Gene expression and genetic diversity of the "*Streptococcus milleri* group"

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LIST OF UNITS AND ABBREVIATIONS

° C	degrees Celsius
%	percent
α	alpha
Å	angstrom (unit of length = 10^{-10} m)
Abs	absorbance
AFLP	amplified fragment length polymorphism
AP	acid stress protein
AP-PCR	arbitrarily primed (PCR)
ATP	adenosine-tri-phosphate
ATPase	adenosine-tri-phosphatase
β	beta
bp	base pair
BA	blood agar
BHI	brain heart infusion
cDNA	complementary DNA
cm	centimetres
CPS	counts per second
CS	chondroitin sulphatase
C.F.U	colony forming units
CHEF	contour clamped homogenous electric field
CSP	complement stimulating peptide
ci	Curies
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide tri-phosphate
dATP	deoxyadenine tri-phosphate
dCTP	deoxycytosine tri-phosphate
dGTP	deoxyguanine tri-phosphate
dTDP	deoxythymine di-phosphate
dTTP	deoxythymine tri-phosphate
dUTP	deoxyuracil tri-phosphate
ddNTP	di-deoxynucleotide tri-phosphate

DEPC	diethylpyrocarbonate
DD RT	differential display reverse transcription
DFI	differential fluorescence induction
DMSO	dimethylsulphoxide
DTT	dithiothreitol
E_h	oxidation / reduction potential
emi	early stage macrophage induced
ERIC	enterobacterial repetitive intergenic consensus
EDTA	ethylenediaminetetra-acetic acid
FACS	fluorescent activated cell sorting
γ	gamma
GSP	general stress protein
GAG	glycosaminoglycan
g	(mass) gram
GAS	group A streptococci
G+C	guanine plus cytosine
GTC	guanine thymine cytosine
h	hour
HA	hyaluronic acid
IgA	Immunoglobulin A
Ir-PCR	inter-repeat (PCR)
IVET	*in-vivo* expression technology
IVIAT	*in-vivo* induced antigen technology
kb	kilobase
kDa	kilodalton
λ	lambda
l	litre
LB	luria broth
ml	millilitre
mRNA	messenger RNA
m	meter
MU	methylumbelliferone
μ	micro
μl	microlitre
μg	microgram
μm	micrometre

mm	millimetre
mM	millimolar
min	minutes
mol	mole
mos	milliosmotic
M	moles per litre
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MMLV	moloney murine leukaemia virus
MOPS	3-(N-Morpholine) propanesulfonic acid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogenase
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate hydrogenase
NCBI	National Centre for Biotechnology Information
ng	nanogram
nm	nanometre
pg	picogram
pmol	picomole
pH	acidity / alkalinity
PMSF	phenylmethylsulphonylflouride
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMNL	polymorphonuclear leukocyte
PFGE	pulsed field gel electrophoresis
PFL	pyruvate formate lyase
QPCR	quantitative PCR
RAP	random amplified polymorphic
RAPD	random amplified polymorphic DNA
REA	restriction endonuclease analysis
REP	repetitive extragenic palindrome
RFLP	restriction fragment length polymorphism
RT	reverse transcriptase
r	ribosomal
rDNA	ribosomal DNA
rRNA	ribosomal RNA

RNA	ribonucleic acid
RNase	ribonuclease
s	second
SSCP	single stranded conformational polymorphisms
STM	signature tagged mutagenesis
SDS	sodium dodecyl sulphate
SLO	streptolysin O
SLS	streptolysin S
SMG	"*Streptococcus milleri* " group
SSC	saline sodium citrate buffer
sub	subgingival
sup	supragingival
Taq	*Thermous aquaticus*
TBE	tris-borate EDTA
TE	tris EDTA
TES	N-tris (hydroxymethyl) methyl-2-aminothane-sulphonic acid
UV	ultra-violet
UWCM	University of Wales College of Medicine
U	unit
UK	United Kingdom
VS	viridans streptococci
V	volts
Xg	times gravity
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-Galactopyranoside
YEPD	yeast extract peptone dextrox

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SUMMARY

The "*Streptococcus milleri*" group (SMG) of bacteria are heterogeneous facultative Gram-positive cocci that are present as commensal organisms in the mouth, oropharynx, gut, and genito-urinary tract. The SMG are also associated with purulent abscesses of the brain, the dentoalveolar region and the liver. They are frequently associated with bacteraemia and have been isolated from cases of endocarditis. However the mechanisms by which the SMG cause infection are little understood. One of the more interesting aspects of the pathogenicity of this group is the switch from apparent commensal to pathogen. The current study seeks to address three novel areas related to the pathogenicity and molecular ecology of this group. 1. The development of differential display reverse transcription polymerase chain reaction for the study of preferential gene expression under varying environmental conditions of heat shock, pH, osmolarity and iron concentration. 2. The study of the dynamics of oral colonisation of two individuals by "species" of the *Streptococcus anginosus* group over time. 3. An attempt to determine the reason for the marked genetic diversity, by studying the stability of the SMG genome over the course of four months subculture *in-vitro* involving sub species typing using ERIC-PCR and Pulsed field gel electrophoresis. A prerequisite for the latter two aims was the development of robust and reproducible typing methods for the SMG.

A protocol for the analysis of preferential gene expression was devised and validated. Analysis revealed possible sequence upregulation of RNA polymerase beta subunits, pyruvate formate lyase activating enzyme and a deoxyglucose epimerase enzyme at pH 7.0. For strain characterisation ERIC-PCR and Pulsed field gel electrophoresis were shown to be discriminatory, robust and reproducible. The colonisation patterns in all sites were shown to be complex with many different clonotypes of a given species present. Interestingly, over time the colonisation patterns were not stable with significant changes in the species representation. The *Streptococcus anginosus* group has been shown to display a significant amount of genomic diversity during *in-vitro* culture.

INTRODUCTION.

The "*Streptococcus milleri*" group (SMG) of bacteria are facultative Gram-positive cocci that can be isolated as commensal organisms from a range of body sites including the mouth (Poole and Wilson, 1977), oropharynx (Ball and Parker, 1979), gut (Mejare and Edwardsson, 1975), and genito-urinary tract (Poole and Wilson, 1979). However, the SMG have also been found at specific sites of purulent disease, both in isolation and in association with other organisms, particularly strict anaerobes (Lewis *et al.,* 1988). The SMG seem to be intimately associated with the disease process at a number of body sites including abscesses of the brain (Molina *et al.*, 1991), the dentoalveolar region (Lewis *et al.*, 1986) and the liver (Edmiston *et al.*, 1991). In addition they are frequently associated with bacteraemia (Bert *et al.*, 1998) and have been documented as the aetiological agents in a significant proportion of cases of endocarditis (Murray *et al.*, 1978). It is been suggested that strains of SMG may be initiators of infection and may do so by creating environments favoured by other pathogenic organisms, in particular anaerobes. However the mechanisms by which the SMG cause infection are little understood despite the fact that a number of putative virulence factors have been proposed (Shinzato and Saito, 1994). One of the more interesting aspects of the pathogenicity of this group is the switch from apparent commensal to pathogen. Also this group of bacteria have a propensity for invasion of the bloodstream to cause bacteraemia and seeding of infection to sites distant from the original infection (Bert *et al.*, 1998). An additional area where understanding is lacking is why in the presence of appropriate antimicrobial therapy (SMG are almost universally susceptible to beta lactam antibiotics) many infections are still slow to resolve (Jacobs and Stobberingh, 1996).

From a microbiological viewpoint, the group has always been known to be phenotypically heterogeneous, and this has contributed to the taxonomic

confusion. However, recently the group has also been shown to be markedly genotypically heterogeneous (Bartie *et al.*, 2000).

The current study seeks to address three novel areas, which may lead either directly or indirectly to a clearer understanding of the pathogenicity of this group of bacteria. Firstly the development of a method to facilitate the study of preferential gene expression under varying environmental conditions. Secondly an analysis of the dynamics and patterns of colonisation of the oral cavity. Thirdly an attempt to address the reason for the marked genetic diversity, through studying the stability of the SMG genome.

The environmental conditions under which bacterial cells grow has long been known to be a contributing factor towards the control of cellular gene expression. Genes associated with virulence may be switched on or off under different environmental conditions including heat shock (Hurme and Rhen, 1998), pH, osmolarity (Badger and Kim, 1997) and iron concentration (McKee *et al.*, 1986). Although the *in-vitro* analysis of virulence factors has been useful, increasingly it is accepted that the study of bacterial pathogenesis under conditions that mimic the *in-vivo* situation is more definitive of the actual pathogenic process. Methods available to detect preferential gene expression under different environmental conditions include *in-vivo* expression technology (IVET), differential fluorescence induction and signature tagged mutagenesis. However, these methods only permit the study of specifically known genes in environmental situations rather than the study of up / down regulation and discovery of previously unknown genes that are induced under specific environmental situations. Differential display reverse transcription polymerase chain reaction (DD RT-PCR), is a technique for the analysis of preferential gene expression that allows the study of up and down regulation of unknown genes. The technique has been applied extensively in eukaryotic systems (Liang and Pardee, 1995) and to an increasing number of bacterial systems (Kwaik and Pederson, 1996; Rindi *et al.*, 1999). Problems encountered when using DD RT-PCR include high rate of false positive results (Milson and Burrows, 1998) and prevalence for reverse transcription of

rRNA's above mRNA's (Nagel *et al.*, 1999). Additional problems with prokaryotic systems include the instability and short half-life of mRNA species in comparison to eukaryotes and the low level of polyadenylation of mRNA, which potentially prohibits the use of 3' dT anchored primers. For Gram-positive bacteria, such as the SMG these problems are further compounded by the difficulties in RNA isolation related to the presence and structure of a thick cell wall.

Although something is known of the species distribution of the SMG, no studies to date have addressed either the clonal diversity or the dynamics of colonisation of the oral cavity by these bacteria. The SMG are known to be heterogeneous in both phenotypic and genotypic terms, however the reasons for this are not understood. The study of genetic diversity has provided a useful framework for the understanding of the genetic structure within bacterial populations (Rolland *et al.*, 1999). The availability of molecular typing tools, which facilitate strain differentiation, has allowed the study of the clonal distribution and persistence of molecular types within a bacterial species. With the exception of *S. mutans* and the "mitis" species group, strain-typing methods have not been extensively applied to study the clonal diversity of oral streptococci (Whiley and Beighton, 1998). However recently, PCR based typing methods, which target repetitive DNA sequences (REP-PCR) have been used in the study of SMG strains (Alam *et al.*, 1999; Clarridge *et al.*, 1999) and have provided further evidence for the significant level of genetic diversity within the group.

In order to study genetic diversity it is imperative that a system of typing be developed and employed that is both robust and reproducible, in order to divide isolates into strain types and gain some understanding as to the pattern of colonisation of the oral cavity and the extent of genetic diversity within the "*Streptococcus milleri*" group. An aim of this thesis is to develop a fast, efficient method of typing multiple isolates of SMG prior to comparison with the currently considered "gold standard" technique - Pulsed field gel electrophoresis.

Increasingly PCR based typing methods are being used to characterise individual strains of bacteria (Alam *et al.*, 1999). Advantages of a PCR based approach include the fact that the methods are generally non-labour intensive and are rapid. PCR typing is based on the amplification of genomic DNA using specific or arbitrary primers to yield a fingerprint, which may be characteristic of an individual strain. Commonly, the primers are based upon the sequence of specific highly conserved centrally inverted repeats present in intercistronic regions of the genome that are present in all strains of the species under study (Kerr, 1994). Although potentially there are many targets for primers, 4 sets of primers have been used frequently for bacterial typing; Rep (Repetitive extragenic palindromic), ERIC (Enterobacterial repetitive intergenic consensus), M13 and BOX.

Pulsed field gel electrophoresis (PFGE) is often cited as the most discriminatory method for the typing of microorganisms (Grothues *et al.*, 1988). The method was developed in 1984 (Schwartz and Cantor, 1984) and enables the separation of large fragments of DNA by the application of an alternating electric field to an agarose gel. Chromosomal DNA is digested by restriction enzymes that cut infrequently and produce between 5 and 20 large fragments (Sader *et al.*, 1995). PFGE has been applied extensively and is considered by many to be the gold standard for epidemiological typing due to its high discriminatory potential (Mendez-Alvarez *et al.*, 1995). However, PFGE is more expensive than conventional typing methods due to the cost of the specialist equipment required. The procedures involved in the extraction and treatment of the genomic DNA from the isolates are time consuming, technically demanding and the optimisation of the running and electrophoresis conditions can be troublesome. Despite this, PFGE has been used on several occasions for the successful typing of strains of streptococci from several species including SMG showing a high level of discrimination and reproducibility (Bartie *et al.*, 2000).

With development of these methods complete, the examination of the patterns and dynamics of oral SMG colonisation and the study of genomic SMG

stability will be facilitated. Many studies have taken place where microorganisms are isolated from selected individuals over set time periods. The main aims of past typing studies have been clinical and epidemiological in nature. Wilson *et al.,* (2001) isolated *Candida albicans* strains from terminally ill patients at regular intervals before and after antifungal treatment. Other similar studies have been performed; Van Belkum *et al.,* (1994) performed a similar longitudinal study on *Candida*, as did Metzgar *et al.,* (1998). Viridans streptococci isolated from cancer patients were studied by Wisplinghoff *et al.*, (1999), but no attention to the study dynamics was made. Changes in the oral colonisation of children by Gram-negative species over time were investigated by Kononen *et al.,* (1994) and Arbitrarily-primed PCR (AP-PCR) was used to examine strain types of *Prevotella intermedia* group organisms in mothers and their children (Kononen *et al.,* 2000).

In summary, the aims of this thesis are the development of a method to facilitate the study of preferential gene expression under varying environmental conditions, the analysis of the dynamics and patterns of colonisation of the oral cavity and analysis of the marked genetic diversity, through studying the stability of the SMG genome.

CHAPTER 1: REVIEW OF THE LITERATURE

1.1. Oral microbial ecology.

1.1.1. The oral environment.

The mouth as an environment for the colonisation of microorganisms is diverse and provides many ecological niches such as epithelial surfaces, enamel dental surfaces, and protected crevices. A range of factors are present that can both limit or promote the growth of the microorganisms. Temperature is one important factor due to an effect on microbial metabolism and enzyme activity, since this can range from a standard 37 °C up to 50 °C during the consumption of food and drink (Marsh and Martin, 2000). Most oral microorganisms require a pH of between 6.5 – 7.5 for optimal growth. However, after sugar consumption the pH of saliva can drop as low as pH 5.0 due to the production of lactic acid by some plaque bacteria. In contrast, the pH can also rise as high as 8.0 during inflammatory responses to periodontal disease (McDermid *et al.*, 1988).

1.1.2. The oral microflora.

Acquisition of the oral microflora begins at birth and the first bacteria found in the mouth are derived from the mother in the first three months of life (Kononen *et al.*, 1999), probably after the first breast feed (Socransky and Mangianello, 1971). Many bacteria considered indigenous to the oral cavity are detected prior to the eruption of teeth, whilst others appear after emergence of the deciduous dentition (Frisken *et al.*, 1990; Kononen *et al.*, 1992). Most organisms within the oral cavity maintain a commensal relationship with the host (Greenstein and Lamster, 1997). There are around 500 different species of bacteria present in the mouth including those that cannot be cultured. Many oral bacterial species colonise at an early stage in human development, these are known as primary colonisers and generally consist of the species most able to bind to the oral surfaces. Those not able

to bind as effectively become adept at attaching to primary colonising species, these are acquired at a later time and are known as secondary colonisers (Lamont *et al.*, 1992).

Colonisation occurs through bacterial attachment to the buccal epithelial surfaces and to the teeth. Members of the oral microflora are able to attach to the epithelial surfaces of the mouth due to salivary components which coat oral surfaces and allow attachment of microorganisms to bind using specific cell surface structures (Jenkinson and Lamont, 1997). Salivary components include secreted bacterial polysaccharides, glycoproteins, and secretory IgA and these are collectively known as the salivary pellicle. The pellicle attaches to free-floating bacteria, to cell surfaces and to teeth, facilitating the attachment of bacterial cells. Some of the main bacterial surface structures used are the antigen I/II family of receptors and M proteins (which are structurally related in oral streptococci (Bleiweis *et al.*, 1992)). However, not all oral bacteria can adhere to all components. The attachment ability of some oral bacteria to epithelial surfaces may also precede intracellular invasion and immune modulation, an important process in the cycle of bacterial virulence (Jenkinson and Lamont, 1997).

Colonisation of the teeth is caused by attachment to the acquired enamel pellicle and glucans that reside on the tooth surface. The "mutans", "mitis" and "salivarius" group of streptococci are primary colonisers of the teeth and are capable of synthesising glucosyltransferase enzymes which catalyse the production of glucans from dietary sugars (Jacques, 1994). Glucans are released into the saliva to be deposited onto other tooth surfaces or attach to other bacterial species (Tsumori and Kuramitsu, 1997). It is likely that the SMG are secondary colonisers as they do not produce the glucosyltransferase enzyme, but can coaggregate to glucans already laid down by primary colonisers to become part of the dental plaque (Fukushima *et al.*, 1992).

A large proportion of the bacteria present in the oral environment consist of members of the *Streptococcus* genus. The streptococci are grouped according to genotypic and phenotypic characteristics (Whiley *et al.*, 1990), and consists of six clusters of species each of which may be characterised by distinct pathogenic potential and other properties such as 16S rRNA typing (Kilian, 2002). The groups are known as the "pyogenic" group, the "mutans" group, "mitis" group, "bovis" group, "salivarius" group and the "anginosus" group (Figure 1.1). The microbes that exist in the oral cavity tend to be site specific. The buccal mucosa of the cheek is colonised mainly by members of the *Streptococcus mitis* group. *Haemophilus parainfluenzae* and *Simonsiella* spp. are found in lower numbers (Frandsen *et al.*, 1991). Obligate anaerobes are not usually found in such exposed areas. The dorsum of the tongue is home to a large number of different bacterial species. Over 40 % of the total culturable microbial mass of the tongue is composed of *Streptococcus* spp. with *S. oralis*, *S. mitis* and *S. salivarius* group organisms predominating (Smith *et al.*, 1993). Anaerobic bacteria, including pigmented and non-pigmented species are frequently isolated from the tongue crevices. Gram-positive bacilli constitute around 16 % of all isolated species including *Actinomyces naeslundii,* and *Actinomyces odontolyticus*. Other organisms include lactobacilli, fusobacteria, spirochaetes and other motile bacteria (Frandsen *et al.*, 1991). Obligate anaerobes are found in high numbers in the gingival crevice where oxygen is limited.

The microflora of the teeth differs between tooth surfaces. "*S. milleri*", "mutans" and "mitis" groups comprise species commonly isolated from the teeth (Frandsen *et al.*, 1991). The SMG are found both subgingivally and supragingivally as well as on the tongue, and are also isolated from saliva. Mejare and Edwardsson, (1975) however, previously noted no SMG growth on the tongue. Whiley *et al.*, (1993) performed a study analysing the distribution of SMG in the oral cavity of 162 subjects. This study found *S. constellatus* to be the least often isolated species out of the three, but the

most commonly isolated species of SMG in the subgingival regions. *S. constellatus* was found alongside *S. anginosus* in supragingival plaque, but was not commonly isolated alongside *S. intermedius* (Whiley *et al.*, 1993). *S. intermedius* was most commonly isolated alongside *S. anginosus* in the supragingival regions and on the tongue, but was also isolated individually and in conjunction with *S. anginosus* in the subgingival plaque. *S. anginosus* was isolated both subgingivally and supragingivally alongside *S. intermedius* and in high numbers in saliva. It was determined that it is not usual to find all three species together at one site or in one individual (Whiley *et al.*, 1993).

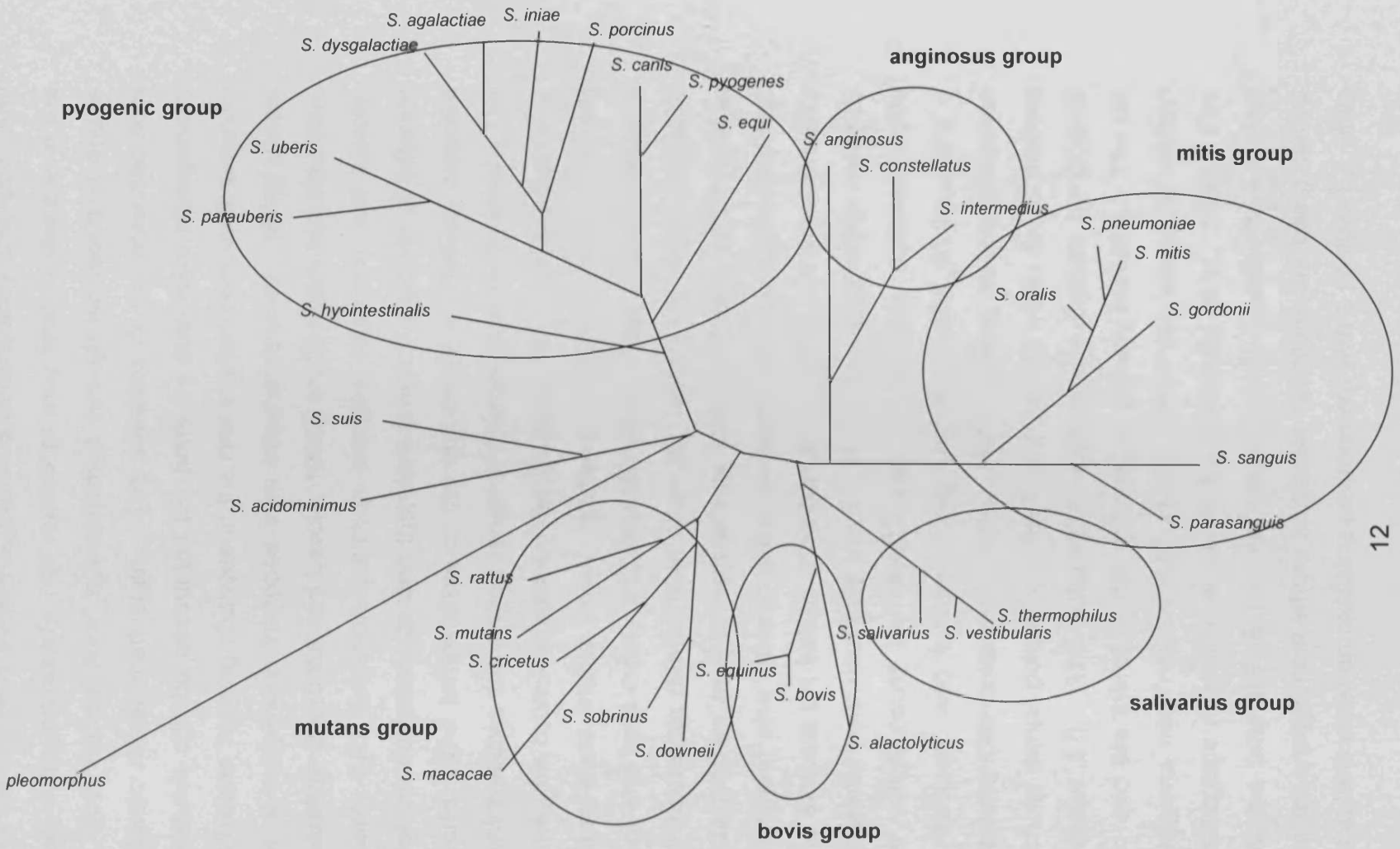

Figure 1. 1 . Phylogenetic relationships among 34 *Streptococcus* species by 16S rRNA sequence analysis. Reproduced from Whiley and Beighton, (1998).

1.1.3. Diversity and stability of the oral flora.

The ecology of the oral environment is diverse and constantly changing. Any drastic change of an individual's oral microflora may lead to disease in the oral tissues. The "normal flora" is maintained through homeostasis and is therefore dynamic rather than static. The variation in the microbial flora between individuals can be accounted for, firstly by both local endogenous and systemic factors causing changes in the oral environment, and secondly by the receipt of exogenous microbes from external sources. Many factors such as temperature (Marcotte and Lavoie, 1998), pH (Bowden and Hamilton, 1987), osmolarity and oxidation-reduction potential (Marcotte and Lavoie, 1998) can alter the surroundings and therefore favour the growth of different microbial species. The temperature of the oral cavity is relatively constant (34-36 °C), which allows for a wide range of microorganisms to grow. The temperature may be more variable on the mucosal and tooth supragingival surface. During food intake, microorganisms colonising these sites are exposed to hot and cold meals and probably must adapt to these extreme variations in temperature (Marcotte and Lavoie, 1998). The pH or hydrogen ion concentration of an environment affects microorganisms and microbial enzymes directly and also influences the dissolution of many molecules that cannot tolerate extreme pH values (Hamilton *et al.*, 1989). In the oral cavity the pH is maintained near neutrality (pH 6.7 to 7.3) by saliva, which removes carbohydrates which could be metabolised by bacteria, removes acids produced by bacteria, and buffers acidity from food and drinks using a bicarbonate system (Marcotte and Lavoie, 1998). Saliva cannot penetrate structures such as dental plaque very well, and the pH within these biofilms may fall to below 5.0. The subgingival regions are bathed by gingival crevicular fluid and are therefore not exposed to salivary buffering. The pH within these regions may rise as high as 8.5, which can select for certain periodontal pathogens (Hamilton *et al.*, 1989; McDermid *et al.*, 1988). The oxidation-reduction potential (E_h) is the proportion of oxidised to reduced components in an environment where enzymatic reactions are being carried out. Aerobic bacteria need an oxidising environment with a positive E_h, while

the opposite can be said of anaerobic bacteria (Theilade, 1990). The redox potentials vary across the different oral environments and changes would alter the variety of bacteria able to colonise those spaces.

There are a variety of host factors influencing the colonisation of the oral cavity such as host defence mechanisms, age, hormonal changes, stress and genetic factors (Marcotte and Lavoie, 1998). Host defence mechanisms are present supragingivally in the saliva as lysozyme, immunoglobulins, mucins, lactoferrin, peroxidase, and salivary glycoprotiens, and subgingivally in the gingival crevicular fluid (Henskens *et al.*, 1993). The composition of the oral microbiota varies with the age of the host due to events such as tooth eruption, changes in dietary habits, salivary flow, or the immune system (Marcotte and Lavoie, 1998). Hormonal changes occur at various stages of the life of a host. It is well documented that pregnancy and puberty are associated with an increase in gingival inflammation which is accompanied by an increase in gingival exudates (Zachariasen, 1993). These exacerbations in gingival inflammation leads to alterations in the microbiota of the gingival crevice (Zachariasen, 1993). Host stress may be associated with changes in hormones, salivary flow, dietary habits and immune system response (Ballieux, 1991; Brain and Nowell, 1970; Dawes, 1987; Landi *et al.*, 1982; Tuli *et al.*, 1994). Genetic factors seem to have an effect upon the host susceptibility to caries (Kurihara *et al.*, 1991; Potter, 1990) and periodontal disease (Gilbert and Sofaer, 1989). The selection of a certain microbiota by the host is dependant on inherited immune factors, physiology, metabolism, mucus composition, or receptor-ligand interactions (Moore *et al.*, 1993).

As well as host factors and internal factors, many external factors can effect the stability and variation of the microbial ecology. Factors such as diet, oral hygiene, and drugs and diseases may impact upon the environment of oral commensals (Greenstein and Lamster, 1997). The high consumption rate of sucrose is known to enhance the development of *S. mutans* and *Lactobacillus*, and this in turn generates a high pH environment favouring acidogenic and acidophilic bacteria (Marcotte and Lavoie, 1998). Oral

hygiene is one of the most important factors in the maintenance of oral homeostasis. Actions such as tooth brushing, the use of antibacterial mouthwashes and dental treatment can have a detrimental effect upon bacteria attempting to adhere to tooth surfaces, and can also limit the production of bacterial proteases (Marsh, 1992). The drugs taken by, and any additional medical problems suffered by the host can have an affect upon the oral environment. Any drugs taken which may limit the amount of saliva produced by the salivary glands would make the host susceptible to increased plaque and low pH tolerant bacteria (Marcotte and Lavoie, 1998). Antibiotics that are given orally or systemically may enter the oral cavity via saliva and gingival crevicular fluid and lead to an imbalance in the oral microbiota (Marsh, 1992). Antibiotics may suppress some resident bacterial populations and result in an overgrowth of antibiotic resistant bacteria or yeast.

The transmission of exogenous pathogens can be explained through personal contact with other individuals or through the sharing of an inanimate object such as a toothbrush (Genco *et al.*, 1988). The evidence of the passing of microorganisms between parent and children is particularly strong. However, the transfer of bacteria does not mean that colonisation will take place, since this is dependant upon many factors including the efficacy of the individuals immune system, dietary influences and oral hygiene measures (Greenstein and Lamster, 1997). A longitudinal study performed by Kononen *et al.,* (1999) on infants established that there is a 'favourable time' for establishment of a bacterial species in the host and concluded that the colonisation by periodontal pathogens does not take place in the early childhood, but later after eruption of primary dentition. The likelihood of colonisation by periodontal pathogens early in childhood increases if the host has parents with periodontal disease.

1.1.4. Clonal populations and diversity.

Bacterial populations within ecological niches have for many years been considered simplistically as clonal with each cell of a strain type reproducing through binary fission to produce two genetically identical daughter cells (Spratt and Maiden, 1999). Since the advent of phenotypic and more recently genotypic identification methods, this simplistic picture has been shown to be far more complicated than once thought (Gupta and Maiden, 2001). Bacteria reproduce, as described above, through asexual means undergoing division to produce two cells containing identical genetic information however, sexual processes can take place after division through a variety of methods to add an element of variation into a cell lineage. This allows evolution and adaptability within bacterial populations which is important if a strain is to survive within a harsh or highly competitive environment. Until recently, little has been known of the tendency towards diversification, and bacterial populations have been considered to be either clonal or panmictic (generating diversity through horizontal gene exchange) whereas the truth is probably somewhere in between the two. Truly clonal or panmictic organisms probably do not exist. However non-clonal organisms display a degree of clonality within a spectrum ranging from clonal to panmictic (Spratt and Maiden, 1999). The closest to true clonality has been shown in strains of salmonella (Spratt and Maiden, 1999), but even these have to adapt to survive. Clonal populations will typically exhibit diversity through *de novo* mutation which will occur over successive generations producing non-random chromosomal polymorphisms which show linkage disequilibrium. However, more panmictic cells exhibit sexual forms of variation including recombination with other related or non-related strains. This level of continually reassorting mutations and the gain and loss of plasmids / transposons results in linkage equilibrium (Spratt and Maiden, 1999). The question of diversity in populations of cells arises due to the clonal or panmictic nature of particular strains within the population. It is generally thought that the more clonal a strain is, the less diverse (Feil *et al.*, 2001). This is a logical assumption as the occurrence of *de novo* mutation in a cell lineage would occur in response to environmental influences in order to

aid fitness of the cell line. The increase in fitness enables the new cell line to out compete older lineages thus decreasing genetic diversity (Atwood *et al.*, 1951; Levin, 1981). The identification of clones within bacterial populations is often taken as evidence for a low rate of recombination, but the validity of this inference is rarely examined (Feil *et al.*, 2001) and a lack of diversity should not be taken as proof of clonality (Spratt and Maiden, 1999). The clonal population theory is pertinent to the oral streptococci, since they exist in tightly packed communities alongside many other related and non-related strains within a highly competitive environment, under constant selective pressure. *S. mutans* and *S. mitis* have been shown to comprise many distinct clones, with different distribution amongst subjects, however little is known of the impact of clonal diversity on caries activity (Bowden, 1997). However, in some bacterial diseases particular clones are associated with virulence. Therefore, possession of a particular clone by an individual could be related to caries activity. The extent of clonal diversity may also reflect the nature of the oral environment (Bowden, 1997).

1.2. The "*Streptococcus milleri*" group.

1.2.1. Taxonomy.

The taxonomy of the "*Streptococcus milleri*" group has always been confusing. The first reference to the "*Streptococcus milleri*" group (SMG) was made in 1906 by Andrews and Horder, who described *S. anginosus* as a variant of *S. pyogenes* that is specifically associated with sore throats. Since the 1920's the terms "*intermedius*" and "*constellatus*" have been introduced to describe isolates of viridans streptococci that required anaerobic culture (Facklam and Carey, 1985). Long and Bliss, (1934) described *S. intermedius* and *S. constellatus* as "minute haemolytic streptococci", while Guthoff, (1956) was the first to use the term "*Streptococcus milleri* group" to describe a group of non-haemolytic streptococci that were isolated from dental abscesses. On

the basis of a numerical taxonomy study, the first proposal for a "*Streptococcus milleri*" species was made (Colman and Williams, 1972). Closely related streptococci were all named members within a single "*Streptococcus milleri*" species, including all group F streptococci, *Streptococcus MG*, certain non-haemolytic streptococci of groups A, C and G, streptococcal strains possessing Ottens typing antigens and the minute haemolytic colony forming streptococci of groups F and G. This scheme was generally accepted in Europe and by oral microbiologists. However, in America, Facklam, (1977, 1984) proposed an alternative classification scheme and favoured the separation of *S. milleri* into *S. intermedius* (non-haemolytic and lactose fermenting biotype), *S. constellatus* (non-haemolytic and lactose negative) and *S. anginosus* (β-haemolytic). Facklam, (1977) even used the terms *Streptococcus anginosus-constellatus* and *S. MG-intermedius* as a replacement for *S. milleri*. Unfortunately, the existence of two opposing schemes caused considerable confusion in the literature.

However, the groups were subsequently selectively changed. Strains relating to *S. anginosus-constellatus* became *S. constellatus* and strains of *S. MG intermedius* were referred to as *S. intermedius*. Strains of *S. anginosus* taxonomically retained their identity. This method is often applied in the identification of members of the SMG, although there are still disagreements in the scientific community over aspects of SMG taxonomy.

In the 1980's and subsequently, molecular techniques such as DNA – DNA hybridisation, were applied to SMG to seek to clarify the taxonomic relationships. A species is generally defined as a group of strains sharing 70 % or greater DNA – DNA relatedness under optimal conditions (Vandamme *et al.*, 1996). However, the degree of nucleic acid relatedness between strains of SMG reported by different workers remained inconclusive. A number of studies have shown sufficient genetic similarity within the SMG, using DNA homology, to support the inclusion of its members into a single species (Welborn *et al.*, 1983; Yajko and Hadley, 1985). Overall, Coykendall *et al.*, (1987) concluded that isolates of the SMG were sufficiently alike both

biochemically and genetically to support the unification of these streptococci into a single species, named *S. anginosus*. The description *S. anginosus* was chosen, since this term is the oldest approved name describing these bacteria (Andrewes and Horder, 1906).

In addition to disagreements about the taxonomy of the SMG, there is also uncertainty about the possible heterogeneity. Lutticken *et al.,* (1978) and French *et al.,* (1989) found many isolates of SMG to be homogenous in phenotypic tests and DNA-DNA hybridisation (Ezaki *et al.*, 1986; Coykendall *et al.*, 1987). Aluyi and Drucker, (1983) found variation in the whole cell trimethylsilyl-sugar profiles of 39 strains of *S. milleri* from various body sites. Cookson *et al.,* (1989) performed qualitative and quantitative analysis of the cellular fatty acids and found no qualitative difference between 21 strains. However, quantitative data analysis showed three distinct groups.

1. *S. anginosus* and *S. constellatus* were indistinguishable.
2. *S. intermedius* (with a wide fermentation pattern).
3. *S. intermedius* (with a narrow fermentation pattern).

Drucker and Lee, (1983) calculated DNA base pair ratios (mol % guanine + cytosine) for eighteen strains of SMG and found values ranging from 36.6 % - 42 % G+C, indicating genetic heterogeneity. Furthermore, Whiley and Beighton, (1991) found significant heterogeneity in the SDS-PAGE patterns of 23 strains whilst Whiley and Hardie, (1989) demonstrated the clustering of twenty-five SMG strains into four homologous groups following DNA-DNA hybridisation analysis. The three distinct groups were *S. intermedius, S. anginosus* and *S. constellatus* and a fourth group comprised of four strains of *S. intermedius* unrelated to those of the first three groups.

Whiley *et al.,* (1990) used a scheme for the phenotypic differentiation of the three DNA homology groups by enzymatic action on fluorogenic substrates. Using this method, Whiley and Beighton, (1991) amended the existence of

four groups into three distinct groups – *S. intermedius, S. anginosus* and *S. constellatus*. However, the results of a combination of biochemical tests and pyrolysis mass spectrometry (Whinstanley *et al.,* 1992) suggested the existence of five clearly distinct groups. The first three groups compared to the three groups as suggested by Whiley and Hardie, (1989), (*S. intermedius, S. constellatus* and *S. anginosus*) a fourth was shown to comprise of three Lancefield group C β-haemolytic strains, and group five represented a second biotype of *S. anginosus*.

As a direct result of the taxonomic difficulties, the methods used by researchers to identify the SMG into individual species have altered over time. The rapid ID32 system (Anyltab products Ltd) of classification was favourably assessed by Ahmet, Warren and Houang, (1995) when compared with the fluorogenic substrate system of phenotypic identification used by Beighton in 1991 (Table 1.1). Flynn and Ruoff, (1995) showed an 88-98 % agreement between traditional methods and the fluo-card milleri system which consists of a fluorogenic substrate impregnated card. Other researchers have also compared methods of identifying the SMG. Limia *et al.*, (2000) compared three different systems including the API system, fluorogenic substrates, and a genotypic method involving line dot blot hybridisation to identify 180 known streptococcal isolates. In this study it was found that the substrate method developed by Whiley was the most accurate even though not all isolates selected could be confidently identified using this method. Goto *et al.*, (2002) developed a rapid PCR method for the identification of *S. intermedius* based upon the *ily* gene (for intermedilysin) being used as a specific marker as only *S. intermedius* is thought to carry this particular gene. The workers compared this method with the phenotypic methods of API and fluorogenic substrates, and the genotypic methods of line blot hybridisation and southern hybridisation. The researchers found that the PCR method proved successful at identifying *S. intermedius* and was very rapid (cycling at <10 min). However, they pointed out that while this would be useful for rapid clinical identification of the one species, there is no way to identify *S. constellatus* or *S. anginosus* using this or a similar method.

Whiley *et al.*, (1995) examined the 16S-23S rDNA intergenic spacer regions of the genome of SMG strains. Griesen *et al.*, (1994) suggested this as being useful in determining phylogenetic relationships between bacteria, thus aiding strain differentiation. Application of this method reliably distinguished between *S. anginosus* and *S. constellatus*, although differentiation was not possible between *S. intermedius* and *S. anginosus* (Whiley *et al.*, 1995). Jacobs *et al.*, (1996) found that only forty-two of 399 SMG strains phenotypically classified as *S. constellatus* hybridised to the 16S rRNA gene sequence of both *S. constellatus* and *S. intermedius* specific probes. The researchers concluded that this classified these 42 organisms as a distinct type within *S. constellatus*. This finding seems to support Jacobs, (1997) who suggested that although the phylogenetic validity of the current taxonomy has been confirmed there may be much yet to discover.

Table. 1.1. Scheme for identification of viridans streptococci (Beighton *et al.*, 1991).

Enzyme Activity	Streptococcal species														
	A 1	A 2	A 3	B	C	D	E	F	G	H	I	J	K	L	M
β-D-fucosidase	-	+	d	d	-	-	-	-	-	d	+	-	-	-	-
β-N-acetylgal-dase	-	-	-	+	d	+	+	-	-	-	+	-	-	-	-
Sialidase	-	-	-	-	-	-	+	d	-	-	+	-	-	-	-
α-L-fucosidase	-	-	-	d	+	+	-	-	-	-	-	-	-	-	-
β-N-acetylglu-dase	-	d	+	+	+	+	+	-	-	-	+	-	-	-	-
α-glucosidase	-	-	-	+	d	-	+	+	d	d	+	+	-	+	+
β-glucosidase	d	+	d	d	+	-	-	-	-	d	d	-	+	+	-
α-arabinosidase	-	-	-	d	-	-	-	-	+	+	-	-	-	-	-
Acid produced from:															
Amygdalin	-	+	-	d	+	-	-	-	d	d	d	d	+	+	-
Inulin	d	d	d	-	+	-	-	d	-	d	-	-	-	+	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
N-acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	d	d	+	-
Raffinose	+	+	-	d	-	-	d	+	-	d	-	-	d	+	-
Hydrolysis of:															
arginine	+	+	+	+	+	d	-	-	-	-	+	+	+	-	-

A1, A2 and A3, *S. sanguis* biotypes 1, 2 and 3. B, *S. parasanguis*; C, *S. gordonii*; D, "tufted fibril group"; E, *S. oralis*; F, *S. mitis*; G, *S. vestibularis*; H, *S. salivarius*; I, *S. intermedius*; J, *S. constellatus*; K, *S. anginosus*; L, *S. mutans*; M, *S. sobrinus* (data for species I to M from Whiley *et al.*, 1990 and Beighton *et al.*, 1991)

+ , >85 % of strains positive; d, 15-85 % of strains positive; - , < 15 % of strains positive.

1.2.2. Bacteriological characteristics.

The SMG are commensal Gram-positive cocci frequently isolated from the mouth, oropharynx, gastrointestinal tract and vagina. Despite their apparent commensal nature, the SMG have been implicated as being responsible for a variety of infections at a range of body sites.

SMG form minute colonies (<0.5 mm in diameter) on blood agar, and require CO_2 for culture (Kambal, 1987). SMG have a distinct caramel – like odour (diacetyl) when grown on blood agar, which has been suggested by Chew and Smith, (1992) as a possible crude detection method. Gram staining reveals spherical positive cells forming chains or pairs.

Strains of SMG may possess a number of different Lancefield groups. Lancefield grouping was devised as a means of classifying streptococci involving the extraction and identification of cell components known as C substances. A number of C substances exist ranging from A to V and can be identified using the antibody to the substance in an agglutination test. Some streptococci don't posess a C substance, however most possess at least one (Table 1.2). Lancefield groups of SMG include groups A, C, F, and G, but around 50 % of SMG cannot be grouped, thus making Lancefield grouping of little value in identification. SMG are heterogeneous with respect to haemolytic and biochemical reactions. In a study of 80 strains Kambal, (1987) found that 54 % were non-haemolytic, 29 % exhibited β-haemolysis, and 17 % were α-haemolytic. Similarly Jacobs *et al.,* (1995) found 33 % of SMG isolates to be β-haemolytic. Whiley *et al.,* (1990) found that within *S. constellatus* β-haemolysis was a feature only of group F strains. This contrasts with strains of *S. intermedius*, where group F strains are non-haemolytic. Jacobs *et al.,* (1995) found that the majority of group F *S. anginosus* strains were non-haemolytic and most *S. anginosus* were β-haemolytic.

Table 1.2. Streptococcal Lancefield group designations (- = absence of Lancefield antigens).

Streptococcal Group	Species	Lancefield group designation
pyogenic group	*S. equi*	C
	S. pyogenes	A
	S. canis	G
	S. porcinus	E, P, U, V
	S. iniae	-
	S. agalactiae	B
	S. dysgalactiae	C
	S. uberis	E, G
	S. parauberis	E
	S. hyointestinalis	-
"mutans" group	*S. rattus*	-
	S. mutans	-
	S. cricetus	-
	S. sobrinus	-
	S. macacae	-
	S. downeii	-
"bovis" group	*S. bovis*	D
	S. equines	D
	S. alactolyticus	D
"salivarius" group	*S. salivarius*	K
	S. vestibularis	-
	S. thermophilus	-
"anginosus" group	*S. intermedius*	C, F, G
	S. constellatus	A, C, F, G
	S. anginosus	A, C, F, G
"mitis" group	*S. mitis*	K, O
	S. pneumoniae	O
	S. oralis	-
	S. gordonii	H
	S. sanguis	H
	S. parasanguis	F, G, C, B
No group	*S. suis*	-
No group	*S. acidominimus*	-

1.2.3. *"Streptococcus milleri"* Group as commensals.

The SMG are generally accepted as being commensal members of the complex mucosal microflora of the mouth, gastrointestinal, respiratory and genitourinary tracts (Poole and Wilson, 1979). The indigenous nature of the SMG in dental plaque has been widely demonstrated by a number of workers (Yakushiji *et al.*, 1988; Frandsen *et al.*, 1991; Whitworth *et al.*, 1991). The SMG have been isolated from dental plaque, particularly at the protected gingival sites on the tooth (Mejare and Edwardsson, 1975). A lower recovery of SMG strains has been detected at other sites within the oral cavity, such as the dorsum of the tongue and buccal mucosa and in the saliva with only 1 % of the total number of isolates originating from those sites.

The SMG have been isolated from the intestinal tract (Poole and Wilson, 1977), the urinary tract and the vagina (Ball and Parker, 1979). Strains of SMG encountered in urine samples are considered to represent contaminants from the microflora of the vagina or perineum. At the present time no pathogenic role has been suggested for the SMG at these sites (Whitworth *et al.*, 1991).

1.2.4. *"Streptococcus milleri"* Group as pathogens.

The SMG have long been associated with pyogenic infection at a range of body sites, including the gastrointestinal system, heart, lungs, liver, kidneys and central nervous system. The exact mechanisms involved in the initiation of infection involving SMG are unknown, although host factors would appear to be a major determinant as to whether disease ensues. The most frequently reported predisposing factor to SMG infection is disease or trauma to the mucosa (Jacobs *et al.*, 1994). The exact source and routes of tissue entry in the pathogenesis of SMG infection have not been established. Infection due to direct local invasion from the adjacent mucosal surfaces and indirectly via

the blood and lymphatics has been proposed (Unsworth, 1989; Sakamoto *et al.*, 1999). However, in a proportion of patients (19 – 33 %) no predisposing factor can be determined (Murray *et al.*, 1978; Gossling, 1988).

1.2.4.1. Orofacial infection.

Dental caries is the predominant cause of tooth loss in children and young adults (Balakrishnan *et al.*, 2000). The causative agents of dental caries are principally known as the *Streptococcus mutans* group, as shown by early animal studies (Keyes, 1960; Fitzgerald and Keyes, 1960), although other groups such as the "mitis" group are known to be involved. This specific grouping of aetiological agents is known as the 'specific plaque hypothesis' (Balakrishnan *et al.*, 2000). Although often present in dental plaque, the SMG are not regarded as important aetiological agents in dental caries (Gossling, 1988). Experimental studies using gnotobiotic rats have shown the SMG to have only mild or moderate cariogenic properties (Drucker and Green, 1978). Dental caries produced by the SMG in animal models tended to be much less severe than that produced by *S. mutans*, being mainly confined to the enamel.

Guthof, (1956) first described an association of the SMG with dental abscesses and this finding has been confirmed by others (Lewis *et al.*, 1986; Fisher and Russell, 1993). Lewis *et al.,* (1988) observed that SMG were found in mixed populations within abscess sites and tested the ability of supposed pathogenic isolates to induce subcutaneous abscess formation in a mouse model. In these studies it was observed that SMG, whilst not being as evasive of host defences as Gram-negative anaerobic bacilli, still produced an inflammatory abscess harbouring viable bacteria.

Along with caries, periodontal disease accounts for the majority of chronic bacterial diseases in man. Periodontal disease affects the attachment apparatus of teeth (gingival, periodontal ligament). The cause of periodontal

disease has been focussed primarily upon subgingival bacterial flora (Asikainen and Alahuusua, 1993). The causative agents of periodontal disease tend to be Gram-negative anaerobic species such as *Fusobacterium* species, *Prevotella intermedia, Porphyromonas gingivalis* (Edwardsson *et al.*, 1999), *Peptostreptococcus micros*, spirochetes (Asikainen and Alajuusua, 1993), *Actinobacillus actinomycetemcomitans*, and eubacterium (Slots and Dahlen, 1985).

Moore *et al.,* (1982) whilst investigating the bacteriology of periodontitis determined that of the SMG species only *S. anginosus* was found in diseased sites while *S. constellatus* and *S. intermedius* were found more often at healthy gingival sites. Rawlinson *et al.*, (1993) found that the SMG constituted 2 % of all microbes isolated from sites of periodontal disease. In those carrying SMG, the group was amongst the most prevalent in terms of c.f.u / g of plaque. The SMG are frequently isolated in high numbers in association with experimental gingivitis, early periodontitis, severe periodontitis and severe generalised periodontitis (Gossling, 1988; Whitworth, 1990). Determination of a pathogenic role for individual bacterial species within periodontal sites is difficult due to the highly complex nature of the microbial flora, and to date, no specific role for the SMG has been suggested. Peltroche-Llacsahuanga *et al.,* (2000) found that 78 % of the facultative anaerobes isolated from pericoronitis were SMG. As a result of this finding the researchers concluded that SMG must be implicated in the infectious process of this condition. Lutticken *et al.,* (1978) recovered a large quantity of SMG samples from abscess sites around the face.

Strains of SMG are frequently isolated from the pharynx and tonsils in mixed culture with little clinical evidence of infection, and their pathogenic significance in the throat remains unresolved (Fox *et al.*, 1993). However, an association of the SMG with peritonsillar abscesses has been established (Mitchelmore *et al.*, 1995).

SMG have not only been isolated from deep infections of the mouth, but also infections of the facial skin. Buckley *et al.,* (1998) reported a case of a pustular mass present on the chin of a 65-year-old Caucasian male and lesional swabs repeatedly yielded SMG. The patient later presented with squamous cell carcinoma of the mandible. It was suggested that the SMG had invaded through the oral tumour to colonise the skin. Furthermore, Shiga *et al.,* (2001) implicated *S. anginosus* as a causative agent of squamous cell carcinoma. These authors used PCR southern blot analysis to detect *S. anginosus* DNA within sample tumours with success. Tateda *et al.,* (2000) further investigated this work and recovered *S. anginosus* DNA from 100 % of tumours studied, while 33 % of tumours showed the presence of *S. anginosus* by Southern blotting.

Synergy has been proposed as a way in which SMG assume a pathogenic role. Kuriyama *et al.,* (2000) found *S. constellatus* to behave synergistically in the presence of *Fusobacterium nucleatum* and it has been suggested that SMG is thought to prepare the site for subsequent infection by obligate anaerobic species. The majority of SMG infections occur as mixed infections usually in combination with Gram-negative strict anaerobes such as *Prevotella* species, *Fusobacterium* species, *Veillonella* species and *Eikenella* species (Van der Auwera, 1985; Shinzato and Saito, 1995). However, in 34 – 44 % of cases of clinically significant infection the SMG organism is the sole isolate (Molina *et al.*, 1991; Shimono *et al.*, 1992).

1.2.4.2. Bacteraemia and endocarditis.

SMG have been cultured from blood samples of patients with bacteraemia and have been isolated from vegetative growths on the heart valves of patients with endocarditis (Salavert *et al.*, 1996). Bacteraemia can occur with or without an associated focus of suppurative infection or as a consequence of endocarditis (Bert *et al.*, 1998). The isolation of the SMG in blood culture

should always be regarded as clinically significant and prompt a search for the source of infection (Bert *et al.*, 1998).

SMG are a relatively rare cause of bacteraemia having a low incidence of 1.6 –1.8 % in the general hospitalised population (Casariego *et al.*, 1996; Salavert *et al.*, 1996). The majority of these cases involved single species with a suspected gastrointestinal origin of infection, although oral, soft tissue and thoracic sources of infection have also been documented (Casariego *et al.*, 1996). The mortality rates of bacteraemia involving SMG are relatively high ranging from 13 – 15 % (Salavert *et al.*, 1996).

Infective endocarditis may be initiated by bacteraemia from a dental source. Streptococci may enter the circulation following tooth brushing, dental procedures and dental disease (Debelian *et al.*, 1996). It is thought that the SMG account for up to 15 % of endocarditis cases involving oral streptococci. Two of the species of streptococci most frequently isolated from bacterial endocarditis are *S. sanguis*, and *S. mutans* (Herzberg *et al.*, 1990). Indeed, the most frequent cause of bacteraemia from an oral source has been shown to be viridans streptococci (Daly *et al.*, 1997). Gastrointestinal disease or trauma may also precede endocarditis (Gossling, 1988). Concern has been raised regarding the association of SMG with suppurative metastases (Murray *et al.*, 1978; Piscitelli *et al.*, 1992). This link to metastatic infection suggests that the SMG are capable of surviving for prolonged periods within the bloodstream, where they can reach target sites of infection. Various complications have been reported including cerebral embolism, septic arthritis, severe disseminated intravascular coagulation, purulent pericarditis, splenic and myocardial abscess (Whitworth, 1990; Casariego *et al.*, 1996).

1.2.4.3. Other sites of infection.

The SMG has been recognised as a significant cause of purulent thoracic infection. Cases of pulmonary abscess, empyema and mediastinitis have

been reported (Jerng *et al.*, 1997). The oropharynx is generally regarded as the source of SMG infection, which is often preceded by the aspiration of oropharyngeal secretions, thoracic trauma or dental infection. It is not uncommon for the SMG to be isolated in pure culture as well as in combination with strict anaerobes (Porta *et al.*, 1998).

Between 7 – 40 % of all infections involving the SMG have been reported to occur within the abdominal cavity (Gossling, 1988). These infections include abscesses of the liver, pelvic and splenic abscess, peritonitis, infected aortic aneurisms, appendicitis and cholangitis (Edmiston *et al.*, 1991). In most cases, the source of infection is thought to be from within the abdomen via the portal circulation (Whitworth, 1990). A mixed aerobic and anaerobic flora is typical of an intra-abdominal abscess. However, pure growth of SMG has been reported particularly in hepatic abscess (Corredoira *et al.*, 1998). Abdominal infections usually develop as a consequence of gastrointestinal disease, perforation, surgery or inappropriate surgical prophylaxis (Edmiston *et al.*, 1991; Molina *et al.*, 1991; Rautio *et al.*, 2000). Poole and Wilson, (1977) found the SMG to be strongly associated with appendicitis and its purulent manifestations. Rautio *et al.*, (2000) also found the SMG to be present in 61 % of purulent specimens from appendicitis in children.

Subcutaneous abscesses involving the SMG within a mixed flora have been associated with human bites, intravenous drug use and post-operative wounds (Whitworth, 1990). In rare cases, such infections have progressed to severe cellulitis with high morbidity and mortality. The majority of abscesses involving the perianal region, as with infections affecting patients with chronic hidradenitis suppurativa of the neck region, occur as a consequence of prior dental disease (Molina *et al.*, 1991; Piscitelli *et al.*, 1992).

The SMG has a strong association with brain abscess formation and may be isolated either in pure culture or in combination with strict anaerobes (Molina *et al.*, 1991). The most common microorganisms reported in brain abscesses are streptococci (60 %), followed by "bacteroides" (30 %) (Li *et al.*, 1999).

Infection is believed to originate from adjacent foci as in cases of sinusitis and otitis media or by haematogeneous spread from distant purulent foci (Melo and Raff, 1978; Li *et al.,* 1999). Dental infection, lung and liver disease, osteomyelitis, congenital heart lesions or direct trauma have all been shown to precede the formation of a brain abscess in individual cases. The SMG has occasionally been associated with meningitis, which is often preceded, by trauma or purulent infection at a nearby site (Cabellos *et al.*, 1999).

1.2.4.4. Treatment of infection.

The treatment of choice for most streptococcal disease is beta-lactam antibiotic therapy. In recent studies intermediate to high level penicillin resistance in over 40 % of *S. mitis* and *S. salivarius* samples and high level resistance to erythromycin has been found. However, drugs with nearly 100 % susceptibility rates include vancomycin, quinuprisin-dalfopristin, teicoplanin, and telithromycin (Tunkel and Sepkowitz, 2002). The majority of SMG strains are susceptible to penicillins, clindamycin, vancomycin, erythromycin and chloramphenicol (Jacobs and Stobberingh, 1996; Teng *et al.*, 1998). Life-threatening infections due to the SMG are treated empirically with beta-lactam antibiotics, and therefore resistance to penicillin can have fatal consequences (Baran *et al.*, 1998). The antibiotics erythromycin and clindamycin are recommended as alternatives to penicillin in subjects with penicillin hypersensitivity or as a second line agent in cases where penicillin fails (Lewis *et al.*, 1995). However, resistance of viridans streptococci to these drugs has become a concern to physicians as they are the drugs of choice in the face of penicillin intolerance (Matsubara *et al.*, 2001). Resistance to all antibiotic groups with the exception of the glycopeptides and chloramphenicol has been observed in occasional isolates of the SMG (Gomez-Garces *et al.*, 1994). Variable sensitivity of the SMG to tetracyclines and cephalosporins have also been observed (Tillotson and Ganguli, 1984; Aracil *et al.*, 1999), and therefore these antibiotics should only be used with prior knowledge of the organisms' susceptibility. Limia *et al.,* (1999) and Tracy *et al.,* (2001) showed that species identification is unhelpful in deciding

upon a course of treatment, as all three species of the SMG seem to have similar resistance patterns.

The majority of infections involving SMG resolve following local abscess drainage, regardless of treatment with antibiotics (Molina *et al.*, 1991). Repeated drainage and prolonged antibiotic therapy of 28 days or longer may be required in cases of persistent deep-seated sepsis (Jacobs *et al.*, 1994; Casariego *et al.*, 1996). Despite increasing resistance, the penicillin's remain the first line agents for the management of acute suppurative oral infection.

The mechanisms of antibiotic resistance by the SMG have been characterised for several antibiotic groups. The mechanism of penicillin resistance in SMG strains has been shown to be independent of beta-lactamase production (Lewis *et al.*, 1995). Regions of DNA sharing high sequence homology to genes which encode for high-level resistance to streptomycin, kanamycin and erythromycin in *Enterococcus faecalis* have been detected in antibiotic resistant SMG strains (Clermont and Horaud, 1990). A conjugative transposon Tn*916* has been found in the majority of the SMG strains resistant to tetracycline (Clermont and Horaud, 1994). This element was carried on the chromosome and contained antibiotic resistance determinants to erythromycin and minocycline in addition to tetracycline. The genes responsible for tetracycline resistance, *tet*(M) and *tet*(O), have been detected in SMG strains by PCR (Olsvik *et al.*, 1995).

1.3. The study of virulence.

1.3.1. Historical perspective.

A pathogen is a microorganism that is able to cause disease in an animal or plant. Pathogenicity of an organism refers to its ability to inflict damage in a host (Mims, 1987). Virulence is usually regarded as a quantitative term used

to indicate the relative degree of pathogenicity of a specific microorganism. Virulence determinants of a pathogen are the genetic, biochemical, and structural features that enable it to inflict damage on the host (Mims, 1987). Smith and Willet, (1968) defined microbial pathogenicity as 'the biochemical mechanisms whereby microorganisms cause disease'.

The study of virulence of microorganisms has traditionally involved the use of general approaches such as strain screening and animal models of human disease processes. Fitzgerald and Keys used mice and rat animal models to study caries discovering evidence of the involvement of *S. mutans* streptococci (Keyes, 1960; Fitzgerald and Keyes, 1960). However, for many human diseases an appropriate animal model is not available. Increasingly, tissue culture systems such as cell monolayers, organ culture and tissue culture multilayers are proving useful in the study of virulence. However, these systems are *in-vitro* and do not represent the complex interactions between the host and infecting microbe (Quinn *et al.*, 1997). In recent times, genetic and molecular methods are supplementing standard phenotypic techniques significantly facilitating our ability to study virulence.

1.3.2. General approaches.

1.3.2.1. Strain screening.

Standard phenotypic techniques for examining virulence in bacteria include cytotoxin assays, adherence assays and animal models. The basis of phenotypic screening rests upon the knowledge of how a particular strain will react *in-vitro* to a specific test, e.g., adherence of a bacterium *in-vitro* to epithelial cell lines, which can then be translated into an animal model to test the potential for adherence *in-vivo*. The results of these tests can help to determine the pathogenic potential of an organism in a living structure.

1.3.3. Molecular approaches.

The basis of molecular approaches to the study of virulence is to detect preferential gene expression in an environment mimicking that of a host to discover pathogenic properties, or to compare the gene expression of virulent and non-virulent strains of bacteria in a model system. Listed in this section are the methods currently employed in the molecular study of virulence.

1.3.3.1. *In-vivo* Expression Technology.

