U_D.

DISCUSSION

The recent demonstrations of nosocomial transmission of enterococcal infection and the increase in their range of resistances to antimicrobial agents underscore the need for the development of reliable and simple methods for the characterization of these organisms. Phenotypic characteristics have been considered for the role of strain differentiation but difficulties arise because biochemical reactions are not stable characteristics, and the variation between isolates is limited. In an attempt to increase discriminatory power, combinations of markers such as phage typing and bacteriocin typing have been used to establish typing schemes for the enterococci [19]. Such methods are complex, and do not address the problem of the variable expression of phenotypic characteristics.

Restriction enzyme analysis of plasmid DNA has been successfully used in combination with phenotypic markers in the epidemiological investigation of enterococcal infection [4]. However, plasmid DNA is mobile between organisms and it is therefore preferable to analyse the bacterial genome.

REA has recently been successfully applied to strain characterization using pulsed field electrophoresis of large DNA fragments of 27 isolates of *E. faecalis*

[20]. The pulsed field technique requires expensive equipment which is not readily available in most routine laboratories. Bingen and colleagues [21] *et al.* have applied REA to show that a small group of nosocomial vancomycin-resistant *E. faecium* isolates were genetically unrelated.

The rapid small-scale DNA extraction method by Pitcher and colleagues [17] has here been successfully applied to a large group of enterococci. The intention in this study was to choose a restriction enzyme which gave a limited number of bands after gel electrophoresis so that comparison with other isolates was relatively simple, and yet to retain the sensitivity needed to distinguish between isolates. After a pilot study the restriction enzyme *Sal* I was selected. The DNA banding patterns obtained were compared over the 1·6–5·0 kb range. It became clear subsequently that the isolate groupings made in this way were, as far as could be observed also indistinguishable beyond this kilobase range. We have considered any REA banding pattern differences between isolates to represent separate strains. However, minor differences such as a single band (Fig. 1, lane 7) could be due to the digestion of a plasmid or transposon in what are otherwise the same strain.

There is no gold standard at the present time for the identification of *Enterococcal* species. We chose the API 20 STREP kit as the most widely available and commonly used product, one that was easily applied to a large group of isolates. A different combination of biochemical and physiological tests may be more accurate in identifying *Enterococcal* species, particularly the most recently described species [16, 22]. However 178/180 enterococcal isolates in this study did satisfy the criteria of the API 20 STREP data base for their identification to species. An incidental finding was that the two isolates not adequately identified to species level by the API 20 STREP system were later identified on the bases of their REA patterns. This suggests that where a collection of banding patterns is available as a reference, there is a place for DNA fingerprinting in the identification of isolates to the species level, as well as to the strain level.

More DNA fingerprint patterns than API profiles (39 compared with 20) were obtained for the 180 enterococcal isolates. As expected there was general lack of marked phenotypic variation with only five different antibiograms, and limited biochemical variation (Table 4). The usefulness of DNA fingerprinting as a typing system is reinforced when the fingerprints of the 7 type strains and 13 out-patient isolates are considered alone, these all being unique in their DNA banding patterns. For example the six out-patient strains of *E. faecalis* were of the same API profile, but each had a unique DNA fingerprint pattern.

The 158 enterococcal isolates typable by DNA fingerprinting from the small, geographically isolated and closed community in the Antarctic with collection over a 7-month period were represented by only 22 of the 39 patterns; these contained between 1 and 34 isolates (Table 3). Thus in a situation where collection of multiple isolates of the same strain would be expected they were detected. When analysed (Table 5) the data represent plausible temporal information.

The DNA fingerprint patterns obtained from the two geographically distinct community-based sources (out-patients and Antarctic) did not display any overlap of patterns, they were also easily distinguished from any of the type strains, suggesting that enterococcal strains in general are genetically diverse; this

is an agreement with the findings of others [20, 23]. However, this genetic heterogeneity was not in evidence when only the isolated Antarctic community was considered. Diversity was evident solely within the *E. faecium* group, while the *E. faecalis* and *E. durans* isolates were largely indistinguishable (Table 3). This is unexpected and may be a demonstration of clonal selection within an isolated community. However, the overall survival of the isolates collected in the Antarctic was low (25%), and the normally higher numbers of *E. faecalis* compared to *E. faecium* isolates in faeces was reversed [19]. These points suggest that this result could also be artifactual and related to isolate storage and survival.

The close correlation between the results for REA with *Sal* I and the rRNA probing of *Eco*R I digests strengthens the conclusion that differences in REA banding patterns reflect significant differences between isolates at the genetic level. However, the rRNA gene restriction patterns are more complex and time-consuming than REA and from the evidence of this study less discriminatory. The poor discrimination of rRNA probing has been noted in other studies [13, 21]; it may be that ribosomal RNA is too highly conserved to be useful at the intra-species level.

In summary the simple DNA extraction methodology and standard gel electrophoresis equipment used for the restriction enzyme analysis described in the present study should make these techniques accessible to most large microbiological laboratories. The diversity and distribution of banding patterns within the different geographical sources of isolates studied suggests that the REA as described is suitable for the epidemiological typing of enterococci.

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