THE CHARACTERISATION OF THE GENUS *MICROCOCCUS* AND THE GENUS *STOMATOCOCCUS* USING PHENOTYPIC AND GENOTYPIC TECHNIQUES.

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Medicine at the University of Glasgow.

The work for this thesis was undertaken at:-

THE DEPARTMENT OF MEDICAL MICROBIOLOGY, ABERDEEN
UNIVERSITY MEDICAL SCHOOL, ABERDEEN.

SUBMITTED 1992

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SUMMARY

This study shows that the biochemical inertness of the genus *Micrococcus* makes the API microtitre strip an unsuitable technique by which to identify species within the genus and that colony pigmentation is an invalid character with which to speciate the genus *Micrococcus*.

The genus *Micrococcus* forms a heterogeneous group of organisms when ribotype group and pulsed-field gel electrophoresis (PFGE) is considered. The use of two restriction enzymes forms different ribotype groupings within the genus. In this study *M.kristinae*, *M.varians* and the majority of *M.sedentarius* formed homogeneous core ribotype profiles. *M.luteus* and *M.lylae* formed heterogeneous ribotype and PFGE groups. The ability of *M.lylae* and *M.sedentarius* to belong to *M.luteus* ribotype groups demonstrates a genetic relationship between these species and *M.luteus*. Restriction enzyme analysis (REA) and PFGE were the most useful techniques for typing the micrococci. By PFGE *M.roseus* has a genome size of 3,387 kilobases (kb) which is approximately 30% larger than other members of the genus, which have genomes of between 1,823 and 2,598 kb, and may warrant generic status.

By REA and ribotyping it was shown that people carry a clonal population of *M.luteus* over a period of time.

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ACKNOWLEDGEMENTS

I would like to acknowledge Professor Pennington for overall supervision, Dr Philip Carter who gave advice and helped with the radioactive probing, Dr Fiona Thomson-Carter who gave advice and direction to this work and also for her helpful suggestions made during the critical reading of this manuscript and to all the staff at the Department of Medical Microbiology in Aberdeen.

This work would not have been possible without the co-operation of my fellow base members who cheerfully allowed me to sample and venesect them on a monthly basis for 13 months. I also acknowledge the support of the British Antarctic Survey.

I would also like to thank the Department of Medical Illustration in Aberdeen Royal Infirmary, and John Tolland in the Communicable Diseases (Scotland) Unit at Ruchill Hospital for helping with the illustrations and tables and for printing the manuscript.

Lastly, I am indebted to Pat who waited, understanding one man's longing to see and experience the Antarctic, and it is to her that I dedicate this work.

Authors declaration.

The work described in this thesis was initiated and carried out by myself, except for the ^{32}P labelling of *E.coli* 16S and 23S rRNA which was performed by Dr Philip Carter, and Figure 16 which was done by Tracy Purcell in my absence.

This work has not been submitted for any other higher degree.

"The boundaries of the species, whereby men sort them are made by men".

John Locke, 1689.

An Essay Concerning Human Understanding. Book III,

Chapter VI.

1.

INTRODUCTION

1.1) The genus *Micrococcus*

The genus *Micrococcus* is a member of the family Micrococcaceae, which contains the Gram-positive catalase positive-cocci. The family Micrococcaceae also includes the genus *Stomatococcus*, the genus *Staphylococcus* and the genus *Planococcus*. The genus *Micrococcus* contains 9 recognised species; *M.luteus*, *M.lylae*, *M.roseus*, *M.nishinomiyaensis*, *M.kristinae*, *M.varians*, *M.sedentarius*, *M.agilis*, and *M.halobius* [1,2,3,4,5]. All (except *M.halobius*) have been isolated from human skin and are considered part of the normal skin flora. Members of the genus *Micrococcus* have cells which are spherical, occuring mostly in pairs or tetrads, are non-motile (except *M.agilis*) and non-sporing. Metabolism is strictly aerobic, although *M.kristinae* and *M.varians* are slightly facultatively anaerobic [6]. Colonies of micrococci are usually circular, entire, convex and smooth. Colonies may be yellow, lemon, orange, pink-red or red pigmented[7]. Differences in colony morphology or pigment are said to reflect differences in other characteristics of the strains [7].

There has been a lack of interest in the genus *Micrococcus* over the years, with the genus as a whole being neglected by medical microbiologists. This is partly due to their low pathogenic potential, leading to small numbers being isolated from clinical material, and

the assumption that when isolated they are culture contaminants. Another difficulty when working with the genus *Micrococcus* is that it is largely a genus defined by negative biochemical criteria. The defining biochemical characteristics of the genus *Micrococcus*, which arose during the 1960's and 70's, define members of the genus as *not* being members of the genus *Staphylococcus*. This was due to the tremendous efforts of medical microbiologists over the decades to differentiate the micrococci from the potentially more pathogenic coagulase-negative staphylococci. The inevitable consequence of this was to admit poorly characterised isolates into the genus to form the "'scrap heap' genus *Micrococcus*" [8].

Within the genus *Micrococcus* speciation presents a problem due to the inability of many members of the genus to produce detectable acid from a wide range of carbohydrates [7,8]. Only *M.varians* and *M.kristinae* show sufficient reactivity with enough carbohydrates to provide an acceptable identification of species [2,7]. The other species among the genus are separated on a few positive biochemical criteria eg, nitrate reduction, *M.roseus* [2]; lysozyme susceptibility, *M.luteus* [7]; antibiotic resistance, *M.sedentarius* [7]; and the major characteristics of colony pigment, cell wall composition, genetic compatibility and the percentage molar

guanine/cytosine (% mol GC) content [9,10,11]. Although an unreliable characteristic with which to speciate bacteria, the biochemical inertness of the genus *Micrococcus* has meant that colony pigment has become an important criterion with which to speciate the genus; *M.luteus* and *M.varians* being classically described as yellow pigmented [1,3,7] *M.kristinae* and *M.lylae* being described as cream or unpigmented [7], *M.roseus* being described as red pigmented [2], *M.nishinomiyaensis* being described as orange pigmented [4] and *M.sedentarius* being either yellow or cream coloured [7].

1.2) The present taxonomic status of the family Micrococcaceae and the genus *Micrococcus*

Although the name "Micrococcus" was used by Ogston in a colloquial sense, in the generic sense the name *Micrococcus* was first used by Cohn (1872) when he described the first *Micrococcus* species *Micrococcus prodigiosus*, which we now know to be a short rod, and is therefore excluded from the genus. The next species described by Cohn was *Micrococcus luteus* (Schroeter) [12]. Since then the genus *Micrococcus*, indeed the family Micrococcaceae, has undergone many transformations, a process that is still continuing. There have been many swings of opinion throughout the 20th century in regard

to the taxonomic position within the family Micrococcaceae, with both the genus *Micrococcus* and the genus *Staphylococcus* being considered invalid at different times [13]. Various genera have been removed from the family Micrococcaceae throughout this century, eg *Sarcinia*, *Rhodococcus*, *Gaffyka* with the inclusion of others, eg *Planococcus* and *Stomatococcus* [15,49]. At present the family Micrococcaceae contains the Gram-positive catalase-positive cocci, which includes the genus *Staphylococcus*, the genus *Micrococcus*, the genus *Planococcus* and the genus *Stomatococcus* each being distinguished from the others by the major characteristics of percentage (%) mol GC content and cell wall structure.

With the realisation that the genetic sequence of an organism is in fact an historical record of evolutionary time, the last decade has seen a fundamental reappraisal of bacterial taxonomics [18]. Comparative oligonucleotide cataloguing of 16S ribosomal RNA (rRNA) has been used to construct bacterial phylogenies. Using this, the family Micrococcaceae was found to be phylogenetically unsound, with its constituent genera being unrelated to each other, each having relatives among the non-spherical bacteria [19,20,21]. The 16S rRNA studies confirm the suggestion from cell wall studies [9], cytochromes [22,23], menaquinones [24,25] and the % mol GC content [22] that the

genus *Micrococcus* is more closely related to the actinomycetes than it is to cocci in the genus *Staphylococcus*. Coccal shape is therefore a useful phylogenetic characteristic only at the very lowest of taxonomic levels [19].

Broadly, *Micrococcus* and *Stomatococcus* are morphologically atypical members of an *Actinomycetes* sub-branch of the Gram-positive Eubacteria that are GC rich (% mol GC > 55%), closely related to those in the genus *Arthrobacter*, *Brevibacterium* and *Cellulomonas* [21,22,26]. These are morphologically complex arthrobacters which exhibit a rod/coccus cycle and are pleomorphic. It has been suggested that the various species of *Micrococcus* should be regarded as degenerate forms of the arthrobacters, which have become locked into the coccoid stage of the arthrobacter life cycle [19]. Although comparative rRNA studies have shown micrococci to be inseparable and intermixed with the arthrobacters [18], numerical taxonomy has shown that the genus *Micrococcus* was phenetically distinct from the arthrobacters, clustering together in a group containing *M.agilis*, *M.luteus*, *M.lylae*, *M.nishinomiyaensis*, *M.sedentarius*, and *M.varians*. *M.kristinae* and *M.roseus* were consistently recovered outside this major *Micrococcus* aggregate cluster.

Planococcus is not a phylogenically valid genus, falling within the genealogical confines of the genus *Bacillus*. The genus *Stomatococcus* represents an independent line of descent within the broad group of Gram-positive bacteria [20].

The genus *Staphylococcus* is considered phylogenetically valid [21,27], belonging to the clostridium-bacillus-streptococcus sub-branch that comprises all the Gram-positive bacteria possessing a DNA with a % mol GC composition lower than 55 %.

The family Micrococcaceae should therefore be considered a defunct phylogenetic group [19]. This has been recognised by the Subcommittee on the Taxonomy of Staphylococci and Micrococci, which took the first steps in dismantling the family Micrococcaceae by removing the genus *Micrococcus* from their remit, suggesting that it be transferred instead to the Committtee on *Cornyebacterium* and Coryneform bacteria [28].

Little is known about the genetic relationships between species in the genus *Micrococcus*. The limited information published suggests considerable heterogeneity. DNA homology values determined from 25 micrococci grouped them into 3 distinct groups [29], corresponding to *M.luteus*, *M.roseus*, and *M.varians*. The homology values between groups was low, 8-17% indicating that there are

no genetic relationships between these three species. This lack of genetic relationship has been confirmed between *M.luteus* and *M.varians*, and *M.luteus* and *M.kristinae* [30]. Transformation experiments also showed no genetic relationship between *M.luteus* and *M.roseus* or between *M.luteus* and *M.varians* [10], although a distant but detectable genetic relationship between *M.sedentarius* and *M.luteus* [7], and a close genetic relationship between *M.luteus* and *M.lylae* was shown. This genetic relationship between *M.luteus* and *M.lylae* has also been demonstrated by immunological studies of their catalases [31]. Furthermore, this study found a weak but significant relationship between *M.luteus* and *M.varians*, but no relationship between *M.luteus* and any other *Micrococcus* species.

This genetic heterogeneity is further supported by the differing % mol GC content among the *Micrococcus* species. The % mol GC range in the genus *Micrococcus* is 66-75% [15]. Two strains differing in % mol GC content by 10% have very little DNA in common and cannot be closely related phylogenetically [31] and a difference of more than 5% is interpreted as implying membership of a different genus [33]. This heterogeneity within the genus *Micrococcus* has fuelled speculation that the genus probably contains the nucleus of at least one new genus [22]. Indeed one study suggests that *M.roseus* may warrant generic status [26].

1.3) Skin carriage of the genus *Micrococcus*

There have been very few attempts to study the skin carriage of the genus *Micrococcus* and early studies were hampered by a poor taxonomic system. The first such study in 1962 [34] showed that *M.luteus* (Baird-Parker's M7 group) formed 7% of the micrococci-staphylococci flora of the human skin. A similar carriage rate for micrococci was again found in a later study [8]. This study however found a carriage rate for *Sarcinia* of 15-30%. Aerobic *Sarcinia* are now considered members of the genus *Micrococcus* [35], thus these early reports of low carriage of micrococci must be interpreted with care.

With the advances in taxonomy a clearer differentiation of the Gram-positive catalase-positive cocci was possible. This led to the recognition of new species [7,36,37], and allowed for a comprehensive reappraisal of the skin ecology of the genus *Micrococcus*.

Members of the genus *Micrococcus* are found more frequently on human skin than the early studies suggested, being found on 96-100% of all subjects sampled [7,38]. This carriage rate is dependent on the age of the carrier as micrococci were only found on 30% of infants (<32 weeks old). In infants *M.luteus* and *M.kristinae* are the prevalent microccocal species found [39]. In adults *M.luteus* is the most prevalent species of micrococci on

the human skin, isolated from 90-95% of all individuals. *M.varians* is the next most prevalent species, isolated in 75-83% of individuals. *M.lylae* was isolated from 33-35% of individuals, *M.nishinomiyaensis* from 28-38% of individuals, *M.kristinae* from 25-35% of individuals, *M.sedentarius* from 13-20% of individuals, and *M.roseus* from 10-15% of individuals [7,38,40]. The most frequently populated skin sites are the head (forehead, chin and cheek), the arms and the legs [38].

1.4) Micrococci isolated from clinical sources

The concept that staphylococci are pathogenic to man is over a century old [41]. For most of this time medical microbiologists have accepted that the coagulase-positive *Staphylococcus aureus* is a pathogen and the coagulase-negative staphylococci and micrococci are commensals or saprophytes. This view has changed with regard to the coagulase-negative staphylococci as their pathogenic potential was reported in the late 1950's, and further confirmed throughout the 1960's.

Interest in the genus *Micrococcus* has often been limited to differentiating these organisms quickly and reliably from the opportunist pathogens in the genus *Staphylococcus*, with little consideration of micrococci in any infective process. This is partly due to the

difficulty in identifying and characterising micrococci easily and quickly in the routine laboratory, and the continuing assumption that the micrococci isolated are contaminants [42,43]. This assumption can lead to delay in diagnosis and effective treatment [44]. Infection due to micrococci may be more prevalent than is commonly believed, because of a general reluctance to consider them pathogenic unless they are isolated repeatedly in pure culture from tissue, blood or other normally sterile body sites. The question of whether micrococci isolated from culture samples originated from those samples or are merely contaminants is irrelevant. The organism when isolated from patients with prosthetic implants assumes importance [45], and appropriate treatment should be given as dictated by antibiotic sensitivities.

Another factor contributing to the lack of data on micrococci in infective processes is the poor and imprecise use of nomenclature, such that *S.epidermidis* has been used interchangeably with *Micrococcus* [46]. There has also been a failure to differentiate between the micrococci and the coagulase-negative staphylococci when considering isolates from clinical sources [47].

A review of the pre-1960's literature is confusing and unhelpful as the framework for the major characteristics separating the coagulase-negative staphylococci from the micrococci had not been established [12]. Micrococci, as

defined by present criteria, were considered as members of various different genera including the genus *Staphylococcus*, the genus *Sarcinia*, the genus *Gaffyka* and the genus *Pediococcus* [48,49]. Indeed the genus *Micrococcus* itself has been considered invalid [13]. Thus some reports of micrococcal infection are now being recognised as due to staphylococci [50,51]. Therefore to know with certainty that the reported pathogenic organism belongs to the genus *Micrococcus*, as understood by modern classification, is impossible. This flux in the genus *Micrococcus* is still continuing with *Micrococcus mucilaginosus*, which has been isolated in bacterial endocarditis, being assigned to the genus *Stomatococcus*[52]. *Stomatococcus mucilaginosus* is now being increasingly recognised in clinical samples [53,54,55].

The normal features of the majority of micrococcal infections are that they are associated with:- surgically implanted artificial prosthetic devices; valvular heart disease; immunocompromised patients; indwelling polythene catheters; and where complex and frequent handling procedures provide opportunities for the entry of organisms, eg, continuous ambulatory peritoneal dialysis (CAPD) [56]. These circumstances are similar to the situations in which coagulase-negative staphylococci are able to cause infection. The lower virulence of the micrococci compared to the coagulase-negative

staphylococci could be due to their increased susceptibility to antiseptic skin cleaning procedures prior to any invasive operative procedure [57]. Unlike some coagulase-negative staphylococci most micrococci do not produce slime [58], although *M.sedentarius* produces a slime similar to that of Gram-negative bacteria [7].

Systemic infections with micrococci can occur in: bacterial endocarditis following cardiovascular surgery [59,60]; cases of valve damage following rheumatic fever [61]; cases of septicaemia associated with intravenous polythene catheters [62]; immunocompromised patients [63] and in the presence of a foci of infection [64]. The frequency of micrococci isolated in bacterial endocarditis varies from 2-10% [65,66,67]. One study however isolated micrococci from 84% of cases of bacterial endocarditis [61]. This study contained patients who had undergone recent cardiac surgery (6 days to 6 months) and patients with endocarditis unrelated to recent cardiac surgey. The latter group contained patients with rheumatic valve disease, tetralogy of Fallot, prosthetic valves, and no previous heart disease. It must be stressed that this higher isolation rate was achieved whilst using the standard oxidation/fermentation test [68] to differentiate between the coagulase-negative staphylococci and the micrococci. The deficiencies of this test will be discussed later.

When speciated *M.flavus*, renamed now as *M.luteus* [66], *M.sedentarius* [69], and a *M.lylae*-like micrococcus [67] have all been isolated in bacterial endocarditis.

Graft infection, which affects 1-6% of patients undergoing vascular reconstructive surgery [70] is a serious complication, with a mortality rate approaching 50%, with associated lower limb amputation amongst survivors. One hypothesis suggests that placement of the graft against an aneurysm wall which harbours bacteria may contribute to post-operative graft infection [45]. When aneurysm contents are culture positive, micrococci are isolated in 5-30% of cases [43,45,70]. In one study [43], 2 out of 12 fatal graft infections had micrococci isolated from the aneurysmal contents. It must be borne in mind that there is a distinction to be made between the simple reporting of the presence of an organism in a pathological condition and its significance to the active disease process, and as such the significance of bacteria in the contents of aneurysms is still disputed. It is claimed they are unimportant in acute infective episodes in the early post-operative period [70].

Micrococci have been recorded as opportunistic pathogens in patients immunocompromised due to malignancy of the lymphoid system [61,71,72] and therapeutic immunosuppressive treatment [73]. In 3 of these cases micrococci caused a pneumonia [71,73]. Micrococci have also been implicated in

causing a condition similar to Whipple's disease in a 13 year old girl with lymphoblastic leukemia involving several central nervous system relapses [72].

Cerebrospinal fluid-diverting ventricular shunts become infected in 6-27% of patients [74,75]. Infection itself was a significant risk factor, raising the mortality in one series from 17% to 40% [74]. Micrococci have been isolated during studies of ventricular shunt infections and infection rates of 3-17% due to micrococci have been reported [74,75,76,77]. The onset of infection within 2 months of an operation suggests contamination of the shunt during the operative procedure by the predominantly low virulence and normally resident skin flora [74,78]. This seems to be the case only in some cases [77], and it is a matter of speculation how many shunt infections are a result of contamination from the patient's nasopharynx, operative personnel and the hospital enviroment. Although micrococci are not usually speciated, there is a report of a shunt infection with *M.varians* [76], a report of *M.sedentarius* causing a cerebral cyst [79], and one report of *M.luteus* causing meningitis [44].

Micrococci, although normal skin inhabitants that usually fulfil the criteria for conditional pathogenicity [41], can initiate the disease process *de novo*.

Pitted keratolysis is a superficial skin infection that causes pitted erosions of the stratum corneum on the soles of the feet and, rarely, the palms. It is generally asymptomatic, but a painful, tender symptomatic form also occurs. In a random sample of men attending a dermatology outpatient clinic, 11% had pitted keratolysis with or without symptoms [80]. *M.sedentarius* was isolated in all cases, and the experimental reproduction of pitted keratolysis was induced on the heel of a healthy volunteer inoculated with an isolated pathogenic strain of *M.sedentarius*. *M.sedentarius* was not isolated from the soles of normal feet in this study. *M.sedentarius* has been found on normal feet [81]. The ability of *M.sedentarius* to degrade the stratum corneum is thought to be due to 2 extracellular proteolytic and callous degrading enzymes [82], which have different activities at different growth rates [83]. There are growth rates for *M.sedentarius* which may be determined by the skin environment, where cells will produce large amounts of extracellular enzymes that can degrade the stratum corneum and lead to pitting. *M.sedentarius* is also found in significant numbers in macerated toe webs [84], and most likely contributes (along with *S.aureus*, Gram-negative bacteria, *C.minutissimum*, and *B.epidermidis*) to the skin erosion and inflammation that occurs due to its ability to cause pitting of the stratum corneum. *M.luteus*,

M.varians and *M.roseus* showed no evidence of involvement in this disease process.

Like pitted keratolysis, a case of septic arthritis of the hip caused by *M.luteus* is unusual in that the micrococcus has produced its pathogenic potential without any of the usual requirements for micrococcal infections [85].

Micrococci have been isolated as part of the normal flora of the conjunctiva and have been shown to be opportunistic in conjunctivitis [86] and in corneal ulcers associated with soft contact lenses [87]. *M.luteus* has also been associated with other ocular pathology. In one study a total of 26 *M.luteus* isolates were collected with 3 being isolated from corneal ulcers, 3 from cases of conjunctivitis, 2 from endophthalmitis, 1 from a contracted socket, and 1 from phthisis bulbi [86]. In trying to assess the pathogenicity of each of these isolates, a rabbit animal model was used. In all cases the isolated bacteria failed to produce disease after intracorneal injection. Micrococci should not be regarded as only opthalmic culture contaminants, as there has been a recorded case of *Micrococcus* endophthalmitis in a young man with intraocular entry of a foreign body after a traumatic wound [89].

1.5) The separation of staphylococci from micrococci

The modern era of structuring the genus *Micrococcus* began with the observation that micrococci and staphylococci could be separated according to the ability of staphylococci to produce acid from glucose anaerobically [90,91,92]. Micrococci either do not produce acid from glucose anaerobically, or produce acid from glucose only aerobically. It was proposed by the International Subcommittee on the Taxonomy of Staphylococci and Micrococci to adopt and standardise this criterion at their first meeting [68,93].

Although now superseded, Baird-Parker's classification of the Micrococcaceae formed a useful framework for grouping the separated micrococci and staphylococci further. Baird-Parker used 56 morphological and physiological characteristics to separate the staphylococci into 6 subgroups and the micrococci into 7 [90], later 8 [91] subgroups.

The standardised oxidation/fermentation test for the separation of the two genera was adopted as a test that was both practical and accurate enough to use in the routine laboratory to differentiate the potentially pathogenic staphylococci from the normally saprophytic micrococci. It soon became clear that this test misclassified weak anaerobic acid producers [94]. The most

frequent misclassification was the characterisation of the slightly facultatively anaerobic micrococci, *M.kristinae* and *M.varians* as staphylococci, and of the weak anaerobic fermenters of glucose *S.xylosus*, *S.cohnii* and *S.saprophyticus* as micrococci [6]. The subcommittee recognised the deficiencies of the standardised oxidation/fermentation test [95], but nevertheless took the pragmatic decision to retain it until a more reliable, simple test became available.

It was shown in 1961 that bacteria could be classified on the basis of their % mol GC content, that this ratio was evolutionarily stable and that phylogenetic relationships could be reflected in the % mol GC content [33]. Using this, Silvestri [96] first stated the hypothesis that the Micrococcaceae could be binominally split into staphylococci and micrococci by the % mol GC ratios of their DNA, with the staphylococci having a % mol GC content of 30-38% and the micrococci having a % mol GC content of 66-75% [15,97].

The correlation between the standard oxidation/fermentation test as recommended by the International Subcommittee on the Taxonomy of Staphylococci and Micrococci and the % mol GC content was tested [11,49,94,96,98,99] and it was shown that there was an anomaly between some Gram-positive catalase-positive cocci that would not produce any detectable acid under

anaerobic conditions and hence would be classified as micrococci, although they had a % mol GC ratio of 30-37%. These were mainly the acetoin-producing micrococci belonging to Baird-Parker's subbgroups 1-4, which have been reclassified by Kloos and Schliefer as *S.cohnii*, *S.saprophyticus* and *S.xylosus* [49,100]. Some facultatively anaerobic micrococci, eg strains of *M.kristinae* would be wrongly identified as staphylococci by relying on the oxidation/ fermentation test [6].

Semi-solid thioglycollate medium was used to try and improve the sensitivity and selectivity of the standard oxidation/fermentation test [101]. This modification however still had the problems inherent in the standard oxidation/fermentation test, in that some staphylococci (bacteria with a % mol GC content of 30-38%) showed only a weak response to growth in glucose anaerobically and would be wrongly classified as micrococci.

With the realisation that the % mol GC content was one of the most reliable and least controversial characteristics capable of dividing the Micrococcaceae [99,102] it was proposed that all Gram-positive catalase-positive cocci formally belonging to the genus *Micrococcus* or the genus *Sarcinia*, which could produce acid from glucose both anaerobically or aerobically, and had a % mol GC content within the range of 30-38%, should be assigned to the genus *Staphylococcus*. Further, that all Gram-positive

catalase-positive cocci that had been formally classified as belonging to the genus *Staphylococcus* or the genus *Sarcinia* but which didn't produce acid from glucose anaerobically or aerobically, and had a % mol GC content between 66-75%, should be assigned to the genus *Micrococcus* [99]. A reappraisal of all published type strains confirmed that % mol GC content could reliably separate these two genera from each other [11], and that it could split the family Micrococcaceae into 3 groups, namely the genus *Micrococcus*, the genus *Staphylococcus*, and the genus *Planococcus* [99]. The genus *Stomatococcus* was later removed from the genus *Micrococcus* and given generic status within the family Micrococcaceae, on the basis of having a % mol GC content of 55-60% [20,26,52].

1.5.1) Cell wall structure

The main components of Gram-positive bacterial cell walls are peptidoglycan, polysaccharides and/or teichoic acids and protein [103].

Peptidoglycan is a heteropolymer built from glycan strands cross-linked through short peptides. The glycan moiety consists of units of alternating β-1,4 linked N-acetylglucosamine and N-acetyl muramic acid. The peptide unit containing alternating L- and D- amino acids is attached to the muramic acid via the peptide's carboxyl

group, forming a bridge between the glycan strands [8]. The structural difference in peptidoglycans between staphylococci and micrococci is a valuable criterion to characterise and distinguish these two genera [104,105]. This difference is also used to speciate bacteria within these two genera [7,104].

Staphylococci have peptidoglycans with a high glycine content in the peptide bridge. *S.aureus* contains a penta- or hexaglycine bridge, whereas coagulase-negative staphylococci have a variable number of glycine residues substituted with lysine, alanine and/or serine residues[9].

The micrococci have peptidoglycans that can be divided into 2 groups. Group 1 contains *M.luteus* which has a polymerised peptide unit (subgroup A2) forming the cross-linkage between peptidoglycans. Group 2 contains the species *M.roseus* and *M.varians*, having a tri- or tetra-alanine peptide (group A3 alpha) as an inter-peptide bridge. Group 1 type peptidoglycans are unique to *M.luteus*, while group 2 type peptidoglycans are also common to streptococci and corynebacteria [9].

This difference in cell wall peptidoglycan has been exploited for the serological identification of staphylococci and micrococci. Immune sera raised against (penta-glycyl-E-amino-hexanoic acid)20-albumin exclusively agglutinated staphylococci while antisera

raised against (tri-L-alanyl-E-amino-n-hexanoic acid)22-albumin reacted strongly to micrococci containing alanine in their peptide linkages, but showed no cross reactivity to staphylococci [106]. Monoclonal antibodies specific for the peptidoglycan of staphylococci have also been used to separate micrococci from staphylococci [107].

The specificity of lysostaphin for the glycine-glycine bonds in the cell wall peptidoglycan is utilised to separate staphylococci and micrococci [108,109,110,111,112]. The most important enzymic mode of action of lysostaphin is its peptidase activity between the glycine-glycine bridges in the cell wall. *S.aureus* with penta- or hexaglycine bridges is therefore very susceptible to the action of lysostaphin [113]. Coagulase-negative staphylococci have a lower molar ratio of glycine in their cell wall peptidoglycans [9,106], thus making coagulase-negative staphylococci less susceptible to the lytic actions of the endopeptidase. This has been confirmed by several studies [114,115,116,117].

It has been stated that lysostaphin sensitivity was the most useful single criterion to separate staphylococci and micrococci [109], that it was more precise and convenient than anaerobic fermentation of glucose [110], and that sensitivity to lysostaphin correlated well with the % mol GC content [109].

A good correlation is found between the % mol GC content of the staphylococci and micrococci and the respective cell wall peptidoglycan types [118]. Within cell wall peptidoglycan groups there is a DNA homology of 48-93%[29]. Further genetic compatibility within groups is confirmed by the genetic transformation of *M.luteus* by organisms containing the subgroup 2A peptidoglycan [119].

Other components of the cell wall useful as taxonomic criteria are the teichoic acids. Teichoic acids are water soluble polymers containing sugar, D-alanine residues and glycerol or ribitol phosphates [120]. Micrococcal cell walls contain no teichoic acids, whereas staphylococcal cell walls contain these acidic polymers, as shown antigenically using the double diffusion technique [121]. Instead of teichoic acids, micrococci contain teichuronic acid which is another acidic polysaccharide consisting of glycosidically linked sugar and uronic acid residues [122]. This difference in the acidic polymers has been shown by using the phage u16, that adsorbs onto the cell wall teichoic acid. Micrococci, having no teichoic acid did not adsorb the phage and were unable to cause any significant phage inactivation [123].

The composition and nature of the aliphatic hydrocarbons has also been used as a differentiating characteristic in the family Micrococcaceae, micrococci having

monosaturated hydrocarbons, whilst the staphylococci have no appreciable quantities of hydrocarbons [124].

1.5.2) Cellular enzymes

The respiratory chain enzymes, namely cytochromes and menaquinones, are distinctive taxonomic characters. Menaquinones are vitamins in the K2 group [24,25], with a side chain of variable length, consisting of polyisoprenoid subunits. Both the length and the nature of this side chain are distinctive for the two genera. Micrococci have "hydrogenated", that is unsaturated isoprenologues, whilst staphylococci have "normal" or saturated isoprenologues [25]. Cytochromes, which act as the terminal electron acceptors in the respiratory chain, also show a difference between the two genera. This forms the basis of the modified benzidine test for the separation of the two genera [125]. Micrococci have cytochromes a,b,c and d, whilst staphylococci have only cytochromes a and b, except for *S.sciuri* and *S.caseolyticus* [6] which has cytochromes a, b, and c, but no d. Cytochrome b-552 appeared in every strain of staphylococcus but is not present in micrococci [23].

There are two classes of D-fructose-1,6-biphosphate aldolases that are distinguished by their catalytic and electrophoretic characteristics. Staphylococci contain

class I aldolases (except *S.caseolyticus* [6]) in contrast to micrococci which contain class II aldolases [126].

1.6) Molecular methods of bacterial characterisation

Bacteria have classically been typed using antisera, biochemical profiles [7,36,37], monoclonal antibodies [107], fimbriation [127], bacteriocin production [128], antibiotic resistance [129], the electrophoretic analysis of whole-cell proteins [130], and multi-locus enzyme electrophoresis [131]. These systems of identification have the disadvantages that they rely on phenotypic characteristics that may not be stably expressed, furthermore the sensitivity of the particular test may not be sufficient to distinguish isolates within clones and species, and the test system may not be universally applicable to all bacteria.

Developments in molecular biology have now enabled the analysis of the genome to be put onto a secure genetic basis. Various typing techniques have been used by the molecular bacteriologist. These are the analysis of bacterial plasmids, the analysis of the chromosomal banding profile produced after digestion with restriction enzymes to produce restriction fragment polymorphisms (DNA fingerprinting), the use of nucleic acid probes which hybridise to specific chromosomal sequences, sequencing of genes and the direct hybridization of DNA

from different organisms.

Bacterial DNA consists of chromosomal and extra-chromosomal DNA. The extra-chromosomal plasmid DNA has been used to characterise strains of bacteria, with or without digestion of the plasmid DNA by a restriction enzyme [132,133]. Characterising bacteria by their extra-chromosomal DNA has the disadvantages that plasmids are potentially mobile between strains, and not all strains carry plasmids. It is therefore preferable to analyse the bacterial chromosome itself using restriction enzyme analysis (REA).