In-vivo Expression Technology (IVET) allowed for the first time the study of the bacterial response to the host environment *in-situ* using a gene expression scheme within the animal host itself for selection of genes that are specifically expressed during the infection (Handfield and Levesque, 1999). The first IVET methods used promoterless reporter genes whose products conferred a phenotype that could be positively selected in the host (Mahan, 1993). Both auxotrophic and antibiotic selection methods were used.

The auxotrophic method included creating transcriptional fusions of random fragments of the *Salmonella typhimurium* chromosome with a promoterless *purA* gene and introducing this into the chromosome of an *S. typhi purA* strain (Mahan, 1993).

The antibiotic selection method included the addition of a reporter gene that conferred resistance to chloramphenicol. The antibiotic can then be used to select out the strains not conferred with resistance (Chiang *et al.*, 1999).

Advantages include a less labour-intensive method compared to individual loci scanning and the forming of high level expression. Disadvantages include strong discrimination against genes that are expressed *in-vitro* as they may have a profound influence on the nature of the genes isolated

1.3.3.2. Differential fluorescence induction.

Differential fluorescence induction (DFI) is an IVET-based system for the detection of genes induced during infection. It uses green fluorescent protein (gfp) as a reporter of promoter activity. It works on the same principle as IVET but relies upon fluorescent activated cell sorting (FACS) to carry out the selection for active gene fusions (Valdivia and Falkow, 1996, 1997).

The ability to separate cells on the basis of fluorescence enables researchers to identify genes induced in complex and poorly defined environments. This method has been used successfully in bacterial systems including *Escherichia coli* (Badger *et al.*, 2000) and *S. pneumoniae* (Bartilson *et al.*, 2001).

Advantages of this technique include automation for initial screening compared to manual screening; the method allows for study of upregulation of genes under certain conditions as opposed to on / off genes (Handfield and Levesque, 1999) and is sensitive and adaptable (Cotter and Miller, 2000).

1.3.3.3. Signature Tagged Mutagenesis.

Signature tagged mutagenesis (STM) is a comparative hybridisation technique that uses a collection of transposons, each one modified by the incorporation of a different DNA sequence tag (Chiang *et al.*, 1999). Individual members of a pool of bacterial cells are exposed to transposons containing a unique labelled tag sequence. The members make up an array, usually in a microtitre plate. The pooled culture can then be introduced into an animal where infection can take place. The infectious agent can then be collected post-infection. The tag probes present in the bacteria can then be replicated by PCR and hybridised to a membrane. A membrane dot / colony blot is then prepared of the original labelled culture array. Attenuated mutants can be singled out by comparison of hybridisation of the input / output tags to the dot blot membranes. This process detects organisms / cells that are

unable to survive the infection process. The strains can then be recovered from the original array and analysis of the nucleotide sequence flanking the insert can be performed (Walsh and Cepko, 1992).

Signature tagged mutagenesis is a valid technique for determining which genes contribute to survival in the host. However, it is important to consider that genes involved in disease may not be linked to the organism's survival in the host.

1.3.3.4. Subtractive hybridisation.

Subtractive hybridisation involves subtraction based on synthesized cDNAs instead of mRNA. In this manner the technique minimizes RNA degradation leading to greater sensitivity. Target cDNAs from the test cells / tissues are hybridised using a vast excess of driver cDNA (from the control tissue) followed by separation of the double stranded nucleic acid hybrids from the single stranded cDNAs (corresponding to differentially expressed mRNAs) by hydroxyapatite or streptivadin–biotin interaction. The resulting subtracted cDNA is then used as a probe to screen libraries (Vedoy *et al.*, 1999).

Subtractive hybridisation has been used to identify virulence genes in strains of *Mycobacterium tuberculosis* (Kinger and Tyagi, 1993) and *Listeria monocytogenes* (Utt *et al.*, 1995).

Limitations of subtractive hybridisation include the requirement for a large amount of starting RNA– a problem for species for which RNA isolation is difficult, and improved methods need to be developed in order to separate bacterial RNA from contaminating host RNA (Quinn *et al.*, 1997).

1.3.3.5. Differential display reverse transcription PCR.

Differential display reverse transcription polymerase chain reaction (DD RT-PCR) is a technique for the analysis of preferential gene expression that has been applied extensively in eukaryotic systems (Liang and Pardee, 1995). Although modifications to the original concept have been described, the underlying principle of the method is the isolation of undegraded cellular RNA; reverse transcription of the RNA to produce DNA complements of a subset of the mRNA, and eventual generation of cDNA fingerprints using random or arbitrary primers on a matrix medium such as a sequencing grade gel (Figure 1.2). By comparing the banding profiles between model systems, bands of potential interest can be identified and excised from the gel. The sequence within the band can then be compared with those in data banks. Representative transcripts, which are either under-expressed or over-expressed are thus identified and available for further analysis (Liang and Pardee, 1995).

DD RT-PCR has been successfully applied to a number of bacteria. Rindi *et al.,* (1999) employed DD RT-PCR to compare gene expression between virulent and avirulent strains of *M. tuberculosis.* In these studies downregulated differentially expressed products related to polyketide kinase and PPE protein were detected. *Legionella* species were examined for response to intracellular infection of eukaryotic macrophages using a modified differential display protocol, (Kwaik and Pederson, 1996). The studies involved isolating samples prior to and several hours post infection of macrophages and comparing gene expression. A chromosomal *emi* locus (early stage macrophage induced locus) was located prior to infection and was found to be missing 12 hours post infection. The *emi* locus would appear to enable the *L. pneumophila* to survive within the macrophages, thus facilitating infection.

In other studies, *Lactobacillus acidophilus* cells were grown at pH 3.5 and sampled at progressive time intervals. The resultant cells were subjected to

RNA extraction and differential display in order to observe any difference in gene expression that may account for the persistence of the organism in the gastrointestinal tract at low, usually uninhabitable pH's. The study revealed the presence of a 0.7 kb fragment corresponding to the bacterial *atpBEF* gene, which regulates ATPase and cytoplasmic pH. The up-regulation of this fragment was verified by Northern blotting (Kullen and Klaenhammer, 1999).

Fislage *et al.,* (1997) set about designing primers specifically for prokaryotic differential display based upon the fact that bacteria have no polyA tail for the attachment of anchored primers. Random primer sets were designed based upon the properties of the original anchored primers used by Liang and Pardee, (1995). The organisms under study included *E. coli, Klebsiella oxytoca, Salmonella enteriditis, Shigella flexneri, Serratia marcescens, Proteus vulgaris, Citrobacter freundii* and *Enterobacter cloacae.* Different selections of random primers were tested in pairs to assess the quality of the PCR products and the number of visible bands produced.

DD RT-PCR is undoubtedly a useful technique, however, it is not without it's limitations. Problems encountered with DD RT-PCR include low reproducibility of results and a bias for high copy number RNA (Ledakis *et al.*, 1998), co-migration of DNA fragments of a similar size to possible differentially displayed products (Miele *et al.*, 1998), high rate of false positive results (Milson and Burrows, 1998) and a prevalence for reverse transcription of rRNA's above mRNA's (Nagel *et al.*, 1999). However, the advantages of this method include the fact that multiple samples and various sample conditions can be tested simultaneously (Nagel *et al.*, 1999), only small amounts of RNA are required for the reverse transcription step and the duration of the process from start to finish is usually around eight working days making it a relatively rapid technique. Low copy number transcripts are also included in the display (Miele *et al.*, 1998). Many of the problems described above can be circumvented to some extent. Increasing the number and combination of primers used can reduce the incidences of mispriming and lower the frequency of false positives (Ledakis *et al.*, 1998). By increasing the

annealing temperature stringency and decreasing the annealing time the specificity of annealing can be increased and the reproducibility of the display can be reinforced (Malhotra *et al.,* 1998). In order to reduce the incidences of rRNA being amplified above mRNA, the antibiotic rifampicin can be incorporated into the culture media. However, the problem can also be addressed at a later stage by the confirmation of differential bands through Northern blotting (Nagel *et al.,* 1999). It should be noted however that a relatively large quantity of RNA (10-20 μg) is needed to perform Northern blotting (Miele *et al.*, 1998).

In addition to the low level of polyadenylation of mRNA, which potentially prohibits the use of 3' dT anchored primers, other problems with prokaryotic systems include the instability and short half-life of mRNA species in comparison to eukaryotes. For Gram-positive bacteria these problems are further compounded by the difficulties in RNA isolation related to the presence and structure of a thick cell wall.

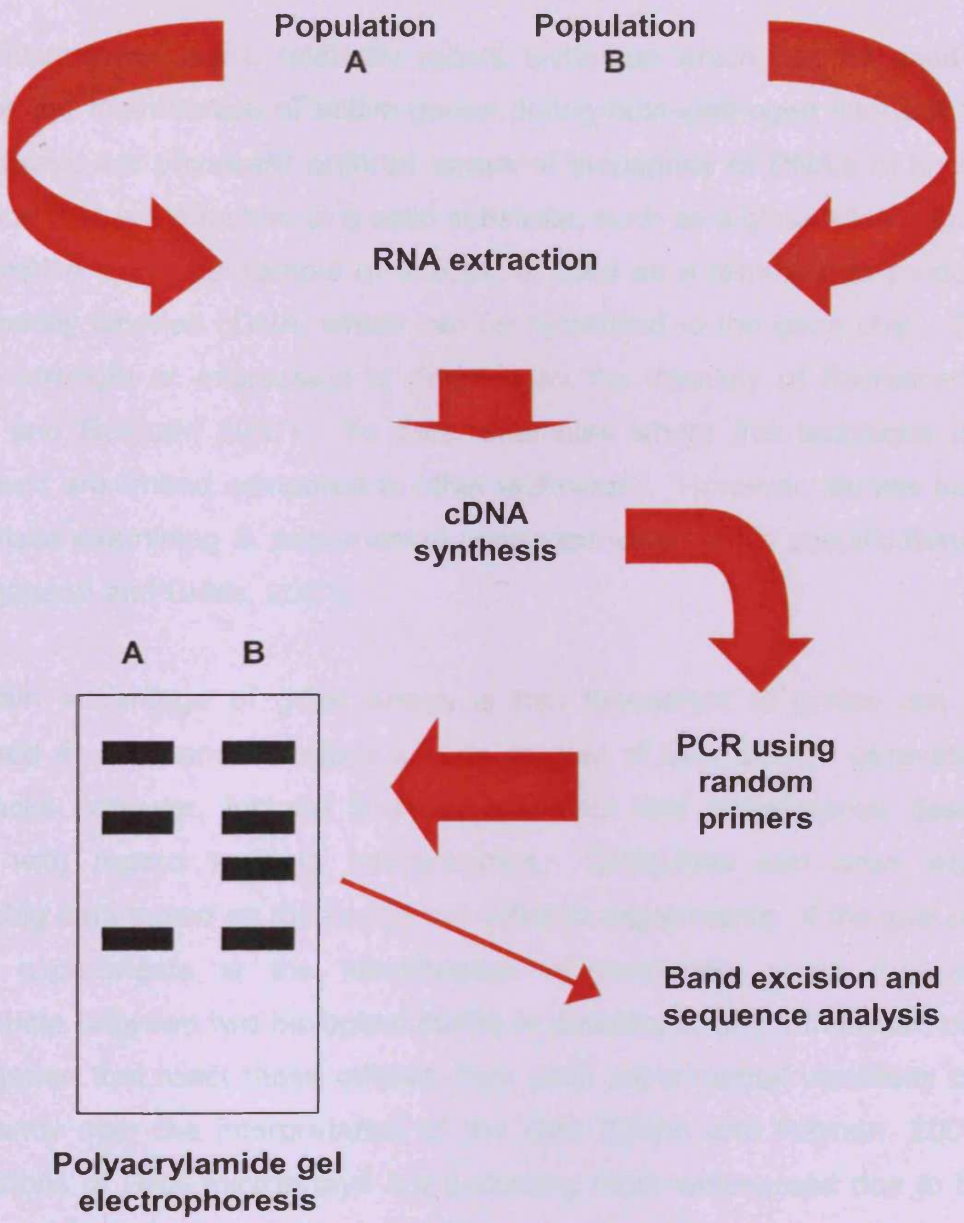

Figure 1.2. Diagrammatic representation of differential display reverse transcription PCR. Populations A and B refer to two groups of identical cells individually subjected to differing environmental stimuli. Differences in banding arrangement post-PCR can be studied by sequence analysis and through bioinformatics.

1.3.3.6. Gene arrays.

Gene microarrays are a relatively recent technique which can be used to facilitate the examination of active genes during host–pathogen interactions. Gene arrays are physically ordered arrays of thousands of DNA's of known sequence that are attached to a solid substrate, such as a glass slide (Figure 1.3). mRNA from the sample of interest is used as a template to produce fluorescently labelled cDNA, which can be hybridised to the gene chip. The relative strength of expression is denoted by the intensity of fluorescence (Diehn and Relman, 2001). To date, examples where this technique has been used are limited compared to other techniques. However, studies have taken place examining *S. pneumoniae* gene expression within specific tissues (Hollingshead and Briles, 2001).

The main advantage of gene arrays is that thousands of genes can be measured at once and therefore a large amount of data can be generated. Drawbacks however, include financial restraints and experimental design issues with regard to data interpretation. Difficulties can arise when comparing data based on the use of two different experiments. If the goal of a set of experiments is the identification of candidate genes that can differentiate between two biological states or conditions, and if there are only a few genes that meet these criteria, then such experimental variations can significantly alter the interpretation of the data (Diehn and Relman, 2001). Applications of DNA microarrays are becoming more widespread due to the rapidity with which the sequences of microorganism genomes are becoming available. Many of the viridans streptococci have been fully sequenced including *S. pyogenes* (Ferretti *et al.*, 2001), *S. pneumoniae* (Tettelin *et al.*, 2001; Hoskins *et al.*, 2001), *S. agalactiae* (Tettelin *et al.*, 2002; Glaser *et al.*, 2002), *S. mutans* (Ajdic *et al.*, 2002), as well as *Lactococcus lactis* (Bolotin *et al.*, 2001). The analysis is *S. pyogenes* has led to the identification of 40 virulence associated genes, and additional genes encoding proteins associated with "molecular mimicry" of host characteristics (Ferretti *et al.*, 2001). *S. pneumoniae* genome analysis has led to the identification of a large

number of new uncharacterised genes predicted to encode proteins that reside on the surface of the cell. These are potential new targets for vaccine and antibiotic development (Hoskins *et al.*, 2001). The analysis of *S. mutans* has revealed virulence genes associated with extracellular adherent glucan production, adhesion, acid tolerance, proteases, and putative haemolysins (Ajdic *et al.*, 2002). The knowledge gained from the entire genome sequences can be employed to develop microarrays, which can be useful in the continuing search for bacterial virulence factors.

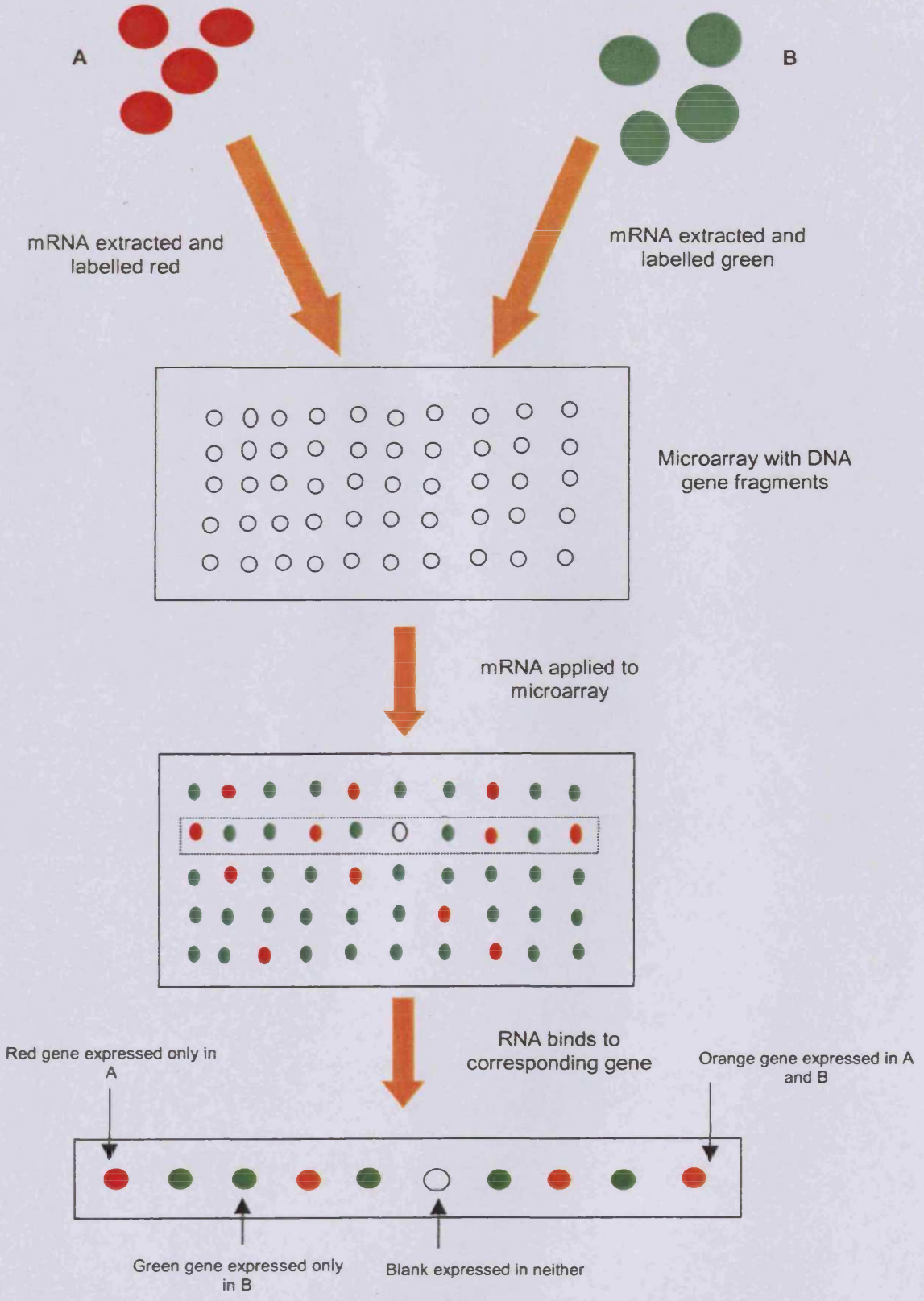

Figure 1.3. Diagrammatic representation of DNA microarrays as used for the analysis of the gene expression of two target cell populations (A and B). Specific colour labelling of mRNA prior to addition to the microarray allows a colour identification of gene expression based upon RNA probe affinity to DNA gene fragments (Modified from the Wellcome trust).

1.4. Streptococcal virulence factors.

Currently, very little is known about SMG pathogenicity, virulence factors possessed by the group and their mode of action. However, in the context of virulence it is interesting to consider the virulence attributes of other related streptococci.

1.4.1. Haemolysins.

Streptococci can cause three different types of haemolysis on blood agar namely α, β and γ. Strains which cause human infection mainly show β-haemolysis. Several enzymes have been identified as being responsible for this.

1.4.1.1. Intermedilysin.

Intermedilysin was first recognised as a cytotoxin responsible for haemolysis caused by the SMG by Nagamune *et al.,* (1996). Intermedilysin was found to be a protein secreted by strains of *S. intermedius* isolated from pyogenic liver abscesses. Structurally it consists of two proteins, 54 kDa and 53 kDa in size, that are active at a temperature range of 10–40 °C, and a pH range of 6-8. Intermedilysin was found to affect the red blood cells of humans, although it also affects cells of other organs such as the liver and the brain, and more importantly has shown itself to cause the lysis of phagocytes contributing to what has been dubbed the "escape factor" (Nagamune *et al.*, 2000). This toxin has been shown to be present only in strains of *S. intermedius* isolated from sites of pyogenic infection, and be absent from other strains of the SMG and other streptococci. Nagamune *et al.,* (2000) has reported that no regulatory mechanism for the toxin has been identified although it has been shown to be inhibited by cholesterol.

1.4.1.2. Streptolysin S.

Streptolysin S (SLS) is the oxygen stable, non-antigenic haemolysin responsible for β-haemolysis around colonies growing on blood agar plates. It is produced by streptococci of Lancefield group A, C, G and others, especially pyogenic strains, but is not known in SMG.

Expression of SLS depends upon interaction between microorganisms and agents such as tween and triton detergents, serum albumin and RNA *in-vitro*. These different inducers give rise to different types of SLS, which act as carrier molecules for the haemolytic moiety, transferring it from one carrier to another (Ginsburg and Harris, 1963). SLS is sensitive to heat and acid and its activity is inhibited by several phospholipids (Elias *et al.*, 1966), zinc ions (Avigad and Bernheimer, 1978), and is sensitive to streptococcal proteinases (Wannamaker, 1983).

SLS has a broader spectrum of action than streptolysin O (SLO), although it is lytic or toxic to all cells that are sensitive to SLO. SLS is also responsible for the leukotoxic properties of group A streptococci, where polymorphonuclear leukocytes (PMNL's) are killed after ingestion of the streptococci (Ofek and Rabinowitz-Bergner, 1972), while lower concentrations inhibit phagocytosis. Unlike SLO, SLS does not create permeations in the membranes of erythrocytes (Hrniewicz and Pryjma, 1977) instead it alters the lipid and / or protein organization in the membrane, which allows free passage of ions. The haemoglobin osmotically draws water into the cell causing it to lyse (Ginsburg, 1999). In animals, infrequent inter-arterial injections of SLS can cause osteoarthritis (Weissmann *et al.*, 1963), whereas frequent injections can cause myocarditis (Ofek *et al.*, 1970). Sublethal concentrations can cause the suppression of mouse peritoneal macrophages (Ofek and Rabinowitz-Bergner, 1972), which suggests that SLS contributes to the invasiveness of streptococci.

Studies on human lymphocytes have shown that T-cells are more sensitive to SLS than B-cells (Ginsburg, 1999).

1.4.1.3. Streptolysin O.

Streptolysin O (SLO) is an oxygen labile, antigenic bacterial toxin and is produced by most group A and some group C and G streptococci, but not SMG. All strains produce an identical toxin (Halbert *et al.*, 1970). Peak SLO production occurs between 6 and 12 hours of incubation, and can be detected in the culture filtrate of Todd Hewitt broth and other complex media. Binding of SLO to cholesterol sites on the host cell membrane is irreversibly inhibited by cholesterol (Hewitt and Todd, 1939).

SLO toxic activity involves several steps: The first stage is the irreversible binding of the reduced form of SLO to membrane cholesterol (Alouf and Raynaud, 1968). After this, permeations in the cell membrane of ~ 500 Å are produced, facilitating in erythrocytes the evacuation of haemoglobin, thus providing a rich supply of iron for the bacterial cells (Ginsburg, 1999).

The toxic effects of SLO have been reviewed in animals by Halbert *et al.*, (1970) and in humans by Hamburger and Lemon, (1953) and Quinn and Singh, (1957). SLO has a number of effects on human cells. It has been shown by Halbert *et al.,* (1961) to be responsible for the pathogenesis of rheumatic fever and for cardiac damage in animals (Halbert *et al.,* 1961). It has also been shown to stimulate light emission in human neutrophils (Anderson and Duncan, 1980), to suppress chemotaxis and mobility of neutrophils (Anderson and Van Epps, 1972), to inhibit phagocytosis by macrophages (Ofek and Rabinowitz-Bergner, 1972) and suppress lymphocyte blast formation (Anderson and Cone, 1974).

1.4.2. Hyaluronidase.

Hyaluronic acid is present in soft tissues such as the gingival tissues and heart valves and contributes to the tissues elastic properties. Hyaluronidase is a putative virulence factor that is responsible for the degradation of hyaluronic acid. Willcox *et al.*, (1995a) reported *S. mitior* and *S. defectivus* to produce the enzyme however, it has also been extensively found in strains of *S. intermedius* and *S. constellatus* isolated from sites of abscess formation. Poole and Wilson, (1979) first showed a positive association between hyaluronidase production in SMG and haemolysis. Hyaluronidase production was most frequent in β-haemolytic strains of Lancefield groups A, C and F and was shown to be reduced in the presence of glucose by Homer *et al.*, (1997). Strains of SMG isolated from systemic abscesses and dental plaque produce type IV hyaluronidase. This observation shows a common link, suggesting a possible oral origin for strains encountered in these abscesses. Unsworth, (1989) showed significant hyaluronidase production in 50-100 % of SMG strains isolated from abscess sites. The sites of deeper infection had a higher frequency of hyaluronidase production than surface sites. In contrast, only 4-52 % of SMG from normal flora sites showed hyaluronidase production. Hyaluronidase has only been found to be produced by *S. intermedius* and *S. constellatus* and is found to have higher activity in the former (Takao *et al.*, 1997), with an optimal activity occurring at pH 5 – 7. Shain *et al.*, (1997) suggested that the role of hyaluronidase was as a proliferator for the SMG in pyogenic infection due to its ability to release carbohydrates useful for bacterial metabolism from the breakdown of the extracellular matrix. The detection of hyaluronidase by SMG is now a supplementary method of identification of the three SMG species and the strong association between hyaluronidase and SMG abscess related strains suggests that the enzyme may have an important role in the disease process.

1.4.3. Sialidase.

Bacterial sialidases are enzymes capable of cleaving terminal non-reducing sialic acid residues from sialoglycoconjugates (Corfield, 1992). The release of sialic acid may act as a virulence factor by exposing cryptic carbohydrate binding sites on host cells to which invading organisms may attach. In turn this may allow the breakdown of the mucosal defence barriers of the host, freeing sialic acid as a fermentable carbohydrate (Gibbons *et al.*, 1990). Sialic acid has been examined in *S. oralis* by Willcox *et al.,* (1995a), and was found to be active at a broad pH range (4–9). Beighton *et al.*, (1990) detected sialidase activity in all three species of the SMG, but mainly in *S. intermedius*. However, they did not detect any activity in many other species of oral streptococci such as *S. parasanguis*, *S. salivarius*, *S. vestibularis*, *S. sanguis*, *S. mutans*, *S. mitis* or *S. sobrinus*.

1.4.4. Chondroitin sulphatase.

Chondroitin sulphatase (CS) is a depolymerising enzyme capable of breaking down the glycosaminoglycan chondroitin sulphate in tissue structures to release carbohydrates that can be utilised metabolically, and contribute to the "spreading" of microorganisms.

Willcox *et al.*, (1995a) found that none of the many isolates from each of the species of viridans streptococci produced CS including the SMG. Homer *et al.,* (1993) found that strains of *S. intermedius* produced chondroitin sulphate depolymerase when the organisms were cultured in the presence of glycosaminoglycan, suggesting that the enzyme is inducible. Jacobs and Stobberingh, (1995) examined the link between the SMG and CS production. They found that only strains of *S. intermedius* and *S. constellatus* isolated from suppurative infection produced the enzyme. Willcox *et al.,* (1995a) failed to find any strain of SMG capable of producing CS. However, Shain *et al.,* (1996) found that *S. intermedius* readily manufactured an A form of the

enzyme when grown in the presence of chondroitin sulphate A. It was also discovered that production of the enzyme was inhibited in the presence of glucose, the latter acting as a more readily catabolisable nutrient.

1.4.5. Nucleases and proteolytic enzymes.

Nucleases (DNase, RNase) are produced by bacteria for defence against other species. Jacobs and Stobberingh, (1995) found that of the SMG only strains of *S. intermedius* and *S. constellatus* seem to produce nuclease enzymes, and DNase is only found in sites of infection. Production of collagenase, gelatinase and RNase / DNase have been observed in SMG (Steffen and Hentges, 1981; Marshall and Kauffman, 1981). However, many other researchers have routinely failed to detect nuclease activity in the SMG. The *Streptococcus sanguis* group and the SMG have been shown to produce proteolytic activity (Homer *et al.*, 1990).

Strauss and Falkow, (1977) detected a strain of *S. intermedius* from infective endocarditis that produced several extracellular enzymes with proteolytic activity. Growth of this strain in conditions mimicking a fibrotic heart lesion resulted in a 4–8 fold increase in proteinases compared with controls. There is evidence to suggest that bacterial proteases are able to subvert protein cascade systems and turn them against the host's own tissues (Lanz, 1997). *S. pyogenes* is able to produce an extracellular cysteine protease capable of doing the above. Group A, C and G streptococci produce a cell surface bound plasminogen protease which can destroy basement membranes (Lanz, 1997).

1.4.6. Synergy.

In episodes of clinical infection it is usual to isolate SMG alongside an obligate anaerobe. The idea of synergy between strains of SMG and anaerobic bacteria in infectious disease has been investigated by Shinzato and Saito, (1994), who infected mice with a mixture of *S. constellatus* and *Prevotella intermedia*. When infected individually, the mice showed only 10 % mortality compared to 60 % when both organisms were present. Evidence also suggests that the anaerobe acts to boost the growth rate of the *Streptococcus* species by acting as a barrier to any bactericidal action from the host. This was first suggested by Mergenhausen *et al.,* (1985) who performed a growth enhancement experiment in a gangrene model using *S. intermedius* and *S. aureus* showing a higher growth rate in mixed infection. Lewis *et al.,* (1988) showed that SMG are often isolated alone, even after inoculation as part of a mixed culture. In clinical practice this situation may occur as a result of bacterial success or due to antibiotic treatment. The antibiotic of choice in the case of anaerobic infection is often metronidazole, but this has no activity against SMG. Synergy has also been shown with other pathogenic streptococci. The group A streptococcus *S. pyogenes* is the principle cause of pharyngitis and has been shown to act synergistically with obligate anaerobic β-lactamase producing species in order to reduce the effectiveness of antibiotic treatment. The anaerobe acts by protecting the *S. pyogenes* strain from the action of penicillin based drugs, allowing colonisation and infection of the throat to continue (Mihajlovic-Ukropina and Roncevic, 1998).

1.4.7. Capsules.

Capsules are a well-established virulence factor. Encapsulated bacterial cells are known to have increased resistance to cellular phagocytosis and offer a degree of protection against serum antibodies when in the host. A small number of SMG have been isolated with capsules, although their structure is as yet unknown. The structure of capsules of the group A streptococci is

known to be composed of hyaluronic acid, a linear polymer of glucuronic acid and N-acetylglucosamine repeating units (Moses *et al.*, 1997). The presence of capsules in the group A and B streptococci is well documented, Kallman *et al.*, (1998) reported the impairment of phagocytosis and opsonisation in group B capsulated streptococci during neonatal infection. It was reported that the virulence was primarily due to the presence of the capsules sialic acid determinants which act to impede the alternative way of complement activation. Schrager *et al.*, (1996) showed that capsulated group A streptococci, while being impeded at epithelial cell invasion were able to produce extensive local necrosis of skin tissue in mouse models whereas uncapsulated cells could not. It may however be a detriment to the survival of the encapsulated cell during infection that invasive capabilities are limited, since gaining access to the intracellular compartment allows protection from complement proteins, specific antibodies and phagocytic leukocytes.

Brook and Walker, (1985) documented that encapsulated SMG strains induced pyogenic abscesses when injected into mice, while strains without capsules required the presence of a capsulated organism to do so. Lewis *et al.,* (1988) also demonstrated that encapsulated SMG strains cause abscess formation in pure culture, and demonstrated the isolation of encapsulated strains from dentoalveolar abscesses. Lewis *et al.,* (1993) studied the influence of the capsule on phagocytosis, and showed that the presence of a capsule did not decrease phagocytosis of the bacterial cells.

1.4.8. Interaction with the host immune system.

Surface structures and streptococcal extracellular products have been found to have immunoprotective properties. The M-protein in group A streptococci has been shown to have an antiphagocytic role (Raeder *et al.*, 2000). Antigen I /II, found in most streptococci (including SMG) has been shown to display antibody suppressive traits (Jenkinson and Demuth, 1997). Streptococcal pyrogenic exotoxin has shown immunolethality in experimental animals

(Leonard and Schlievert, 1992). Evidence has emerged to show that strains of SMG have the ability to produce extracellular products with the ability to reduce the effectiveness of the immune system. Higerd *et al.,* (1978) was the first to identify an extracellular product named EP–Si. This protein was originally isolated in a crude form, and found to suppress fibroblast formation and altered lymphocytic responses *in-vivo*. Arala–Chaves *et al.,* (1979) expanded on this by examining the extract and finding an immunosuppressive non–cytotoxic protein of 90 Kd in size named F3' EP–Si. The authors found that the protein could induce T–cell suppressor lymphocytes and also possessed B–cell mitogenic activity (Arala–Chaves *et al.*, 1981). The protein was renamed P 90 by Limia *et al.,* (1992) who performed tests using mice to determine its activity. The authors showed that treating mice with P 90 made them 50-fold more vulnerable to infection with strains of *S. intermedius*. B–cells obtained from these mice were less able to respond to antigenic challenge. Furthermore, T–cells were found to actively suppress the specific immune response produced by B–cells. This finding may not be an entirely accurate picture of events however, since the authors used a much higher concentration of P 90 than *S. intermedius* would actually be able to produce.

Toyoda *et al.*, (1995) found a virulent strain of *S. constellatus* isolated from infectious conditions. This was found to exert an inhibitory effect upon phagocytic killing when compared with cells of a commensal origin.

1.4.9. Surface associated properties.

The majority of research on the surface properties of the SMG has been in the area of adherence and surface binding. Willcox and Knox, (1990) studied the characteristics of the SMG that are possibly associated with pathogenicity and compared them to type strains of other streptococci. They found that abscess related strains adhered to buccal epithelial cells to a greater extent than commensal strains, giving adherence values similar to those of *S. salivarius*, which shows preferential attachment. The SMG were not readily aggregated

by human saliva and exhibited low levels of binding to hydroxyapatite, which correlates with their low population levels in plaque. The ability of the SMG to bind fibrinogen is low in comparison to other species (SMG binding is 2 % of isolates whilst *S. pyogenes* binding is 22 % of isolates). However, this is still a considerable amount and may contribute towards avoidance of the host defence mechanisms. Yang *et al.,* (1988) found that SMG strains isolated from sites of infection managed to bind fibronectin to a greater capacity than other strains of commensal origin. Willcox *et al.,* (1995b) characterised a fibronectin receptor from *S. anginosus* strain F4. The receptor was found to be a 14 kDa protein, and exhibited specific binding. The cell surface was found to contain 770 such attachment sites. Other species of oral streptococci are well known for their binding abilities. *S. mutans* and *S. mitis* groups have been extensively studied with regard to their ability to adhere to the salivary pellicle and form plaque biofilms (see section 1.1.2) (Jacques, 1994). Oral streptococci express a family of structurally and antigenically related surface proteins termed antigen I/II which are capable of binding to soluble extracellular matrix glycoproteins and to host cell receptors. They are also responsible for coaggregation with other microbes, interaction with salivary glycoproteins and activation of monocytic cells (Petersen *et al.*, 2001). Antigen I/II as well as being found in cariogenic streptococci has been found in other species, particularly of note is *S. intermedius* (Jenkinson and Demuth, 1997).

As well as antigen I/II, glucosyltransferases are responsible for the attachment of cariogenic oral streptococci to the tooth surface through the production of glucans. While glucans and glucosyltransferases are not surface structures, the glucan binding proteins (present on the surface of the cell) enable attachment of the extracellular products and consequently allow the adherence of the cell to the tooth surface. While SMG are found to have the essential binding proteins present in their cell wall, they exhibit no glucosyltransferase activity (Kato and Kuramitsu, 1991)

When it comes to pathogenic streptococcal attributes *S. pyogenes* displays a rare ability to bind to and invade epithelial cells using a novel fibronectin-binding protein. Fibronectin binding plays an important role in infection as it mediates specific and non-specific interactions between bacterial surface components and epithelial cell receptors (Terao *et al.*, 2001).

Lutticken *et al.,* (1978) observed antigens on the surface of SMG strains isolated from pyogenic infections. An M–protein function was suggested. Handley *et al.,* (1985) studied M–protein fimbrial structures on the surface of oral strains of SMG. These fimbrial structures are typically found on *S. pyogenes*. In group A streptococci they are found to have antiphagocytic properties (Raeder *et al.*, 2000), although their role in SMG is not known.

Strains of *S. anginosus* have been shown to exhibit a gliding type of motility as demonstrated from spreading on culture media; this was found to be attributed to the overproduction of glycocalyx (Bergman *et al.*, 1995).

Cell surface hydrophobicity can affect the likelihood of the cell undergoing phagocytosis during immune attack (Absolom, 1988). Wilcox and Knox, (1990) found that the SMG vary in hydrophobicity with no relation to site of isolation or to species. Willcox, (1995) also noticed that an increase in hydrophobicity correlated with an increase in binding to fibronectin.

The aggregation of platelets in the blood is important in the induction and progression of endocarditis and in abscess formation, where bacteria are localized in a platelet / fibrin clot. Willcox *et al.,* (1994) noticed that Lancefield group C members of the SMG aggregated human platelets mediated by cell surface proteins. The aggregation was however reduced by disruption of platelet membrane stability. Binding was found to be due to a cell surface protein as a result of treatments with proteinases, lipases and antisera to Lancefield group C antigens. The effect was still apparent after treatment to remove cell surface structures leaving only proteins, however the effect was found to be reduced upon treatment with proteinases. Willcox, (1995) found

that strains of SMG produced thrombin activity. Thrombin can convert fibrinogen to fibrin to produce blood clotting. The SMG may produce clotting to seal off areas of systemic colonisation and can lead to a platelet / fibrin clot, effectively producing a pyogenic abscess structure. This serves to offer protection from the circulating host defences.

1.5. Bacterial typing methods.

Bacterial typing is the separation of a related group of microorganisms into subgroups based upon biochemical and biological differences, which may be structural or genetic in origin (Singleton and Sainsbury, 1993). Traditionally, typing of bacterial species has been used in routine microbiology for the characterisation and identification of disease-causing microorganisms and in outbreak situations to elucidate the origin and route of transmission of an infecting organism. However, bacterial typing has also become a useful tool in the research laboratory, since the data collected can provide information regarding the physiological and ecological nature of individual strains (Vanndamme *et al.,* 1996). Traditional phenotypic methods, although still of value in some microbial systems are often labour intensive, slow to provide results and may not always be robust due to variable gene expression (Sader *et al.*, 1995).

1.5.1. Phenotypic approaches.

Phenotypic techniques are based on the detection of an expressed characteristic unique to an individual strain of microorganism (Maslow and Mulligan, 1996). This group of techniques has become the generally accepted way of typing microorganisms. However, such systems are limited by the ability of bacteria to alter the expression of a particular characteristic under examination in an unpredictable way. In addition, the methods are limited since some strains may not have obvious identifying phenotypic

characteristics, and consequently cannot be typed by a given phenotypic approach. (Maslow *et al.*, 1993).

1.5.1.1. Biochemical characteristics (Biotyping).

Biotyping involves the analysis of a series of biochemical reactions, the results of which can be summarised as a specific numerical code for identification (Maslow and Mulligan, 1996). The method looks at the pattern of activity of approximately 20 metabolic enzymes (Maslow *et al.*, 1993). Such an approach is considered highly reliable and the detection of multiple isolates of an unusual species can effectively identify an outbreak (Maki *et al.*, 1977). However, depending on the species under study the discriminatory power may be poor and the technique may not be able to distinguish individual strains due to changes in gene expression and random mutations (Maslow and Mulligan, 1996). Tenover *et al.,* (1994) found biotyping to be ineffective at grouping known outbreak related strains of *S. aureus* due to the identification of multiple subtypes.

1.5.1.2. Resistance to antimicrobial agents.

Differences in antimicrobial susceptibility among strains of the same species can be used as a method for typing (Maslow *et al.*, 1993). Tenover *et al.*, (1994) found that although this technique was not clearly superior to any other testing method, it was still relatively reliable and accurate. Kariuki *et al.,* (1999) used antimicrobial susceptibility alongside phage typing and Pulsed field gel electrophoresis (PFGE) in the epidemiological typing of *Salmonella enterica*. Unfortunately, problems arise with this method of typing when multiple resistance patterns are encountered. Therefore, the technique is only reliable when individual strains show reproducible results and retain their profile from one test to the next (Maslow and Mulligan, 1996). The environment under which individual strains are tested is critical for expression

of resistance. Furthermore, due to the ease with which antibiotic resistance genes can be exchanged between bacteria, the results may be difficult to interpret.

1.5.1.3. Antigenic factors (Serotyping).

Serotyping is based on antigenic factors and is one of the first forms of typing used for bacteria. The method relies on the presence of antigenic determinants expressed on the surface of microorganisms which differ between strains of a given species (Maslow *et al.*, 1993). Serotyping is a rapid and reproducible approach to the typing of microorganisms that has been widely applied to medically important microbes, for example, *E. coli* 0157:H7 (Maslow and Mulligan, 1996). However, not all bacteria are amenable to typing using this method.

1.5.1.4. Susceptibility to phage infection.

The basis of phage typing is the characteristic susceptibility patterns of bacterial isolates to individual types of bacteriophage. Phage types have been discovered that are specific for several species of bacteria (Blair and Carr, 1960). The technique has been applied to the typing of *Salmonella enterica* (Kariuki *et al.*, 1999) and *S. aureus* (Tenover *et al.*, 1994; Bannerman *et al.*, 1995). The method has been compared with other more contemporary techniques and found to be satisfactory by Tenover *et al.,* (1994) and unsatisfactory by Bannerman *et al.,* (1995) when compared to PFGE. Phage typing was found to have low reproducibility and an inability to type a high percentage of samples was reported. The method is technically demanding and its use is mainly restricted to reference laboratories. Furthermore, some bacterial species cannot be typed using this method (Maslow and Mulligan, 1996).

1.5.2. Genotypic approaches.

1.5.2.1. Plasmid analysis.

Plasmids are extrachromosomal genetic elements, the number and size of which in a given strain can be used for characterisation purposes. Related isolates tend to have the same number and type of plasmids and therefore yield the same plasmid profile (Sader *et al.*, 1995). Plasmids can be isolated from bacterial cells and analysed following electrophoresis on agarose gels. Discrimination between organisms is enhanced with the use of restriction enzymes to digest plasmids prior to electrophoresis (Williams *et al.*, 1999). Plasmid profiling was the earliest DNA technique to be applied for epidemiological studies (Sadowski *et al.*, 1979). The technique has been used successfully in the typing of *S. aureus* (Tenover *et al.*, 1994). Advantages of the technique include its rapidity and ease of use, (Williams *et al.*, 1999). Limitations of the technique include the absence of plasmid DNA in many bacterial species. Conformational difficulties can also arise due to differences in plasmid structure, giving discrepancies between gel runs (Archer *et al.*, 1985).

1.5.2.2. Whole genome analysis.

1.5.2.2.1. Restriction endonuclease analysis.

Restriction endonuclease analysis (REA) is a method based on estimating differences in nucleotide sequence of different chromosomes indirectly. The method involves the digestion of chromosomal DNA with restriction enzymes, such as *EcoR* I, *Hind* III and *BstN* I, which are "frequent cutting" enzymes. The fragments produced are separated by electrophoresis usually on agarose. Banding patterns often specific for a given strain can be compared.

Related isolates will display similar patterns, whereas unrelated isolates will not. Any differences between related isolates may be accounted for by sequence rearrangements and insertion or deletion of DNA (Sader *et al.,* 1995). REA typing has been employed with success for a wide range of medically important bacteria, including enterococci (Savor *et al.,* 1998). The technique is relatively inexpensive but the complex DNA profiles can be difficult to interpret under some circumstances due to overlapping of unresolved bands (Savor *et al.,* 1998). Southern blotting is a modification of REA typing involving the detection of a subset of restriction fragments by the annealing and subsequent detection of labelled nucleic acid probes. Whiley *et al.,* (1995) studied this method as a possible typing method for the SMG, but found that it was of little benefit for individual species of the SMG when compared to more simple phenotypic tests. The technique is labour intensive with many steps involved, a lot of good quality DNA is required and in general a number of restriction endonucleases must be examined for optimal discrimination.

1.5.2.2.2. Pulsed field gel electrophoresis (PFGE).

Pulsed field gel electrophoresis (PFGE), which was developed in the 1980's (Schwartz and Cantor, 1984), achieves the separation of large fragments of DNA by the application of an alternating electric field to an agarose gel (Figure 1.4). Chromosomal DNA is digested by restriction enzymes that cut infrequently and thus produce between 5 and 20 large fragments (Sader *et al.*, 1995). PFGE has been widely used and is considered by many to be the 'gold standard' for epidemiological typing due to its high discriminatory potential (Mendez-Alvarez *et al.*, 1995). However, PFGE is more expensive than conventional typing methods due to the cost of specialist equipment required. The procedures involved in the extraction and treatment of the genomic DNA from the isolates are time consuming, technically demanding and the optimisation of the running and electrophoresis conditions can be troublesome.

Adjustment of each step of PFGE, depending on the organism under investigation can influence the success of the method. The steps of PFGE are considered below.

The first stage of PFGE is DNA extraction. The main method employed for DNA extraction is the one described by Maule, (1998). This approach requires a two-day incubation with proteinase K, and is therefore time consuming. Quicker methods for DNA extraction of bacterial cells have been suggested. Matushek *et al.,* (1996) shortened the incubation times for cell lysis and proteinase K digestion allowing the extraction procedure to take place in a day. However, this may not be acceptable depending upon the organism in use. Bartie *et al.,* (2000) modified this for use with streptococci with the addition of mutanolysin to the extraction solution, which facilitated the breakdown of the streptococcal cell wall.

In order for the genomic profile to be displayed, it must be restricted using one of a number of restriction enzymes. Prior to this however, the plugs must be pure and free of M ethylenediaminetetra-acetic acid (EDTA) and proteinase K from the digestion, since these agents would inhibit any enzyme activities. Maule, (1998) advised the use of phenylmethylsulphonylflouride (PMSF), which is a potent protease inhibitor and causes inactivation of the proteinase K, followed by multiple rinses in Tris-EDTA (TE) buffer. The restriction digest should take place after incubation of the plug in the appropriate buffering solution provided with the enzyme. Restriction itself involves incubation of between 4 h and overnight. Matushek *et al.*, (1996) incubated plugs for 2 h at 25 °C in a rapid digestion and overnight at 25 °C in an extended protocol, and found that neither method was superior. Bartie *et al.,* (2000) favoured the use of PMSF, followed by multiple TE buffer rinses prior to equilibration of the plugs in distilled water. The elongated method of digestion was used (overnight at 25 °C) with *Sma* I and *Apa* I enzyme to good effect.

Once the DNA has been restricted it must be separated using electrophoresis. This is possibly the most technically demanding step of the process (Pfaller *et al.*, 1994), since it requires strict optimisation of pulse time and voltage.

Pulse time refers to the interval at which the polarity of the current running the gel is altered to allow the DNA fragments to linearize (Olive and Bean, 1999). The pulse time, also known as switchover time, varies from organism to organism depending upon the size of the fragments being separated. A long pulse time allows better separation of larger fragments that take longer to reorient themselves in the gel matrix, whereas a short pulse time allows the separation of smaller fragments ahead of larger ones. Bartie *et al.,* (2000) determined that a pulse time of 5-35 s was optimal for SMG.

A relationship between resolution and voltage (in V/cm) exists for the separation of large fragments of DNA. Higher voltages give greater migration, but at the cost of resolution of the bands (Maule, 1998) and possible total loss of some fragments. However, a higher voltage serves to reduce the run time, which in turn reduces the exposure to higher temperatures and likelihood of exposure to damaging nucleases. It is clear that a compromise has to be made between voltage and resolution, whereas temperature can be controlled (the optimal being ~ 14 °C). Bartie *et al.,* (2000), reported that a voltage of 6 V/cm for 20 h was optimal, while controlling the temperature at between 4 °C and 10 °C, for SMG.

Advantages of PFGE include the fact that large molecules of DNA (up to 12 Mb) can be separated on one gel allowing the entire genome to be displayed (Maule, 1998) in a highly reproducible and discriminating manner (Matushek *et al.*, 1996). Unfortunately, PFGE is technically demanding and expensive to perform (Bannerman *et al.*, 1995). In addition, interpretation of banding profiles can be difficult; although criteria for strain typing using this method have been suggested (Tenover *et al.*, 1995).

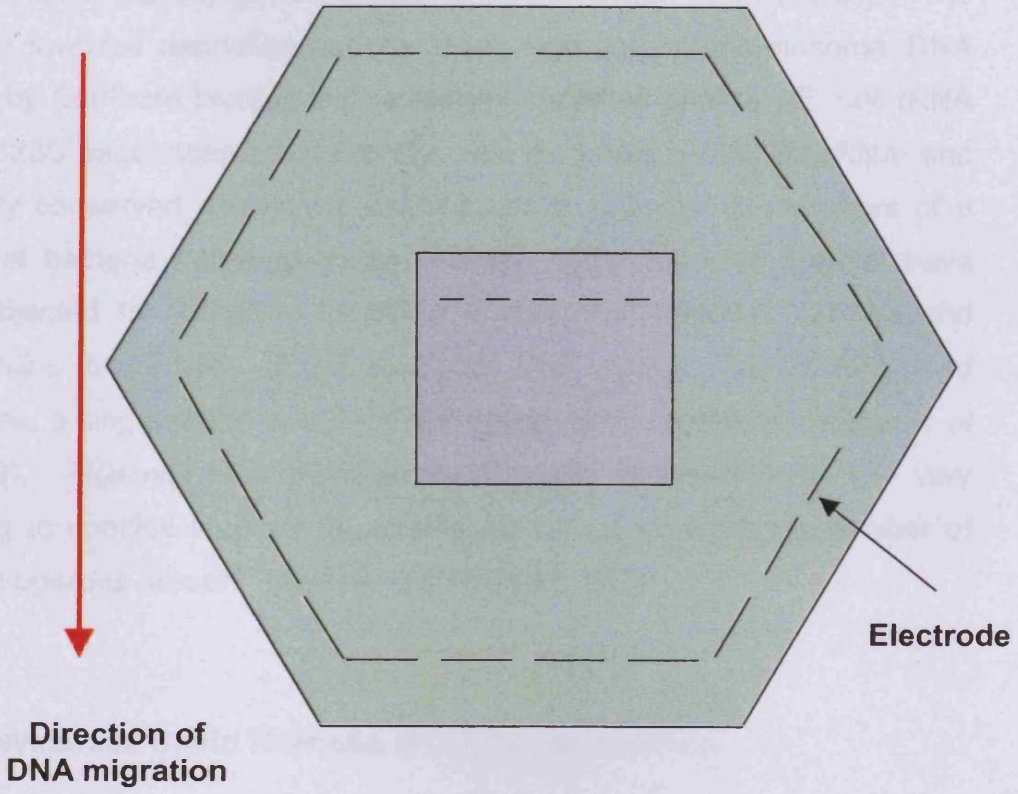

Figure 1.4. Schematic representation of Pulsed field gel electrophoresis using a CHEF (contour clamped electric field) system. The gel can be seen sitting in the centre of a hexagonal array of electrodes, which cause migration of DNA through the creation of a homogenous electric field.

1.5.2.3. Ribotyping.

Ribotyping is an approach that makes use of restriction polymorphisms within ribosomal DNA (rDNA) genes encoding for ribosomal RNA (rRNA). The technique involves restriction endonuclease digestion of chromosomal DNA followed by Southern blotting with a range of labelled probes. *E. coli* rRNA 16S and 23S sequences are generally used as genes coding for rRNA, and are highly conserved allowing a single probe to subtype all members of a species of bacteria (Vaneechhoutte, 1996). Many bacterial species have been subjected to ribotyping including *E. coli, Pseudomonas cepacia* and *Haemophilus influenzae*. Since ribosomal DNA contains highly conserved sequences, a single probe is suitable for typing many organisms (Williams *et al.*, 1999). However, the discriminatory power of the method will vary according to species studied, depending on factors such as the number of ribosome operons present (Maslow and Mulligan, 1996).

1.5.3. Polymerase Chain Reaction (PCR) based methods.

PCR based typing methods are the most frequently employed form of typing in research, and are becoming more prevalent in the epidemiological lab. These techniques involve the production of multiple copies of areas of the genome using primers designed specifically for the region under study (Figure 1.5). DNA based typing relies upon the fact that isolates of the same strain produce the same typing pattern when displayed on electrophoresis gels, and epidemiologically unrelated isolates have distinctly different patterns (Sader *et al.*, 1995). The use of PCR provides one of the most convenient and rapid methods for typing (Kerr, 1994). DNA may be amplified by the selection of primers specific to conserved regions of the bacterial genome. The primers anneal to the DNA and produce multiple copies in the presence of DNA polymerase. Differences between strains may be detected by analysis of the banding profiles following electrophoresis of PCR products. The disadvantages of PCR based typing include the fact that variations in the final

profile can occur due to imperfections in the method of DNA extraction or due to differences in the conditions of electrophoresis. In addition, there can be difficulty in the analysis of complex profiles. It can also be difficult to compare results between laboratories and methods due to a lack of standardisation of methodology and interlab variation particularly where low stringency primers are used (Sader *et al.*, 1995). However, methods that rely on the indirect detection of DNA sequence polymorphisms have been shown to be the most reproducible, discriminatory and universally applicable (Kerr, 1994).

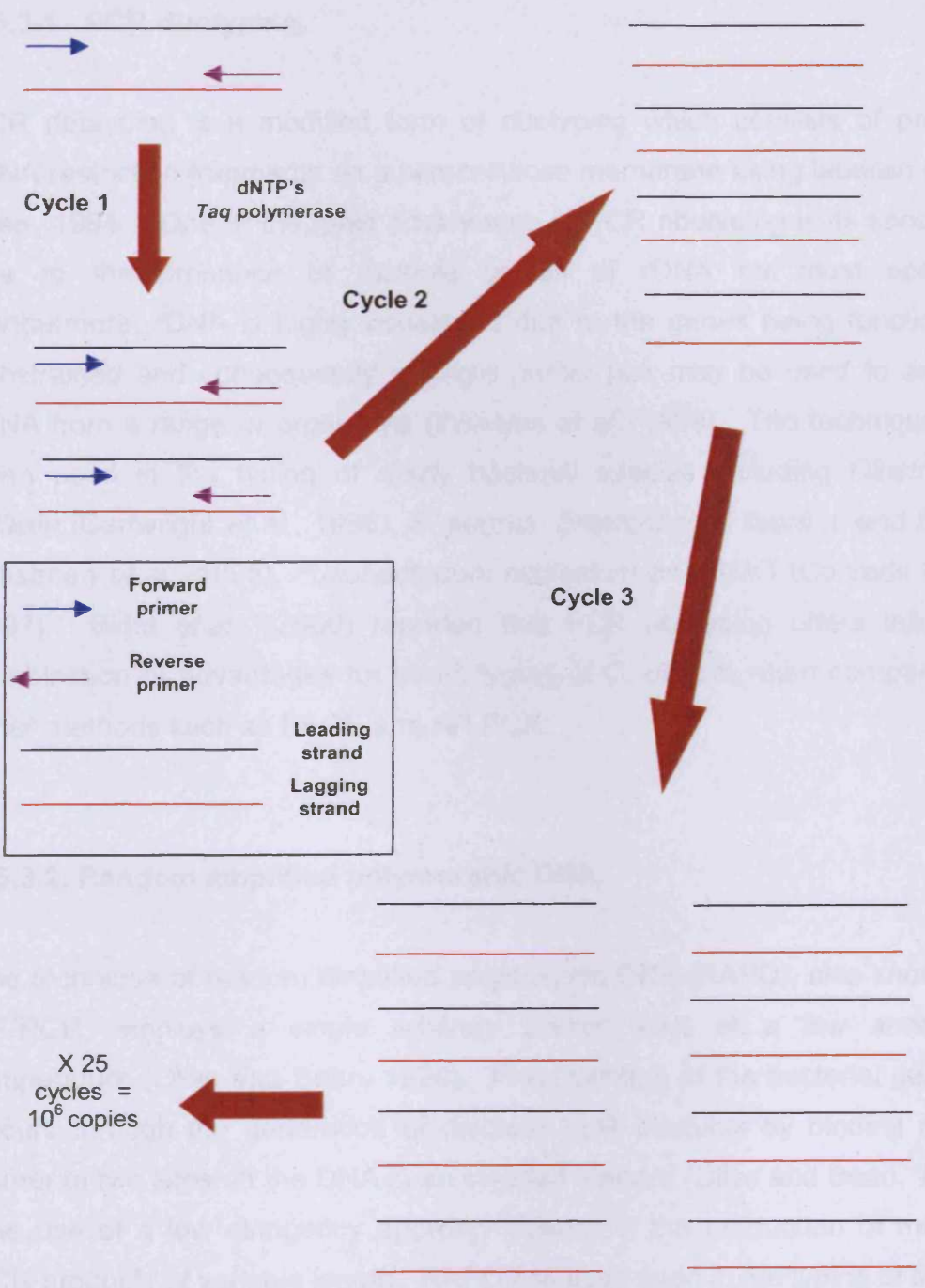

Figure 1.5. Diagrammatic representation of the polymerase chain reaction (PCR). The diagram demonstrates forward and reverse primers being used to highlight regions of the genome on the leading and lagging DNA strands. Using *Taq* DNA polymerase, dDNTP's and successive heating using a thermocycler, many copies of the specific region can be produced. The number of DNA copies produced after each cycle increases exponentially, allowing a sufficient number to be produced so that visualisation can be performed using electrophoresis media.

1.5.3.1. PCR ribotyping.

PCR ribotyping is a modified form of ribotyping which consists of probing rDNA restriction fragments on a nitrocellulose membrane using labelled rRNA (Kerr, 1994). One of the main advantages of PCR ribotyping is its sensitivity due to the presence of multiple copies of rDNA for most species. Furthermore, rDNA is highly conserved due to the genes being functionally constrained and consequently a single primer pair may be used to amplify rDNA from a range of organisms (Williams *et al.*, 1999). The technique has been used in the typing of many bacterial species including *Clostridium difficile* (Cartwright *et al.*, 1995), *S. aureus*, *Enterococcus faecium* and *E. coli* (Kostman *et al.*, 1995), *Fusobacterium nucleatum* and SMG (Conrads *et al.*, 1997). Bidet *et al.*, (2000) reported that PCR ribotyping offers the best combination of advantages for strain typing of *C. difficile* when compared to other methods such as PFGE and AP-PCR.

1.5.3.2. Random amplified polymorphic DNA.

The technique of random amplified polymorphic DNA (RAPD), also known as AP-PCR, employs a single arbitrary primer used at a low annealing temperature (Olive and Bean, 1999). Fingerprinting of the bacterial genome occurs through the generation of discrete PCR products by binding of the primer to two sites on the DNA in an inverted manner (Olive and Bean, 1999). The use of a low stringency approach results in the production of multiple PCR products of variable length. RAPD has been used in the typing of strains of *Acinetobacter* species (Koelman *et al.*, 1998) and *Pseudomonas stutzeri* (Sikorski *et al.*, 1999). Considerable advantages of this technique include the fact that no previous knowledge of the organism's genome is required and it is a relatively simple technical method (Williams *et al.*, 1999). However, lack of reproducibility has been reported due to the susceptibility to variation by primer and DNA concentration, DNA template quality, gel electrophoresis and the type of DNA polymerase used (Koelman *et al.*, 1998). As with other

arbitrary approaches reproducibility between different laboratories is also a problem (Olive and Bean, 1999).

1.5.3.3. Inter-repeat PCR.

The application of inter-repeat PCR (IR-PCR) is based on the design of primers complementary to interspersed repetitive sequences (Figure 1.6). This form of PCR based fingerprinting can be classified into groups depending on the nature of the target DNA sequence. Although potentially there are many targets for primers, four sets of primers have been used frequently for bacterial typing (enterobacterial repetitive intergenic consensus, ERIC; repetitive extragenic palindrome, REP, BOX and M13). Although originally detected in enteric bacteria, repetitive PCR typing based on the presence of repetitive sequences within the genome is also suitable for a wide selection of genera (Versalovic *et al.,* 1991). Recently, REP-PCR and ERIC-PCR have been applied to the genotyping of the SMG (Alam *et al.*, 1999; Clarridge *et al.*, 1999). The resultant DNA patterns were found to be unique for each strain, although strains of a given species did not yield similar patterns so species identification was not possible. The length of the primers also enables higher annealing temperatures and therefore higher stringency. Van Belkum *et al.,* (1996) included strains of *S. intermedius*, which were subjected to PCR typing using BOX A, BOX B and BOX C primers. However, these workers found that none of the BOX primers gave rise to amplimers. Clarridge *et al.,* (1999) used primers Rep1R-I and Rep2-I, in a study solely involving "*Streptococcus milleri*" group species. Good results were obtained with a low percentage similarity between isolates demonstrated.

