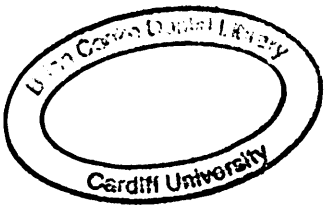

A Cultural and Molecular Analysis of the Microorganisms Present Within Human Oral Squamous Cell Carcinoma

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SUMMARY

There is increasing interest in the relationship between bacteria and the different stages of cancer development, yet the association of bacteria with cancer of the oral cavity has yet to be adequately examined.

The main objective of this thesis was to characterise the bacteria present within oral squamous cell carcinoma (OSCC) tissue by a combination of standard culture and molecular techniques.

Portions of tissue were harvested at the time of surgery from deep within the tumour mass using fresh blades for each cut. Whenever possible, "superficial" portions from the mucosa overlying the tumour and non-tumourous control specimens from at least 5 cm away from the primary tumour site were also obtained. Twenty deep tissue specimens, 19 corresponding superficial tissues and 12 control tissues were studied. Surface contamination was successfully eliminated by immersion in Betadine® and washing with PBS.

Viable microorganisms were isolated by culturing aseptically macerated specimens on non-selective media under both aerobic and anaerobic conditions. Concurrently, PCR using *Bacteria*-specific universal primers was undertaken on DNA extracted from the specimens. The products were singularised by TA cloning. All isolates, cultivated and cloned, were identified to species-level by sequence analysis of the 16S rRNA gene. Different species were detected by the culture and the culture-independent techniques, highlighting the importance of a combined approach. Both methods revealed the presence of a diversity of species within both tumourous and non-tumourous tissue, including some putatively novel taxa.

Denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments was used to generate profiles of the bacterial populations within each tissue specimen. Differences in the banding patterns provided evidence that the composition of the microflora is significantly different in OSCC than in non-tumourous tissue.

The hypothesis that bacteria are present within OSCC tissue was further supported by performing *in situ* hybridisation on sections from one of the specimens with a fluorescently-labeled oligonucleotide probe, designed to bind to all *Bacteria*.

This work has demonstrated for the first time the existence of viable bacteria within OSCC tumour tissue. A diversity of bacterial species was detected and a degree of restriction in comparison to control sites demonstrated. The significance of these bacteria within the tumour tissue warrants further study.

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FIGURES USED IN THIS THESIS

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ABBREVIATIONS USED IN THIS THESIS

Abbreviation	Meaning
16S rRNA	Bacterial small subunit ribosomal RNA
8-OHdG	8-Hydroxy-deoxyguanosine
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
ATCC	American type culture collection
BA	Blood agar
bp	(Nucleotide) base pairs
CLSM	Confocal laser scanning microscope
CNF	Cytotoxic necrotizing factor
COX	Cyclooxygenase
CYP	Cytochrome P450
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAA	Fastidious anaerobic agar
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
gDNA	Genomic DNA
GST	Glutathione S-transferase
H and E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HA	Heterocyclic aromatic amine
HCl	Hydrochloric acid
HIF	Hypoxia-inducible transcription factor
HPV	Human papillomavirus
IFN	Interferon
IL	Interleukin
IQ	2-Amino-3-methylimidazo[4,5,f]quinoline
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NADPH	Nicotinamide adenosine dinucleotide phosphate (reduced form)
NBMA	N-nitroso-benzylmethylamine
NCTC	National collection of type cultures
NF	Nuclear factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
O_2-	Superoxide free radical

OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
PAH	Polynuclear aromatic hydrocarbon
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PGE	Prostaglandin E
PMT	*Pasteurella multocida* toxin
RNA	Ribonucleic acid
rpoB	RNA polymerase beta subunit
RT	Reverse transcriptase
RTM	Reduced transport medium
Sab	Sabouraud's agar
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
sodA	Manganese-dependent superoxide dismutase
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra-violet
VBNC	Viable but non-culturable
VEGF	Vascular endothelial growth factor

CHAPTER ONE

INTRODUCTION

1.1 Oral Cancer

1.1.1 Incidence of Oral Cancer

Oral cancer is estimated to be the sixth most common malignant neoplasm in the world (Sugerman *et al.*, 1995; Zakrzewska, 1999). This corresponds to 3 percent of cancers in males and 2 percent in females worldwide (Kujan *et al*, 2005). It is particularly prevalent in developing countries, such as on the Indian subcontinent where up to 30 – 40 percent of all malignancies are located within the oral cavity (Cawson & Odell, 1998; Llewellyn *et al*, 2001).

In the U.K., approximately 1 – 4 percent of all malignancies are oral cancers (Rodrigues *et al*, 1998), representing around 2000 – 3500 new cases per annum (Hindle *et al.,* 2000; Llewellyn *et al*, 2001; Zakrzewska, 1999).