Restriction enzymes cleave DNA at specific recognition sequences provided the recognition sequence has not been modified by methylation. There are currently more than 100 commercially available restriction enzymes with different specific recognition sequences. When bacterial DNA is digested with a chosen restriction enzyme, a specific number of fragments are produced which can then be separated according to size by agarose gel electrophoresis. Conventional horizontal agarose gel electrophoresis (CHAGE) can resolve DNA fragments up to 20 kilobases (kb). The choice of restriction enzyme is important in REA. DNA fingerprints consisting of more than 40 bands are difficult to compare and interpret, requiring rigorous side-by-side comparisons before an understanding of the genetic relationship between

isolates can be reached. A simple pattern can be achieved by using restriction enzymes which cleave chromosomal DNA relatively infrequently, or alternatively a restriction enzyme which cleaves DNA frequently can be used to form a pattern containing a large number of fragments. A pattern of large fragments is desirable when the presence of plasmid bands may complicate the interpretation of the DNA profile.

REA has been used to distinguish between strains in various bacteria including *Staphylococcus epidermidis* [134], *Staphylococcus aureus* [135,136], *Streptococcus suis* [137], *Mycobacterium paratuberculosis* [138], *Neisseria meningitidis* [139], *Haemophilus influenzae* [140], and *Clostridium difficile* [141]. REA provides a sensitive means of characterising individual isolates as minor genomic differences can be seen. The usefulness of REA is however limited by the large number of DNA fragments produced, making interpretation difficult.

Two ways of simplifying the DNA fingerprint produced are firstly, the use of nucleic acid probes which hybridise to specific sequences in the bacterial chromosome, and secondly, the use of rare cutting restriction enzymes to produce large DNA fragments which can be separated by pulsed-field gel electrophorsis (PFGE).

The use of nucleic acid probes to highlight restriction site heterogeneity within selected parts of the bacterial chromosome reduces the complexity of the banding pattern produced, therefore facilitating interpretation. Nucleic acid probes have been used which hybridise to random chromosome sequences [142], toxin production genes [143,144], and antibiotic resistance genes [145]. These nucleic acid probes have the disadvantage of being specific for the species for which they were produced, and are therefore not universally applicable. The use of 16S and 23S ribosomal RNA (rRNA) circumvents this problem. rRNA operons which encode 16S, 23S and 5S rRNA exist in a high copy number in a variety of genetic arrangements within the bacterial chromosome [145,147]. rRNA genes are highly conserved, and are common to all bacteria. The sequence conservation in rRNA genes has been used as a measure of genetic relatedness to construct stable phylogenetic trees [18,19]. It is reasonable to expect that some changes in the rRNA gene sequences between closely related taxa would lead to taxa specific restriction fragment polymorphisms.

The analysis of restriction fragment polymorphisms in the rRNA genes of bacteria has shown that the restriction pattern produced is useful both epidemiologically and taxonomically. The discriminatory level of the technique is dependent on the restriction enzyme used to create the

restriction fragment polymorphisms. When an rRNA restriction profile is identical the isolates producing that pattern are highly related with insignificant genetic divergence [146]. Within a species a differing rRNA restriction profile signifies significant genetic diversity. The finding of species specific restriction profiles has proved the usefulness of rRNA probing in the taxonomy of staphylococci [148,149,150,151], *Mycobacterium leprae* [152], *Candida* species [153] and *Leptospira* [154]. These taxon specific restriction profiles can be utilised for characterising bacteria in biochemically unreactive species and species that present practical problems in typing such as *Leptospira* [154]. The variation in the restriction profile has been able to show the genetic variability within the species *Leptospira interrogans* and *Haemophilus influenzae* [155,183] and to etablish the epidemiology of *Pseudomonas aeruginosa* [144], *Pseudomonas cepacia* [157], *Escherichia coli* [158], *Haemophilus influenzae*[159] and *Vibrio cholerae* [160].

The second way of simplifying the DNA profile is the use of pulsed-field gel electrophoresis to separate the large fragments produced by a rare cutting restriction enzyme. The largest DNA fragment that CHAGE is able to separate is approximately 20 kb. In CHAGE the ability to separate the differently sized DNA fragments produced after restriction enzyme digestion is dependent on the sieving

properties of the gel matrix. DNA fragments above 50 kb are able to enter the gel, but show essentially the same mobility through the pores of the gel matrix and therefore these molecules remain unseparated by conventional electrophoresis. Early attempts to separate these larger DNA fragments relied on reducing the agarose concentration of the gel, therefore increasing the effective pore size of the gel matrix, or using lower voltage gradients [161,162].

In PFGE the DNA fragments are continually forced to change the direction in which they are moving. The principle is as follows: when an electric field is applied to the gel, DNA fragments elongate and migrate in the direction of the field. This field is then removed, and a second field at an angle to the first is applied. The DNA molecule must reorientate before it can migrate in the direction of the second field. The time required for this reorientation is dependent on the molecular weight of the DNA fragment, with larger fragments taking more time to fully reorientate, therefore these fragments will have a concomitant decrease in the available time left for the molecule to migrate through the gel [163,164]. The time that the DNA is subjected to the 2 electric fields is called the pulse time. The size of the largest resolved DNA fragment increases with increasing pulse time [165]. The separation of the DNA fragments is therefore

based on retarding the net forward motion of DNA fragments through the gels, which explains the requirement for long run times [166]. PFGE can separate DNA large fragments of 6 megabases.

Since the first description of PFGE in 1982 [163], many synonymous variations have been described, eg field inversion gel electrophoresis (FIGE), contour-clamped homogeneous electric field (CHEF), pulsed homogeneous orthogonal field gel electrophoresis (PHOGE), orthogonal-field-alteration gel electrophoresis (OFAGE) and programmable, autonomously controlled electrodes (PACE). These different systems rely on the same principles to separate large DNA fragments; only their methods of doing so vary.

Although still a relatively new technique, PFGE has been used to size and make a physical map of the bacterial genome [167,168,169] and isolate genes [170]. By being able to directly visualise restriction fragment polymorphisms from the whole genome, PFGE will become a powerful epidemiological tool. It has already been used to elucidate the epidemiology of *Pseudomonas aeruginosa* [171,172] *Pseudomonas cepacia* [173], *Candida* species [174], staphylococci [175] and lactococci [176].

There is also evidence that PFGE may have a useful role in the taxonomy of bacteria with the observation that for

several staphylococcal species there is a reproducible species specific PFGE profile [177].

1.7) The clone model of population structure.

Much evidence has accumulated to support the hypothesis that many bacterial species have a clonal population structure. The word clone was first used to designate a population in which all members have been derived fron one and the same progenitor by non-sexual multiplication. For prokaryotes the word clone is used to denote "bacterial cultures isolated from different sources, in different locations and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin" [178]. This concept was originally used to describe organisms from a clear-cut outbreak of disease caused by a phenotypicaly well characterised pathogen, but the concept has now been applied to population studies in both eukaryotes [179] and prokaryotes [180]. Implicit in the clone concept is the requirement that the specific combinations of chromosomal genes coding for characters identifying clones are not rapidly broken down by recombination and mutation [181].

Various techniques have been utilised to show the clonal population of prokaryotes. Most work has been done using

multilocus enzyme electrophoresis [131], in which strains are characterised by the relative elctrophoretic mobility of a number of different water-soluble cellular enzymes. This technique equates the electrophoretic movement of the enzymes with genotype. Because evolutionary convergence to the same multilocus genotype is highly improbable, strains exhibiting the same multilocus genotype are considered to be clones. Other techniques used are outer-membrane protein patterns, sodium dodecyl sulphate polyacrylamide electrophoresis, restriction fragment length polymorphisms and rRNA probing. In one study with *Haemophilus influenzae* comparing these different techniques, there was complete concordance between the groupings derived using the different methods, providing strong evidence of clonality [183].

The evidence for clonality within a population is indicated by;
i) overrepresented, widespread identical genotypes,
ii) the absence of recombinant genotypes,
iii) linkage disequilibrium,
iv) correlation between independant sets of genetic markers [181].

The occurance of strong and complex nonrandom associations of alleles over loci (linkage disequilibrium) is an expectation of clonal reproduction, with the presence of a particular genotype in great

excess as one of the most robust and significant criteria of clonality [181]. It has been pointed out that that this kind of finding is particularly telling when the same genotype appears in excess in various localities at or at different times, years apart, indicating that this genotype has replicated as a unit over time and space [180,184]. Further evidence of the clonal nature of bacterial populations is that the number of clones of a species is moderate, hundreds, as opposed to the astronomical theoretical numbers that would be possible given the existing levels of allelic diversity at an enzyme loci [180].

The above techniques have been used to illustrate the clonal nature of *Legionella spp.*, *Bordetella spp.*, *Haemophilus influenzae*, *E.coli*, and *Neisseria meningitidis* [181,185].

1.8) The Antarctic Base

Faraday (latitude 65.15'S, longitude 64.16'W) is situated about 5 miles from the Antarctic Peninsula on a small island called Galindez Island, which itself forms part of an island group called the Argentine Islands. Faraday, established in 1947, is the oldest operational station in the Peninsular area. It is one of two British Antarctic Survey (BAS) bases responsible for atmospheric research,

with active meteorological and geomagnetic research programmes. It has a normal summer complement of 16-17 men and a winter complement of 10 men. The normal tour of duty in the Antarctic is two and a half years.

The Antarctic base offers a unique opportunity to study the normal human bacterial flora due to its very uniform environment, with all base personnel being young, fit, healthy, eating the same diet and exposed to the same internal and external climatic conditions. It also allows for the study of a very tightly controlled population of bacteria, as the base is completely isolated from contact for 9 months of the year during the winter months.

1.9) The aims of the study

There has been little work done on the genus *Micrococcus* within the last decade and most present day knowledge about the genus was collected throughout the 1960's and 1970's. The heterogeneity found within the genus reflects the problems of characterising the species within the genus *Micrococcus* on only a few poorly defined biochemical characteristics.

The use of molecular biological techniques for bacteriological studies has obviated the need to define bacteria biochemically, allowing instead a genotypic as

opposed to a phenotypic definition of a bacterial species.

This study aims to evaluate the use of biochemical tests, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), restriction enzyme analysis, ribosomal RNA probing and pulsed field gel electrophoresis as taxonomic and epidemiological tools in regard to a temporal collection of the genus *Micrococcus* from a small isolated community.

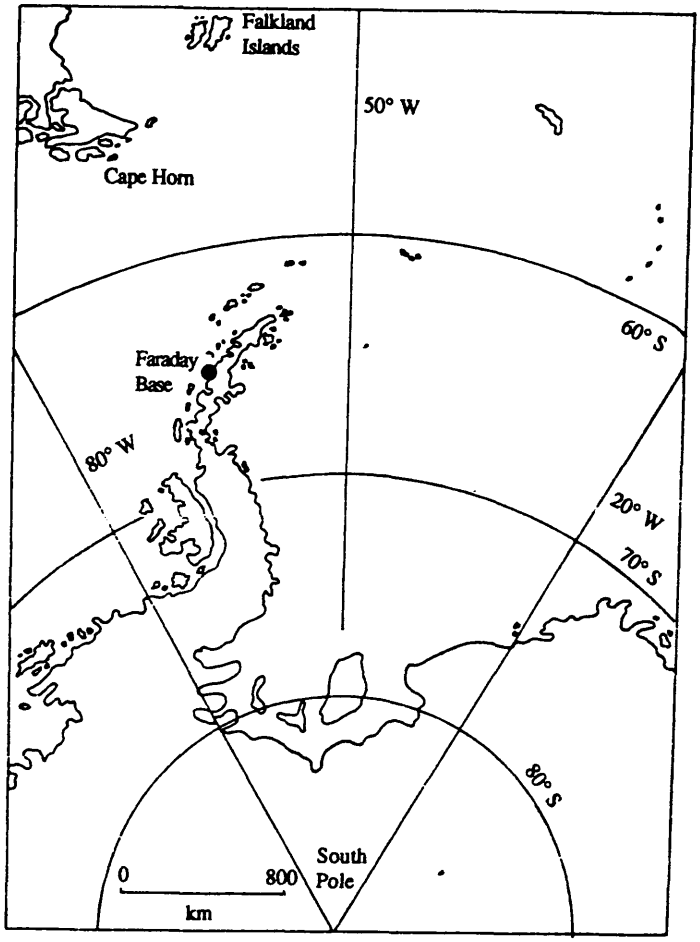

Figure 1. Map of the Antarctic Peninsula showing the position of Faraday base.

Figure 2. Faraday Base.

2.

MATERIALS AND METHODS

2.1) Sampling

Sampling took place monthly from the first week in March 1989 till the first week of March 1990, thus representing a 13 month collecting period. Initially 10 out of 11 base members took part. After August 1989 (8 months into the collection period) all base members participated as the non-participating base member was repatriated. In the summer with the influx of new personnel, the sample population increased to 17. The study was approved by the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen. All members taking part in the study gave their informed consent and signed a consent form (see Appendix A, p231). All participating base members were sampled on the same day, unless they were off the base travelling, in which case they were sampled when they returned to the base. All nutrient agar was autoclaved the day prior to sampling, with the plates and bijou slopes being incubated overnight (16 hours) to check sterility. To collect the isolates a sterile cotton wool swab moistened in sterile water was rubbed for about 15 seconds over an $8cm^2$ area of the skin and used to inoculate a nutrient agar plate (Oxoid). Samples were collected from 8 skin sites on the right hand side of the body:

1) the crown of the head
2) the forehead

3) the angle of the mandible

4) the anticubital fossa

5) the hairy part of the forearm along the radial border

6) the umbilicus

7) the hairy part of the shin midway along the anterior tibial border

8) the interdigital toe web between the big toe and the fourth toe.

The agar plate was incubated for 4 days at 37^0C and 3 representative colonies from any one plate were chosen on the basis of colony morphology, colour and Gram staining. These presumptive micrococci were subcultured onto nutrient agar plates, incubated for 4 days at 37^0C and then inoculated onto nutrient agar slopes in 7ml plastic bijous. These were incubated for 4 days at 37^0C and then stored at 4^0C. A 5ml sample of blood was also taken at the time of sampling for serum to use in Western blotting. Every three months, 10 bijou slopes which had been stored at 4^0C were selected randomly from each preceding three month period and regrown to test survival rates. Survival was 100% for 9 months of storage and then dropped to 66-75% thereafter. This was deemed acceptable as on return to the UK the last 7 sampling months would still be 100% viable. Therefore the isolates were not subcultured onto fresh slopes. The bijou slopes were returned to the UK via ship at 4^0C and finally to the

Bacteriology Department in Aberdeen at 4^0C.

In all, approximately 2,800 isolates were collected and stored throughout the sampling period.

Type strains used were;

Micrococcus luteus: NCIMB 9278
NCIMB 8166
NCIMB 8553

Micrococcus lylae: NCTC 11037
ATCC 27567
ATCC 27568
ATCC 27569

Micrococcus sedentarius: NCTC 11040

Micrococcus kristinae: NCTC 11038

Micrococcus varians: NCIMB 11697

Micrococcus roseus: NCIMB 11696

Micrococcus nishinomiyaensis: NCTC 11039

Micrococcus mucilaginosus (*Stomatococcus mucilaginosus*): NCTC 10663

Planococcus citreus: NCIMB 1493

Staphylococcus aureus: NCTC 6571

Staphylococcus cohnii: DSM 20261

Staphylococcus warneri: ATCC 27837

Staphylococcus epidermidis: ATCC 14490

Staphylococcus capitis: ATCC 27840

Staphylococcus saprophyticus: NCTC 07292

Abbreviations: NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen; NCTC, National Collection

of Type Cultures, London; ATCC, American Type Culture Collection, Rockville, Maryland, USA; DSM, Deutsche Sammlung fur Mikroorganismen, Gottingen, Germany. Thirteen isolates from clinical samples were gratefully received from Dr J Magee, Department of Bacteriology, Royal Hallamshire Hospital, Sheffield. Six strains were isolated from blood cultures all from patients with line infections, 5 strains were isolated patients with CAPD, 1 strain was isolated from cerebro-spinal fluid (CSF) and 1 strain was isolated from CSF from a patient with a ventriculo-peritoneal shunt.

2.2) Differentiation of staphylococci from micrococci

This was done according to the susceptibility of the isolates to lysozyme at 25 µg/ml, lysostaphin at 200 µg/ml, with the production of acid from glycerol in the presence of 0.4 µg of erythromycin as described by Kloos and Schliefer [108]. Nutrient agar was used instead of P-agar. The overlay plates were examined daily for 3 days, and the erythromycin/glycerol plates were examined for 7 days before being discarded. Acid production from glycerol was recorded as positive if the purple agar changed colour to yellow under the colony streak. Sensitivity to either lysostaphin or lysozyme was recorded as positive if a clear lytic area was produced.

M.luteus NCIMB 9278, *S.epidermidis* ATCC 14990, *S.capitis* ATCC 27840, and *S.saprophyticus* NCTC 07292 were used as positive and negative controls.

2.3) Identification to the species level

API (API-bioMerieux (UK) Ltd) ATB Staph 32 kits (now renamed ID 32 Staph) were used to attempt to identify the isolates to species level. Manufacturer's instructions were followed, and after 24 hours incubation the strips were read and a 9 digit code was produced. This was analysed by the manufacturer's manual and the API computer service to give a species identification [186].

2.4) Antibiotic sensitivities

Disc susceptibility testing was performed when required, by using drug susceptibility test agar plates (Oxoid CM 261) inoculated with a distilled water suspension of bacteria (10^6 colony forming units/ml). They were incubated at 37^0C (30^0C for methicillin testing) overnight. The discs used and concentration are shown below:-

methicillin 5µg

ciprofloxacin 1µg

trimethoprim 2.5µg

vancomycin 30µg

cefotaxime 30µg

amoxycillin 30µg

piperacillin 75µg

gentamicin 10µg

ampicillin 10µg

cefuroxime 30µg

penicillin 1IU

erythromycin 5µg

fusidic acid 10µg

2.5) Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

To prepare samples for SDS-PAGE, isolates were plated out to give a confluent growth and grown overnight at 37^{0}C on nutrient agar. The plate was harvested in the morning and the cells washed twice in 10ml sterile distilled water, the cells being sedimented by centrifugation at 13,500rpm for 15 minutes between washes. After washing the pellet was resuspended in 1ml sterile distilled water and transferred to an Eppendorf tube. The cells were disrupted in distilled water by an ultrasonic probe (Lucas Dawe Ultrasonics Soniprobe) whilst on ice. The sonicated samples were stored at -70^{0}C until required. The separating gel [acrylamide solution (acrylamide 30%,

w/v, bis-acrylamide 0.8%, w/v) to a final acrylamide concentration of 10%, w/v; 0.375M Tris pH 8.8 ; SDS 0.1%, w/v; ammonium persulphate 0.5%, w/v; TEMED (Sigma) 0.05%, v/v] was made, and allowed to set overnight after overlaying the surface of the gel with a layer of distilled water. Next day the water was poured off and stacking gel (acrylamide solution to a final acrylamide concentration of 3.6%, w/v; 0.12M Tris pH 6.8; SDS 0.1%, w/v; ammonium persulphate 0.1%, w/v; 0.1% TEMED (Sigma), v/v) was overlaid. The samples for electrophoresis were boiled for 5 minutes in one part boiling mix (glycerol 30%, v/v; 2-mercaptoethanol 15%, v/v; SDS 8.2%, w/v; spacer gel buffer 30%, v/v (0.5M Tris-HCl pH 6.8, SDS 0.4%, w/v); pinch of bromophenol blue to colour) to one part sample, centrifuged at 13,500 rpm for 5 minutes and then loaded into the wells. Approximately 300µg of protein was added to each well. Molecular weight standards (Pharmacia, 94-14.4 kilodaltons) were run in each gel. The gels were run in electrode buffer (0.04M glycine; 0.05M Tris; SDS 0.1%, w/v), on a vertical Protean II gel kit (Bio-Rad) with water cooling, at 30 mA per gel until the dye front reached the end of the gel which took 4-4.5 hours. The gels were stained in Coomassie Brilliant Blue dye (Coomassie Brilliant Blue 0.25%, w/v; 5 parts methanol: 5 parts distilled water: 1 part glacial acetic acid) overnight, destained in 1 part

7% (v/v) acetic acid; 1 part methanol and stored in 7% (v/v) acetic acid.

2.6) Protein estimation

This was done using a Sigma protein assay kit, by the technique of colorimetric protein binding using Coomassie Brilliant Blue [187].

2.7) DNA extraction

This was performed using a modification of Pitcher's method [188]. Cells were harvested after overnight growth on nutrient agar and suspended in an Eppendorf tube containing 150 µl of 150mM NaCl, 10mM EDTA, pH8. A 150 µl solution of 150mM NaCl, 10mM EDTA, pH8, containing 1 mg of lysozyme, was added and the resultant solution was incubated at 37^{0}C for 60 minutes. Then 500 µl GES [5M guanidium thiocyanate (Sigma), 100 mM EDTA, Sarkosyl 0.5%, v/v (Sigma)] was added and mixed gently for 15 minutes, or until clearing occurred. Once clearing had occurred the Eppendorf tube was placed on ice. A 270 µl aliquot of cold (4^{0}C) 7.5M ammonium acetate was added, the tube mixed briefly and allowed to stand for 10 minutes on ice. Then 250 µl of chloroform:isoamyl alcohol (24:1 v/v) was added and the tube shaken vigorously for 5

minutes until a stable emulsion had formed. This was spun at 13,500 rpm for 20 minutes. A maximum of 900 µl of the clear supernatant was transferred to a clean Eppendorf tube and 486 µl of isopropanol at -20^{0}C was added. The tube was inverted several times until a precipitate was observed, the DNA was pelleted by centrifuging at 13,500 rpm for 2 minutes. The isopropanol was poured off, the last remnants being removed by a capillary tube. The pellet was resuspended in 400 µl TE buffer (10mM Tris, 1mM EDTA, pH8) and then an equal volume of water saturated phenol was added and the tube shaken vigorously for 5 minutes. It was then spun at 13,500 rpm for 2 minutes, the clear supernatant was removed to a clean tube and NaCl (final concentration, 0.1M) was added along with 2 volumes of absolute (99.9%) alcohol at -20^{0}C. The tube was inverted until the DNA precipitated out. If no precipitate was obvious the Eppendorf tube was left at -70^{0}C for 30 minutes. The DNA was then pelleted by spinning at 13,500 rpm for 2 minutes, the alcohol was removed and the pellet redissolved in 100 µl of TE buffer. The final DNA concentration was measured spectrophotometrically at 260nm. Total DNA yields varied from 61 µg-13,400 µg.

M.sedentarius isolates and *M.lylae* ATCC 27569 consistently gave poor DNA yields by this method and the more labour intensive method of Bjorvatn [189] had to be used. Cells were harvested after overnight growth on

nutrient agar and suspended in an Eppendorf tube containing 150 µl TE buffer. To this was added 300 µl lysis mix (10mM EDTA; 50mM Tris; 2% Triton X-100 (Sigma) pH 8), 100 µl lysozyme (50mg/ml stock solution, final concentration 77 µg/µl) and 50 µl PB buffer [0.5m EDTA, pH 9; 1% Sarkosyl (Sigma); 1mg/ml Proteinase K (Boehringer Mannheim), final concentration 77 µg/µl]. This mixture was incubated at 37^{0}C for three hours and then phenol:chloroform extracted as described by Maniatis[190] . This method gave total DNA yields of 120-1,100 µg.

2.8) Calculation of theoretical cutting frequencies

This was done by the method of Forbes [191] using published DNA sequence data from the European Molecular Biological Laboratories (EMBL), the frequencies of mono-, di-, and trinucleotides (see Appendix B, p233) were determined by computer (Composition ProGramme, Sequence Analysis Software Package for VAX computers, Genetics Computer Group, University of Wisconsin). The micrococcal DNA sequence used for the calculated cutting frequencies was UN:X12578 (a gene homologous to *Escherichia coli uvr A*) from *M.luteus*. The % mol GC content of the total sequence used was calculated from the mononucleotide frequencies and compared to the % mol GC content of the species to

determine how representative the particular fragment used, was to the genome as a whole. This sequence of the genome has a % mol GC of 69%, which is lower than published % mol GC contents of *M.luteus*, but it has the advantage of being a useful representative for the genus as a whole, lying within the range of the micrococcal % mol GC content of 66-75%. The frequencies (p) of the di- and trinucleotides were used to determine the mean fragment lengths for a particular restriction enzyme using the formula: for restriction enzymes with a tetranucleotide recognition sequence ($N_1N_2N_3N_4$) then mean fragment length in nucleotides =

$$\frac{p(N_2N_3)}{p(N_1N_2N_3).p(N_2N_3N_4)}$$

Similarly for the hexanucleotide $N_1N_2N_3N_4N_5N_6$, mean fragment length in nucleotides =

$$\frac{p(N_2N_3).p(N_3N_4).p(N_4N_5)}{p(N_1N_2N_3).p(N_2N_3N_4).p(N_3N_4N_5).p(N_4N_5N_6)}$$

2.9) Restriction enzyme digestion

This was performed overnight with 5 µg of extracted DNA to which 5 units of the appropriate restriction enzyme (Boehringer-Mannheim and Pharmacia) was added, along with 1 µl of the manufacturers buffer and 0.5 µl of ribonuclease (10mg/ml stock solution, final concentration

50 ng/µl) and the final volume made up to 10 µl with distilled water. The sample was loaded onto a gel after the addition of 2µl 6x loading buffer (6X loading buffer contains bromophenol blue 0.25%, w/v; sucrose 40%, w/v, in distilled water).

2.10) Agarose gel electrophoresis

This was carried out in a 0.8% (w/v) agarose gel (Type II medium EEO, Sigma), at 5V/cm on a Bio-Rad horizontal gel tank containing 1x TBE (0.089M Tris, 0.089M boric acid, 0.002M EDTA) until the dye front reached the end of the gel. This took 4.5-5 hours. The gel was stained for 30 minutes in ethidium bromide (5 µg/ml), destained for 60 minutes in distilled water, and photographed under UV light through both a red and a UV filter with a Polaroid Land camera using Polaroid type 655 or 677 film. A 1 kilobase ladder (Bio-Rad) was used as a marker.

2.11) Estimation of molar percentage GC content

This was performed as described by Frederiq [192]. DNA was extracted as described above, and the pellet redissolved in 400 µl of TE buffer, 1 µl of ribonuclease (10mg/ml stock solution, final concentration 25 pg/µl) was added, mixed briefly, and incubated overnight at 37^0C. Then 400

µl of water-saturated phenol was added and the tube was shaken vigorously for 5 minutes and spun at 13,500 for 5 minutes. The clear supernatant was removed and placed in a clean Eppendorf tube. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the tube shaken vigorously for 5 minutes and spun at 13,500rpm for 5 minutes. The DNA was precipitated with 2 volumes of absolute (99.9%) alcohol and the tube placed at -70^0C for 10 minutes. The DNA was pelleted by centrifuging at 13,500rpm for 2 minutes and the pellet washed once in 70% ethanol. The pellet was then redissolved overnight in 0.1M NaCl and the ratio of absorbance at 260/280nm read by spectrophotometer the following morning in 0.1M acetic acid pH 2.9.

2.12) Southern blotting

After electrophoresis and staining (using ethidium bromide 2.5 µg/ml) as described above, a photograph was taken to note the position of the kilobase markers. Transfer of DNA to nylon or nitrocellulose membranes (Hybond-N or Hybond-C, Amersham) was done according to the method of Southern [193]. The gel was washed in two changes of 0.25M HCL for 30 minutes, and then neutralised with two changes of 1.5M NaCl/0.5M NaOH for 30 minutes. Finally the gel was washed for 30 minutes in 3M NaCl/0.5M

Tris, pH 7.4, using two changes of solution. The DNA was transferred to the membrane by capillary action overnight using 20x SSC as the mobile phase (1x SSC is 0.15M NaCl, 0.015M tri-sodium citrate), as described by Southern [185]. The membrane was air dried and the DNA fixed by either UV irradiation for 4 minutes (nylon) or baking for 2 hours at 80^{0}C (nitrocellulose). If required the membranes could be stored between 2 sheets of blotting paper for 2-8 weeks prior to probing.

2.13) 5'-end labelling of the 16S and 23S ribosomal RNA

5'-end labelling of the ribosomal RNA (rRNA) was done following the forward reaction procedure [182] with 10µg 16S and 23S rRNA from *E.coli* MRE 600 (Boehringer Mannheim). This was added to 2µl of 10x kinase buffer (10x kinase buffer contains 0.5M Tris, 0.1M $MgCl_2$, 50mM dithiothreitol, 1mM spermidine, 1mM EDTA), 1µl polynucleotide kinase (Sigma, 10 units/µl) and 5µl γ^{32}P-ATP (Amersham, 110 TBq, 3000 Ci/mmol) and incubated at 37^{0}C for 30 minutes. The reaction was stopped by placing on ice for 30 minutes. To concentrate the label and remove unreacted P^{32}, 100µl ammonium acetate 2.5M pH 7.5, 1µl transfer RNA (tRNA) (Sigma) and 400µl absolute (99.9%) alcohol (-20^{0}C) was added to the Eppendorf tube, mixed by inversion and put in a dry ice/isopropanol bath

for 15 minutes at -70^{0}C, then spun at 13,500 rpm for 20 minutes. The pellet was washed twice in 500µl 70% ethanol (-20^{0}C) and then dissolved in 200µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8).

2.14) Ribosomal RNA probing

The filters were bathed at 65^{0}C for 3 hours in polythene bags containing 15 ml of pre-hybridising solution with 100µg/ml of sonicated, boiled salmon sperm (Sigma) added. Pre-hybridising solution is made up of; 2x standard saline citrate buffer (1xSSC is 0.15M NaCl, 0.015M tri-sodium citrate); 5xFPG (1xFPG is Ficoll 400, 0.02%, w/v; polyvinylpyrrolidone 350, 0.02%, w/v; glycine, 0.02%, w/v), SDS, 0.5%, w/v; Poly A 1µg/ml, and the whole made up to 100ml with distilled water. The pre-hybridising buffer was replaced with hybridising solution (2xSSC; 1XFPG; 25mM KH_2PO_4; 2mM EDTA; SDS, 0.5%, w/v; dextran sulphate, 10%, w/v; and made up to 30ml with distilled water) containing 100µg/ml sonicated, boiled salmon sperm, and 50µl of ^{32}P labelled rRNA ($3x10^5$ cpm.ml^{-1}/ specific activity $1.3x10^6$ cpm.µg^{-1}). Excess air was removed and the bags were heat sealed. They were checked for leaks whilst mixing the contents by rubbing gently and then incubated at 65^{0}C for 24 hours. The hybridising solution was discarded and the blots were washed three

times at 65^{0}C in 2xSSC; SDS 0.1%, w/v, pre-heated to 65^{0}C, for 15 minutes each and then once at 65^{0}C in the final wash solution (0.1xSSC; SDS, 0.1%, w/v). The membranes were air dried, wrapped in Saran wrap and autoradiographed with Kodak XAR-5 film between intensifying screens at -70^{0}C for the appropriate time (approximately 36 hours).

2.15) Preparation of DNA inserts for pulsed field gel electrophoresis (PFGE)

Half a plate of overnight culture grown on nutrient agar was inoculated into 10ml of nutrient broth and incubated whilst shaking at 37^{0}C overnight. A 2 ml aliquot of this overnight culture was inoculated into 8ml of fresh nutrient broth and incubated for 1 hour at 37^{0}C prior to the addition of 2 µl penicillin (10mg/ml stock solution, final concentration 0.2 ng/µl). The cell suspension was incubated for a further hour at 37^{0}C. The cells were harvested by spinning at 3,000rpm for 15 minutes and the pellet suspended in 1ml of EET buffer (100mM EDTA, 10mM EGTA, 10mM Tris, pH 8) and transferred to an Eppendorf tube. This was spun at 13,500rpm for 5 minutes and the pellet resuspended in 800 µl EET buffer. The cell suspension was pre-warmed to 37^{0}C and 200µl of this suspension was added to 800µl of 1.5% (w/v) low melting

point agarose (FMC Incert) made with sterile distilled water. This mixture was dispensed into moulds (220µl volume) and allowed to cool and set. The insert was removed from the mould and placed in an Eppendorf tube containing 1ml EET, 1mg lysozyme (Sigma) and 2µl ribonuclease (10mg/ml stock solution, final concentration 1.67 ng/µl) and incubated overnight at 37^{0}C. The insert was washed twice in 1ml EET prior to the addition of 1 ml of ESP solution containing 960µl 0.5M EDTA, 40µl 25% SDS (w/v), 1mg proteinase K (Boehringer Mannheim) and incubated overnight (16 hours) at 50^{0}C. The insert was then dialysed in 20ml TE (10mM Tris-HCl, 1mM EDTA, pH 8) containing 1mM PMSF (Sigma) for 2 hours and then dialysed in two changes of TE buffer for a further 4 hours. The inserts were stored at 4^{0}C in TE buffer.