Inter-repeat primers have proven successful in the typing of a range of bacterial species, including many types of streptococci. M13 primers have been used to type *S. mitis*, *S. oralis* and *S. salivarius* (Wisplinghoff *et al*, 1999). BOX primers, although discovered within *S. pneumoniae*, have not been successfully used to type other closely related species.

The advantages of IR-PCR include rapid preparation and completion times (Woods *et al.*, 1993), the use of DNA that has undergone minimal preparation and the ability to use standard laboratory equipment (Alam *et al.*, 1999). Since the primers are designed to be complementary to endogenous interspersed repetitive bacterial sequences they enable a non-arbitrary approach to primer design and use of a limited set of primers with a wide variety of bacteria. Since the primers are relatively long in length (18-22mers) it is possible to use higher annealing temperatures that enable greater stringency in the PCR (Sader *et al.*, 1995).

Disadvantages of IR-PCR include uneven band intensities making interpretation and reproducibility difficult, and problems with interpretation of banding profiles due to the lack of guidelines for analysis (Grundmann *et al.*, 1997). Even with the use of a computer analysis program, it is still reliant upon a degree of subjective interpretation as to the profile (Bartie *et al.*, 2000).

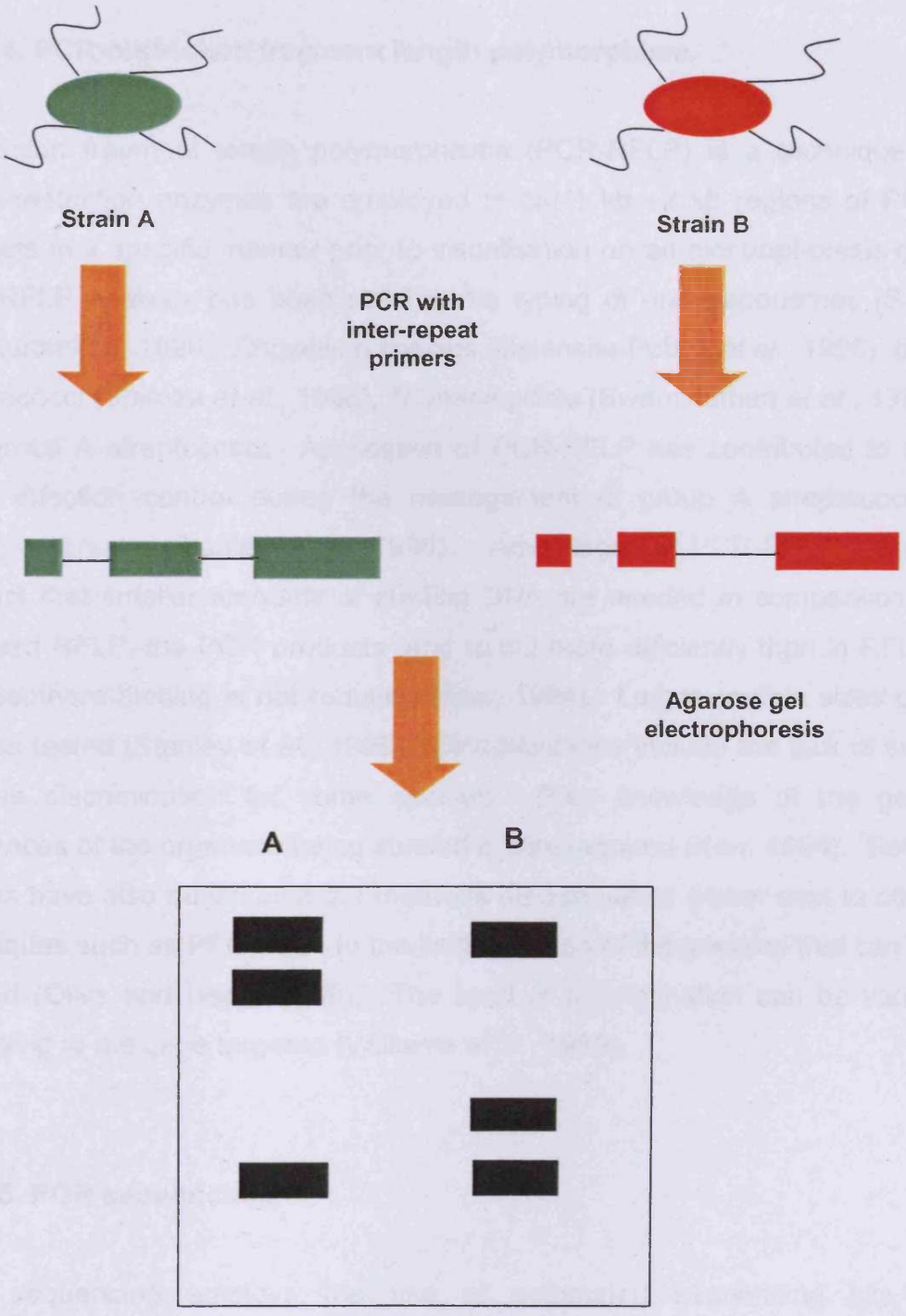

Figure 1.6. Diagrammatic representation of inter-repeat PCR. The diagram shows two individual bacterial strains (Strain A and B). The strain's DNA with the inter-repeat fragments present (coloured blocks) undergoing PCR amplification can be seen. The PCR products run out on an agarose gel show a unique fingerprint for each strain composed of different sized fragments. This is produced due to the unique sizes of the inter-repeat fragments present on the strains genome. Strain A can be seen to have two large fragments and a small fragment, where strain B has a large fragment and two small fragments.

1.5.3.4. PCR-restriction fragment length polymorphism.

Restriction fragment length polymorphisms (PCR-RFLP) is a technique in which restriction enzymes are employed to cut 1 kb –2 kb regions of PCR products in a specific manner prior to visualisation on an electrophoresis gel. PCR-RFLP analysis has been used in the typing of oral treponemes (Sato and Kuramitsu, 1999), *Rhizobium* species (Selenska-Pobell *et al.*, 1996), oral streptococci (Shiroza *et al.*, 1998), *N. meningitidis* (Swaminathan *et al.*, 1996) and group A streptococci. Application of PCR-RFLP has contributed to the cross infection control during the management of group A streptococcal (GAS) outbreaks (Stanley *et al.*, 1996). Advantages of PCR-RFLP include the fact that smaller amounts of starting DNA are needed in comparison to standard RFLP, the PCR products tend to cut more efficiently than in RFLP, and Southern blotting is not required (Kerr, 1994). Larger sample sizes can also be tested (Stanley *et al.*, 1996). Disadvantages include the lack of sub-species discrimination for some species. Prior knowledge of the gene sequences of the organism being studied is also required (Kerr, 1994). Some studies have also questioned the methods discriminatory power next to other techniques such as PFGE due to the limited region of the genome that can be studied (Olive and Bean, 1999). The level of discrimination can be varied according to the gene targeted (Williams *et al.*, 1999).

1.5.3.5. PCR sequencing.

PCR sequencing employs the use of automated sequencing kits in conjunction with a PCR reaction to incorporate labelled di-deoxynucleoside tri-phosphates (ddNTP's) into the PCR products. The extension of the product is halted by the incorporation of the labelled nucleotide producing a band for each base in the sequence. This approach is considered to be the most sensitive method for detection of polymorphisms in PCR products (Kerr, 1994). Although this method is highly sensitive and reproducible, its use is limited due to the strict nature of the area to be studied. The area in question

must consist of a variable region flanked by highly conserved regions, but must also be variable enough to be able to detect different strains of a species (Olive and Bean, 1999). Although it is a rapid method, it is still a relatively expensive technique.

1.5.3.6. PCR-single strand conformational polymorphism analysis.

Single stranded conformational polymorphism (SSCP) analysis is based upon the altered mobility of DNA fragments when run in a polyacrylamide gel depending upon how the folding structure of a strand of DNA will be affected by the sequence (Hayashi, 1991). PCR products are typically labelled with fluorescent or radioactive nucleotides and separated on a polyacrylamide gel with a denaturing gradient. The bands are then detected using autoradiography or a fluorescent alternative. SSCP has been used with *E. coli* (Maxam and Gilbert, 1980). The method can be highly discriminatory and it is possible to detect a single base substitution within a DNA fragment.

1.5.3.7. Amplified fragment length polymorphism.

Amplified fragment length polymorphism (AFLP-PCR) analysis involves the digestion of genomic DNA with two restriction enzymes. The restricted sites are then ligated with adaptor DNA sequences. A PCR is then performed using radiolabelled primers, which are specific for the adaptor fragments. The resulting PCR products are separated on a polyacrylamide gel and are detected by autoradiography (Janssen *et al.*, 1996). AFLP-PCR has been used to type *Vibrio cholerae* (Jiang *et al.*, 2000a), *Pseudomonas* species (Geornaras *et al.*, 1999) and *Acinetobacter* species (Koelman *et al.*, 1998). The method is rapid, requiring only two working days to complete, is ideal for fingerprinting a large number of strains simultaneously, and can be performed without prior sequence knowledge (Jiang *et al.*, 2000b). It has been suggested that the method should be highly reproducible due to the

stringency of the PCR conditions. However, technical difficulties including a lack of reproducibility dependant on DNA and primer concentration, and variable PCR conditions have been reported (Koelman *et al*, 1998).

1.6. Summary.

The SMG are commensal, Gram-positive bacteria of great clinical relevance due to their presence in systemic infection and deep-seated sepsis at a variety of body sites including the heart (Salavert *et al.*, 1996), liver (Edmiston *et al.*, 1991) and dentoalveolar region (Lewis *et al.*, 1988). The taxonomy of the SMG has been a source of much debate (Whiley and Hardie, 1989). This taxonomic confusion is due mainly to their marked heterogeneity and genomic diversity that has been demonstrated by Drucker and Lee, (1983) and more recently Bartie *et al.*, (2000). This has made their identification from oral sites difficult. Perhaps as a direct consequence of this confusion only a few studies of the ecology of SMG in the oral cavity have been performed. These have been restricted to the distribution of the three species in different sites in the mouth. Only a few studies have been reported in which strains of SMG are subjected to typing. A discriminatory and reproducible method of typing is required for the SMG prior to any detailed analysis of oral ecology.

The marked heterogeneity exhibited by the SMG, in addition to generating difficulties in taxonomy and identification, is of interest in itself. Population studies can facilitate understanding of the nature of this group of commensal bacteria which are nevertheless pathogenic. Although molecular epidemiological studies suggest that horizontal gene exchange is the main cause of bacterial diversity (Gupta and Maiden, 2001), it is possible that intragenomic recombination may also be of importance (Harrington *et al.*, 1997). However very little work of this nature has been undertaken and no streptococci have been examined to date.

Only a little is known about the pathogenicity of the SMG. For example, *S. intermedius* can produce intermedilysin (Nagamune *et al.*, 1996), *S. intermedius* and *S. constellatus* are able to produce chondroitin sulphatase (Jacobs and Stobberingh, 1995) and nucleases (Jacobs and Stobberingh, 1995) and all species have been shown to produce sialidase (Beighton *et al.*, 1990), and hyaluronidase (Unsworth, 1989). Synergy would seem to be a factor contributing to virulence (Shinzato and Saito, 1994) as is the presence of a capsule (Brook and Walker, 1985). Surface associated properties of the SMG showed that clinical strains possessed greater binding abilities than commensal strains (Yang *et al.*, 1988) and SMG have been shown to possess antigen I/II polypeptides for binding (Jenkinson and Demuth, 1997), however, they have no glucosyltransferase production capabilities (Kato and Kuramitsu, 1991). The thrombin conversion of fibrinogen to fibrin has been observed in SMG strains (Willcox, 1995). This could enable the production of a platelet/fibrin clot which could lead to abscess formation. However to date the properties studied rely on the analysis of phenotypic properties which are subject to variability in expression. The study of a number of bacterial systems has demonstrated that a more productive line of research might be the analysis of preferential gene expression (Mahan *et al.*, 1993; Badger *et al.*, 2000; Utt *et al.*, 1995; Rindi *et al.*, 1999; Kwaik and Pederson, 1996). Of the molecular methods available at this time DD RT-PCR, would appear to be the best method for the analysis of differential gene expression of the SMG.

CHAPTER II: GENERAL MATERIALS AND METHODS

2.1. Standard agarose gel electrophoresis.

A 1.5 % standard agarose gel was prepared in a 5 X Tris-Borate EDTA (1 X TBE; 89 mM Tris-HCl [pH 7.4], 89 mM boric acid, 25 mM EDTA [pH 8.0]) buffer (Sigma, UK). After heating, the agarose was cooled to 55 °C prior to the addition of 10 μl of ethidium bromide at 500 μg/ml. The gel was poured into a casting tray and allowed to set at room temperature. PCR products (5 μl), and 4 μl DNA loading dye VI (Life Technologies, UK) were loaded into the wells of the gel and a 100 bp DNA ladder (Life Technologies, UK) was loaded at either end of the gel. The gel was subjected to electrophresis in 300 ml of 0.5 X TBE buffer at 80 V for 1.5 h. Analysis was performed using UV light and Gel Doc 1000 molecular analyst software version 1.5 (Bio-Rad, UK).

2.2. Modified agarose gel electrophoresis.

A modified version of the agarose gel electrophoresis method involved the production of a 2.0 % agarose gel using 1.0 % molecular grade agarose, and 1.0 % Nusieve agarose (Flowgen, UK) in a volume of 200 ml. This method employed a larger casting tray (25 x 35 cm). The PCR products (10 μl) were loaded together with 4 μl of DNA loading dye VI, and a 100 bp DNA step ladder (1 μl) was loaded every 8th lane. After loading, the gel was subjected to electrophoresis in pre-chilled (4 °C) 0.5 X TBE at 175 V for 4 h at a temperature of 4 °C prior to analysis using UV light and the Gel Doc 1000 software.

2.3. DNA extraction from SMG.

2.3.1. Heat extraction.

DNA was extracted from test strains of SMG as detailed by Alam, (1999). A 5 µl loopful of 'wet' cells was harvested from a blood agar plate that had been incubated at 37 °C for 18 h anaerobically in an atmosphere of 20 % Hydrogen, 10 % Carbon dioxide and 70 % Nitrogen and the sample resuspended in 300 µl of nuclease free water prior to vortexing for 20 s. The suspension was immersed in a waterbath at 100 °C for 10 min. Following centrifugation at 15,000 X g for 10 s the supernatant was stored at –20 °C.

2.3.2. "Chelex" extraction.

A 5 µl loop of bacterial cells were harvested from blood agar plates previously incubated for 18 h in an anaerobic chamber. The wet cells were placed directly into 20 µl of a 5 % Chelex-100 DNA extraction suspension (Bio-Rad, UK). The suspension was heated to 100 °C for 12 min then centrifuged at 12,000 X g for 15 min prior to the supernatant being stored at -20 °C.

2.3.3. Phenol:chloroform / isoamyl alcohol extraction.

DNA extraction was performed as described by Keay *et al.*, (1998). A 5 µl loopful of cells, harvested from a blood agar plate following incubation at 37 °C for 18 h in an anaerobic chamber was suspended in 0.4 ml of digestion buffer (5 M NaCl, 2 M Tris-HCl [pH 8.0], 0.5 M EDTA [pH 8.0], 10 % SDS, 10 mg/ml Proteinase K) and incubated at 50 °C for 18 h in a waterbath. An equal volume of phenol:chloroform / isoamyl alcohol (25:24:1; Sigma, UK) was added and the solution centrifuged at 11,000 X g for 10 min. A 0.5

volume of 7.5 M ammonium acetate (Sigma, UK) and two volumes of absolute ethanol was added to the supernatant, followed by centrifugation at 11,000 X g for 10 min. The pellet was washed twice with a 75 % solution of ethanol prior to lyophilisation and resuspension in 20 μl of nuclease free water. The pellet was dissolved by heating at 65 °C for 10 min and the resulting extract solution stored at – 20 °C.

2.4. Pulsed field gel electrophoresis.

2.4.1. DNA preparation.

The genomic DNA of SMG strains was prepared in agarose plugs according to the rapid DNA extraction procedure described by Matushek *et al.,* (1996) and subsequently modified by Bartie *et al.,* (2000). All biochemicals used in the macrorestriction study were purchased from Sigma unless otherwise stated. A 5 ml portion of an overnight bacterial culture in BHI broth was harvested by centrifugation. Cells were resuspended in 0.5 ml of 2 X lysis solution (12 mM Tris-HCl [pH 7.4], 2 M NaCl, 20 mM EDTA [pH 7.5], 1.0 % Brij, 0.4 % sodium deoxycholate, 1.0 % sodium lauroyl sarcosine) containing lysozyme at 1.0 mg/ml, mutanolysin at 10 U/ml and RNase A at 20 μg/ml and mixed with an equal volume of 2.0 % low-melting-point agarose (Bio-Rad, UK) tempered at 56 °C. The agarose mixture was placed in 100 μl plug moulds (Bio-Rad, UK). Following solidification, the resulting agarose blocks were then incubated sequentially in the following solutions: 3 ml 1 X lysis buffer (6 mM Tris-HCl [pH 7.4], 1 M NaCl, 10 M EDTA [pH 7.5], 0.5 % Brij, 0.2 % sodium deoxycholate, 0.5 % sodium lauroyl sarcosine) containing lysozyme at 0.5 mg/ml, mutanolysin at 5 U/ml and RNase A at 20 μg/ml for 2 h at 37 °C; 3 ml of proteinase K buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) to which proteinase K at 100 μg/ml and 1.0 % sodium dodecyl sulphate (SDS) was freshly added before incubation for 1 h at 50 °C; 5 ml of dilute TE (10 mM

Tris-HCl [pH 7.4], 0.1 mM EDTA) for 1 h at 50 °C. The plugs were washed three times in 5 ml of TE buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF; stock solution freshly made at 100 mM in absolute ethanol) for 1 h at room temperature. The agarose blocks were finally washed in 5 ml of TE buffer and stored at 4 °C until restriction digestion.

2.4.2. Restriction digestion of DNA.

A 5 mm slice of agarose plug was cut using sterilised coverslips (Chance Propper Ltd., Warley, UK) and equilibrated in DNase free water for 1 h at 4 °C. Restriction digestion was then performed using 10 U *Sma*I in 100 μl of appropriate buffer (New England Biolabs UK Ltd., Hitchin, UK) according to the manufacturer's instructions. Each digestion was carried out for 18 h. Following the digestion, the plug slices were placed in dilute TE for 1 h at 4 °C before loading into the gel.

2.4.3. Pulsed field gel electrophoresis.

The restricted gel slices were loaded into a 20 well 1 % pulsed field certified agarose gel in 0.5 % TBE (Bio-Rad, UK). Typically, each PFGE gel consisted of 17 DNA sample lanes. A bacteriophage concatemer with 48.5 kb increments (Bio-Rad, UK) was used as a molecular size marker and was loaded in the outermost and middle lane in each electrophoresis run to facilitate band alignment. The macrorestriction fragments were separated using a contour-clamped homogeneous field electrophoresis apparatus (CHEF-DRII; Bio-Rad). PFGE was performed in 0.5 X TBE buffer at 4 °C. The pulsed field parameter for *Sma*I was 20 h at 6 V/cm (200 V) with switch times ramped from 5 - 35 s. Following electrophoresis, the gel was stained in 300 ml distilled water containing 0.5 μg/ml ethidium bromide and destained in distilled water for at least 3 h. The DNA bands were visualised under

ultraviolet illumination and the gel image captured and digitised by Molecular Analyst software.

CHAPTER III: DIFFERENTIAL GENE EXPRESSION OF *S. ANGINOSUS*

3.1. Introduction.

The "*Streptococcus milleri*" group (SMG) of bacteria have been found at specific sites of purulent disease, both in pure culture and in association with other organisms, often strict anaerobes (Lewis *et al.,* 1988). The SMG appear to be intimately associated with the disease process at a number of body sites. It is thought that strains of SMG are not only capable of inducing infection themselves but also create favourable environments for the proliferation of other potential disease-causing organisms (Shinzato and Saito, 1994). Although a range of virulence factors have been studied to date, there is a significant lack of understanding of the mechanisms involved (Shinzato and Saito, 1994).

SMG are frequently isolated in association with strict anaerobes (Shinzato and Saito, 1994). In a murine orofacial model the virulence of *S. constellatus* was shown to increase during co-infection with *Fusobacterium nucleatum* (Kuriyama *et al.*, 2000). Strains of SMG have been shown to reduce the oxygen concentration at sites of abscess formation, thus lowering the REDOX potential (Shinzato and Saito, 1994). In addition, *in-vitro* studies have shown that the facultative anaerobe *S. aureus* has a growth enhancing effect upon *S. intermedius* (Mergenhagen *et al.*, 1985).

Thrombin-like activity has been demonstrated in strains of *S. constellatus*. Thrombin coverts fibrinogen to fibrin causing the coagulation of plasma, which tends to encapsulate infected sites via a platelet-fibrin clot, thus protecting infecting bacteria from the host's immune system (Willcox *et al.*, 1994). In some cases, the treatment of purulent infection involving mixed anaerobes may produce an environment that increases the pathogenic potential of SMG. Metronidazole, a drug used for treatment of anaerobic abscesses, has been shown to have little effect upon some strains of SMG, and may even enhance their pathogenicity (Tresadern *et al.*, 1983).

The production of factors by the SMG that inhibit phagocytosis and degrade surrounding tissues has also been studied. Strains of SMG have been shown to produce hyaluronidase (Whiley *et al.*, 1990), collagenase, gelatinase and DNase (Marshall and Kauffman, 1981).

The adherence of SMG to a number of substances has also been investigated. Willcox and Knox, (1990) tested the ability of SMG to bind to hydroxyapatite, buccal epithelial cells, fibrinogen and fibrin, although the mechanism of adhesion is currently unknown. This study reported that strains recovered from abscesses showed significantly increased binding to buccal epithelial cells in comparison to commensal strains. The ability to bind to hydroxyapatite, fibronectin and fibrinogen was also found in small numbers of strains. Fibronectin binding protein has been discovered to be more frequently found in isolates of SMG of a systemic origin (Willcox and Knox, 1990). The platelet binding ability of SMG appears to be related to possession of the Lancefield group C polysaccharide, present in some strains (Willcox *et al.*, 1994).

The possession of capsular material may be an important factor in virulence. Gossling, (1988) reported that strains of SMG possessing a capsule were capable of producing an abscess when injected in pure culture into an animal model when uncapsulated strains needed the presence of another organism to produce a similar effect.

In the study of virulence factors there has been a trend towards attempting to study microorganisms under conditions which mimic the *in-vivo* situation. For example, a technique has been developed that uses patients sera to identify microbial genes that are expressed during an actual human infection. The technique is called *in-vivo* induced antigen technology (IVIAT) (Lee, 2000). Secondly, whilst the phenotypic analysis of presumed virulence factors is important, the importance of the study of gene expression is also being realised. Genes associated with virulence may be switched on or off under different environmental conditions. Recently, methods have become available

that facilitate the detection of preferential gene expression under different environmental conditions such as *In-vivo* expression technology (IVET), differential fluorescence induction and signature tagged mutagenesis. However, these methods only permit the study of previously characterised genes in environmental situations rather than the study of up / down regulation and the discovery of previously recognised genes that are induced under specific environmental situations (see section 1.3.3).

Differential display reverse transcription polymerase chain reaction (DD RT-PCR) is a technique widely used for the analysis of preferential gene expression in eukaryotes, and more recently, in prokaryotes (Kullen and Klaenhammer, 1999; Rindi *et al.,* 1999; Bonass *et al.,* 2000; Chia *et al.*, 2000; Du and Kolenbrander, 2000). The principle of the method is the isolation and reverse transcription of undegraded cellular RNA and the subsequent generation of fingerprints using random or arbitrary primers by electrophoresis of cDNA fragments on a matrix medium such as a sequencing grade gel. Comparisons can be made between the RT-PCR profiles of model systems, and differentially expressed bands, which can be excised from gels and subjected to sequence analysis. Rindi *et al.,* (1999) employed DD RT-PCR in the comparison of gene expression of virulent and avirulent strains of *Mycobacterium tuberculosis* leading to the discovery of polyketide kinase expression which is thought to play a part in *M. tuberculosis* infection or disease. *Legionella* species have been examined by Kwaik and Pederson, (1996) and genes involved in the response to intracellular infection of eukaryotic macrophages identified. In this work gene expression was compared prior to and several hours post infection. A chromosomal locus was identified which appears to enable *L. pneumophila* to survive within macrophages.

The use of DD RT-PCR by Kullen and Klaenhammer, (1999) revealed the presence of a 0.7 kb fragment in *L. acidophilus*, responsible for the regulation of ATPase and cytoplasmic pH. Bonass *et al.,* (2000) identified an operon (*ragAB*) in strains of *Porphyromonas gingivalis*, which was shown to be

regulated according to the temperature within periodontal pockets. Chia *et al.*, (2000) incubated *S. mutans* under a range of environmental conditions and performed DD RT-PCR using random primers. These workers found both up and downregulation of GSP-781 (General stress protein) and AP-185 (Acid stress protein) under conditions of high temperature, osmolarity and acid shock. Du and Kolenbrander, (2000) performed random amplified polymorphic PCR (RAP-PCR) using a modified differential display method on strains of *S. gordonii* and found upregulation of salivary agglutinin glycoprotein-binding proteins SspA and SspB following incubation in saliva. Chakerbortty *et al.,* (2000) applied RAP-PCR to strains of *Vibrio cholerae* grown in a rabbit ileal loop model. These strains showed increased motility compared to *in-vitro* grown bacteria. It was found that the strains grown *in-vivo* had an upregulated *MurE* gene encoding for a protein making the cells less susceptible to lysis within the gut.

Problems encountered with the technique of DD RT-PCR include the abundance of rRNA in comparison to mRNA's resulting in a high rate of false positive results (Milson and Burrows, 1998; Nagel *et al.,* 1999). Additional problems with prokaryotic systems include the instability and short half-life of mRNA species, the low level of polyadenylation of mRNA, and difficulties in isolating RNA from thick cell walled Gram-positive bacteria.

Fislage *et al.,* (1997) designed primers specifically for prokaryotic differential display by testing them on a variety of bacterial species including *E. coli, Salmonella enteriditis* and *Enterobacter cloacae*. Different random primers were tested in pairs to assess the quality of the PCR products and the degree of genomic coverage.

The environmental conditions under which bacterial cells are incubated is known to be an important factor in the control of cellular gene expression. Conditions that have been found to effect preferential gene expression include heat shock (Hurme and Rhen, 1998), pH, osmolarity (Badger and Kim, 1997) and iron concentration (McKee *et al.*, 1986). Differences in these conditions

are likely to be experienced by SMG in the oral cavity. Temperature is intimately associated with gene regulation, causing DNA supercoiling and changes in RNA conformation (Hurme and Rhen, 1998). Changes in *E. coli* growth temperature have been shown to induce the transcription of a number of proteins (Yamamori *et al.*, 1978).

In this context the aim of this study was to devise a means of studying the preferential gene expression of *S. anginosus* under different environmental conditions with a view to facilitating the identification of genes associated with virulence. *S. anginosus* was chosen due to it's greater level of diversity when compared with *S. intermedius* and *S. constellatus*. Previously published evidence indicates that there is a considerable level of intraspecies diversity within *S. anginosus*, as well as heterogeneity within the species being studied. This has been shown using a variety of techniques including 16S rRNA sequences (Bergman *et al.*, 1995; Jacobs *et al.*, 2000), 16S-23S rRNA intergenic spacer size polymorphisms (Whiley *et al.*, 1995), and ribotypes (Doit *et al.*, 1994). A high level of heterogeneity / diversity would suggest a higher than normal possibility of virulence according to clonal population theory (Gupta and Maiden, 2001). The more specific aims are listed below.

3.2. Aims.

The aims of the work described in this chapter were as follows:

1. To establish a reproducible method of RNA extraction from *S. anginosus* that would provide RNA of a suitable quality and quantity for RT-PCR.

2. To establish a method for differential display RT-PCR analysis of *S. anginosus*.

3. To study preferential gene expression of *S. anginosus* after exposure to defined environmental stimuli.

3.3. Materials and methods.

3.3.1. Test strains

A total of seven isolates of *S. anginosus,* from a range of sources were studied (Table 3.1). Strains were isolated from clinical samples in routine diagnostic laboratories on non-selective (Blood agar and fastidious anaerobic agar) plates. Both disease-associated and one commensal strain were studied. Test strains were inoculated either directly from pure cultures on fresh blood agar (Lab M, UK) or from Microbank frozen beads (Pro-Lab diagnostics) into 20 ml of Brain heart infusion broth (BHI) and cultured anaerobically (Anaerobic work station, Don Whitley Scientific, UK) in an atmosphere of 20 % Hydrogen, 10 % Carbon dioxide and 70 % Nitrogen. Growth was harvested at mid-log phase after approximately 18 h. Purity of culture was confirmed by subsequent plating onto blood agar.

A strain of *Candida albicans* (strain 71- Cardiff Oral Microbiology Unit strain collection) was used as a control in the RNA extraction procedure, since RNA has been successfully extracted from this species in our laboratory on a routine basis. This strain was inoculated into Yeast Extract Peptone Dextrox (YEPD) broth (Lab M, UK) and incubated aerobically at 37 °C for 18 h.

Table 3.1. Origin of strains of *S. anginosus* taken from the Cardiff Oral Microbiology Unit culture collection used for the optimisation of RNA extraction and differential display RT-PCR.

Reference Number	Origin of isolate
Strain 8	dentoalveolar abscess
322/96	dentoalveolar abscess
7K	brain abscess
CDC 2236-81	blood
CDC 2405-81	blood
SL 34/W	subphrenic abscess
Strain 56	dental plaque

3.3.2. RNA extraction from *S. anginosus*.

Two RNA extraction methods were used in order to determine which approach would produce RNA of sufficient quantity and adequate purity for further manipulation. Six extractions were performed on all seven strains using each method.

Strains were cultured as described above (3.3.1) and cells harvested by centrifugation (speed and duration of centrifugation were dependant upon method). RNA was extracted using either the Ultraspec® RNA isolation system (see section 3.3.2.1. for method) or the Qiagen RNeasy kit (see section 3.3.2.2. for method). The RNA solution obtained by each method was subjected to enzymatic degradation of DNA with Qiagen RNeasy filter columns using the Qiagen DNase I treatment solution (see section 3.3.3 for method). After extraction, RNA samples were run on a denaturing agarose gel (see section 3.3.4. for method) and subjected to quantification (see section 3.3.3. for method). After cDNA synthesis (see section 3.3.6.5 for method) the RNA was tested for its suitability for PCR by application of the 16S rRNA gene (see section 3.3.5. for method) followed by agarose gel electrophoresis (see section 2.1. for method).

3.3.2.1. Ultraspec® RNA extraction.

Streptococcus anginosus growth (5 ml) from an 18 h Brain heart infusion (BHI – Lab M, UK) broth culture was concentrated by centrifugation (5 min, 2000 x g.) and the pellet resuspended in 1 ml BHI broth. The resulting suspension was transferred to a 1.5 ml Eppendorf tube (Thermoquest, UK) prior to further centrifugation (5 min, 12,000 X g) and the supernatant discarded. An approximate 50 μl volume of glass beads 150-212 μm or 425-600 μm in diameter (Sigma, UK) was added. A 1 ml volume of Ultraspec® solution (Biotecx Laboratories Ltd, Houston, Texas) was added and the suspension subjected to continuous agitation for 30 min at 4 °C to fragment

the cell pellet. Chloroform (Sigma UK, 0.2 ml) was added and the tubes agitated for 15 s. The preparation was stored on ice for 5 min and then centrifuged at 12,000 X g for 15 min. The upper aqueous layer was dispensed into a clean 1.5 ml Eppendorf tube and 400 μl of isopropanol (Sigma, UK) added. The tubes were inverted twice and kept at 4 °C for 5 min prior to centrifugation at 12,000 X g for 10 min. The nucleic acid pellet and tubes were then washed twice with 75 % ethanol and the pellet dissolved in 30 μl of RNase treated water at 50 °C for 5 min. DNase I treatment followed.

3.3.2.2. Qiagen RNeasy Minikit.

Streptococcus anginosus growth from an 18 h BHI broth culture was concentrated by centrifugation (5 min 2,000 X g) and the pellet resuspended in 100 μl of Tris-EDTA (TE) buffer with 3 mg/ml lysozyme (Sigma, UK), 350 μl of buffer RLT (25-50 % guanidinium thiocyanate, β-mercaptoethanol 10 μl/ml) and 250 μl ethanol (Sigma, UK). The mixture was applied to a "spin column" (Qiagen, UK) and centrifuged for 15 s at 12,000 X g. The column was then washed with 700 μl of buffer RW1 (2.5-10 % guanidinium thiocyanate) and 500 μl of buffer RPE (constituents unknown) was added. The spin tube was inserted into a clean 1.5 ml collection tube and 30 μl of RNase-free water deposited on to the spin column to elute the nucleic acid.

3.3.3. DNase I treatment on column.

Eluted RNA samples were made up to 100 μl with RNase-free water. RLT buffer (350 μl) was added containing β-mercaptoethanol (Sigma, UK) at 10 μl /ml and thoroughly mixed. Absolute ethanol (250 μl) was then added and mixed by pipetting. The sample was applied to an RNeasy spin column (placed in a 2 ml collection tube) and centrifuged at 12,000 X g for 15 s. Buffer RW1 (700 μl) was added to the tubes, which were then centrifuged for

15 s at 12,000 X g. DNase I (10 μl) was added to 70 μl of buffer RDD (constituents unknown), 80 μl of this mix was added to the column, which was then incubated at room temperature for 15 min. Buffer RDD (350 μl) was added and the columns centrifuged at 12,000 X g for 15 s. Buffer RPE (500 μl) was added and the tubes were centrifuged for a further 15 s. The addition of RPE was repeated prior to a further 15 s centrifugation step. The tubes were then centrifuged for 2 min. RNase-free water (30 μl) was added directly to the membrane and the tubes were centrifuged for 1 min at 12,000 X g. The RNA was quantified spectrophotometrically (Abs 260 : 280 nm) prior to aliquotting and storage at –20 °C.

3.3.4. RNA denaturing agarose gel electrophoresis.

A 1% gel was prepared using molecular grade agarose (Sigma, UK) by dissolving 1 g of agarose in 20 ml of 5 X 3-[N-Morpholine] propanesulfonic acid (MOPS, Sigma, UK) buffer (0.1 M MOPS, 0.025 M sodium acetate, and 0.005 M Ethylenediaminetetra-acetic acid [EDTA] [pH 5.5]) and 80 ml of diethylpyrocarbonate (DEPC) treated water. Following cooling to approximately 40 °C, 5.5 ml of 37 % formaldehyde was added. An 8 μl volume of each sample was added to 16 μl of Northern max RNA loading buffer (Ambion, UK). The mixture was heated to 65 °C for 10 min and immediately placed on ice. A 1 μl volume of ethidium bromide (Sigma, UK) at 500 μg/ml was added, prior to loading the sample onto the gel.

Electrophoresis (Flowgen, UK) was performed in 0.5 X MOPS buffer, at 80 V for 45 min. Gel images were viewed using UV transillumination and recorded using the Gel Doc 1000 system and Molecular Analyst Software version 1.5 (Bio-Rad, UK).

3.3.5. 16S rRNA PCR.

RNA was tested as a suitable template for PCR using 16S rRNA primers K1 (5'3'GAAGTCGTAACAAGGTAAGCCGT) and K2 (5' 3'GCCAGGCATCCACC) (Whiley *et al.*, 1995). Positive and negative controls were included consisting of a non-reverse transcribed *E. coli* RNA sample, H_2O and 1.0 μg of non-RT *S. anginosus* RNA. The reaction mixture consisted of 1.5 mM $MgCl_2$ (Promega, UK), 1X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40 - Promega, UK), 125 mM Deoxynucleotide Tri-Phosphate (dNTP) mix (Life Technologies, UK), 2.5 U *Taq* DNA polymerase (Promega, UK), and 50 pmol of primers K1 and K2 (Life Technologies, UK) in 25 μl H_2O per reaction. PCR was performed in a Hybaid PCR Express thermocycler (Hybaid, UK) using 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. PCR products were analysed on an agarose gel (Section 2.1).

3.3.6. DD RT-PCR

Seven strains of *S. anginosus*, representing clinical isolates from both oral and extra-oral sites and a commensal isolate were chosen for use in the development of DD RT-PCR (Table 3.1). After development of the method two strains representing both a clinical isolate (strain 8) and a commensal isolate (strain 56) were selected for further study. Both strains were subjected to growth under varying conditions prior to RNA extraction and RT-PCR. The conditions included heat shock, different pH's, osmolarity and iron concentration. After growth, all cultures were subjected to RNA extraction using the Ultraspec® system (as described in section 3.3.2.1.) and cDNA synthesis.

3.3.6.1. Heat shock.

Individual test strains were inoculated into duplicate volumes (10 ml) of BHI at 37 °C in an anaerobic chamber (Don-Whitley Scientific, UK). Following incubation at 37 °C for 18 h under anaerobic conditions, one preparation of each duplicate was subjected to heat shock in a waterbath at 50 °C for 10 min whilst the other remained at 37 °C. After heat shock, the bacterial cells from each sample were harvested at mid-log phase growth by centrifugation for 5 min at 12,000 X g.

3.3.6.2. pH.

Differences in pH were achieved by the addition of acidic and alkaline reagents to BHI broth. A pH of 5.0 or 6.0 was achieved by dissolving MOPS buffer in BHI. pH's of 8.0 and 9.0 were obtained by the addition of sodium hydroxide (Fischer bioreagents, UK). A pH of 7.5 (the normal pH of BHI) was used as a control. Duplicate cultures of each test strain at each pH were inoculated anaerobically at 37 °C for 18 h.

3.3.6.3. Osmolarity.

Differences in the osmotic potential of the culture medium were achieved by the addition of sodium chloride (Fischer bioreagents, UK) or sucrose (Sigma, UK). Individual cultures were prepared at a final osmolarity of 200 milliosmotic moles per litre (mos mol / l) and 400 mos mol / l. Duplicate cultures were prepared for 200 mos mol / l with sucrose, 400 mos mol / l with sucrose, 200 mos mol / l with sodium chloride and 400 mos mol / l with sodium chloride.

3.3.6.4. Haemin/iron concentration.

Test strains were cultured in minimal salts media containing no iron. Basal medium (2.5 g peptone [Lab-M, UK], 0.5 g trypticase [Lab-M, UK], 2.5 g yeast extract [Lab-M, UK], 0.4 ml resazurin [Sigma, UK]), 4.0 ml salts solution (0.2 g calcium chloride [anhydrous], 0.2 g magnesium sulphate [Sigma, UK] [anhydrous], 1.0 g dipotassium hydrogen orthophosphate [Fischer bioreagents, UK], 1.0 g potassium dihydrogen orthophosphate [Fischer bioreagents, UK], 10 g sodium hydrogen carbonate, 2.0 g sodium chloride in 1 l distilled water) and 100 ml distilled water. After autoclaving at 121 °C for 15 min 0.4 g cysteine hydrochloride (Sigma, UK), 1 mg of filter sterilised Vitamin K1 (Sigma, UK) and an amount of filter-sterilised haemin (Sigma, UK) was added. Haemin concentrations of 0.5 μmol / l, 1 μmol / l, 8 μmol / l and 15 μmol / l were used (Stubbs *et al.*, 1999).

3.3.6.5. cDNA synthesis.

Reverse-transcription of RNA was performed by mixing 1 μg of source RNA with 50 mM of a primer from the primer pair employed for the prospective PCR reaction (section 3.3.6.6). This mixture was then denatured at 75 °C for 5 min prior to the addition of 50 μl of the reverse transcription reaction mixture, containing (all constituents PE Biosystems, UK) 300 U MMLV-reverse transcriptase, 5 μM DTT, 10 μl 5 X reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 10 μM dNTP's, 7.5 U RNase inhibitor and 17.8 μl of water. The mixture was stored at 37 °C in an incubator for 1 h, followed by further inoculation at 95 °C for 10 min in a waterbath. Samples were stored at -20 °C.

3.3.6.6. Polymerase chain reaction

A range of reaction mixtures and cycling conditions were used in order to determine the optimal conditions for PCR. An "in house" method and the method of Fislage *et al.,* (1997) were used. The "in-house" method was taken from a collaborating research group on-site with a proven track record of consistent differential display results, and consisted of a pre-mix containing 1.5 mM $MgCl_2$ (Perkin Elmer, UK), 1X PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl) (Perkin Elmer, UK), 100 mM GTC mix (Perkin Elmer, UK), 0.5 U *Taq* DNA Polymerase (Perkin Elmer, UK), 50 mM of primer Ea7 and Es1 (Life Technologies, UK) and 0.25 μl α-^{33}P dATP (Amersham, UK) with 2 μl of cDNA template and H_2O to 20 μl per reaction. Water controls and non-reverse transcribed controls were prepared to ensure the purity of RNA samples and the reaction mix. Reactions were subjected to 1 cycle at 94 °C for 1 min, 40 °C for 4 min, and 70 °C for 1 min, followed by 35 cycles of 94 °C for 45 s, 60 °C for 2 min, and 72 °C for 1 min. The method of Fislage consisted of an identical PCR mix except for primer Es1, which was present in a X 10 lower concentration (5 mM). Thermocycling was performed with 40 cycles at 94 °C for 30 s, 40 °C for 60 s and 70 °C for 30 s followed by 1 cycle at 70 °C for 5 min. In addition to the two basic methods, the reaction mix from the method of Fislage *et al.,* (1997) was combined with the cycling conditions of the "in-house" method and vice versa. A 2 μl volume of the reverse transcription mixture was subjected to PCR in a 20 μl total volume of pre-mix. The following primer pairs were used, Opa-01/Opa-02 (Operon technologies, Alameda, CA), RT, RT2, 3 and 5 (Life Technologies, UK) and Ea7/Es1 (Life Technologies, UK) (see Table 3.2. for sequence data).

Table 3.2. Sequences of random primers used in DD RT-PCR.

Primer	Sequence 5' – 3'	Reference
Opa-01	CAGGCCCTTC	Operon technologies inc, Alameda, CA
Opa-02	TGCCGAGCTG	
RT	TTTTTTTTTTGG	
RT2	TTTTTTTTTTGC	Benito, *et al.*, (1996)
RT3	TTTTTTTTTTCC	
RT5	TTTTTTTTTTAA	
Ea7	TCTTTTTTACC	Fislage *et al.*, (1997)
Es1	GCTGGAAAAA	

3.3.6.7. Detection of PCR products.

3.3.6.7.1. Non-radioactive detection.

Three non-radioactive methods of product detection were examined, namely ethidium bromide, silver staining and SYBR green II staining (Sigma, UK). Staining with ethidium bromide was achieved by the addition of 10 μl of a 500 μg/ml preparation of ethidium bromide to a 100 ml amount of agarose prior to pouring followed by analysis using UV light. SYBR green II staining was performed by soaking of the gel in a 1 in 10,000 solution of SYBR green II for ten minutes prior to analysis using UV light. Silver staining was performed using a silver staining kit. The gel was soaked in 300 ml of fixing solution for 10 min (10 % glacial acetic acid, 30 % ethanol per litre) with two changes of solution. The gel was then subjected to three 5 min 300 ml ddH_2O rinses. Silver solution (300 ml) was added and the solution stirred by gentle agitation for 30 min followed by a rapid water rinse with 300 ml ddH_2O for 20 s. To develop the gel, 150 ml of developer solution was poured over the gel and left for 8 min. The development solution was poured off and 150 ml stop solution was added (1 % acetic acid solution) and left for 5 min. Three 5 min 300 ml ddH_2O washes followed, prior to the addition of 300 ml of reducer solution (30 s) and a 1 min tap water rinse. A further three 5 min ddH_2O rinses took place prior to drying.

3.3.6.7.2. Radioactive detection.

Both experimental protocols used α-^{33}P dATP as the radiolabel. Four different experimental sets were used in which the PCR pre-mix and the running conditions were exchanged between the two methods. A negative control using water in place of template nucleic acid was included for each set of conditions. Resulting PCR products were run on a sequencing gel for 2.5 h

and exposed to Kodak X-ray film overnight. The following forms of electrophoresis were used: 1.5 % agarose gels (Sigma, UK), 4.5 % polyacrylamide mini-gels and sequencing grade 4.5 % polyacrylamide gels (Amersham, UK).

3.3.6.8. Detection and isolation of differentially expressed bands visualised using radioactive method.

Differences in the fingerprints were determined by viewing the X-ray autoradiograph of the gel. The autoradiograph was then used as a template to lay over the mounted polyacrylamide while the positions of the selected bands were marked into the gel using a sterile needle. The area of gel containing the band of interest was excised using a sterile scalpel, placed into 50 μl of sterile distilled water and the gel macerated using a sterile pipette tip. Following incubation overnight at room temperature the mixture was centrifuged at 15,000 X g in order to separate out the electrophoresis medium. The aqueous DNA containing layer was drawn off and re-amplified using the primer sets as for the original amplification by PCR reaction (4 μl DNA, 10 μM of primer Ea7/Es1, 1 mM dNTP mix, 1 X PCR buffer II [100 mM Tris-HCl [pH 8.3], 500 mM KCl], 25 mM $MgCl_2$, 1 U *Taq* DNA polymerase in a total volume of 40 μl). The PCR was run for 40 cycles at 94 °C for 30 s, 40 °C for 60 s and 70 °C for 30 s and for 1 cycle at 70 °C for 5 min. The products were separated on a 1.5 % low melting temperature agarose (Bio-rad, UK) gel in 1 X TBE buffer at 80 V for 1 h. Molecular weight marker VI (Life Technologies, UK) was used as a reference for fragment size.

3.3.6.9. Band excision from agarose.

The gel segments containing the bands of interest were excised from the agarose gel using a sterile scalpel as before. The gel slices were weighed,

and 3 X volume of QC buffer (constituents unknown) was added to 1 X volume of agarose gel slice. The tubes were incubated in a water bath at 50 °C for 10 min with vortexing every 2-3 minutes to aid dissolution. Following the addition of a 1 X gel volume of isopropanol the mixture was applied to a Qiaquick filter column (Qiagen, UK) and the columns centrifuged at 12,000 X g for 1 min. The column was washed with 0.5 ml of the QG buffer (50-100 % guanidinium thiocyanate) provided and then 0.75 ml of the provided PE buffer (constituents unknown) each time collecting the wash buffer by centrifugation for 1 min at 12,000 X g. A final centrifugation for 1 min followed to remove any residual buffer. The column was placed into a clean 1.5 ml Eppendorf tube and the DNA eluted with 50 μl water. The DNA solution was stored at -20 °C.

3.3.6.10. Purification of amplified DNA.

Amplified DNA was purified to remove excess primer using the Qiaquick PCR purification kit (Qiagen, UK). A 5 X volume of the buffer PB (25-50 % guanidinium thiocyanate, 25-50 % propan-2-ol) provided was added to 1 X volume of PCR product. The mix was added to a filter spin column and centrifuged at 15,000 X g for 30 – 60 s. The flow through was discarded. PE wash buffer (0.75 ml) was added prior to further centrifugation at 15,000 X g for 30 – 60 s. The filter column was centrifuged for an additional 60 s at 15,000 X g in order to remove any residual buffer. The filter column was transferred to a 1.5 ml Eppendorf tube and 50 μl of the buffer EB (constituents unknown) provided was added directly to the filter. The column was left to stand at room temperature for 1 min prior to elution of the PCR products by centrifugation for 1 min at 15,000 X g. The DNA was stored at –20 °C.

3.3.6.11. Direct sequencing of PCR products (without cloning).

A PE biosystems sequencing kit (PE biosystems, UK) was used for direct sequencing of PCR products. Three sequencing reactions were set up including a control (20 ng pGEM nucleic acid, 3.2 pmol M13 primer, 8 µl terminator reaction mix), a forward (20 ng DNA, 3.2 pmol Ea7 primer, 8 µl terminator reaction mix) and a reverse (20 ng DNA, 3.2 pmol Es1 primer, 8 µl terminator reaction mix) reaction. Mixes were heated at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min for 25 cycles.

3.3.6.12. Removal of labelled nucleotides.

Labelled nucleotides were removed using Qia Ex II spin columns.

For resuspension of the resin, the Qia Ex removal kit spin columns (Qiagen, UK) were spun at 750 X g for 3 min. The caps were loosened by a quarter turn and the bottom closure snapped off. The spin column was centrifuged at 750 X g for 3 min in order to separate the liquid from the resin. The spin columns were then transferred to fresh 1.5 ml Eppendorf tubes and the sequencing reaction mixtures loaded carefully to the gel resin bed. The gel bed was centrifuged at 750 X g to elute the sequencing reaction product. The DNA was then analysed at a dedicated sequencing unit, which employs an ABI Prism sequencer (PE Biosystems, UK). Sequences were supplied as SEQ files, which were analysed in Microsoft Word for Windows 2000 (Microsoft, USA).

3.3.6.13. Cloning and sequencing of reamplified bands.

Cloning was performed using the TOPO TA cloning kit (Invitrogen). Freshly prepared PCR products were produced for cloning from the original

reamplified bands using the same protocol as for DD RT-PCR (Section 3.3.6.6), with the exception of a final seven minute extension step. Analysis of 10 μl of the PCR product was performed on 1.5 % agarose gels. PCR products were purified using the QIAquick PCR purification kit (Qiagen, UK) according to manufacturers instructions prior to cloning. PCR products were purified by the addition of 5 volumes of buffer PB to 1 volume of product in a spin column provided. The column was spun at 12,000 X g for 60 s after which 0.75 ml of buffer PE was added. Two consecutive 1 min spins took place prior to elution of the product into a clean 1.5 ml tube with 50 μl of H_2O. Cloning was performed using the chemical transformation method recommended by the manufacturer. The cloning mix was prepared using 2 μl of fresh PCR product, 1 μl of salt solution and 1 μl of the TOPO vector, made up to 5 μl with sterile water. This mix was incubated at room temperature for 30 min prior to 2 μl being added to a 1 X volume of competent *E. coli* cells, which had been thawed on ice. The mixture was incubated on ice for between 5 and 30 min prior to the addition of 250 μl of SOC medium (20 g tryptone, 5.0 g yeast extract, 10 mM NaCl, 10 mM KCl, 20 mM MgCl, 20 mM glucose in 1 l H_2O). After shaking at 37 °C for 1 h, 40 μl of the transformation was spread onto Luria broth (LB) agar (10 g tryptone, 5.0 g yeast extract, 10 g NaCl, 15 g agar in 1 l H_2O) (Lab-M, UK) containing 5-bromo-4-chloro-3-indolyl-β -D-galactopyranoside (X-Gal), and kanamycin (Sigma, UK) at 50 μg / ml. Plates were incubated in air at 37 °C for 18 h. A total of 10 white/light blue colonies were selected randomly for analysis and plated onto LB agar plates containing 50 μg / ml kanamycin.

Recombinant plasmids obtained were analysed to determine the nature of the insert by performing PCR (1 X buffer [100 mM Tris-HCl [pH 8.3], 500 mM KCl], 1.5 mM $MgCl_2$, 100 mM dNTP's, 4 pmol of each forward and reverse M13 primer (Invitrogen), 0.625 U DNA polymerase and made up to a final volume of 10 μl per reaction). A single colony was added to each reaction, the suspension subjected to 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by one cycle at 72 °C for 10 min prior to inspection

of products on a 1.5 % agarose gel and sequencing using a ABI Prism Sequencing kit (Section 3.3.6.11) (PE Biosystems, UK).

Sequences were aligned using the Clustal W program (http://www.ebi.ac.uk/clustalw/), and referenced using the nr database within blast N version 2.2.1 on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/) and Tblastx on the NCBI website.

3.3.6.14. Northern blotting of sequenced transcripts.

The RNA for Northern blotting was extracted using three methods. Initially the Ultraspec® method was used with the RNA undergoing DNase I treatment using the DNA Free kit (Ambion, UK). Alternative methods used included the addition of an RNA stabilisation agent to the cells prior to extraction. This included the addition of X 5 volumes of RNAlater® (Ambion, UK) reagent to a 50 µl cell pellet for storage at –20 °C prior to RNA extraction using the Ultraspec® isolation system. Other methods used included a modification to the Qiagen RNeasy kit and the use of a cryogenic cell smasher. The RNeasy modification involved incubating a 5 µl cell pellet with 100 µl lysis buffer (lysozyme 20 mg / ml and mutanolysin 100 U / ml) for 30 min at 37 °C prior to extraction of the RNA using the spin column (see section 3.3.2.2) and DNase I treatment using the DNA free kit. This involved adding the provided DNase I reaction buffer (constituents unknown) into the RNA solution at a 1:10 ratio followed by 0.5 µl of the kit DNase I solution. The mixture was then incubated at 37 °C for 30 min prior to the addition of the provided DNase I deactivation buffer (constituents unknown) in a ratio of 1:10, followed by gentle mixing at room temperature for a further 2 min. The second alternative method involved smashing a cell pellet using a freezer mill prior to RNA extraction using the Ultraspec® method. A cell pellet (50 µl) was frozen at –70 °C for 10 min prior to placement within a containment vial beneath a steel impactor,

which was sealed and placed into the freezer mill 6750 (Glen Creston Ltd, Stanmore, UK) containing 7 l liquid nitrogen. The pellet was ground for 2 min, removed and treated using Ultraspec® RNA isolation reagent. The RNA was then DNase I treated using the DNA free kit.

A 2 % denaturing agarose gel containing ethidium bromide at 2 μg/ml was used to display 20 μg of each RNA sample to be blotted. RNA was dissolved in 20 μl of loading buffer (500 μl formamide, 166.4 μl formaldehyde, 100 μl 10 X MOPS in 1 ml) and denatured at 55 °C for 15 min prior to the addition of 5 μl of running dye (0.25 % bromophenol blue [Sigma, UK], 0.25 % xylene cyanol [Sigma, UK], 50 % glycerol [Sigma, UK], 0.2 M EDTA pH 8.0). The samples were run alongside a pGEM marker at 30 V for 0.5 h, followed by 18 V for 18 h. The gel was photographed, and rinsed twice in DEPC treated water for 10 min prior to equilibration in 10 X SSC (0.15 M sodium chloride, 0.015 M sodium citrate) for 10 min. Six sheets of 3MM paper (Whatman, UK) were cut to the size of the gel and soaked in SSC until required. A glass plate was assembled on top of four rubber bungs within a pyrex dish. A sheet of 3MM paper was placed over the glass plate to act as a wick. Three sheets of 3MM paper were placed on top of the construction; any air present under the paper was rolled out after each addition. The RNA gel was placed onto the 3MM paper. Hybond N Northern blotting membrane (Hybond, UK) was soaked in SSC prior to being placed on top of the gel. A further 3 sheets of 3MM paper were placed on top of the membrane. Saran wrap was placed up to each side of the gel to prevent short-circuiting. A stack of paper towels were placed on top of the final 3MM paper and kept in place by a final glass plate and a 500 g weight. The blotting apparatus was soaked in SSC, and the blotting took place over 24 h. Following blotting, the membrane orientation was marked and the Hybond membrane was inspected for RNA bands under UV illumination. The membrane was air dried between two sheets of 3MM paper for 30 min and nucleic acid fixed by exposure to UV light for a few seconds. The membrane was placed in Saran wrap and stored at 4 °C in the dark until needed. Cloned DNA (25 ng) was diluted to a volume of 9 μl in

nuclease free water, which was then denatured at 95 °C for 3-5 min and snap frozen in a dry ice/ethanol mixture prior to thawing and placing on ice at 4 °C. Labelling of the probe was performed using the Strip-EZ kit (Ambion, UK) according to manufacturers instructions. The primer extension mix was made up with the 9 μl of template (1 X decamer, 1 X buffer [DTT, 10 mM dGTP, 10 mM dUTP] minus dATP / dCTP, 2 mM dCTP, 3000 Ci / mmol α-^{32}P dATP, 1 μl Exonuclease-free Klenow polymerase) to a final volume of 25 μl. The primer extension cocktail was mixed gently at 37 °C for 10 min prior to the addition of 1μl of 0.5 M EDTA in order to stop the reaction. The mix was then cleaned by passing through a push column, protected by a β-shield in order to remove unincorporated nucleotides. The column containing the membrane was pre-wetted with 1 X STE buffer (constituents unknown). The probe was diluted to 70 μl with STE prior to its addition to the membrane. A further 70 μl of STE buffer was then applied to the membrane. The membrane was pre-wetted with DEPC water and placed in a hybridisation tube (RNA face inwards), and pre-hybridised by the addition of 10 ml of Ultrahyb™ solution (50 % formamide) (Ambion, UK). The membrane was then incubated in a rolling oven at 42 °C for 30 min. The eluted probe (50 μl) was diluted 1 in 10 with 10 mmol EDTA, denatured at 90 °C for 10 min, and added directly to the prehybridisation tube where hybridisation was allowed to occur for 18 h. The blot was treated with 2 X 5 min washes in wash solution 1 (2 X SSC, 0.1 % SDS in 20 ml) at 42 °C followed by 2 X 15 min washes in wash solution 2 (0.1 X SSC, 0.1 % SDS in 20 ml) at 42 °C. The film was monitored during the process and once the film was reading 20-50 cps, it was removed from the tube, sealed in a plastic bag and exposed to X-ray film in an AUTORAD cassette (Kodak, UK) for 18 h prior to development using Kodak film.

3.4. Results.

3.4.1. RNA extraction.

RNA was extracted from all of the seven test strains of *Streptococcus anginosus* on at least six separate occasions. Both methods of RNA extraction provided RNA of sufficient quantity and quality for subsequent analysis. However, the Ultraspec® extraction method provided better quality RNA more consistently when compared to those obtained using the Qiagen RNeasy kit (Figure 3.1 and Table 3.3). The mean amounts of RNA obtained were 3.76 μg/ml for the Qiagen extraction kit and 7.5 μg/ml for the Ultraspec® method. The absorbance ratios (260:280 nm) of the RNA obtained using the Ultraspec® method, in comparison to the Qiagen method, were more consistently within the ideal range of 1.8 – 2.0. Six examples from each method can be seen in Table 3.3. RNA extracted using both methods served adequately as a cDNA template in the amplification of 16S rRNA genes using universal primers (results not shown).

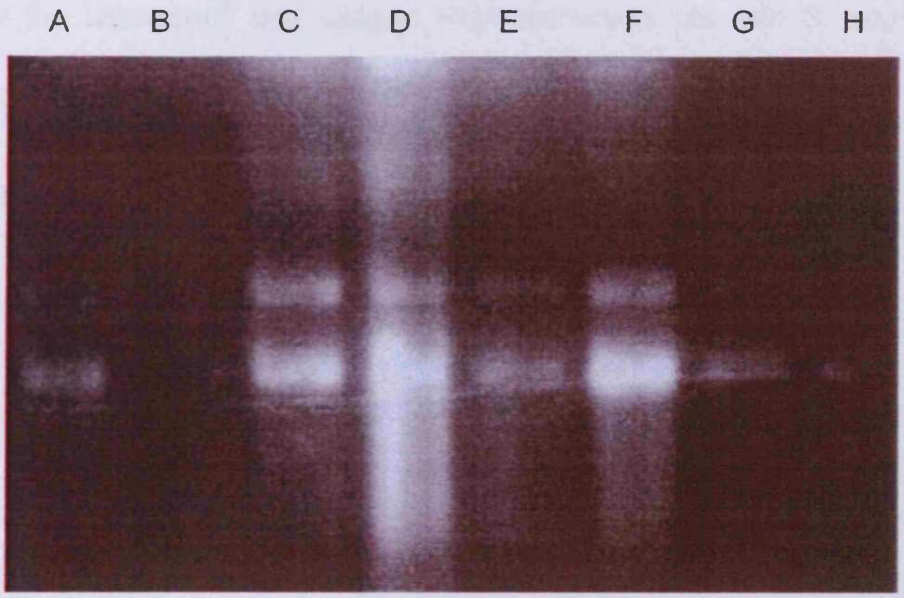

Figure 3.1. Denaturing agarose RNA gel demonstrating the RNA extraction abilities of the Qiagen and Ultraspec® methodologies with *S. anginosus* cells. A = *E. coli* RNA control, B - D = Qiagen method, E - H = Ultraspec® method.