There is evidence that the incidence of cancer of the oral cavity is increasing in many parts of the world, including the U.K. (Hindle *et al.,* 2000; Llewellyn *et al.*, 2001; Macfarlane *et al.*, 1996). Traditionally, oral cancer has been a disease predominantly affecting men in their sixth or seventh decade (Llewellyn *et al*, 2004). In young adults the disease remains relatively uncommon, with only about 0.4 – 6 percent of cases occurring in patients under 45 years old worldwide (Annertz *et al*, 2002; Llewellyn *et al*, 2003; Mathew Iype *et al*, 2004; Oliver *et al*, 2000). Whilst the highest incidence is still seen in patients aged 50 years or over (Llewellyn *et al*, 2001), there is, however, strong evidence that the incidence in the under 45 age group is on the increase (Popovtzer *et al*, 2004; Shiboski *et al*, 2005). Also, in recent years, the difference between male and female patient numbers has reduced dramatically,

and there has been a noteworthy increase in incidence in women under 45 years old (Hyde & Hopper, 1999; Worrall, 1995).

Oral cancer has one of the lowest survival rates of all malignant neoplasms, worldwide (Kujan *et al*, 2005). The five-year survival rate for oral cancer is 65 percent of patients with tumours of the smallest clinical stage (T1), and only 16 percent of those of T4 stage (Hyde & Hopper, 1999). Survival rates also worsen with increasing age. For instance, for carcinoma of the tongue in the U.S., a 10-year increase in the age of the patient was associated with an 18 percent increase in risk of death (Davidson *et al*, 2001). The average all-stage survival is approximately 50 percent (Kujan *et al*, 2005), putting the disease on a par with carcinoma of the cervix and malignant melanoma in terms of annual mortality figures (Hyde & Hopper, 1999).

1.1.2 Presentation of Oral Cancer

Many different types of tumour or lesion, benign, premalignant or malignant, can present in the oral cavity. Some of the key conditions associated with oral cancer are described here in greater detail.

1.1.2.1 Premalignant lesions

Premalignant lesions are defined as any type of lesion in which the incidence of malignant change is greater than in normal tissues (Dimitroulis & Avery, 1998). It is thought that most malignancies in the oral cavity are preceded by a visible change in the mucosa, normally presenting as red or white patches (Mignogna *et al*, 2004b). One of the most important markers of potential malignant change is the presence of epithelial dysplasia, the

histopathological description of epithelium in which features of cellular atypica are present (Dimitroulis & Avery, 1998). Oral lesions with epithelial dysplasia develop into carcinoma more often than those without (Reibel, 2003). Severe dysplasia appears to signify at least double the risk of cancer compared to mild dysplasia (Scully *et al*, 2003). However, not all oral cancers go through a recognisable premalignant stage and not all premalignant lesions in the mouth become malignant. Similarly, not all precursor lesions are associated with epithelial dysplasia, and not all dysplasias will develop into malignancies (Reibel, 2003; Zakrzewska, 1999).

The best-known precursor lesion is oral leukoplakia, a clinical term describing a white patch with no obvious cause and which cannot be wiped off (Auluck, 2004; Cawson, 1969; Neville & Day, 2002; Sapp *et al*, 1997; van der Waal *et al*, 1997) (see Figure 1.1). The overall lifetime rate of malignant transformation for such lesions is an estimated 4 – 6 percent, although this may be higher at certain high-risk sites. For leukoplakias on the floor of the mouth, when coupled with smoking and/or alcohol, the rate may be up to 40 percent (Hyde & Hopper, 1999).

Erythroplakia is the clinical term describing predominantly red lesions that cannot be characterised clinically or pathologically as being due to any other condition (Dimitroulis & Avery, 1998; Hyde & Hopper, 1999) (see Figure 1.2). It is a relatively rare condition, with an estimated range of prevalence between 0.02 and 0.83 percent. Oral erythroplakia has a high rate of malignant transformation, generally varying between 14 and 50 percent (Reichart & Philipsen, 2005).

Figure 1.1 Oral leukoplakia

(A) Localised leukoplakia affecting the soft palate (reproduced from Langlais & Miller, 1998); (B) Localised leukoplakia affecting the lower alveolar ridge; (C) Extensive leukoplakia affecting the ventral surface of the tongue and the floor of the mouth (B – C reproduced from Lamey & Lewis, 1997).