2.16) Digestion and electrophoresis of DNA inserts

The inserts (approx 50µl) were pre-incubated in a BSA/DTT solution [1x enzyme buffer, 1mM dithiothreitol (Sigma), 5µl BSA (Sigma) 2mg/ml] for 2 hours at 37^{0}C. The pre-incubated insert (approx 50µl) was placed in 10µl of the appropriate enzyme buffer (according to manufacturer's instructions, Boehringer Mannheim and Pharmacia), 2µl of the appropriate enzyme and 38µl of sterile distilled water, mixed gently and incubated at 37^{0}C overnight. Then

25µl 0.5M EDTA was added and the insert washed with TE buffer once and stored at 4^0C until use. The appropriate amount (approximately 2-3µl) of digested insert was loaded into the wells of a 1% (w/v) agarose gel ensuring that the insert fragment is in contact with the bottom and front of the well. The wells were then flooded with 0.7% (w/v) low melting point agarose (Bio-Rad) made using 0.5x TBE (1x TBE is 0.089M Tris, 0.089M boric acid, 0.002M EDTA). This was allowed to set and the gel was then run in the PFGE apparatus (Bio-Rad CHEF II) containing 0.5x TBE pre-cooled to 10^0C and circulating at 0.5 litres/minute, with a variable pulse time (usually ramped from 15-40 seconds), at 200V for 24 hours. The gels were then stained and photographed as described above.

3.

RESULTS

3.1) Revival of isolates in the United Kingdom

A total of 2,834 isolates were shipped back to the United Kingdom from the Antarctic. It was immediately apparent that this was too large a number to process with the time and funds available.

To reduce numbers, isolates collected from the shin only were chosen for further study. Throughout the study period this site yielded a consistently good growth of presumptive micrococci from all members of the base.

The total number of isolates collected from the shin for the whole year amounted to 458. This was still too large a number to study, so it was further reduced by choosing isolates collected from the eighth month of the study onwards. After that period all members of the base were participating in the study, following the repatriation of the one non-participating base member. This would provide an 8 month sampling period which would encompass two sampled groups. The first group would consist of the 10 winterers completing the last 4 months of the winter period, whilst the second group would include the first group who had wintered, and the 7 newly arrived summer personnel.

This gave a sample size of 226 isolates collected in the Antarctic. Of these 226 isolates, 209 (92%) were successfully regrown on arrival in the UK. These isolates

had been in storage for 9-11 months before being recultured. The survival rate of 92% is higher than reported previously for isolates collected in the Antarctic [186,187].

3.2) Identification of isolates to the genus level

Of the 209 surviving isolates, 201 (96%) were Gram-positive catalase-positive cocci. Seven bacilli (3%) were removed from the study, and one isolate which initially survived died after subculture and could not be further studied. Of the 201 Gram-positive catalase-positive cocci, 191 isolates (95.5%) were resistant to lysostaphin at 200 µg/ml, did not produce detectable acid from glycerol in the presence of 0.4 µg/ml erythromycin, and were therefore classified as belonging to the genus *Micrococcus* (Table 1). Of these 191 isolates 54 (28%) were lysozyme sensitive.

Ten isolates (5.5%) were sensitive to lysostaphin at 200 ug/ml and were classed as belonging to the genus *Staphylococcus*. All 10 staphylococcal isolates were resistant to lysozyme, with 9 isolates producing detectable acid from glycerol in the presence of 0.4 mg/ml erythromycin. The isolates identified as staphylococci at this point were removed from the study and considered no further.

3.3) Identification of isolates to the species level

191 isolates were identified as belonging to the genus *Micrococcus*. It was decided to study the first 4 months of the initially selected group of 191 micrococci, and if time allowed to continue with the isolates collected in the last 4 months of this initially selected group. This reduced the number of isolates collected from the Antarctic to 88.

API bio-Merieux (UK) make a microtitre strip method (ATB 32 Staph) for the identification of Gram-positive catalase-positive cocci using 20 biochemical tests. This microtitre strip was used to speciate the isolates which had been placed into the genus *Micrococcus*.

A total of 114 isolates belonging to the genus *Micrococcus* and genus *Stomatococcus* were tested by the API microtitre strip method, of which 88 isolates were from the Antarctic sample group, 13 were isolates collected from clinical material, 12 isolates were *Micrococcus* type strains and one isolate was the type strain *Stomatococcus mucilaginosus* (Tables 2 and 3).

Using biochemical criteria the API microtitre strip was unable to identify 54% (61/114) of all isolates, gave a poor identification (< %id 90%) of species in 14% (15/114) of all isolates and made a positive identification to species (> %id 90) in 33% (38/114) of

all isolates. If colony pigmentation is used as a criterion then 56% (64/114) of all isolates (including type strains) would be speciated by the API microtitre strip. The %id is the percentage of identification of a taxon derived from the frequencies of occurence of the reactions to known substrates for a given isolate relative to other members of the taxon [178].

The group of isolates which were not identified contained 2 sub-groups. The first sub-group contained isolates which were either non-reactive to all substrates in the microtitre strip, or were able to react to one substrate only, whereas the second sub-group contained isolates with reactive API profiles which could not be identified by the API database because of their conflicting biochemical reactions. These isolates were classed as having "unacceptable profiles" by the API database.

In the group where no identification was made, the most frequent API profile was 000000000, (no biochemical reactivity shown with any substrate). This occurred in 61% (37/61) of isolates. The next most frequent API profile was 000001000 (arginine arylamidase positive), occurring in 21% (13/61) of isolates. If colony pigment is included as the other identifying characteristic, 9 isolates producing yellow colonies would be classed as *M.luteus* and 4 isolates producing white or cream colonies would be classed as *M.lylae*.

18% (11/61) of the non-identified group had a reactive profile but no identification was possible, all profiles being considered unacceptable when compared against the API database. Of the 11 isolates which were not speciated, but gave a biochemical reaction, the API database gave 8 isolates a designation of staphylococcal species as their "best guess".

Of the 15 isolates in the poorly identified group, 80% (12/16) of isolates had the API profile 000005000 which gave an identification of either *M.luteus* (%id 78), or *M.lylae* (%id 22). The characteristic used in the API database to differentiate between these 2 species is colony pigment. If the colony is yellow pigmented then the isolate would be considered as *M.luteus*, and *M.lylae* if not yellow pigmented. By this criterion 9 isolates would be identified as *M.luteus* and 3 isolates as *M.lylae*. This group includes the type strains *M.luteus* NCIMB 8166 and *M.lylae* ATCC 27569 both of which were correctly identified to species level when the criterion of colony pigment was applied. In this poorly identified group there was one indentification of *Staphylococcus warneri*, isolate 21214. Two isolates, isolate 4910 and *M.kristinae* type strain NCTC 11038 were poorly identified as *M.varians* and *Stomatococcus mucilaginosus* respectively.

Of the 38 isolates identified to species level (%id >90), 74% (28/38) of them were identified as *M.luteus*. This included type strain *M.luteus* NCIMB 9278. When using colony pigment as one of the speciating characteristics it is notable that 3 isolates positively identified as *M.luteus* produced colonies which were the classically described cream colour normally attributed to *M.lylae*. There was one identification each of *M.varians* (isolate 282), *M.kristinae* (isolate A545), *M.nishinomiyaensis* (isolate 661), and *M.roseus* which was type strain *M.roseus* NCIMB 11696. A positive identification to staphylococcal species was made in 16% (6/38) of all positive identifications. They were identified as *S.capitis* (isolate 972), *S.lentus* (isolate T886), *S.auricularis* (*M.luteus* type strain NCIMB 8553), *S.cohnii* (isolates 373, 382), and *S.warneri* (isolate 6921).

There were no positive identifications of *M.sedentarius* by the API microtitre test.

Table 2 shows the API identification of the type strains used in the study. Of 13 type strains used, 15% (3/13) gave a positive identification. *M.roseus* NCIMB 11696 and *M.luteus* NCIMB 9278 were correctly, positively identified as *M.roseus* (%id 94.8) and *M.luteus* (%id 99.9) respectively, whereas *M.luteus* NCIMB 8553 was positively identified as *S.auricularis* (%id 98.1).

23% (3/13) of isolates were poorly identified (%id <80) to species level with 2 isolates, *M.luteus* NCIMB 81663 and *M.lylae* 27569, relying on the colony pigment to make a correct positive identification. *M.kristinae* NCIMB 11038 was poorly identified as *Stomatococcus mucilaginosus*.

Over half (7/13) of the type strains could not be identified by the API microtitre strip. These were *M.lylae* NCIMB 11037, *M.lylae* ATCC 27568, *M.lylae* ATCC 27569, *M.varians* NCIMB 11697, *M.sedentarius* NCIMB 11040, *Stomatococcus mucilaginosus* NCTC 10663, and *M.nishinomiyaensis* NCIMB 11039. Of these non-identified isolates, an identification of staphylococci was chosen by the API database as the "best guess" for the 2 isolates *Stomatococcus mucilaginosus* 10663 and *M.lylae* ATCC 27568.

In summary, from the sample group of 101 unspeciated isolates belonging to the genus *Micrococcus* (ie non-type strains), the API microtitre strip using colony pigment as an additional characteristic made a positive micrococcal identification to the species level in 55 (54%) isolates, and a positive staphylococcal identification in 5 (5%) isolates. There were 41 (40%) isolates still not identified to the species level after testing with the API microtitre strip.

All the 41 non-identified non-type strain isolates were tested for the *M.sedentarius* species specific characteristic of methicillin and penicillin resistance. Penicillin and methicillin resistance is a characteristic of 12 isolates and these were classed as *M.sedentarius*. Of the 12 isolates, 9 produced colonies which were cream pigmented, one of the classically described colony pigments for *M.sedentarius*. Three isolates produced colonies which were orange, a colony pigment normally associated with *M.nishinomiyaensis* and never before reported for *M.sedentarius*. Of these 12 isolates identified as *M.sedentarius* all gave an API profile of 000000000. It is noteworthy that API bio-Merieux do not include *M.sedentarius* in their identification database.

After excluding the methicillin and penicillin resistant organisms from the group not identified to species level, 29 (29%) isolates from the 101 non-type strains were still not identified to the species level. Of these, 22 (76%) isolates gave an API profile of 000000000 therefore the biochemical non-reactivity of micrococci is the greatest factor in the failure to identify this genus to the species level.

Table 3 summarises the species identified from the 101 unknown isolates. When the API microtitre strip, antibiotic resistance and colonial morphology were used as criteria for speciation, of the 101 unknown isolates

from the Antarctic group and the clinical group, 67 (66%) isolates were positively identified to the species level while 29 (29%) remained non-speciated. There were no positive identifications of *M.roseus* from the sample population of 101 isolates.

	Resistant to			
No. of isolates (%)	Lysozyme (25 ug/ml)	Lysostaphin (200 ug/ml)	Acid	Genus
54 (28%)	-	+	-	MICROCOCCUS
137 (72%)	+	+	-	
9 (5%)	+	+	+	STAPHYLOCOCCUS
1 (0.5%)	+	-	-	

Table 1. The separation of 201 Gram-positive catalase-positive cocci to the genus level using sensitivity to lysozyme (25µg/ml), lysostaphin (200µg/ml) and the production of detectable acid (aerobically) from glycerol in the presence of 0.4 µg/ml of erythromycin.

TYPE STRAIN		API INDENTIFICATION (id%)
M.luteus	NCIMB 9278	*M.luteus* (99.9%)
	NCIMB 8166	*M.luteus* (78.0%) / *M.lylae* (22.0%)
	NCIMB 8553	*S.auricularis* (98.1%)
M.lylae	NCTC 11037	Not identified
	ATCC 27567	*M.nishinomiyaensis* (77.7%) / *M.lylae* (21.1%)
	ATCC 27568	Not identified
	ATCC 27569	*M.luteus* (78.0%) / *M.lylae* (22.2%)
M.roseus	NCIMB 11696	*M.roseus* (94.8%)
M.kristinae	NCTC 11038	*S.mucilaginosus* (82.3%) / *M.varians* (17.7%)
M.varians	NCIMB 11697	Not identified
M.sedentarius	NCTC 11040	Not identified
M.nishinomiyaensis	NCTC 11039	Not identified
S.mucilaginosus	NCTC 10663	Not identified

Table 2. The identification of type strains of the genus *Micrococcus* and *Stomatococcus* by the API microtitre biochemical test strip.

Identification is defined as an API profile of id >90%. Poorly identified is defined as an API identification of id <80%. Not identified is defined as an API profile that was nonreactive or was classed as unacceptable by the API database. Abbreviations as before.

Identification	No. of Isolates (%)
Genus Micrococcus:	67 (66%)
Species	
M.luteus	46 (45%)
M.sedentarius	12 (12%)
M.lylae	6 (6%)
M.varians	1 (1%)
M.kristinae	1 (1%)
M.nishinomiyaensis	1 (1%)
Genus Staphylococcus:	5 (5%)
Species	
S.cohnii	2 (2%)
S.warneri	1 (1%)
S.lentus	1 (1%)
S.capitis	1 (1%)
Not identified	29 (29%)

Table 3. The identification of 101 non-type strain isolates to the species level by biochemical, morphological and physiological characteristics as described in Section 3.3, p74.

Identification is defined as an API profile with an %id >90.

3.4) Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

All 88 Antarctic isolates and 13 type strains were examined by whole-cell protein SDS-PAGE to assess its potential in the epidemiology and taxonomy of micrococci. The 13 isolates collected from clinical material were not examined by SDS-PAGE as they entered the study at a later stage.

The only obvious groupings apparent were with isolates that showed indistinguishable banding patterns. In Figure 3 *M.luteus* isolates in lanes 2-6 and 9 have indistinguishable profiles; *M.luteus* isolates in lanes 7 and 8 are different by the 4 bands (arrows) with molecular weights >94 kilodaltons; the *M.luteus* isolate in lane 9 also differs from lanes 7 and 8 by the protein band at 67 kilodaltons; *M.luteus* isolates in lanes 10 and 11 have profiles which are indistinguishable, and *M.luteus* isolates in lanes 12 and 13 and an isolate that has the API profile 000000000 in lane 14 have several obvious differences.

All isolates identified as *M.sedentarius* gave very poorly defined protein profiles.

Figure 4 shows the heterogeneity of the profiles produced by SDS-PAGE.

The usefulness of the SDS-PAGE profiles at the initial stage of the study was reduced because of the number of isolates which remained unidentified to the species level and the lack of any obvious species specific SDS-PAGE protein profiles making interpretation of the electrophoretic profiles difficult. The heterogeneity within the genus *Micrococcus* made SDS-PAGE difficult and time consuming to analyse. The additional problem of inter-gel variability necessitating rigorous side-by-side comparisons of isolates makes this a time intensive technique. Lack of isolate speciation by biochemical and physiological methods, complexity and heterogeneity of the SDS-PAGE profiles, poorly defined protein profiles of isolates identified as *M.sedentarius*, and subsequent difficulty in interpreting the protein profiles made SDS-PAGE a difficult technique with which to try and characterise the sample population of poorly biochemically characterised micrococci. It was therefore considered no further in the study.

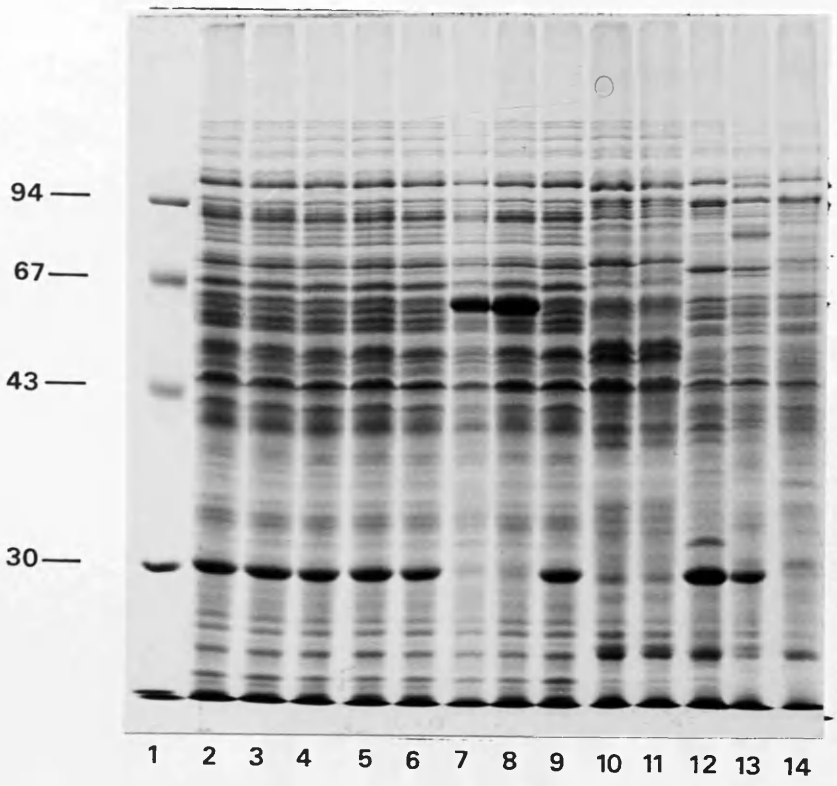

Figure 3. SDS-PAGE of whole-cell proteins of 13 micrococci. Samples were prepared as described in the methods and run in a 10% polyacrylamide gel at 35 mA for 4.5 hours. The gel was stained in Coomassie Brilliant Blue dye and destained in 1 part methanol; 1 part 7% (v/v) acetic acid. Lane 1 molecular weight marker (kilodaltons). Lane 2, isolate 471; lane 3, isolate 472; lane 4, isolate 861; lane 5, isolate 1361; lane 6, isolate 1365; lane 7, isolate 771; lane 8, isolate 763; lane 9, isolate 1362; lane 10, isolate 562; lane 11, isolate 563; lane 12, isolate 661; lane 13, isolate 662; lane 14, isolate 762. Lanes 2-6 and 9 have indistinguishable profiles; lanes 7 and 8 are different by 4 bands; lanes 10 and 11 are indistinguishable, and lanes 12-14 have several obvious differences.

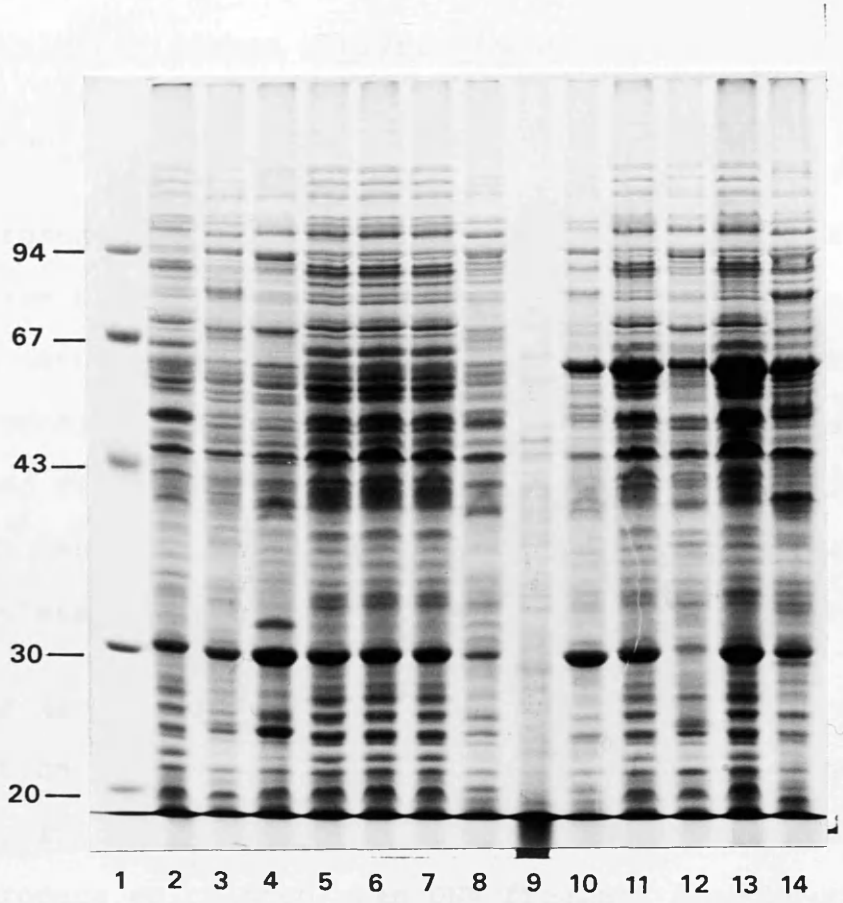

Figure 4. SDS-PAGE of 13 micrococcal isolates showing the heterogeneity that was apparent in their SDS-PAGE profile. Lane 1 molecular weight marker (kilodaltons). Isolates are: Lane 2, *M.luteus* 463; Lane 3, *M.luteus* 673; Lane 4, *M.luteus* 661; Lane 5, *M.luteus* 861; Lane 6, *M.luteus* 1361; Lane 7, *M.luteus* 1365; Lane 8, *M.luteus* 1364; Lane 9, *M.sedentarius* 1382; Lane 10, *M.luteus* 671; Lane 11, *M.luteus* 1363; Lane 12, *M.luteus* 871; Lane 13, *M.luteus* 6919; Lane 14, not identified 6920. Running conditions are as for Figure 1.

3.5 Restriction enzyme analysis (REA)

3.5.1) Choice of restriction enzyme for REA

Eleven restriction enzymes were assessed to establish which gave the most easily interpreted and discriminatory banding pattern for REA. The enzymes tried were *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, and *Taq*I. These enzymes gave calculated mean DNA fragment lengths of between 0.098 to 240 kilobases (kb) as calculated using di- and trinucleotide frequencies.

The calculated mean DNA fragment lengths and the recognition sequences of the 11 enzymes are shown in table 4. It is apparent that there are only 2 enzymes which produce calculated mean DNA fragment lengths within the 10-20 kb range. These enzymes however had different cutting frequencies in practice, possibly due to methylation of the DNA.

Figure 5 shows *M.luteus* isolate 972 digested with *Bam*HI, *Sal*I, *Bgl*II, *Hind*III, *Eco*RI and *Eco*RV. The enzymes *Hae*III and *Taq*I which produced calculated mean DNA fragment lengths less than 200 base pairs digested the genome into DNA fragments less than 300 base pairs, with no large fragments produced. Under the electrophoretic conditions used, all these DNA fragments ran before the dye front. All enzymes which produce calculated mean DNA fragment lengths less than 2 kb (*Bam*HI, *Kpn*I, *Sac*I, *Sal*I and *Pst*I)

gave a DNA profile similar to tracks 1 and 2. BglII produced a DNA profile which gave fewer, more discrete bands but was still considered too complex to interpret easily. *Hind*III, *Eco*RI, and *Eco*RV gave profiles with 2, 8 and 5 fragments respectively.

The ability of each restriction enzyme to discriminate between isolates was assessed against the SDS-PAGE profile in Figure 3. Figure 6 shows the DNA profile of 13 isolates digested with the restriction enzymes BamHI (Fig. 6(a)), *Pst*I (Fig. 6(b)), *Hind*III (Fig. 6(c)), and *Eco*RI (Fig. 6(d)). Using SDS-PAGE, isolates in lanes 2-6 and 9 have indistinguishable profiles; lanes 7 and 8 are different by 4 bands; lanes 10 and 11 are indistinguishable, and lanes 12-14 have several obvious differences.

The restriction enzymes *Bam*HI and *Pst*I produce large fragments in the 10-20 kb range which can be useful for REA (Fig. 6(a) and (b)). Both enzymes differentiate among the 6 isolates with indistinguishable SDS-PAGE profiles and the isolates showing different SDS-PAGE profiles in lanes 12-14. *Pst*I differentiates between the 2 isolates in lanes 7 and 8 whilst *Bam*HI does not. The 2 isolates in lanes 10 and 11 have indistinguishable DNA profiles with both enzymes. *Pst*I gives a better separation of large fragments and is easier to interpret than the *Bam*HI digest. The calculated mean DNA fragment lengths for *Pst*I

is greater by a factor of 10, 0.209 kb for *Bam*HI versus 1.918 kb for *Pst*I, therefore larger DNA fragments are expected to be produced using this restriction enzyme.

In the 1-20 kb range *Hind*III and *Eco*RI produce between 0-9 and 3-22 DNA fragments respectively (Fig. 6(c) and (d)). *Hind*III differentiates between the 6 isolates with indistinguishable SDS-PAGE profiles and the isolates showing different SDS-PAGE profiles in lanes 12-14. Isolates in lanes 7 and 8 are not differentiated with *Hind*III. *Eco*RI does not differentiate the 8 isolates with indistinguishable SDS-PAGE profiles. This restriction enzyme is able to differentiate the 3 isolates with different SDS-PAGE profiles and differentiates between the isolates in lanes 7 and 8.

It was decided to use the restriction enzymes *Hind*III and *Eco*RI for REA. All 114 isolates were digested with both restriction enzymes. The DNA profiles produced by these two restriction enzymes were easier to interpret than the protein profile produced by SDS-PAGE, or the restriction enzymes *Bam*HI and *Pst*I. *Hind*III had a higher number of isolates non-restricted, 4, all *M.sedentarius*, compared to *Eco*RI which had no incidence of non-restriction.

3.5.2) Analysis of restriction enzyme profiles

Using *Eco*RI 68 isolates produced unique REA profiles. Using *Hind*III 75 isolates produced unique REA profiles.

Tables 5 and 6 show the isolates which have indistinguishable DNA profiles after digestion with the restriction enzymes *Eco*RI and *Hind*III, the sensitivity of the isolate to lysozyme at 25 µg/ml, the API code number of the isolate and the person from which the isolate was collected and the month of isolation.

The restriction enzyme *Eco*RI produced 15 REA groups, each group having its own unique DNA profile and all members of an REA group having indistinguishable DNA profiles. Each group contains between 2 and 7 isolates (Table 5).

In the *Eco*RI grouping, clone 1 is present on persons 4, 8 and 13 in months 6 and 7; whilst clone 2 is present on person 5 in months 6, 7 and 8. Clone 3 is present on persons 6 and 13 during months 6, 7 and 8; clone 4 is present on 3 people, persons 3, 8, and 9 in the same month, namely month 7. Clone 5 was isolated during month 7 and 9 from person 7. Clone 6 was isolated from persons 5 and 7 in months 7 and 6 respectively. Clone 6 and isolate 7918 are clonally related, with isolate 7918 having one extra DNA band of 5.6 kb. Clonal groups 7, 8, 9, and 10 represent isolates collected from the same person on 2 separate months. Clonal groups 11, 12, 13,

14, 15 are isolates collected from the same person in the same month.

In the *Eco*RI groups there is good agreement between colony pigment and the DNA fingerprint groupings. There is also broad agreement with lysozyme sensitivity and the DNA fingerprint groupings, although isolates 1361, 1362 and 472 show lysozyme resistance whilst the other isolates in group 1 show lysozyme sensitivity. Isolate 572 in group 6 and the paired isolates in groups 8, 9, 13 and 14 also show different sensitivity to lysozyme compared to the other members of the groups. When lysozyme sensitivity was retested on group 1, isolates 463 and 471 were lysozyme resistant (formerly sensitive) and isolates 1361 and 1362 were lysozyme sensitive (formerly resistant). The test for lysozyme sensitivity using the overlay plate technique [108], under the experimental conditions used, is not a reproducible method of determining sensitivity to lysozyme. This is a reflection of the difficulties in standardisation of the variables present in this technique, most critically, the number of colony forming units incorporated within the agar forming the overlay plate.

Isolates showing the same DNA fingerprint showed variations in their API profile number. This was most noticeable in group 1 with isolate 472, in group 2 with

isolate 583, in group 4 with isolate 972, in group 6 with isolate 572 and in group 15 with isolate 782.

The restriction enzyme *Hind*III produced 12 groups of isolates with indistinguishable DNA profiles (Table 6). Within each group there are 2 to 5 isolates. The *Hind*III groups 3, 4, 6, 7, 8 and 10 have the same isolates within them and correspond to the *Eco*RI groups 3, 4, 7, 13, 9, and 11 respectively. The *Hind*III groups 1, 2, 5, and 9 are similar to the *Eco*RI groups 1, 2, 5, and 10. The *Eco*RI group 1 has been split into the *Hind*III groups 1 and 11, with *Hind*III groups 1 and 11 gaining isolates 572 (a member of *Eco*RI group 6) and isolate 13917 respectively. The *Eco*RI group 5 has retained the isolates 772 and 773 and added the isolates 6920 and 7918 (in *Eco*RI group 6) to form the *Hind*III group 5. The *Eco*RI group 10 has gained isolates 1371 and 1373 to form the *Hind*III group 9.

In the *Hind*III groups, clone 1 was isolated from persons 4, 5 and 13 in months 6, and 7; clone 2 was isolated from person 5 in months 6, 7, and 8; clone 3 was isolated from person 6, and 13 in months 6, 7 and 8. Clone 4 was isolated from persons 3, 8, and 9 in month 7; clone 5 was isolated from persons 6, and 7 in months 7, and 9; clone 9 was isolated from person 13 in months 6, 7, and 9; clone 12 was isolated from persons 8, and 13 in months 6, and 9. Clone 6 represents isolates collected from person

3 in months 7, and 8; clone 8 represents isolates collected from person 2 in months 8, and 9. Clones 7, 10 and 11 represent isolates collected from the same person in the same month.

As with the *Eco*RI groups the *Hind*III groups show good correlation between colony pigment and DNA profile group although *Hind*III group 5 contains 4 isolates which have the 3 different colony pigments of cream, yellow and lemon. Isolates 472 and 572 are lysozyme resistant whilst the other isolates in group 1 are lysozyme sensitive. Group 5, group 12 and the paired groups 7 and 8 also show differences in the lysozyme sensitivity of their individual isolates. The variations in the API profile number are the same as for the *Eco*RI groups.

Assuming that an indistinguishable DNA profile indicates a clonal relationship, it is possible to say that the same isolates are present on the same person throughout the study period and also on several people during both the same month and sequential months.

Figure 7 shows the restriction enzyme *Eco*RI DNA profiles of clonal groups 2 and 3.

Figure 8 shows isolates identified as *M.luteus* by the API microtitre strip digested with *Eco*RI. The restriction enzymes *Eco*RI and *Hind*III did not produce *M.luteus* species specific DNA profiles. Figure 9 shows the DNA

profiles of isolates speciated as *M.sedentarius* after digestion with *Eco*RI. These isolates had a similar profile by the restriction enzyme *Eco*RI, with one DNA fragment of 3 kb common to all isolates. Isolate 783 in lane 8 showed an intermediate resistance to methicillin (zone of inhibition 13 mm) and was resistant to penicillin. It therefore did not meet the full criteria of methicillin and penicillin resistance that characterises *M.sedentarius*. Isolate 783 clearly has a different DNA profile from the other isolates classed as *M.sedentarius*.

Enzyme	Recognition sequence	Mean nucleotide length (kilobases)
*Hae*III	GGCC	0.098 kb
*Taq*I	TCGG	0.112 kb
*Bam*HI	GGATCC	0.209 kb
*Kpn*I	GGTACC	0.687 kb
*Sac*I	GAGGTC	0.803 kb
*Sal*I	GTCGAC	1.237 kb
*Pst*I	CTGCAG	1.918 kb
*Bgl*II	AGATCT	9.8 kb
*Hind*III	AAGCTT	14.3 kb
*Eco*RI	GAATTC	57.8 kb
*Xba*I	TCTAGA	220.8 kb
*Eco*RV	GATATC	240 kb
*Hpa*I	GTTAAC	340 kb
*Dra*I	TTTAAA	Not Cut

Table 4. The theoretical, calculated mean micrococcal DNA fragment lengths produced by 11 restriction enzymes. The mean calculated frequencies were computed using di- and trinucleotide frequencies from sequence UN:X12578 from *M.luteus* as described in the methods.