Table 3.3. Six examples of data comparing the concetration and purity of RNA for the Ultraspec® and Qiagen RNA extraction kits with *S. anginosus* cells.

Test	260 nm value	Ratio	μg/ml RNA
Ultraspec®	0.122	1.8	4.88
	0.094	2.0	3.76
	0.3451	1.8	13.804
	0.1362	2.0	5.448
	0.2174	1.9	8.696
	0.2140	1.9	8.56
Qiagen	0.074	2.1	2.96
	0.003	1.5	0.12
	0.19	1.6	7.6
	0.086	1.8	3.44
	0.105	1.8	4.2
	0.106	1.7	4.24

3.4.2. Optimisation of DD RT-PCR methodology.

No bands were detected using the ethidium bromide, SYBR green II or the silver staining methods. It was therefore determined that the three methods were not sufficiently sensitive to allow detection of RT-PCR products. However, many bands were detected using radioisotope labelling.

Fislage *et al.*, (1997) cycling conditions when used with the in-house PCR mix yielded up to 30 bands by autoradiography using primers Ea7 and Es1, however, Fislage cycling conditions and PCR mix yielded up to 40 detectable bands by autoradiography with the same primer set. Use of in-house cycling conditions failed to yield any PCR products (Figure 3.2). Only primers Ea7 and Es1 succeeded in producing detectable PCR products.

3.4.3. The effect of heat shock and pH on the gene expression of *S. anginosus.*

After 18 h of inoculation the pH of the BHI broth used in the pH experiment was recorded. The results of this are summarised in Table 3.4.

Upon electrophoresis using the cycling parameters and PCR mix of Fislage *et al.*, (1997), the RT-PCR products representing *S. anginosus* strains 8 and 56 subjected to heat shock or pH alteration yielded profiles with over 100 bands of detectable products per lane (Figure 3.3) using primers Ea7 and Es1. Reproducibility of the method was shown by the demonstration of similar banding arrangements using different RNA extracts. No detectable product was obtained from non-reverse transcribed RNA and the water control. Analysis of the autoradiograph revealed many candidate bands worthy of further analysis such as bands present in strain 8 that were absent after heat-shocking. The bands selected for further study are summarised in Table 3.5 and can be seen in Figure 3.3.

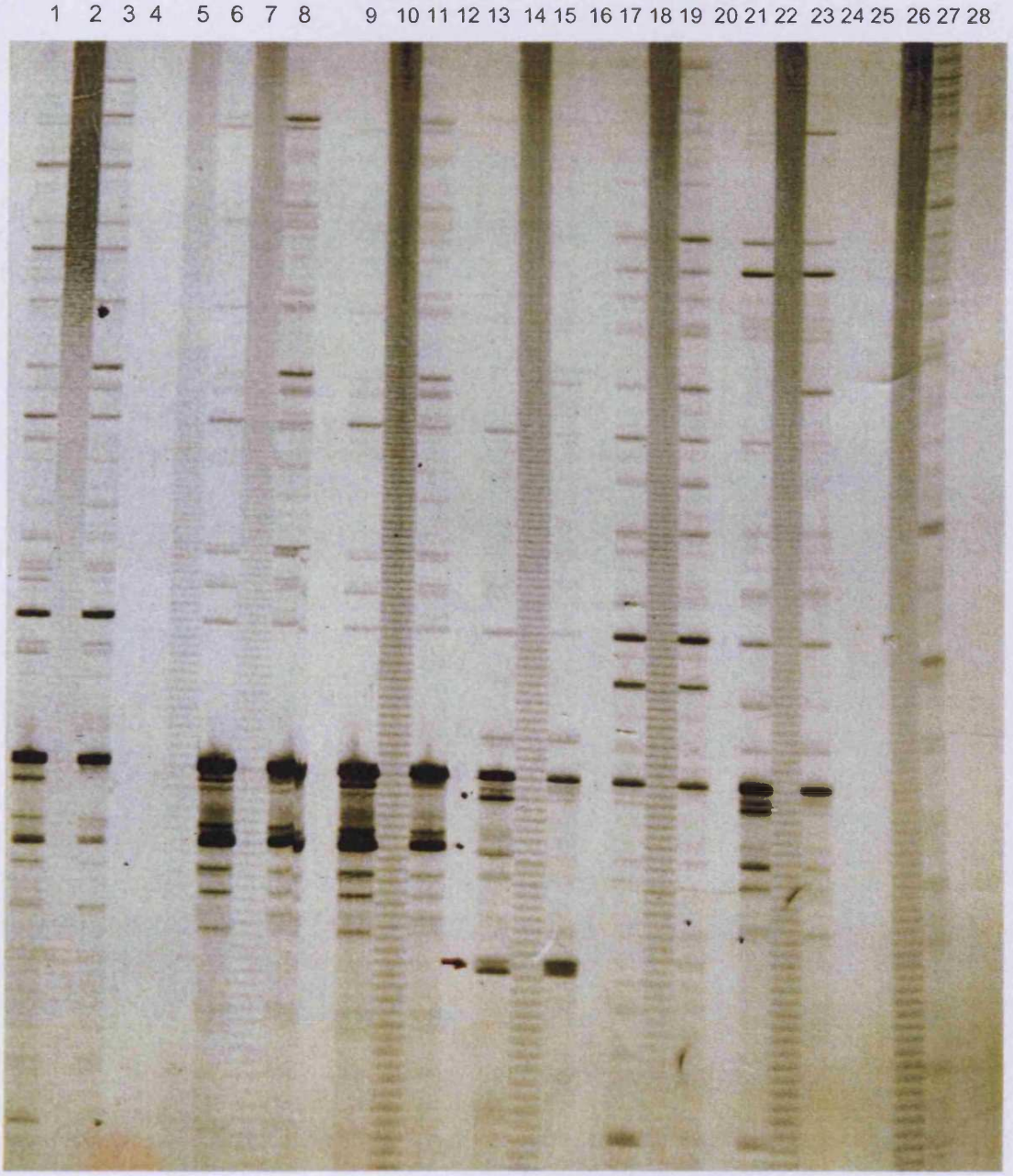

Figure 3.2. DD RT-PCR polyacrylamide gel showing successful random amplification of six strains of *S. anginosus* under different experimental conditions relating to the PCR mix using random primers Ea7 and Es1 (Fislage *et al.*, 1997). 1 = Strain SL 34/W $(a,x)^q$, 2 = Strain SL 34/W $(a,y)^q$, 3 = Strain SL34/W $(b,x)^q$, 4 = Strain SL34/W $(b,y)^q$, 5 = Strain CDC 2405-81 $(a,x)^q$, 6 = Strain CDC 2405-81 $(a,y)^q$, 7 = Strain CDC 2405-81 $(b,x)^q$, 8 = Strain CDC 2405-81 $(b,y)^q$, 9 = Strain CDC 2236-81 $(a,x)^q$, 10 = Strain CDC 2236-81 $(a,y)^q$, 11 = Strain CDC 2236-81 $(b,x)^q$, 12 = Strain CDC 2236-81 $(b,y)^q$, 13 = Strain 7K $(a,x)^q$, 14 = Strain 7K $(a,y)^q$, 15 = Strain 7K $(b,x)^q$, 16 = Strain 7K $(b,y)^q$, 17 = Strain 322/96 $(a,x)^q$, 18 = Strain 322/96 $(a,y)^q$, 19 = Strain 322/96 $(b,x)^q$, 20 = Strain 322/96 $(b,y)^q$, 21 = Strain 8 $(a,x)^q$, 22 = Strain 8 $(a,y)^q$, 23 = Strain 8 $(b,x)^q$, 24 = Strain 8 $(b,y)^q$, 25 = *E. coli* $(b,y)^q$, 26 = *E. coli* $(b,x)^q$, 27 = *E. coli* $(a,x)^q$, 28 = *E. coli* $(a,y)^q$. Profiles were obtained by performing RT-PCR on RNA samples extracted on 3 separate occasions.
^{q}a = Fislage mix. x = Fislage parameters. b= In-house mix. y = In-house parameters.

Table 3.4. Starting pH and final pH of the culture broths used to grow *S. anginosus* strains 8 and 56 for the pH gene expression study after 18 h of inoculation.

	Final pH	
Starting pH	**Strain 8**	**Strain 56**
6.00	4.70	4.84
7.20	5.27	5.90
8.00	6.33	6.10

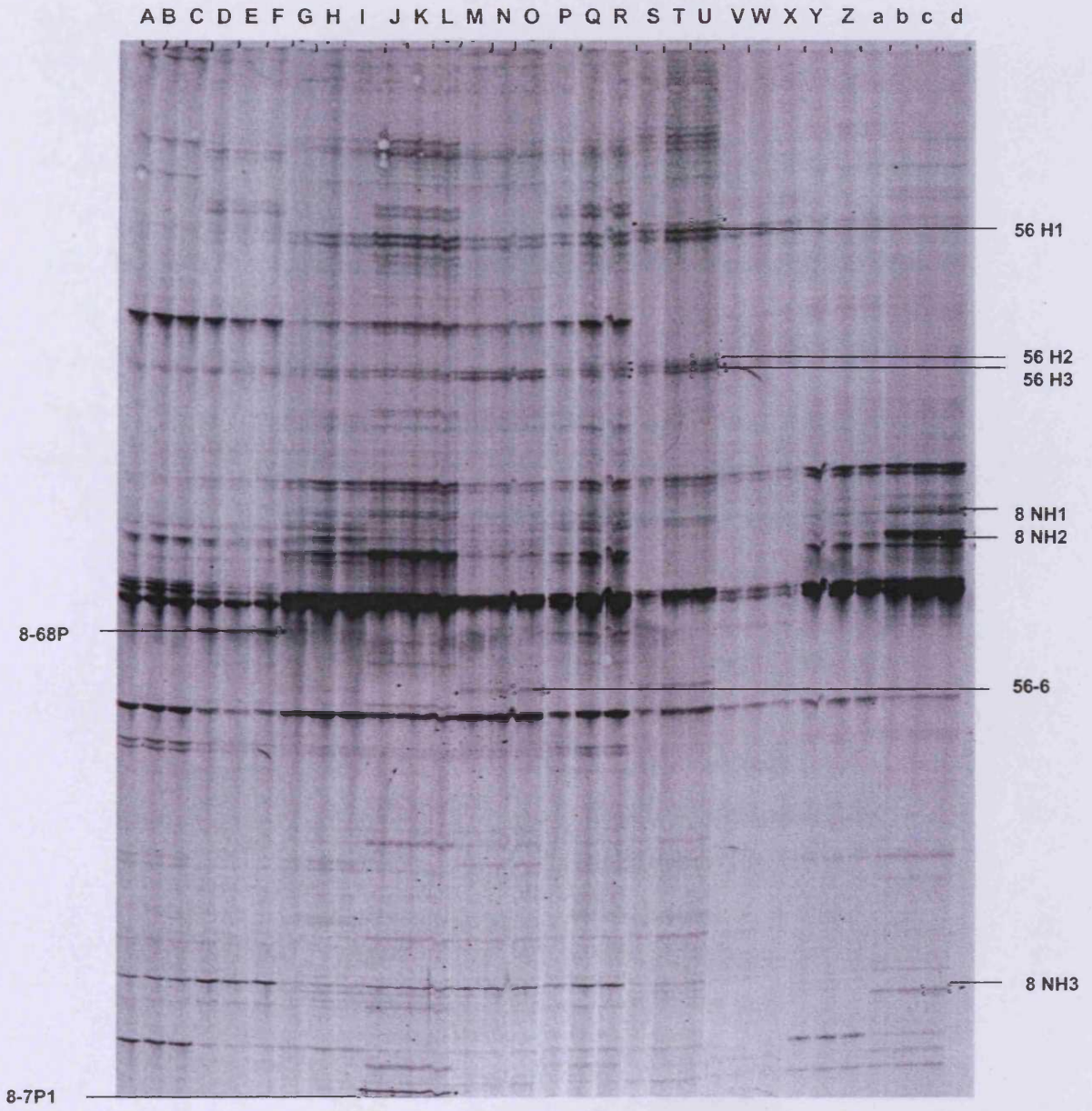

Figure 3.3. Differential display RT-PCR sequencing gel showing differences in the RNA fingerprints obtained for two strains of SMG grown at different temperatures and pH. (A-C: Strain 56 pH 8.0, D-F: Strain 8 pH 8.0, G-I: Strain 56 pH 7.2, J-L: Strain 8 pH 7.2, M-O: Strain 56 pH 6.0, P-R: Strain 8 pH 6.0, S-U: Strain 56 heatshock, V-X: Strain 8 heatshock, Y-a: Strain 56 non-heatshock, b-d: Strain 8 non-heatshock). The reverse transcription and PCR are performed in duplicate, showing reproducibility of the method. Profiles were obtained by performing RT-PCR on RNA samples extracted on 3 separate occasions.
Nine of the eleven extracted bands are highlighted and labelled. The additional two bands are missing from the above figure.

Table 3.5. Nomenclature for differential transcripts and conditions.

Band reference	Conditions under which differential transcripts appear
8 NH1	8 no heatshock
8 NH2	8 no heatshock
8 NH3	8 no heatshock
56 H1	56 following heatshock
56 H2	56 following heatshock
56 H3	56 following heatshock
8-68P	8 pH 8.0 + 6.0
8-7P1	8 pH 7.2
8-7P2	8 pH 7.2
56-6	56 pH 6.0
56-8	56 pH 8.0

Of the transcripts extracted from the DD RT-PCR gel (Figure 3.4), only transcripts 8-68P, 8-7P1, 8-7P2, 56-6 and 56-8 (see Table 3.5 for nomenclature) yielded products following PCR with the original primers (Figure 3.4) despite repeated attempts at amplification. Direct sequencing failed to provide sequence data, therefore the reamplified bands were cloned prior to sequencing. The clones of each transcript were selected on the basis of the size of the insert corresponding with the known size of the transcript upon PCR amplification (Figure 3.5a and 3.5b). Good sequencing profiles were obtained for bands 8-68P and 8-7P1. All other transcripts repeatedly yielded ambiguous sequence data. These sequences could not therefore be analysed further. The sequences are shown in Table 3.6.

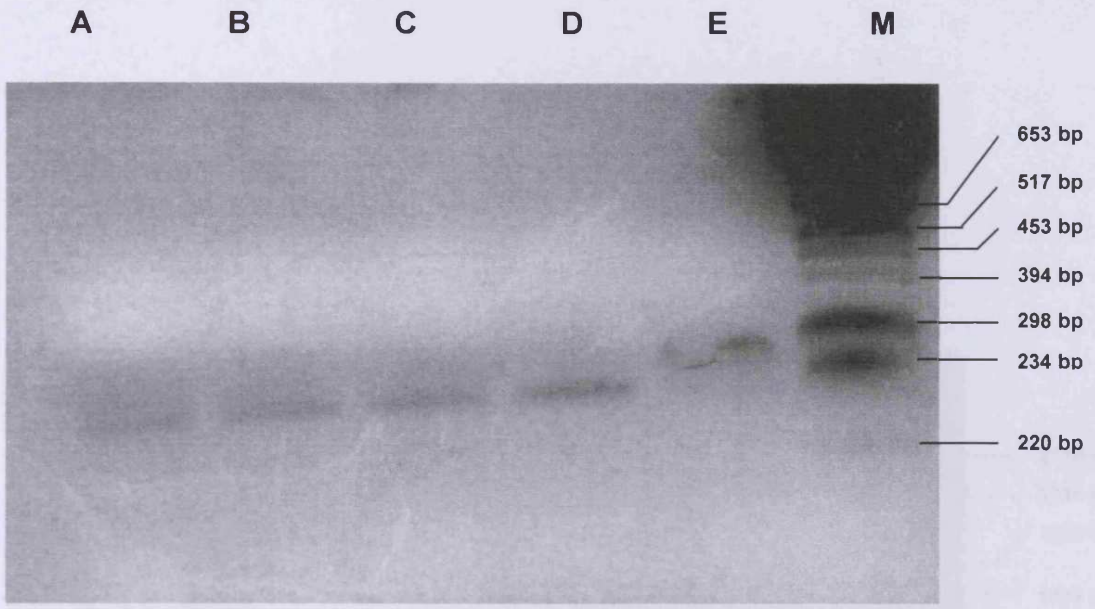

Figure 3.4. An agarose gel demonstrating the successful reamplification by PCR of five transcripts of interest from a DD RT-PCR gel using primers Ea7 and Es1. A = 56-8, B = 56-6, C = 8-7P2, D = 8-7P1, E = 8-68P. Lane M contains molecular weight marker VI.

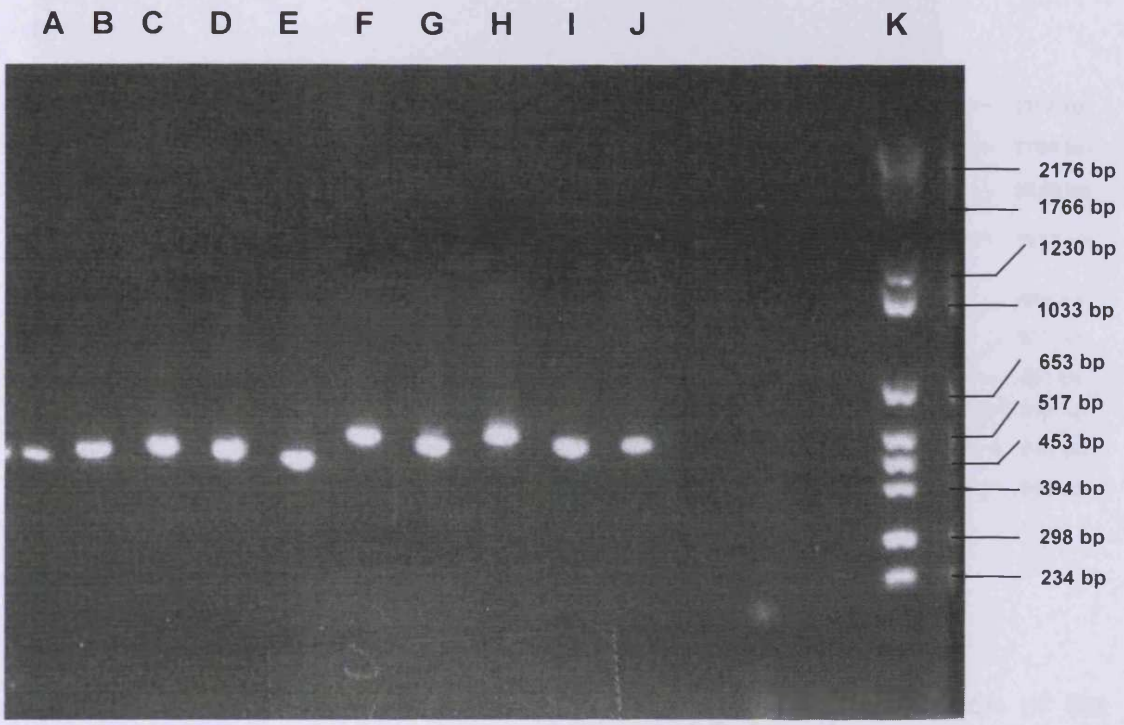

Figure 3.5a. Agarose gel showing the PCR amplification of transcripts after cloning using primers Ea7 and Es1. The image shows amplified DNA extracted from clones of 8-7P1 (A-E) followed by 8-68P (F-J). A number of different clones of each band were run in order to select the correct insert size (plus size of vector). The size of the insert was previously identified in Figure 3.4. A molecular weight marker is present in lane K.

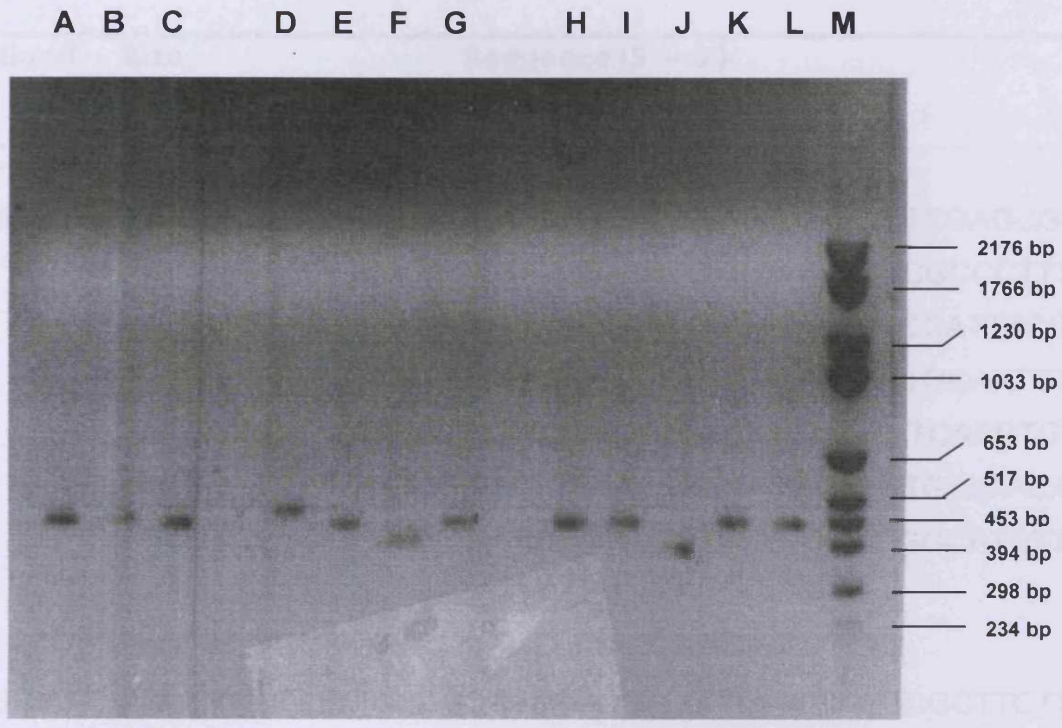

Figure 3.5b. Agarose gel showing the successful reamplification of the remaining clones extracted from the DD RT-PCR gel using primers Ea7 and Es1. A number of different clones of each band were run in order to select the correct insert size (plus size of vector). The size of the insert was previously identified in Figure 3.4. Molecular weight marker VI is marked M. A – C = 56-8, D – G = 56-6, H – L = 8-7P2.

Table 3.6. Sequences of bands extracted from the DD RT-PCR gel.

Band	Size (bp)	Sequence (5' – 3')
8-68P	298	ATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCG GGCCGGCAGTGTGATGGATATCTGCAGAATTCGCCCTTT CTTTTTTACCTCAGCACCAATGCGGATAATTCCCATTTCG TCAAGGTCTCTCAAAGCATCTTCCCCAACATTTGGAATTT CGCGCGTAATTTCTTCAGGTCCAAGCTTTGTATCACGTGT TTCTGATTCAAATTCTTCCAAGTGAACAGACGTGTAAACA TCGTCTTTCACTAAACGTTCACTCATGATAACGGCATCTT CAAAGTTGTAACCTTCCCATGT
8-7P1	234	TCTTTTTTACCCCAAAAGCCACGATAACGAAGCGCTTCTT GCAAAACGTCTTCAACCGTTCGCTCTCTTGAATTATTTGT TTCCATTGCCCATGTATCTGGATTGTGGCAATATTGGCAA CGCATTTTACAACCCTGTAAAAAGACAATGAAACGGATAC CAGGCCCATCAACAGCTCCAAAACTTTCAGTCGAATGGA CCATACCTGTTACTTTTCCATAATCAATGACTTCTTTTTCC AGCA

3.4.3.1. Sequence analysis

8-68P:

Base pairs 1-71 showed highest (97 %) homology to regions of the 16S rRNA gene in species such as bradyrhizobium (97 %), Flexistyphes (97 %) and Rhodococcus (96 %) (Table 3.7). Base pairs 266-299 showed 100 % homology to a gene for RNA polymerase beta subunit of two different species of streptococci: *S. intermedius*, *S. anginosus*, and 97 % for two species of streptococci: *S. constellatus* and *S. sanguis* (Table 3.8).

Due to the low number of bases being matched in the Blast searches a Tblastx search was performed on the entire sequence to attempt to match more of it to the predicted product. Tblastx works by translating the nucleotide sequence and matching it directly with translations of the nucleotide databases.

The Tblastx analysis revealed a similar result to that of the original Blast searches. The transcript showed homology to RNA polymerase beta subunits of *S. mutans* and *S. pneumoniae* (both 100 % homology) and *S. intermedius* (94% homology) (Table 3.9).

8-7P1:

Base pairs 50-65 showed 100 % homology to a *Streptococcus agalactiae* NEM316 gene coding for dTDP-4-keto-6-deoxyglucose-3,5-epimerase enzyme (Table 3.10).

Base pairs 84-127 showed 93 % homology to a *S. mutans* gene for a PFL (Pyruvate Formate Lyase) activating enzyme (Table 3.10).

Base pairs 178-225 showed 91 % homology to a *S. pyogenes* MGAS315 section of the genome containing gene *hlyX*, known to code for a putative haemolysin (Table 3.10).

To some extent the Tblastx analysis correlated with the results of the original Blast analysis. High homology was demonstrated to the pyruvate formate lyase activating enzyme gene of *Streptococcus bovis* (97 %). A fragment of the transcript showed 94 % homology to the putative *S. pyogenes hlyX* haemolysin (Table 3.11).

A diagrammatic representation of the blast analysis of band 8-7P1 can be seen in Figure 3.6 illustrating the relevant sections of the sequence.

Table 3.7. Blast sequence homology data for transcript 8-68P of 96 % or greater.

Bradyrhizobium sp. PAC50 gene for 16S rRNA, partial sequence
Length = 390
Accession number: ABO69653
Score = 117 bits (59), Expect = 6e-25
Identities = 69/71 bp (97 %), Gaps = 1/71 (1 %)

```
Query:1 atagggcgaattgggccctctagatgcatgctcgagcgggccggcagtgtgatggatatc
        ||||||||||||||||||||||||||||||||||||||| ||||| ||||||||||||||||
sub:386 atagggcgaattgggccctctagatgcatgctcgagc-ggccgccagtgtgatggatatc

Query: tgcagaattcg 71
       ||||||||||||
Sub:   tgcagaattcg 317
```

Flexistipes-like sp. oral clone BB062 16S ribosomal RNA gene, partial sequence
Length = 1604
Accession number AY005445
Score = 115 bits (58), Expect = 2e-24
Identities = 68/70 bp (97 %), Gaps = 1/70 (1%)

```
Query:50 atagggcgaattgggccctctagatgcatgctcgagcgggccggcagtgtgatggatatc
         |||||||||||||||||||||||||||||||||||||| ||||| ||||||||||||||||
S:1546   atagggcgaattgggccctctagatgcatgctcgagc-ggccgccagtgtgatggatatc

Query:  tgcagaattc 70
        ||||||||||
Sbjct:  tgcagaattc 1478
```

Rhodococcus opacus gene for 16S rRNA, partial sequence
Accession number AB060974
Length = 696
Score = 105 bits (53), Expect = 2e-21
Identities = 63/65 bp (96 %), Gaps = 1/65 (1%)
Strand = Plus / Plus

```
Query:7  cgaattgggccctctagatgcatgctcgagcgggccggcagtgtgatggatatctgcaga
         |||||||||||||||||||||||||||||| ||||| ||||||||||||||||||||||
Sbjct:1  cgaattgggccctctagatgcatgctcgagc-ggccgccagtgtgatggatatctgcaga

Query: attcg 71
       |||||
Sbjct: attcg 64
```

Table 3.8. Blast sequence homology data for transcript 8-68P of 97 % or greater.

Streptococcus intermedius RNA polymerase beta subunit (rpoB) gene, partial cds
Accession number AF194516.1
Length = 871
Score = 67.9 bits (34), Expect = 5e-10
Identities = 34/34 bp (100 %)
Strand = Plus / Minus

```
Query: 266 taacggcatcttcaaagttgtaaccttcccatgt 299
           ||||||||||||||||||||||||||||||||||
Sbjct: 871 taacggcatcttcaaagttgtaaccttcccatgt 838
```

Streptococcus anginosus RNA polymerase beta subunit (rpoB) gene, partial cds
Accession number AF194510.1
Length = 871
Score = 67.9 bits (34), Expect = 5e-10
Identities = 34/34 bp (100 %)
Strand = Plus / Minus

```
Query: 266 taacggcatcttcaaagttgtaaccttcccatgt 299
           ||||||||||||||||||||||||||||||||||
Sbjct: 871 taacggcatcttcaaagttgtaaccttcccatgt 838
```

Streptococcus constellatus RNA polymerase beta subunit (rpoB) gene, partial cds
Length = 871
Accession number AF194512.1
Score = 60.0 bits (30), Expect = 1e-07
Identities = 33/34 bp (97 %)
Strand = Plus / Minus

```
Query: 266 taacggcatcttcaaagttgtaaccttcccatgt 299
           |||||||||||||||| ||||||||||||||||
Sbjct: 871 taacggcatcttcaaaattgtaaccttcccatgt 838
```

Streptococcus sanguinis RNA polymerase beta subunit (rpoB) gene, partial cds
Length = 871
Accession number AF194528.1
Score = 60.0 bits (30), Expect = 1e-07
Identities = 33/34 bp (97 %)
Strand = Plus / Minus

```
Query: 266 taacggcatcttcaaagttgtaaccttcccatgt 299
           |||||||||||||||| ||||||||||||||||
Sbjct: 871 taacggcatcttcaaaattgtaaccttcccatgt 838
```

Table 3.9. Sequence homology data for band 8-68 P using Tblastx.

gi|17225227|gb|AF325869.1| *Streptococcus intermedius* RNA polymerase B-subunit (rpoB) gene, partial cds
Length = 3845
Score = 148 bits (318), Expect = 1e-33
Identities = 57/73 (78%), Positives = 69/73 (94%)
Frame = -1 / +3

```
299 TWEGYNFEDAVIMSERLVKDDVYTSVHLEEFESETRDTKLGPEEITREIPNVGEDALRDL
    TW+GYN+EDAVIMSERLVKDDVYTS+H+EE+ESE RDTKLGPEEITR+IPNVE+ AL++L
    TWDGYNYEDAVIMSERLVKDDVYTSIHIEEYESEARDTKLGPEEITRDIPNVSENALKNL

Query: 119  DEMGIIRIGAEVK 81
             D+ GI+ +GAEVK
Sbjct: 2391 DDRGIVYVGAEVK 2429
```

gi|24378349|gb|AE015022.1| *Streptococcus mutans* UA159 section 170 of 185 of the complete genome
Length = 10029
Score = 173 bits (373), Expect = 3e-41
Identities = 72/74 (97%), Positives = 74/74 (100%)
Frame = -1 / -2

```
299  TWEGYNFEDAVIMSERLVKDDVYTSVHLEEFESETRDTKLGPEEITREIPNVGEDALRDL
     TWEGYNFEDAVIMSERLVKDDVYTSVHLEEFESETRDTKLGPEEITREIPNVGEDAL+DL
     TWEGYNFEDAVIMSERLVKDDVYTSVHLEEFESETRDTKLGPEEITREIPNVGEDALKDL

Query: 119  DEMGIIRIGAEVKK 78
             DEMGIIRIGAEVK+
Sbjct: 1238 DEMGIIRIGAEVKE 1197
```

>gi|15459456|gb|AE008542.1|AE008542 *Streptococcus pneumoniae* R6 section 158 of 184 of the complete genome
Length = 10902
Score = 172 bits (370), Expect = 8e-41
Identities = 71/74 (95%), Positives = 74/74 (100%)
Frame = -1 / -3

```
299  TWEGYNFEDAVIMSERLVKDDVYTSVHLEEFESETRDTKLGPEEITREIPNVGEDALRDL
     TWEGYNFEDAVIMSERLVKDDVYTSVHLEE+ESETRDTKLGPEEITREIPNVGEDAL+DL
     TWEGYNFEDAVIMSERLVKDDVYTSVHLEEYESETRDTKLGPEEITREIPNVGEDALKDL

Query: 119  DEMGIIRIGAEVKK 78
             DEMGIIRIGAEVK+
Sbjct: 6283 DEMGIIRIGAEVKE 6242
```

Table 3.10. Blast sequence homology data for transcript 8-7P1.

Streptococcus agalactiae NEM316 complete genome, segment 8
Length = 199050
Score = 32.2 bits (16), Expect = 6.8
Identities = 16/16 (100%)
Strand = Plus / Minus

```
Query: 50     cttcaaccgttcgctc 65
              ||||||||||||||||
Sbjct: 104375 cttcaaccgttcgctc 104360
```

Streptococcus mutans YtqB (ytqB) gene, partial cds; ABC transporter (abcX), putative permease (perM), putative haemolysin (hlyX), pyruvate-formate lyase activating enzyme (pflC), D-alanine-D-alanyl carrier protein ligase (dltA), integral membrane protein
Length = 11202
Accession number AF051356
Score = 63.9 bits (32), Expect = 6e-09
Identities = 41/44 bp (93%)
Strand = Plus / Minus

```
Query: 84   cattgcccatgtatctggattgtggcaatattggcaacgcattt 127
             ||||||||| ||||| ||||||||||||||||| ||||||||||
Sbjct: 3943 cattgcccaagtatcaggattgtggcaatattgacaacgcattt 3900
```

Streptococcus mutans genes for PFL-activating enzyme and PFLAE-5'ORF, partial and complete cds
Length = 1502
Accession number AB018417
Score = 63.9 bits (32), Expect = 6e-09
Identities = 41/44 bp (93%)
Strand = Plus / Minus

```
Query: 84  cattgcccatgtatctggattgtggcaatattggcaacgcattt 127
            ||||||||| ||||| ||||||||||||||||| ||||||||||
Sbjct: 454 cattgcccaagtatcaggattgtggcaatattgacaacgcattt 411
```

Streptococcus pyogenes MGAS315, section 7 of 37 of the complete genome
Length = 50259
Score = 61.9 bits (31), Expect = 1e-08
Identities = 43/47 (91%)
Strand = Plus / Minus

```
Query: 51   ccaaaactttcagtcgaatggaccatacctgttacttttccataatc 97
             |||||||||| |||||||| ||||| |||||||||| ||||||||
Sbjct: 6736 ccaaaactttctgtcgaatgcaccatccctgttacttgtccataatc 6690
```

Table 3.11. Sequence homology data for band 8-7P1 using Tblastx.

gi|14141681|db|AB061728.1| *Streptococcus bovis* act gene for pyruvate formate-lyase activating enzyme, complete cds
Length = 1147
Score = 176 bits (378), Expect = 1e-42
Identities = 63/74 (85%), Positives = 72/74 (97%)
Frame = -3 / +1

```
IDYGKVTGMVHSTESFGAVDGPGIRFIVFLQGCKMRCQYCHNPDTWAMETNNSRERTVED
IDYGKVTGM+HSTESFG+VDGPG+RF++F+QGCKMRCQYCHNPDTWA+ETNNSRERTV+D
IDYGKVTGMIHSTESFGSVDGPGVRFVIFMQGCKMRCQYCHNPDTWALETNNSRERTVDD

Query: 47  VLQEALRYRGFWGK 6
           VL EALRYR FWG+
Sbjct: 454 VLAEALRYRHFWGE 495
```

>gi|13621628|gb|64AE006501.1|AE006501 *Streptococcus pyogenes* M1 GAS strain SF370, section 30 of 167 of the complete genome
Length = 11447
Score = 168 bits (361), Expect = 3e-40
Identities = 62/74 (83%), Positives = 70/74 (94%)
Frame = -3 / +3

```
224     DYGKVTGMVHSTESFGAVDGPGIRFIVFLQGCKMRCQYCHNPDTWAMETNNSRERTVEDV
        DYG+VTGMVHSTESFG+VDGPGIRFI+FLQGCK+RCQYCHNPDTW METNNS+ RTV DV
        DYGQVTGMVHSTESFGSVDGPGIRFIIFLQGCKLRCQYCHNPDTWEMETNNSKIRTVNDV

Query: 44   LQEALRYRGFWGKK 3
            L+EAL+Y+ FWGKK
Sbjct: 1701 LKEALQYKHFWGKK 1742
```

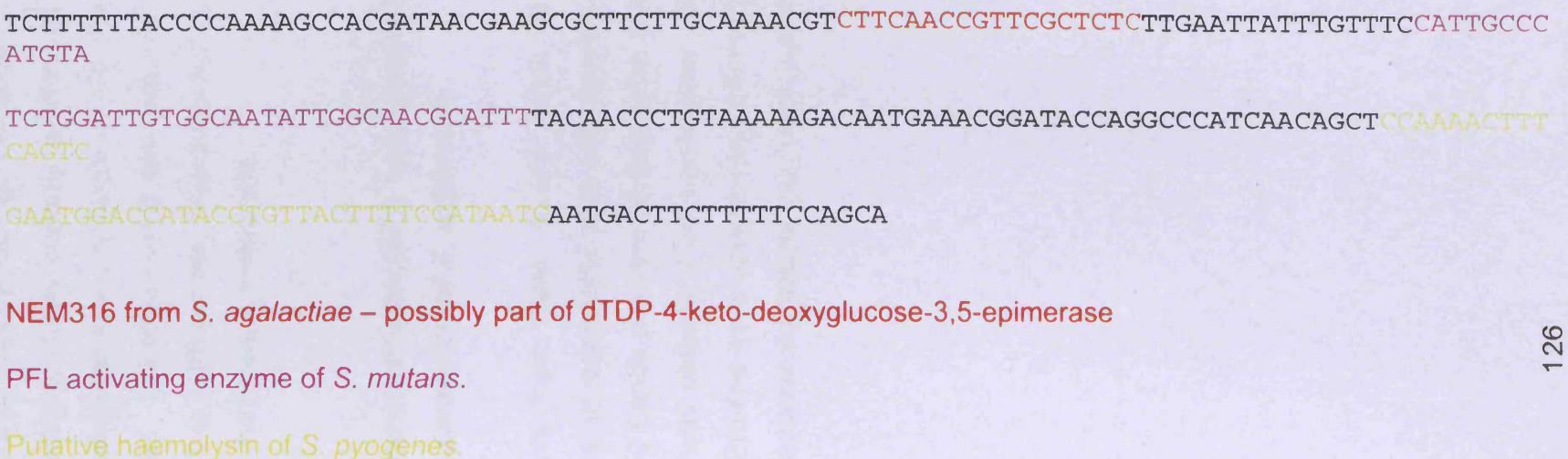

Figure 3.6. Diagrammatic representation of band 8-7P1 showing the NEM316 fragment from *S. agalactiae*, the PFL activating enzyme from *S. mutans* and the putative haemolysin from *S. pyogenes*. The sequences can be seen superimposed onto the original sequence. It should be noted that this is based upon blastN sequence analysis and not TblastX.

3.4.4. Northern blotting.

While a sufficient amount of RNA (10-20 μg) was obtained for the purpose of Northern analysis, the Northern blots routinely failed to work. RNA was found to degrade upon initial electrophoresis and consistently failed to transfer to the nitrocellulose membrane. Extraction using alternate methods succeeded in producing enough RNA to perform the procedure, but consistently failed to successfully transfer to the Hybond membrane.

3.4.5. Effect of environmental osmolarity and haemin/iron concentration upon the gene expression of *S. anginosus*.

The RT-PCR transcripts from Strain 8 and Strain 56 of *S. anginosus* subjected to alterations in osmolarity and iron concentration produced a fingerprints with over 50 bands per lane of detectable products (Figure 3.7). No detectable product was obtained from non-reverse transcribed RNA and the water only control. Profiles were obtained by performing RT-PCR on RNA samples extracted on separate occasions, but failed to show reproducibility.

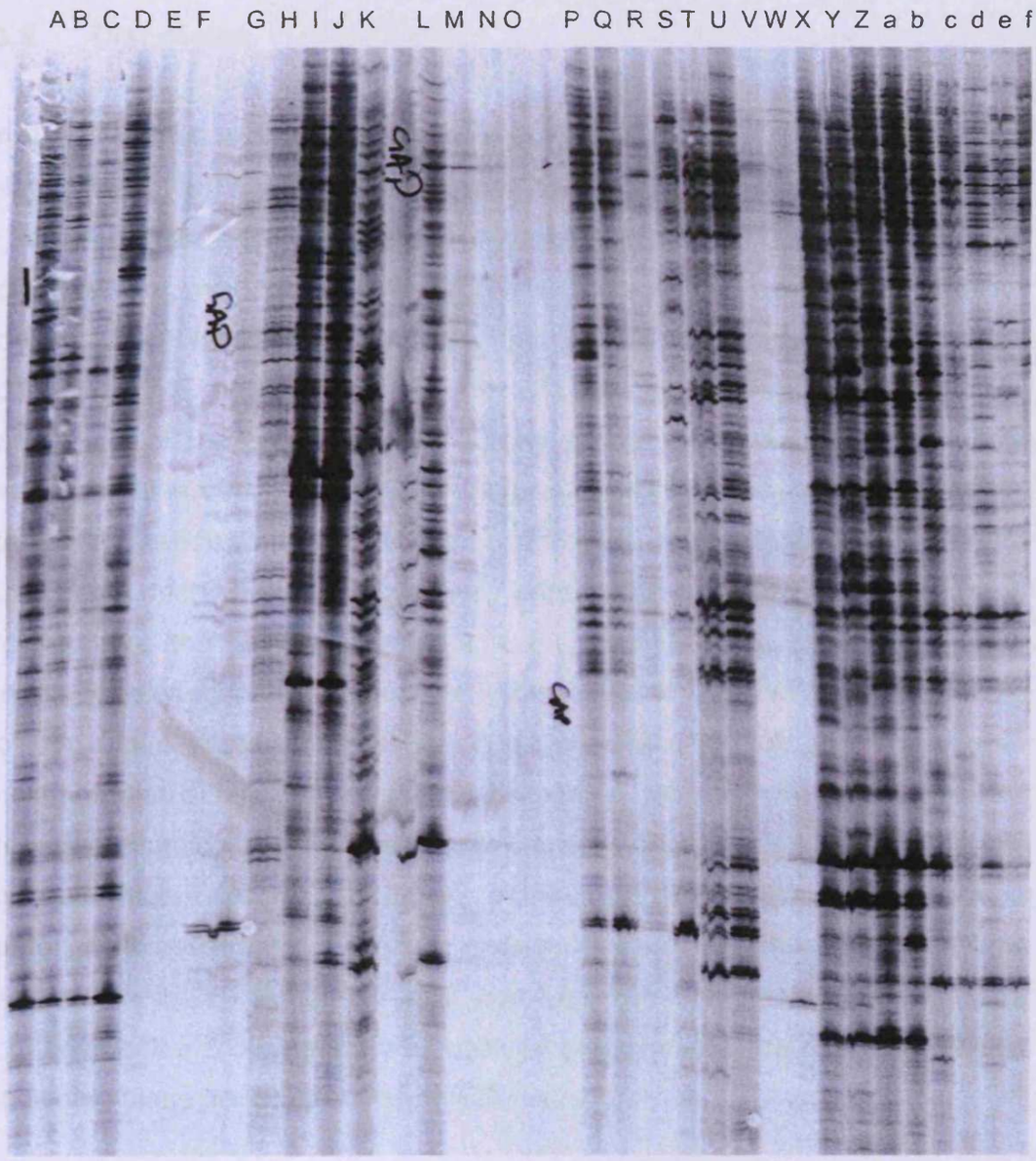

Figure 3.7. Polyacrylamide gel showing DD RT-PCR products of *S. anginosus* strains 8 and 56 grown at different osmolarities[p] and iron concentrations[q]. A and B = 56 (2.0), C and D = 56 (0.2), E and F = 56 (0.14), G and H = 56 (1.4), I and J = 56 (700), K and L = 56 (234), M and N = 56 (117), O = 56, P and Q = 8 (4.0), R and S = 8 (2.0), T and U = 8 (0.2), V and W = 8 (0.14), X and Y = 8 (1.4), Z and a = 8 (700), b and c = 8 (234), d and e = 8 (117), f = 8.
The reverse transcription and PCR are performed in duplicate, but fail to show reproducibility. Profiles were obtained by performing RT-PCR on RNA samples extracted on 2 separate occasions.
[p]117 and 234 indicates level of NaCl in mg, 700 and 1.4 indicates level of sucrose in mg.
[q]0.14, 0.2, 2.0 and 4.0 indicate the haemin levels in µl).

3.5. Discussion

An association between the environment and the expression of virulence determinants of a microorganism has long been recognised. Mekalanos, (1992) reported a link between environmental conditions and the inducement of virulence genes such as the effect of osmolarity, pH, temperature and iron on *Vibrio cholerae*. Badger and Kim, (1998) showed that the invasion capability of *E. coli* was increased by changes in the environmental pH and osmolarity of the growth media. Using a basic nutrient broth and adding varying concentrations of NaCl or sucrose to alter the osmolarity, and altering the pH they showed that the tissue invasiveness of *E. coli* increased at pH 5.5 to 6.5, but decreased at a higher pH. Altering the osmolarity was also shown to suppress the ability of the cells to invade tissue. Stubbs *et al.,* (1999) studied the effect of haemin concentration in basal media on the biochemistry of *Prevotella intermedia* and found an increased growth rate and protein concentration at higher haemin concentrations. The pH in the mouth is known to vary according to diet, and can become as high as pH 8.5 in periodontal pockets (Marsh and Martin, 2000). Likewise, when members of the oral flora enter the bloodstream, a marked change in many environmental conditions and in particular osmolarity and iron concentration is observed. These changes to the environment may have an effect on bacterial gene expression, including those genes associated with virulence.

The study of up or down regulation and the discovery of previously unknown genes that are induced under specific environmental situations can be undertaken by subtractive hybridisation or DD RT-PCR. DD RT-PCR was chosen as the method to study preferential gene expression of *S. anginosus* for a number of reasons; multiple samples and sample conditions can be tested simultaneously (Nagel *et al.*, 1999); low amounts of RNA can be used for the reverse transcription step; the speed of the process from start to finish is usually around 8 working days making it fast when compared to other techniques; and low copy number transcripts are included in the display, (Miele *et al.,* 1998).

A critical element in DD RT-PCR is the ability to extract RNA in sufficient quantity and of adequate purity and integrity for further genetic manipulation. This is a problem in Gram-positive bacteria due to the fact that they have cell walls that are difficult to lyse, and a short RNA half life. Successful extraction of RNA from microorganisms with thick cell walls, for example mycobacteria and Gram-positive organisms has been documented previously with the use of a range of agents including Trizol reagent (Kullen and Klaenhammer, 1999), guanidinium thiocyanate (Chakrabortty *et al.,* 2000; Rindi *et al.*, 1999), CTAB detergent (Cheung *et al.*, 1994; Mangan *et al.*, 1997), lysozyme and Proteinase K buffers (Bashyam and Tyagi, 1994). However, these methods are time consuming and labour intensive. The present study has demonstrated that commercially available methods can offer a simple and rapid method of RNA isolation from *S. anginosus*. The RNA obtained was of sufficient quantity and quality for further genetic analysis, specifically RT-PCR with random primers. The Ultraspec® method was both more time consuming (1.5 h compared to 45 min for the RNeasy kit) and labour intensive, requiring more hands-on time, however it proved more reliable at extracting RNA when compared to the Qiagen system. The Ultraspec® was more successful at providing the RNA possibly due to a mechanical method of lysis, which may facilitate the breakdown of the cell wall with more efficiency. The RNeasy kit used only a lysozyme-based method of lysis, which may be insufficient for some organisms.

For DD RT-PCR, differential transcripts are typically resolved by polyacrylamide gel electrophoresis and detected using radio-isotopic methods (Sturtevant, 2000). However, there have been reports of the successful use of agarose gel electrophoresis and a range of non-radioisotope detection methods (An *et al.*, 1996; Hsu *et al.,* 1997; Sokolov and Prockop, 1994). In the current study, agarose gel electrophoresis and the use of non-radioactive methods (Ethidium bromide, SYBR green II, and silver staining) proved too insensitive for the detection of cDNA species by the methods used. The

detection limit of SYBR green II (20 pg DNA) is approximately 20 – 25 times more sensitive than that of ethidium bromide. Adding to this the proposed detection limits of silver staining (20 – 50 pg DNA) and it is evident that a detection method more sensitive than those mentioned is required. Radiolabelling with [α-^{33}P] dATP in conjunction with polyacrylamide gel electrophoresis was required for the optimal resolution and detection of transcripts.

Experimentation with four variations of the PCR method determined that the protocol outlined by Fislage *et al.,* (1997) for differential display with *E. coli* was optimal for differential display with *S. anginosus*. The in-house method with the Fislage cycling parameters, while producing transcripts, seemed to produce fewer bands than the former method, and use of the in-house cycling parameters with either PCR mix resulted in no detectable product.

Primer design is critical for efficient and accurate DD RT-PCR. Primers should be designed that will fully represent the mRNA species population under study. The lack of a large polyadenylated tail on the 3' end of bacterial mRNA has necessitated the use of random primer sets for the generation of cDNA and a randomly primed PCR step (Fislage *et al.,* 1997). The primers used in the present study were those designed by Fislage *et al.,* (1997) to provide adequate coverage of the genome of the Gram-negative bacteria *E. coli.* The findings presented here have demonstrated that these primers may be usefully employed in the analysis of *S. anginosus*. The reproducibility of the technique is of critical importance and experiments performed in triplicate on RNA samples from different extraction experiments for heat shock and pH were similar. However, a comparison of RNA fingerprints derived for bacterial strains subjected to varying environmental conditions of osmolarity and iron concentration showed that differences were apparent. It is important in every model system to incorporate a number of controls to ensure that there is no DNA contamination of the RNA. The non-reverse transcribed control ensures that this is the case. A water control is also incorporated to ensure that there is no contamination of the nuclease free water being used in

the PCR reactions. RNA is also extracted twice for each sample and is processed simultaneously to ensure that a matching result is obtained ensuring reproducibility. Reproducibility is important, as a non-reproducible result is more likely to be a false positive and therefore is not valid. The reasons for the differences in reproducibility are unclear, but may be due to heat shock and pH change being a more extreme form of stress to the cells, and therefore producing a more standard, uniform response whereas osmolarity and iron concentration involve smaller changes taking place gradually and this producing a more inhibited reaction by the cells that is difficult to accurately reproduce.

Problems typically encountered with the DD RT-PCR method include low reproducibility of results and a bias for high copy number RNA (Ledakis *et al.*, 1998), co-migration of DNA fragments of a similar size to possible differentially displayed products (Miele *et al.*, 1998), a high rate of false positive results (Milson and Burrows, 1998) and a prevalence for the reverse transcription of rRNA's above mRNA's (Nagel *et al.,* 1999). The prevalence of reverse transcription of rRNA over mRNA was observed in the sequence analysis of band 8-68P with the discovery of rRNA sequences for SMG.

Many of the problems associated with differential display can be potentially circumvented. Increasing the number of primers used and their combinations can reduce the incidences of mispriming and lower the frequency of false positives (Ledakis *et al.*, 1998). By increasing the annealing temperature stringency and decreasing the annealing time the specificity of annealing can be increased and the reproducibility of the display can be reinforced (Malhotra *et al.,* 1998). In order to reduce the incidences of rRNA being amplified above mRNA, the antibiotic rifampicin can be used to culture the bacteria. However, the problem can also be dealt with at a later stage by confirmation of differential bands through Northern blotting (Nagel *et al.,* 1999). Additional problems with prokaryotic systems include the instability and short half-life of mRNA species in comparison to eukaryotes and the low level of polyadenylation of mRNA, which potentially prohibits the use of 3' dT

anchored primers. For Gram-positive bacteria, these problems are further compounded by the difficulties in RNA isolation related to the presence and structure of a thick cell wall. The current study shows how problems can be overcome through the use of a commercial extraction method.

Sequencing of band 8-68P revealed sequence data with similarity to RNA polymerase beta subunits present within the "*Streptococcus milleri*" group. This is a common problem in differential display due to the abundance of rRNA present in streptococci. Tblastx results also showed homology to RNA polymerase beta subunits, but in a wider variety of organisms. Closer homology was found in *S. mutans* and *S. pneumoniae*. Band 8-7P1 was more interesting, showing sequence homology to *S. mutans* PFL (pyruvate formate lyase) activating enzyme at bases 84 to l27. Tblastx analysis showed a closer homology to the PFL activating enzyme of *S. bovis.* This is responsible for the post translational activation of the stored inactive form of PFL to initiate the mixed acid fermentation pathway in the production of NAD/$NADH^+$ and ATP (Conradt *et al.*, 1984) (Figure 3.8). This pathway has been shown to form the major route of pyruvate catabolism in anaerobically growing *E. coli* (Weidner and Sawers, 1996). It is regarded as important in anaerobic metabolism in most anaerobes as it is considered to take over ATP synthesis from lactate dehydrogenase (Yamamoto *et al.*, 2000). The enzyme in *S. bovis* was deduced to consist of 261 amino acids with a molecular mass of 29.9 kDa and appeared to be a monomer protein (Asanuma and Hino, 2001). The enzyme appeared to be upregulated in *S. anginosus* at pH 7.2. In *S. bovis* it was found that low pH's (< 6.0) resulted in an increase in Lactate dehydrogenase transcription and a lowering in PFL transcription, and pH's of 7.0 (an optimal of 7.5) resulted in activation of PFL and the mixed acid fermentation pathway (Asanuma and Hino, 2000). The flanking regions to this section showed homology to a putative *hlyX* haemolysin at base pairs 178 to 225, and the dTDP-4-keto-6-deoxyglucose-3,5-epimerase enzyme at base pairs 50 to 65 in *S. pyogenes.* The dTDP-4-keto-6-deoxyglucose-3,5-epimerase enzyme has been shown to be involved in the process of dTDP-rhamnose production through its reversible epimerisation of the C-3' and C-5'

atoms of dTDP-4-keto-6-deoxyglucose to produce dTDP-4-keto-Rhamnose (Stern *et al.*, 1999) (Figure 3.9). L-rhamnose is a deoxy-sugar found widely in bacteria and plants (Giraud and Naismith, 2000). The deoxy-sugar is found in serotype-specific cell wall polysaccharides in almost all streptococci (Schleifer and Kilpper-Balz, 1987) and in the O antigen of lipopolysaccharide in many Gram-negative bacteria (Reeves, 1993). Evidence continues to emerge about its essential role in many pathogenic bacteria (Giraud and Naismith, 2000). Rhamnose has been suggested as a virulence factor in *Streptococcus suis* (Charland *et al.*, 1998) and is considered essential for virulence in *Pseudomonas aeruginosa* (Ochsner and Reiser, 1995). The serotype-specific polysaccharide antigens of *S. mutans* are composed of backbone structure of 1,2 and 1,3-linked rhamnosyl units (Linzer *et al.*, 1986; Michalek *et al.*, 1984). Identical backbone units have also been found in Lancefield A, C and E streptococci (Coligan *et al.*, 1978; Pritchard, 1985) and the rhamnose-glucose polysaccharide antigen of *S. sobrinus* (Linzer *et al.*, 1985). These backbone units have been proposed as putative mediators for the colonisation of tooth surfaces (Michalek *et al.*, 1984) and for its binding to heart, kidney and muscle tissues (Stinson *et al.*, 1980). Tsukioka *et al.*, (1997) took information concerning the gene loci for rhamnose in Gram-negative species and applied it to a similar search in *S. mutans*. The researchers cloned the three genes of the serotype C *S. mutans* chromosome which showed homology to the same genes in Gram-negative bacteria associated with dTDP-L-rhamnose anabolism (*rmlA, rmlB, rmlC*). The function of the cloned genes were analysed and an involvement with dTDP-rhamnose synthesis was found. As well as this, the homologous genes were identified in other streptococci by Southern blotting. The genes responsible for the dTDP-rhamnose synthesis pathway have been therefore identified in *S. mutans* as *rmlA*, *rmlB* and *rmlC* (Figure 3.9). However the final gene *rmlD* was not found in the *rml* locus alongside the other genes. A wider search was initiated and the gene found using a probe constructed from a sequence obtained from the *S. pyogenes* sequencing database (which produced the blast sequence homology in band 8-7P1). The gene responsible for the deoxyglucose epimerase enzyme (*rmlC*) has been proposed as a potential drug target in several genera of

bacteria (Stern *et al.*, 1999). Tblastx analysis confirmed homology to a putative *hlyX* haemolysin in *S. pyogenes*. Unfortunately, the gene has not been characterised in streptococci. However, in *E. coli* it is known to accompany the fumarate and nitrate reduction (*FNR*) gene in responding to decreases in environmental oxygen levels and stimulating anaerobic metabolism, as a further function it stimulates the transcription of the *hlyE* gene that codes for a haemolytic protein in some strains (Green and Baldwin, 1997). Although it has not been characterised, its position in the *S. mutans* genome is known to lie next to that of the PFL gene within an open reading frame adjacent to the *dlt* operon (Boyd *et al.*, 2000). The band producing these fragments appeared to be upregulated at pH 7.2 (standard growth pH), and was absent from other strains grown at higher or lower pH's. These results may indicate a rise in the rate of anaerobic metabolism at pH 7.2.

Whilst it was possible to obtain RNA of sufficient quantity for RT-PCR, it was more difficult to collect RNA of an adequate quality for Northern analysis. The main disadvantage of Northern blotting is the requirement of a large amount of starting material, while this was possible, the stability problem made the task of performing Northern blots and thus proving / disproving the authenticity of the reamplified bands difficult. For Northern blotting to work successfully 10 – 20 μg of RNA of high stability and good quality must be available (Miele *et al.*, 1998). While Northern blots can be performed using as little as 2 μg of material (Petersen *et al.*, 2001), this would appear to be a minimal requirement and may not be suitable for Gram-positive bacteria. Alternative methods of RNA extraction were attempted including a method previously employed by Petersen *et al.,* (2001) on *S. intermedius* where a lysis solution of TE buffer, lysozyme and mutanolysin was used prior to extraction using Qiagen RNeasy columns. This method was successful for *S. intermedius* but unsuccessful for *S. anginosus*. Another method employing mechanical lysis at extremely low temperature using a cryogenic cell grinder also proved unsuccessful. Other methods are available that have previously been successfully used for RNA extraction in streptococci. However, these

employ machinery and reagents that are very specialised and expensive. The Q-biogene Bio101 system of nucleic acid extraction reagents when used with the Fastprep® instrument has been shown to be successful at fast, clean RNA preparation for difficult to lyse bacteria (Bolano *et al.*, 2001). The only other way of modifying existing methods would be to try using a different RNA stabiliser prior to extraction as one of the stabilising agents available (RNAlater®) failed to increase RNA quality. Alternatives to Northern blotting include RNA dot blots and real time PCR however, due to time constraints neither of these methods could be attempted.

DD RT-PCR has been successfully used for a range of bacteria including strains of *Mycobacterium tuberculosis* (Rindi *et al.*, 1999), Legionella (Kwaik and Pederson, 1996), *P. gingivalis* (Bonass *et al.*, 2000), *S. gordonii* (Du and Kolenbrander, 2000) and *S. mutans* (Chia *et al.*, 2000). This work demonstrates the successful application of DD RT-PCR to *S. anginosus* and shows that it can be reproducible under different environmental conditions, as similar profiles have been obtained with different RNA extracts. It has shown that with careful attention to experimental protocol and appropriate controls; candidate preferentially expressed gene fragments may be detected. This paves the way for further work to increase our understanding of factors that may be associated with the virulence of this group of pathogens.