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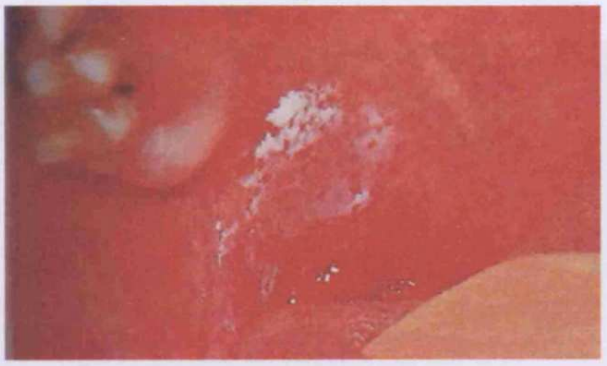

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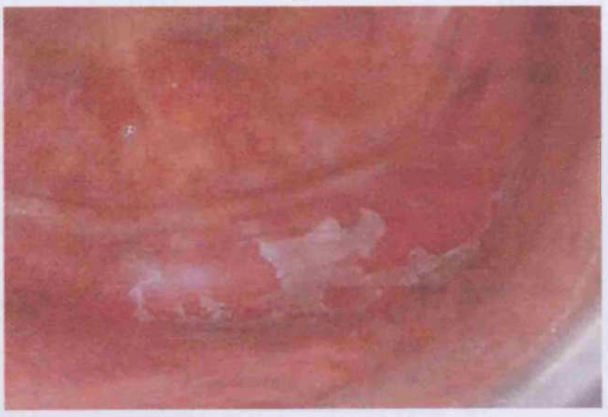

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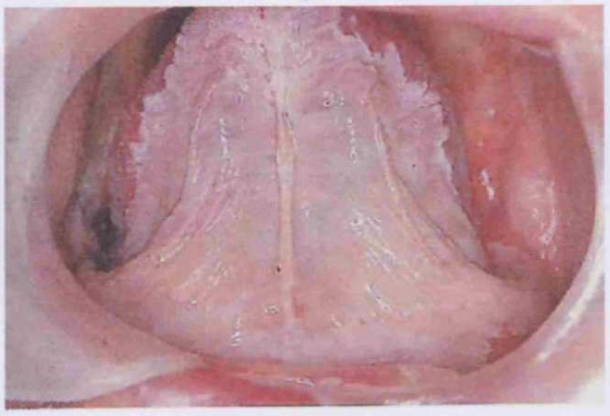

Figure 1.2 Oral erythroplakia

(A) Mucosal "speckled" erythroplakia with a leukoplakic border; (B) Erythroplakia involving the sublingual caruncle; (C) Erythroplakia affecting the floor of the mouth, which was later found to be SCC (all images reproduced from Langlais & Miller, 1998).

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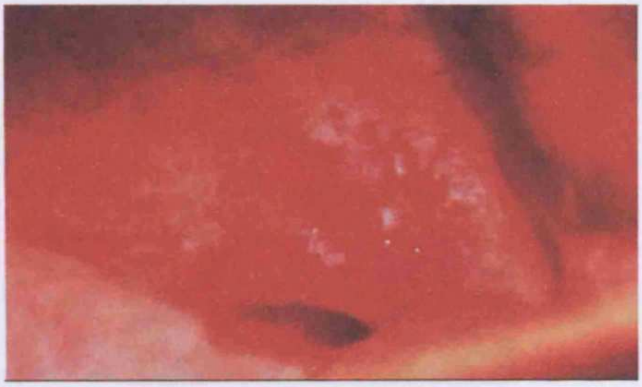

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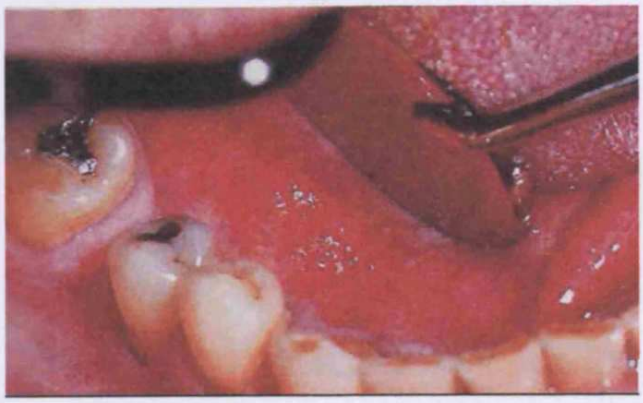