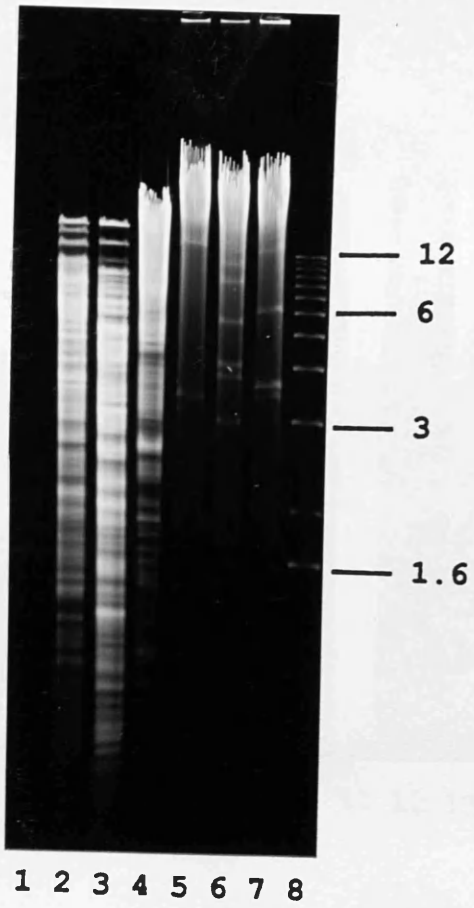

Figure 5. *M.luteus* isolate 972 digested with the restriction enzymes *Bam*HI, *Sal*I, *Bgl*II, *Hind*III, *Eco*RI and *Eco*RV. The DNA was extracted as described in the methods. A 5ug aliqout of DNA was digested overnight (16 hours) at 37^0C with the appropriate enzyme. The DNA fragments produced were separated by electrophoresis in 1x TBE at 5V/cm in a 0.8% (w/v) agarose gel for 4.5-5 hours. Lanes 1 and 8 are 1 kilobase markers.

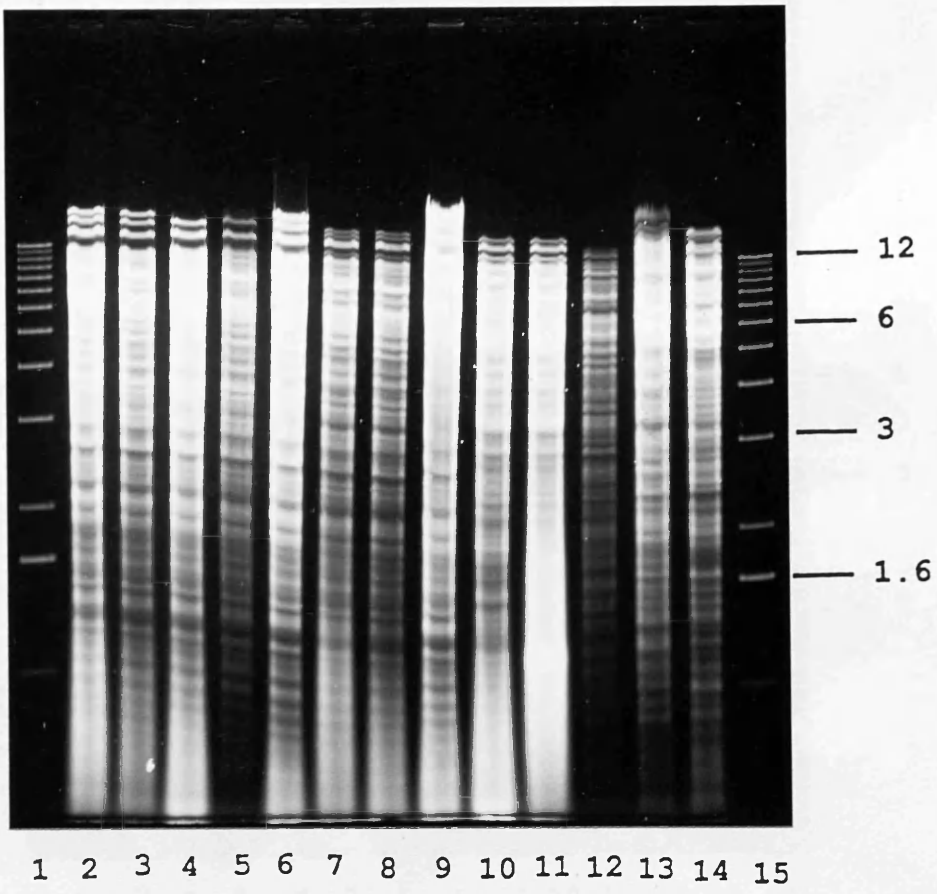

Figure 6a. Isolates as for Figure 3 digested with the restriction enzyme *Bam*HI. Running conditions as for Figure 3. Fragment sizes are in kilobases.

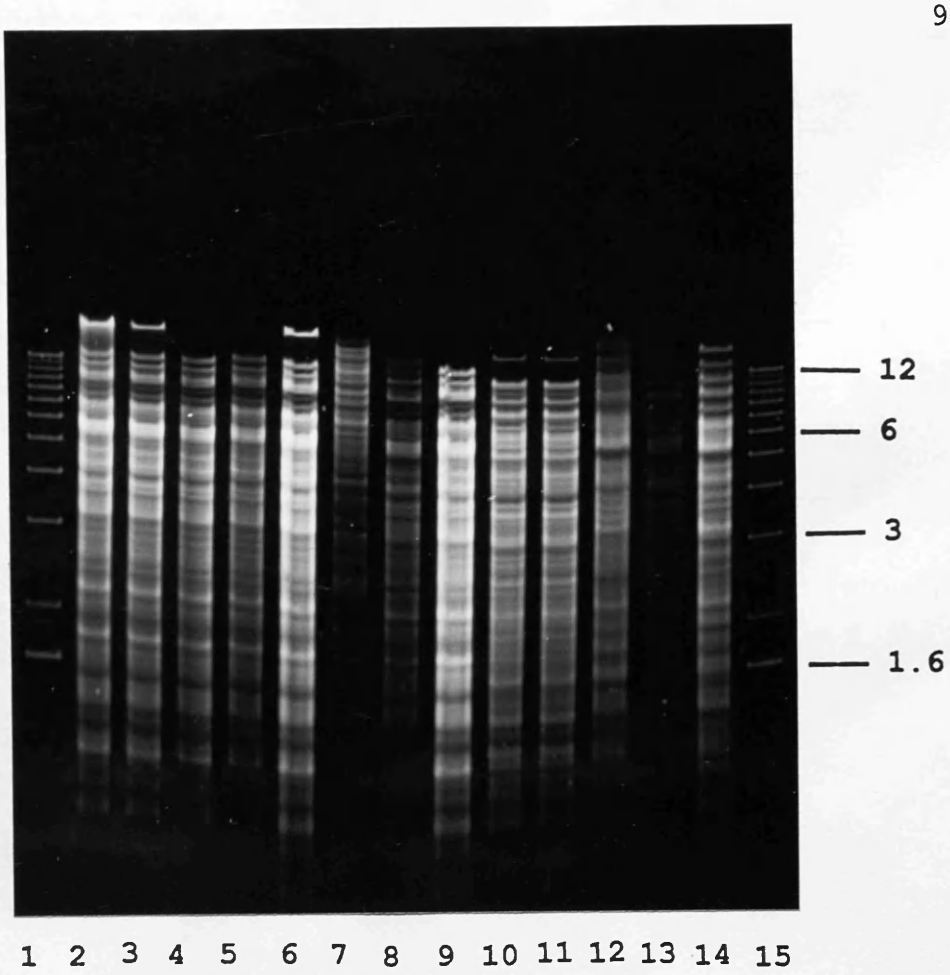

Figure 6b. Isolates as for Figure 3 digested with the restriction enzymes *Pst*I. Running conditions as for Figure 3. Fragment sizes are in kilobases.

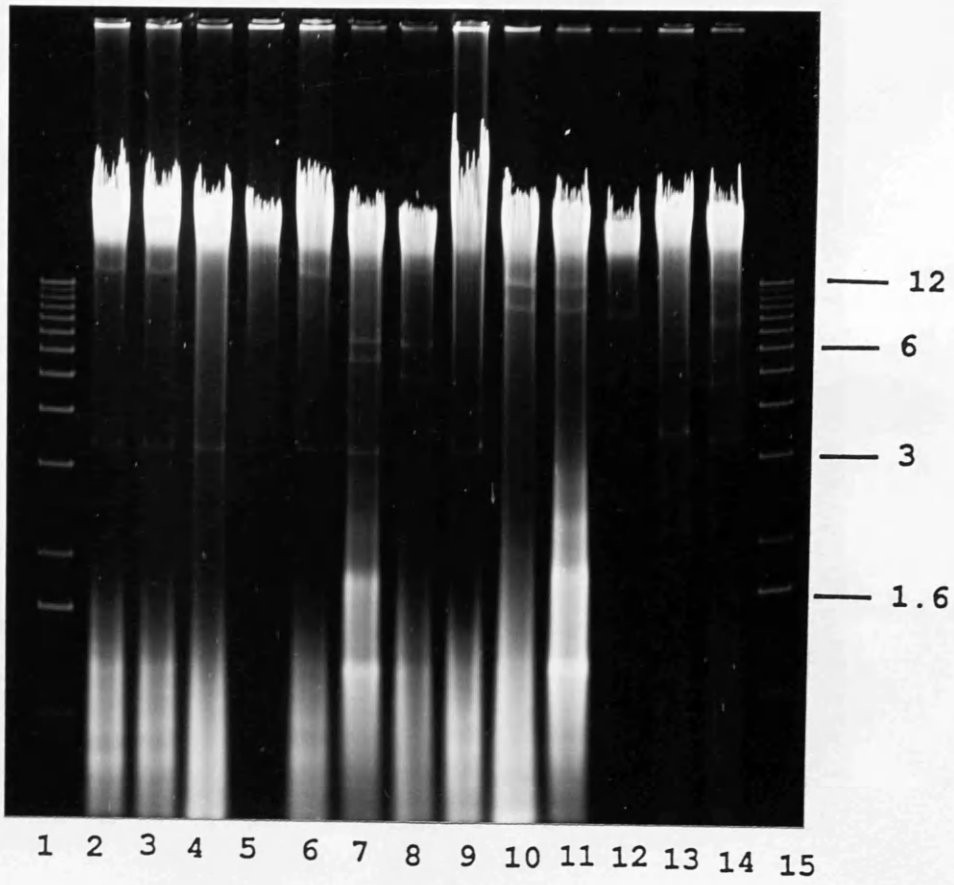

Figure 6c. Isolates as for Figure 3 digested with the restriction enzymes *Hind*III. Running conditions as for Figure 3. Fragment sizes are in kilobases.

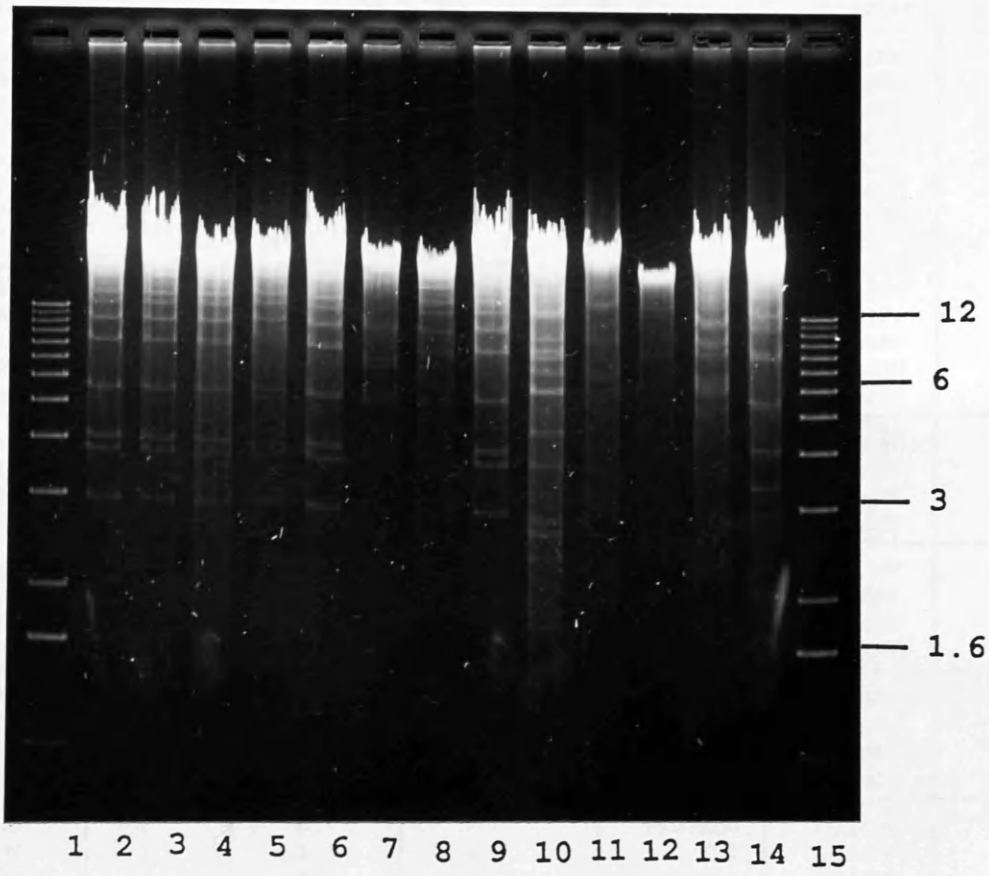

Figure 6d. Isolates as for Figure 3 digested with the restriction enzymes *Eco*RI. Running conditions as for Figure 3. Fragment sizes are in kilobases.

ISOLATE NUMBER	PERSON	MONTH OF ISOLATION	LYSOZYME RESISTANCE	API NUMBER	COLONY PIGMENT	GROUP
861	8	6	-	100005200	YELLOW	1
1361	13	6	+	100001200	YELLOW	
1362	13	6	+	100027000	YELLOW	
1365	13	6	-	100007000	YELLOW	
463	4	6	-	100005000	YELLOW	
471	4	7	-	100005000	YELLOW	
472	4	7	+	100000000	YELLOW	
561	5	6	-	000000000	L.LEMON	2
562	5	6	-	000001000	L.LEMON	
563	5	6	-	000020000	L.LEMON	
571	5	7	-	000005000	LEMON	
582	5	8	-	000010000	L.LEMON	
583	5	8	-	076761612	L.LEMON	
662	6	6	+	000025000	YELLOW	3
673	6	7	+	000025000	YELLOW	
681	6	8	+	000020000	YELLOW	
1381	13	8	+	000001000	YELLOW	
871	8	7	+	100021000	YELLOW	4
971	9	7	+	100020000	YELLOW	
972	9	7	+	360030200	YELLOW	
372	3	7	+	100021000	YELLOW	
772	7	7	+	000000000	CREAM	5
773	7	7	+	000005000	CREAM	
7919	7	9	+	000000000	CREAM	
7920	7	9	+	000000000	CREAM	
763	7	6	-	100025000	YELLOW	6
572	5	7	+	102107210	YELLOW	
7918	7	9	-	100000000	YELLOW	
373	3	7	+	060020040	WHITE	7
382	3	8	+	060020040	WHITE	
1384	13	8	-	000000000	CREAM	8
3916	13	9	+	000000000	CREAM	
281	2	8	+	000020000	L.LEMON	9
2915	2	9	-	000000000	L.LEMON	
1364	13	6	+	000003000	L.LEMON	10
13918	13	9	+	000001000	YELLOW	
762	7	6	+	000000000	YELLOW	11
764	7	6	+	000021200	YELLOW	
1383	13	8	-	000000000	ORANGE	12
1386	13	8	-	000000000	ORANGE	
261	2	6	+	000000000	L.YELLOW	13
263	2	6	-	000020000	L.YELLOW	
2914	2	9	-	000001000	YELLOW	14
2916	2	9	-	000025000	YELLOW	
782	7	8	-	100025000	CREAM	15
783	7	8	+	000000000	CREAM	

Table 5. Isolates with identical DNA profiles after digestion with *Eco*RI, showing species designation by API, lysosyme sensitivity of the isolate, the person from which the isolate was collected from and the month of isolation.

ISOLATE NUMBER	PERSON	MONTH OF ISOLATION	LYSOZYME RESISTANCE	API NUMBER	COLONY PIGMENT	GROUP
1365	13	6	-	100007000	YELLOW	1
463	4	6	-	100005000	YELLOW	
471	4	7	-	100005000	YELLOW	
472	4	7	+	100000000	YELLOW	
572	5	7	+	102107210	YELLOW	
562	5	6	-	000001000	L.LEMON	2
563	5	6	-	000020000	L.LEMON	
571	5	7	-	000005000	LEMON	
582	5	8	-	000001000	L.LEMON	
583	5	8	-	076761612	L.LEMON	
662	6	6	+	000025000	YELLOW	3
673	6	7	+	000025000	YELLOW	
681	6	8	+	000020000	YELLOW	
1381	13	8	+	000001000	YELLOW	
372	3	7	+	100021000	YELLOW	4
871	8	7	+	100021000	YELLOW	
971	9	7	+	100020000	YELLOW	
972	9	7	+	360030200	YELLOW	
772	7	7	+	000000000	CREAM	5
773	7	7	+	000005000	CREAM	
6920	6	9	-	000000000	LEMON	
7918	7	9	-	100000000	YELLOW	
373	3	7	+	060020040	WHITE	6
382	3	8	+	060020040	WHITE	
261	2	6	+	000000000	L.YELLOW	7
263	2	6	-	000020000	L.YELLOW	
281	2	8	+	000020000	L.LEMON	8
2915	2	9	-	000000000	L.LEMON	
1364	13	6	+	000003000	L.LEMON	9
1371	13	7	+	000020000	LEMON	
1373	13	7	+	000001000	LEMON	
13918	13	9	+	000001000	YELLOW	
762	7	6	+	000000000	YELLOW	10
764	7	6	+	000021200	YELLOW	
7919	7	9	+	000000000	CREAM	11
7920	7	9	+	000000000	CREAM	
861	8	6	-	100005200	YELLOW	12
1362	13	6	+	100027000	YELLOW	
13917	13	9	+	100001000	YELLOW	

Table 6. Isolates with identical DNA profiles after digestion with *Hind*III, showing species designation by API, lysosyme sensitivity of the isolate, the person from which the isolate was collected from and the month of isolation.

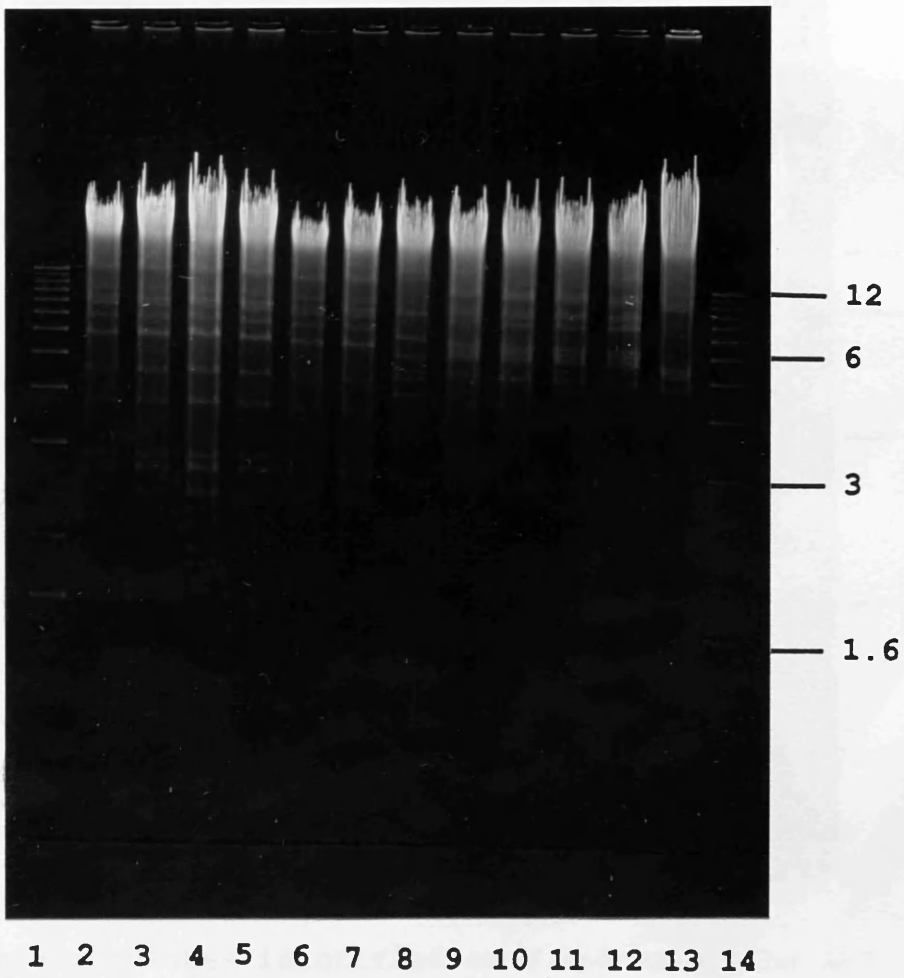

Figure 7. *Eco*RI REA groups 2 (Lanes 2-7) and 3 (Lanes 9-12). Lanes 1 and 14 are 1 kilobase molecular weight markers. Isolates are: Lane 2, 561; Lane 3, 562; Lane 4, 563; Lane 5, 571; Lane 6, 582; Lane 7, 583; Lane 8, 661; Lane 9, 662; Lane 10, 673; Lane 11, 681; Lane 12, 1381; Lane 13, *M.luteus* NCIMB 9278.

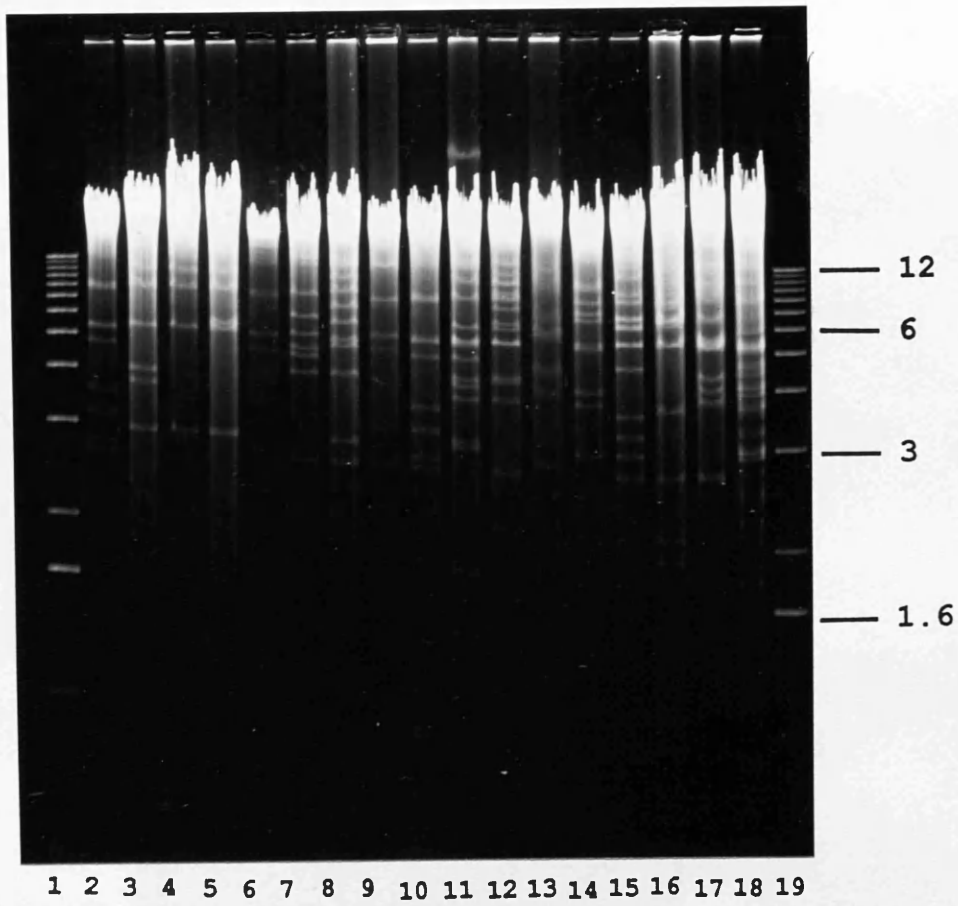

Figure 8. Isolates identified as *M.luteus* by the API microtitre strip digested with *Eco*RI. Lanes 1 and 19 are 1 kilobase molecular weight markers. Isolates are: Lane 2, NCIMB 9278; Lane 3, 463; Lane 4, 572; Lane 5, 7918; Lane 6, 972; Lane 7, 661; Lane 8, 671; Lane 9, 672; Lane 10, 372; Lane 11, 6919; Lane 12, 761; Lane 13, 765; Lane 14, 771; Lane 15, 782; Lane 16, 8919; Lane 17, 13917; Lane 18, 1363.

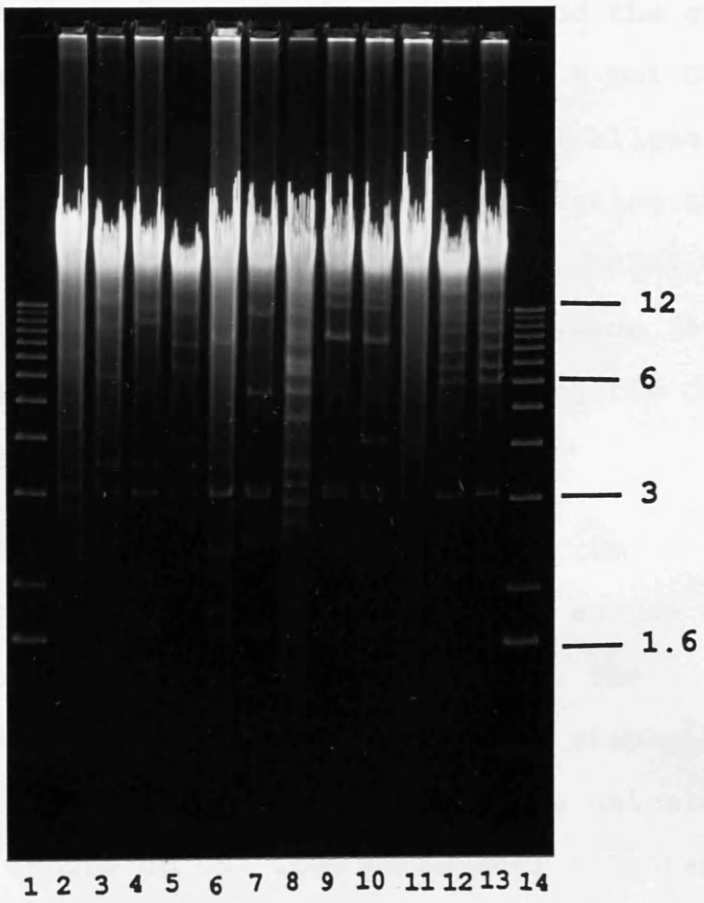

Figure 9. Isolates identified as *M.sedentarius* digested with *Eco*RI. Lanes 1 and 14 are 1 kilobase molecular weight markers. Isolates are: Lane 2, NCTC 11040; Lane 3, 383; Lane 4, 3910; Lane 5, 483; Lane 6, 498; Lane 7, 781; Lane 8, 783; Lane 9, 13916; Lane 10, 1384; Lane 11, 1382; Lane 12, 1383; Lane 13, 1386.

3.5.3) Differentiation of the genus *Staphylococcus* and the genus *Micrococcus* by the use of restriction enzymes.

The specific recognition sequences of restriction enzymes can be used to differentiate the Gram-positive catalase-positive cocci into the genus *Micrococcus* and the genus *Staphylococcus*. The large difference in the % mol GC content between staphylococci and micrococci allows for a visual differentiation to be made after digesting the extracted DNA with a restriction enzyme containing a guanine/cytosine (GC) rich or an adenine/thymine (AT) rich recognition sequence and then separating the DNA fragments by gel electrophoresis.

The calculated mean fragment length of DNA from micrococci digested by *Eco*RI, a restriction enzyme with the recognition sequence GAATTC, is 57.8 kb. The calculated mean fragment length of DNA from staphylococci digested with the same enzyme is 3.3 kb. The calculated mean fragment length of DNA from micrococci digested by *Bam*HI, which has the restriction site recognition sequence GGATCC, is 0.209 kb, whilst the calculated fragment length of DNA from staphylococci digested with the same enzyme is 16.0 kb. Figure 10 lanes 2 and 3, and 6 and 7 show the characteristic DNA profile produced by micrococci and staphylococci respectively when digested with *Bam*HI. Lanes 9 and 10, and 13 and 14 shows the DNA

fingerprint for micrococci and staphylococci respectively when digested with *Eco*RI.

All 114 isolates and *Planococcus citreus* were digested with *Eco*RI. Of these 91% (104/114) gave a DNA profile that was visually indicative of a micrococcal % mol GC content. Included amongst these were 2 isolates, *M.luteus* type strain NCIMB 8553 and isolate 972, positively identified as staphylococci by the API microtitre strip. Ten isolates gave a DNA fingerprint indicative of a lower % mol GC content than micrococci when digested with *Eco*RI. Three of these isolates were *Stomatococcus mucilaginosus* type strain NCTC 10663, *Planococcus citreus* type strain NCIMB 1493, and *M.sedentarius* type strain NCIMB 555, two were isolates collected from clinical material which were received as *Stomatococcus mucilaginosus* and 5 were collected from the Antarctic sample group (Figure 10 lanes 4 and 5; and 11 and 12). When the five non-type strain isolates were digested with the GC rich recognition site restriction enzyme *Bam*HI, 4 isolates gave a fingerprint indicative of a higher % mol GC content than staphylococci, whilst isolate 6921 gave a profile indicative of a staphylococcal % mol GC content. Isolate 6921 had an API profile that identified it as *S.warneri* and a GC content of 36.5%. Isolate 6921 was the only lysostaphin resistant staphylococcus found in this study. The 4 isolates produced DNA profiles as seen in

Figure 10 lanes 4 and 5, and 11 and 12 which are indicative of a % mol GC content intermediate between staphylococci and micrococci. These 4 isolates will be discussed later.

M.sedentarius type strain NCIMB 555 had a DNA profile indicating that it has a lower % mol GC content than micrococci. The isolate was a Gram-positive catalase-positive coccus which formed rounded, convex, glistening, orange colonies on nutrient agar, formed diplococcal pairs and tetrads, was non-motile, gave an API profile of 000000000, was resistant to lysozyme and lysostaphin, and was sensitive to methicillin, penicillin, amoxycillin, piperacillin, erythromycin, fusidic acid, vancomycin, cefotaxime, ciprofloxacin, cefuroxime, gentamicin and trimethoprim. After further tests carried out at NCIMB the strain *M.sedentarius* NCIMB 555 was withdrawn from the collection.

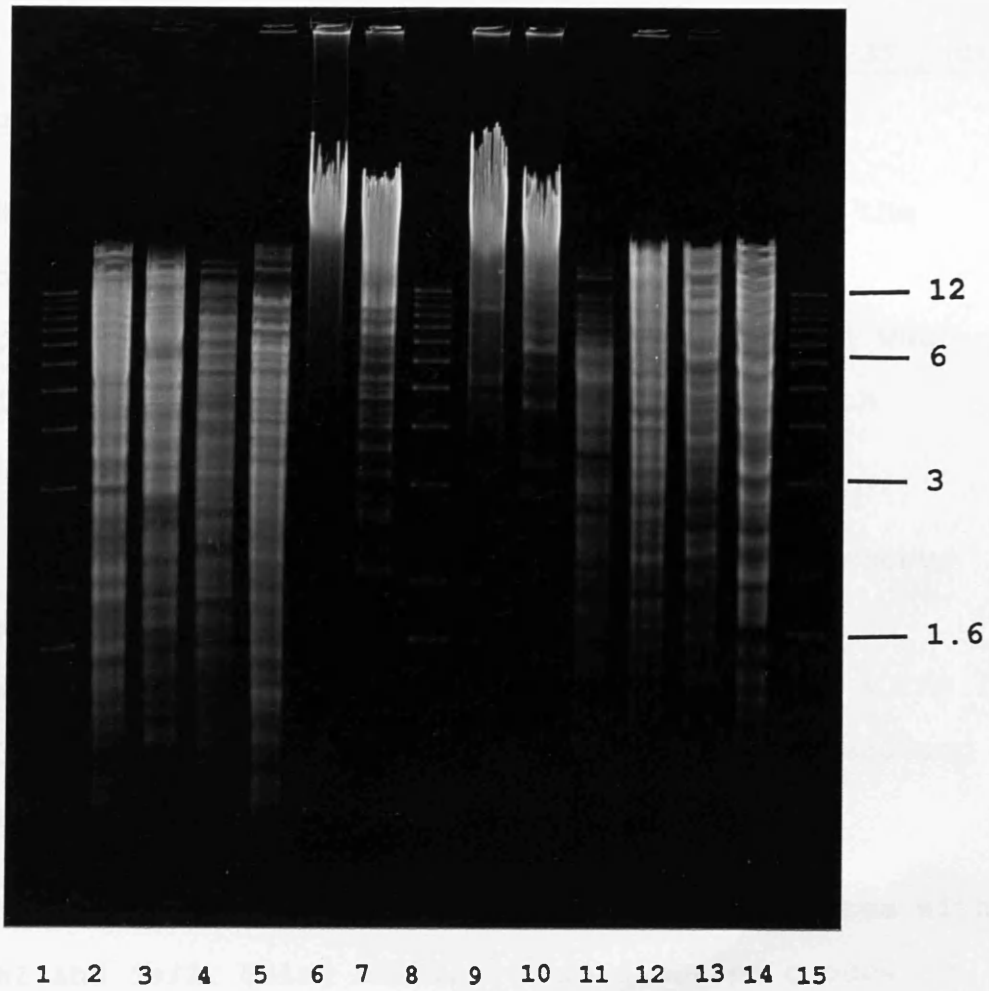

Figure 10. The use of restriction enzyme digestion to estimate the % mol GC content. Lanes 2-7 isolates digested with *Eco*RI, Lanes 9-14 isolates digested with *Bam*HI. Isolates are: Lane 2, *M.luteus* NCIMB 9278; Lane 3, *M.lylae* NCTC 11037; Lane 4, isolate 373; Lane 5 isolate 21213; Lane 6, *S.aureus* NCTC 6571; Lane 7, *S.epidermidis* ATCC 14490. Lanes 9-14 as for lanes 2-7. Lanes 1,8 and 15 are 1 kilobase molecular weight markers.