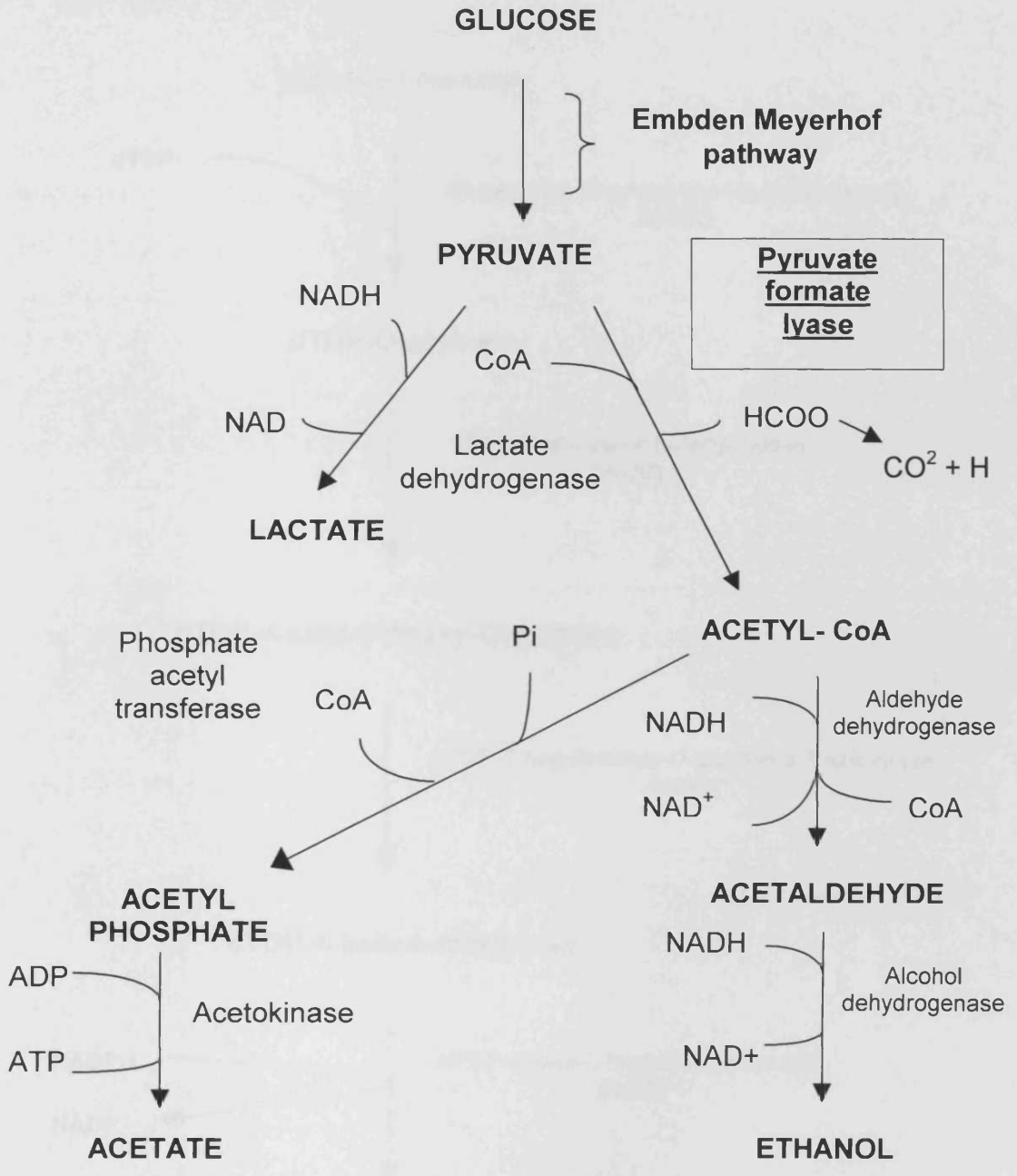

Figure 3.8. Mixed acid fermentation pathway showing Pyruvate Formate Lyase activating enzyme producing Acetyl-CoA from Pyruvate. Adapted from Prescott, *et al.*, (1995).

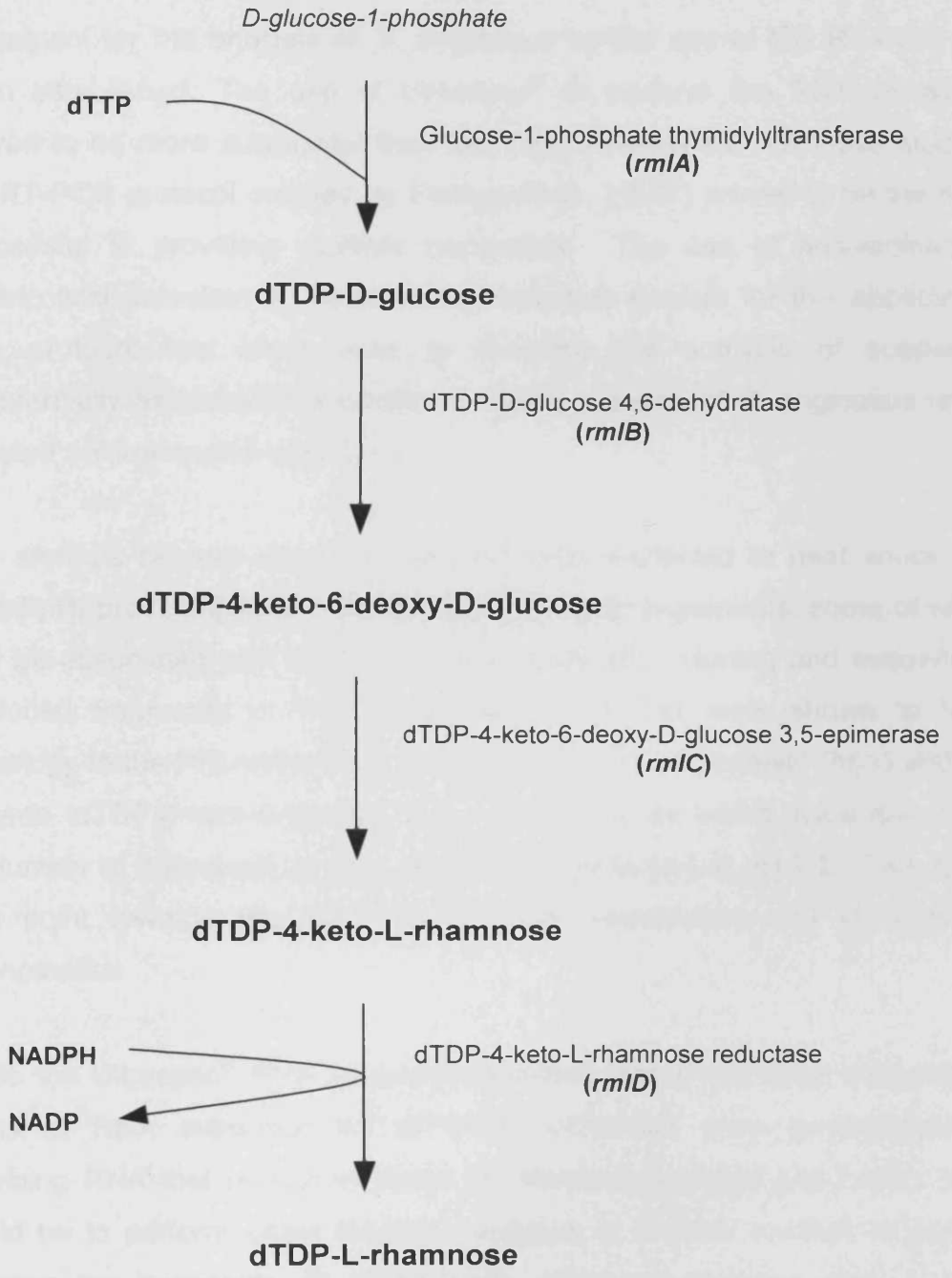

Figure 3.9. Biosynthesis pathway of dTDP-L-rhamnose illustrating the function of the dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase enzyme. The genes encoding the pathway enzymes are in parentheses

3.6. Conclusions.

A protocol for the analysis of *S. anginosus* by the use of DD RT-PCR has been established. The use of Ultraspec® to perform the RNA extraction proved to be more successful than the rapid RNeasy kit. Of those studied, the RT-PCR protocol outlined by Fislage *et al.,* (1997) proved to be the most successful at providing multiple transcripts. The use of non-radioactive nucleic acid detection is, however not sensitive enough for this application. This protocol has been used to facilitate the analysis of suspected preferentially expressed transcripts in model systems of *S. anginosus* under different environmental conditions.

The analysis centred upon the bacterial cells subjected to heat shock and varied pH, producing many differential banding arrangements, some of which may be associated with virulence. Band extraction, cloning and sequencing produced fragments of transcripts of strain 8 that were shown to have homology to the PFL-activating enzyme, a putative haemolysin (*hlyx*) and the enzyme dTDP-4-keto-6-deoxyglucose-3,5-epimerase which has a role in the production of rhamnose (a suspected virulence factor) at pH 7.2. This result may point towards the role of pH in the metabolism and virulence of *S. anginosus*.

While the Ultraspec® RNA extraction protocol proved the most adequate in terms of RNA extraction for RT-PCR, difficulties were encountered in obtaining RNA that remained stable for Northern analysis and further work would be to perform either Northern analysis or another method to prove / disprove the transcripts as differentially expressed products and examine further their role in these bacteria.

CHAPTER IV: DEVELOPMENT OF MOLECULAR FINGERPRINTING METHODS FOR ANALYSIS OF THE "*STREPTOCOCCUS MILLERI*" GROUP

4.1. Introduction.

The focus of this section of the thesis is the optimisation of molecular fingerprinting methods for SMG strains. This was a pre-requisite to a larger study into the molecular ecology of SMG in the oral cavity and a study of the genomic stability of oral SMG strains (Chapter 5).

Commonly used genotypic typing methods include pulsed field gel electrophoresis (PFGE), ribotyping, restriction fragment length polymorphisms (RFLP), repetitive extragenic palindromic-PCR (Rep-PCR), BOX-PCR and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) (see section 1.5). Of these, the typing methods based on PCR are increasingly being used to characterise individual strains of bacteria (Alam *et al.*, 1999). Advantages of PCR-based approaches include the fact that they are generally non-labour intensive and provide results rapidly. PCR techniques are based upon the amplification of genomic DNA using specific or arbitrary primers to yield a fingerprint that is ideally characteristic of an individual strain. The primers used are frequently based upon the sequence of specific, highly conserved, centrally inverted repeats present in intercistronic regions of the genome, that are present in all strains of the species under study (Kerr, 1994). Although there are potentially many targets for primers, four sets have been employed frequently for bacterial typing; Rep, ERIC, M13 and BOX.

ERIC-PCR: The ERIC element is 126 bp long, occurs in multiple copies at each chromosome location (Kerr, 1994) and are located in the extragenic regions of the bacterial genome (Olive and Bean, 1999). This element has been used in the typing of many different groups of bacteria including streptococcal species. A number of primers targeting the region have been used successfully, such as ERIC1R (1246) and ERIC2 (1245) - a primer pair used in the typing of viridans streptococci (Alam *et al.,* 1999). These workers demonstrated that each species of viridans streptococci yielded multiple bands over a wide range of sizes and that this approach was highly

discriminatory. Cluster analysis showed that no two strains were more than 88 % similar. Wisplinghoff *et al.,* (1999) used ERIC2 primer with *S. mitis*, *S. sanguis* and *S. oralis*. They documented that each of 19 isolates yielded unique fingerprint patterns, a finding that was shown to correlate with the results of PFGE. It has been reported that a DNA extraction step is not required for this type of PCR, and that whole cell products can be used (Woods *et al.*, 1993). Indeed, both Alam *et al*, (1999) and Grundmann *et al.*, (1997) successfully used a crude boiling method to prepare template for use with these primers. Other ERIC primers (1245 and 1246) have been used to target prokaryotic repeat motifs at a higher annealing temperature of 56 °C (Alam *et al.*, 1999). Van Belkum *et al.,* (1994) used the same primers at a lower annealing temperature (25 °C) with strains of *Candida*.

Rep PCR: Rep elements are 38 bp long and occur in multiple copies at each chromosome location (Kerr, 1994). The Rep elements are palindromic, have an ability to form stem loop structures and have a highly conserved nature (Olive and Bean, 1999). Fingerprinting by Rep-PCR has been widely used in the analysis of different groups of bacteria. The original primers as designed by Gillings and Holley, (1997) were used for the typing of Gram-negative organisms but have also proven to be useful for Gram-positive bacteria. Coffey *et al.,* (1998) used Rep primers successfully to examine strains of *S. pneumoniae*. Alam *et al.,* (1999) demonstrated the use of the primers Rep1RDT and Rep2DT with viridans streptococci including *S. oralis*, *S. sanguis*, *S. parasanguis*, *S. mitis*, *S. mutans*, *S. sobrinus*, *S. salivarius, S. anginosus, S. intermedius* and *S. constellatus* amongst others. The results of this study indicated that the Rep sequences present in these bacterial groups could be employed for highly discriminatory intra-species comparisons.

In the case of both Rep and ERIC-PCR, the bands represent amplification of DNA between adjacent repetitive elements within the limit of polymerase extension (approximately 3 kb). Differences in band sizes result from

polymorphisms in the distance between Rep / ERIC sequences in different genomes, and since Rep / ERIC primers complement the conserved central inverted repeat sequences of the adjacent elements, single primers may be substituted for primer pairs without loss of typability or discriminative power (Kerr, 1994).

M13-PCR: M13-PCR has been used in the fingerprint typing of *Acinetobacter* spp. (Grundmann *et al.*, 1997) and viridans streptococci (Wisplinghoff *et al.*, 1999). In the latter study, the strains of *S. mitis*, *S. oralis* and *S. salivarius* studied all yielded a number of distinct profiles, suggesting that M13-PCR has a potential for fingerprint typing of the *"Streptococcus milleri"* group.

BOX-PCR: BOX elements were originally discovered in the early 1990's as interspersed repetitive DNA sequences within the intergenic regions of *S. pneumoniae.* BOX elements are mosaic repetitive sequences composed of various combinations of three subunits; BOX A, BOX B and BOX C. Individual subsequences within BOX A have been located in other species of streptococci, whereas BOX B and BOX C are predominantly found only in *S. pneumoniae* (Koeuth *et al.*, 1995). Van Belkum *et al.,* (1996) used BOX A primer in the typing of *S. pneumoniae*, and found that it generated amplicons of adequate size and frequency for DNA typing. BOX primers have also been used to type species of viridans streptococci. Alam *et al.,* (1999) employed the BOX A primer for the typing of oral isolates. It was found that none of the streptococcal species studied produced amplicons using this primer. Interestingly *S. oralis*, an organism phylogenetically closely related to *S. pneumoniae* failed to yield amplimers. BOX A1R primers (a subsequence within the BOX A region) has been used for successful typing of a number of organisms including *Salmonella enterica, E. coli* and *Proteus* species (Johnson and Clabots, 2000; Johnson and O' Bryan, 2000; Serwecinska *et al.,* 1998). BOX A2R primer (a subsequence within the BOX A region) has also been used by Serwecinska *et al.,* (1998) in the typing of *Proteus*, and by Malathum *et al.,* (1998) in the successful typing of *Enterococcus faecalis.* However, primers BOX A1R and BOX A2R have not been used in the typing

of any other streptococcal species other than *S. pneumoniae*. More information on PCR-typing is available in section 1.5.3.3.

Members of the *"Streptococcus milleri"* group of bacteria have been subjected to PCR typing previously. Van Belkum *et al.,* (1996) included a strain of *S. intermedius*, which was subjected to PCR typing using BOX A, BOX B and BOX C primers. In this study none of the BOX primers gave rise to amplimers. Alam *et al.,* (1999) studied four strains of *S. intermedius*, four strains of *S. constellatus* and six strains of *S. anginosus* by typing using ERIC1R and ERIC2 primers, Rep1RDT and Rep2DT primers and a BOX A primer. No products were obtained for any of the strains with the BOX A primer. However all the other primer pairings studied produced amplimers. Clarridge *et al.*, (1999) performed a study only involving SMG species, using primers Rep1R-I and Rep2-I. Clear banding profiles were obtained with a low percentage similarity between isolates indicating good discrimination.

Pulsed field gel electrophoresis (PFGE) is often cited as the most discriminatory method for the typing of microorganisms (Grothues *et al.*, 1988). PFGE was developed in 1984 (Schwartz and Cantor, 1984) and is based on the digestion of chromosomal DNA using infrequent cutting enzymes after which the large fragments are separated on an agarose gel using an alternating electrical field. PFGE is highly discriminatory and is regarded by many as the current "gold standard" for bacterial typing (Mendez-Alvarez *et al.*, 1995). However, the expense of the equipment and running costs limit its application in both diagnostic and research laboratories. PFGE is also a time consuming and technically demanding method that has shown itself to be user sensitive. Despite these factors PFGE has been used for the successful typing of strains of streptococci including SMG, providing a high level of discrimination and reproducibility, and showing heterogeneity within SMG strains (Bartie *et al.*, 2000).

The general aim of this chapter was the identification and optimisation of a suitable PCR method for fingerprinting of SMG and the further optimisation of

PFGE. In addition to the optimisation, a considered selection of test strains would allow comparisons of disease and commensal isolates and enable analysis at inter and intraspecies levels.

4.2. Aims.

In summary, the aims of the work described in the chapter were:

1. To develop a protocol for the extraction of DNA from SMG isolates that was amenable to typing by a range of primers.

2. To establish a discriminatory and reproducible PCR-based typing method for SMG.

3. To further optimise an existing in-house PFGE method for SMG isolates.

4. To study the profiles obtained in the context of site of origin of strain and at inter and intraspecies levels.

4.3. Materials and methods.

4.3.1. Test strains.

A representative sample of SMG test strains was selected that incorporated members of each of the 3 species, and included both commensal and clinical isolates from each species. Clinical samples were isolated from routine diagnostic laboratory specimens in the Oral Microbiology Unit, UWCM and at the National Public Health Service, Cardiff (previously Public Health Laboratory Service). Commensal isolates were isolated from the oral cavities of otherwise healthy individuals, see section 3.3.1 for isolation details.

The identity and source of the 30 test strains is shown in Table 4.1.

4.3.2. DNA extraction from SMG.

Three methods of DNA extraction were tested in order to ascertain which was the most suitable method to provide template for subsequent PCR based typing studies. Heat extraction (see section 2.3.1 for method), "Chelex" extraction (see section 2.3.2 for method) and the phenol: chloroform / isoamyl alcohol method of extraction (see section 2.3.3 for method) were studied. Extraction was performed for each method using at least ten strains and was repeated on at least two occasions.

4.3.3. PCR based methods.

All PCR amplification reactions were carried out using the Hybaid PCR Express thermal cycler. All primer sequences are shown in Table 4.2. The most successful method was then used to type all of the test strains in Table 4.1.

Table 4.1. Identity and source of 30 test strains from the Oral Microbiology Unit, UWCM used in the development of the PCR typing methods and Pulsed field gel electrophoresis (*).

Species	Strain	Site of isolation[a]
S. intermedius	*82c	commensal dental plaque
	*69c	commensal dental plaque
	59c	commensal dental plaque
	68c	commensal dental plaque
	58c	commensal tongue
	*54252/95	ethmoidal fluid
	*240B/95	dentoalveolar abscess
	313A/95	dentoalveolar abscess
	HW69	brain abscess
	R87/3972	blood
S. constellatus	*57c	commensal tongue
	*34c	commensal tongue
	72c	commensal tongue
	48c	commensal tongue
	50c	commensal throat
	*4515/96	perianal abscess
	27647/96	wound swab from appendix
	*313B/95	dentoalveolar abscess
	3395/97	perianal swab
	229/98	dentoalveolar abscess
S. anginosus	NCTC10713	type strain (no source given in catalogue)
	*19c	commensal plaque
	*17c	commensal tongue
	52c	commensal throat
	56c	commensal tongue
	240A/95	dentoalveolar abscess
	*7K	brain abscess
	CDC2236-81	blood
	SL34/W	subphrenic abscess
	323/96	dentoalveolar abscess

[a] The term commensal in this context is used to indicate that the isolates were from volunteers who were in general good health and not taking any medication. No formal oral health assessment of the volunteers was undertaken however, it was noted that none of them demonstrated oropharyngeal disease other than chronic marginal gingivitis.

Table 4.2. Primer sequences for PCR typing methods.

Source	Primer	Sequence 5' – 3'
ERIC primers (Van Belkum *et al.*, 1994).	1245	AAGTAAGTGACTGGGGTGAGCG
	1246	ATGTAAGCTCCTGGGGATTCAC
	1251	TGGGTGTGTGGGTGTGTGGGTGTG
Rep primers (Serwecinska *et al.*, 1998).	Rep1RDT	*IIINCGNCGNCATCNGCC
	Rep2DT	NCGNCTTATCNGGCCTAC
M13 primer (Grundmann *et al.*, 1997).	M13	GAGTGGCGGTTCT
Box primers (BOX A: Alam *et al.*, 1999; BOX A1R and BOX A2R: Koeuth *et al.*, 1995).	BOX A	ATACTCTTCGAAAATCTCTTCAAAC
	BOX A1R	CTACGGCAAGGCGACTGACG
	BOX A2R	ACGTGGTTTGAAGAGATTTCG

* I = Inosine

4.3.3.1. ERIC-PCR.

Different reaction mixtures and cycling conditions were used in order to determine optimal parameters. Different primers (all from MWG Biotech, Germany) were also employed; 1245, 1246, 1251 and 1245/1246. A 2 μl volume of extraction supernatant was used in a total PCR reaction volume of 25 μl.

The PCR reaction mix for primers 1245/1246 consisted of 18 mM $MgCl_2$, 1 X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40; Promega, UK), 125 mM dNTP mix, 2.5 U of *Taq* DNA polymerase / Hotstart *Taq* DNA polymerase (Qiagen, UK), and 100 pmol of each primer per reaction. The mix was subjected to 1 cycle at 95 °C for 5 min, and 35 cycles of 92 °C for 45 s, 52 °C for 1 min, and 70 °C for 10 min.

The reaction mix for primer 1251 consisted of 2.5 μM $MgCl_2$, 1 X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40; Promega, UK), 0.2 μM dNTP mix, 1.25 U of *Taq* DNA polymerase / Hotstart *Taq* DNA polymerase, and 0.5 μM of primer per reaction. The mix was subjected to 40 cycles at 94 °C for 1 min, 25 °C for 2 min, and 74 °C for 3 min. In an alternate cycling parameter, the annealing temperature was altered to 52 °C for 2 min.

The reaction mix for primer 1245 consisted of the same mix as used for primers 1245 and 1246 with cycling parameters indicating 1 cycle at 95 °C for 7 min, 30 cycles at 90 °C for 30 s, 52 °C for 1 min, 65 °C for 8 min and 1 cycle at 65 °C for 16 min.

The reaction mix and cycling parameters for primer 1246 were identical to those for primer 1245.

In some cases Hotstart *Taq* DNA polymerase was used instead of standard *Taq* polymerase for optimisation purposes.

4.3.3.2. Rep-PCR.

The reaction mixture and cycling conditions for the Rep primers were those previously described by Alam *et al.,* (1999). The reaction mix consisted of 18 mM $MgCl_2$, 1 X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40; Promega, UK), 125 μM dNTP mix, 2.5 U *Taq* DNA polymerase, and 100 pmol of each primer per reaction. The mix was subjected to 1 cycle at 95 °C for 5 min, followed by 35 cycles at 95 °C for 45 s, 52 °C for 1 min, 70 °C for 10 min and a final 1 cycle at 70 °C for 20 min. The conditions were modified by alteration of the annealing temperature over a 15 °C gradient and the concentration of magnesium chloride over a gradient of 1.5 – 3.5 μM. The primer set used was Rep1RDT / Rep2DT (MWG Biotech, Germany). A 2 μl volume of extraction supernatant was used in a total volume of 25 μl.

4.3.3.3. M13-PCR.

The reaction mixture and cycling conditions as described by Grundmann *et al.*, (1997) were employed. The mix consisted of 3.5 mM $MgCl_2$, 1 X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40; Promega, UK), 20 μM dNTP mix, 1.25 U *Taq* DNA polymerase, and 1 μM of primer. The mix was subjected to 1 cycle at 94 °C for 1 min, 35 cycles at 94 °C for 45 s, 50 °C for 1 min, 72 °C for 20 s, and 1 cycle at 72 °C for 5 min. The primer used was M13 (MWG Biotech, Germany). A 2 μl volume of extraction supernatant was used in a total reaction volume of 25 μl. Optimisation was performed using Hotstart *Taq* DNA polymerase in the place of standard DNA polymerase.

4.3.3.4. BOX-PCR.

A combination of reaction mixtures and cycling conditions including annealing temperature gradients ranging from 45.5 °C to 59 °C and Hotstart *Taq* DNA polymerase (Qiagen, UK) were tested in order to achieve optimal PCR products. The primers used were BOX A (MWG Biotech, Germany), BOX A1R (MWG Biotech, Germany) and BOX A2R (MWG Biotech, Germany). The reaction mix for primer BOX A (Alam, 1999) consisted of 3.75 mM $MgCl_2$, 1 X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40; Promega, UK), 125 μM dNTP mix, 2.5 U *Taq* DNA polymerase, and 50 pmol primer per reaction. The mix was subjected to 1 cycle at 94 °C for 4 min, 40 cycles at 94 °C for 1 min, 45 °C for 45 s, 74 °C for 2 min and 1 cycle at 74 °C for 5 min. Hotstart *Taq* DNA polymerase was used in order to optimise the PCR.

The reaction mix for primer BOX A1R (Johnson and O'Brian, 2000) consisted of 1.5 mM $MgCl_2$, 1 X PCR buffer, 200 μM dNTP mix, 2.5 U *Taq* DNA polymerase, and 20 pmol primer per reaction. The mix was subjected to 1 cycle at 95 °C for 7 min, 30 cycles at 90 °C for 30 s, 52 °C for 1 min, 65 °C for 8 min, and 1 cycle at 65 °C for 16 min. Hotstart *Taq* DNA polymerase was used in order to optimise the PCR.

The reaction mix for primer BOX A2R (Malathum *et al.*, 1998) consisted of 2.5 μM $MgCl_2$, 1 X PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween 20 and 0.5 % Nonidet - P40; Promega, UK), 200 μM dNTP mix, 10 % DMSO, 2.5 U *Taq* DNA polymerase, and 50 pmol of primer per reaction. The mix was subjected to 1 cycle at 95 °C for 7 min, 35 cycles at 90 °C for 30 s, 40 °C for 1 min, 65 °C for 8 min, and 1 cycle at 65 °C for 10 min. Hotstart *Taq* DNA polymerase was used in order to optimise the PCR.

2 μl volume of extraction supernatant was used in a total reaction volume of 25 μl.

4.3.4. Modified agarose gel electrophoresis.

See section 2.2 for protocol.

4.3.5. Pulsed field gel electrophoresis.

Pulsed field gel electrophoresis was performed on strains listed in Table 4.1 (marked with *) using the method as stated in section 2.4 with modifications listed in the following sub-sections.

4.3.5.1. DNA preparation.

A modification to the method included the storage of the bacterial cells on ice prior to being resuspended in 0.5 ml of 2 X lysis solution (12 mM Tris-HCl [pH 7.4], 2 M NaCl, 20 mM EDTA [pH 7.5], 1.0 % Brij, 0.4 % sodium deoxycholate, 1.0 % sodium lauroyl sarcosine) containing lysozyme at 1.0 mg/ml, mutanolysin at 10 U/ml and RNase A at 20 μg/ml.

A modification to the original method included reducing the incubation time in 5 ml TE buffer containing 1 mM PMSF from 1 h to 0.5 h.

4.3.5.2. Restriction of DNA.

A modification to the restriction method included incubation of the plugs in restriction buffer for 30 min at 4 °C prior to equilibration in distilled water for 1 h at 4 °C followed by restriction.

4.3.5.3. Electrophoresis of samples.

The pulsed field parameter for *Sma*I was increased from 20 h to 22 h at 6 V/cm (200 V) with switch times ramped from 5 - 35 s.

4.4. Results.

4.4.1. DNA extraction.

No product was obtained for any of the four PCR protocols for any of the ten strains tested when using supernatant from "Chelex" extractions as the PCR template.

Using the supernatant obtained from the heat extraction as template, PCR products were consistently obtained for all strains tested with ERIC 1245/46 primers and 1251 primers. However the bands obtained were poorly resolved and diffuse despite attempts to improve resolution by an increase of electrophoresis time and reduction of the voltage. In addition, template DNA appeared to be unstable and no product was obtained by PCR following storage of the template for 1-2 weeks at –20 °C as recommended (Grundmann *et al.*, 1997).

The phenol: chloroform/isoamyl alcohol method of DNA extraction consistently provided DNA of a quality and quantity that yielded sharp, clear well resolved bands using PCR typing for all strains tested with ERIC primer 1245 (see Figure 4.2a and 4.2b).

4.4.2. PCR based fingerprinting.

4.4.2.1. PCR fingerprinting using ERIC primers.

ERIC primers 1245/1246 when used in combination were successful at providing a fingerprint consisting of up to 6 bands with a small number of strains. However, the results were not reproducible in that the fingerprint for a given strain was not identical upon repetition. No improvement in reproducibility was observed when an annealing temperature of 25 °C was employed, as described by Hermans *et al.*, (1995) in the successful typing of *S. pneumoniae*.

ERIC primer 1251 failed to produce profiles for any of the strains at an annealing temperature of 25 °C. At an annealing temperature of 52 °C approximately three bands were obtained for the majority of the test strains (Figure 4.1). Unfortunately, the profiles were consistently faint, poorly resolved and tended to be non-reproducible when repeated with the same strains. The use of Hotstart *Taq* DNA polymerase, higher percentage agarose gels and Nusieve gels failed to improve either resolution or reproducibility.

Typing with ERIC 1245 as a single primer consistently yielded reproducible profiles of approximately five bands when subjected to electrophoresis on standard agarose mini gels. When resolved upon larger gels constructed from a mix of 1.0 % standard agarose and 1.0 % Nusieve agarose the resolution improved further and up to approximately ten bands were visible for

some strains (Figure 4.2a and 4.2b). All test strains were amenable to typing using this primer and the fingerprinting profiles are represented by schematics in Figures 4.3a and 4.3b.

This method proved to be highly discriminatory for the strains studied with only 1 group of strains giving identical profiles. Only a limited number of shared bands were apparent. There did not appear to be distinct profiles or signatures characterising the source of the isolate, that is of commensal or disease origin. There are a number of bands in common for most of the lower molecular weight region, and many strains of the same species share additional bands of lower molecular weight, e.g. 50c (*S. constellatus*) and 27647/96 (*S. constellatus*). However there was not enough similarity of which to base any robust species distinction. Isolates 82c (*S. intermedius*) and 69c (*S. intermedius*) appear to have the same profile. The interesting aspect of this appears to be similar nature of 2 commensal isolates. Similar profiles were obtained for isolates 52c (*S. anginosus*) and 7K (*S. anginosus*), with only 3 minor profile differences. Apparent relationships between strains can be seen in Table 4.3.

No products were obtained with use of the single primer ERIC 1246 even following the modifications listed in the materials and methods section.

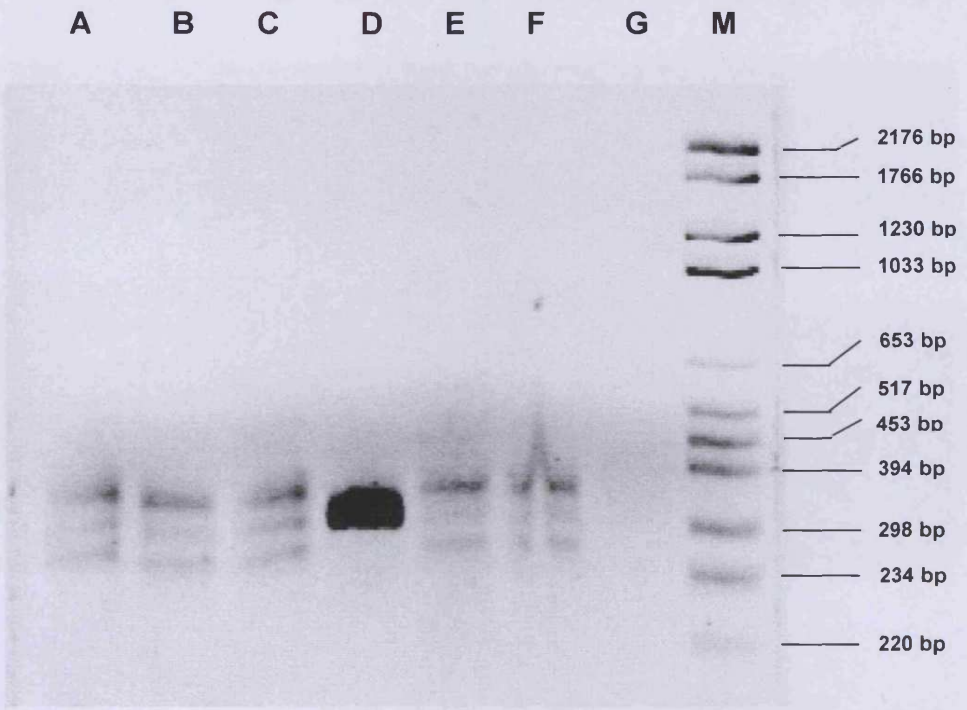

Figure 4.1. Agarose gel showing products obtained for strains of SMG using ERIC primer 1251 with an annealing temperature of 52 °C. Molecular weight marker VI is present in lane M. The negative control is denoted by the letter G. A-F represents isolates of the "*Streptococcus milleri*" group of bacteria. A = 229/98 (*S. constellatus*; abscess), B = 57c (*S. constellatus*; commensal), C = 7K (*S. anginosus*; brain abscess), D = 19c (*S. anginosus*; commensal), E = 240B/95 (*S. intermedius*; abscess), F = 69c (*S. intermedius*; commensal) .

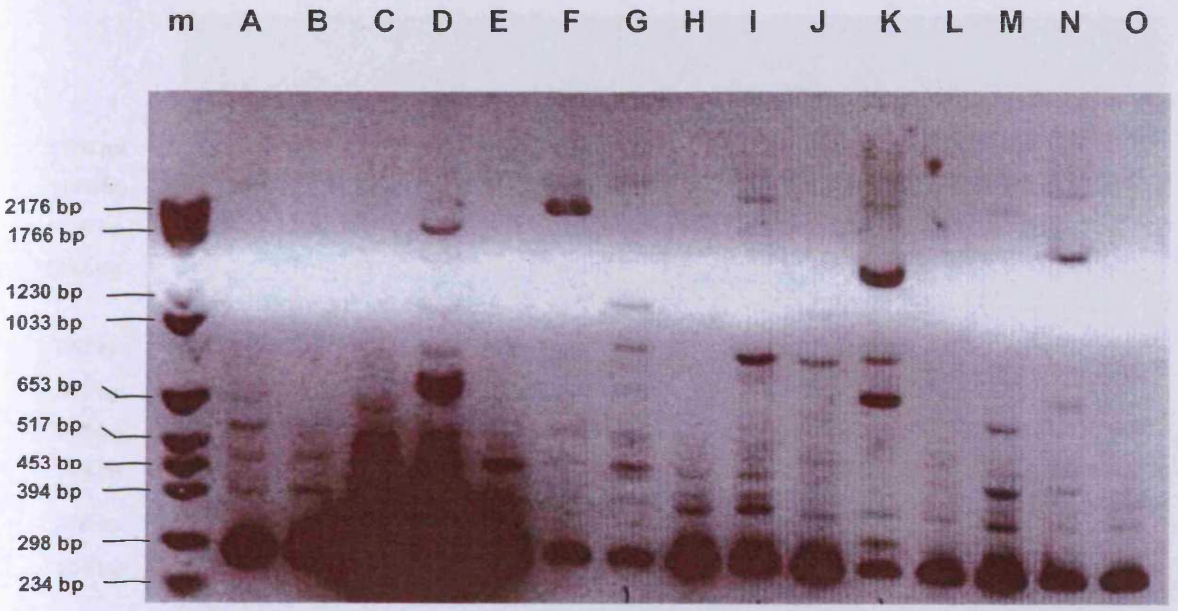

Figure 4.2a. Fingerprinting of SMG strains by PCR with ERIC primer 1245. The molecular weight marker VI is denoted by the small letter m. The isolates used are test strains of the "*Streptococcus milleri*" group. A = R87/3972, B = 313A/95, C = 68c, D = NCTC 10713, E = 54252/95, F = 57c, G = 58c, H = 323/96, I = 59c, J = 240B/95, K = SL34/W, L = 82c, M = HW 69, N = 19c, O = 69c.

A.	*S. intermedius*	blood
B.	*S. intermedius*	dentoalveolar abscess
C.	*S. intermedius*	commensal
D.	*S. anginosus*	type strain
E.	*S. intermedius*	ethmoidal fluid
F.	*S. constellatus*	commensal
G.	*S. intermedius*	commensal
H.	*S. anginosus*	dentoalveolar abscess
I.	*S. intermedius*	commensal
J.	*S. intermedius*	dentoalveolar abscess
K.	*S. anginosus*	subphrenic abscess
L.	*S. intermedius*	commensal
M.	*S. intermedius*	brain abscess
N.	*S. anginosus*	commensal
O.	*S. intermedius*	commensal

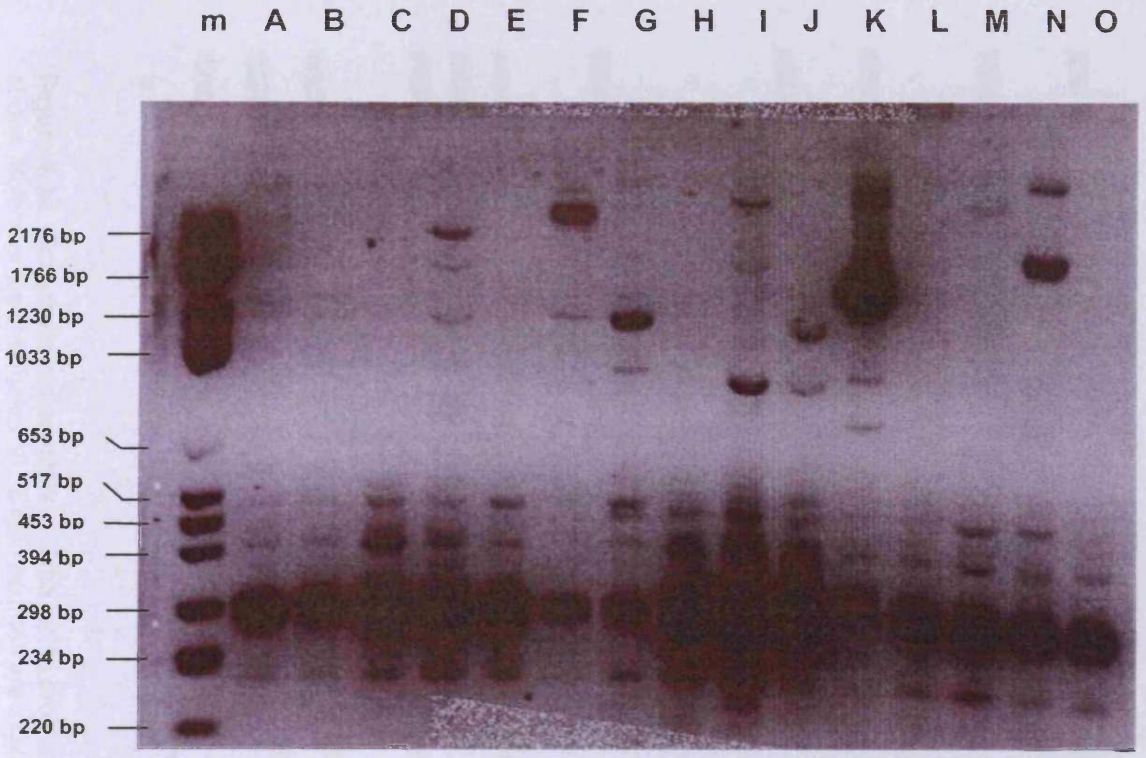

Figure 4.2b. Fingerprinting of SMG strains by PCR with ERIC primer 1245. The molecular weight marker VI is denoted by the small letter m. The isolates used are test strains of the "*Streptococcus milleri*" group. A =17c, B =56c, C =52c, D =240A/95, E =7K, F =34c, G =72c, H =48c, I =50c, J =27647/96, K =313B/95, L =3395/97, M =229/98, N =4515/96, O =CDC2236-81.

A.	*S. anginosus*	commensal
B.	*S. anginosus*	commensal
C.	*S. anginosus*	commensal
D.	*S. anginosus*	dentoalveolar abscess
E.	*S. anginosus*	brain abscess
F.	*S. constellatus*	commensal
G.	*S. constellatus*	commensal
H.	*S. constellatus*	commensal
I.	*S. constellatus*	commensal
J.	*S. constellatus*	appendix wound swab
K.	*S. constellatus*	dentoalveolar abscess
L.	*S. constellatus*	perianal swab
M.	*S. constellatus*	dentoalveolar abscess
N.	*S. constellatus*	perianal abscess
O.	*S. anginosus*	blood

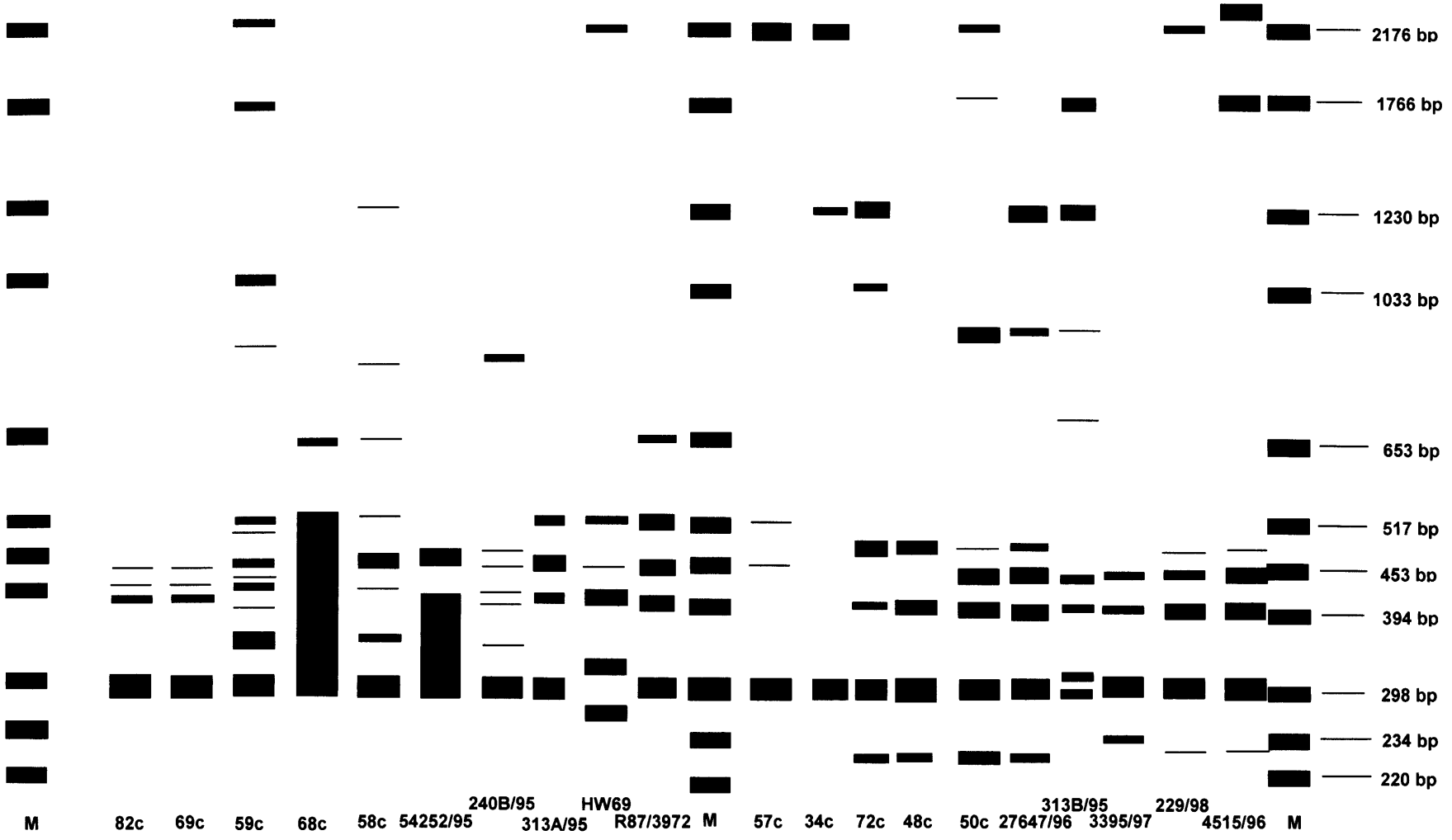

Figure 4.3a. Schematic representation of the typing profiles of *S. intermedius* and *S. constellatus* strains obtained using ERIC 1245 primer. M shows Molecular weight marker VI. Strain sources are indicated on Table 4.1.

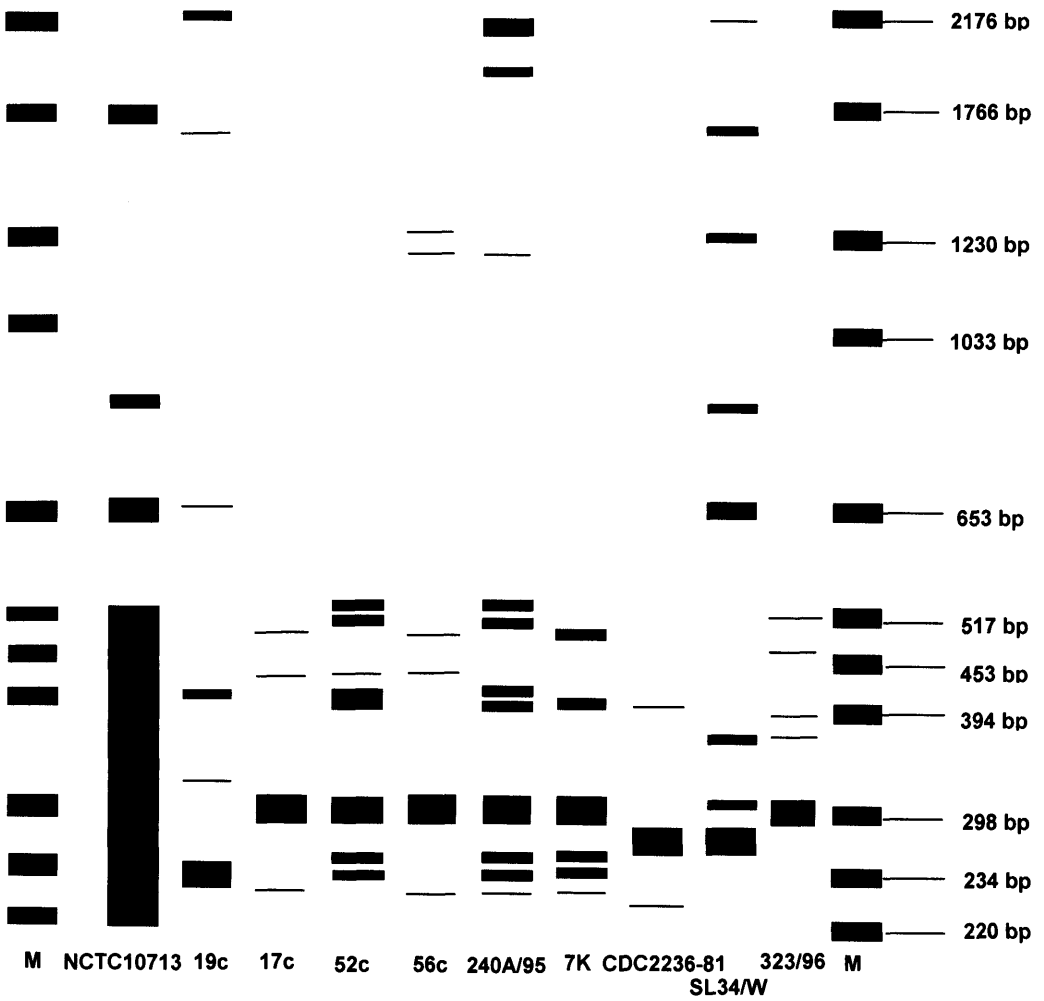

Figure 4.3b. Schematic representation of the typing profiles of *S. anginosus* strains obtained using ERIC 1245 primer. M shows Molecular weight marker VI. Strain sources are indicated on Table 4.1.

Table 4.3. Table highlighting isolates with identical and similar profiles after fingerprinting using ERIC 1245 primer.

Relationship	Strain	Species
Identical fingerprint	82c	*S. intermedius* commensal
	69c	*S. intermedius* commensal
Related fingerprint (3 band differences)	52c	*S. anginosus* commensal
	7K	*S. anginosus* brain

4.4.2.2. PCR fingerprinting using Rep primers.

The Rep primers Rep1RDT and Rep2DT, in combination yielded profiles of up to three bands per test strain. However, these profiles were not reproducible. Modifications to the protocol as outlined in the materials and methods did not improve reproducibility.

4.4.2.3. PCR fingerprinting using M13 primer.

No products were obtained for any of the test strains using the M13 primer. Modifications of the protocol as outlined in the materials and methods, failed to improve results.

4.2.2.4. PCR fingerprinting using BOX primers.

No amplimers were obtained with any of the strains by PCR typing with the BOX A primer. When Tween 80 was omitted from the PCR mix a number of bands were obtained. However, the water control repeatedly appeared contaminated with a single band of consistent size. Measures to remove this (such as primer replacement, fresh reagents) failed to correct the problem.

Similar problems were encountered with BOX A2R in that the water control appeared contaminated. Measures taken to rectify the problem as mentioned in section 4.3.3.4. were unsuccessful.

BOX A1R primer failed to produce any amplimers with any of the conditions listed.

4.4.3. Pulsed field gel electrophoresis.

Modifications made to the method of PFGE employed by Bartie *et al.*, (2000) were shown to increase the resolution and reliability of the approach. Well-resolved banding patterns were routinely obtained (Figure 4.4). Adjustments made to the method are described below.

Prior to production of the agarose plugs, cell suspensions and pellets were stored on ice. During the plug processing, PMSF rinses were reduced from 1h to 30 min. Prior to restriction digestion, ½ of a plug was incubated in 50 µl of the relevant restriction buffer for 30 min at 4 °C. The electrophoresis run time was extended from 20 h to 22 h and the temperature was kept at 4 °C. The electrophoresis buffer (0.5 X TBE) was replaced following each run, and pre-chilled at 4 °C prior to each electrophoresis run.

Due to the labour intensive nature of the method in comparison to ERIC-PCR, a smaller number of strains were studied for optimisation purposes. Pulsed field profiles were obtained for eleven strains of SMG (Figure 4.4) and are shown in diagrammatic form (Figure 4.5). The profiles were found to be reproducible upon repeat testing.

Profile analysis of these strains did not reveal any inter or intra species characteristics, nor was there evidence of profile differences or signatures relating to source of isolate.

A pair of strains which had been shown to yield either identical or related profiles by ERIC-PCR were studied by PFGE. Strains 82c and 69c did not show any relation in profile.

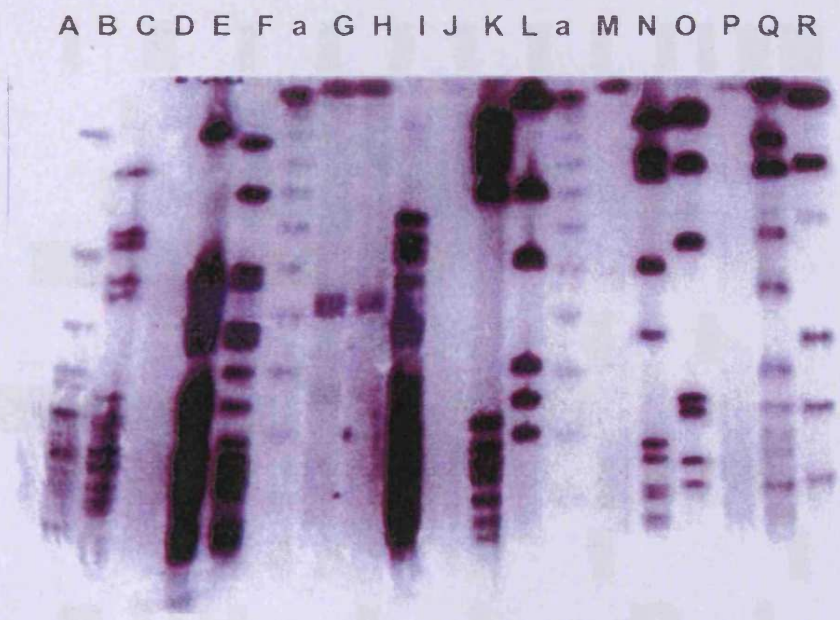

Figure 4.4. Pulsed field gel electrophoresis of eleven strains of SMG taken from the test strain group. The lambda ladder marker is denoted by the symbol a. Lane A = 82c*, B = 82c, C = 69c, D = 69c*, E = 54252/95, F = 240B/95, G = 240B/95*, H = 57c*, I = 57c, J = 34c*, K = 34c, L = 4515/96, M = 313B/95*, N = 313B/95, O = 19c, P = 17c*, Q = 17c, R = 7K. * = undigested DNA.

82c	*S. intermedius*	commensal
69c	*S. intermedius*	commensal
54252/95	*S. intermedius*	ethmoidal fluid
240B/95	*S. intermedius*	dentoalveolar abscess
57c	*S. constellatus*	commensal
34c	*S. constellatus*	commensal
4515/96	*S. constellatus*	perianal abscess
313B/95	*S. constellatus*	dentoalveolar abscess
19c	*S. anginosus*	commensal
17c	*S. anginosus*	commensal
7K	*S. anginosus*	brain abscess

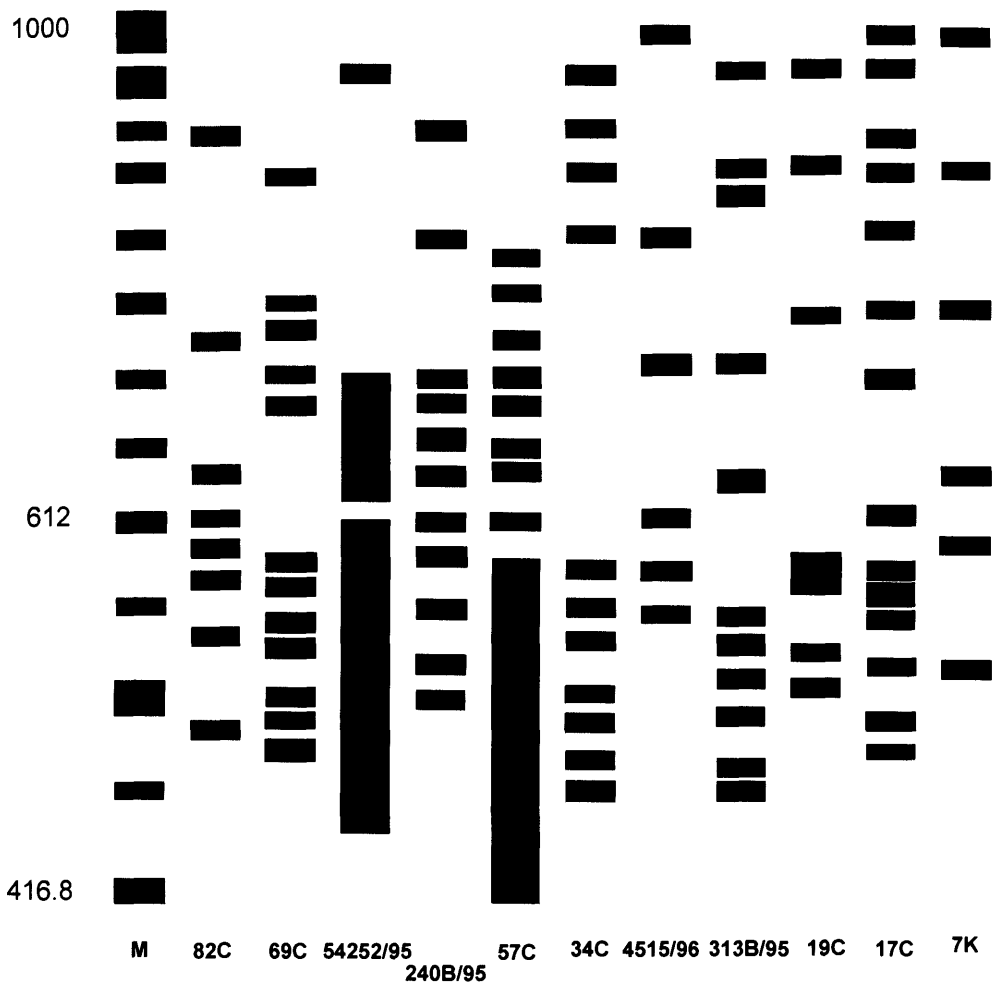

Figure 4.5. Banding diagram representing the PFGE typing arrangements of eleven SMG isolates. The 1000 bp DNA lambda ladder marker is designated M

4.5. Discussion.

A rapid PCR-based typing method has been successfully developed in this chapter for the purpose of characterisation of SMG strains. Preliminary typing of a range of SMG test strains from a diversity of sources has been accomplished and the method has been shown to be discriminatory. This has allowed further work to be performed as described in Chapter 5.

A number of methodological issues are worthy of note. It has previously been reported (Woods *et al.,* 1993) that template prepared crudely from cell disruption could be used in Rep / ERIC-PCR without loss of sensitivity or reproducibility. However, the present study has shown that extraction using the phenol: chloroform / isoamyl alcohol method provided DNA of better quality than the crude method as assessed by PCR typing using the extracts as templates. Furthermore, the DNA obtained by cell disruption appears to degrade readily even at low temperatures. This makes the crude heat extraction impractical as part of a large scale longitudinal typing study where DNA may be required as template over a long space of time for additional retrospective confirmation of profiles.

ERIC primers have been used frequently in the typing of microorganisms. The ERIC 1245 primer when used alone (Johnson and O' Brian, 2000) produced multi-banded fingerprints useful for typing purposes for all strains tested. Furthermore, the bands proved reproducible when repeated with the same isolates of SMG using the same PCR thermal cycler. The pairing of 1245 and 1246 reportedly worked well for Alam *et al.,* (1999) when used with viridans streptococci including strains of SMG. However, the combination of primers 1245 and 1246 failed to produce adequate amplimers for typing of SMG isolates in this study. A 25 °C annealing temperature was assessed (as used by Van Belkum *et al.*, 1994) with use of the 1251 primer, and perhaps not surprisingly proved unsuccessful as it represents conditions used for the eukaryotic repeat motifs. Although more PCR products were obtained at an

annealing temperature of 52 °C, the fingerprints were non-reproducible. The 1246 primer, when used as a single primer, (Johnson and O' Brian, 2000) failed to produce any bands even after modifications of the conditions.

The profiles obtained with the use of Rep primers for typing were found to be non-discriminatory and non-reproducible with for example only three similar bands being produced in the fingerprints and changes in profiles occurring upon repetition of the procedure. The Rep primers (Rep1RDT and Rep2DT) have previously been used to successfully type SMG species by Alam *et al.*, (1999). However, no verifiable results for these experiments were presented in the publication. Other Rep primers (Rep1R-I and Rep2-I) have proven successful when used with SMG species (Clarridge *et al.*, 1999), although the Rep1R-I and Rep2-I primer sequences used have many copies of the nucleoside inosine. Inosine is a pyrimidine that will bind to any of the bases within the DNA structure and therefore its presence results in low stringency (Singleton and Sainsbury, 1993). This may explain why the typing data obtained by Clarridge *et al.*, (1999) showed a low percentage similarity between isolates, and as such appeared discriminatory, as the areas of the primers containing the inosine may have bound randomly to any area of the template and therefore will have low sensitivity. For this reason the alternative primers Rep1RDT and Rep2DT were chosen in the present study.

Although the M13 primer theoretically showed potential in the typing of SMG based on the successful fingerprinting of other streptococcal species (Wisplinghoff, 1999) these primers routinely failed to yield any products upon PCR amplification in the present study. It is difficult to explain this result in view of the close relationship of the SMG to the other streptococci studied. However, the reason must be related to species specificity of the primer.

The use of BOX primers initially seemed a promising prospect for streptococcal typing due to its use with other streptococcal species, although the BOX primer BOX A had previously found to be unsuccessful for SMG (Van Belkum *et al.*, 1996; Alam *et al.*, 1999). However, the primers BOX A1R

and BOX A2R had not been used for streptococcal typing previously other than for *S. pneumoniae*. The results have revealed that banding products were consistently present in the negative controls even when all reagents (including the primers on a number of occasions) were replaced. This may have been due to repeated contamination of the primers at source.