3.6) Identification of micrococci using 16S and 23S rRNA probing

*Bam*HI and *Sal*I, which produce DNA fragments with the calculated mean lengths of 0.209 kb and 1.237 kb respectively, were used to digest the micrococcal whole-cell DNA. All 114 isolates were digested with both restriction enzymes.

Two of the clinical isolates received as *Stomatococcus mucilaginosus*, the type strains *Stomatococcus mucilaginosus* NCTC 10663 and *Planococcus citreus* NCIMB 1493, and 4 of the Antarctic sample group are discussed later in Section 3.7, p131.

Table 7 shows the ribotype grouping of the isolates with *Bam*HI and *Sal*I. Using *Bam*HI, 7 main ribotype groups containing 98 isolates are apparent, with 8 isolates producing unique ribotypes. The 7 main ribotype groups are shown in Figure 11 with the unique *Bam*HI ribotypes produced by *M.nishinomiyaensis*, *M.roseus* and *M.lylae* also shown.

The largest ribotype group, group 1, contained 37 isolates and was characterised by rDNA fragments of 9.7 and 6.5 kb. Group 1 contained 18 isolates identified as *M.luteus* by the API microtitre strip, 1 isolate identified as *M.lylae* by the API microtitre strip, 2 *M.luteus* type strains NCIMB 8166 and NCIMB 8553, and 2

M.lylae type strains NCTC 11037 and ATCC 27569. In group 1, 15 isolates were not identified by the API microtitre strip, 12 of those isolates having the API profile 000000000. This *Bam*HI ribotype group contained 7 different *Sal*I ribotypes, with 14 isolates belonging to *Sal*I ribotype group 1, 8 isolates belonging to *Sal*I ribotype group 2, 8 isolates belonging to *Sal*I ribotype group 3, 3 isolates belonging to *Sal*I ribotype group 6, one isolate from *Sal*I ribotype group 5 and 2 isolates, 882 and 961, with unique ribotypes.

*Bam*HI group 2 contained 15 isolates and was characterised by rDNA fragments of 12 and 9.7 kb. Eight isolates were identified as *M.luteus* by the API microtitre strip, 1 isolate was identified as *Staphylococcus auricularis* and 1 isolate was identified as *M.lylae*. Five isolates from this ribotype group have the API profile number of 000000000 and therefore remained unidentified. This *Bam*HI ribotype group contained 3 different *Sal*I ribotype groups. Thirteen of the 15 isolates belonged to *Sal*I ribotype group 1, while 2 isolates, 5915 and 5917, belonged to *Sal*I ribotype group 7 and a clinical isolate, C87, belonged to *Sal*I ribotype group 4.

*Bam*HI group 3 contained 12 isolates and was characterised by rDNA fragments of 9.7 and 7.2 kb. Seven of these isolates were identified as *M.luteus* by the API microtitre strip. Included in this group is *M.luteus* type

strain NCIMB 9278. Four isolates were unidentified by the API microtitre strip, 3 because they have the API profile 000000000 and 1 because it had the profile 076761612 which, although reactive, was regarded as an unacceptable API profile code when compared with API's database. This *Bam*HI ribotype group contained the members of the *Eco*RI REA groups 2 and 3. *Bam*HI ribotype group 3 contained 3 different *Sal*I ribotype groups. The *Eco*RI REA group 2 isolates belong to *Sal*I ribotype group 4, whereas the *Eco*RI REA group 3 isolates belong to *Sal*I ribotype group 1, with one isolate, *M.luteus* type strain NCIMB 9278 belonging to *Sal*I ribotype group 10.

*Bam*HI group 4 contained 16 isolates and was characterised by rDNA fragments of approximately 14 kb and 9.7 kb. Using the API microtitre strip 12 of these isolates were identified as *M.luteus*, 1 isolate was identified as *M.lylae*, and 3 isolates remained unidentified by the API microtitre strip, 2 isolates having the API profile 000000000 and 1 isolate having the API profile 076771612 which is deemed unacceptable by the API database. *Bam*HI ribotype group 4 contained 6 different *Sal*I ribotype groups. These are *Sal*I:- ribotype group 1 with 5 members; group 2 with 2 members; group 4 with 2 members; group 5 with 5 members (three of which form *Hind*III REA group 9); group 10 with one member, and one isolate producing a unique *Sal*I ribotype.

*Bam*HI group 5 was characterised by 2 rDNA fragments of >13 and 13 kb and contains 11 of the 13 isolates characterised as *M.sedentarius* by methicillin and penicillin resistance. Two isolates, 1382 and *M.sedentarius* type strain 11040, have rDNA fragments at >13, 13 and 6.0 kb. The 2 other isolates, A208 and B705, identified as *M.sedentarius* by the characteristic of methicillin and penicillin resistance were isolated from clinical material and each have a unique ribotype. Isolates A208 and B705 have rDNA fragments of 6.3 kb and 10.3, and, 9.2 and 6.0 kb respectively. rRNA probing after digestion with *Bam*HI therefore differentiates *M.sedentarius* as characterised by antibiotic resistance into 3 main ribotypes. Isolates in *Bam*HI ribotype group 5 belong to 4 different *Sal*I ribotype groups. Ten of the isolates belong to *Sal*I ribotype group 4, one isolate, 781, belongs to *Sal*I ribotype group 10 and the 2 *M.sedentarius* isolates producing unique *Bam*HI ribotypes have unique *Sal*I ribotypes.

*Bam*HI group 6 contains the 2 isolates from the Antarctic sample group identified as *M.varians* by the API microtitre strip, the *M.varians* type strain NCIMB 11697, and one isolate, 481, which was not identified by the API microtitre strip. Isolates 282 and 481 have 3 rDNA fragments of 7.2, 7.1, 6.9 kb and 7.1, 6.9 and 5.6 kb respectively. Isolates 4910 and the type strain NCIMB

11697 produce 2 rDNA fragments of 7.1 and 6.9 kb and 6.9 and 6.3 kb respectively. All the isolates in this group have a common rDNA fragment of 6.9 kb. All isolates with *Bam*HI ribotype group 6 belong to *Sal*I ribotype group 8.

*Bam*HI group 7 contained all the isolates identified as *M.kristinae*. This included *M.kristinae* type strain NCTC 11038 containing rDNA fragments of 6.2 and 5.6 kb, and the isolate collected from clinical material, typed by the API microtitre strip as *M.kristinae*, which had rDNA fragments of 7.1, 6.2 and 5.6 kb. Both isolates have common rDNA fragments of 6.2 and 5.6 kb, similar *Sal*I ribotypes and belong to *Sal*I ribotype group 9.

Eight isolates showed unique *Bam*HI ribotypes. Two, isolates A208 and B705 have previously been discussed as *M.sedentarius* (p110). The remaining isolates are 682 (API profile 000001000) with rDNA fragments of 7.1 and 7.5 kb, 862 (API profile 000005000) with rDNA fragments of approximately 13 and 9.7 kb, and the type strains *M.nishinomiyaensis* NCTC 11039 with rDNA fragments of >13, 10.5 and 8.0 kb, *M.lylae* ATCC 27567 with rDNA fragments of >13 and 6.9 kb, *M.lylae* ATCC 27568 with rDNA fragments of 7.3 and 6.0 kb, and *M.roseus* NCIMB 11696 with a rDNA fragment of 6.9 kb.

M.luteus in this study forms a heterogenous population of bacteria belonging to 4 different *Bam*HI ribotype groups.

Within *Bam*HI ribotype groups 1, 2, 3 and 4 there are isolates identified as *M.luteus* by the API microtitre strip. A common rDNA fragment of 9.7 kb is shared by *Bam*HI ribotype groups 1, 2, 3, and 4. Figure 12 shows the *Bam*HI ribotypes of isolates identified as *M.luteus* by the API microtitre strip. *M.nishinomiyaensis* type strain NCIMB 11696, *M.lylae* type strain ATCC 27567 and all isolates identified as *M.varians* in *Bam*HI ribotype group 6 share a common 6.9 kb rDNA fragment. *M.varians* type strain NCIMB 11697 and isolate B705 share a common rDNA fragment of 6.3 kb.

Within *Bam*HI ribotype groups 1, 2, 3 and 4 there are 3 API microtitre strip identifications of *M.lylae*, and 2 *M.lylae* type strains are members of group 1. Apart from the *M.lylae* type strains, the 3 API identifications of *M.lylae* were made solely on the cream colony pigment produced by the isolate. Each isolate would be identified as a member of the species *M.luteus* if it produced yellow pigmented colonies.

Of the 4 commercially available *M.lylae* type strains two, *M.lylae* NCIMB 11037 and ATCC 27569, are members of the largest *Bam*HI ribotype group 1, whilst the other 2 *M.lylae* type strains ATCC 27567 and 27568 have unique BamHI ribotypes.

In the *Bam*HI ribotype groups, colony pigmentation was no predictor of isolate grouping, with isolates belonging to identical ribotype groups producing differently pigmented colonies. From Table 7 it is also apparent that the API biochemical identification is no predictor of ribotype groups, with isolates having identifiable API profiles, and isolates which are biochemically non-reactive belonging to the same ribotype groups.

With *Sal*I 11 main ribotype groups containing 96 isolates were apparent. Eight isolates produced unique ribotypes, with 2 isolates *M.lylae* ATCC 27569 and *M.roseus* NCIMB 11696 not digested by *Sal*I. Figure 13 shows some of these *Sal*I ribotype groups.

The largest *Sal*I ribotype group, group 1, contains 37 isolates. It is characterised by rDNA fragments of 9.6 and 6.3 kb. One isolate, 1363, contains an extra rDNA fragment of 0.8 kb. Group 1 contains 21 isolates identified as *M.luteus*, 1 isolate identified as *M.lylae*, one isolate poorly identified as *M.nishinomiyaensis* and one isolate identified as *Staphylococcus auricularis* by the API microtitre strip and colony pigment. Thirteen isolates were not identified by the API microtitre strip. There were no type strains within this group. This *Sal*I ribotype group contains 15 members of *Bam*HI ribotype group 1, 12 members of *Bam*HI ribotype group 2, and 5 each of *Bam*HI ribotypes group 3 and 4.

*Sal*I group 2 contains 9 isolates and is characterised by rDNA bands of 10.6 and 6.4 kb. *M.luteus* type strains NCIMB 8166 and 8553 are members of this *Sal*I ribotype group. There are a further 5 isolates identified as *M.luteus* by the API microtitre strip in this group and 2 isolates are not identified, having the API profile 000000000. This SalI ribotype group contains 7 and 2 members from *Bam*HI ribotype groups 1 and 4 respectively.

*Sal*I group 3 contains 7 isolates and is characterised by rDNA fragments of 9.7 and 7.1 kb. All the isolates were identified by the API microtitre strip as *M.luteus*. The *Sal*I ribotype group 3 comprises the same isolates as REA *Hind*III group 1 and all isolates are members of *Bam*HI ribotype group 1.

*Sal*I group 4 containing 21 isolates is characterised by rDNA fragements of 6.4 kb (Figure 14). When lightly loaded this single fragment resolves into 2 rDNA fragments of 6.5 and 6.4 kb. This ribotype group contains all the isolates identified as *M.sedentarius*, except for isolate B705 and A208, and includes *M.sedentarius* type strain NCIMB 11040. This group also contains the *M.lylae* type strain ATCC 27567 and 6 isolates which were identified as *M.luteus* by the API microtitre strip and colony pigment. This *Sal*I ribotype group contains the 8 different *Bam*HI ribotype groups: 1, 2, 3, 4, 5, 6, 7, and a unique *Bam*HI ribotype. Six of the isolates in *Sal*I

group 4 belong to the REA *Eco*RI group 2, with all the REA group 2 members sharing the same *Bam*HI ribotype group, group 3. *M.lylae* type strain ATCC 27567 has a unique *Bam*HI ribotype.

*Sal*I group 5 contains 5 isolates characterised by rDNA fragments of 10.6 and 7.1 kb. All isolates in *Sal*I group 4 share the same *Bam*HI ribotype group 4. Four out of the 5 isolates were identified as *M.luteus* by colony morphology and the API microtitre strip.

*Sal*I group 6 contains 4 isolates characterised by rDNA fragments of 9.6 and 7.9 kb, which are members of the same *Bam*HI ribotype group, group 1. All isolates were cream or white coloured and remained unidentified, having the API profile 000000000.

*Sal*I group 7 contains 3 isolates with rDNA fragments of 6.0, 4.6, 4.0 and 2.9 kb and isolate 961 with rDNA fragments of 4.6, 4.0, 2.9 and 2.2 kb. Two isolates were identified as *M.lylae* by the API microtitre strip and colony morphology, with one isolate, 862, producing a unique *Bam*HI ribotype and the other, isolate 5915, belonging to *Bam*HI ribotype group 2. One isolate, 5917, was not identified by the API microtitre strip and belonged to *Bam*HI ribotype group 2. Isolate 961 was identified as *M.luteus* by the API microtitre strip and belonged to *Bam*HI ribotype group 1.

*Sal*I group 8 contains the 2 isolates identified as *M.varians* by the API microtitre strip, *M.varians* type strain NCIMB 11697 and one isolate which gave a reactive API profile but was not identified. Isolates 481 and 4910 have 2 rDNA containing fragments of 8.0 and 7.1 kb and 7.1 and 5.7 kb respectively. Isolates 282 and *M.varians* type strain NCIMB 11697 have 3 rDNA fragments of 8.0, 7.1 and 6.2 kb and 10.6, 9.9 and 7.1 kb respectively (Figure 15). All members of *Sal*I ribotype group 8 have a common rDNA fragment of 7.1 kb. All *Sal*I ribotype group 8 isolates are members of *Bam*HI ribotype group 6.

*Sal*I group 9 contains the 2 *M.kristinae* isolates *M.kristinae* NCTC 11038 and isolate A545 collected from clinical material. *M.kristinae* NCTC 11038 and isolate A545 have rDNA fragments of 10, 9.6 and 7.1 kb. Both the *Sal*I ribotype group 9 isolates are members of *Bam*HI ribotype group 9.

*Sal*I group 10 contains 3 isolates characterised by 2 rDNA fragments of 7.7 and 6.2 kb. *M.luteus* type strain NCIMB 9278 is a member of *Bam*HI ribotype group 3, with one isolate identified as *M.luteus* by the API microtitre strip as a member of *Bam*HI ribotype group 4, and one isolate, 781, with the API profile 000000000 as a member of *Bam*HI ribotype group 5.

Eight isolates showed unique *Sal*I ribotypes. These included isolate 682 (*Bam*HI unique) producing rDNA fragments of 7.7 and 6.4 kb (see Figure 14); *M.nishinomiyaensis* type strain NCTC 11039 (*Bam*HI unique) producing rDNA fragments of 7.9 and 7.1 kb; *M.lylae* type strain ATCC 27568 (*Bam*HI unique) producing rDNA fragments of 7.1 and 6.2 kb; and the 2 *M.sedentarius* clinical isolates B705 (*Bam*HI unique) and A208 (*Bam*HI unique) producing rDNA fragments of 6.0 and 5.4 kb and 7.0 and 6.4 kb respectively. Isolate 882, producing rDNA fragments of 6.4 and 6.1 kb, and *M.lylae* type strain NCTC 11037, producing rDNA fragments of 10.6, 4.7 and 3.4 kb which are members of *Bam*HI ribotype group 1, showed unique *Sal*I ribotypes. Isolate 8919 produced rDNA fragments of 7.9 and 6.4 kb and is a member of *Bam*HI ribotype group 4.

M.luteus, as identified by the API microtitre strip on purely biochemical criteria, were members of SalI ribotype groups 1, 2, 3, 4, 5 and 10. *M.luteus* also produced 2 unique SalI ribotypes.

*Sal*I ribotype groups 1 and 6 and *M.kristinae* type strain NCTC 11038 share a common rDNA fragment of 9.6 kb; *Sal*I ribotype groups 2 and 5 and *M.lylae* type strain 11037 share a common 10.6 kb rDNA fragment; *Sal*I ribotype groups 3 and 5, *M.kristinae* type strain NCTC 11038, and isolates identified as *M.varians* share a common 7.1 kb

rDNA fragment; *Sal*I ribotype groups 2 and 4, isolate A205, isolate 882, isolate 682 and isolate 8919 share a common 6.4 kb rDNA fragment; isolate 8919 shares a common 7.9 kb rDNA fragment with *Sal*I ribotype group 6 and *M.nishinomiyaensis* type strain 11039.

As with the *Bam*HI ribotype groups the colony pigment and API profile were no predictors of *Sal*I ribotype group. Within the *Hind*III and *Eco*RI REA groups all members of each group have the identical *Bam*HI and *Sal*I ribotypes except for *Hind*III group 12 containing the isolates 861, 1362 and 13917. This *Hind*III REA group has *Bam*HI ribotype groups 1 and 2 and *Sal*I ribotype groups 1 and 3 within it.

When assigning the micrococci to species the conservative view was taken whereby both the biochemical and ribotype characteristics of the isolates were used as criteria. All biochemically (without the criterion of colony pigmentation) identified *M.luteus* were found in the *Bam*HI ribotype groups 1, 2, 3 and 4 and *Sal*I ribotype groups 1, 2, 3, 4, 5 and 10. Therefore "double" ribotype fingerprints belonging to *Bam*HI groups 1-4 and *Sal*I groups 1, 2, 3, 4, 5, or 10 were identified as *M.luteus*. This meant that 67% (59/88) of the isolates collected in the Antarctic are *M.luteus*.

If the *Bam*HI ribotype groups 5, 5A and 5B and *Sal*I ribotype group 4 are assumed to be *M.sedentarius* then 10% (9/88) are *M.sedentarius*. One isolate, 781, with *Bam*HI ribotype group 5 and *Sal*I ribotype group 10 was not identified as *M.sedentarius*. *M.varians* formed 3% (3/88) of the sampled population.

Using the biochemical identification and ribotype group as speciating characteristics it was not possible to speciate 12 (14%) isolates. This number included isolates in *Bam*HI ribotype group 1: and *Sal*I ribotype group 6, isolates in *Sal*I ribotype group 7 and isolates 682 and 882 which produced unique *Sal*I ribotypes.

ISOLATE	API NUMBER	COLONY PIGMENT	*Bam*HI GROUP	*Sal*I GROUP	SPECIES
261	000000000	L.YELLOW	2	1	*M.luteus*
263	000000000	L.YELLOW	2	1	*M.luteus*
271	076771612	LEMON	4	1	*M.luteus*
281	000000000	L.LEMON	2	1	*M.luteus*
2914	000001000	LEMON	4	1	*M.luteus*
2915	000000000	L.LEMON	2	1	*M.luteus*
2916	000005000	LEMON	4	1	*M.luteus*
372	100001000	YELLOW	2	1	*M.luteus*
461	103025201	YELLOW	1	1	*M.luteus*
572	102107210	YELLOW	1	1	*M.luteus*
581	000001000	YELLOW	1	1	*M.luteus*
5916	000000000	YELLOW	1	1	*M.luteus*
661	100000200	YELLOW	3	1	*M.luteus*
662	000005000	YELLOW	3	1	*M.luteus*
672	100040300	LEMON	2	1	*M.luteus*
673	000005000	YELLOW	3	1	*M.luteus*
681	000000000	YELLOW	3	1	*M.luteus*
6919	100007000	YELLOW	1	1	*M.luteus*
761	100005000	YELLOW	2	1	*M.luteus*
762	000000000	YELLOW	1	1	*M.luteus*
763	100005000	YELLOW	1	1	*M.luteus*
764	000001200	YELLOW	1	1	*M.luteus*
765	100000000	YELLOW	2	1	*M.luteus*
771	100002000	L.YELLOW	1	1	*M.luteus*
772	000000000	CREAM	1	1	*M.luteus*
773	000005000	CREAM	1	1	*M.luteus*
7918	100000000	YELLOW	1	1	*M.luteus*
7919	000000000	CREAM	1	1	*M.luteus*
7920	000000000	CREAM	1	1	*M.luteus*
871	100001000	YELLOW	2	1	*M.luteus*
971	100000000	YELLOW	2	1	*M.luteus*
972	360030200	YELLOW	2	1	*M.luteus*
1161	000000000	CREAM	4	1	*M.luteus*
1363	100004000	YELLOW	1	1	*M.luteus*
1381	000001000	YELLOW	3	1	*M.luteus*
13917	100001000	YELLOW	2	1	*M.luteus*
C1779	100007000	YELLOW	4	1	*M.luteus*
671	100005000	L.YELLOW	1	2	*M.luteus*
6920	000000000	LEMON	1	2	*M.luteus*
782	100005000	CREAM	1	2	*M.luteus*
783	000000000	CREAM	1	2	*M.luteus*
8553	060025000	YELLOW	1	2	*M.luteus*
8166	000025000	LEMON	1	2	*M.luteus*
C156	100005000	YELLOW	1	2	*M.luteus*
C425	000005000	YELLOW	4	2	*M.luteus*
C2634	000007000	YELLOW	1	2	*M.luteus*
463	100005000	YELLOW	1	3	*M.luteus*
471	100005000	YELLOW	1	3	*M.luteus*
472	100000000	YELLOW	1	3	*M.luteus*
861	100005200	YELLOW	1	3	*M.luteus*
1361	100001200	YELLOW	1	3	*M.luteus*
1362	100007000	YELLOW	1	3	*M.luteus*
1365	100007000	YELLOW	1	3	*M.luteus*
383	000000000	CREAM	5	4	*M.sedentarius*
3910	000000000	CREAM	5	4	*M.sedentarius*
498	000000000	CREAM	5	4	*M.sedentarius*

Table 7. The API profile, colony pigmentation and rRNA groups with the restriction enzyme *Bam*HI and *Sal*I of 106 micrococci and the species identification by ribotyping.

UNI=unique ribotype.

N/A=not avaiable.

ISOLATE	API NUMBER	COLONY PIGMENT	*Bam*HI GROUP	*Sal*I GROUP	SPECIES
1384	000000000	CREAM	5	4	*M.sedentarius*
1383	000000000	ORANGE	5	4	*M.sedentarius*
1386	000000000	ORANGE	5	4	*M.sedentarius*
13916	000000000	CREAM	5	4	*M.sedentarius*
11040		CREAM	5	4	*M.sedentarius*
1382	000000000	ORANGE	5	4	*M.sedentarius*
483	000000000	WHITE	5	4	*M.sedentarius*
27567	000020000	CREAM/LEM	UNI	4	*M.luteus*
A211	000001000	CREAM	4	4	*M.luteus*
A506	000003000	CREAM	4	4	*M.luteus*
A366	100007000	CREAM	1	4	*M.luteus*
C87	000005000	YELLOW	2	4	*M.luteus*
561	000000000	L.LEMON	3	4	*M.luteus*
562	000001000	L.LEMON	3	4	*M.luteus*
563	000000000	L.LEMON	3	4	*M.luteus*
571	000005000	LEMON	3	4	*M.luteus*
582	000001000	L.LEMON	3	4	*M.luteus*
583	076761612	L.LEMON	3	4	*M.luteus*
1364	000003000	L.YELLOW	4	5	*M.luteus*
1371	000000000	LEMON	4	5	*M.luteus*
1373	000001000	LEMON	4	5	*M.luteus*
1385	000001000	LEMON	4	5	*M.luteus*
13918	000001000	YELLOW	4	5	*M.luteus*
881	000000000	CREAM	1	6	
3912	000000000	CREAM	1	6	
883	000000000	CREAM	1	6	
8920	000000000	WHITE	1	6	
862	000005000	CREAM	UNI	7	
5915	000001000	CREAM	2	7	
5917	000000000	CREAM	2	7	
282	160010000	CREAM	6	8	*M.varians*
481	172110200	L.LEMON	6	8	*M.varians*
4910	160012000	WHITE	6	8	*M.varians*
11697	167150311	L.YELLOW	6	8	*M.varians*
11038	063127310	CREAM	7	9	*M.kristinae*
A545	063126210	CREAM	7	9	*M.kristinae*
9278	100005000	YELLOW	3	10	*M.luteus*
3911	000001000	YELLOW	4	10	*M.luteus*
781	000000000	WHITE	5	10	
11037	000001000	CREAM	1	UNI	*M.lylae*
27568	000077000	CREAM	UNI	UNI	*M.lylae*
27569	000025000	CREAM	1	N/A	*M.lylae*
682	000001000	WHITE	UNI	UNI	
A208	000000000	CREAM	UNI	UNI	*M.sedentarius*
B705	000000000	CREAM	UNI	UNI	*M.sedentarius*
961	000005000	L.YELLOW	1	UNI	
882	000000000	CREAM	1	UNI	
8919	100003000	YELLOW	4	UNI	
11039	000000000	ORANGE	UNI	UNI	*M.nishinomyaensis*
11696	040010000	PINK	UNI	N/A	*M.roseus*

Table 7. Continued.

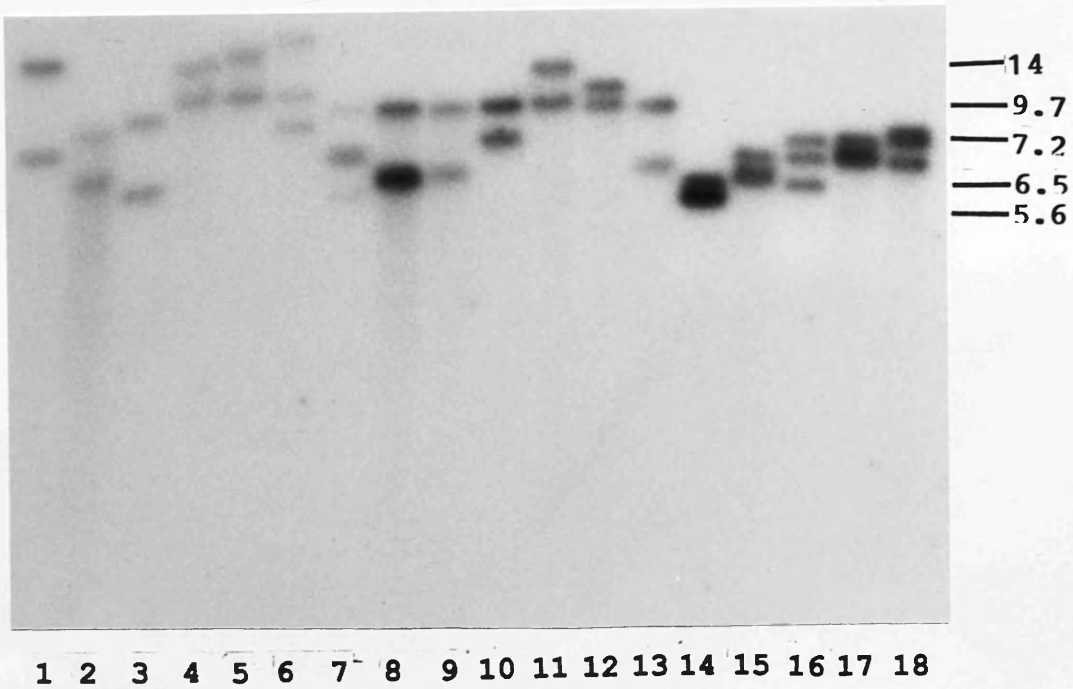

Figure 11. *Bam*HI ribotype profiles of the genus *Micrococcus*. Isolates, with ribotype group in brackets, are: Lane 1, *M.lylae* ATCC 27567 (Uni); Lane 2, *M.lylae* ATCC 27568 (Uni); Lane 3, *M.lylae* NCTC 11037 (1); Lane 4, *M.sedentarius* 383 (5); Lane 5, *M.sedentarius* NCTC 11040 (5A); Lane 6, *M.nishinomiyaensis* NCTC 11039 (Uni); Lane 7, *M.roseus* NCIMB 11696 (Uni); Lane 8, *M.luteus* NCIMB 8553 (1); Lane 9, *M.luteus* NCIMB 8166 (1); Lane 10, *M.luteus* NCIMB 9278 (3); Lane 11, *M.luteus* 8919 (4); Lane 12, *M.luteus* 761 (2); Lane 13, *M.luteus* 463 (1); Lane 14, *M.kristinae* NCTC 11038 (7); Lane 15, *M.varians* NCIMB 11697 (6); Lane 16, *M.varians* 481 (6); Lane 17, *M.varians* 4910 (6); Lane 18, *M.varians* 282 (6). Fragment sizes are in kilobases. Abbreviations as before, see Methods Section 2.1, p53. Uni=unique ribotype.

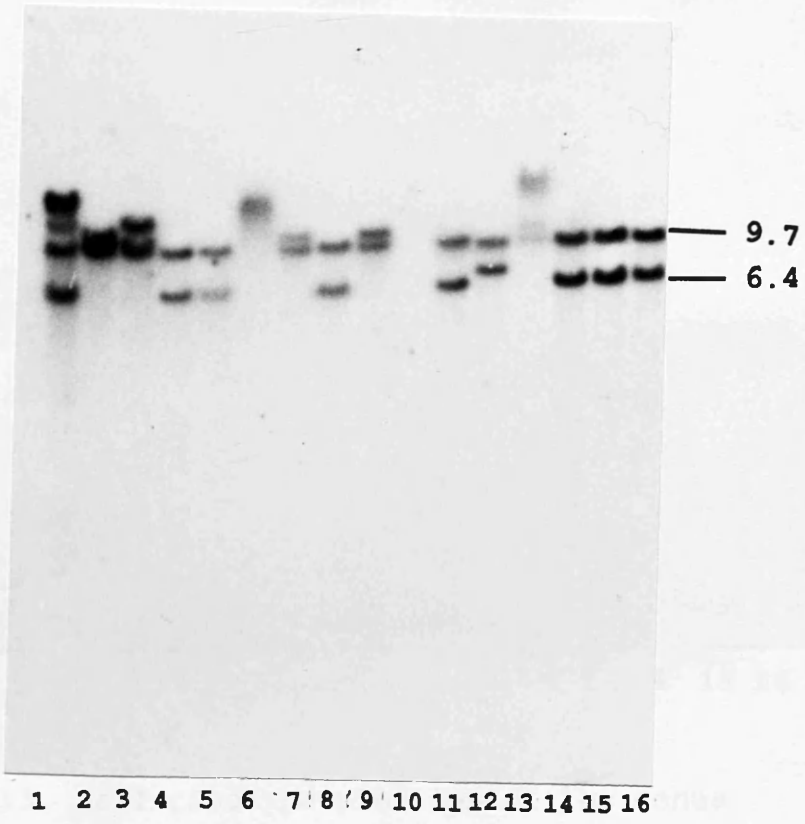

Figure 12. *M.luteus* isolates digested with *Bam*HI. Isolates, with ribotype groups in brackets, are: Lane 1, 1363 (1); Lane 2, 13917 (2); Lane 3, 8919 (4); Lane 4, 782 (1); Lane 5, 771 (1); Lane 6, 765 (2); Lane 7, 761 (2); Lane 8, 6919 (1); Lane 9, 372 (2); Lane 10, blank; Lane 11, 671 (1); Lane 12, 661 (3); Lane 13, 972 (2); Lane 14, 7918 (7918); Lane 15, 572 (1); Lane 16, 463 (1). All isolates were identified as *M.luteus* by the API microtitre strip. Abbreviations as before, see Methods Section 2.1, p53. Lane 6 is partially digested. Fragment sizes are in kilobases.

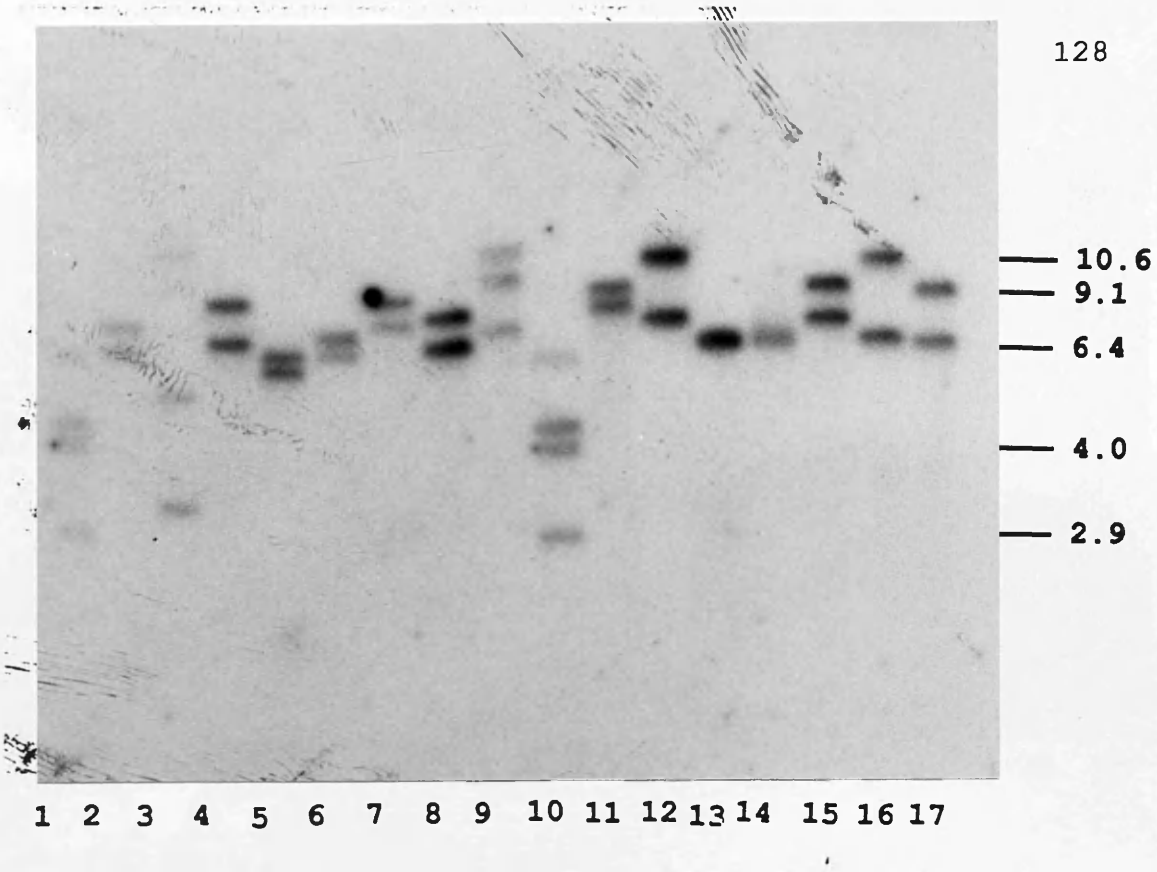

Figure 13. *Sal*I ribotype profiles of the genus *Micrococcus*. Isolates, with ribotype groups in brackets, are: Lane 1, isolate 862 (7); Lane 2, *M.lylae* ATCC 27568 (Uni); Lane 3, *M.lylae* NCTC 11037 (Uni); Lane 4, *M.luteus* 8919 (Uni); Lane 5, *M.sedentarius* B705 (Uni); Lane 6, *M.sedentarius* A208 (Uni); Lane 7, *M.nishinomiyaensis* NCTC 11039 (Uni); Lane 8, *M.luteus* NCIMB 9278 (10); Lane 9, *M.varians* NCIMB 11697 (8); Lane 10, isolate 5915 (7); Lane 11, isolate 883 (6); Lane 12, *M.luteus* 1364 (5); Lane 13, *M.luteus* 561 (4); Lane 14, *M.sedentarius* 383 (4); Lane 15, *M.luteus* 463 (3); Lane 16, *M.luteus* 782 (2); Lane 17, *M.luteus* 771 (1). Abbreviations as before, see Methods Section 2.1, p53. Uni=unique ribotype. Fragment sizes are in kilobases.

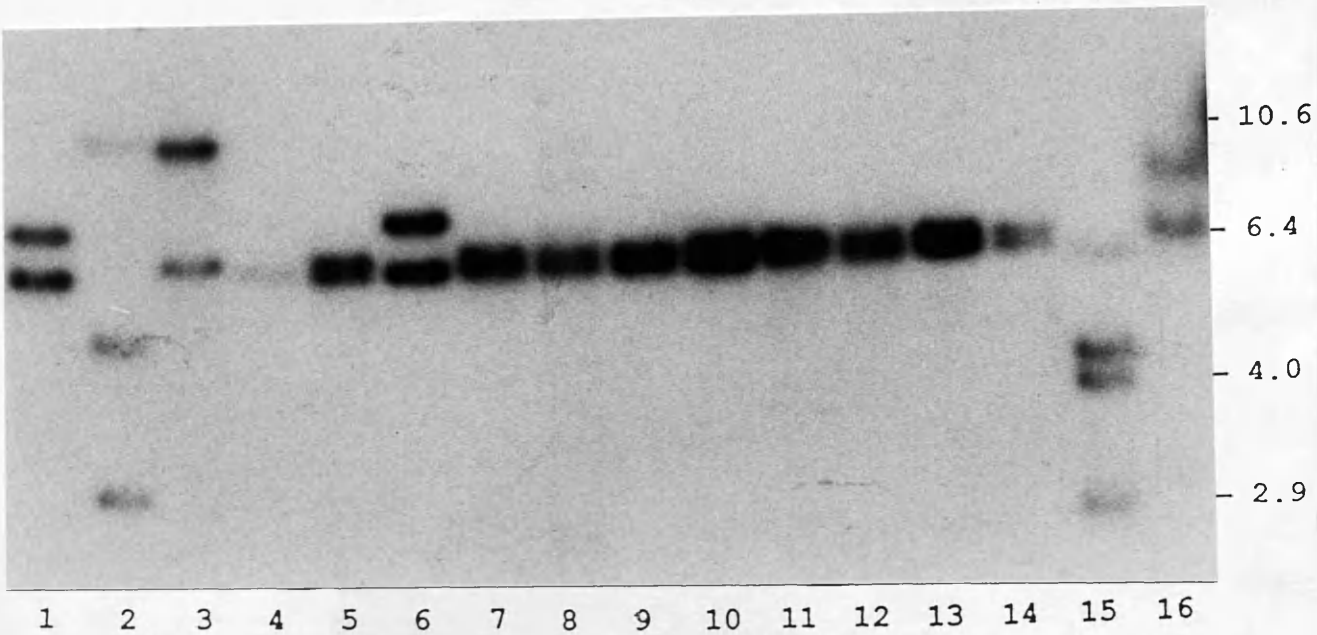

Figure 14. *Sal*I ribotype profiles of *M.sedentarius* (Lanes 4-14). Isolates, with ribotype group in brackets, are: Lane 1, *M.luteus* NCIMB 9278 (10); Lane 2, *M.lylae* NCTC 11037 (Uni); Lane 3, *M.luteus* 783 (2); Lane 4, *M.sedentarius* NCTC 11040 (4); Lane 5, *M.sedentarius* 383 (4); Lane 6, *M.sedentarius* 781 (4); Lane 7, *M.sedentarius* 1384 (4); Lane 8, *M.sedentarius* 3910 (4); Lane 9, *M.sedentarius* 498 (4); Lane 10, *M.sedentarius* 1383 (4); Lane 11, *M.sedentarius* 1386 (4); Lane 12, *M.sedentarius* 13916 (4); Lane 13, *M.sedentarius* 1382 (4); Lane 14, *M.sedentarius* 483 (4); Lane 15, isolate 862 (7); Lane 16, isolate 682 (Uni). Abbreviations as before, see Methods Section 2.1, p53. Uni=unique ribotype. Fragment sizes are in kilobases.

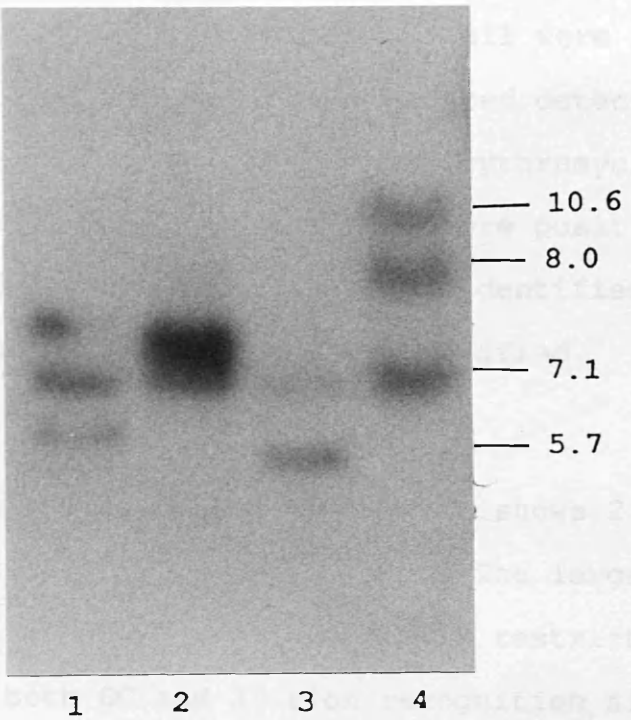

Figure 15. *M.varians* digested with *Sal*I. All isolates are members of group 8. Isolates are: Lane 1, isolate 282; Lane 2, isolate 4910; Lane 3, isolate 481; Lane 4, NCIMB 11697. Abbreviations as before, see Methods Section 2.1, p53. Fragment sizes are in kilobases.

3.7) Characterising presumptive *Stomatococcus* isolates

Of the 4 non-type strain isolates identified as having a low % mol GC content after digestion with *Eco*RI, all were resistant to lysostaphin at 200 µg/ml, all were resistant to lysozyme at 25 µg/ml, and none produced detectable acid from glycerol in the presence of erythromycin. Using the API microtitre strip two isolates were positively identified as *S.cohnii*, one was poorly identified as *S.warneri* and one remained as an unidentified staphylococcal species.

Figure 10 (lanes 4 and 5 and, 11 and 12) shows 2 of these isolates digested with *Bam*HI and *Eco*RI. The large number of restriction fragments produced with 2 restriction enzymes having both GC and AT rich recognition sites, suggests a % mol GC content intermediate between that of staphylococci and micrococci. As measured by spectrophotometer the % mol GC content of these 4 isolates varied from 55.5-58% (Table 8). Known micrococci and staphylococci were used as controls. *M.luteus* NCIMB 9278 gave a measured % mol GC content of 72.0%, *M.kristinae* NCIMB 11038 gave a measured % mol GC content of 66.8% and *S.aureus* NCTC 6571 gave a measured % mol GC content of 30.5%. The published results for *M.luteus* NCIMB 9278 is 72.5% [3] and for *M.kristinae* NCIMB 11038 it is 66.8% [7]. Isolates 373 and 382 gave indistinguishable banding patterns when digested with the restriction

enzymes used, but their % mol GC content was measured as 55.5% and 58.0%. This discrepancy can be explained by remaining traces of RNA contaminating the DNA sample. Isolates 21213 and 21214 gave a % mol GC content of 57.0% and 57.5% respectively.

This % mol GC content would place these isolates in the genus *Stomatococcus* which has a defined % mol GC content of 56-60.4% [15]. Further tests were therefore employed to attempt to characterise these isolates.

Table 8 shows the characteristics of these 4 isolates. Introduced into table 8 is the type strain *Stomatococcus mucilaginosus* NCTC 10663 and 2 isolates collected from clinical material, received as *Stomatococcus mucilaginosus*.

These 4 isolates produced lemon white colonies 1mm in size after 24 hours' growth on nutrient agar. The colony pigment deepened to lemon (isolates 373, 382) or orange (21213, 21214) after 5 days incubation at 37^0C on nutrient agar. They predominantly formed tetrads, and measured between 0.9-1.2 μm on Gram-staining. All 4 isolates had a creamy colony consistency, were not adherent to the agar surface, were strongly catalase positive, non-motile, lysozyme resistant, grew well on nutrient agar (+/- 5% NaCl). Two of the isolates, 373 and 382, grew on nutrient agar at 45^0C. Isolates 21213 and

21214 produced detectable acid from sucrose aerobically. None of the 4 isolates produced detectable acid aerobically from salicin, glycerol, adenitol, sorbitol, xylose or from glucose anaerobically when tested by the tube method using Anrades indicator. The positive API reactions of isolates 373, 382, 211213 and 211214 can be seen in table 8. Two of the isolates, 21213 and 21214, reduced nitrate to nitrite. All isolates were sensitive to methicillin, penicillin, amoxycillin, piperacillin, erythromycin, fusidic acid, vancomycin, cefotaxime, ciprofloxacin, cefuroxime and gentamicin. Isolates 373, 382 and 21214 were sensitive to trimethoprim. Isolate 21213 was resistant to trimethoprim.

The API microtitre strip misidentified all the *Stomatococcus* isolates including the type strain NCTC 10663 as staphylococci. This group of *Stomatococcus* spp. are phenotypically dissimilar. Not all isolates in the *Stomatococcus* group show all the classical biochemical and morphological characteristics of the genus *Stomatococcus*. The 4 Antarctic isolates had different colony morphology and biochemical characteristics from the type strain *Stomatococcus mucilaginosus* and the 2 *Stomatococcus* spp. from clinical material. The isolates 373 and 382 also show different biochemical properties from the other *Stomatococcus* spp. in the group.

The stomatococcus is a capsulated organism. All 7 isolates showed a thin capsule using India ink staining. All the micrococci and staphylococci used as negative controls also showed the presence of a thin capsule.

Figure 16 shows the banding pattern of the 4 isolates after digesting total chromosomal DNA with *Bam*HI and *Hind*III, and probing with labelled ^{32}P *E.coli* 16S and 23S rRNA. Due to the lower % mol GC content *Hind*III was used instead of *Sal*I. Isolate 6921 (discussed earlier Section 3.5.3, p107) shows a ribotype similar to *S.warneri* ATCC 27837 when digested with the restriction enzyme *Hind*III. This is consistent with a measured % mol GC content of 36.5%, production of acid from glucose anaerobically and the API profile identifying the isolate as *S.warneri*.

The four isolates 373, 382, 21213 and 21214 show a ribotype pattern dissimilar to *M.luteus*, *M.lylae*, *S.warneri*, *S.cohnii* and *Planococcus citreus*. In the *Bam*HI digest they share no common rDNA fragments. In the *Hind*III digest a common rDNA fragment of 1.2 kb is present in all the 4 isolates. These 4 isolates also have ribotypes dissimilar to the type strain *S.mucilaginosus* NCTC 10663, although a common rDNA fragment of 3.6 kb is present in isolates 373 and 382 and the type strain. The isolates 373 and 382 have a different ribotype from 21213 and 21214 with both *Bam*HI and *Hind*III. This difference in ribotype is reflected in differences in phenotype (Table

8). The two clinical isolates A3587 and T886 share 2 common rDNA fragments of 4.0 and 3.2 kb. These 2 isolates share no common fragments with the 4 Anarctic isolates, whilst isolate A3587 shares 3 common rDNA fragments of 5.0, 4.0 and 3.2 kb and isolate T886 shares 2 common rDNA fragments of 4.0 and 3.2 kb (data not shown).

Using biochemical and physiological criteria and the % mol GC content the 4 isolates 373, 382, 21213 and 21214 are deemed to belong to the genus *Stomatococus*. By ribotype pattern they show significant divergence from the type strain *Stomatococcus mucilaginosus* NCTC 10663.

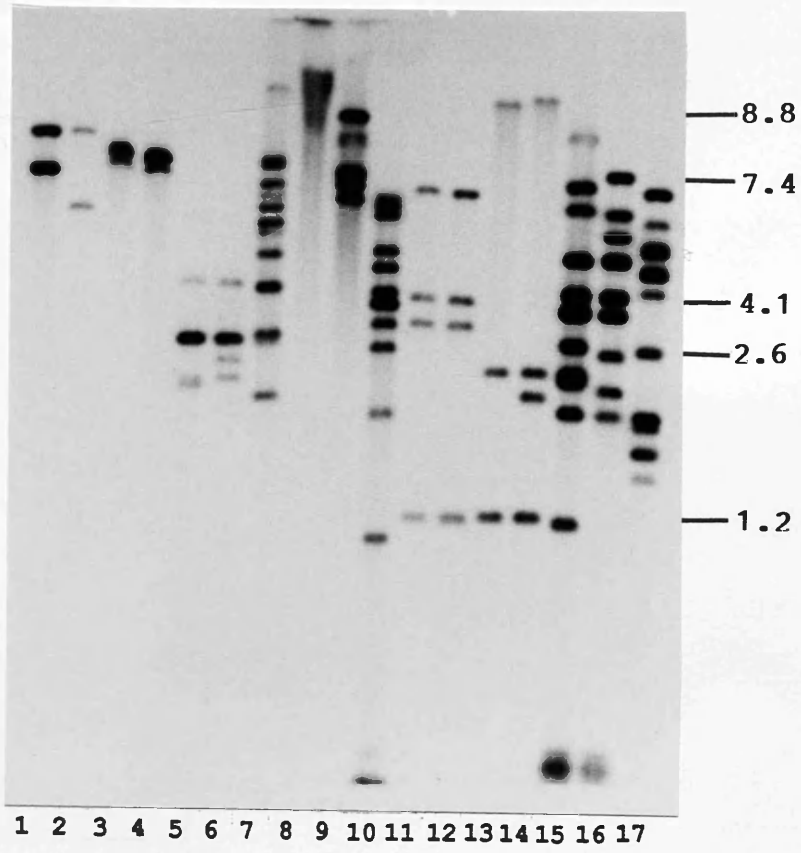

Figure 16. Presumptive *Stomatococcus* digested with *Bam*HI (Lanes 1-8) and *Hind*III (Lanes 9-17). Isolates are: Lane 1, *M.luteus* NCIMB 9278; Lane 2, *M.lylae* NCTC 11037; Lane 3, isolate 373; Lane 4, isolate 382; Lane 5, 21213; Lane 6, 21214; Lane 7, *Planococcus citreus* NCIMB 1493; Lane 8, *Stomatococcus mucilaginosus* NCTC 10663; Lane 9, *Stomatococcus mucilaginosus* NCTC 10663; Lane 10, *Planococcus citreus* NCIMB 1493; Lane 11, isolate 373; Lane 12, isolate 382; Lane 13, isolate 21213; Lane 14, isolate 21214; Lane 15, isolate 6921; Lane 16, *S.warneri* ATCC 27837; Lane 17, *S.cohnii* DSM 20261. Abbreviations as before, see Methods Section 2.1, p53. Fragment sizes are in kilobases. Run at 1.5V/cm for 16 hours.

ISOLATE	API NUMBER	COLONY PIGMENT	CATALASE	GROWTH IN 5% NaC1	GROWTH AT 45 C	CIP 1ug	W 2.5ug	SUCROSE	SALACIN	NITRATE REDUCTION	% MOL GC CONTENT
373	060020040	LEMON	+	+	+	-	-	-	-	-	58.0%
382	060020040	LEMON	+	+	+	-	-	-	-	-	55.5%
21213	060010250	ORANGE	+	+	-	-	+	+	-	+	57.0%
21214	062110250	ORANGE	+	+	-	-	-	+	-	+	57.5%
A3587	067715212	WHITE	-	-	-	-	-	+	+	+	N/A
T886	077774212	WHITE	-	-	-	-	+	+	+	+	N/A
10663	226116710	WHITE	+	+	+	+		+	-	+	59.0a

Table 8. The phenotypic characteristics of 7 *Stomatococcus* spp.

3.8) Choice of restriction enzyme for PFGE

High-molecular weight genomic DNA of *M.luteus* isolate 972 was digested with the restriction enzymes *Eco*RI, *Eco*RV, *Hind*III, *Xba*I, *Hpa*I and *Dra*I. These enzymes are rich in A+T recognition sequences and give a calculated mean fragment length of 14.3-340 kb (Table 4). One enzyme, *Dra*I, had a mean fragment length calculated as infinity, due to the absence of the *Dra*I recognition site (TTTAAA) in the sequence of DNA used to calculate the mean fragment lengths.

High molecular weight genomic DNA suitable for digestion with restriction enzymes was obtained from all micrococcal species except *M.sedentarius*. The failure to obtain genomic DNA from this species was due to the lack of effective lysis of the bacterial cell wall whilst *in situ* within the agarose insert. This was not unexpected, as all *M.sedentarius* isolates were moderately resistant to normal DNA extraction methods and gave low yields of low molecular weight DNA.

Figure 17 shows the selected restriction enzymes assessed for usefulness in PFGE using a ramp time of 30-50 seconds. Digestion of DNA with *Hind*III (Lane 1) gave the most useful DNA profile with DNA fragments ranging from 500 to >50 kb separated over the length of the gel. The restriction enzyme *Eco*RI produced fragments between 250

and >50 kb (Lane 2) with most of the fragments concentrated below 100kb. The restriction enzyme *Eco*RV gave a good separation of fragments from 350 to >50 kb (Lane 3) and *Xba*I gave fragments between 500-50 kb (Lane 4). The restriction enzyme *Hpa*I (Lane 5) gave too few fragments to be useful and *Dra*I (Lane 6) did not cut the genomic DNA of *M.luteus* 972.

*Hind*III was used for all further digestions in PFGE as it gave DNA fragments which separated over the length of the gel at the chosen pulse time and gave a profile that was easy to analyse and compare.

In figure 17, Lane 3 shows DNA fragments that are partially digested by the restriction enzyme *Eco*RV. To reduce the number of partial DNA fragments produced, the agarose inserts were pre-incubated in a BSA/DTT buffer prior to digestion with the restriction enzyme (see Methods Section 2.16, p69). This effectively prevented partial digestion of the genomic DNA.

For PFGE the pulse time is an important variable in finding the most useful and discriminatory restriction enzyme. This is because only those fragments with a mean length which are able to reorientate and migrate at the chosen pulse time will be separated sufficiently. The pulse time is a retardative element in PFGE. Pulse times should be chosen both above and below the range first

considered when choosing a new restricton enzyme to ensure that all DNA fragments are separated. The pulse time chosen for all digests was 15-40 seconds. This ramp time was capable of separating DNA fragments between 450-30 kb.

Figure 18 shows genomic DNA from *M.lylae*, *M.luteus*, *M.roseus*, *M.varians*, *M.kristinae* and *M.sedentarius* digested with *Hind*III. There is no obvious species specific DNA profile for those species where more than one isolate was analysed.

Lanes 1-4 contain *M.lylae* type strains NCTC 11037, ATCC 27567, ATCC 27568 and ATCC 27569 respectively. By PFGE DNA profile the *M.lylae* type strains represent 2 distinct groups consisting of NCTC 11037 and ATCC 27569 and ATCC 27568 and ATCC 27569. This is consistent with their published % mol GC content and biochemical profiles [6]. *M.lylae* type strains NCTC 11037 and ATCC 27569 have indistinguishable DNA profiles. No restriction fragment polymorphisms were apparent between these 2 type strains using a ramp time of 2-15 seconds.

Lanes 5-8 contain the *M.luteus* isolates NCIMB 8553, 463, 1373 and 271 respectively. There is no obvious species specific DNA profile present in these 4 *M.luteus* isolates.

Lanes 10-12 contain *M.varians* isolates 282, 4910 and NCIMB 11697 respectively. Like *M.lylae* and *M.luteus* there is no apparent species specific DNA profile.

Lanes 9 and 13 contain *M.roseus* NCIMB 11696 and *M.kristinae* NCTC 11038 respectively. Lane 14 contains *M.sedentarius* NCTC 11040 which has only been partially digested by *Hind*III.

Isolates selected by colony pigmentation and API profile were taken from within each *Bam*HI ribotype group and their genomic DNA was digested with *Hind*III. There was no ribotype specific PFGE DNA profile in any of the *Bam*HI ribotype groups. Figure 19 shows PFGE DNA profiles of representative isolates from *Bam*HI ribotype groups 1 and 2.

3.9) Micrococcal genome size as determined by PFGE

Sizing the micrococcal genome by PFGE was done over 3 different pulse times. By using 3 different sets of pulse times it was possible to resolve all the *Hind*III DNA fragments between 600 and 17 kilobases. The pulse times chosen were 2-15 seconds to enhance the separation of DNA fragments in the 11-200 kb range, 15-40 seconds for fragments in the 150-400 kb range and 25-60 seconds for fragments in the 400-600 kb range.

The sizes of the *Hind*III DNA fragments and the deduced size of the genomes of *M.luteus*, *M.lylae*, *M.roseus* and *M.varians* are shown in table 9. The genome size of members of the genus *Micrococcus* as determined by PFGE after digestion with *Hind*III are heterogeneous with size ranging from 1,823 to 3,397 kilobases.

The genome size of *M.luteus* varies from 2,184 to 2,598 kb with a mean of 2,366 kb. *M.lylae* had a genome size of between 2,138 and 2,584 kb. *M.lylae* type strain NCIMB 11037 and ATCC 27569 had indistinguishable DNA profiles and therefore had the same genome size. The 4 *M.lylae* type strains represent 2 distinct groups on the basis of genome size and DNA restriction pattern. *M.varians* has a genome size of 1,823 to 2,334 kb. *M.roseus* type strain NCIMB 11696 has a genome size of 3,397 kb.

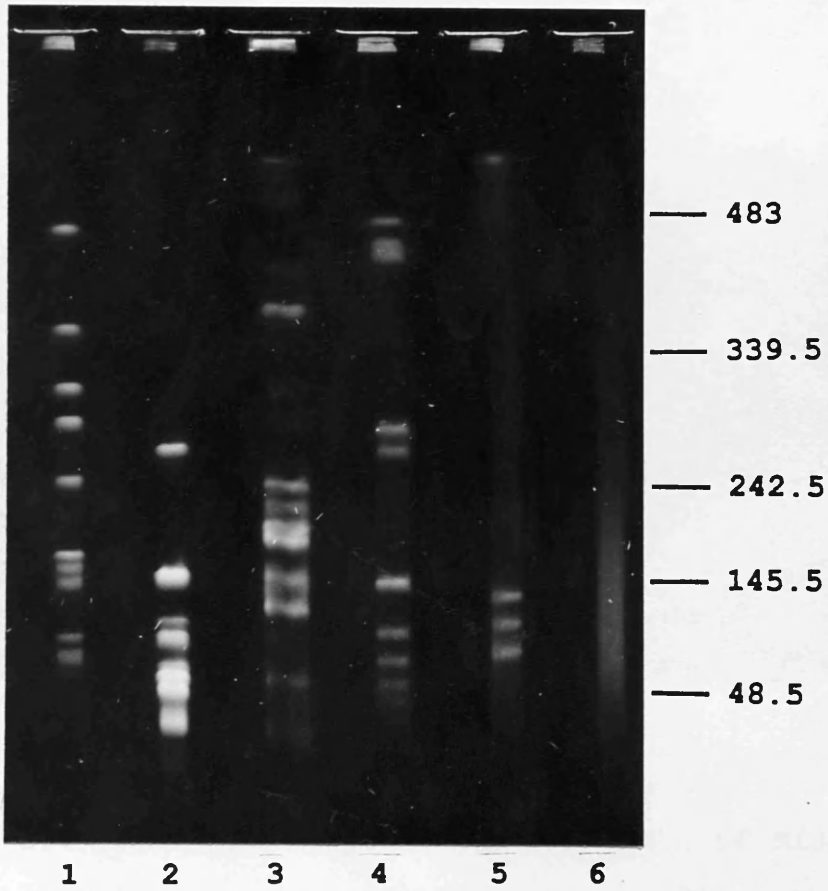

Figure 17. PFGE patterns of restriction enzyme digests of genomic DNA of *M.luteus* isolate 972. High molecular weight DNA in agarose blocks was digested with the following restriction enzymes: *Hind*III (Lane 1), *Eco*RI (Lane 2), *Eco*RV (Lane 3), *Hpa*I (Lane 4), *Xba*I (Lane 5) and *Dra*I (Lane 6). Electrophoresis was for 24 hours at 200V, with a pulse time of 30-50 seconds. λ phage concatamers were used as molecular weight markers (kb).

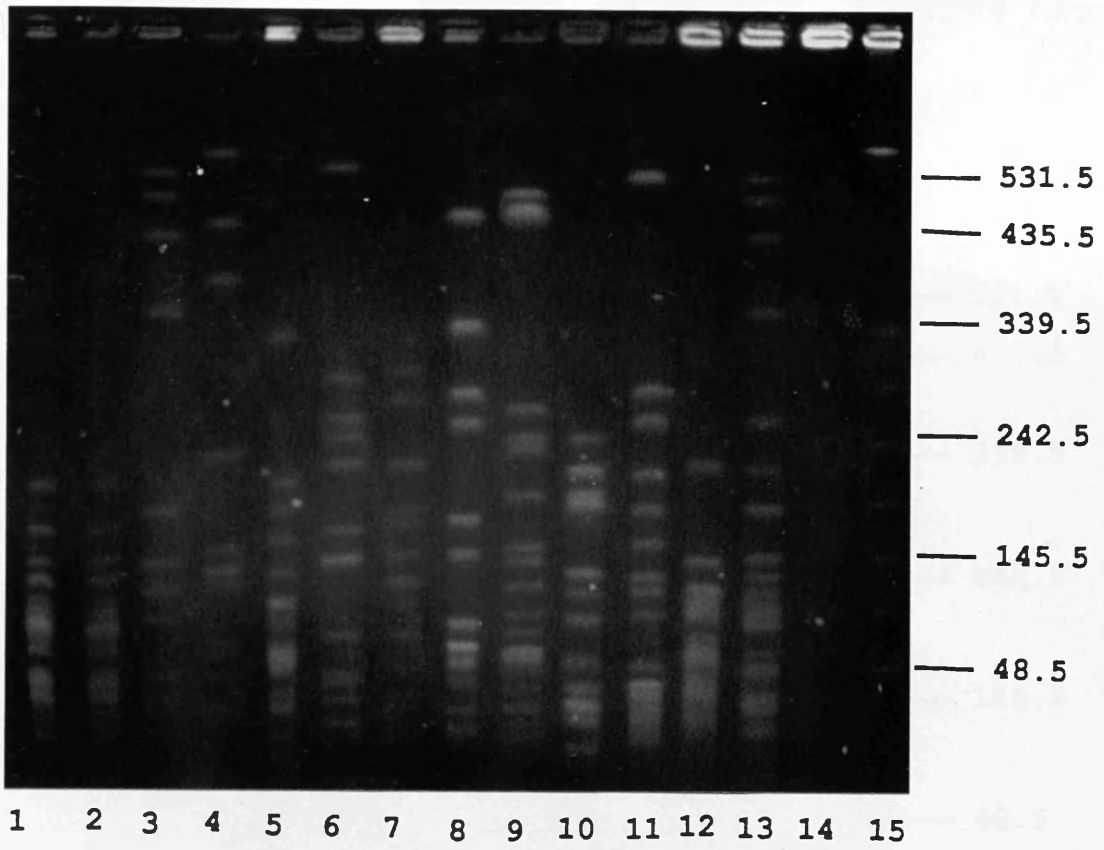

Figure 18. Pulsed field gel electrophoresis of *Hind*III digested whole-cell DNA extracted from *M.lylae*, *M.luteus*, *M.roseus*, *M.varians*, *M.kristinae* and *M.sedentarius*. Lane: 1 *M.lylae* NCIMB 11037; Lane 2 *M.lylae* ATCC 27569; Lane 3 *M.lylae* ATCC 27567; Lane 4 *M.lylae* ATCC 27568; Lane 5 *M.luteus* NCIMB 8553; Lane 6 *M.luteus* 463; Lane 7 *M.luteus* 1373; Lane 8 *M.luteus* 271; Lane 9 *M.roseus* NCIMB 11696; Lane 10 *M.varians* 282; Lane 11 *M.varians* 4910; Lane 12 *M.varians* NCIMB 11697; Lane 13 *M.kristinae* NCTC 11038; Lane 14 *M.sedentarius* NCIMB 11040. Markers are λphage concatamers (kb).