The primer ERIC 1245 was found to produce well resolved fingerprints of up to 10 bands for some isolates. The fingerprints were easy to read visually and highly discriminatory with 29 different profiles being produced out of 30 test strains. Although inter-species identification was not possible due to the lack of any signature bands or profiles within the strains, some general observations are worth noting. *S. constellatus* and *S. anginosus* showed some similarity in banding. The two species shared similar banding profiles between 234 and 394 bp. Interestingly, *S. anginosus* and *S. constellatus* are considered by some on the basis of DNA homology to be as similar as a single species, (Welborn *et al.*, 1983; Coykendall *et al.*, 1987). Furthermore, Cookson *et al.*, (1989) reported that *S. constellatus* and *S. anginosus* were indistinguishable by analysis of cellular fatty acids. However, other researchers such as Griesen *et al.*, (1994) reported that *S. anginosus* and *S. constellatus* demonstrate only a low similarity using 16S rDNA profiling. Other researchers have found *S. constellatus* to be more closely related to *S. intermedius* than to *S. anginosus* on the basis of DNA-DNA reassociation studies (Jacobs *et al.*, 1996). No profile signature was apparent on which to base a distinction between clinical or commensal origin of strain. The identical nature of strains 82c and 69c was interesting as both were *S. intermedius* commensals. Although the profiles were related, they were isolated from the plaque of different subjects not known to be related in any way. Strains 52c and 7K were *S. anginosus* strains found to be similar in fingerprint. They had a difference of 3 bands separating them. 52c was a commensal isolate taken from an oral site, whereas 7K was taken from the brain of a separate clinical subject. These findings indicate that there is a possible dissemination of strain types between individual subjects and more

interestingly a possible dissemination of similar strain types throughout the oral cavity in individual subjects.

It would be interesting as a future study to take a larger known sample of both commensal and clinical SMG strains and use ERIC-PCR and PFGE to produce fingerprints and then use a computer program to produce dendrograms to analyse for similarities and show the degree of relatedness of isolates.

The PFGE method had already been developed within the laboratory for use with SMG. However, it was not considered a routine method as it was time consuming, laborious and seemed to be highly operator sensitive (personal communications). In the present study a number of modifications to the previously established protocol were explored and these resulted in increasing reliability and were therefore more conducive to the processing of a large number of strains as required in the succeeding study reported in Chapter 5. It was determined that cells kept on ice to prevent degradation prior to plug production survived the process much better and produced clearer profiles with less degradation when later examined. PMSF rinsing was reduced from 1 h to 0.5 h thus decreasing the turnover time for plug production. The ½ plug to be restricted was pre-incubated in restriction buffer for 0.5 h prior to restriction as advised by Maule, (1998). It was found that changing the buffer (0.5 X TBE) after each use improved the quality of the resulting gel. Furthermore increasing the running time at high voltage, and lowering the running temperature improved the separation of the fragments. The time taken to produce, process, restrict and run the plugs amounted to approximately three working days, a period similar to that previously reported (Olive and Bean, 1999).

In view of the labour intensive nature a smaller number of strains were selected to optimise PFGE and check for reproducibility. These included some of the pairs shown to yield identical or similar profiles by ERIC-PCR. However, it was interesting to study the results of some strains in context of

the PCR results. Eleven distinct strain profiles were produced by PFGE, however as with the ERIC-PCR results no profile signatures characteristic of either species or clinical source were apparent. The high discriminatory power of PFGE for SMG has been noted previously (Bartie *et al.*, 2000). Strains 82c and 69c while previously yielding identical profiles with PCR typing exhibited different fingerprint patterns using PFGE.

4.6. Conclusions.

The optimal method of extracting DNA from SMG strains to serve as template for PCR typing proved to be a chemical method involving phenol: chloroform / isoamyl alcohol. The boiling method succeeded in producing nucleic acid that was amenable to PCR, but the resulting fingerprints were typically of poor quality and resolution.

Reproducible well resolved profiles were obtained for all 30 test strains using ERIC primer 1245. Use of the primer proved to be highly discriminatory with 29 unique profiles for 30 strains. It was not possible to distinguish between species on the basis of the profiles. ERIC 1245/46, ERIC 1251, M13, Rep1RDT / Rep2DT, BOX A, BOX A1R and BOX A2R primers either failed to produce any amplimers, were non-reproducible, or were found to be too poorly resolved for typing purposes. Furthermore, no signatures were identified to allow discrimination between commensal or clinically associated isolates.

The PFGE method of Bartie *et al.,* (2000) for SMG was optimised further to enable its routine use on SMG strains. The method was improved by extending electrophoresis times, lowering the running temperature and by a number of measures associated with handling of bacterial cells prior to agarose plug production and handling of the plugs during processing.

In summary, two genotypic methods (PCR typing with ERIC primer 1245 and PFGE) have been optimised and shown to be highly discriminatory for SMG. These methods will now be used for further analysis of the SMG as described in the following chapter.

CHAPTER V: MOLECULAR FINGERPRINTING AND GENOMIC STABILITY OF THE "*STREPTOCOCCUS MILLERI*" GROUP

5.1. Introduction.

Members of the SMG are frequently isolated from oral sites. However, little is known regarding the distribution of microbial species within the oral cavity or of the stability of colonisation patterns. Marsh, (1994) found a level of microbial homeostasis in the composition of plaque, although he also found that changes in environmental factors, such as pH, could easily upset this homeostasis. Investigation of the endogenous flora is a difficult process, as highlighted by Carman *et al.*, (1993), who encountered a number of difficulties whilst studying the normal intestinal flora which included the inherent complexity of the endogenous microflora when sampling.

Previous investigations have shown that the SMG are present in the oral cavity of all subjects studied (Whiley *et al.*, 1993). SMG are isolated frequently in small numbers from a range of sites including the throat, tongue, gingival crevice and within subgingival and supragingival plaque. Mejare and Edwardsson, (1975) found that SMG accounted for 41 % of all oral streptococcal isolates, 33 % of the streptococcal population of the gingival crevice and 11 % of the supragingival plaque flora. However, SMG were not shown to be present in saliva or on the tongue. These findings were confirmed to an extent by a study performed by Michalek and McGhee, (1977), in which it was reported that SMG constitute between 14 – 56 % of the bacterial population of the gingival crevice. However, in this study 1 – 10 % of the flora in the saliva and 5 – 10 % of the flora of the tongue were shown to be SMG. Stratton, (1999) found *S. intermedius* to be the predominant species in dental plaque. A study of the species distribution within the group was performed by Whiley *et al.,* (1993) who found *S. anginosus* to be the most frequently isolated species, constituting 60 % of SMG recovered from oral sites. The species isolated least often was *S. constellatus* (13 %) whilst *S. intermedius* constituted the rest. The subgingival plaque was shown to harbour equivalent proportions of *S. constellatus, S. intermedius* and *S. anginosus*. *S. intermedius* and *S. constellatus* were rarely isolated together from one site, and all three species were never isolated together

from one subject. Only one phenotype of a species as determined by biochemical test results and colonial morphology was isolated from a specific site.

It is possible that many of the studies into the distribution of oral isolates are inaccurate due to difficulties in the identification of oral streptococcal species. According to conventional identification schemes, streptococci can be initially separated by their ability to haemolyse erythrocytes (Lebrun *et al.*, 1986). In addition to this, colony size, serological tests and metabolic tests may be used. The majority of haemolytic streptococci can be distinguished by possession of Lancefield group antigens and through simple biochemical or enzymatic tests designed to separate haemolytic SMG strains from large-colony type pyogenic streptococci which possess similar antigens (Kaufhold and Ferrieri, 1993). It is likely that in many studies, the SMG have remained undetected due to their fastidious growth requirements preventing isolation or have been disregarded due to a lack of haemolysis or Lancefield antigen markers (Ruoff, 1988). In addition, the differentiation of *S. anginosus* and *S. constellatus* is difficult due to the lack of discriminatory phenotypic characteristics.

In 1990 a microtitre plate assay based on the glycosidic activities against fluorogenic substrates proved highly successful in the differentiation of the SMG taxa (Whiley *et al.*, 1993). Additional differential phenotypic characteristics included the degradation of a glycosaminoglycan (GAG) substrate and hyaluronic acid (HA) incorporated into an agar growth medium (Whiley *et al.*, 1990). Strains of *S. constellatus* and *S. anginosus* can be distinguished by the production of α-glucosidase and hyaluronidase by the former and the production of β-glucosidase by the latter. *S. intermedius* strains show β-D-fucosidase, β-N-acetylgalactosaminidase, β-N-acetylglucosaminidase, sialidase, α-glucosidase, β-galactosidase and hyaluronidase activity. Subsequently these substrates have been used extensively in the differentiation of SMG and indeed the viridans streptococci

(Whiley *et al.*, 1993; Whiley *et al.*, 1992; Bantar *et al.*, 1996; Pearce *et al.*, 1995).

The study of genetic diversity has provided a useful framework for the understanding of the genetic structure within bacterial populations (Rolland *et al.*, 1999). The availability of molecular typing tools which facilitate strain differentiation has allowed the study of the clonal distribution and persistence of molecular types within both a given individual or a particular environment. The theory of clonality within populations has proved a useful framework for a better understanding of diversity. The widely accepted theory is that the more clonal an organism is, the less diverse, and the more panmictic, the more diverse (Spratt and Maiden, 1999) (see section 1.1.4). Clonal theory has implications for virulence and pathogenicity as pathogens are frequently strains of commensal organisms causing disease for unknown reasons. The theory that more diversity increases opportunities for acquisition of virulence would account for this in many ways. Studies have been performed with this in mind, but very often they are poorly designed, with unrepresentative sampling regimens and test strains made up of clinical isolates only. This approach fails to take into account the fact that pathogens do not rely upon the generation of a pathological condition for their evolutionary survival and merely cause disease opportunistically or accidentally (Gupta and Maiden, 2001). The level of diversity within a population can be analysed using molecular strain typing methods. With the exception of *S. mutans* and the "mitis" group, strain typing methods have not been extensively applied in the study of the diversity of oral streptococci (Whiley and Beighton, 1998).

The level of heterogeneity within SMG has been studied for decades. Lutticken *et al.*, (1978) and French *et al.*, (1989) showed heterogeneity using phenotypic methods, while genotypic studies have been performed using a variety of methods. Ezaki *et al.*, (1986), Coykendall *et al.*, (1987) and Whiley and Hardie, (1989) all employed DNA-DNA hybridisation to study diversity, while Drucker and Lee, (1983) used DNA base pair ratios (C + G). Whiley

and Beighton, (1991) used SDS-PAGE analysis and in the most comprehensive study performed to date on SMG phenotypic and genotypic diversity Whiley *et al.*, (1997) used SDS-PAGE, 16S rRNA intergenic spacers and DNA-DNA hybridisation to show *S. anginosus* as the most diverse species within SMG. Evidence suggesting that the level of genetic diversity may be significant within the SMG has recently been obtained through Rep-PCR genotyping (Clarridge *et al.*, 1999) and PFGE (Bartie *et al.*, 2000). While the SMG appear to be highly diverse, the mechanism underlying this diversity have been little studied. Although there is undoubtedly a role for horizontal gene exchange in the generation of intergenomic diversity in the streptococci a second mechanism is intragenomic recombination about which very little is known.

The theories regarding genomic plasticity have been tested in organisms such as *H. pylori*, which has been shown to have a genomic arrangement incorporating a "plasticity zone", containing almost half of the strain-specific genes (Alm *et al.*, 1999). The genes within this regions are thought to undergo greater genetic reorganization than any other region of the genome, and this is thought to account for the diversity of the phenotypes seen in the *H. pylori* species (Nakagawa *et al.*, 2003). At the present time the extent of genomic plasticity within most of the groups of bacteria present in the oral cavity, including the SMG is unknown.

There have been relatively few studies on the stability of the endogenous microflora and the longitudinal colonisation of individuals with individual species or strains. The majority of longitudinal strain characterisation studies have been performed for clinical epidemiological reasons to determine routes of the spread of infection within the hospital environment. Other longitudinal studies involving strain characterisation have been undertaken to determine the elimination and / or re-appearance of colonising strains of microbes following antimicrobial treatment. For example, Wilson *et al.,* (2001) isolated *Candida albicans* strains from terminally ill patients at regular intervals before and after antifungal treatment and showed re-emergence of original strains in

most cases. Other similar studies include those by Van Belkum *et al.,* (1994) and Metzgar *et al.,* (1998) who performed longitudinal studies on *Candida* species. Viridans streptococci isolated from cancer patients were studied by Wisplinghoff *et al.,* (1999) who performed a prospective study on 57 patients experiencing streptococcal bacteraemia over a three year period using IR-PCR and PFGE. It was found that all isolates from different patients produced unique fingerprint patterns suggesting that streptococcal bacteraemia derives from an endogenous source. Wisplinghoff demonstrated that typing using these methods was reproducible and robust, yielding unique fingerprint patterns in each case. Matto *et al.*, (1996) performed a study to examine the genetic diversity of oral *Prevotella* species using ribotyping and reported extensive genetic heterogeneity within the group. Hannula *et al.*, (1999) performed a study examining the stability of colonisation of oral yeasts in 22 young children of various ages over a course of 22 months using AP-PCR typing. The study determined that yeast species are transient colonisers, with the same yeast species rarely being detected in follow up sampling. Changes in the oral colonisation of 21 children at varying ages by Gram-negative species were investigated by Kononen *et al.,* (1994). Samples were taken from saliva and the gingival margin showing that in young children many species of Gram-negative bacteria have a low colonisation stability. However, *Prevotella* strains proved to be an exception – showing a high colonisation stability. Kononen *et al.*, (2002) performed a longitudinal study on the indigenous microflora of the upper respiratory tract of 50 young infants of various ages. It was concluded that streptococcal colonisation was more stable in the oral cavity than in the oropharynx.

5.2. Aims.

The aims of the research presented in this chapter were to:

1. Study the site variability and dynamics of oral colonisation by SMG over time in two volunteers.

2. Examine the genetic stability of strains of SMG during prolonged subculture using two methods of molecular typing previously optimised in Chapter 4.

5.3. Materials and methods.

5.3.1. Isolation of the SMG.

5.3.1.1. Isolation of streptococci.

Samples were taken on a monthly basis over a period of four months from the mouths of two volunteers (MW and DW). Volunteer MW was a 34-year-old female, with no systemic disease, no periodontal disease, no periodontal pockets, and no inflammation at sampling sites. Volunteer DW was a 35-year-old male with no systemic disease, no periodontal disease, no periodontal pockets, and no inflammation at sampling sites. Three samples were taken; 1. subgingival plaque from a 1-3 mm deep gingival site using a sterile spoon excavator, 2. supragingival plaque using a sterile spoon excavator and 3. tongue flora using a sterile cotton swab. Samples taken from volunteers were transferred aseptically into 3 ml of BHI broth and resuspended by vortexing for 30 s. A spiral plater (Don Whitley Scientific, UK) was used to evenly distribute 50 μl of inoculum onto a semi-selective agar medium for viridans streptococci. Modified MC-agar was produced using 40 g

DST agar base (Difco, UK), 5.0 g sucrose (Sigma, UK), 0.075 g trypan blue (Sigma, UK) and 0.8 mg crystal violet (Sigma, UK) in 1000 ml distilled water. The agar differed from that originally described (Carlsson, 1967) by the replacement of sulphadimetine with sulfadiazine (Sigma, UK) at 100 mg/L. The mix was autoclaved at 121 °C for 15 min prior to cooling at 56 °C. A 1.0 ml volume of Bacto-chapman tellurite solution was added (Difco, UK), and 1 g of sulfadiazine was added at 100 mg/L prior to pouring. The inoculated plates were incubated at 37 °C anaerobically for 48 h in an atmosphere of 20 % hydrogen, 10 % carbon dioxide and 70 % nitrogen. On MC-agar, SMG isolates appear as small (approximately 1 mm in diameter) dark blue colonies with both rough and smooth colonial forms evident (Yakushiji *et al.*, 1988).

Up to 30 colonies were selected from each plate and transferred to blood agar for further analysis using the reference identification scheme adopted by Whiley *et al.*, (1990) and as described below.

5.3.1.2. Arginine hydrolysis.

Two methods were employed to differentiate streptococcal strains by arginine hydrolysis. The first involved inoculation of 50 µl of an 18 h bacterial suspension into 200 µl of arginine broth (5.0 g proteose peptone [Lab-M, UK], 5.0 g yeast extract [Lab-M, UK], 2.0 g dipotassium hydrogen phosphate [pH 7.0, Fischer Bioreagents, UK], 0.5 g glucose [Sigma, UK], 3.0 g arginine monohydrochloride [Sigma, UK], in 1000 ml H_2O) in a microtitre plate. Plates were incubated at 37 °C for four days in an anaerobic chamber in an atmosphere of 20 % hydrogen, 10 % carbon dioxide and 70 % nitrogen. After incubation, 50 µl of ammonia colour reagent (Sigma, UK) was added to each well. A positive reaction was denoted by a colour change from yellow to orange. The second method involved the inoculation of approximately 10 bacterial colonies from a blood agar plate into 5 ml of arginine broth followed by incubation for 18 h at 37 °C anaerobically. Nesslers reagent (100 µl)

(Fischer Bioreagents, UK) was added and a positive result denoted by a colour change from yellow to brown.

5.3.1.3. Glycosidase assays.

Arginine positive strains were selected and glycosidase assays were performed according to the method of Whiley *et al.*, (1990) using (4-MU) linked fluorogenic substrates (α- glucosidase, β-glucosidase, β-fucosidase, β-glucosaminidase, β-galactosaminidase – Sigma, UK) in flat-bottomed microtitre plates. Colonies were harvested from blood agar plates with sterile swabs and suspended in 50 mM N-tris (hydroxymethyl) methyl-2-aminoethane-sulphonic acid (TES) buffer [pH 7.5] to a turbidity of a McFarland number four standard. The substrates were dissolved in a minimum volume of dimethyl sulphoxide (DMSO; Sigma, UK) and further diluted in TES buffer to a concentration of 100 μg/ml. A 100 μl aliquot of the bacterial suspension was mixed with 50 μl of each substrate solution in a flat-bottomed 96 well microtitre plate. The mixture was aerobically incubated for 4 h at 37 °C. Wells containing substrate solution or the bacterial suspension alone in TES buffer served as negative controls and were incubated simultaneously. Substrate hydrolysis resulted in the release of 4-methylumbelliferone, seen as a distinct blue fluorescence under ultraviolet illumination.

5.3.2. Collection and storage of strains.

SMG strains were isolated on a monthly basis over the course of four months resulting in 5 sampling times, M0, M1, M2, M3, M4. All strains identified as SMG were stored on Microbank storage beads at -70 °C. The first set of isolates from volunteers (Month 0) were subcultured into 5 ml BHI bi-weekly and grown at 37 °C anaerobically. Continuous subcultures were plated out onto blood agar at regular intervals to check for purity. In addition, at monthly

intervals colonies from the blood agar purity plates were transferred to beads for storage.

5.3.3. PCR fingerprinting.

All SMG strains were subjected to DNA extraction, and ERIC-PCR fingerprint analysis as described in section 4.3.3.1.

5.3.3.1. Growth of SMG strains.

All strains of SMG were grown on blood agar plates at 37 °C anaerobically for 18 h prior to DNA extraction.

5.3.3.2. DNA extraction using phenol:chloroform / isoamyl alcohol method.

As described in section 2.3.3.

5.3.3.3. ERIC PCR fingerprinting.

As described in section 4.3.3.1.

5.3.3.4. Modified agarose gel electrophoresis.

As described in section 2.3.3.

Some strains were selected for further typing study out of interest in order to examine the intra-species diversity. See section 5.4.2.2.

5.3.4. Pulsed field gel electrophoresis.

As described in section 4.3.5.

5.3.4.1. Selection of test strains.

Due to the laborious nature of PFGE small numbers of strains were studied. They were selected (n = 34) to some extent on the nature of the ERIC-PCR results.

5.4. Results.

5.4.1. Colonisation of oral cavity by SMG.

5.4.1.1. Isolation and identification of SMG.

The identity and frequency of isolation of strains of SMG obtained from subject MW can be seen in Table 5.1. The number and identification of false positive viridans streptococcal isolates for subject MW can be seen in Table 5.2. The species identifications and number of SMG isolates for subject DW can be seen in Table 5.3, while the viridans streptococcal false positives for subject DW can be seen in Table 5.4. Since the media is not completely selective the isolation frequencies cannot be accurately compared, however some trends and observations are worth noting. At this stage (i.e. prior to strain characterisation by molecular typing) a simple comparison of the number of colonies of a given species is being discussed.

Of the SMG, *S. intermedius* was isolated most frequently in both subjects (see Tables 5.1 and 5.3) representing 68 % of SMG isolates in MW and 64 % of SMG isolates in DW. *S. constellatus* represented 17 % of SMG isolates in MW and 8 % of isolates in DW. *S. anginosus* represented 15 % of SMG isolates in MW and 28 % of isolates in DW.

At some point each of the 3 species were isolated from each of the 3 sites in subject DW. However, in subject MW *S. anginosus* was never isolated from the tongue.

In subject MW, *S. intermedius* was isolated most often in the supragingival and subgingival plaque (Table 5.5 / 5.6). *S. constellatus* was isolated in the highest numbers on the tongue (Table 5.5 / 5.6). *S. anginosus* was isolated most frequently from the subgingival plaque (Table 5.5 / 5.6).

In subject DW, *S. intermedius* was isolated in equal numbers from the subgingival and supragingival plaque (Table 5.7 / 5.8). *S. constellatus* was isolated most often from the subgingival plaque (Table 5.7 / 5.8). *S. anginosus* was isolated in highest numbers from the supragingival plaque (Table 5.7 / 5.8). The predominant finding when the isolation frequencies for the 2 subjects are compared is that for both *S. intermedius* is most often isolated from the subgingival and supragingival sites.

Numbers of SMG isolated over the four month period can be seen in Figure 5.1. The graph shows a fall in both subjects at Month 2 and Month 4 of total numbers of SMG isolated from all sites to 4 (MW) and 1 (DW) at Month 2 , 5 and 6 (MW and DW) at Month 4. Between M1 and M2 both individuals received a beta-lactam antibiotic for treatment of upper respiratory tract infections. It is possible that this factors explains the drop in the numbers of SMG isolated.

Other viridans streptococci were present as false positives in large numbers (Tables 5.2 and 5.4), particularly *S. oralis* and *S. parasanguis*.

Table 5.1. Total number of SMG by species by site from subject MW on five occasions over a four month period. Samples were isolated from the tongue, subgingival plaque (Sub) and supragingival plaque (Sup). These fragments represent total number of colonies of each species isolated prior to further analysis by typing.

	Month 0			Month 1			Month 2			Month 3			Month 4			
Organism	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Total**
S. intermedius	3	9	2	0	6	6	0	2	1	0	0	7	0	0	5	**41**
S. constellatus	9	0	0	0	0	0	0	1	0	0	0	0	0	0	0	**10**
S. anginosus	0	0	1	0	0	0	0	0	0	0	7	0	0	1	0	**9**
Total	**12**	**9**	**3**	**0**	**6**	**6**	**0**	**3**	**1**	**0**	**7**	**7**	**0**	**1**	**5**	**60**

Table 5.2. Identity of viridans streptococcal colonies present on the modified MC-agar isolated from subject MW over a four month period.

Viridans streptococcal species	Total isolated
S. mutans	1
S. gordonii	3
S. oralis	28
S. parasanguis	14
S. salivarius	7
S. vestibularis	4
Total	**57**

Table 5.3. Total number of SMG by species by site from subject DW on five occasions over a four month period. Samples were isolated from the tongue, subgingival plaque (Sub) and supragingival plaque (Sup). These figures represent total number of colonies of each species isolated prior to further analysis by typing.

	Month 0			Month 1			Month 2			Month 3			Month 4			
Organism	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Tongue**	**Sub**	**Sup**	**Total**
S. intermedius	0	13	26	0	1	0	1	0	0	0	12	0	0	0	0	**53**
S. constellatus	0	0	0	1	3	2	0	0	0	0	0	0	0	1	0	**7**
s. anginosus	0	0	0	1	0	8	0	0	0	0	1	9	1	0	3	**23**
Total	**0**	**13**	**26**	**2**	**4**	**10**	**1**	**0**	**0**	**0**	**13**	**9**	**1**	**1**	**3**	**83**

Table 5.4. Identity of viridans streptococcal colonies present on the modified MC-agar isolated from subject DW over a four month period.

Viridans streptococcal species	Total isolated
S. mutans	3
S. gordonii	7
S. oralis	25
S. parasanguis	14
S. salivarius	0
S. vestibularis	1
Total	**50**

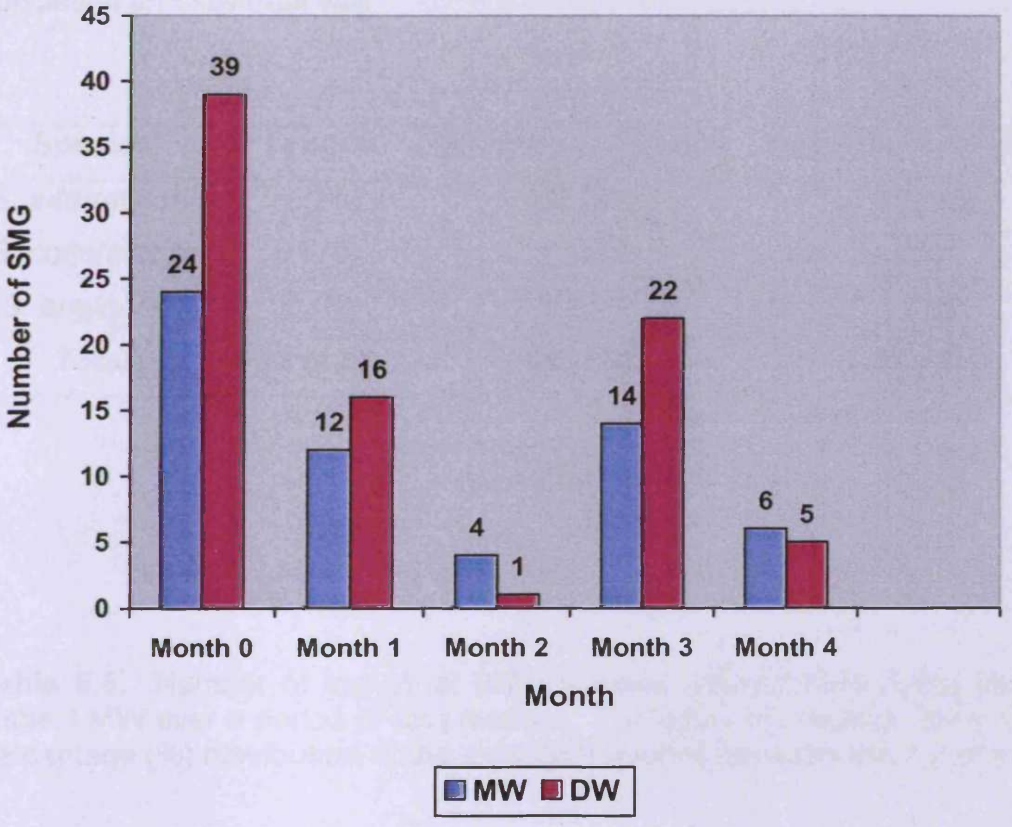

Figure 5.1. Numbers of SMG isolated from subjects MW and DW over a four month period. During Month 2 both subjects were receiving treatment for a clinical condition with a beta-lactam antibiotic. Numbers on the graph correspond to isolation numbers.

Table 5.5. Number of individual SMG species isolated from each oral site of subject MW over a period of four months. The figure in brackets refers to the frequency of isolates expressed as a percentage (%) of the total of all 3 species in an individual site.

Species	Tongue	Subgingival plaque	Supragingival plaque
S. intermedius	3 (25)	17 (65)	21 (95)
S. constellatus	9 (75)	1 (4.0)	0 (0)
S. anginosus	0 (0)	8 (31)	1 (5)
Total	**12 (100)**	**26 (100)**	**22 (100)**

Table 5.6. Number of individual SMG species isolated from 3 oral sites of subject MW over a period of four months. The figure in brackets refers to the percentage (%) distribution of the individual species between the 3 oral sites.

Species	Tongue	Subgingival plaque	Supragingival plaque	Total
S. intermedius	3 (7)	17 (41)	21 (51)	**41 (100)**
S. constellatus	9 (90)	1 (10)	0 (0)	**10 (100)**
S. anginosus	0 (0)	8 (89)	1 (11)	**9 (100)**

Table 5.7. Number of individual SMG species isolated from each oral site from subject DW over a period of four months. The figure in brackets refers to the frequency of isolation expressed as a percentage (%) of the total of all 3 species in an individual site.

Species	Tongue	Subgingival plaque	Supragingival plaque
S. intermedius	1(25)	26 (84)	26 (54)
S. constellatus	1(25)	4 (13)	2 (4)
S. anginosus	2 (50)	1(3)	20 (42)
Total	**4 (100)**	**31 (100)**	**48 (100)**

Table 5.8. Number of individual SMG species isolated from 3 oral sites of subject DW over a period of four months. The figure in brackets refers to the percentage distribution of the individual species between the 3 oral sites.

Species	Tongue	Subgingival plaque	Supragingival plaque	Total
S. intermedius	1(2)	26 (49)	26 (49)	**53 (100)**
S. constellatus	1(14)	4 (57)	2 (29)	**7 (100)**
S. anginosus	2 (9)	1(4)	20 (87)	**23 (100)**

5.4.1.2. PCR fingerprinting using ERIC 1245 primer.

All isolates were amenable to fingerprinting using ERIC-PCR. A visual representation of some of the results can be seen in Figure 5.2. The fingerprinting revealed 14 strain types to be present at Month 0 in subject MW as compared to three at Month 1, two at Month 2, three at Month 3 and four at Month 4. At Month 0, 3 and 4 variants of the A2 *S. anginosus* clonotype were isolated. *S. intermedius* clonotype I11b was detected in the subgingival plaque in Month1 and Month 2 and in the supragingival plaque at Month 1, 2 and 3. I1 clonotype was discovered on the tongue, subgingivally and supragingivally at Month 0. Results for MW can be seen in Table 5.9.

Fingerprinting results for subject DW revealed 19 strain types to be present at Month 0 as compared to six at Month 1, one at Month 2, seven at Month 3 and four at Month 4. Clonotype A2b was found in the supragingival plaque at both Month 3 and 4 and clonotype I1d was found both subgingivally and supragingivally at Month 0. Clonotype A2 was discovered at the supragingival plaque at Month 1, the subgingival plaque of Month 3, and on the tongue at Month 4. Results for DW can be seen in Table 5.10.

For both subjects there appears to be more clonotypes present at Month 0 than at any other month.

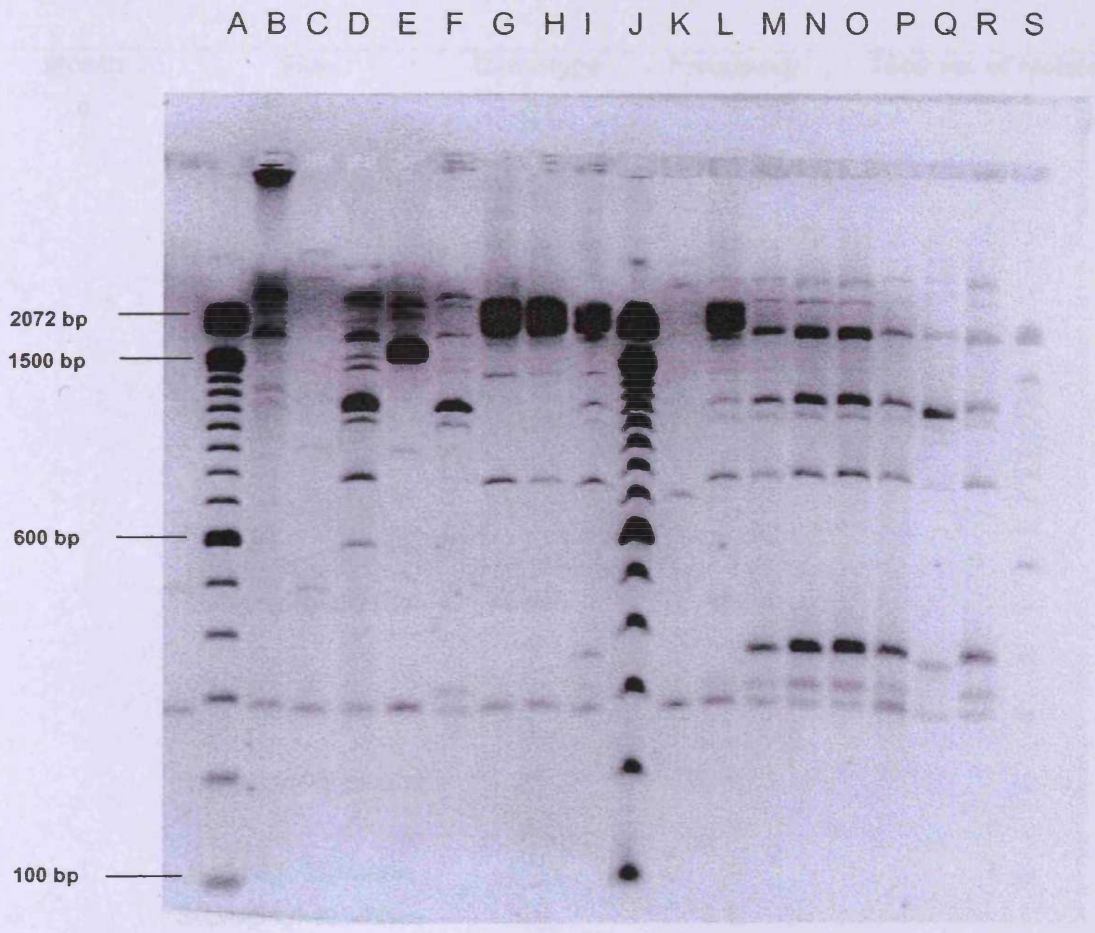

Figure 5.2. A 2 % agarose / Nusieve gel showing the ERIC-PCR banding profiles of a selection of strains from MW and DW *in-vivo*. M = Month.

A = 100 bp DNA ladder			
B = 10M4	DW supragingival	*S. anginosus*	A3
C = 23 M0	MW supragingival	*S. anginosus*	A2
D = 2 M4	MW supragingival	*S. intermedius*	I13
E = 13 M1	DW tongue	*S. anginosus*	A1
F = 15M0	MW subgingival	*S. intermedius*	I6d
G = 7 M1	MW supragingival	*S. intermedius*	I1i
H = 8 M1	MW supragingival	*S. intermedius*	I1i
I = 9 M3	MW supragingival	*S. intermedius*	I11a
J = 100 bp DNA ladder			
K = 51 M0	DW supragingival	*S. intermedius*	I12d
L = 54 M0	DW supragingival	*S. intermedius*	I12a
M = 1 M1	MW subgingival	*S. intermedius*	I11b
N = 2 M1	MW subgingival	*S. intermedius*	I11b
O = 3 M1	MW subgingival	*S. intermedius*	I11b
P = 4 M1	MW subgingival	*S. intermedius*	I11b
Q = 5 M1	MW subgingival	*S. intermedius*	I11b
R = 6 M1	MW subgingival	*S. intermedius*	I11b
S = 100 bp DNA ladder.			

Table 5.9. Strain types present in MW *in-vivo* as determined by ERIC-PCR typing with primer 1245. A = *S. anginosus*, I = *S. intermedius*, C = *S. constellatus*.

Month	Site	Clonotype	Frequency	Total no. of isolates
0	Tongue	C1	1	
		I1	2	
		I1a	1	
		C2	1	
		C3a	1	
		C4a	1	12
		C2a	1	
		C6	1	
		C4b	1	
		C4c	1	
		C4d	1	
	Subgingival plaque	I6d	2	
		I7	2	
		I1	3	9
		I1a	2	
	Supragingival plaque	I1	2	
		A2	1	3
1	Subgingival plaque	I11b	6	6
	Supragingival plaque	I1i	2	
		I11b	2	6
		I6g	2	
2	Subgingival plaque	I11b	2	
		C4a	1	3
	Supragingival plaque	I11b	1	1
3	Subgingival plaque	A2b	7	7
	Supragingival plaque	I11b	3	
		I11a	4	7
4	Subgingival plaque	A2	1	1
	Supragingival plaque	I1n	1	
		I4	2	5
		I13	2	

Table 5.10. Strain types present in DW *in-vivo* as determined by ERIC-PCR typing with primer 1245. A = *S. anginosus*, I = *S. intermedius*, C = *S. constellatus*.

Month	Site	Strain type	Frequency	
0	Subgingival plaque	I6d	1	
		I5a	2	
		I1b	1	
		I1c	3	13
		I1d	2	
		I2b	2	
		I7	2	
	Supragingival plaque	I1d	2	
		I12a	2	
		I1g	3	
		I1i	7	
		I12d	1	
		I11	1	
		I6	1	26
		I7	1	
		I5b	1	
		I7g	1	
		I1k	1	
		I1n	3	
		I4	1	
		I8	1	
1	Tongue	A1	1	
		C4e	1	2
	Subgingival plaque	C4a	3	
		I1	1	4
	Supragingival plaque	A2	1	
		A2a	7	10
		C4a	2	
2	Tongue	I1a	1	1
3	Subgingival plaque	I12d	4	
		I11b	3	

		I1o	2	
		I1n	3	13
		A2	1	
	Supragingival plaque	A2b	5	
		A2a	4	9
4	Tongue	A2	1	1
	Subgingival plaque	C4a	1	1
	Supragingival plaque	A3	2	
		A2b	1	3

5.4.2. *In-vitro* longitudinal study.

5.4.2.1. PCR fingerprinting using ERIC 1245 primer.

The original isolates from Month 0 were subcultured *in-vitro* over the course of four months and analysed using ERIC 1245 primer. All tested isolates were amenable to PCR fingerprinting and a selection can be seen in Figures 5.3a and 5.3b. Out of the original Month 0 strains collected subject MW produced 13 strains that retained the same profile throughout, 5 strains that changed profile then reverted back, and 6 strains that exhibited constant change throughout. Subject DW produced 25 strains that retained the same profile throughout, 8 strains that exhibited constant change, 2 strains that changed prior to reverting back, 2 strains that changed and retained the new profile, and 2 strains that retained the same profile prior to changing.

5.4.2.2. Selection of strains for pulsed field analysis.

The strains chosen for Pulsed field gel electrophoresis were selected according to the PCR fingerprints generated each month. The selection included strains that had undergone alterations to the fingerprint pattern shown on the gel, strains that had undergone visible change but had reverted back to their original configuration, and strains that had not undergone any change. The ERIC-PCR fingerprints of the final selected isolates are shown in Figures 5.3a and 5.3b, the ERIC-PCR banding profiles are shown in Figures 5.4a and 5.4b and isolates are listed in Table 5.11.

The fingerprint for isolate 3 showed a number of changes over a three month period (Month 0 to Month 2) similarly for the profiles for isolates 8 and 9. Strain 6 showed two strain types over a four month period (Month 0 to Month 4), as did Strain 16. Strain 7 showed 3 changes to a new type over a four month period. Strain 18 remained the same over a three month period as did Strain 26 and 43. Strain 30 showed two strain types over three months as did Strain 56.

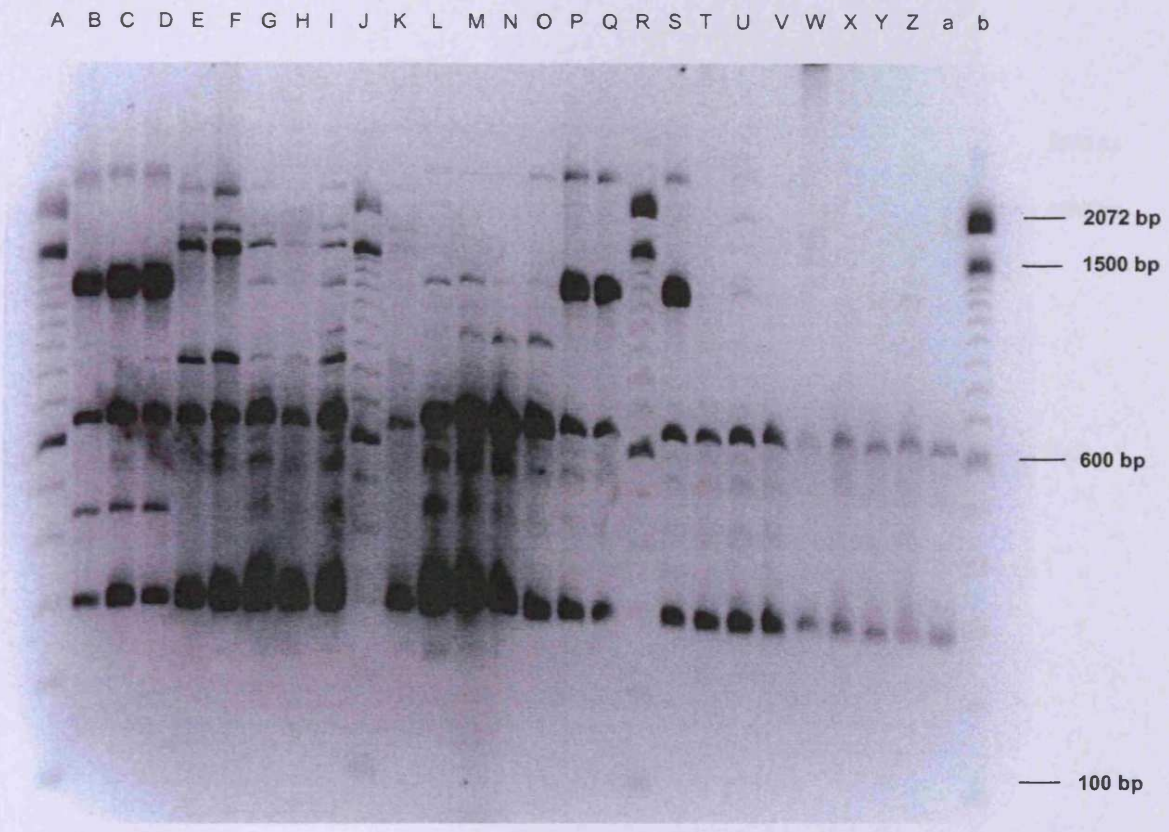

Figure 5.3a. A 2 % agarose / Nusieve gel showing the ERIC-PCR banding profiles of a selection of 24 *S. intermedius* and *S. constellatus* SMG strains (3 M0-26 M1) subcultured over the course of four months. A, J, R and b = 100 bp DNA ladder, B = 3 M0, C = 3 M1, D = 3 M2, E = 6 M0, F = 6 M1, G = 6 M4, H = 7 M0, I = 7 M1, K = 7 M2, L = 7 M4, M = 8 M0, N = 8 M1, O = 8 M2, P = 9 M0, Q = 9 M1, S = 9 M2, T = 16 M1, U = 16 M2, V = 16 M4, W = 18 M0, X = 18 M1, Y = 18 M2, Z = 26 M0, a = 26 M1. M = Month.

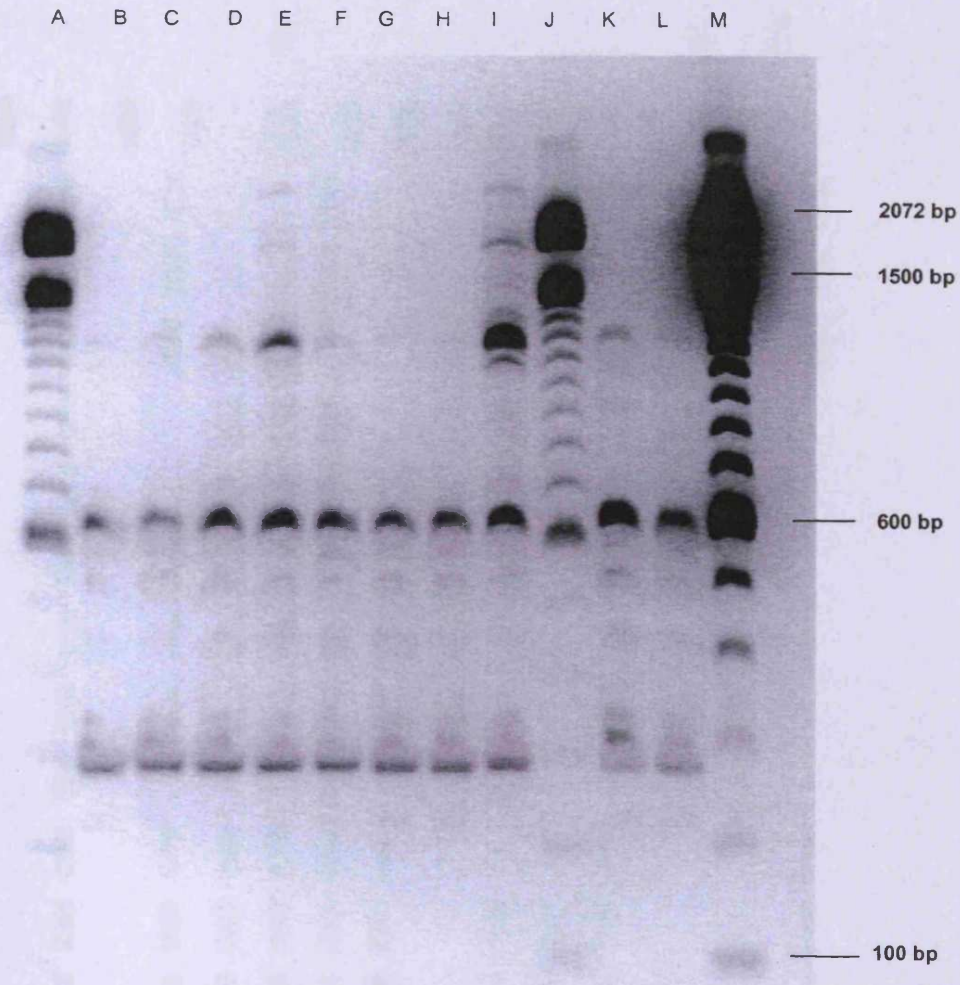

Figure 5.3b. A 2 % agarose / Nusieve gel showing the ERIC-PCR banding profiles of the final selection of ten *S. intermedius* and *S. constellatus* SMG strains subcultured over the course of four months. B = 26 M2, C = 30 M0, D = 30 M1, E = 30 M2, F = 43 M0, G = 43 M1, H = 43 M2, I = 56 M0, K = 56 M1, L = 56 M2. A, J and M = 100 bp DNA ladder.

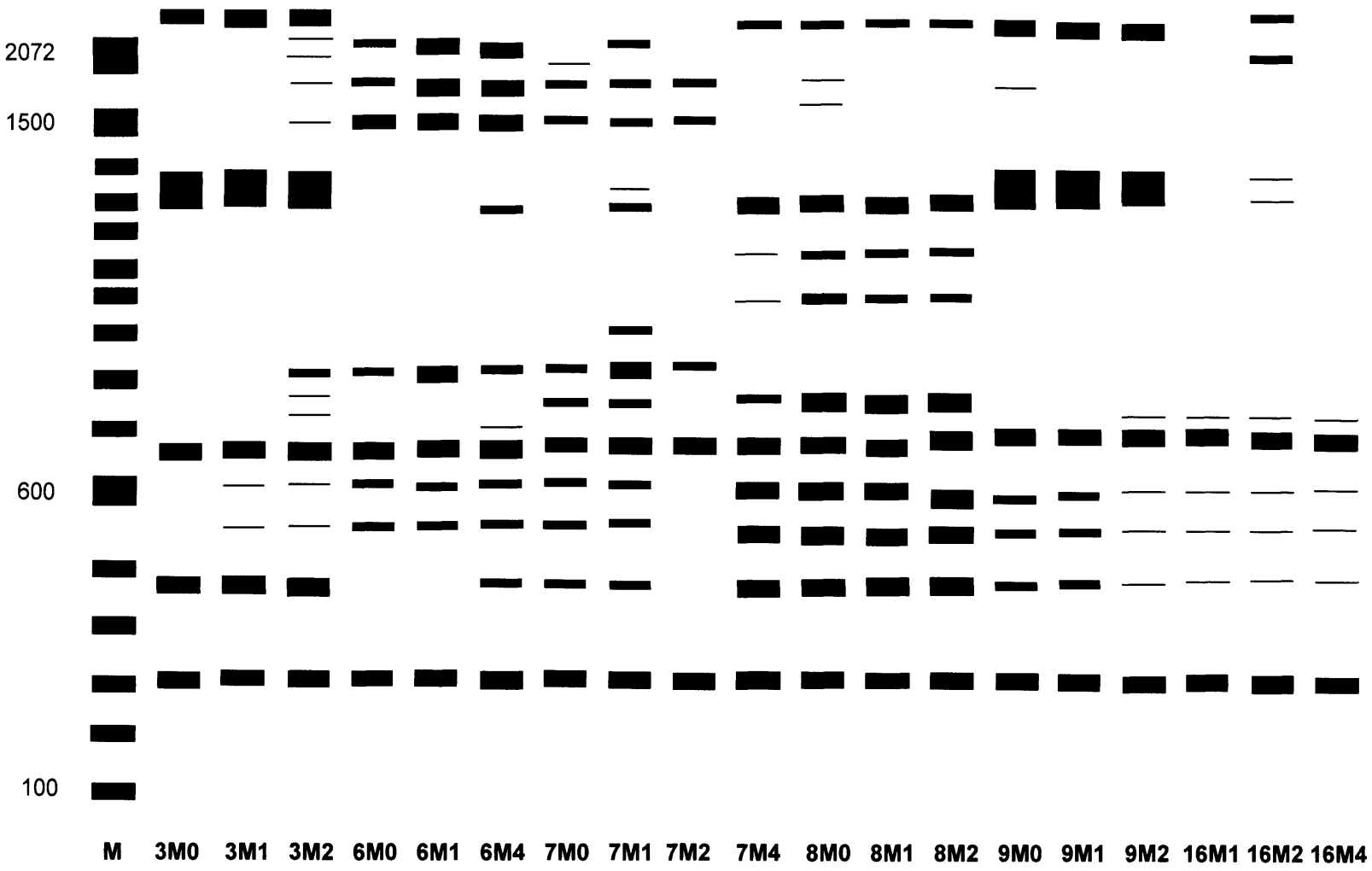

Figure 5.4a. Banding diagram representing the ERIC-PCR typing arrangements of *in-vitro* isolates 3 month 0 to 16 month 4. The 100 bp marker is designated M.

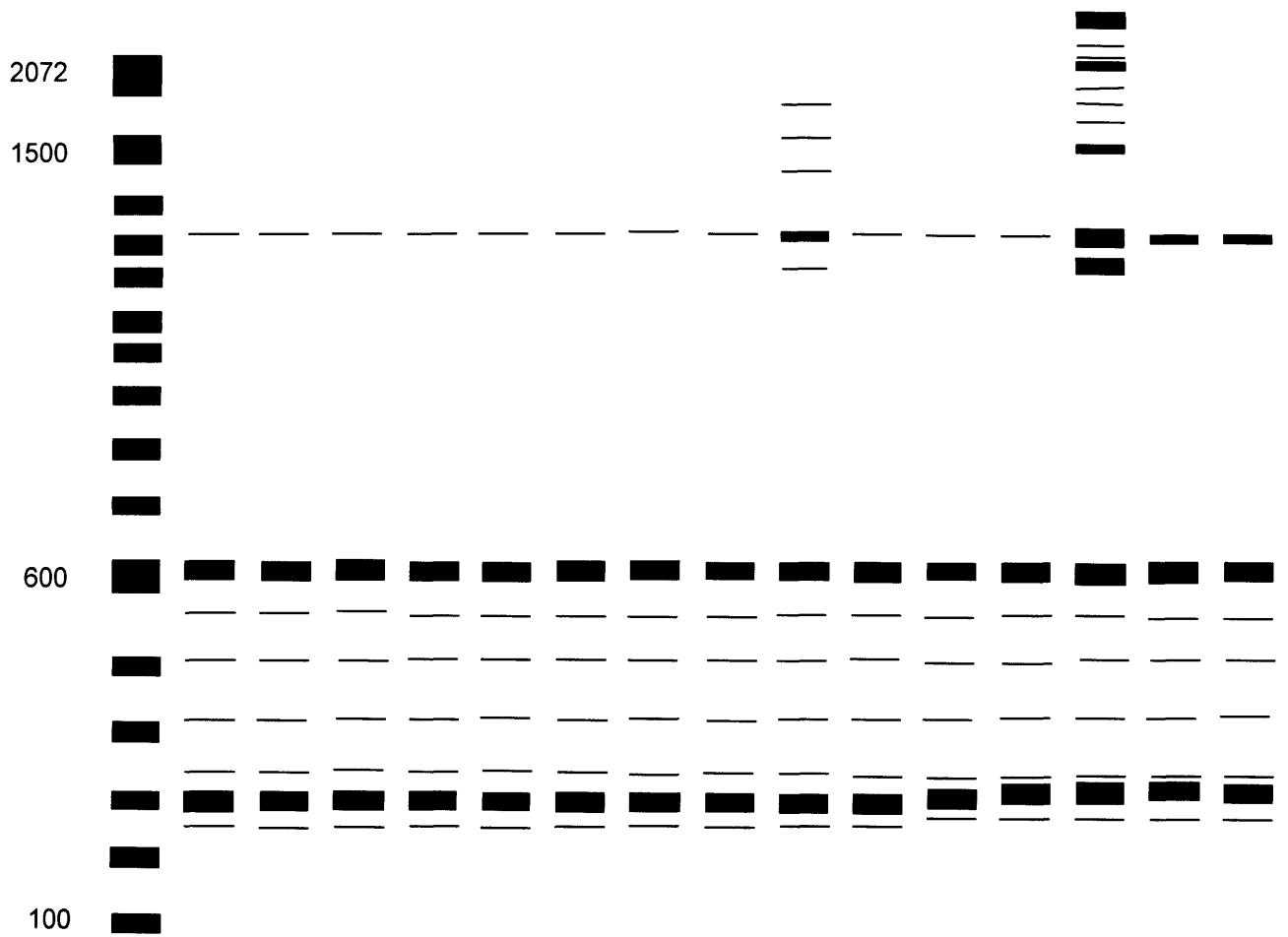

Figure 5.4b. Banding diagram representing the ERIC-PCR typing arrangements of *in-vitro* isolates 18 month 0 to 56 month 2.

Table 5.11. Identity and site of isolation of 34 strains of SMG subjected to ERIC-PCR and chosen for PFGE fingerprinting as part of a four month *In-vitro* subculture study. M = month.

Isolate	Species	Area of isolation	Subject	Strain type
3 M0	*S. intermedius*	Tongue	MW	I1a
3 M1	*S. intermedius*	Tongue	MW	I1b
3 M2	*S. intermedius*	Tongue	MW	I1c
6 M0	*S. constellatus*	Tongue	MW	C3a
6 M1	*S. constellatus*	Tongue	MW	C3a
6 M4	*S. constellatus*	Tongue	MW	C5
7 M0	*S. constellatus*	Tongue	MW	C4a
7 M1	*S. constellatus*	Tongue	MW	C5a
7 M2	*S. constellatus*	Tongue	MW	C7
7 M4	*S. constellatus*	Tongue	MW	C8
8 M0	*S. constellatus*	Tongue	MW	C6
8 M1	*S. constellatus*	Tongue	MW	C6a
8 M2	*S. constellatus*	Tongue	MW	C6b
9 M0	*S. constellatus*	Tongue	MW	C2a
9 M1	*S. constellatus*	Tongue	MW	C2c
9 M2	*S. constellatus*	Tongue	MW	C2d
16 M1	*S. intermedius*	Subgingival plaque	MW	I4
16 M2	*S. intermedius*	Subgingival plaque	MW	I5
16 M4	*S. intermedius*	Subgingival plaque	MW	I4
18 M0	*S. intermedius*	Subgingival plaque	MW	I7
18 M1	*S. intermedius*	Subgingival plaque	MW	I7
18 M2	*S. intermedius*	Subgingival plaque	MW	I7
26 M0	*S. intermedius*	Subgingival plaque	DW	I7
26 M1	*S. intermedius*	Subgingival plaque	DW	I7
26 M2	*S. intermedius*	Subgingival plaque	DW	I7
30 M0	*S. intermedius*	Subgingival plaque	DW	I7
30 M1	*S. intermedius*	Subgingival plaque	DW	I7
30 M2	*S. intermedius*	Subgingival plaque	DW	I9
43 M0	*S. intermedius*	Supragingival plaque	DW	I7
43 M1	*S. intermedius*	Supragingival plaque	DW	I7
43 M2	*S. intermedius*	Supragingival plaque	DW	I7
56 M0	*S. intermedius*	Supragingival plaque	DW	I8
56 M1	*S. intermedius*	Supragingival plaque	DW	I7
56 M2	*S. intermedius*	Supragingival plaque	DW	I7

5.4.2.3. Pulsed field gel electrophoresis.

All strains were amenable to typing using PFGE and showed banding trends. However, Strain 30 and Strain 43 after repeated analysis showed poorly resolved fingerprints (Figures 5.5a and 5.5b).

The results using this method showed the same trend as the PCR method for Strain 7, Strain 18, Strain 26 and Strain 56 (Figure 5.5a, 5.5b, 5.6a, 5.6b and Table 5.12).

Strain 3 showed a minor change in profile between Month 0 and Month 1 and a further minor change in profile between Months 1 and 2 with PCR fingerprinting, whereas with PFGE the profile between Month 0 and Month 1 was unchanged followed by a minor change in profile between Month 1 and Month 2 (Figure 5.5a, 5.6a and Table 5.12).

Strain 6 showed a similar trend in the banding profiles between the Months 0 to 4 using PFGE when compared to the profiles using PCR typing. However, between Month 0 and Month 1 showed no change in profile with PCR, major changes occur between these months with PFGE. (Figure 5.5a, 5.6a and Table 5.12).

Strain 8 showed a minor change between Month 0 and Month 1 and a minor change between Month1 and Month 2 with PCR typing. The PFGE analysis however shows that the strain retains the same profile between Month 0 and Month 1 and a minor change in profile between Month 1 and month 2 (Figure 5.5a, 5.6a and Table 5.12).

Strain 9 showed a minor change in profile between Month 0 and Month 1 and showed a further minor change in profile between Month 1 and Month 2 with PCR typing, however the PFGE analysis shows that a small change occurred between Month 0 and Month 1 producing a profile that remained constant thereafter (Figure 5.5a, 5.6a and Table 5.12).

Strain 16 showed a change in profile between Month 1 and Month 2 and a reversion back to its original profile between Month 2 and Month 4 using PCR typing. The PFGE analysis shows that the profile is unchanged between Month 1 and Month 2 and a major change in profile occurs between Month 2 and Month 4 (Figure 5.5b, 5.6a and Table 5.12).

The most stable *in-vitro* strains (strains 18 and 26) were compared to the *in-vivo* tables (Tables 5.9 and 5.10) to see if their apparent genomic stability showed a greater isolation stability. Table 5.13 shows that both strains do not recur at all throughout 4 months of isolations in any isolation site.