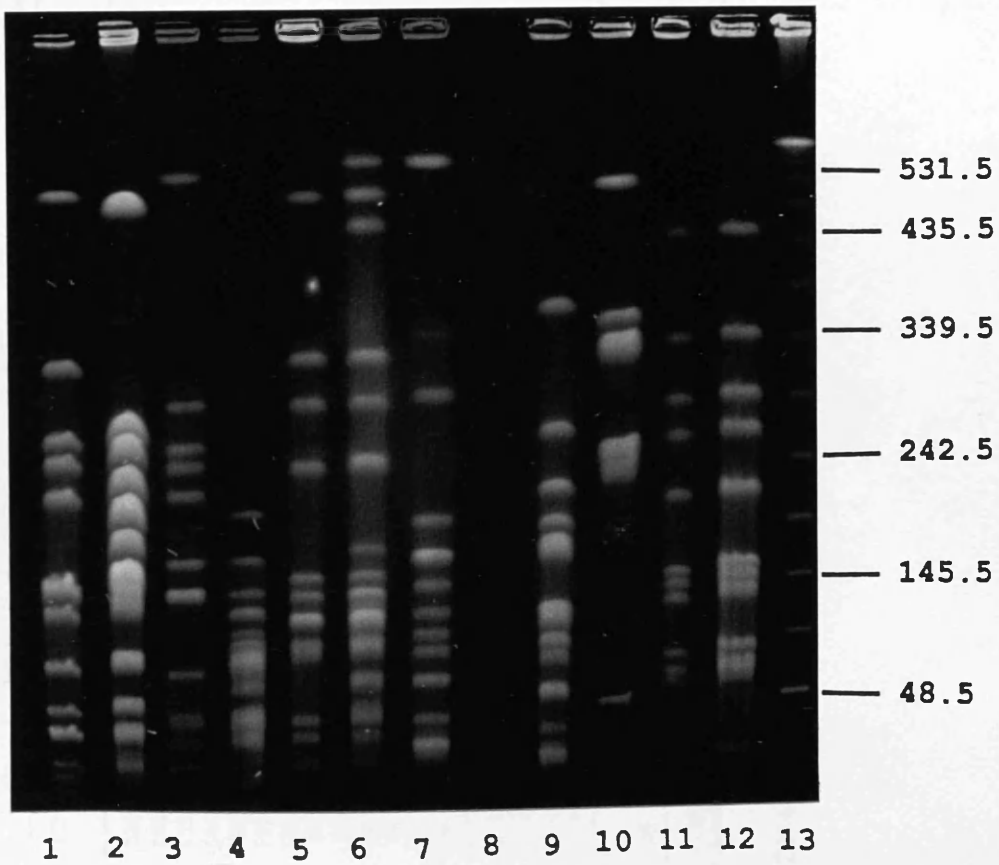

Figure 19. PFGE patterns of *Hind*III restriction enzyme digests of genomic DNA of *Bam*HI ribotype group 1 (Lanes 1-7) and 2 (Lanes 9-12). Isolates are: Lane 1, 572; Lane 2, 7918; Lane 3, 463; Lane 4, NCTC 11037; Lane 5, 783; Lane 6, 882; Lane 7, 883; Lane 9, 5915; Lane 10, 672; Lane 11, 372; Lane 12, 972. All isolates are *M.luteus* except for Lane 4 which is *M.lylae*. Markers are λ phage concatamers (kb).

Isolate	*M.varians*		*M.roseus*	*M.lylae*			*M.luteus*						
	282	4910	NCIMB 11696	NCTC 11037	ATCC 27567	ATCC 27568	463	783	972	372	582	1364	1161
Fragment size (kb)	275 238 218 205 150 132 110 103 77 73 57 53 42 40 33 17	537 305 272 225 195 165 145 132 110 69 53 42 35 28 21	500 457 453 295 253 210 170 156 141 109 93 85 72 62 43 21 14	200 180 155 141 (x2) 125 112 110 98 (x2) 95 88 79 77 64 60, 53 50, 45 42, 33 27, 26 14, 11	560 500 430 375 200 160 141 110 45 32	620 445 375 250 170 155 153 144 93 83 64 32	575 285 313 253 185 185 155 93 62 51 35 23	600 372 321 268 208 153 135 105 60 39 21	460 350 310 285 235 177 163 147 102 89 81 26 13	480 380 298 275 223 180 163 147 105 90 83 26 12	395 355 275 245 223 215 185 135 87 76 70 45 35 30 14	317 285 235 211 200 147 140 130 102 90 81 65 52 38 31 25 23 12	555 328 290 280 211 196 172 144 127 98 71 45 35 26 20
Number of fragments	16	15	18	25	10	12	12	11	13	13	15	18	15
Genome size (kb)	1,823	2,334	3,397	2,138	2,553	2,584	2,251	2,282	2,438	2,362	2,385	2,184	2,598

Table 9. The size of single *Hind*III DNA fragments and the derived total genome size of *Micrococcus* species.

3.10) Characterisation of micrococci and stomatococci isolated from clinical material.

Thirteen micrococcal isolates which had been collected from clinical material were received from Dr J Magee, Department of Bacteriology, Royal Hallamshire Hospital, Sheffield.

Six strains were isolated from blood cultures, all from patients with line infections, 5 strains were isolated from patients using CAPD, 1 strain was isolated from cerebro-spinal fluid (CSF) and 1 strain was isolated from the CSF of a patient with a ventriculo-peritoneal shunt.

Table 10 shows the antibiotic sensitivities of the 13 clinical isolates. Isolates B705 and A208 are resistant to both methicillin and penicillin and are classed as *M.sedentarius*. All isolates were sensitive to cefuroxime, ampicillin, amoxycillin, gentamicin, cefotaxime and erythromycin.

When digested with *Eco*RI no isolates had identical REA profiles. Isolates A3587 and T886 which were received as *Stomatococcus mucilaginosus* have a DNA profile consistent with a % mol GC content lower than micrococci.

Table 11 shows the source of the isolate, the colony pigmentation, the API profile number, the species designation, and the *Bam*HI and *Sal*I ribotype groups.

Of the 13 isolates collected from clinical material 6 were identified as *M.luteus*, 2 as *M.sedentarius*, 2 as *M.lylae*, 1 as *M.kristinae* and 2 as stomatococci.

These 13 clinical isolates belonged to 7 *Bam*HI ribotype groups. Isolate A366 isolated from venous blood and isolate C425 isolated from a patient using CAPD belonged to *Bam*HI ribotype group 1. Isolate C87 isolated from a patient using CAPD belonged to *Bam*HI ribotype group 2. Isolates A211 and A506 isolated from venous blood, along with isolates C425, C1779 and C2634 isolated from patients using CAPD belonged to *Bam*HI ribotype group 4. Isolates A545 isolated from venous blood culture and identified as *M.kristinae* by the API microtitre strip, belonged to *Bam*HI group 7 and *Sal*I ribotype group 9, which identifies this isolate as *M.kristinae*. Isolates B705 and A208 produced unique ribotype patterns with both *Sal*I and *Bam*HI. The 2 isolates received as stomatococci, isolate T886 isolated from a ventriculo-peritoneal drain, and isolate A3587 isolated from venous blood, both having unacceptable API profiles, had ribotypes similar to that of *Stomatococcus mucilaginosus* NCTC 11036 as discussed in Section 3.7, p131.

Using *Sal*I, the 13 isolates were placed into 5 ribotype groups. The isolates identified as stomatococci were not digested by *Sal*I. Isolates A208, B705 and A545 have been discussed above. Isolate C1779 belongs to *Sal*I ribotype

group 1. Isolates C156, C425 and C2634 belong to *Sal*I ribotype group 2. Isolates A211, A366, A506 and C87 belong to *Sal*I ribotype group 4.

Figure 20 shows the ribotype profiles of the 11 micrococci isolated from clinical material, after digestion with the restriction enzyme *Bam*HI.

3.11) The skin carriage and prevalence of the *Micrococcus* ribotypes.

Micrococci belonging to *Sal*I ribotype group 1 were present in all individuals on the base. Micrococci belonging to *Bam*HI ribotype group 1 were present in 8 out of 10 base members. These 2 single ribotypes were the most prevalent in the sample population (Table 12).

When the combination of the *Sal*I ribotype group and the *Bam*HI ribotype group is taken as a fingerprint of any one micrococcus, then persons 5, 6, 8, 9 and 13 carry micrococci not seen in the rest of the sample population. The micrococci with unique fingerprints in persons 6 and 9 represent single isolates. In person 5, the 6 micrococci with the fingerprint *Bam*HI ribotype group 3: *Sal*I ribotype group 3 (B3:S3) and 2 micrococci with *Bam*HI ribotype group 2: *Sal*I ribotype group 7 (B2:S7) are unique to that person. The 6 micrococci (B3:S3)

correspond to the 6 isolates in *Eco*RI REA group 2. In person 13, 5 micrococci with the fingerprint *Bam*HI ribotype group 4: *Sal*I ribotype group 5 (B4:S5) are unique to that person. Four of these micrococci form *Hind*III REA group 9.

Isolates not common to the sample population are also found on 2 or 3 people. Persons 4 and 11 carried micrococci with the fingerprint *Bam*HI ribotype group 4: *Sal*I ribotype group 1 (B4:S1). Three micrococci, 2 from person 2 and 1 from person 11, were isolated in months 1, 2 and 4 of the study period. Persons 4, 8 and 13 carried micrococci with the fingerprint, *Bam*HI ribotype group 1: *Sal*I ribotype group 3 (B1:S3), 5 micrococci being isolated over 2 months corresponding to *Eco*RI REA group 1. Persons 4 and 13 shared sleeping quarters. Persons 3 and 8 also shared sleeping quarters and 4 micrococci collected over 2 months shared the common fingerprint *Bam*HI ribotype group 1: *Sal*I ribotype group 6 (B1:S6). Over 3 months persons 6 and 13 carried micrococci with the fingerprint *Bam*HI ribotype group 3: *Sal*I ribotype group 1 (B3:S1). *M.sedentarius* was isolated from persons 3, 4, 7 and 13 over the study period. *M.varians* was isolated from persons 2 and 4 over 2 months. *M.kristinae*, *M.nishinomiyaensis* and *M.roseus* were not isolated from any of the Antarctic samples.

Isolate	Taxon	MET 5ug	P 1IU	FD 10ug	E 5ug	CIP 1ug	W 2.5ug	CTX 30ug	AML 30ug	PRL 75ug	CN 10ug	AMP 10ug	CXM 30ug
B705	M.sedentarius	R	R	S	S	S	S	S	S	S	S	S	S
A208	M.sedentarius	R	R	R	S	R	R	S	S	S	S	S	S
A545	M.kristinae	S	S	S	S	R	R	S	S	S	S	S	S
A211	M.lylae	S	S	S	S	R	S	S	S	S	S	S	S
A366	M.luteus	S	S	S	S	R	R	S	S	S	S	S	S
A506	M.lylae	S	S	S	S	S	S	S	S	S	S	S	S
C87	M.luteus	R	S	S	S	R	S	S	S	S	S	S	S
C156	M.luteus	S	S	S	S	S	S	S	S	S	S	S	S
C425	M.luteus	S	S	S	S	S	S	S	S	S	S	S	S
C1779	M.luteus	S	S	S	S	R	S	S	S	S	S	S	S
C2634	M.luteus	S	S	S	S	S	S	S	S	S	S	S	S
T886	Stomatococci	S	S	S	S	R	R	S	S	S	S	S	S
A3587	Stomatococci	S	S	S	S	R	S	S	S	S	S	S	S

Table 10. The antibiotic sensitivities of 13 micrococci isolated from clinical material.

Abbreviations are: Met, methicillin; P, penicillin; FD, fusidic acid; E, erythromycin; CIP, ciprofloxacin; CTX, cefotaxime; AML, amoxycillin; PRL, piperacillin; CN, gentamicin; AMP, ampicillin; CXM, cefuroxime.

Isolate	Source	API Number	Colony Pigment	Taxon	BamHI	SalI
B705	CSF	000000000	CREAM	*M.sedentarius*	UNI	UNI
A208	BC	000000000	CREAM	*M.sedentarius*	UNI	UNI
A545	BC	063126210	CREAM	*M.kristinae*	9	9
A211	BC	000001000	CREAM	*M.luteus*	4	4
A366	BC	100007000	CREAM	*M.luteus*	1	4
A506	BC	000003000	CREAM	*M.luteus*	4	4
C87	CAPD	000005000	YELLOW	*M.luteus*	2	4
C156	CAPD	100005000	YELLOW	*M.luteus*	1	2
C425	CAPD	000005000	YELLOW	*M.luteus*	4	2
C1779	CAPD	100007000	YELLOW	*M.luteus*	4	1
C2634	CAPD	000007000	YELLOW	*M.luteus*	4	2
T886	V-P	077774212	WHITE	*Stomatococci*		
A3587	BC	067715212	WHITE	*Stomatococci*		

Table 11. The characteristics of 13 micrococci collected from clinical material, showing source of isolation, colony pigment, API profile, *Sal*I and *Bam*HI ribotype groups and the species designation.

Abbreviations: CSF, cerebral spinal fluid; BC, blood culture; CAPD, continuous ambulatory peritoneal dialysis; V-P, ventriculo-peritoneal shunt.

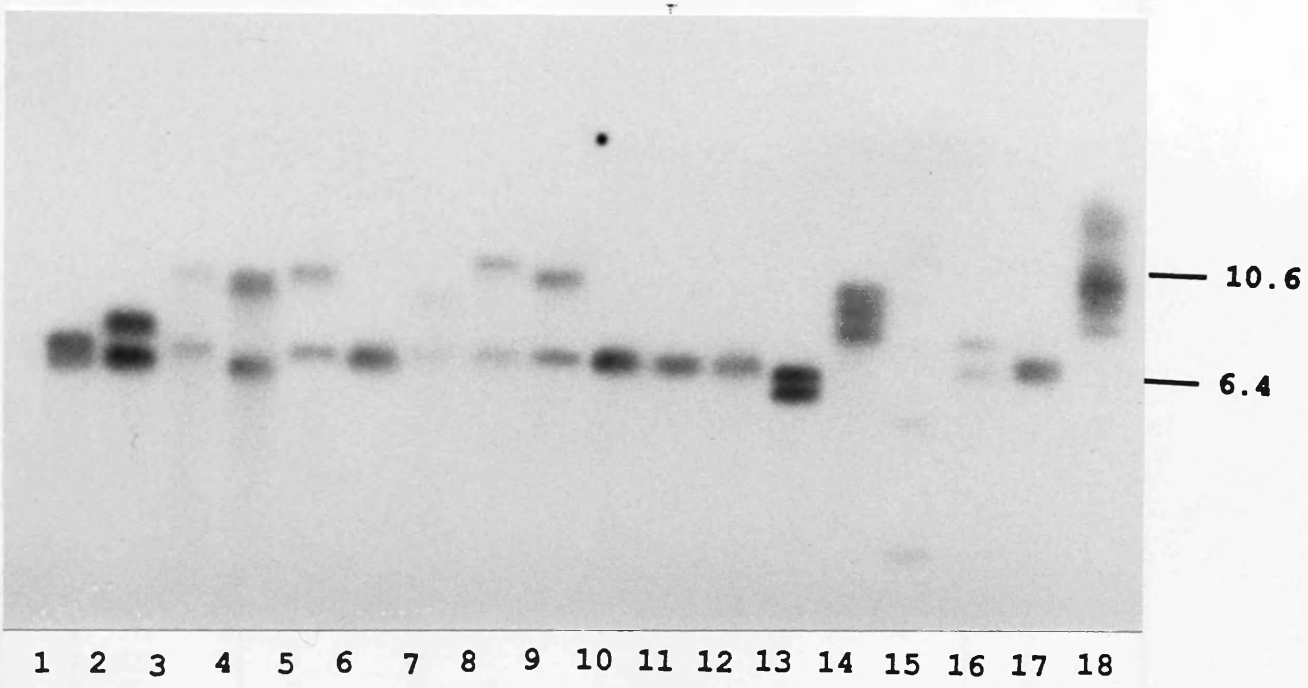

Figure 20. The *Sal*I ribotype profiles of 13 micrococci isolated from clinical material. Isolates are: Lane 1, isolate A208; Lane 2, *M.luteus* NCIMB 9278; Lane 3, *M.luteus* NCIMB 8166; Lane 4, *M.luteus* NCIMB 8553; Lane 5, isolate C2634; Lane 6, isolate C87; Lane 7, isolate C1779; Lane 8, isolate C156; Lane 9, isolate C425; Lane 10, isolate A506; Lane 11, isolate A366; Lane 12, isolate A211; Lane 13, isolate B705; Lane 14, isolate B5454; Lane 15, *M.lylae* NCTC 11037; Lane 16, *M.lylae* ATCC 27568; Lane 17, *M.lylae* ATCC 27567; Lane 18, *M.kristinae* NCTC 11038. Abbreviations as before, see Methods Section 2.1, p53. Fragment sizes are in kilobases.

ISOLATE NUMBER	PERSON	MONTH	SPECIES	*Bam*HI	*Sal*I
261	2	6	*M.luteus*	2	1
263	2	6	*M.luteus*	2	1
271	2	7	*M.luteus*	4	1
282	2	8	*M.varians*	6	8
281	2	8	*M.luteus*	2	1
2914	2	9	*M.luteus*	4	1
2915	2	9	*M.luteus*	2	1
2916	2	9	*M.luteus*	4	1
372	3	7	*M.luteus*	2	1
383	3	8	*M.sedentarius*	5	4
3910	3	9	*M.sedentarius*	5	4
3911	3	9	*M.luteus*	4	10
3912	3	9		1	6
461	4	6	*M.luteus*	1	1
463	4	6	*M.luteus*	1	3
471	4	7	*M.luteus*	1	3
472	4	7	*M.luteus*	1	3
481	4	8	*M.varians*	6	8
483	4	8	*M.sedentarius*	5	4
498	4	9	*M.sedentarius*	5	4
4910	4	9	*M.varians*	6	8
561	5	6	*M.luteus*	3	4
562	5	6	*M.luteus*	3	4
563	5	6	*M.luteus*	3	4
571	5	7	*M.luteus*	3	4
572	5	7	*M.luteus*	1	1
581	5	8	*M.luteus*	1	1
582	5	8	*M.luteus*	3	4
583	5	8	*M.luteus*	3	4
5915	5	9		2	7
5917	5	9		2	7
5916	5	9	*M.luteus*	1	1
661	6	6	*M.luteus*	3	1
662	6	6	*M.luteus*	3	1
671	6	7	*M.luteus*	1	2
672	6	7	*M.luteus*	2	1
673	6	7	*M.luteus*	3	1
682	6	8		UNI	UNI
681	6	8	*M.luteus*	3	1
6919	6	9	*M.luteus*	1	1
6920	6	9	*M.luteus*	1	2

Table 12. The person, month of isolation and *Bam*HI and *Sal*I ribotype group of 88 micrococci collected in the Antarctic from 10 subjects.

ISOLATE NUMBER	PERSON	MONTH	SPECIES	*Bam*HI	*Sal*I
761	7	6	*M.luteus*	2	1
762	7	6	*M.luteus*	1	1
763	7	6	*M.luteus*	1	1
764	7	6	*M.luteus*	1	1
765	7	6	*M.luteus*	2	1
771	7	7	*M.luteus*	1	1
772	7	7	*M.luteus*	1	1
773	7	7	*M.luteus*	1	1
781	7	8	*M.sedentarius*	5	10
782	7	8	*M.luteus*	1	2
783	7	8	*M.luteus*	1	2
7918	7	9	*M.luteus*	1	1
7919	7	9	*M.luteus*	1	1
7920	7	9	*M.luteus*	1	1
861	8	6	*M.luteus*	1	3
862	8	6		UNI	7
871	8	7	*M.luteus*	2	1
881	8	8		1	6
882	8	8		1	UNI
883	8	8		1	6
8919	8	9	*M.luteus*	4	UNI
8920	8	9		1	6
961	9	6		1	UNI
971	9	7	*M.luteus*	2	1
972	9	7	*M.luteus*	2	1
1161	11	6	*M.luteus*	4	1
1361	13	6	*M.luteus*	1	3
1362	13	6	*M.luteus*	1	3
1363	13	6	*M.luteus*	1	1
1364	13	6	*M.luteus*	4	5
1365	13	6	*M.luteus*	1	3
1371	13	7	*M.luteus*	4	5
1373	13	7	*M.luteus*	4	5
1381	13	8	*M.luteus*	3	1
1382	13	8	*M.sedentarius*	5Â	4
1383	13	8	*M.sedentarius*	5	4
1384	13	8	*M.sedentarius*	5	4
1385	13	8	*M.luteus*	4	5
1386	13	8	*M.sedentarius*	5	4
13916	13	9	*M.sedentarius*	5	4
13917	13	9	*M.luteus*	2	1
13918	13	9	*M.luteus*	4	5

Table 12. Continued.

Assuming that isolates showing the same "double" ribotype are clonally related, then where a sufficient number of isolates from a person was collected it is possible within the confines of the sampling method used, to state the skin carriage of a particular micrococcal clone. Only *M.luteus* carriage is considered due to insufficient numbers of the other micrococcal species being collected.

Person 2 carried 2 resident *M.luteus* clones, B2:S1 and B4:S1, over the 4 months of the study; person 5 carried 2 resident *M.luteus* clones, B1:S1 and B3:S4, for 3 out of the four months studied. Person 6 carried 2 resident *M.luteus* clones, B3:S1, isolated over 3 months and B1:S2, isolated in the second and last month of the study. Person 7 carried 1 resident *M.luteus* clone, B1:S1, which was isolated over the 4 study months. Person 7 also had the micrococcal clones B1:S2 and B2:S1 isolated in one month only. Person 13 carried 1 resident *M.luteus* clone, B5:S5 throughout the 4 months of the study.

Several general trends are noticeable from Table 12. Firstly there are several occasions where a single micrococcal clone is isolated from a person in any month ie person 4 clone B1:S1, person 6 clone B2:S1, which signifies these isolates could be transients. The clone B1:S1 was isolated from several people during different months as a single isolate. However it was resident only on person 7. The paucity of *M.luteus* isolates from

persons 3, 8, 9 and 11 makes it impossible to state *M.luteus* carriage in these people. Person 8 does however carry the micrococcal clone B1:S6 for 2 months of the study. *M.sedentarius* was also isolated from 3 people on 2 consecutive months and this may represent residence of this species.

4.

DISCUSSION

4.1) Skin sampling and isolate survival.

After storage and transport to the United Kingdom, the overall survival rate of bacterial isolates was 92%. This is double the survival rate recorded in previous studies involving the storage and transportation of staphylococci from the Antarctic [194,195].

That micrococci are normally saprophytic and have simple nutritional requirements may account for this high survival rate. Micrococci are able to undergo storage at 4^0C for 9 months with almost no loss of viability. By the time the collected isolates had returned to the United Kingdom and been recultured, only 2 months of the 8 month sampling period used in this study fell outside this 9 month viability window. There was no correlation between the length of isolate storage, or from whom the isolate was collected, and isolate mortality.

The isolates were collected by the moist swab technique described by Kloos and Schliefer, which is capable of collecting between 100 and 700 colonies per plate [7]. Skin culture swab techniques sample the surface and not deeper skin sites, but superficial organisms are continuously being shed from the deeper sites and, thus, are usually representative of the population of the deeper flora. The hair root is sterile, and bacteria are not seen in the depths of the hair follicles nor in the sebaceous glands or

ducts on Gram stained serial section biopsies [196]. Aggregates of Gram-positive bacteria are however found in the infundibulum, the uppermost portion where the hair follicle opens onto the surface. The epithelium lining this funnel shaped opening keratinises and exfoliates, passively carrying bacteria to the surface [196].

The moist swab technique is a very simple and convenient method for collecting skin bacteria. It is more efficient than tape stripping and direct contact plate methods [197] and although not as accurate as the glass cup method, can nevertheless provide a reasonably good estimate of skin bacterial density devoid of gross errors [198]. When swabbing techniques were compared to skin biopsy, both produced the same qualitative distribution of organims although skin biopsy recovered approximately six times more organisms [197].

It is important to emphasise that the sampling technique used in this study selected three colonies from any one plate to subculture onto bijou slopes and to store subsequently. Assuming that the number of aerobic colony forming units collected from the Antarctic sample population is similar to the numbers previously published [7,38] and that micrococci form 20% of the aerobic colony forming units [38], then it can be shown using binomial statistics that if colonies are chosen at random, for all the 3 chosen isolates to be unique from an assumed micrococcal population of 20, the chosen unique isolate must number between 2 and 11

colonies of the original population of 20 colonies. If 2 chosen isolates are identical, then these chosen isolates must number between 9 and 17 colonies from the micrococcal population of 20 colonies. This assumes random selection of isolates. In practice this never occurred. If a culture plate had three morphologically different micrococci on it, then one representative from each morphologically different group was collected and stored on bijou slopes. Therefore cases where an identical isolate has been collected twice from the same person, in the same month, from the same site must represent a micrococcal colony consisting mostly of clonally derived bacteria.

From the initial sample of 2,438 isolates brought back from the Antarctic, only 88 were finally studied in detail. The cost and complexity of the techniques used demanded that the number of organisms to be studied in detail be reduced. From the 8 body sites sampled monthly the shin was chosen as the study site because it gave a consistently high yield of presumptive micrococci. Although not quantified in this study, another study has shown the lower leg to be heavily colonised by micrococci [38].

At each sampling session blood taken from each volunteer was spun down and the serum stored at $-20^{0}C$. This was intended to be used in Western blotting experiments to assess the technique's usefulness in characterising the genus

Micrococcus. This experiment was not performed due to lack of time.

4.2) Assignation of isolates to the genus *Micrococcus*.

Of the 201 Gram-positive catalase-positive cocci, 95.5% were identified as belonging to the genus *Micrococcus*. This high percentage of micrococci in the original sample population is due to the selective collection of isolates with the colony morphology and Gram-staining characteristics of the genus *Micrococcus*.

Of these 191 isolates only 88 were identified to the species level. To these 88 isolates were added 13 isolates which were isolated from clinical material, 12 *Micrococcus* type strains and 1 *Stomatococcus* type strain.

4.3) Identification of isolates to the species level using the API microtitre strip.

The introduction of commercial microtitre methods for the biochemical characterisation of bacteria provides a rapid and convenient method of identifying bacteria. These microtitre methods have been used to separate the staphylococci from the micrococci and to speciate the separated staphylococci. There have been no previous

attempts to use a microtitre method to speciate micrococci. Prior to the release of the ATB 32 STAPH, no commercial microtitre strip was able to differentiate the species within the genus *Micrococcus*, however a comparison can be made with studies using a microtitre system to speciate staphylococci.

The API Staph-Ident system, designed to speciate staphylococci using 10 biochemical tests, is quick, convenient and gives a 90% congruence with the conventional tests [199] and DNA:DNA hybridisation [200]. Studies identifying both coagulase-positive and coagulase-negative staphylococci have shown an 80-96% agreement rate between the API Staph-Ident system, and conventional methods of staphylococcal identification [201,202,203,200,205]. However, in these and subsequent studies there was only a 44.9-90.4% correct identification of coagulase-negative staphylococci of human origin obtained [206]. Furthermore one study showed that only 14.4% of 111 coagulase-negative staphylococci isolates belonging to species other than *S.epidermidis* were correctly identified [203]. Therefore for some bacterial species microtitre methods of identification may cause mis-identifications and non-identifications.

The API ATB 32 STAPH microtitre system using 20 test substrates claims to identify 24 *Staphylococcus* species, 6 *Micrococcus* species, *Stomatococcus mucilaginosus* and *Aerococcus viridans*. When the API ATB 32 microtitre strip

was used to identify staphylococci, an identification to the correct species was made in 91.8% of isolates, with a misidentification rate of 1.7% [207]. In the present study the ATB 32 STAPH strip positively identified 33% of all micrococcal isolates (including type strains) when biochemical criteria alone were used. When colony pigmentation was used as a further criterion as recommended by the API database, 56% of all isolates were positively identified. The API microtitre strip does not identify *M.sedentarius* and this species was identified by antibiotic resistance profile [7,81].

Of the 56% (64/114) of all isolates identified by the API microtitre strip, 14% (9/64) were misidentified, either as staphylococci, *Stomatococcus mucilaginosus*, *M.nishinomiyaensis* or *M.lylae*, when the % mol GC content as determined by REA pattern and species as determined by ribotype grouping were considered. When compared against ribotype group and the % mol GC content the combination of the API microtitre strip, colony pigmentation and antibiotic resistance correctly identified 59% (67/114) of all isolates belonging to the genus *Micrococcus* or genus *Stomatococcus*.

M.luteus NCIMB 9278 and *M.roseus* NCIMB 11696 were the only *Micrococcus* type strains correctly identified by the API microtitre strip. The misidentifications and non-identifications of the 13 micrococcal type strains and 1 stomatococcus type strain by the API ATB 32 STAPH microtitre

system is in contrast to the 100% correct identification rate when staphylococcal type strains were tested [207].

The biochemical inertness of the genus *Micrococcus* was the single most important determinant for a lack of species identification by the API STAPH 32 microtitre strip. This is a major limitation for a testing system that relies on phenotypic biochemical criteria to identify species.

Apart from the 2 biochemically reactive species, *M.kristinae* and *M.varians*, the majority of species identifications using the API microtitre strip are made on only 3 to 5 positive reactions, therefore a limited number of positive characters identify a species. This limited number of positive characters can change a species identification if one test is negative. The difference between the species *M.luteus* and *M.lylae* by the API profile number is a positive urease test generating the profile 100005000 which, irrespective of colony pigmentation, is identified as *M.luteus*. If the urease test is negative, then the profile 000005000 is generated identifying the isolate as either *M.luteus* or *M.lylae*. In this case the colony pigmentation is used as a further characteristic to speciate the isolate. If the isolate produces yellow pigmented colonies then it is identified as *M.luteus*, however if the isolate produces cream-pigmented colonies it is identified as *M.lylae*. To identify isolates the colony pigment produced by that isolate had to be considered in 25 cases. The present study

and others [7] shows that colony pigmentation is not a reliable characteristic with which to speciate micrococci. This study shows that urease positive cream-pigmented micrococci are classed as *M.luteus* and that urease-negative micrococci which would be classed as *M.lylae*, belong to the same ribotype groups as isolates identified as *M.luteus*. Colony pigment in the genus *Micrococcus* therefore does not correlate with the genotypic grouping as determined by rRNA probing.

The objective manual reading of the strips is also a problem in deriving a satisfactory species identification as the test readings are dependent on subtle colour changes between straw, yellow, yellow-orange, orange, red-orange and red.

Although Bio Merieux claim that the ATB 32 STAPH microtitre system can identify and distinguish 6 micrococcal species (*M.sedentarius* is not included) the results of this study do not support this claim. Due to the biochemical inertness of the genus *Micrococcus* and the paucity of positive biochemical reactions, the API STAPH 32 microtitre strip cannot be recommended for the speciation and differentiation of the genus *Micrococcus*.

4.4) Differentiation of the Micrococcaceae by restriction enzymes.

There have been numerous efforts to find a quick, easy and reliable method of differentiating staphylococci from micrococci. Methods which have been evaluated to separate these two genera include menaquinone type [24,25], novobiocin resistance [208], bacitracin resistance [209], serological tests [106], phage typing [210], anaerobic production of acid from glucose [98], a modified oxidase and benzidine test [125], microtitre API testing [201], lysostaphin sensitivity either alone [111], or in combination with lysozyme susceptibility and a glycerol/erythromycin media [108], and furazolidone sensitivity [211].

Currently the most reliable means of differentiating between staphylococci and micrococci is the determination of the cell wall peptidoglycans or the DNA composition of the organisms. The second of these criteria for the separation of the two genera, namely ascertaining the % mol GC content requires specialised equipment and/or labour intensive techniques [212,213]. Since the DNA base composition of micrococci is 65-75% and differs greatly from that of staphylococci at 30-40%, even an approximation of this value is sufficient to distinguish between the 2 genera [214]. The marked difference in base composition between these two genera allows for the selective use of restriction enzymes with their specific base recognition sequences. This large

difference in % mol GC content means that a chosen restriction enzyme will restrict the genome over a greater range of fragment sizes. Those restriction enzymes with GC-rich recognition sequences, eg *Bam*HI (GGATCC), would be expected to cut an isolate with a GC-rich genome frequently, and an isolate with a GC-poor, ie AT-rich, genome less so, whereas a restriction enzyme with an AT-rich recognition sequence, eg *Eco*RI (GAATTC), will cut a GC-rich genome infrequently and an AT-rich genome frequently. The use of restriction enzymes to digest extracted total chromosomal DNA allows for a quick visual method of determining whether a Gram-positive catalase-positive cocci belongs to the genus *Micrococcus* or the genus *Staphylococcus*.

Of the 6 lysostaphin resistant isolates biochemically identified as staphylococci, only one, isolate 6921, was a true staphylococcus with a low % mol GC. This represents 1% of all the isolates collected. One type strain, *M.luteus* NCIMB 8553, and 4 isolates identified as belonging to the genus *Stomatococcus* comprised the other misidentifications.

This low percentage of lysostaphin-resistant coagulase-negative staphylococci in the sample population is due to the active collection of colonies with a colony morphology consistent with the genus *Micrococcus*. However, the lowered sensitivity of coagulase-negative staphylococci to the lytic effects of lysostaphin, because of the lower molar ratio of glycine in their cell wall, would allow coagulase-negative

staphylococci to be misidentified as micrococci [108]. This is a limitation of using lysostaphin susceptibility as a means of differentiating the staphylococci from the micrococci.

The near equal number of fragments produced by both *Bam*HI and *Eco*RI with 4 of the Gram-positive catalase-positive cocci isolates suggested that these isolates have a % mol GC content that is intermediate between that of staphylococci and micrococci. This % mol GC would place them in the genus *Stomatococcus*. As the % mol GC content of an organism approaches 50%, with the resultant equality of all four nucleotides in the genome, the frequency of restriction with most restriction enzymes approaches parity, tempered only by the frequencies of di- and trinucleotides in that species[191].

The identification in this study of stomatococci which were lysostaphin-resistant as staphylococci by the API microtitre strip, means that studies relying on the API system to identify an isolate to the genus *Staphylococcus* and the subsequent finding of a large proportion of lysostaphin-resistant staphylococci within that study must be viewed with caution [215].

The use of 2 restriction enzymes therefore allows the Gram-positive catalase-positive cocci to be visually differentiated into the genus *Staphylococcus*, the genus *Micrococcus* and the genus *Stomatococcus* after digestion of

total chromosomal DNA and the electrophoretic separation of the resultant DNA fragments.

This technique requires that 2 restriction enzymes be used to separate the Micrococcaceae. One restriction enzyme will only be able to differentiate one genus, ie the genus *Staphylococcus* from the genus *Micrococcus* and the genus *Stomatococcus;* and conversely the genus *Micrococcus* from the genus *Staphylococcus* and the genus *Stomatococcus*.

The use of restriction enzymes to differentiate between staphylococci and micrococci will only be applicable if the genome has not undergone significant methylation. Although *Hind*III restricted at a lower frequency than predicted, there were no observations of greater frequencies of restriction from that predicted in the use of restriction enzymes. Restriction frequency depends primarily on the sequence composition of the DNA and of the restriction enzyme recognition sequence, and secondarily, in a few cases, on the presence of protective methylation of an enzyme recognition sequence in a particular isolate [191]. Therefore for a given % mol GC content, micrococcal or staphylococcal, a restriction enzyme will be able to differentiate one genus from another by the number and size of the DNA fragments produced after digestion, primarily as a result of the sequence composition of the DNA.

This method of differentiating between staphylococci and micrococci was used to show that the type strain *M.sedentarius* NCIMB 555 did not belong to the genus *Micrococcus*. The restriction enzyme profile suggested it had a low % mol GC, although it was non-reactive biochemically, formed diplococcal pairs and tetrads, and was resistant to lysostaphin and lysozyme. NCIMB were contacted and carried out further tests; this type strain was eventually removed from the collection.

4.5) Analysis of restriction enzyme profiles.

For REA the ideal DNA profile is achieved with 10-20 DNA fragments in the 1-25 kb size range, which gives both a discriminatory, and an easily interpreted DNA profile. This number of DNA fragments can be achieved by using an enzyme that cuts relatively rarely producing a few small DNA fragments (1-12kb). Alternatively a frequently cutting enzyme may be used to produce many large DNA fragments (12-25 kb) that will separate at the top of the agarose gel. Large fragments would be desirable when the interpretation of the DNA profile may be complicated by the presence of plasmid bands.

*Hind*III and *Eco*RI were the enzymes of choice for REA. These two enzymes were selected because they gave a DNA profile that produced between 4 and 22 DNA fragments. This enabled

easy analysis and comparison of the DNA profiles from the large number of isolates studied, without the need for rigorous side-by-side comparisons. These 2 restriction enzymes differentiated between different isolates, hence both were used.

Within the limitations of the sampling method used, as discussed earlier (Section 4.1, p159), it is possible to say that over the period of the study an individual can carry a particular clone for several months, and also that different individuals can carry the same clone for several months. The skin epidemiology is discussed more fully later in Section 4.10, p189.

The instances where the same clone is collected from an individual twice in the same month represents a situation where that particular clone forms between 45% to 85% of the micrococcal colonies theoretically available. This is suggestive of a clonally derived population where that clone will predominate. The episodes where a particular clone is collected from the same person in 2-3 different months, represents a situation where that isolate is resident [186].

Micrococci are known to contain plasmids [216]. With the low number of DNA fragments produced with the 2 chosen restriction enzymes the possibility exists that these DNA fragments represent DNA fragments produced from the digestion of plasmid DNA, as opposed to the chromosomal DNA.

To exclude this possibility 3 different plasmid extraction methods were tried on 5 different occasions and on different isolates throughout the study period [217,218,219]. No plasmid DNA was isolated by any of these methods. There could be 2 reasons for this, firstly there were no plasmids present in any of the micrococci tested, or secondly that the methods used for plasmid isolation were not capable of extracting any micrococcal extra-chromosomal DNA present. Another way of isolating plasmid DNA is to use PFGE where plasmid DNA, because of its structure, is capable of entering the agarose gel. When selected isolates were subjected to PFGE no plasmid DNA was seen. Isopycnic centrifugation of extracted DNA in caesium chloride would be an alternative method by which isolation of micrococcal plasmid DNA colud be attempted [190].

If these DNA fragments are plasmid bands the use of *Pst*I would be recommended to produce large DNA fragments useful for REA.

4.6) Analysis of 16S and 23S rRNA probing.

Micrococci are biochemically inert bacteria with few phenotypic criteria by which to characterise them. rRNA gene restriction patterns produced after hydridising DNA fragments with ^{32}P-labelled *E.coli* 16S and 23S rRNA have been proposed as a taxonomic tool [146,220]. Using this method,

species- and subspecies-specific core restriction fragment patterns are observed. Thus, organisms usually considered as difficult to identify because they are closely related, or lack sufficient phenotypic characteristics with which to type them, can be differentiated using this technique. The use of genotypic methods to characterise an organism removes the dependence placed on detecting phenotypes which can be variably expressed.

A high degree of homology exists among various bacteria in the DNA sequences encoding rRNA. This homology allows the use of *E.coli* 16S and 23S rRNA sequences to be used as a broad spectrum probe for a large number of bacteria [146,220]. Where organisms shared a single rDNA pattern there was insignificant genetic divergence as shown by DNA:DNA hybridisation studies. Different ribotype patterns corresponded to significant divergences in thermal stability studies of DNA:DNA hybrids [146,221]. Thus if a collection of isolates show a single rDNA pattern, or a variation of a core rDNA pattern, they are related [146,220]. This has been shown for staphylococci where the rDNA profiles are species-specific, with a particular species sharing an identical or similar restriction pattern [146,147,150].

Two restriction enzymes, *Bam*HI and *Sal*I were evaluated for their ability to digest micrococcal DNA and produce restriction patterns after hybridisation with ^{32}P-labelled *E.coli* 16S and 23S rRNA. With the exceptions of *M.lylae* type

strain ATCC 27569 and *M.roseus* type strain NCIMB 11696, complete digestion of micrococcal DNA was achieved in all cases with the restriction enzyme *Sal*I, whereas *Bam*HI produced complete DNA digestion for all micrococci except for DNA extracted from *M.sedentarius* which was only partially digested by this restriction enzyme, leaving some large DNA fragments incompletely separated following electrophoresis.

When micrococcal DNA was digested with *Bam*HI, a ribotype profile consisting of no more than 4 rDNA-containing fragments was produced, with the majority of isolates giving a profile containing 2 rDNA fragments. With *Sal*I all micrococci produced no more than 4 rDNA fragments with the majority giving a profile containing 2 rDNA fragments. Hence, it is estimated that micrococci contain a minimum of 2 copies of the 16S and 23S rRNA operon within the genome.

With the majority of isolates producing a limited number of rDNA fragments, a difficulty arises when trying to identify a common core pattern and variants of that pattern from an rDNA hydridisation pattern forming a different and separate core pattern. With isolates producing between 2 and 4 rDNA fragments the possibility of common rDNA fragments arising coincidentally is greater than if a larger number of rDNA fragments were produced. In this study the observation of common rDNA fragments forming a core pattern is taken as evidence of a genetic relationship [146,220]. In this study the

biochemical characterisation of an isolate and membership of a particular ribotype group were used to ascribe a species definition. In some cases this definition is overly conservative and leaves some isolates unspeciated.

A variety of rDNA restriction patterns were observed within the genus *Micrococcus* depending on the restriction enzyme used. Cleavage with *Bam*HI produced 7 main groups and 8 unique ribotypes, whereas cleavage with *Sal*I produced 11 main groups and 8 unique ribotypes. The specificity of ribotyping was therefore greater with *Sal*I. The different specificity between the two restriction enzymes used confirms the observations of other studies regarding the use of several restriction enzymes to produce an rDNA restriction pattern [150,220,222]. *Sal*I ribotype groups 3, 5, 6, 8 and 9 had only one *Bam*HI ribotype present within them, therefore these *Sal*I groups represent *Bam*HI subgroups. No other *Bam*HI or *Sal*I ribotype groups were mutually exclusive. Several *Bam*HI ribotypes belonged to one *Sal*I ribotype group, and vice versa. That different ribotype groupings are formed with different restriction enzymes is consistent with the findings on the use of two restriction enzymes on *S.aureus* [150].

When both the *Sal*I and *Bam*HI ribotypes are taken together to form a "double" fingerprint of an isolate, the epidemiological sensitivity of the technique is increased, producing 17 combination ribotypes and 12 unique ribotypes.

Within a ribotype group the phenotypic characteristics of colony pigment and biochemical reactivity are heterogeneous. Colony pigmentation was no predictor of ribotype group or genotypic species. Isolates classed as *M.lylae*, by the API microtitre strip, on the basis of colony pigmentation belong to the same *Bam*HI and *Sal*I ribotype groups as *M.luteus*. Colony pigmentation is therefore an invalid characteristic with which to speciate micrococci.

Isolates identified as *M.luteus* by the API microtitre strip using only biochemical characteristics were only found in *Bam*HI ribotype groups 1, 2, 3 and 4 and in *Sal*I ribotype groups 1, 2, 3, 4, 5 and 10. The *M.luteus Bam*HI ribotype groups, ie *Bam*HI groups 1, 2, 3 and 4, produce 2 rDNA fragments and share a common fragment of 6.9 kb. If a common restriction pattern shows insignificant divergence, then *M.luteus* represents 4 *Bam*HI *M.luteus* subpopulations. Within the limits of accuracy (see later Section 4.6, p181) the *Sal*I ribotype groups, 1, 2, 3, 4, 5, and 10 share common rDNA fragments. If the combination of a biochemical identification of *M.luteus*, and a ribotype containing common bands are used as criteria to define the species *M.luteus*, then all isolates belonging to *Bam*HI ribotype group 1, 2, 3 or 4 and *Sal*I ribotype group 1, 2, 3, 4, 5 or 10 can be identified as *M.luteus*. Although *Sal*I ribotype group 7 contains isolates identified as *M.luteus*, this ribotype group was not speciated as *M.luteus* since the identification

of species in this ribotype group required the further character of colony pigmentation. Furthermore, this ribotype shared no common rDNA fragments with *Sal*I ribotypes 1 to 5.

If the 2 restriction enzyme patterns are combined to form a unique "double" ribotype then *M.luteus*, as defined by biochemical characteristics, forms a heterogeneous population of organisms producing 15 different "double" ribotypes. Within these *M.luteus* groups there are organisms that produce a urease-negative API profile and cream-pigmented colonies, and are therefore identified as *M.lylae* when using the additional characteristic of colony pigmentation. This confirms the invalidity of using colony pigmentation as the critical differentiating characteristic in the speciation of the genus *Micrococcus*.

The characterisation of *M.lylae* is more problematic. This is due firstly to the inability of the API microtitre strip to make a positive identification of *M.lylae* without considering the colony pigmentation, and secondly to the fact that all the isolates identified as *M.lylae* cluster within *M.luteus* ribotype groups, or show unique ribotype patterns.

Examination of the ribotype profiles produced from isolates identified as *M.lylae* (by biochemical criteria and colony pigmentation) and the 4 *M.lylae* type strains show that they form a heterogeneous group of bacteria. Two type strains,

M.lylae NCIMB 11037 (the type strain for the species) and ATCC 27569, were members of *Bam*HI ribotype group 1. This ribotype group contains the largest number of biochemically defined *M.luteus*, including the two *M.luteus* type strains NCIMB 8166 and NCIMB 8553. The other two type strains produced unique *Bam*HI ribotypes. Of the 3 *M.lylae* type strains digested by *Sal*I, ATCC 27567 was a member of *Sal*I ribotype group 4 and the other 2 *M.lylae* type strains produced unique *Sal*I ribotypes. Three further isolates were identified as *M.lylae* by the API microtitre strip and colony pigmentation. All of these isolates were members of the *M.luteus* *Bam*HI and *Sal*I ribotype groups and must therefore be considered as genotypic *M.luteus* species. One other isolate was identified as *M.lylae* which produced unique *Bam*HI and *Sal*I ribotypes and was therefore not speciated. Apart from the *M.lylae* type strains there are no positive identifications of *M.lylae* in this study when colony pigment, biochemical reactivity and ribotype are considered.

The finding of *M.lylae* type strains belonging to ribotype groups containing predominantly *M.luteus* supports studies using DNA:DNA hybridisation [30], immunological cross reactivity [31], and transformation studies [7] showing a close, but distinct, genetic relationship between *M.luteus* and *M.lylae*. The close genetic relationship between *M.luteus* and *M.lylae* is reflected in their phenotypic similarities [15] and the fact that it is only cell wall peptidoglycan type that

definitively separates these two species [7]. Although the *M.lylae* strains clustered within *M.luteus* *Bam*HI or *Sal*I ribotype groups, all four *M.lylae* type strains produced unique "double" ribotypes, distinct from the *M.luteus* "double" ribotype groups.

Isolates identified as *M.sedentarius* belonged to 1 *Bam*HI ribotype group and 4 *Sal*I ribotype groups. The majority belonged to *Bam*HI group 5 and *Sal*I ribotype group 4. This *Sal*I ribotype group also contains isolates identified as *M.luteus*. This is consistent with the finding using transformation of reference *M.luteus* auxotrophs that there exists a distant, but detectable, genetic relationship between *M.sedentarius* and *M.luteus* [7].

M.varians forms a homogeneous group of organisms with a core ribotype pattern sharing common rDNA fragments when both *Bam*HI and *Sal*I were used. Using *Sal*I a shared rDNA fragment of 7.1 kb is produced which is common to *Sal*I *M.luteus* ribotypes 2 and 5, supporting the finding of a genetic relationship between *M.luteus* and *M.varians* [31]. Only two *M.kristinae* isolates were in this study. Both isolates had a common ribotype pattern. The type strains of *M.roseus* and *M.nishinomiyaensis* produced unique ribotypes.

Fifteen isolates (15%) of the 101 non-type strain isolates were not speciated. Isolates in *Sal*I groups 6 and 7, along with isolates showing unique ribotypes were not speciated.

This was either because colony pigment had to be taken into account to assign a species to a biochemically poorly defined group of organisms, the group of organisms were biochemically non-reactive and produced a unique ribotype with no shared rDNA fragments with any other ribotype group or because these organisms were single isolates producing unique ribotypes. In the case of *Sal*I group 6 this may be conservative as all isolates were members of *Bam*HI ribotype group 1 (a *M.luteus* ribotype group) and produced a common rDNA fragment of 9.6 kb with the *Sal*I *M.luteus* ribotype groups. This group however did not fulfil both the biochemical and genotypic criteria for speciation.

The finding of common *Bam*HI rDNA fragments between isolates belonging to *M.nishinomiyaensis*, *M.lylae* and *M.varians* and between *M.varians* and *M.sedentarius* isolate B705, and common *Sal*I rDNA fragments between *M.varians*, *M.kristinae*, and various *Sal*I ribotype groups needs to be further evaluated as to the genetic relationships between each of these ribotype groups. Also the question of whether common rDNA fragments outwith a core pattern shows a significant genetic relationship between two isolates is unknown.

In view of the difficulties inherent in speciating organisms within the genus *Micrococcus* using rRNA probes, it will be necessary to use DNA reassociation of hybrid molecules from different strains to assist in defining species within the genus *Micrococcus* [223].

When running a series of electrophoretic gels the problem of inter-gel variability must be addressed. The sizes of the rDNA fragments reported in this study were means of between 3 and 12 size determinations. The differences between gels for large fragments (> 10 kb) varied by 2-4 kb, corresponding to 1 to 2 millimetres difference in the measured mobility of the fragment. To try and lessen the effect of this variability on fragment size two reference strains were always run on each gel to accurately align fragments of the same size. A further problem with fragment sizing is the upper limit of size resolution in the electrophoretic system used. As conventional gel electrophoresis with 0.8% agarose can only resolve fragments up to 8 kb [190], the co-migration of larger fragments gives an artificial upper size limit to fragments. Therefore the large rDNA fragments sized in this study are subject to a source of error.

4.7) Analysis of *Stomatococcus* spp. isolated from skin.

Four of the isolates from the Antarctic sample group and two isolates received from clinical material had REA profiles suggestive of a % mol GC content lying between that of staphylococci and micrococci. The measured % mol GC content of 4 of these isolates was 55.5% to 58% which was consistent with their REA profiles. This % mol GC content places these

Gram-positive catalase-positive cocci in the genus *Stomatococcus*.

Stomatococcus mucilaginosus has undergone several taxonomic changes since its original description and designation as *Micrococcus mucilaginosus* by Migula in 1900 [53]. This micro-organism currently resides in its own genus and has unique biochemical characteristics distinguishing it from the genus *Micrococcus* and the genus *Staphylococcus*. *Stomatococcus mucilaginosus* is considered part of the normal human oral and upper respiratory tract flora and has not been previously isolated from skin [53,56]. It is now a recognised pathogen, becoming more frequently isolated from clinical sources [54,55].

The primary characteristics which differentiate *Stomatococcus mucilaginosus* from members of the genus *Micrococcus* and the genus *Staphylococcus* are: a weak or absent catalase reaction; the presence of a capsule; the ability to grow on 5% NaCl; lysostaphin resistance; and the % mol GC content [15,53].

In the present study the 7 *Stomatococcus* spp. formed a heterogeneous phenotypic population with isolates 373 and 382 which are clonal, having different carbohydrate utilisation, and growth requirements from the other *Stomatococcus* spp.

Ribotyping was done using*HindIII*which replaced *SalI*, due to the inability of *SalI* to completely digest *Stomatococcus* DNA which has a lower % mol GC content than micrococci. These 7 *Stomatococcus* spp. form different ribotype groups, with the 4 *Stomatococcus* spp. isolated from the Antarctic producing a different ribotype profile from both the *Stomatococcus* spp. isolated from clinical material and the type strain *Stomatococcus mucilaginosus*. It is apparent that differences in phenotype are consistent with different ribotypes.

The heterogeneity of ribotype profiles found within the *Stomatococcus* spp. in this study contrasts with the high degree of homology found with *Stomatococcus mucilaginosus* isolated from the upper respiratory tract or oropharynx, where all the *Stomatococcus* isolates represented one species [20]. By rRNA probing this present study has found that the *Stomatococcus* spp. isolated from human skin have similar ribotypes and have different ribotype profiles from the *Stomatococcus* type strain and the *Stomatococcus* spp. isolated from clinical material. The similarity between isolates collected in the Antarctic could be the result of a small sample population living in close proximity. However, that these skin isolates produced a ribotype profile different from the *Stomatococcus* type strain is indicative of a group of organisms with genetic differences from the type strain. By ribotype profile *Stomatococcus* isolated from

the skin are different and distinct from the *Stomatococcus* type strain which represents *Stomatococcus* isolated from the oropharynx. Clearly a larger collection of organisms needs to be studied to elucidate the amount of heterogeneity within the genus *Stomatococcus*.

4.8) Pulsed-field gel electrophoresis to size genomes and determine genomic heterogeneity of members of the genus *Micrococcus*.

The restriction enzyme *Hin*dIII was used for the determination of the genome size and for the typing of micrococci using PFGE. This restriction enzyme gave a good range of DNA fragments over the length of the gel that were both well separated and easy to analyse at a pulse time of 15-40 seconds.

The genome size of the genus *Micrococcus* varied from 1,823 to 3,397 kb.

It has been stated that a difference in band intensity denotes the presence of more than one DNA fragment in a separated DNA band, all fragments in that DNA band co-migrating. If suspected, these DNA fragments can be resolved by varying the pulse times used or by laser densitometric measurement [166]. It has been shown however that the presence of multiple fragments within a DNA band could not be

detected by differences in band intensity [170]. The two *M.varians* isolates gave a measured genome size of 1,823 kb and 2,334 kb. This difference in genome size may be due to additional fragments present in the *Hind*III digest that have not been resolved, and therefore the genome size of *M.varians* must be regarded as preliminary until more isolates from this species can be studied.

M.lylae has a genome size of 2,138 kb to 2,584 kb. The 4 *M.lylae* type strains formed two groups. *M.lylae* NCTC 11037 and ATCC 27569 had a genome size of 2,138 kb and had DNA profiles that were indistinguishable when ramp times of 2-15 seconds and 15-40 seconds were used. These 2 isolates also share the same *Bam*HI ribotype. The genotypic similarity of these two isolates is supported by the original study which shows that they share the same phenotypic characteristics [7]. The *M.lylae* type strains ATCC 27567 and 27568 have a genome size of 2,553 kb and 2,584 kb respectively and gave profiles that were different from the the 2 former *M.lylae* isolates, producing fewer DNA fragments and having a number of DNA fragments larger than 200 kb. This difference in the frequency of digestion of the genome is supported by the published % mol GC content of NCTC 11037 at 67.1% and of ATCC 27568 at 72.2% [7]. The dichotomy of the % mol GC content, the genome size and the rRNA probing shows these 4 *M.lylae* type strains to be a heterogeneous group of organisms forming 2 separate groups.

M.luteus has a genome size of 2,184 kb to 2,598 kb. Within *Bam*HI ribotype groups 1 and 2 the genome sizes of *M.luteus* correlate. More isolates will have to be studied to identify whether this is coincidental or if membership of a ribotype group reflects a difference in genome size. The difference of approximately 400 kb between the smallest and largest genome size for isolates characterised as *M.luteus* suggests that either multiple DNA fragments within bands exist or it is a reflection of the heterogeneity existing within the species. That isolates from both *M.luteus* and *M.lylae* have a similar genome size is consistent with the finding that the same isolates are clustered within the same *Bam*HI and *Sal*I ribotype groups. That *M.lylae* NCTC 11037 and ATCC 27569 with a different % mol GC content from *M.luteus* (reflected in a different PFGE profile) should belong to the same *Bam*HI ribotype group shows a high degree of conservation of the *Micrococcus* rRNA operon.

M.roseus NCIMB 11696 has a genome size of 3,397 kb. This is approximately 30% larger than the largest genome of the other micrococcal species studied. It has already been stated that *M.roseus* may warrant generic status [26]. The finding of a larger genome supports the need for further work to elucidate the taxonomic position of *M.roseus*.

Selected isolates were taken from each *Bam*HI ribotype group and subjected to PFGE after digestion with *Hind*III to attempt to establish if PFGE was a useful taxonomic tool. It

has been shown that species in the genus *Staphylococcus* have species specific DNA profiles [177] and that both rRNA probing and PFGE separates *P.cepacia* into the same groups [173]. In this study no species or ribotype specific DNA profiles were evident with the use of PFGE. This is consistent with SDS-PAGE work showing that micrococci formed a heterogeneous population of organisms a situation analogous to that observed in *Haemophilus influenzae* [195]. The heterogeneity in PFGE profiles means this technique will be a useful tool to elucidate the epidemiology of the micrococci.

This heterogeneity amongst the *M.luteus* isolates, indeed amonst the genus as a whole, is one of the reasons why SDS-PAGE was not a useful technique in the initial stages of the study. SDS-PAGE although to sensitive to form broad isolate groupings, would be a more suitable technique to elucidate individual isolate differences within a clonal group.

4.9) Micrococci isolated from clinical material.

As mentioned in the introduction (Section 1.4, p22) micrococci are infrequently isolated from clinical material. Thirteen isolates collected from blood cultures, CSF or people using CAPD were added to the isolates collected in the Antarctic.

The 13 isolates belonged to the 4 species *M.sedentarius*, *M.kristinae*, *M.luteus* and *Stomatococcus* spp. No one species predominated from any one source. Three of these 4 species have been recognised as opportunistic pathogens in the literature [44,53,66,69,81]. This study is the first time that *M.kristinae* has been reported causing infection. With the exception of two isolates, all the isolates belonging to *M.luteus* produced "double ribotypes" which were not found in the Antarctic sample group. This could be a reflection of the small confined study population in the Antarctic, alternatively the "double ribotypes" found on the clinical samples may reflect isolates which have a higher capacity for pathogenicity.

Certainly the isolation of micrococci from clinical material should not be immediately dismissed as contamination, as the *Micrococcus* under certain circumstances has the capacity to become an opportunistic pathogen.

4.10) The temporal skin carriage of micrococci in an isolated community.

All subjects in this study carried *M.luteus*. It is therefore only possible to comment on the temporal carriage of this organism. Although only one skin site was looked at in detail it was apparent that certain people supported the same clone of *M.luteus* over the duration of the study. It is

also apparent that the same clone of *M.luteus* was present on more than one person. In the few cases where a unique clone was found on two people it was found on people who had lived in close proximity on base and shared sleeping quarters.

There were periods where colonisation persisted for the length of the study but in many cases there were lengthy periods where the clones could not be isolated. This is a limitation of the sampling method used in the study whereby only a few selected colonies are collected for storage.

It has been proposed that there are three categories of colonisation status for skin microbes: *transients*, those contaminating organisms which are not multiplying; *temporary residents*, contaminants which multiply and stay resident for short periods of time: and lastly *residents*, the "permanent" inhabitants of the skin [224]. Colonising status has been defined as the repeated isolation of an organism over a period of time, with transiency or temporary residence defined as a single isolation of an organism [194]. It has been demonstrated that staphylococci form discrete clonal colonies on a particular area of skin with a dominant clone proliferating [194]. Where sufficient organisms were collected the present study shows that people carry the same resident clone(s) of *M.luteus* over time and in only one case was a particular clone dominant for the site sampled. The clone B1:S1 was only resident on persons 5 and 7, but was isolated from several people on single months over the study period.

These random isolations of a clone may represent *transient* resident status as this clone was never isolated from these people on consecutive months, alternatively these isolates may represent *resident* clones that have been shed from sites outwith the studied area. The survival of a particular clone may represent the importance of an established strain in interclonal competition, or clonal specialisation that allows only one clone to thrive within a particular microenvironment.

The low isolation rate of *M.sedentarius*, *M.varians* and the absence of *M.kristinae* and *M.nishinomiyaensis* may represent a very low level of colonisation of the site sampled, or colonisation of a site outwith the study, with the isolates isolated representing transient or temporary residents shed from the principal reservior.

Considering the low numbers of organisms isolated over the 4 months of the study period, and the limitations of the selective sampling technique used more work needs to be done to elucidate the temporal skin epidemiology of the micrococci.

4.11) Future work

This study has shown that the genus *Micrococcus* is a genus composed of a heterogeneous population of organisms.

Although a genetic relationship by rRNA probing has been shown between *M.luteus* and *M.lylae*, and *M.luteus* and *M.sedentarius* the extent of this genetic relationship is unknown. Furthermore, ribotyping showed heterogeneity within the species *M.luteus* and *M.lylae*. Again the extent of the genetic diversity within these two species is unknown. The finding that *M.roseus* has a significantly larger genome than the other members of the genus *Micrococcus* warrants more work on its taxonomic position.

Intrageneric relationships can be determined with the use of 16S rRNA sequencing to obtain a measure of how closely or distantly a group of organsims are related to each other [225,226,227]. 16S rRNA sequencing would help answer the following questions:

i) What is the genetic relationship between the different species in the genus *Micrococcus*?

ii) What is the genetic relationship between ribotype subgroups in the species *M.luteus* and how heterogeneous is this species?

iii) What heterogeneity exists within the species *M.lylae*?

iv) What is the taxonomic position of *M.roseus*?

The PFGE derived genome size of *M.varians* presented in this study must be considered as provisional until more organisms from this species can be studied by PFGE. The species *M.kristinae* was not sized in this study, therefore this species, and organisms from different ribotype groups

representing different *Micrococcus* species should be studied further using PFGE to answer the following questions:

i) What is the genome size of *M.kristinae*?

ii) Do different ribotype groups represent organisms with differing sized genomes?

iii) What is the heterogeneity within species of the genus *Micrococcus*?

This study has shown that *Stomatococcus* spp. isolated from the skin differ from the type strain *Stomatococcus mucilaginosus* NCTC 10663. Previous work has indicated that *Stomatococcus mucilaginosus* formed a homogeneous group of organisms that were highly related [20]. More isolates of *Stomatococcus* need to be collected from both the oropharynx and skin sites to allow characterisation by biochemical criteria, SDS-PAGE, rRNA probing, and PFGE to elucidate the heterogeneity within this genus.

5.

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6.

APPENDICES

Appendix A.

THE BACTERIOLOGICAL STUDY AT FARADAY

You are probably aware that the body serves as a vast store of resident bacterial species, each of which has been able to adapt to allow it to live in every part of the body. These organisms are not harmful to the body (indeed many of them may be positively helpful) and are normal residents either on or within the body.

One such organism is a small round bacteria called a micrococci which is normally resident on our skin. At present very little is known about these bacteria, mainly because they have thus far not been implicated in any major disease processes or infections, and have largely been ignored.

What little we do know about these bacteria includes, what they look like as they grow, what they grow on, what sugars they metabolise and what antibiotics they are sensitive to. All this information allows us to classify the organisms into different species.

This study will hopefully broaden our knowledge of the micrococci and try to answer some fairly basic but important questions, such as:

1) What is the normal bacterial population like?
2) Can new techniques of identification tell slightly different bacteria apart?
3) Do individuals mount an immune response against

these normally resident bacteria and if so, how large is this response.

To answer these questions I will be asking for volunteers who will be sampled every month by taking a wet sterile cotton wool swab and wiping selected easily accesible areas of the body. The swabs will be plated and any bacteria grown will be stored and analysed in the United Kingdom.

I cannot stress to strongly that participation in this study is entirely voluntary and that if you decide to participate but wish to withdraw for whatever reason you are free to do so withoutany obligation to continue.

I ..... having read the accompanying explanatory sheet and having had the implications of inclusion into the study explained to me by Dr A. Leanord do give my full and informed consent to any procedures necessary for completion of the above study. I also fully realise that I am under no obligation to continue in the study and that I can withdraw at any time without stating a reason.

Signed.....

Date.....

Witness.....

Appendix B.
Number of nucleotides in the gene sequence UN: X12578.
Total number of nucleotides 2,286.

A: 391	C:789	G:790	T:316
	Total number of nucleotides: 2,286		
GG:234	GA:188	GT:102	GC:265
AG:134	AA:40	AT:65	AC:152
TG:125	TA:26	TT:24	TC:141
CG:296	CA:137	CT:125	CC:231
	Total number of nucleotides: 2,285		
GGG:54	GGA:62	GGT:35	GGC:82
GAG:72	GAA:14	GAT:32	GAC:70
GTC:45	GTA:13	GTT:10	GTC:34
GCG:94	GCA:53	GCT:46	GCC:72
AGC:43	AGA:30	AGT:15	AGC:46
AAG:22	AAA:0	AAT:1	AAC:17
ATG:29	ATA:0	ATT:0	ATC:36
ACG:59	ACA:16	ACT:21	ACC:56
TGG:44	TGA:20	TGT:12	TGC:49
TAG:1	TAA:0	TAT:1	TAC:24
TTG:1	TTA:0	TTT:0	TTC:23
TCG:65	TCA:19	TCT:11	TCC:46
CGG:93	CGA:76	CGT:39	CGC:88
CAG:39	CAA:26	CAT:31	CAC:41
CTG:50	CTA:13	CTT:14	CTC:48
CCG:78	CCA:49	CCT:47	CCC:57
	Total number of nucleotides: 2,284		