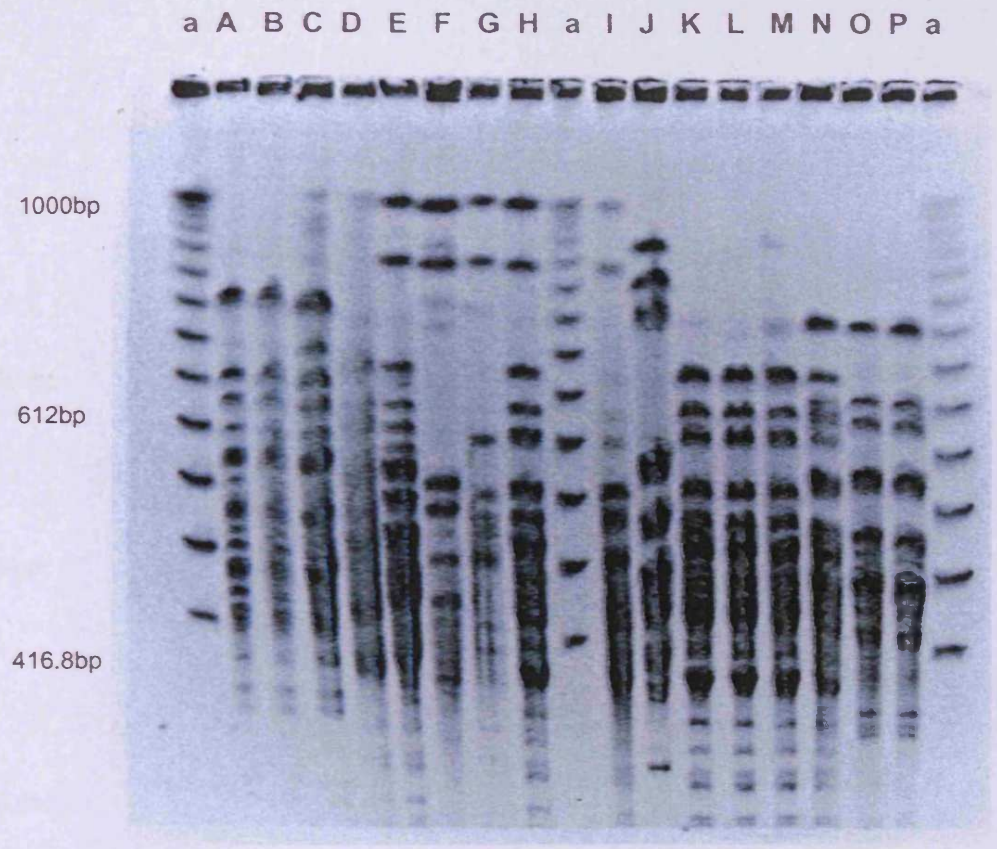

Figure 5.5a. An agarose gel showing the pulsed field gel electrophoresis profiles of strains 3, 6, 7, 8 and 9. A = 3 M0, B = 3 M1, C = 3M2, D = 6 M0, E = 6 M1, F = 6 M4, G = 7 M0, H = 7 M1, I = 7 M2, J = 7 M4, K = 8 M0, L = 8 M1, M = 8 M2, N = 9 M0, O = 9 M1, P = 9 M2. The marker (lambda ladder) is shown labelled a.

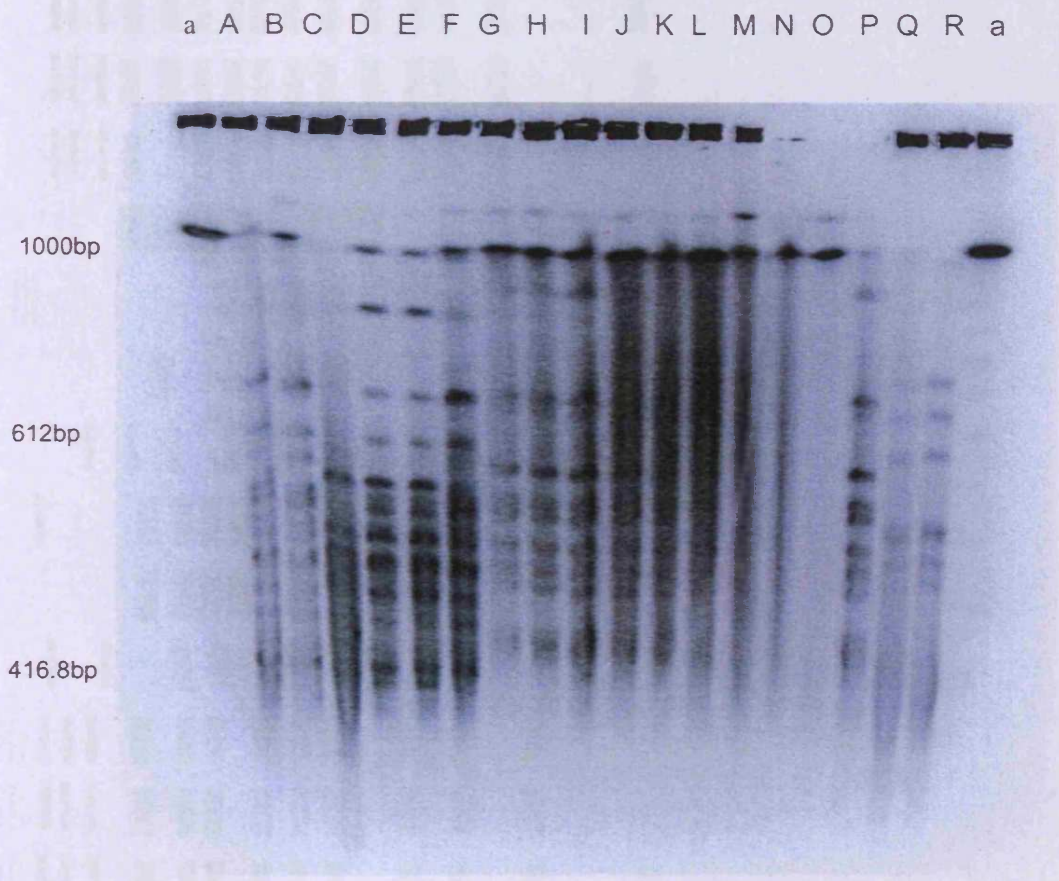

Figure 5.5b. An agarose gel showing the pulsed field gel electrophoresis profiles of strains 16, 18, 26, 30, 43 and 56. A = 16 M0, B = 16 M1, C = 16 M4, D = 18 M0, E = 18 M1, F = 18 M2, G = 26 M0, H = 26 M1, I = 26 M2, J = 30 M0, K = 30 M1, L = 30 M2, M = 43 M0, N = 43 M1, O = 43 M2, P = 56 M0, Q = 56 M1, R = 56 M2. The Lambda ladder marker is shown labelled a.

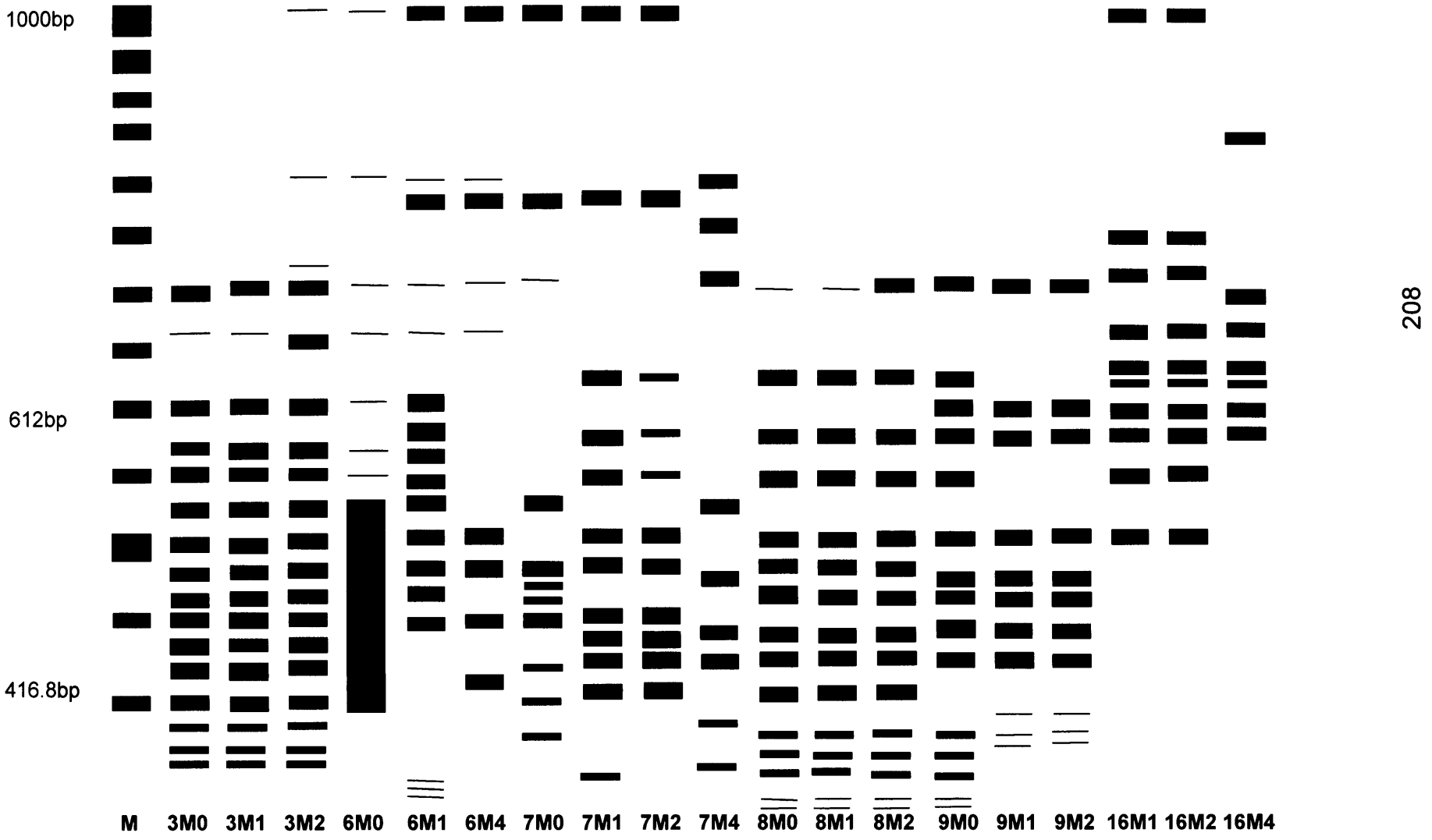

Figure 5.6a. Banding diagram representing the PFGE typing arrangements of isolates 3 month 0 to 16 month 4. The 1000 bp DNA lambda ladder marker is designated M.

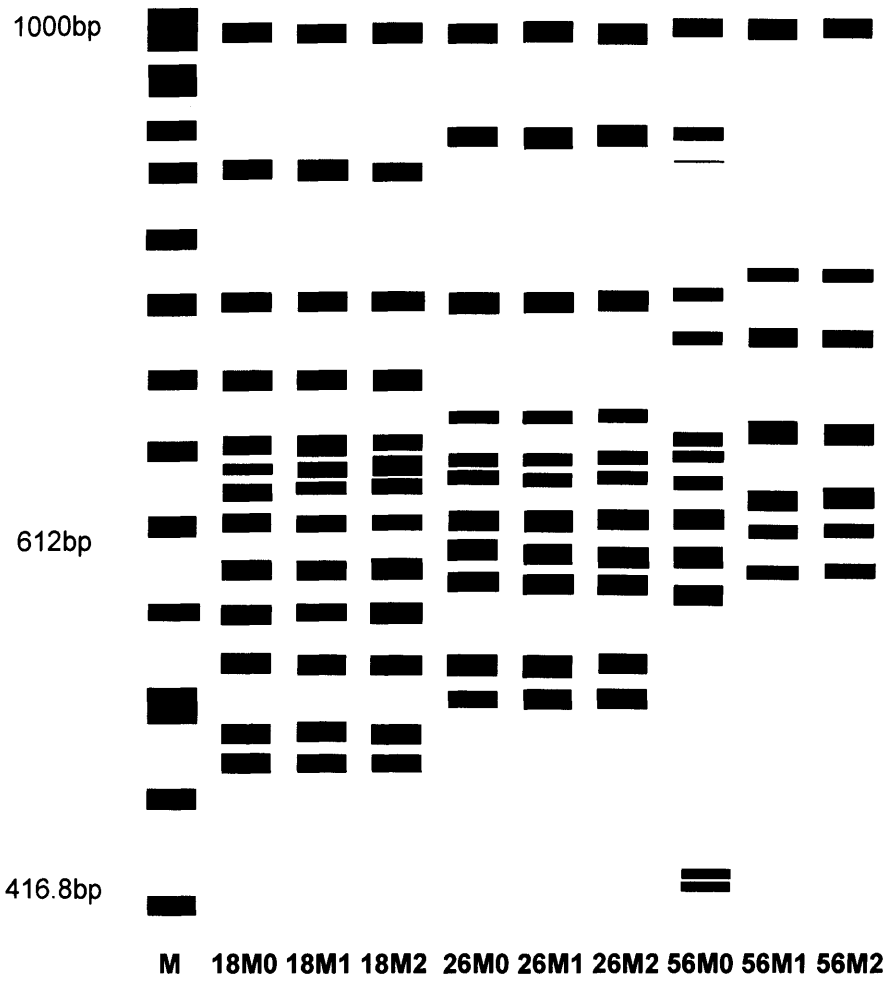

Figure 5.6b. Banding diagram representing the PFGE typing arrangements of isolates 18 month 0 to 56 month 2. The 1000 bp DNA lambda ladder marker is designated M.

Table 5.12. Table comparing the changes in banding profiles of selected strains at indicated time intervals when analysed using ERIC-PCR and pulsed field gel electrophoresis as part of a four month longitudinal study.

Strain	Time	ERIC-PCR	PFGE
	0-1	Minor change in profile	Profile unchanged
3	**1-2**	Minor change in profile	Minor change in profile
	0-1	Profile unchanged	Major change in profile
6	**1-4**	Major change in profile	Major change in profile
	0-1	Major change in profile	Major change in profile
7	**1-2**	Major change in profile	Major change in profile
	2-4	Major change in profile	Major change in profile
	0-1	Minor change in profile	Profile unchanged
8	**1-2**	Minor change in profile	Minor change in profile
	0-1	Minor change in profile	Loses major bands
9	**1-2**	Minor change in profile	Profile unchanged
	1-2	Minor change in profile	Profile unchanged
16	**2-4**	Reversion to former profile	Major change in profile
	0-1	Profile unchanged	Profile unchanged
18	**1-2**	Profile unchanged	Profile unchanged
	0-1	Profile unchanged	Profile unchanged
26	**1-2**	Profile unchanged	Profile unchanged
	0-1	Profile unchanged	No profile
30	**1-2**	Major change in profile	No profile
	0-1	Profile unchanged	No profile
43	**1-2**	Profile unchanged	No profile
	0-1	Major change in profile	Major change in profile
56	**1-2**	Profile unchanged	Profile unchanged

Table 5.13. Table to show *in-vivo* distribution of genomically stable *in-vitro* strains. The table shows strains 18 and 26, which have shown to be the most stable *in-vitro*, compared with their *in-vivo* distributions over the period of 4 months.

Strain	Site of isolation	Clonotype	*In-vivo* frequency
18	MW subgingival	I7	No recurrence at any other months
26	DW subgingival	I7	No recurrence at any other months

5.5. Discussion.

Very little is currently known about the patterns and dynamics of colonisation by SMG within individuals. For this reason, a longitudinal study has been performed to examine the distribution of species of the SMG in three sites in two individuals over a four month period. It was also the intention to look for intraspecies diversity at sites and where possible, follow this on a longitudinal basis.

There are a number of limitations to this type of ecological study, particularly relating to sampling. A semi selective media was used and up to 30 colonies thought to represent members of the SMG were selected for study. Although initially a high isolation was obtained, this was significantly reduced on subsequent time periods. The reasons for this are unclear, but may be related to the fact that coincidentally both individuals had taken a beta-lactam antibiotic between sampling time M1 and M2. There was a high level of false positives i.e. isolates selected for study based upon colony morphology which turned out to be other species of streptococci and in particular *S. oralis*. A useful addition to any future study would be the development of a new selective media specifically for SMG. Even considering the difficulties of this

kind of study, many useful observations can be made from the results, since the present study is the largest of its kind to focus solely on SMG.

Previous studies on the ecology of SMG have shown *S. anginosus* to be the most frequently isolated species and *S. constellatus* to be the species encountered least often at oral sites (Whiley *et al.*, 1993). *S. constellatus* has however been shown to dominate the subgingival regions, isolated alongside *S. anginosus* but never alongside *S. intermedius*. *S. intermedius* has shown to be dominant in the supragingival regions and on the tongue and, has been isolated alongside *S. anginosus* more often than alone. *S. anginosus* has been isolated frequently both alone and in conjunction with *S. intermedius* and *S. constellatus* at subgingival and supragingival sites, but all three species have never been isolated together at one site or in one subject (Whiley *et al.*, 1993). Mejare and Edwardsson, (1975) failed to detect SMG on the tongues of their 18 subjects. The current study has attempted to improve and update the current knowledge of SMG ecology at oral sites by focusing on SMG and omitting viridans streptococcal species. It is therefore difficult to directly draw comparisons between this study and previous studies, however there are a number of interesting points to note.

The results of the present study reveal *S. intermedius* to be the most commonly isolated of the SMG species overall, with this species constituting 68 % of the total SMG in subject MW and 64 % of the total SMG in subject DW. *S. anginosus* was the second most frequently isolated species in subject DW constituting 28 % of the total SMG. *S. constellatus* was the SMG species least often isolated in DW. This in contrast to previous studies that found *S. anginosus* to be the dominant species and *S. constellatus* to be the species least often isolated. This study supports the observation of Whiley *et al.*, (1993) that *S. intermedius* and *S. constellatus* are hardly ever isolated together in the same site. Interestingly, both that study and this study yielded a combination of these species once in the subgingival plaque, although only in small amounts. Interestingly, the prior observation that all three species were never isolated from the same subject was not supported since both

subjects in this study yielded all three species. *S. intermedius* was consistently isolated in higher numbers in plaque samples when compared to *S. constellatus.* *S. anginosus* was isolated from subgingival plaque in small numbers in subject MW, but in considerably larger numbers in subject DW. The present study found SMG species to be isolated in small numbers on the tongue of both subjects with *S. constellatus* predominating in subject MW and *S. anginosus* predominating in subject DW.

It was evident from isolations that a great SMG species variation exists within oral sites. Although this is the case, certain isolates of a given species were characterised by typing to determine the subspecies diversity.

When designating the clonotypes of strains a minor change was denoted by the loss or gain of a weak band and a major change in profile the loss or gain of a major band. This system was used throughout in order to be consistant with the typing comparisons. In the monthly *in-vivo* sampling study, certain trends in the strain types isolated became apparent. In subject MW, variants of the A2 "*anginosus*" clonotype were found both at Month 0 (A2), Month 3 (A2b) and Month 4 (A2). Variants of I1 and I6 *S. intermedius* species were found in the supragingival plaque at Month 1. I11b *S. intermedius* was found in the subgingival plaque at both Month 1 and Month 2 and was found in the supragingival plaque at Month 1, 2 and 3. C4a *S. constellatus* species was found on the tongue at Month 0 and in the subgingival plaque at Month 2 so would seem to be quite widespread. Variants of I1 *S. intermedius* species were detected on the tongue (I1a and I1 at Month 0) and in the supragingival plaque (I1i at Month 1 and I1n at Month 4). In subject DW variants of C4 were detected on the tongue (C4e at Month 1), in the subgingival plaque (C4a at Month 1) and in the supragingival plaque (C4a at Month 1). *S. anginosus* strain A2b was found in the supragingival plaque at both Month 3 and Month 4. Variants of *S. intermedius* strain I1 were found in the subgingival plaque at Month 1 (I1) and Month 3 (I1o). I1d was found both subgingivally and supragingivally in the plaque at Month 0. It is interesting to note that a wider variety of strain types exist at Month 0 than any other isolation month. MW *in-*

vivo showed 14 different clonotypes at Month 0, compared to very few at the proceeding months, most of these clonotypes were varieties of *S. constellatus*, which were absent from future months perhaps suggesting a greater diversity within *S. constellatus*. In DW *in-vivo* 19 different clonotypes were isolated at Month 0 with very few in the following months. Interestingly, the majority of clonotypes at Month 0 were strains of *S. intermedius*, which were in the minority in future months. This perhaps suggests a greater variation in *S. intermedius* populations within the oral cavity of that subject.

Out of the original 63 strain collected at Month 0, the majority retained the same profile throughout the study *in-vitro* (38). The second highest category was the isolates that exhibited constant change in profile (14) followed by isolates changing profile prior to changing back (7), isolates changing profile and keeping the profile for the remainder of the study (2) and finally isolates retaining the same profile until the final month (2). The strains chosen for further study with PFGE were a selection from the above catagories to best represent the spread of results.

In-vitro isolates from subject MW's tongue showed four strains exhibiting constant change between strain types with ERIC-PCR typing. Other strains included one that remained the same until the final month. In the subgingival plaque one strain underwent a change to a variant and reverted back and one strain exhibited constant change. Isolates from subject DW's subgingival plaque showed one strain that remained the same until the final month and one strain that was unchanged. In the supragingival plaque one strain remained unchanged and one strain showed a major change in profile followed no change in profile. Overall, five strains kept changing throughout, two strains changed in the final month, one strain changed and then reverted back to original type and three strains changed to variants of type (minor change) each month throughout the study. It seems that many changes have taken place, although not all of them major. The minor changes to variants of a type may just be accounted for by inconsistencies in the agarose gel PCR band intensities. A larger casting tray was used which enables greater

running time and thus greater spacing of typing PCR bands in order to reduce any possibility of obscuring relevant banding arrangements. A minor band may show up in one lane and be missing in another due to a low intensity. It is a recognised limitation of PCR agarose gel typing that uneven band intensities can make interpretation of gels difficult (Grundmann *et al.*, 1997). Another potential limitation of the technique is the lack of any consensus guidelines for interpretation of the banding patterns. However, the same rules have been applied when interpreting each strain's pattern and assigning type, therefore the degree of randomness of the technique has been limited as much as possible.

It is interesting to note that the large dark bands present at 300 bp on the gels performed in Chapter 4 were only present on a small number of strains in this chapter. The darkness of the band may be representative of an enlarged number of 300 bp ERIC 1245 fragments being amplified in some strains compared to a lower frequency of amplification in others. This can best be explained by hypothesising that the test strains used in Chapter 4 and some strains (3M0 – 16M4) from Chapter 5 all had an excess level of 300 bp fragments undergoing amplification, whereas remaining Chapter 5 strains (18M0 – 56M2 and strain in Figure 5.2) showed lower amplification resulting in less prominent bands on the gels. This does not present a problem with strain typing as the level of amplification of a specific band may simply be a feature of a particular strain.

Pulsed field gel electrophoresis (PFGE) was used as a second technique of strain typing for determining the genetic stability of selected SMG strains. The macrorestriction method detected sufficient DNA polymorphism within the fingerprint patterns to be able to differentiate between the SMG strains. The restriction patterns were mostly clear, reproducible and readable by visual inspection with a few exceptions. The *Sma* I restrictions yielded profiles of between six to eleven bands with the majority producing eleven bands. It has been proposed that the reliability of PFGE in strain typing is dependant upon the selection of restriction enzymes that produce at least ten bands so that

inter-strain comparisons may be interpreted with confidence (Tenover *et al.*, 1995). The results of the PFGE when compared to the PCR typing results for the selected *in-vitro* strains show marked differences in the trends from one month to the next. Strain 7, 18, 26 and 56 showed the same trend between the months, however, all other strains had a difference - whether minor or major. The trends of ERIC-PCR and PFGE patterns across the months did not correlate completely. Many of the banding profile differences within a presenting strain are so marked in their alteration that the change must be deemed considerable, seemingly involving a major change in the chromosomal arrangement of the organism. It has been suggested that DNA rearrangements – not point mutations are responsible for observed RFLP within enterococci and *S. pneumoniae* (Hall, 1994; Hall and Duke, 1998). It may be hypothesised that the more genomically stable a strain is, the more stable its colonisation of a site may be. This study however, has shown that it is not necessarily the case with the two most stable strains (as shown by PCR typing and PFGE) showing low potential colonisation stability in both subjects.

It would have been interesting to have included *S. anginosus* strains in the typing study to see if they exhibited more molecular instability than the other SMG species, however, unfortunately this was not possible due to a lack of interesting *S. anginosus* profiles available. It would also be interesting to investigate the level of diversity exhibited further by performing tests to measure the levels of recombination in the strains.

According to the theories of clonal populations, the higher the level of diversity the less clonal the population (Spratt and Maiden, 1999). If this is the case then the large amount of diversity shown by the SMG in this study would suggest a low level of clonality, pointing towards a more panmictic existence where a state of linkage equilibrium is present and a significant degree of horizontal gene transfer occurs (Gupta and Maiden, 2001). If this is a true picture then this would have implications for the potential virulence of the group (Spratt and Maiden, 1999). It would be interesting to examine the strains for levels of recombination between cells.

The level of horizontal gene transfer within the *in-vivo* samples was expected as it was theorised that a high level of diversity would exist in a population as dense and under as large a degree of selective pressure as the oral cavity. However, the level of diversity present in the *in-vitro* longitudinal strains is more difficult to explain as the cells had been removed from their natural habitat into an environment rich in nutrients and low in selective pressure. The only explanation available is that intragenomic diversity was taking place and the strains were diverse beyond any natural contributing factors.

5.6. Conclusions.

The research presented in this chapter describes a study of population structure and ecological dynamics of the SMG and the genomic stability of strains subcultured *in-vitro* over time. The study provided data on SMG populations and the subspecies variation in the oral cavity of two healthy subjects over a period of four months. The study demonstrated the complexity of colonisation of the tongue, sub and supra-gingival plaque by species of the SMG. In comparison to other studies on SMG populations (Whiley *et al.*, 1995) this study indicates that *S. intermedius* is the predominant species of SMG in oral sites. The study confirmed previous reports that *S. intermedius* and *S. constellatus* are rarely found together in the same oral site at any one time but observations were also made that had not been noted previously. The levels of *S. constellatus* were not found to equal those of *S. intermedius* in subgingival plaque. As well as this SMG species were found on the tongue in small numbers (*S. anginosus* and *S. constellatus*) and all three species were isolated from each subject which has not been previously reported.

The characterisation of multiple isolates by ERIC-PCR typing revealed a high level of diversity of subspecies colonising the oral cavity. This diversity varied

over the time period and for reasons that are unclear was greatest for both individuals at the start of the study. A number of clonotypes were seen to persist in a given site but many were not isolated repeatedly.

The longitudinal analysis of genomic stability as assessed by ERIC-PCR typing showed that in the majority of cases strains yielded similar profiles over the time period of *in-vitro* subculture. A few changes in profile were apparent and these together with a selected number of strains that remained stable were compared by macro-restriction fingerprinting. There were more changes from one month to the next with the ERIC-PCR fingerprinting than with the PFGE suggesting that the latter is a more stable approach to typing, however, this may also suggest that ERIC-PCR is more sensitive than PFGE in detecting differences in fingerprint. Although there were a number of interesting discrepancies which require further analysis, typically the two methods correlated for most of the strains studied. The reasons for and mechanisms behind the profile changes are unclear but suggest that for this group of bacteria intragenomic recombination may be a contributory factor to the great heterogeneity of the group.

GENERAL DISCUSSION.

As described throughout this thesis, the SMG are isolated as commensal organisms from a range of body sites, however, they also seem to be intimately associated with the disease process at a number of body sites including purulent abscesses of the brain (Molina *et al.*, 1981), the dentoalveolar region (Lewis *et al.*, 1986) and the liver (Edmiston *et al.*, 1991). They are also frequently associated with bacteraemia (Bert *et al.*, 1998) and have been documented as the aetiological agents in a significant proportion of cases of endocarditis (Murray *et al.*, 1978). SMG seem to have a propensity for invasion of the bloodstream to cause bacteraemia and seeding of infection to sites distant from the original infection (Bert *et al.*, 1998) and an ability to resist antimicrobial therapy even when susceptible to most broad spectrum agents (Jacobs and Stobberingh, 1996). One of the more interesting aspects of the pathogenicity of this group is the switch from apparent commensal to pathogen. It is been suggested that strains of SMG may be initiators of infection and may do so by creating environments favoured by other pathogenic organisms, in particular anaerobes.

This study has sought to address the three novel areas, which may lead either directly or indirectly to a clearer understanding of the pathogenicity of this group of bacteria. Firstly the development of a method to facilitate the study of preferential gene expression under varying environmental conditions. Secondly an analysis of the dynamics and patterns of colonisation of the oral cavity. Thirdly an attempt to address the reason for the marked genetic diversity, through studying the stability of the SMG genome.

The results of these studies attempt to answer two important questions: Are the SMG genetically diverse / genomically unstable, how can the SMG 'switch' from being commensal organisms to being associated with clinical infection and how these factors impact upon virulence within the group?

The work of chapter 3 was the development of a method to detect putative virulence factors within *S. anginosus* after exposure to a range of environmental stimuli mimicking the host environment. The DD RT-PCR method was successfully developed. RNA was obtained and RT-PCR was achieved using radiolabelling. Three interesting sequences were discovered within the clinical isolate at a neutral pH that were not present in other samples. Confirmation of the importance of up/down regulation of these sequences was frustrated by inability to obtain RNA amenable to Northern blotting. Other extraction methods (e.g. Q-biogene bio101 extraction system) alternative methods to Northern blotting such as quantitative PCR and dot-blots should be investigated for further continuation of this aspect of the work. Once this problems area has been resolved, the analysis of differential gene expression would be significantly facilitated by gene chip technology which has now largely superseded the use of DD RT-PCR gene expression analysis.

Interestingly, by DD RT-PCR three sequence homologies were identified using the TblastX program. Sequences included pyruvate formate lyase (PFL) activating enzyme, dTDP-4-keto-6-deoxyglucose-3,5-epimerase enzyme and a putative haemolysin *hlyX*. Even though the upregulation of these sequences is not certain as they may have proven to be false positive results it is still interesting to speculate upon what the results may mean in the context of SMG virulence. It is interesting that all three of these sequences seemed to be upregulated at pH 7.2. PFL is a switch to anaerobic metabolism, *hlyX* is activated in low oxygen conditions in *E. coli* to produce a haemolysin and the epimerase enzyme (used in the production of rhamnose) is employed in the production of antigen specific polysaccharide chains on the cell surface. The fact that upregulation occurred at pH 7.2 suggests that it may be possible that all three substances could be produced at a higher rate upon entry into an pH neutral environment such as the bloodstream (which is pH neutrally buffered). If this were the case then a haemolysin would be useful for the breakdown of red blood cells for nutrition, while specific cell surface polysaccharides would prove useful for the colonisation of interior

surfaces of the heart or the surface of the kidneys. The anaerobic environment would be present as a consequence of the deoxygenation of the blood as it flows away from the tissues of the bacterial entry point (the gums). The ability of the cell to form or colonise anaerobic abscess sites is increased as well. This could go part of the way to explaining why SMG are able to survive for elongated periods within the bloodstream. It is interesting that the possible gene upregulation occurred in the clinically isolated *S. anginosus* strain and not the commensal isolate. Indeed, Strain 8 – already isolated within an abscess site – seemed to exhibit the characteristics that would be crucial for the survival of a bacterial strain within the bloodstream: haemolysin activity for nutrient assimilation, anaerobic metabolic switching for adaptation to purulent environments and polysaccharide cell wall structures using in the cellular attachment to internal structures such as heart valves and kidneys in other pathogenic streptococci. The commensal strain didn't seem to copy this activity, so is it the case that some strains are more able to take advantage of environmental situations to exhibit pathogenic traits than others and if so what is it that gives them this capability? The effect of pH on the gene expression of oral streptococci has been speculated upon before now, Vriesema *et al.*, (2000) studied the viridans streptococci in pH environments and discovered a pH induced promoter upregulated by the shift from mouth pH to blood pH. The promoter showed sequence homology to a putative hydrogenase from *Clostridium acetobutylicum*.

The fourth and fifth chapters were concerned with the development of methods of molecular typing and their use in the analysis of the genomic stability of the SMG over the space of four months of *in-vitro* subculture as well as the analysis of clonal diversity in the oral environments of two volunteers over four months of *in-vivo* isolations. The study within chapter four culminated in the establishment of a successful DNA extraction method for the SMG coupled with an improvement on the current protocol for PFGE with SMG and the development of ERIC-PCR for rapid typing of isolates. During the course of the evolution of the typing protocols interesting observations were made concerning the fingerprinting of control SMG strains

from both clinical sites and commensal sites. Interestingly, no correlation could be found (using either method) within the fingerprinting profiles to relate to either species or site of origin. This finding tends to support the commensal and panmictic status of the SMG. Chapter five describes *in-vivo* and *in-vitro* longitudinal study into both colonisation dynamics and genomic stability. Although there was disappointingly low recovery of SMG subsequent to the second month an interesting picture of colonisation emerged both agreeing with and contradicting previous studies. The colonisation of both individuals in the study was shown to be complex with many different clonotypes of a given species present. This is significant and demonstrates for the first time the diversity of isolates of SMG present within a given individual. On a longitudinal basis the colonisation patterns were not stable with significant changes in the species representation. The reasons for this are unclear particularly in view of the documented stability of other members of the oral microflora. It is still the case that sampling variations account for some of the findings, however it is unlikely to explain the results wholly. The rise and fall of clonotypes might be explained by changes in the micro-environment in which the bacteria find themselves. In view of the genetic instability documented for these bacteria, it is possible that colonisation by a given strain is stable but that the fingerprint of the strain changes. However, this suggestion cannot be proven by the results of the present study and furthermore it would be extremely difficult to devise a means of testing this hypothesis. The important aspect of this chapters results was the level of genomic diversity displayed by some strains of SMG. This is important from the point of view of SMG virulence since there is some evidence that genomic instability may be associated with pathogenicity under certain circumstances. When changes occur to the genome of a SMG cell, transcription factors may be upregulated that enable the production of previously dormant virulence factors. Tenover *et al.*, (1995) proposed that two or three differences represented an independent genetic event and that seven or more band differences represented three or more genetic events. In a number of cases there was apparent reversion to a previous fingerprint perhaps suggesting that at least in some cases the genetic changes are not of a random nature.

The level of change in fingerprint can only point to a significant event causing an alteration in the chromosomal arrangement of the organism. As mentioned in section 5.5, Hall and Duke, (1998) suggested DNA rearrangements not point mutations to be responsible for changes in typing patterns in *S. pneumoniae*. It is difficult to explain the genetic events underlying these changes in genomic fingerprints. For PFGE, attempts have been made to correlate band differences with genetic events.

It is thought that genomic change may be accounted for by recombination or mutation, indeed transformation (in intergenomic diversity) has been demonstrated *in-vitro* using cariogenic strains of SMG (Jacobs *et al.*, 1989). Dobrindt and Hacker, (2001) reported that certain bacterial species including *S. pneumoniae* and *S. pyogenes* have an unusually high frequency of recombination that may result in a breakdown of the 'clonal frame'. Another possible explanation for diversity amongst strains is adaptive mutation. The term is used to describe mutations of specific loci required for survival under non-lethal conditions such as environmental stress (Chandler and Claverys, 2001). The phenomenon is thought to involve either gene amplification or recombination dependant point mutation. In a study based upon multi-locus sequence typing (MLST), it was found that recombination played a bigger role than point mutation in clonal diversification of five bacterial species including *S. pneumoniae* (Spratt *et al.*, 2001). Havarstein *et al.*, (1997) found that a novel receptor exists in some strains of streptococci including *S. anginosus* called strain specific peptide pheromone receptor or complement-stimulating peptides (CSP's). Each CSP represents a separate pheromone type (pherotype), which is recognised by the signalling domain of the downstream histadine kinase Com D. Thus all bacteria that are induced to competence by a particular CSP belong to the same pherotype. Any recombination events will lead to switches in pherotype for the strains involved. By looking at strains with related pherotypes, the researchers demonstrated that horizontal gene transfer and point mutation often occurs between naturally competent strains of streptococci under certain growth conditions and suggests a novel

mechanism for the adaptation of naturally competent streptococci to new environmental situations.

Future work could include analysis of the fingerprints of strains 8 and 56 to analyse their respective level of genomic diversity to see if a link exists between diversity and virulence factor transcription. The question could reasonably be asked does strain 8 show a high genomic diversity as well as (possibly) coding for probable virulence determinants at neutral pH? It would be interesting to create primers based upon the complete sequences of the PFL, *hlyX* and the glucose epimerase gene and perform a quantitative PCR on cDNA taken from representatives of all three SMG species grown under defined environmental conditions. If this is successful, primers could be created based upon the sequences of other known streptococcal virulence determinants and quantitative PCR performed on SMG to test for upregulated mRNA products. This would be a quicker method than differential display for searches involving known gene products presuming that the QPCR is reproducible.

The next stage in the process would be to perform this study on a larger scale involving both clinical isolates and commensal isolates over a longer time period to that used in this study. It would be interesting to see which origin of isolate displays the most instability (diversity) that is for example clinical isolates or those of commensal origin. Following on from this would be the discovery of the mechanism of instability for example (recombination, or point mutation), using the pherotype CSP method. Of further interest would be to test the 'adaptive mutation' theory by growing identical strains under different conditions to discover what growth situations, if any, more readily induce genome change.

It would also be interesting to bring the two strands of this work together, to do this, any strains showing diversity could be subjected to differing pH levels. DD RT-PCR could be performed on the cDNA and differential band sequences analysed to test whether more genomically diverse strains exhibit

a higher tendency to upregulate putative virulence determinants when compared to less diverse strains.

In closing, the work presented has demonstrated that the SMG are genetically diverse and colonise the oral cavity in a complex manner which tends not to be stable. Furthermore, evidence in some strains of a tendency towards intragenomic instability over prolonged subculture has been documented, A method for analysis of preferential gene expression has been developed which could be to identify and characterise potential virulence factors. Further work can be performed to build on this research and answer more of the questions regarding SMG virulence and if there is a link between diversity and pathogenic capability.

BIBLIOGRAPHY.

Absolom D. R. 1988. The role of surface hydrophobicity in infection: bacterial adhesion and phagocyte ingestion. *Can. J. Microbiol.* **34**: (3); 287-298.

Ahmet Z., M. Warren., and E. T. Houang. 1995. Species identification of members of the "*Streptococcus milleri* " group isolated from the vagina by ID 32 Strep system and differential phenotypic characteristics. *J. Clin. Microbiol.* **33**: 1592-1595.

Ajdic D., W. M. McShan., R. E. McLaughlin., G. Savic., J. Chang., M. B. Carson., C. Primeaux., R. Tian., S. Kenton., H. Jia., S. Lin., Y Qian., S. Li., H. Zhu., F. Najar., H. Lai., J. White., B. A. Roe., and J. J. Ferretti. 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *PNAS.* **99**: (22); 14434-14439.

Alam S., S. R. Brailsford., R. A. Whiley., and D. Beighton. 1999. PCR-based methods for genotyping viridans group streptococci. *J. Clin. Microbiol.* **37**: 2772-2776.

Alm R. A., L. S. Moir., D. T. King., B. L. Brown., E. D. Doig., P. C. Smith., D. R. Noonan., B. Guild., B.C. Dejonge. 1999. Genome sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature*. **397**: 176-180.

Alouf J. E., and M. Raynaud. 1968. Action of streptolysin O in cellular membranes II – kinetics of erythrocyte lysis. *Ann. Inst. Pasteur (Paris).* **115**: (1); 97-121.

Aluyi H. S., and D. B. Drucker. 1983. Trimethylsilyl-sugar profiles of "*Streptococcus milleri*" and *Streptococcus mitis*. *J. Appl. Bacteriol.* **54**: 391-397.

An G., G. Luo., R. W. Veltri., and S. M. O Hara. 1996. Sensitive, non-radioactive differential display method using chemiluminescent detection. *BioTechniques*. **20**: (3); 342-348.

Anderson B. R., and D. Van Epps. 1972. Suppression of chemotactic activity of human neutrophils by streptolysin O. *J. Infect.* **25**: 353-359.

Anderson B. R., and R. Cone. 1974. Inhibitors of human lymphocyte blast transformation by streptolysin O. *J. Lab. Clin. Med.* **84**: (2); 241-248.

Anderson B. R., and J. L. Duncan. 1980. Activation of human neutrophil metabolism by streptolysin O. *J. infect. Dis.* **141**: (5); 680-685.

Andrewes F. W., and T. J. Horder. 1906. A study of streptococci pathogenic for man. *Lancet*. **2**: 703-713.

Aracil B., J. L. Gomez-Garces., and J. I. Alos. 1999. A study of susceptibility of 100 clinical isolates belonging to the "*Streptococcus milleri*" group to 16 cephalosporins. *J. Antimicrob. Chemother*. **43**: (3); 399-402.

Arala – Chaves M. P., T. B. Higerd., M. T. Porto., J. Munoz., J. M. Goust., H. H. Fudenberg., and E. C. B. Leadholt. 1979. Evidence for the synthesis and release of strongly immunosuppressive substances by *Streptococcus intermedius*. *J. Clin. Microbiol.* **64**: 871-883.

Arala – Chaves M. P., M. T. Porto., P. Arnaud., M. J. Saraiva., H. Geada., C. C. Patrick., H. H. Fudenberg. 1981. Fractionation and characterisation of the

immunosuppressive substance in crude extracellular products released by *Streptococcus intermedius*. *J. Clin. Microbiol.* **68**: 294-302.

Archer G. L., D. R. Dietrick., and J. L. Johnston. 1985. Epidemiology of transmissible gentamycin resistance among coagulase negative staphylococci in a cardiac surgery unit. *J. infect. Dis.* **151**: (2); 243-249.

Asanuma N., and T. Hino. 2000. Effects of pH and energy supply on activity and amount of Pyruvate formate lyase in *Streptococcus bovis*. *Appl. Environ. Microbiol.* **66**: (6); 3773-3777.

Asanuma N., and T. Hino. 2001. Molecular characterization and expression of Pyruvate formate lyase-activating enzyme in a ruminal bacterium, *Streptococcus bovis*. *Appl. Environ. Microbiol.* **68**: (7); 3352-3357.

Asikainen S., and S. Alaluusua. 1993. Bacteriology of dental infections. *European Heart Journal*. **14**: 43-50.

Atwood K. C., L. K. Schneider., and F. J. Ryan. 1951. Periodic selection in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **37**: 146-155.

Avigad L. S., and A. W. Bernheimer. 1978. Inhibition of haemolysis by zinc and its reversal by L – Histadine. *Infect. Immun*. **19**: (3); 1101-1103.

Badger J. L., and K. S. Kim. 1997. Environmental growth conditions influence the ability of *Escherichia coli* K1 to invade brain microvascular endothelial cells and confer serum resistance. *Infect. Immun*. **66**: (12); 5692-5697.

Badger J. L., C. A. Wass., and K. S. Kim. 2000. Identification of *Escherichia coli* K1 genes contributing to human brain microvascular endothelial cell invasion by differential fluorescence induction. *Mol. Microbiol.* **36**: (1); 174-182.

Balakrishnan M., R. S. Simmonds., and J. R. Tagg. 2000. Dental caries is a preventable infectious disease. *Australian Dental Journal.* **45**: (4); 235-245.

Ballieux R. E. 1991. Impact of mental stress on the immune response. *J. Clin. Periodontol.* **18**: 427-430.

Ball L. C., and M. T. Parker. 1979. The cultural and biochemical characters of "*Streptococcus milleri*" strains isolated from human sources. *J. Hyg.* **82**: 63-78.

Bannerman T. L., G. A. Hancock., F. C. Tenover., and J. M. Miller. 1995. Pulsed field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *J. Clin. Microbiol.* **33**: (3); 551-553.

Bantar, C., L. Fernandez Cariega., S. Relloso., A. Lanza., H. Biarichini., and J. Smayevsky. 1996. Species belonging to the "*Streptococcus milleri*" group: antimicrobial susceptibility and comparative prevalence in significant clinical specimens. *J. Clin. Microbiol.* **34**: (8); 2020-2021.

Baran J., W. M. Abdo., K. W. Merritt., and R. Khatib. 1998. Tricuspid valve endocarditis due to a moderately susceptible *Streptococcus constellatus*: persistent bacteraemia and fatal outcome despite penicillin plus gentamycin therapy. *Scand. J. Infect. Dis.* **30**: 420-421.

Bartie K. L., M. J. Wilson., D. W. Williams., and M. A. O. Lewis. 2000. Macrorestriction fingerprinting of the "*Streptococcus milleri*" group bacteria by pulsed field gel electrophoresis. *J. Clin. Microbiol.* **38**: (6); 2141-2149.

Bartilson M., A. Marra., J. Christine., J. S. Asundi., W. P. Schneider., and A. E. Hromocky. 2001. Differential fluorescence induction reveals *Streptococcus pneumoniae* loci regulated by competence stimulatory peptide. *Mol. Microbiol.* **39**: (1); 126-135.

Bashyam M. D., and A. K. Tyagi. 1994. An efficient and high-yielding method for isolation of RNA from mycobacteria. *BioTechniques.* **17**: (5); 834-837.

Beighton D., R. A. Whiley., and K. A. Homer. 1990. Transferrin binding by *Streptococcus oralis* and other oral streptococci. *Microb. Ecol. Health. Dis.* **3**: 145-150.

Beighton D., J. M. Hardie., and R. A. Whiley. 1991. A scheme for the identification of viridans streptococci. *J. Med. Microbiol.* **35**: (6); 367-372.

Benito E. P., T. Prins., and J. A. Van Kan. 1996. Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Mol. Biol.* **32**: (5); 947-953.

Bergman S., M. Selig., M. D. Collins., J. A. E. Farrow., E. J. Baron., G. R. Dickerson., and K. L. Ruoff. 1995. "*Streptococcus milleri*" strains displaying a gliding type of motility. *Int. J. Syst. Bacteriol.* **45**: 235-239.

Bert F., M. Bariou-Lancelin., and N. Lambert-Zechovsky. 1998. Clinical significance of bacteraemia involving the "*Streptococcus milleri*" group: 51 cases and review. *Clin. Infect. Dis.* **27**: (2); 385-387.

Bidet P., V. Lalande., B. Salauze., B. Burghoffer., V. Avesari., M. Delmee., A. Rossier., P. Barbut., and J. C. Petit. 2000. Comparison of PCR ribotyping, AP-PCR and pulsed field gel electrophoresis for typing *Clostridium difficile*. *J. Clin. Microbiol.* **38**: (7); 2484-2487.

Blair J. E., and M. Carr. 1960. The techniques and interpretation of phage typing of staphylococci. *J. Lab. Clin. Med.* **55**: 650-662.

Bleiweis A. S., P. C. F. Oysten., and L. J. Brady. 1992. Molecular immunological and functional characterization of the major surface adhesin of *Streptococcus mutans*. p. 229-241. *In* J. E. Ciardi., J. E. McGeerland. (ed.), Genetically engineered vaccines, Plenum press, New York.

Bolano A., S. Stinchi., R. Preziosi., F. Bistoni., M. Allegrucci., F. Baldelli., A. Martini., and G. Cardinali. 2001. Rapid methods to extract DNA and RNA from *Cryptococcus neoformans*. *FEMS Yeast Res.* **1**: (3); 221-224.

Bolotin A., P. Wincker., S. Mauger., O. Jaillon., K. Malarme., J. Weissenbach., S. D. Ehrlich., and A. Sorokin. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* spp. *Lactis* IL1403. *Genome Research*. **11**: 731-753.

Bonass W. A., P. D. Marsh., R. S. Percival., J. Aduse-Opaku., S. A. Hanley., D. A. Devine., and M. A. Curtis. 2000. Identification of RagAB as a temperature – regulated operon of *Porphyromonas gingivalis* W50 using differential display of randomly primed RNA. *Infect. Immun.* **68**: (7); 4012-4017.

Bowden G. H. 1997. Does assessment of microbial composition of plaque/saliva allow for diagnosis of disease activity of individuals? *Community. Dent. Oral. Epidemiol.* **25**: (1); 76-81.

Bowden G. H., and I. R. Hamilton. 1987. Environmental pH as a factor in the competition between strains of the oral streptococci *Streptococcus mutans*, *Streptococcus sanguis*, and "*S. mitior*" growing in continuous culture. *Can. J. Microbiol.* **33**: 824-827.

Boyd D. A., D. G. Cvitkovitch., A. S. Bleiweis., M. Y. Kiriukhin., D. V. Debabov., F. C. Neuhaus., and I. R. Hamilton. 2000. Defects in D-Alanyl-Lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *J. Bacteriol.* **182**: (21); 6055-6065.

Brain P. F., and N. W. Nowell. 1970. The effect of differential grouping on endocrine function of mature male albino mice. *Physiol. Behav.* **5**: 907-910.

Brook I., and R. I. Walker. 1985. The role of encapsulation in the pathogenesis of anaerobic Gram-positive cocci. *Can. J. Microbiol.* **31**: 176-180.

Buckley D. A., A. Murphy., P. Dervan., R. Hone., T. O'Dwyer., and S. O'Loughlin. 1998. Persistent infection of the chin with an unusual skin pathogen ("*Streptococcus milleri*"): a sign of intraoral carcinoma. *Clinical and experimental dermatology*. **23**: 35-37.

Cabellos C., P. F. Viladrich., J. Corredoira., R. Verdaguer., J. Ariza., and F. Gudiol. 1999. Streptococcal meningitis in adult patients: current epidemiology and clinical spectrum. *Clin. Infect. Dis.* **30**: (1); 238-239.

Carman R. J., R. L. Van Tassel., and T. D. Wilkins. 1993. The normal intestinal microflora: ecology, variability and stability. *Vet. Hum. Toxicol.* **35**: Suppl 1; 11-14.

Carlsson J. 1967. A medium for isolation of *Streptococcus mutans*. *Arch. Oral. Biol.* **12**: 1657-1658.

Cartwright C. P., F. Stock., S. E. Beekmann., E. C. Williams., and V. J. Gill. 1995. PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile*. *J. Clin. Microbiol.* **33**: 184-187.

Casariego E., A. Rodriguez., J. C. Carredoira., P. Alonso., A. Coira., H. Bal., M. J. Lopez., and J. Varela. 1996. Prospective study of "*Streptococcus milleri*" bacteraemia. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**: 194-200.

Chakerbortty A., S. Das., S. Majumder., K. Mukhopadhyay., S. Raychoudhury., and K. Chadudhuri. 2000. Use of RNA arbitrarily primed PCR fingerprinting to identify *Vibrio cholerae*. *Infect. Immun.* **68**: (7); 3878-3887.

Chandler M., and J. P. Claverys. 2001. Genome diversity: sources and forces. *Curr. Opin. Microbiol.* **4**: 547-549.

Charland N., J. Harel., M. Kobisch., S. Lacasse., and M. Gottschalk. 1998. *Streptococcus suis* serotype 2 mutants deficient in capsular expression. *Microbiology*. **144**: 325-332.

Cheung A. L., K. J. Eberhardt., and V. A. Fischetti. 1994. A method to isolate RNA from Gram-positive bacteria and mycobacteria. *Analyt. Biochem.* **222**: 511-514.

Chew T. A., and J. M. Smith. 1992. Detection of diacetyl (caramel odor) in presumptive identification of the "*Streptococcus milleri*" group. *J. Clin. Microbiol.* **30**: (11); 3028-9.

Chia J. S., C. Y. Yeh., and J. Y. Chen. 2000. Identification of a fibronectin binding protein from *Streptococcus mutans*. *Infect. Immun.* **68**: (4); 1864-70.

Chiang S. L., J. J. Mekalanos., and D. W. Holden. 1999. *In-vivo* genetic analysis of bacterial virulence. *Annu. Rev. Microbiol.* **53**: 129-154.

Clarridge J. E. 3rd., C. Osting., M. Jalali., J. Osborne., and M. Waddington. 1999. Genotypic and phenotypic characterization of "*Streptococcus milleri*" group isolates from a veteran's administration hospital population. *J. Clin. Microbiol.* **37**: (11); 3681-3687.

Clermont D., and T. Horaud. 1990. Identification of chromosomal antibiotic resistance genes in *Streptococcus anginosus* ("*S. milleri*"). *Antimicrob. Agents. Chemother.* **34**: 1685-1690.

Clermont D., and T. Horaud. 1994. Genetic and molecular studies of a composite chromosomal element (Tn*3705*) containing a Tn*916*-modified structure (Tn*3704*) in *Streptococcus anginosus* F22. *Plasmid.* **31**: 40-48.

Coffey T. J., M. C. Enright., M. Daniels., R. Morona., W. Hryniewicz., J. C. Paton., and B. G. Spratt. 1998. Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. *Mol. Microbiol.* **27**: (1); 73-83.

Coligan J. E., T. J. Kindt., and R. M. Krause. 1978. Structure of the streptococcal groups A, A-variant and C carbohydrates. *Immunohistochemistry*. **15**: 755-760.

Colman G., and R. E. O. Williams. 1972. Taxonomy of some human viridans streptococci. p. 282-299. *In* L. W. Wannamaker., and J. M. Matsen. (ed.), Streptococci and streptococcal diseases. Academic Press, Inc., New York.

Conrads G., S. E. Gharbia., K. Gulabivala., F. Lampert., and H. N. Shah. 1997. The use of a 16S rDNA directed PCR for the detection of endodontopathogenic bacteria. *J. Endodont.* **23**: 433-438.

Conradt H., M. Hohmann-Berger., H. P. Hohmann., H. P. Blaschkowski., and J. Knappe. 1984. Pyruvate formate-lyase (inactive form) and pyruvate formate lyase activating enzyme of *Escherichia coli*: isolation and structural properties. *Arch. Biochem. Biophys.* **228**: (1); 133-42.

Cookson B., H. Talsania., S. Chinn., and I. Phillips. 1989. A qualitative and quantitative study of the cellular fatty acids of "*Streptococcus milleri*" with capillary gas chromatography. *J. Gen. Microbiol.* **135**: 831-838.

Corfield T. 1992. Bacterial sialidase – roles in pathogenicity and nutrition. *Glycobiology.* **2**: (6); 509-521.

Corredoira J., E. Casariego., C. Moreno. L., Villanueva Lopez., J. Varela., A. Rodriguez., P. Alonso., and A. Coira. 1998. Prospective study of "*Streptococcus milleri* " hepatic abscess. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**: 556-60.

Cotter P. A., and J. F. Miller. 2000. *In-vivo* and *ex-vivo* regulation of bacterial virulence gene expression. *Curr. Opin. Microbiol.* **1**: (1); 17-26.

Coykendall A. L., P. M. Wesbecher., and K. B. Gustafson. 1987. "*Streptococcus milleri*," *Streptococcus constellatus*, and *Streptococcus intermedius* are later synonyms of *Streptococcus anginosus*. *Int. J. Syst. Bacteriol.* **37**: 222-228.

Daly C., D. Mitchell., D. Grossberg., J. Highfield., and D. Stewart. 1997. Bacteraemia caused by periodontal probing. *Australian Dental Journal.* **42**: (2); 77-80.

Dawes C. 1987. Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. *J. Dent. Res.* **66**: 648-653.

Debelian G. J., I. Olsen., and L. Tronstad. 1996. Electrophoresis of whole-cell soluble proteins of microorganisms isolated from bacteraemia in endodontic therapy. *Eur. J. Oral. Sci.* **104**: 540-546.

Diehn M., and D. A. Relman. 2001. Comparing functional genomic datasets: lessons from DNA microarray analyses of host-pathogen interactions. *Curr. Opin. Microbiol.* **4**: 95-101.

Dobrindt U., and J. Hacker. 2001. Whole genome plasticity in pathogenic bacteria. *Curr. Opin. Microbiol.* **4**: 550-557.

Doit C., F. Grimont., R. A. Whiley., B. Regnault., P. A. D. Grimont., J. M. Hardie., and A. Bouvet. 1994. Ribotypes of the "*Streptococcus milleri* group" allow discrimination between strains of *Streptococcus constellatus*, *Streptococcus intermedius* and *Streptococcus anginosus*. P. 531-532. *In*. A. Totolian (ed.). Pathogenic streptococci present and future. Lancer Publication, St Petersburg, Russia.

Drucker D. B., and R. M. Green. 1978. The relative cariogenicities of "*Streptococcus milleri*" and other viridans group streptococci in gnotobiotic hooded rats. *Arch. Oral. Biol.* **23**: 183-187.

Drucker D. B., and S. M. Lee. 1983. Possible heterogeneity of "*Streptococcus milleri*" determined by DNA mol % (guanine plus cytosine) measurement and physiological characterization. *Microbios.* **38**: 151-157.

Du L. D., and P. E. Kolenbrander. 2000. Identification of a saliva-regulated gene of *Streptococcus gordonii* DL1 by differential display using random arbitrarily primed PCR. *Infect. Immun.* **68**: (4); 4834-4837.

Edmiston C. E. Jr., A. P. Walker., C. J. Krepel., C. M. Gohr., G. R. Seabrook., and C. T. Frantzides. 1991. "*Streptococcus milleri*" group (*Streptococcus anginosus*): recovery from intra-abdominal and soft tissue sites. *Ann. Clin. Lab. Sci.* **21**: (1); 56-61.

Edwardsson S., M. Bing., B. Axtelius., B. Lindberg., B. Soderfeldt., and R. Attstrom. 1999. The microbiota of periodontal pockets with different depths in therapy-resistant periodontitis. *J. Clin. Microbiol.* **26**: 143-152.

Elias N., M. Heller., and I. Ginsburg. 1966. Binding of streptolysin S to red blood cell ghosts and ghost lipids. *Isr. J. Med. Sci.* **2**: (3); 302-309.

Ezaki T., R. Facklam., N. Takeuchi., and E. Yabuuchi. 1986. Genetic relatedness between the type strain of *Streptococcus anginosus* and minute-colony-forming beta-haemolytic streptococci carrying different Lancefield grouping antigens. *Int. J. Syst. Bacteriol.* **36**: 345-347.

Facklam R. R. 1977. Physiological differentiation of viridans streptococci. *J. Clin. Microbiol.* **5**: 184-201.

Facklam R. R. 1984. The major differences in the American and British streptococcus taxonomy schemes with special reference to "*Streptococcus milleri*". *Eur. J. of Clin. Microbiol.* **3**: 91-93.

Facklam R. R., and R. B. Carey. 1985. Streptococci and aerococci. p. 154-175. *In* E. M. Lenette., A. Balows., W. J. Jr. Hausler., and H. J. Shadomy. (ed.). Manual of clinical microbiology. 4th edition, American society for microbiology. Washington DC.

Feil E. J., E. C. Holmes., D. E. Bessen., M. S. Chan., N. P. J. Day., M. C. Enright., R. Goldstein., D. W. Hood., A. Kalia., C. E. Moore., J. Zhou., and B. G. Spratt. 2001. Recombination within natural populations of pathogenic bacteria: Short-term empirical estimates and long-term phylogenetic consequences. *PNAS.* **98**: (1); 182-187.

Ferretti, J. J., W. M. McShan., D. Ajdic., D. J. Savic., G. Savic., K. Lyon., C. Primeaux., S. Sezate., A. N. Suvorov., S. Kenton., H. S. Lai., S. P. Lin., Y. Qian., H. G. Jia., F. Z. Najar., Q. Ren., H. Zhu., L. Song., J. White., X. Yuan., S. W. Clifton., B. A. Roe., and R. McLaughlin. 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes. PNAS.* **98**: (8); 4658-4663.

Fisher L. E., and R. R. B. Russell. 1993. The isolation and characterisation of Milleri group streptococci from dental periapical abscesses. *J. Dent. Res.* **72**: 1191-1193.

Fislage R., M. Berceanu., Y. Humboldt., M. Wendt., and H. Oberender. 1997. Primer design for a prokaryotic differential display RT-PCR. *Nucl. Acids. Res.* **25**: (9); 1830-1835.

Fitzgerald R. J., and P. H. Keyes. 1960. Demonstration of the etiologic role of streptococci in experimental caries in the hamster. *J. Am. Dent. Assoc.* **61**: 9-19.

Flynn C. E., and K. L. Ruoff. 1995. Identification of "*Streptococcus milleri*" group isolates to the species level with a commercially available rapid test system. *J. Clin. Microbiol.* **33**: (10); 2704-2706.

Fox K., J. Turner., and A. Fox. 1993. Role of beta-haemolytic group C streptococci in pharyngitis: incidence and biochemical characteristics of *Streptococcus equisimilis* and *Streptococcus anginosus* in patients and healthy controls. *J. Clin. Microbiol.* **31**: 804-807.

Frandsen E. V. G., V. Pedrazzoli., and M. Kilian. 1991. Ecology of viridans streptococci in the oral cavity and pharynx. *Oral. Microbiol. Immunol.* **6**: 129-133.

French G. L., H. Talsania., J. R. H. Charlton., and I. Phillips. 1989. A physiological classification of viridans streptococci by use of the API-20 STREP system. *J. Med. Microbiol.* **28**: 275-286.

Frisken K. W., T. Higgins., and J. M. Palmer. 1990. The incidence of periodontopathic microorganisms in young children. *Oral. Microbiol. Immunol.* **5**: 43-45.

Fukushima K., T. Ikeda., and H. K. Kuramitsu. 1992. Expression of *Streptococcus mutans gtf* genes in *Streptococcus milleri*. *Infect. Immun.* **60**: 2815-2822.

Genco R. J., J. J. Zambon., and L. A. Christersson. 1988. The origin of periodontal infections. *Adv. Dent. Res.* **2**: 245-259.

Georgnaras I., N. F. Kanene., A. Von Holy., and J. W. Hastings. 1999. AFLP fingerprinting of pseudomonas strains from a poultry processing plant. *Appl. Environ. Microbiol.* **65**: (9); 2828-3833.

Gibbons R. J., D. I. Hay., W. C. Childs., G. Davis. 1990. Role of cryptic receptors (cryptitopes) in bacterial adhesion to oral surfaces. *Arch. Oral. Biol.* **35**: Suppl 1075-1145.

Gilbert A. D., and J. A. Sofaer. 1989. Neutrophil function, genotype and periodontal bone loss in the mouse. *J. Periodontal. Res.* **24**: 412-414.

Gillings M., and M. Holley. 1997. Repetitive element PCR fingerprinting (Rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers. *Lett. Appl. Microbiol.* **25**: 17-21.

Ginsburg I., and T. N. Harris. 1963. Oxygen stable haemolysins of group A streptococci. *J. Exp. Med.* **118**: 919-934.

Ginsburg I. 1999. Is streptolysin S of group A streptococci a virulence factor? *APMIS.* **107**: 1051-1059.

Giraud M. F., and J. H. Naismith. 2000. The rhamnose pathway. *Curr. Opin. Struct. Biol.* **10**: (6); 687-696.

Glaser P., C. Rusnoik., C. Buchreiser., F. Chevailier., L. Frangeul., T. Msadek., M. Zouine., E. Couve., L. Lalioui., C. Poyart., P. Trieu-Cuot., and F. Kunst. 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol.* **45**: (6); 1499-1513.

Gomez-Garces J. L., J. I. Alos., and R. Cogollos. 1994. Bacteriologic characteristics and antimicrobial susceptibility of 70 clinically significant isolates of "*Streptococcus milleri*" group. *Diag. Microbiol. Infect. Dis.* **19**: (2); 69-73.

Gossling J. 1988. Occurrence and pathogenicity of the "*Streptococcus milleri*" group. *Rev. Infect. Dis.* **10**: (2); 257-285.

Goto T., H. Nagamune., A. Miyazaki., Y. Kawamura., O. Ohnishi., K. Hattori., K. Ohkura., K. Miyamoto., S. Akimoto., T. Ezaki., K. Hirota., Y. Miyake., T. Maeda., and H. Kourai. 2002. Rapid identification of *Streptococcus intermedius* by PCR with the *ily* gene as a species marker gene. *J. Med. Microbiol.* **51**: 178-186.

Green J., and M. L. Baldwin. 1997. The molecular basis for the differential regulation of the *hlyE*-encoded haemolysin of *Escherichia coli* by FNR and *HlyX* lies in the improved activating region 1 contact of *HlyX*. *Microbiology.* **143**: 3785-3793.

Greenstein G., and I. Lamster. 1997. Bacterial transmission in periodontal diseases: a critical review. *J. Periodontol.* **68**: 421-431.

Griesen K., M. Loeffelholtz., A. Purohit., and P. Leong. 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria including bacteria found in cerebrospinal fluid. *J. Clin. Microbiol.* **32**: 335-351.

Grothues D., U. Koopmann., T. Van der Hardt., and B. Tummler. 1988. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonisation of cystic fibrosis siblings with closely related strains. *J. Clin. Microbiol.* **26**: 1977-1979.

Grundmann H. J., K. J. Towner., L. Dijkshoorn., P. Gerner-Smidt., M. Maher., H. Seifert., and M. Vaneechoutte. 1997. Multicenter study using standardised protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. *J. Clin. Microbiol.* **35**: 3071-3077.

Gupta S., and M. C. J. Maiden. 2001. Exploring the evolution of diversity in pathogen populations. *TRENDS in Microbiology*. **9**: (4); 181-185

Guthof O. 1956. Uber pathogene, vergrunende "streptokokken": Streptokokkenbefunde bei dentog enen abszessen und infiltraten im bbereich der mundhohle. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankhe.* **166**: 553-564.

Halbert S. P., R. Birchen., and E. Dahle. 1961. Cardiac effects of streptolysin O in rabbits. *Nature*. **189**: (4760); 232-233.

Halbert S. P., R. Bircher., and E. Dahle. 1970. The analysis of streptococcal infections: cardiotoxicity of streptolysin O for rabbit's *in-vivo*. *J. Exp. Med.* **113**: 759-185.

Hall L. M. 1994. Are point mutations or DNA rearrangements responsible for the restriction fragment length polymorphisms that are used to type bacteria? *Microbiology*. **140**: 197-204.

Hall L. M. C., and B. Duke. 1998. Conservation of restriction sites in isolates of *Streptococcus pneumoniae* with diverse restriction fragment patterns. *J. Clin. Microbiol.* **36**: 1805-1807.

Hamburger M., and H. M. Lemon. 1953. The failure of antistreptolysin to protect against streptococcal infection. *J. Lab. Clin. Med.* **42**: (1); 140-144.

Hamilton I. R., A. S. McKee., and G. H. Bowden. 1989. Growth and metabolic properties of *Bacteroides intermedia* in anaerobic continuous culture. *Oral Microbiol. Immunol.* **4**: 89-97.

Handfield M., and R. C. Levesque. 1999. Strategies for isolation of *in-vivo* expressed genes from bacteria. *FEMS Microbiology reviews*. **23**: 69-91.

Handley P. S., P. L. Carter., J. E. Wyatt., and L. M. Hesketh. 1985. Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to coaggregate with other oral genera. *Infect. Immun*. **47**: 212-227.

Hannula J., M. Saarela., H. Jousimies-Somer., A. Takala., R. Syrjaneri., E. Kononen., and S. Asikaineri. 1999. Age-related acquisition of oral and nasopharyngeal yeast species and stability of colonization in young children. *Oral. Microbiol. Immunol*. **14**: (3); 176-82.

Harrington C. S., F. M. Thomson-Carter., and P. E. Carter. 1997. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: Implications for the flagellin gene typing scheme. *J. Clin. Microbiol*. **35**: (9); 2386-2392.

Havarstein L. S., R. Hakenbeck., and P. Gaustad. 1997. Natural competence in the genus streptococcus: Evidence that streptococci can change pherotype by interspecies recombinational exchanges. *J. Bacteriol*. **179**: (21); 6589-6592.

Henskens Y. M., U. Van der Velden., E. C. Veerman., and A. V. Nieuw Amerongen. 1993. Protein, albumin, and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. *J. Periodontal Res*. **28**: 43-48.

Hermans P. W. M., M. Sluijter., T. Hoogenboezem., H. Heersma., A. Van Belkum., and R. De Groot. 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. *J. Clin. Microbiol*. **30**: (6); 1606-1612.

Herzberg M. C., K. Gong., G. D. MacFarlane., P. R. Erickson., A. H. Soberay., P. H. Kresbach., G. Manjula., K. Schilling., and W. H. Bowen. 1990. Phenotypic characterization of *Streptococcus sanguis* virulence factors associated with bacterial endocarditis. *Infect. Immun.* **58**: (2); 515-522.

Hewitt L. F., and E. W. Todd. 1939. The effect of cholesterol and of sera contaminated with bacteria on the haemolysis produced by haemolytic streptococci. *J. Path. Bacteriol.* **49**: 49-51.

Higerd T. B., D. H. Vesole., and J. Goust. 1978. Inhibitory effects of extracellular products from oral bacteria on human fibroblasts and stimulated lymphocytes. *Infect. Immun.* **21**: 567-574.

Hollingshead S. K., and D. E. Briles. 2001. *Streptococcus pneumoniae*: new tools for an old pathogen. *Curr. Opin. Microbiol.* **4**: 71-77.

Homer K. A., R. A. Whiley., and D. Beighton. 1990. Proteolytic activity of oral streptococci. *FEMS. Microbiol. Letts.* **67**: 257-260.

Homer K. A., L. Denbow., R. A. Whiley., and D. Beighton. 1993. Chondroitin sulphate depolymerase and hyaluronidase activities of viridans streptococci determined by a sensitive spectrophotometric assay. *J. Clin. Microbiol.* **31**: 1648-1651.

Homer K., H. Shain., and D. Beighton. 1997. The role of hyaluronidase in growth of *Streptococcus intermedius* on hyaluronate. *Adv. In. Exp. Med. and Biol.* **418**: 681-683.

Hoskins J., W. E. Alborn Jr., J. Arnold., L. C. Blaszczak., S. Burgett., B. S. DeHoff., S. T. Estrem., L. Fritz., D. J. Fu., W. Fuller., C. Geringer., R. Gilmour., J. S. Glass., H. Khoja., A. R. Kraft., R. E. Lagace., D. J. LeBlanc., L. N. Lee., E. J. Lefkowitz., J. Lu., P. Matsushima., S. M. McAhren., M.

McHenney., K. McLeaster., C. W. Mundy., T. I. Nicas., F. H. Norris., M. O'Gara., R. B. Peery., G. T. Robertson., P. Rockey., P. Sun., M. E. Winkler., Y. Yang., M. Young-Bellido., G. Zhao., C. A. Zook., R. H. Baltz., S. R. Jaskunas., P. R. Rosteck Jr., P. L. Skatrud., and J. L. Glass. 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J. Bacteriol.* **183**: (19); 5709-5717.

Hrniewicz W., and J. Pryjma. 1977. Effects of streptolysin S on human and mouse T and B-lymphocytes. *Infect. Immunol.* **16**: (3); 730-733.

Hsu C. C., B. C. Yang., M. H. Wu., and K. E. Huang. 1997. Enhanced interleukin-4 expression in patients with endometriosis. *Fertility and Sterility.* **67**:(6);1059-1064.

Hurme R., and M. Rhen. 1998. Temperature sensing in bacterial gene regulation - what it all boils down to. *Mol. Microbiol.* **30**: (1); 1-6.

Hayashi K. 1991. PCR-SSCP: A simple and sensitive method for detection of mutation in the genomic DNA. PCR methods and applications. *PCR. Methods. Appl.* **1**: (1); 34-38.

Jacobs A. E., W. A. Horton., and D. B. Drucker. 1989. Genetic transformation in some cariogenic "*Streptococcus milleri*". *Microbios.* **60**: 167-175.

Jacobs J. A., H. G. Pietersen., E. E. Stobberingh., and P. B. Soeters. 1994. Bacteraemia involving the "*Streptococcus milleri*" group: analysis of 19 cases. *Clin. Infect. Dis.* **19**: (4); 704-713.

Jacobs J. A., H. G. Pietersen., E. E. Stobberingh., and P. B. Soeters. 1995. *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. Clinical relevance, haemolytic and serologic characteristics. *Am. J. Clin. Pathol.* **104**: 547-553.

Jacobs J. A., and E. E. Stobberingh. 1995. Hydrolytic enzymes of *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius* in relation to infection. *Eur. J. Clin. Microbiol. Infect. Dis.* **14**: 818-820.

Jacobs J. A., C. S. Schot., A. E. Bunschoten., and L. M. Schouls. 1996. Rapid species identification of "*Streptococcus milleri*" strains by line blot hybridisation: identification of a distinct 16S rRNA population closely related to *Streptococcus constellatus*. *J. Clin. Microbiol.* **34**: 1717-1721.

Jacobs J. A., and E. E. Stobberingh. 1996. *In-vitro* antimicrobial susceptibility of the "*Streptococcus milleri*" group (*Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*). *J. Antimicrob. Chemother.* **37**: (2); 371-375.

Jacobs J. A. 1997. The "*Streptococcus milleri*" group: *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. *Rev. Med. Microbiol.* **8**: (2); 73-80.

Jacobs J. A., C.S. Schot., and L. M. Schouls. 2000. The *Streptococcus anginosus* species comprises five 16S rRNA ribogroups with different phenotypic characteristics and clinical relevance. *Int. J. Syst. Evol. Microbiol.* **50**: (3); 1073-1079.

Jacques N. A. 1994. The glucosyltransferases of *Streptococcus salivarius*. *Australian Dental Journal.* **39**: (2); 111-114.

Janssen P., R. Coopman., G. Huys., J. Sings., M. Bleeker., P. Vos., M. Zabaeu., and K. Kersters. 1996. Evaluation of the DNA fingerprinting method

AFLP as a new tool in bacterial taxonomy. *Microbiology*. **142:** (7); 1881-1893.

Jenkinson H. F. and R. O. Lamont. 1997. Streptococcal adhesion and colonisation. *Crit. Rev. Oral Biol. Med.* **8**: (2); 175-200.

Jenkinson H. F., and D. R. Demuth. 1997. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. *Mol. Microbiol.* **23**: (2); 183-190.

Jerng J. S., P. R. Hsueh., L. J. Teng., L. N. Lee., P. C. Yang., and K. T. Luh. 1997. Empyema thoracis and lung abscess caused by viridans streptococci. *Am. J. Respir. Crit. Care. Med.* **156**: 1508-1514.

Jiang S. C., M. Matte., G. Matte., A. Hug., and R. R. Colwell. 2000a. Genetic diversity of clinical and environmental isolates of *Vibrio cholerae* determined by AFLP fingerprinting. *Appl. Environ. Microbiol.* **66**: (1); 148-153.

Jiang S. C., V. Louis., N. Choopun., A. Sharma., A. Hug., and R. R. Colwell. 2000b. Genetic diversity of *Vibrio cholerae* in cheapspeak bay determined by AFLP fingerprinting. *Appl. Environ. Microbiol*. **66**: (1); 140-7.

Johnson J. R., and T. T. O' Bryan. 2000. Improved repetitive element PCR fingerprinting for residing pathogenic and non-pathogenic phylogenetic groups within *Escherichia coli*. *Clin. Diag. Lab. Immunol.* **7**: (2); 265-273.

Johnson J. R., and C. Clabots. 2000. Improved repetitive element PCR fingerprinting of *Salmonella enterica* with the use of extremely elevated annealing temperatures. *Clin. Diag. Lab. Immunol.* **7**: (2); 258-264.

Kallman J., J. Schollin., C. Schalen., A. Erlandsson., and E. Kihlstrom. 1998. Impaired phagocytosis and opsonisation towards group B streptococci in preterm neonates. *Arch. Dis. Child. Fetal. Neonatal. Ed.* **78**: F46-F50.

Kambal A. M. 1987. Isolation of *"Streptococcus milleri"* from clinical specimens. *J. Infect.* **14**: 217-223.

Kariuki S., C. Gilks., and J. Kimari. 1999. Analysis of *Salmonella enterica* serotype Typhimurium by phage typing, antimicrobial susceptibility and pulsed-field gel electrophoresis. *J. Med. Microbiol.* **48**: 1037-1042.

Kaufhold A., and P. Ferrieri. 1993. The microbiologic aspects, including diagnosis, of β-haemolytic streptococcal and enterococcal infections. *Lab. Diag. Infect. Dis.* **7**: 235-256.

Kato C., and H. K. Kuramitsu. 1991. Molecular basis for the association of glucosyltransferases with the cell surface of oral streptococci. *FEMS Microbiol. Letts.* **79**: 153-158.

Keay S., C. O. Zhang., B. R. Baldwin., S. C. Jacobs., and J. W. Warren. 1998. Polymerase chain reaction amplification of bacterial 16S rRNA genes in interstitial cystitis and control patient bladder biopsies. *J. Urology.* **159**: (1); 280-283.

Kerr K. G. 1994. The rap on REP - PCR-based typing systems. *Rev. Med. Microbiol.* **5**: 233-244.

Keyes P. H. 1960. The infectious and transmissible nature of experimental dental caries: findings and implications. *Arch. Oral. Biol.* **1**: 304-320.

Kilian M., 2002. Streptococcus and enterococcus. P. 174-188. *In* D. Greenwood., R. C. B. Slack., and J. F. Peutherer (ed.). Medical Microbiology. 16th edition, Churchill Livingstone, UK.

Kinger A. K., and J. S. Tyagi. 1993. Identification and cloning of genes differentially expressed in the virulent strains of *Mycobacterium tuberculosis*. *Gene.* **131**: (1); 113-117.

Koeleman J. G., J. Stoof., D. J. Biesmans., P. H. Savelkoul., and C. M. Vandenbroucke-Grauls. 1998. Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of acinetobacter genomic species and typing of *Acinetobacter baumannii*. *J. Clin. Microbiol.* **36**: 2522-2529.

Koeuth T., J. Versalovic., and J. R. Lupski. 1995. Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome. Res.* **5**: (4); 408-418.

Kononen E., S. Asikainen., and H. Jousimies-Somer. 1992. The early colonisation of Gram-negative anaerobic bacteria in edentulous infants. *Oral. Microbial. Immunol.* **7**: 8-31.

Kononen E., S. Asikainen., M. Saarela., J. Karjalainen., and H. Jousimies-Somer. 1994. The oral Gram-negative anaerobic microflora in young children: longitudinal changes from edentulous to dentate mouth. *Oral Microbiol Immunol.* **9**: (3); 136-141.

Kononen E., A. Kanervo., A. Takala., S. Asikainen., and H. Jousimies-Somer. 1999. Establishment of oral anaerobes during the first year of life. *J. Dent. Res.* **78**: (10); 1634-1639.

Kononen E., J. Wolf., J. Matto., E. V. Frandsen., K. Poulson., H. Jousimies-Somer., and S. Asikainen. 2000. The *Prevotella intermedia* group organisms in young children and their mothers as related to maternal periodontal status. *J. Periodontal. Res.* **35**: (6); 329-334.

Kononen E., H. Jousimies-Somer., A. Bryk., T. Kilp., and M. Kilian. 2002. Establishment of streptococci in the upper respiratory tract: longitudinal changes in the mouth and nasopharynx up to 2 years of age. *J. Med. Microbiol. 51*: (9); 723-30.

Kostman J. R., M. B. Alden., M. Mair., T. D. Edlind., J. J. LiPuma., and T. L. Stull. 1995. A universal approach to bacterial molecular epidemiology by polymerase chain reaction ribotyping. *J. Infect. Dis.* **171**: 204-208.

Kullen M. J., and T. R. Klaenhammer. 1999. Identification of the pH inducible, protein-translocating F_1 F_0 – ATPase (atp BEFHAGDC) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterisation. *Mol. Microbiol.* **33**: (6); 1152-1161.

Kurihara Y., T. Naito., K. Obayashi., M. Harasawa., Y. Kurihara., and K. Moriwaki. 1991. Caries susceptibility in inbred mouse strains and inheritance patterns in F1 and backcross (N2) progeny from strains with high and low caries susceptibility. *Caries Res.* **25**: 341-346.

Kuriyama T., K. Nakagawa., S. Kawashiri., E. Yamamoto., S. Nakamura., and T. Karasawa. 2000. The virulence of mixed infection with *Streptococcus constellatus* and *Fusobacterium nucleatum* in a murine orofacial infection model. *Microbes and Infection.* **2**: 1425-1430.

Kwaik Y. A., and L. L. Pederson. 1996. The use of differential display PCR to isolate and characterise a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. *Mol. Microbiol.* **21**: (3); 543-556.

Lamont R. J., S. G. Hersey., and B. Rosen. 1992. Characterization of the adherence of *Porphyromonas* (*Bacteroides*) *gingivalis* to oral streptococci. *Oral Microbiol. Immunol.* **7**: 193-197.

Landi M. S., J. W. Kreider., C. M. Lang., and L. P. Bullock. 1982. Effects of shipping on the immune function in mice. *Am. J. Vet. Res.* **9**: 1654-1657.

Lanz M. S. 1997. Are bacterial proteases important virulence factors? *J. Periodont. Res.* **32**: 126-132.

Lebrun L., M. Guibert., P. Wallet., M. M. De Maneville., and J. Pillot. 1986. Human Fc(v) receptors for differentiation in throat cultures of Group C *Streptococcus equisimilis* and Group C "*Streptococcus milleri*". *J. Clin. Microbiol.* **24**: 705-707.

Ledakis P., H. Tanimura., and T. Fojo. 1998. Limitations of differential display. *Biochem. Biophys. Res. Comm.* **251**: 653-656.

Lee C. A. 2000. New and old ways of understanding microbial pathogenesis. *Trends in Microbiology*. **8**: (2); 53-54.

Leonard B. A., and P. M. Schlievert. 1992. Immune cell lethality induced by streptococcal pyrogenic exotoxin A and endotoxin. *Infect. Immun.* **60**: (9); 3747-3755.

Levin B. R. 1981. Periodic selection, infectious gene exchange and the genetic structure of *E. coli* populations. *Genetics.* **99**: 1-23.

Lewis M. A. O., T. W. MacFarlane., and D. A. McGowan. 1986. Quantitative bacteriology of acute dento-alveolar abscesses. *J. Med. Microbiol.* **21**: 101-104.

Lewis M. A. O., T. W. MacFarlane., D. A. McGowan., and D. G. MacDonald. 1988. Assessment of the pathogenicity of bacterial species isolated from acute dentoalveolar abscesses. *J. Med. Microbiol.* **27**: 109-116.

Lewis M. A. O., S. G. Milligan., T. W. Macfarlane., and F. A. Carmichael. 1993. Phagocytosis of bacterial strains isolated from acute dentoalveolar abscesses. *J. Med. Microbiol.* **38**: 151-154.

Lewis M. A. O., C. L. Parkhurst., C. W. I. Douglas., M. V. Martin., E. G. Absi., P. A. Bishop., and S. A. Jones. 1995. Prevalence of penicillin resistant bacteria in acute suppurative oral infection. *J. Antimicrob. Chemother.* **35**: 785-791.

Li X., L. Tronstad., and I. Olsen. 1999. Brain abscesses caused by oral infection. *Endod. Dent. Traumatol.* **15**: 95-101.

Liang P., and A. B. Pardee. 1995. Recent advances in differential display. *Curr. Opin. immunol.* **7**: 274-280.

Limia M., A. Banderia., D. Portnoi., A. Ribero, and M. Arala-chaves. 1992. Protective effect of a T-cell immunosuppressive, cell mitogenic protein (F3' Ep-Si, or P90) produced by *Streptococcus intermedius*. *Infect. Immun.* **60**: (9); 3571-3578.

Limia A., M. L. Jimenez., T. Alarcon., and M. Lopez-Brea. 1999. Five-year analysis of antimicrobial susceptibility of the "*Streptococcus milleri*" group. *Eur. J. Clin. Microbiol. Infect. Dis.* **18**: (6); 440-444.

Limia A., T. Alarcon., M. L. Jimenez., and M. Lopez-Brea. 2000. Comparison of three methods for identification of *Streptococcus milleri* group isolates to species level. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**: 128-131.

Linzer R., S. Reddy., and M. J. Levine. 1985. Structural studies of the rhamnose-glucose polysaccharide antigen from *Streptococcus sobrinus* B13 and 6715-T_2. *Infect. Immun.* **50**: 583-585.

Linzer R., S. Reddy., and M. J. Levine. 1986. Immunochemical aspects of serotype carbohydrate antigens of *Streptococcus mutans.* p. 29-38. *In* S. Hamada, S. M. Michalek., H. Kiyono., L. Menaker., and J. R. McGhee (ed.). Molecular microbiology and immunology of *Streptococcus mutans.* Elsevier Science Publishers, Amsterdam, The Netherlands.

Long P. H., and E. A. Bliss. 1934. Studies upon minute haemolytic streptococci I. The cultural characteristics of minute beta haemolytic streptococci. *J. Exp. Med.* **60**: 619-631.

Lutticken R., U. Wendorff., D. Lutticken., E. A. Johnson., and L. W. Wannamaker. 1978. Studies on streptococci resembling "*Streptococcus milleri*" and on an associated surface-protein antigen. *J. Med. Microbiol.* **11**: 419-431.

Mahan M. J., J. M. Slauch., and J. J. Mekalanos. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science.* **259**: 686-688.

Maki D. G., F. S. Rhame., D. C. Mackel., and J. V. Bennet. 1977. Nationwide epidemic of septicaemia caused by contaminated intravenous products. *American journal of medicine.* **60**: 471-485.

Malathum K., K. V. Singh., G. M. Weinstock., and B. E. Murray. 1998. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J. Clin. Microbiol.* **36**: 211-215.

Malhotra K., L. Foltz., W. C. Mahoney., and P. A. Schneider. 1998. Interaction and effects of annealing temperature on primers used in differential display RT-PCR. *Nucl. Acids. Res.* **26**: (3); 854-856.

Mangan J. A., K. M. Sole., D. A. Mitchison., and P. D. Butcher. 1997. An effective method of RNA extraction from bacteria refractory to disruption, including mycobacteria. *Nucleic Acids Res.* **25**: (3); 675-676.

Marcotte H., and Lavoie M. C. 1998. Oral microbial ecology and the role of salivary Immunoglobulin A. *Microbiology and Molecular Biology Reviews.* **62**: (1); 71-109.

Marsh P. D. 1992. Microbiological aspects of the chemical control of plaque and gingivitis. *J. Dent. Res.* **71**: 1431-1438.

Marsh P. D. 1994. Microbial ecology of dental plaque and its significance in health and disease. *Adv. Dent. Res.* **8**: (2); 263-271.

Marsh P. D., and M. Martin. 2000. p. 14 – 26. *In* Oral microbiology 4th edition. Chapman and Hall, London.

Marshall R., and A. K. Kauffman. 1981. Production of deoxyribonuclease, ribonuclease, coagulase and haemolysins by anaerobic Gram-positive cocci. *J. Clin. Microbiol.* **13**: 787-788.

Maslow J. N., M. E. Mulligan., and R. D. Arbeit. 1993. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. *Clin. Infect. Dis.* **17**: 153-162.

Maslow J. N., and M. E. Mulligan. 1996. Epidemiologic typing systems. *Infect. Control. Hosp. Epidemiol.* **17**: 595-604.

Matsubara K., Y. Nishiyama., K. Katayama., G. Yamamoto., M. Sugiyama., T. Murai., and K. Baba. 2001. Change of antimicrobial susceptibility of group B streptococci over 15 years in Japan. *JAC.* **48**: 579-582.

Matto J., M. Saarela., B. von Troil-Linden., S. Alaluusua., H. Jousimies-Somer., and S. Asikainen. 1996. Similarity of salivary and subgingival *Prevotella intermedia* and *Prevotella nigrescens* isolates by arbitrarily primed polymerase chain reaction. *Oral. Microbiol. Immunol.* **11**: (6); 395-401.

Matushek M. G., M. J. M. Bonten., and M. K. Hayden. 1996. Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **34**: 2598-2600.

Maule J. 1998. Pulsed field gel electrophoresis. *Mol. Biotechnol.* **9**: 107-126.

Maxam A. M., and W. Gilbert. 1980. Sequencing end-labelled DNA with base specific chemical cleavages. *Methods. enzymol.* **65**: 499-560.

McDermid A. S., A. S. McKee., and P. D. Marsh. 1988. Effect of environmental pH on enzyme activity and growth of *Bacteroides gingivalis* W50. *Infect. Immun.* **56**: (5); 1096-1100.

McKee A. S., A. S. McDermid., A. Baskerville., A. B. Dawsett., D. C. Ellwood., and P. D. Marsh. 1986. Effect of haemin on the physiology and virulence of *Bacteroides gingivalis* W50. *Infect. Immun.* **52**: (2); 349-355.

Mejare B., and S. Edwardsson. 1975. "*Streptococcus milleri*" (Guthof): an indigenous organism of the human oral cavity. *Arch. Oral. Biol.* **20**: 757-762.

Mekalanos J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**: (1); 1-7.

Melo J. C., and M. J. Raff. 1978. Brain abscess due to *Streptococcus MG-intermedius* ("*Streptococcus milleri*"). *J. Clin. Microbiol.* **7**: 529-532.

Mendez-Alvarez S., V. Paron., I. Esteve., R. Guernero., and N. Gaju. 1995. Analysis of bacterial genomes by Pulsed field gel electrophoresis. *Microbiologia. SEM.* **11**: 323-336.

Mergenhagen S. E., J. C. Theonard., and H. W. Scherp., *et al.* 1985. Studies on synergistic infection I. Experimental infection with anaerobic streptococci. *J. Infect. Dis.* **103**: 33-34.

Metzgar D., A. Van Belkum., D. Field., R. Haubrich., and C. Wills. 1998. Random amplification of polymorphic DNA and microsatellite genotyping of pre-and post treatment isolates of *Candida* spp. from human immunodeficiency virus-infected patients on different fluconazole regimens. *J. Clin. Microbiol.* **36**: (8); 2308-2313.

Michalek S. M., and J. R. McGhee. 1977. Virulence of *Streptococcus mutans*: an antibiotic-suppressed rat model for studies of pathogenesis. *J. Dent. Res.* **56**:(3);205-211.

Michalek S. M., I. Morisaki., R. L. Gregory., S. Kimura., C. C. Harmon., S. Hamanda., S. Kotani., and J. R. McGhee. 1984. Oral adjuvants enhance salivary IgA responses to purified *Streptococcus mutans* antigens. *Protides Biol. Fluids Proc. Colloq.* **32**: 47-52.

Miele G., L. MacRae., D. McBride., J. Manson., and M. Clinton. 1998. Elimination of false positives generated through PCR re-amplification of differential display cDNA. *BioTechniques.* **25**: 138-144.

Mihajlovic-Ukropina M., and N. Roncevic. 1998. Significance of normal oropharyngeal flora in the development of streptococcal pharyngitis and outcome of penicillin therapy. *Med. Pregl.* **51**: (5-6); 275-278.

Milson J. L., P. D. Burrows. 1998. Minimizing false positives in differential display. *BioTechniques.* **24**: (5); 766-770.

Mims C. 1987. Pathogenesis of infectious disease. 3rd edition. Academic Press, San Diego CA.

Mitchelmore I. J., A. J. Prior., P. Q. Montgomery., and S. Tabaqchali. 1995. Microbiological features and pathogenesis of peritonsillar abscesses. *Eur. J. Clin. Microbiol. Infect. Dis.* **14**: 870-877.

Molina J. M., C. Leport., A. Bure., M. Wolff., C. Michon., and J. L. Vilde. 1991. Clinical and bacterial features of infections caused by "*Streptococcus milleri*". *Scand. J. Infect. Dis.* **23**: 659-666.

Moore W. E. C., L. V. Holdeman., R. M. Smibert., D. E. Hash., J. A. Burmeister., and R. R. Rannis. 1982. Bacteriology of severe periodontitis in young adult humans. *Inf. Immun.* **38**: (3); 1137-1148.

Moore W. E. C., J. A. Burmeister., C. N. Brooks., R. R. Ranney., K. H. Hinkelman., R. M. Schieken., and L. V. H. Moore. 1993. Investigation of the influences of puberty, genetics, and environment on the composition of subgingival periodontal floras. *Infect. Immun.* **61**: 2891-2898.

Moses A. E., M. R. Wessels., K. Zalcman., S. Alberti., S. Natanson-Yaron., T. Menes., and E. Hanski. 1997. Relative contributions of hyaluronic acid capsule and M protein to virulence in a mucoid strain of the group A *Streptococcus*. *Infect. Immun.* **65**: (1); 64-71.

Murray H. W., K. C. Gross., H. Masur., and R. B. Roberts. 1978. Serious infections caused by "*Streptococcus milleri*". *Am. J. Med.* **64**: 759-764.

Nagamune H., C. Ohnishi., A. Katsuura., K. Fushitani., R. A. Whiley., A. Tsuji., and Y. Matsuda. 1996. Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. *Infect. Immun.* **64**: (8); 3093-3100.

Nagamune H., R. A. Whiley., T. Goto., Y. Inai., T. Maeda., J. M. Hardie., and H. Kourai. 2000. Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with *Streptococcus intermedius*. *J. Clin. Microbiol.* **38**: (1); 220-226.

Nagel A., J. T. Fleming., and G. S. Sayler. 1999. Reduction in false positives in prokaryotic mRNA differential display. *BioTechniques*. **26**: 641-648.

Nakagawa I., K. Kurokawa., A. Yamashita., M. Nakata., Y. Tomiyasu., N. Okahshi., S. Kawabata., K. Yanazaki., T. Shiba., T. Yasunaga., H. Hayashi., M. Hattori., and S. Hamada. 2003. Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in

invasive strains and new insights into phage evolution. *Genome Research.* **13**: (6A); 1042-1055.

Ofek I., S. Bergner-Rabinowitz., and I. Ginsburg. 1970. The relation of oxygen stable haemolysins of group A streptococci VII: The relation of the leukocytotoxic factors to streptolysin S. *J. Infect. Dis.* **122**: (6); 517-522.

Ofek I., S. Bergner-Rabinowitz. 1972. Oxygen stable haemolysins of group A streptococci VIII leukocytotoxic and antiphagocytic effects of streptolysin S and O. *Infect. Immunol.* **6**: 459-464.

Olive D. M., and P. Bean. 1999. Principles of DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37**: (6); 1661-1669.

Olsvik B., I. Olsen., and F. C. Tenover. 1995. Detection of tet (M) and tet (O) using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. *Oral. Immunol.* **10**: (2); 87-92.

Ochsner U. A., and J. Reiser. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA.* **92**: 6424-6428.

Pearce C., G. H. Bowden., M. Evans., S. P. Fitzsimmons., J. Johnson., M. J. Sheridan., R. Wientzen., and M. F. Cole. 1995. Identification of pioneer viridans streptococci in the oral cavity of human neonates. *J. Med. Microbiol.* **42**: 67-72.

Peltroche-Llacsahuanga H., E. Reichhart., W. Schmitt., R. Lutticken., and G. Haase. 2000. Investigation of infectious organisms causing periocoronitis of the mandibular third molar. *J. Oral. Max. Surg.* **58**: 611-616.

Petersen F. C., S. Pasco., J. Ogier., J. D. Klein., S. Assey., and A. A. Scheie. 2001. Expression and functional properties of the *Streptococcus intermedius* surface protein antigens I/II. *Infect. Immun.* **69**: (7); 4647-4653.

Pfaller M. A., R. J. Hollis., and H. J. Sader. 1994. Chromosomal restriction fragment analysis by Pulsed field gel electrophoresis. P. 10.5c.1 – 1 10.5c.12. *In* H. D. Isenberg (ed.). Clinical microbiology procedures handbook Vol 2: Suppl 1. American society for microbiology, Washington DC.

Piscitelli S. C., J. Shwed., P. Schreckenberger., and L. H. Danziger. 1992. "*Streptococcus milleri*" group: renewed interest in an elusive pathogen. *Eur. J. Clin. Microbiol. Infect. Dis.* **11**: (6); 491-498.

Poole P. M., and G. Wilson. 1977. "*Streptococcus milleri*" in the appendix. *J. Clin. Pathol.* **30**: 937-942.

Poole P. M., and G. Wilson. 1979. Occurrence and cultural features of "*Streptococcus milleri*" in various body sites. *J. Clin. Pathol.* **32**: 764-768.

Porta G., M. Rodriguez-Carballeira., L. Gomez., M. Salavert., N. Fseixas., M. Xercarins., and J. Garau. 1998. Thoracic infection caused by "*Streptococcus milleri*". *Eur. Respir. J.* **12**: 357-362.

Potter R. H. 1990. Twin half-sibs: a research design for genetic epidemiology of common dental disorders. *J. Dent. Res.* **69**: 1527-1530.

Prescott L. M., J. P. Harley., and D. A. Kline. 1995. Microbiology (2nd Ed.). William. C. Brown Publishers. Oxford, England.

Pritchard D. G. 1985. Structure of the group-specific polysaccharide of group E *Streptococcus*. *Carbohydr. Res.* **144**: 289-296.

Quinn R., and K. P. Singh. 1957. Antigenicity of hyaluronic acid. *Biochem. J.* **95**: 290-291.

Quinn F. D., G. W. Newman., and C. H. King. 1997. In search of virulence factors of human bacterial disease. *TRENDS in microbiology.* **5**: (1); 22-27.

Raeder R., E. Harokopakis., A. Hollingshead., and M. D. P. Boyle. 2000. Absence of SpeB production in virulent large capsular forms of group A streptococcal strain 64. *Infect. Immun.* **68**: (2); 744-751.

Rautio M., H. Saxen., A. Sitonen., R. Nikku., and H. Jousimies-Somer. 2000. Bacteriology of histopathologically defined appendicitis in children. *Paediat. Infect. Dis. J.* **19**: 1078-1083.

Rawlinson A., B. I. Duerden., and L. Goodwin. 1993. New findings on the microbial flora associated with adult periodontitis. *J. Dent.* **21**: 179-184.

Reeves P. 1993. Evolution of Salmonella O antigen variation by interspecific gene transfer on a large scale. *Trends. Genet.* **9**: 17-22.

Rindi L., N. Lan., and C. Garzelli. 1999. Search for genes potentially involved in *Mycobacterium tuberculosis* virulence by mRNA differential display. *Biochem. Biophys. Res. Comm.* **258**: 94-101.

Rolland K., C. Marois., V. Siquier., B. Cattier., and R. Quentin. 1999. Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-field gel electrophoresis and *hylB* gene analysis. *J. Clin. Microbiol.* **37**: 1892-1898.

Ruoff K. L. 1988. *Streptococcus anginosus* ("*Streptococcus milleri*"): the unrecognised pathogen. *Clin. Microbiol. Rev.* **1**: 102-108.

Sader H. S., R. J. Hollis., and M. A. Pfaller. 1995. The use of molecular techniques in the epidemiology and control of infectious diseases. *Clin. Lab. Med.* **15**: 407-431.

Sadowski P. L., B. C. Peterson., and D. N. Gerding. 1979. Physical characterisation of ten R plasmids obtained from an outbreak of nosocomial *Klebsiella pneumoniae* infection. *Antimicrob. Agents. Chemother.* **15**: (4); 616-624.

Sakamoto H., H. Naito., Y. Ohta., R. Tanakna., N. Maeda., J. Sasaki., and C. E. Nord. 1999. Isolation of bacteria from cervical lymph modes in patients with oral cancer. *Arch. Oral. Biol.* **44**: 789-793.

Salavert M., L. Gomez., M. Rodriguez-Carballeira., M. Xercavins., N. Freixas., and J. Garau. 1996. Seven-year review of bacteraemia caused by "*Streptococcus milleri*" and other viridans streptococci. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**: (5); 365-371.

Sato T., and H. K. Kuramitsu. 1999. Restriction fragment-length polymorphism analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction for rapid identification of cultivatable oral treponemes. *Oral. Microbiol. Immunol.* **14**: 117-121.

Savor C., M. A. Pfaller., J. A. Kruszynski., R. J. Hollis., G. A. Noskin., and L. R. Peterson. 1998. Comparison of genomic methods for differentiating strains of *Enterococcus faecium*: Assessment using clinical epidemiological data. *J. Clin. Microbiol.* **36**: (11); 3327-3331.

Schleifer K. H., and R. Kilpper-Balz. 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. *Syst. Appl. Microbiol.* **10**: 1-19.

Schrager H. M., J. G. Rheinwald., and M. R. Wessels. 1996. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J. Clin. Invest.* **98**: (9); 1954-1958.

Schwartz D. C., and C. Cantor. 1984. Separation of Yeast chromosome sized DNA's by Pulsed field gel electrophoresis. *Cell.* **37**: 67-75.

Selenska-Pobell S., E. Evgnericera-Hackenberg., G. Radera., and A. Squartini. 1996. Characterisation of *Rhizobium hedysari* by RFLP analysis of PCR amplified rDNA and by genomic PCR fingerprinting. *J. Appl. Bacteriol.* **80**: 517-528.

Serwecinska L., T. Cleslikowski., M. Pytlosi., A. Jaworski., and W. Kaca. 1998. Genomic fingerprinting of Proteus species using repetitive sequence based PCR (Rep-PCR). *Acta. Microbiol. Pol.* **47**: (3); 313-319.

Shain H., K. A. Homer., and D. Beighton. 1996. Degradation and utilisation of chondroitin sulphate by *Streptococcus intermedius*. *J. Med. Microbiol.* **44**: 372-380.

Shain H., K. Homer., J. Aduse-Opaku., and D. Beighton. 1997. A conserved region of a hyaluronidase gene from *Streptococcus intermedius*. *Adv. Exp. Med. Biol.* **418**: 769-772.

Shiga K., M. Tateda., S. Saijo., T. Hori., I. Sato., H. Tateno., K. Matassuura., T. Takasaki., and T. Miyagi. 2001. Presence of *Streptococcus* infection in

extra-oropharyngeal head and neck squamous cell carcinoma and its implication in carcinogenesis. *Oncology reports.* **8**: 245-248.

Shimono N., T. Ishimaru., K. Takaki., K. Okada., S. Nagafuchi., K. Takemori., T. Tsutsui., and Y. Sawae. 1992. Clinical features of patients suffering from *"Streptococcus milleri"* infections - a retrospective analysis. *Fukuoka. Acta. Med.* **83**: 420-425.

Shinzato T., and A. Saito. 1994. A mechanism of pathogenicity of "*Streptococcus milleri*" group in pulmonary infection: synergy with an anaerobe. *J. Med. Microbiol.* **40**: 118-123.

Shinzato T., and A. Saito. 1995. The "*Streptococcus milleri*" group as a cause of pulmonary infections. *Clin. Infect. Dis.* **21**: Suppl 3; S238-243.

Shiroza T., N. Shinozaki., T. Watanabe., T. Ikemi., K. Fukushima., and Y. Abiko. 1998. Rapid isolation of chromosomal DNA from oral streptococci and polymerase chain reaction-oriented restriction fragment-length polymorphism analysis for genetic heterogeneity. *Oral. Microbiol. Immunol.* **13**: 11-16.

Sikorski J., R. Rossello-Mora., and M. G. Lorerrz. 1999. Analysis of genotypic diversity and relationships among *Pseudomonas stuteri* strains by PCR-based genomic fingerprinting and multilocus enzyme electrophoresis. *System. Appl. Microbiol.* **22**: 393-402.

Singleton P., and D. Sainsbury. 1993. Dictionary of microbiology and molecular biology. 2nd edition. Wiley-interscience, Chichester, UK.

Slots J., and G. Dahlen. 1985. Subgingival microorganisms and bacterial virulence factors in periodontitis. *Scand. J. Dent. Res.* **93**: 119-127.

Smith R. F., and N. P. Willett. 1968. Rapid plate method for screening hyaluronidase and chondroitin sulphatase-producing microorganisms. *Appl. Microbiol.* **16**: 1434-1436.

Smith D. J., J. M. Anderson., W. F. King., J. Van Houte., and M. A. Taubman. 1993. Oral streptococcal colonisation of infants. *Oral. Microbiol. Immunol.* **8**: 1-4.

Socransky S. S., and A. P. Mangienello. 1971. The oral microbiota of man from birth to senility. *J. Periodontol.* **42**: 485-494.

Sokolov B. P., and D. J. Prockop. 1994. A rapid and simple PCR-based method for isolation of cDNA's from differentially expressed genes. *Nucl. Acids. Res.* **22**: (19); 4009-4015.

Spratt B. G., and C. J. Maiden. 1999. Bacterial population genetics, evolution and epidemiology. *Phil. Trans. R. Soc. London.* **354**: 701-710.

Spratt B. G., W. P. Hanage., and E. J. Feil. 2001. The relative contributions of recombination and point mutation to the diversification of bacterial clones. *Curr. Opin. Microbiol.* **4**: 602-606.

Stanley J., M. Desai., J. Xerry., A. Tanna., A. Efstratiova., and R. George. 1996. High-resolution genotyping elucidates the epidemiology of group A *Streptococcus* outbreaks. *Clin. Infect. Dis.* **174**: 500-506.

Steffen E. K., and D. J. Hentges. 1981. Hydrolytic enzymes of anaerobic bacteria isolated from human infection. *J. Clin. Microbiol.* **14**: 153-156.

Stern R. J., T. Y. Lee., T. J. Lee., W. Yan., M. S. Scherman., V. D. Vissa., S. K. Kim., B. L. Wanner., and M. R. McNeil. 1999. Conversion of dTDP-4-keto-6-deoxyglucose to free dTDP-4-keto-rhamnose by the *rmlC* gene products of *Escherichia coli* and *Mycobacterium tuberculosis*. *Microbiology*. **145**: 663-671.

Stinson M. W., R. J. Nisengard., and E. J. Bergey. 1980. Binding of streptococcal antigens to muscle tissue *in-vitro*. *Infect. Immun*. **27**: 604-613.

Stratton C. W. 1999. *Streptococcus Intermedius* group. p. 1861-1864. *In* Murray P. R., E. J. Baron., and M. A. Pfaller (ed.). Manual of Clinical Microbiology. 7th edition: American Society for Microbiology, Washington DC.

Strauss E. J., and S. Falkow. 1977. Microbial pathogenesis genomes and beyond. *Science*. **276**: 707-712.

Stubbs S. L., J. A. Hobol., R. J. Waddington., G. Embry., and M. A. O. Lewis. 1999. Effect of environmental haemin upon the physiology and biochemistry of *Prevotella intermedia* R78. *Lett. Appl. Microbiol*. **29**: (1); 31-36.

Sturtevant J. 2000. Application of differential display RT-PCR to molecular pathogenesis and medical mycology. *Clin. Infect. Dis*. **13**: (3); 408-427.

Swaminathan B., G. M. Matar., M. W. Reeves., L. M. Graves., G. Ajello., W. F. Bibb., L. O. Helsel., M. Morales., H. Dronavalli., M. El-Swify., W. DeWitt., and S. B. Hunter. 1996. Molecular subtyping of *Neisseria meningitidis* serogroup B: comparison of five methods. *J. Clin. Microbiol*. **34**: 1468-1473.

Takao A., H. Nagashima., H. Usui., F. Sasaki., N. Maeda., K. Ishibashi., and H. Fujita. 1997. Hyaluronidase activity in human pus from which *Streptococcus intermedius* was isolated. *Microbiol. Immunol*. **41**: 795-798.

Tateda M., K. Shiga., S. Saijo., M. Sone., T. Hori., J. Yokohama., K. Matjuura., T. Takasaka., and T. Mijagi. 2000. *Streptococcus anginosus* in head and neck squamous cell carcinoma: implications in carcinogenesis. *Int. J. Mol. Med.* **6**: 699-703.

Teng L. J., P. R. Hsueh., Y. C. Chen., S. W. Ho., and K. T. Luh. 1998. Antimicrobial susceptibility of viridans group streptococci in Taiwan with an emphasis on the high rates of resistance to penicillin and macrolides in *Streptococcus oralis*. *J. Antimicrob. Chemother.* **41**: 621-627.

Tenover F. C., R. Arbeit., G. Archer., J. Biddle., S. Byrne., R. Goering., G. Hancock., G. A. Hebert., B. Hill., and R. Hollis. 1994. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J. Clin. Microbiol.* **32**: 407-415.

Tenover F. C., R. D. Arbeit., R. V. Goering., P. A. Mickelsen., B. E. Murray., D. H. Persing., and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by Pulsed field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**: 2233-2239.

Terao Y., S. Kawabata., E. Kunitomo., J. Murakami., I. Nakagawa., and S. Hamada. 2001. Fba, a novel fibronectin-binding protein from *Streptococcus pyogenes*, promotes bacterial entry into epithelial cells, and the *fba* gene is positively transcribed under the Mga regulator. *Mol. Microbiol.* **42**: (1); 75-86.

Tettelin H., K. E. Nelson., I. T. Paulsen., J. A. Read., S. Peterson., J. Heidelberg., R. T. DeBoy., D. H. Haft., R. J. Dodson., A. S. Durkin., M. Gwinn., J. F. Kolonay., W. C. Nelson., J. D. Peterson., L. A. Umayam., O. White., S. L. Salzberg., M. R. Lewis., D. Radune., E. S. Holtzapple., T. Dickinson., E. K. Hickey., I. E. Holt., B. J. Loftus., F. Yang., H. O. Smith., J. C.

Venter., B. A. Dougherty., D. A. Morrison., S. K. Hollingshead., and C. M. Fraser. 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science*. **293**: 498-506.

Tettelin H., V. Masignani., M. J. Cieslewicz., J. A. Eisen., S. Peterson., M. R. Wessels., I. T. Paulsen., K. E. Nelson., I. Margarit., T. D. Read., L. C. Madoff., A. M. Wolf., M. J. Beanan., L. M. Brinkac., S. C. Daugherty., R. T. DeBoy., A. S. Durkin., J. F. Kolonay., R. Madupu., M. R. Lewis., D. Radune., N. B. Fedorova., D. Scanlan., H. Khouri., S. Mulligan., H. A. Carty., R. T. Cline., S. E. Van Aken., J. Gill., M. Scarselli., M. Mora., E. T. Lacobini., C. Brettoni., G. Galli., M. Mariani., F. Vegni., D. Maioni., D. Rinaudo., R. Rappuoni., J. L. Telford., D. L. Kasper., G. Grandi., and C. M. Fraser. 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. *PNAS*. **99**: 12391-12396.

Theilade E. 1990. Factors controlling the microflora of the healthy mouth. P. 2-56. *In*. M. J. Hill and P. D. Marsh (ed.). Human Microbial Ecology. CRC Press, Inc., Boca Raton, Fla.

Tillotson G. S., and L. E. Ganguli. 1984. Antibiotic susceptibilities of clinical strains of "*Streptococcus milleri*" and related streptococci. *J. Antimicrob. Chemother.* **14**: 557-560.

Toyoda K., N. Kusano., and A. Saito. 1995. Pathogenicity of the "*Streptococcus milleri*" group in pulmonary infections-effect on phagocytic killing by human polymorphonuclear neutrophils. *Kansenshogaku Zasshi - Journal of the Japanese Association for Infectious Diseases*. **69:** (3); 308-315.

Tracy M., A. Wanahita., Y. Shuhatora., E. A. Goldsmith., J. E. 3rd. Clarridge., and D. M. Musher. 2001. Antibiotic susceptibilities of genetically characterised "*Streptococcus milleri*" group strains. *Antimicrob. Agents. Chemother.* **45**: (5); 1511-1514.

Tresadern J. C., R. J. Farrand., and M. H. Irving. 1983. "*Streptococcus milleri*" and surgical sepsis. *Ann. R. Coll. Surg. Engl.* **65**: (2); 78-79.

Tsukioka Y., Y. Yamashita., T. Oho., Y. Nakano., and T. Koga. 1997. Biological function of the dTDP-Rhamnose synthesis pathway in *Streptococcus mutans. J. Bacteriol.* **179**: (4); 1126-1134.

Tsumori H., and H. Kuramitsu. 1997. The role of *Streptococcus mutans* glucosyltransferases in the sucrose-dependant attachment to smooth surfaces: essential role of the *GtfC* enzyme. *Oral Microbiol. Immunol.* **12**: 274-280.

Tuli J. S., J. A. Smith., and D. B. Morton. 1994. Stress measurements in mice after transportation. *Lab. Anim.* **29**: 132-138.

Tunkel A. R., and K. A. Sepkowitz. 2002. Infections caused by viridans streptococci in patients with neutropenia. *CID.* **34**: 1524-1529.

Unsworth P. F. 1989. Hyaluronidase production in "*Streptococcus milleri*" in relation to infection. *J. Clin. Pathol. 42*: (5); 506-510.

Utt E. A., J. P. Brousal., L. C. Kikuta-Oshima., and F. D. Quinn. 1995. The identification of bacterial gene expression differences using mRNA-based isothermal subtractive hybridisation. *Can. J. Microbiol.* **41**: 152-156.

Valdivia R. H., and S. Falkow. 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol. Microbiol.* **22**: (2); 367-378.

Valdivia R. H., and S. Falkow. 1997. Fluorescence based isolation of bacterial genes expressed within host cells. *Science*. **277**: 2007-2011.

Van Belkum A., W. Melchen., B. E. de Pauw., S. Scherer., W. Quint., and J. F. Meis. 1994. Genotypic characterisation of sequential *Candida albicans* isolates from fluconazole treated neutrogenic patients. *J. I. D.* **169**: 1062-1070.

Van Belkum A., M. Sluijter., R. de Groot., H. Verbrugh., and P. W. Hermans. 1996. Novel Box repeat PCR assays for high resolution typing of *Streptococcus pneumoniae* strains. *J. Clin. Microbiol.* **34**: (5); 1176-1179.

Van der Auwera P. 1985. Clinical significance of *"Streptococcus milleri"*. *Eur. J. Clin. Microbiol.* **4**: 386-390.

Vandamme P., B. Pot., M. Gillis., P. De Vos., K. Kersters., and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.* **60**: 407-438.

Vaneechoutte M. 1996. DNA fingerprinting techniques for microorganisms. *Mol. Biotechnol.* **6**: 115-142.

Vedoy C. G., M. H. Bengtson., and M. C. Soyagar. 1999. Hunting for differentially expressed genes. *Braz. J. Med. Biol. Res.* **32**: (7); 877-884.

Versalovic J., T. Koeuth., and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic. Acids. Res.* **19**: 6823-6831.

Vriesema A. J. M., R. Brinkman., J. Kok., J. Dankert., and S. A. J. Zaat. 2000. Broad host-range shuttle vectors for screening of regulated promoter activity in viridans group streptococci: isolation of a pH-regulated promoter. *Appl. Environ. Microbiol.* **66**: (2); 535-542.

Walsh C., and C. L. Cepko. 1992. Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science.* **255**: 434-440.

Wannamaker L. W. 1983. Streptococcal toxins. *Rev. Infect. Dis.* Suppl **4**: s723-732.

Weidner, G., and G. Sawers. 1996. Molecular characterization of the genes encoding Pyruvate formate-lyase and its activating enzyme of *Clostridium pasteurianum*. *J. Bacteriol.* **178**: (8); 2440-2444.

Weissmann G., H. Keiser., and A. W. Bernheimer. 1963. Studies on lysosomes III. The effects of streptolysin O and S on the release of acid hydrolases from a granular fraction of rabbit liver. *J. Exp. Med.* **118**: 205-222.

Welborn P. P., W. K. Hadley., E. Newbrun., and D. M. Yajko. 1983. Characterisation of strains of viridans streptococci by deoxyribonucleic acid hybridisation and physiological tests. *Int. J. Syst. Bacteriol*. **33**: 293-299.

Whiley R. A., and J. M. Hardie. 1989. DNA-DNA hybridisation studies and phenotypic characteristics of strains within the "*Streptococcus milleri*" group. *J. Gen. Microbiol.* **135**: 2623-2633.

Whiley R. A., H. Fraser., J. M. Hardie., and D. Beighton. 1990. Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the "*Streptococcus milleri*" group. *J. Clin. Microbiol.* **28**: (7); 1497-1501.

Whiley R. A., and Beighton D. 1991. Emended descriptions and recognition of *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus* as distinct species. *Int. J. Syst. Bacteriol* . **41**: 1-5.

Whiley R. A., D. Beighton., T. G. Whinstanley., H. Y. Fraser., and J. M. Hardie. 1992. *Streptococcus intermedius*, *Streptococcus constellatus* and *Streptococcus anginosus* (the "*Streptococcus milleri*" group): association with different body sites and clinical infections. *J. Clin. Microbiol.* **30**: (1); 243-244.

Whiley R. A., L. Freemantle., D. Beighton., J. R. Radford., J. M. Hardie., and G. Tillotsen. 1993. Isolation, identification and prevalence of *Streptococcus anginosus*, *S. intermedius* and *S. constellatus* from the human mouth. *Microbial Ecology in Health and Disease*. **6**: 285-291.

Whiley R. A., B. Duke., J. M. Hardie., and L. M. Hall. 1995. Heterogeneity among 16S-23S rRNA intergenic spacers of species within the *"Streptococcus milleri"* group. *Microbiology*. **141**: (6); 1461-1467.

Whiley R. A., L. M. C. Hall., J. M. Hardie., and D. Beighton. 1997. Genotypic and phenotypic diversity within *Streptococcus anginosus*. *Int. J. Syst. Bacteriol.* **47**: (3); 645-650.

Whiley R. A., and D. Beighton. 1998. Current classification of the oral streptococci. *Oral. Microbiol. Immunol.* **13**: 195-216.

Whinstanley T. G., J. T. Magee., D. I. Limb., T. M. Hindmarch., R. C. Spencer., R. A. Whiley., D. Beighton., and J. M. Hardie. 1992. A numerical taxonomic study of the "*Streptococcus milleri*" group based upon conventional

phenotypic tests and pyrolysis mass spectrometry. *J. Med. Microbiol.* **36**: (3); 149-155.

Whitworth J. M. 1990. Lancefield group F and related streptococci. *J. Med. Microbiol.* **33**: 135-151.

Whitworth J. M., P. W. Ross., and I. R. Poxton. 1991. Use of rapid carbohydrate utilisation test for identifying "*Streptococcus milleri*" group. *J. Clin. Pathol.* **44**: 329-333.

Willcox M. D., and K. W. Knox. 1990. Surface-associated properties of "*Streptococcus milleri*" group strains and their potential relation to pathogenesis. *J. Med. Microbiol.* **31**: (4); 259-270.

Willcox M. D., H. J. Oakey., D. W. Harty., M. Patrikakis., and K. W. Knox. 1994. Lancefield group C "*Streptococcus milleri*" group strains aggregate human platelets. *Microb. Path.* **16**: (6); 451-457.

Willcox M. D. 1995. Potential pathogenic properties of members of the "*Streptococcus milleri*" group in relation to the production of endocarditis and abscesses. *J. Med. Microbiol.* **43**: 405-410.

Willcox M. D., M. Patrikakis., and K. W. Knox. 1995a. Degradative enzymes of oral streptococci. *Aust. Dent. J.* **40**: (2); 121-128.

Willcox M. D., C. Y. Loo., D. W. Harty., and K. W. Knox. 1995b. Fibronectin binding by "*Streptococcus milleri*" group strains and partial characterisation of the fibronectin receptor of *Streptococcus anginosus* F4. *Microb. Path.* **19**: 129-137.

Williams D. W., M. J. Wilson., and M. A. O. Lewis. 1999. Deoxyribonucleic acid typing methods for medically important microorganisms. *Brit. J. Biomed. Sci.* **56**: 56-65.

Wilson M. J., D. W. Williams., M. D. L. Forbes., I. G. Finlay., and M. A. O. Lewis. 2001. A molecular epidemiological study of sequential oral isolates of *Candida albicans* from terminally ill patients. *J. Oral. Pathol. Med.* **30**: 206-212.

Wisplinghoff H., R. R. Reinert., O. Cornely., and H. Seifert. 1999. Molecular relationships and antimicrobial susceptibilities of viridans group streptococci isolated from blood of neutropenic cancer patients. *J. Clin. Microbiol.* **37**: 1876-1880.

Woods C. R., J. Versalovic., T. Koeuth., and J. R. Lupski. 1993. Whole cell repetitive element sequence based PCR allows rapid assessment of clonal relationships of bacterial isolates. *J. Clin. Microbiol.* **31**: (7); 1927-1931.

Yajko D. M., and W. K. Hadley. 1985. Support of the single species concept for *"Streptococcus milleri"* by DNA hybridisation data. *Eur. J. Clin. Microbiol.* **4**: 355-356.

Yakushiji T., M. Katsuki., A. Yoshimitsu., J. Mizuno., and M. Inoue. 1988. Isolation and physiological characterization of "*Streptococcus milleri*" strains from human dental plaque. *Microbios.* **55**: 161-171.

Yamamori T., K. Ito., Y. Nakamura., and T. Yura. 1978. Transient regulation of protein synthesis in *Escherichia coli* upon shift-up of growth temperature. *J. Bacteriol.* **134**: (3); 1133-1140.

Yamamoto Y., Y. Sato., S. Takahashi-Abbe., N. Takahashi., and H. Kizaki. 2000. Characterisation of the *Streptococcus mutans* Pyruvate formate lyase (PFL) activating enzyme gene by complimentary reconstruction of the *in-vitro* PFL-reactivating system. *Infect. Immun.* **68**: (8); 4773-4777.

Yang K. D., N. H. Augustine., L. A. Gonzalez., J. F. Bohnsack., and H. R. Hill. 1988. Effects of fibronectin on the interaction of polymorphonuclear leukocytes with unopsonised and antibody-opsonised bacteria. *J. Infect. Dis.* **158**: 823-830.

Zachariasen R. D. 1993. The effect of elevated ovarian hormones on periodontal health: oral contraceptives and pregnancy. *Women Health* **20**: 21-30.