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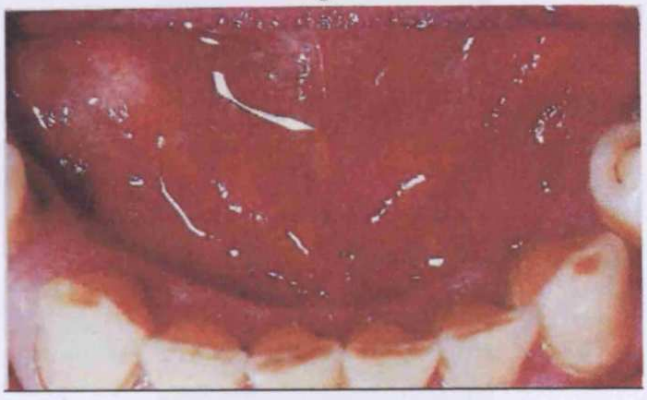

Figure 1.3 Other oral premalignant lesions

(A) Oral submucous fibrosis affecting the lip and soft palate, which has produced an intense paleness; (B) Widespread oral lichen planus displaying sharply defined white striae, which involves the buccal mucosa; (C) Oral lichen planus affecting the dorsum of the tongue and displaying plaque-like lesions (All images reproduced from Cawson *et al,* 1994).

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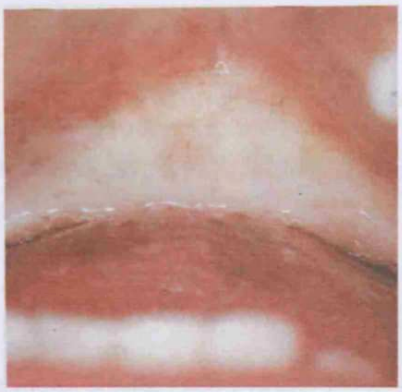

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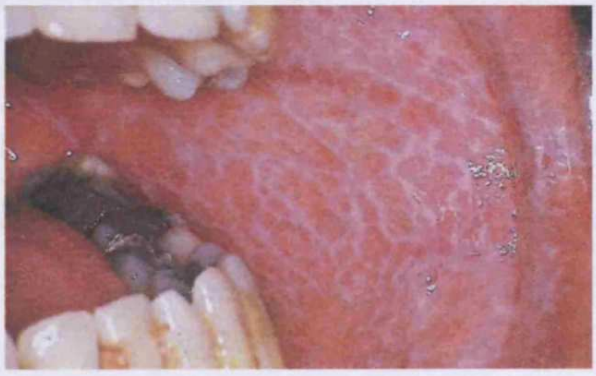

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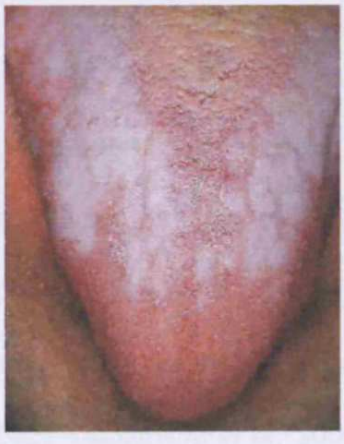


Other existing pan-oral conditions that may confer an increased risk of oral cancer development include oral submucous fibrosis and lichen planus. Oral submucous fibrosis (OSF) (see Figure 1.3) is a progressive disease in which the oral mucosa becomes gradually firmer, paler and stiffer, fibrous bands form and mouth opening is reduced. In OSF, the epithelium is atrophic, with dysplasia in 13 percent of cases. Atrophy and dysplasia are both signs of pre-malignancy (Dimitroulis & Avery, 1998; Hyde & Hopper, 1999; Sapp *et al*, 1997).

Lichen planus is a relatively common, chronic inflammatory disease that affects mucosal and cutaneous tissue (see Figure 1.3). Oral lichen planus (OLP) is more prevalent than the cutaneous form, and is generally more persistent and more resistant to treatment (Edwards & Kelsch, 2002). The cause of OLP is unknown, as is the mechanism by which OLP in some patients develop into OSCC (Rödström *et al*, 2004). The rate of malignant transformation is approximately 0.5 – 2 percent over a 5-year period (Dimitroulis & Avery, 1998; Larsson & Warfvinge, 2003).

1.1.2.2 Malignancies found in the oral cavity

Most types of malignant tumour that are found in and around the human oral cavity are also found in other parts of the body (Dimitroulis & Avery, 1998). Melanomas (Gu *et al*, 2003), salivary gland carcinomas (Toida *et al*, 2005), and both soft- and hard-tissue sarcomas (Bennett *et al*, 2000; do Nascimento Souza *et al*, 2005; Yamaguchi *et al*, 2004) are occasionally seen in the oral cavity, but by far the most common oral malignancy is squamous cell carcinoma arising from the oral mucosal membrane. Squamous cell carcinoma

(SCC) accounts for 90-95% of oral malignancies (Daley & Darling, 2003; Johnson, 2001) and so, in the clinic and in the literature, the term 'oral cancer' is often used synonymously with oral SCC (Dimitroulis & Avery, 1998).

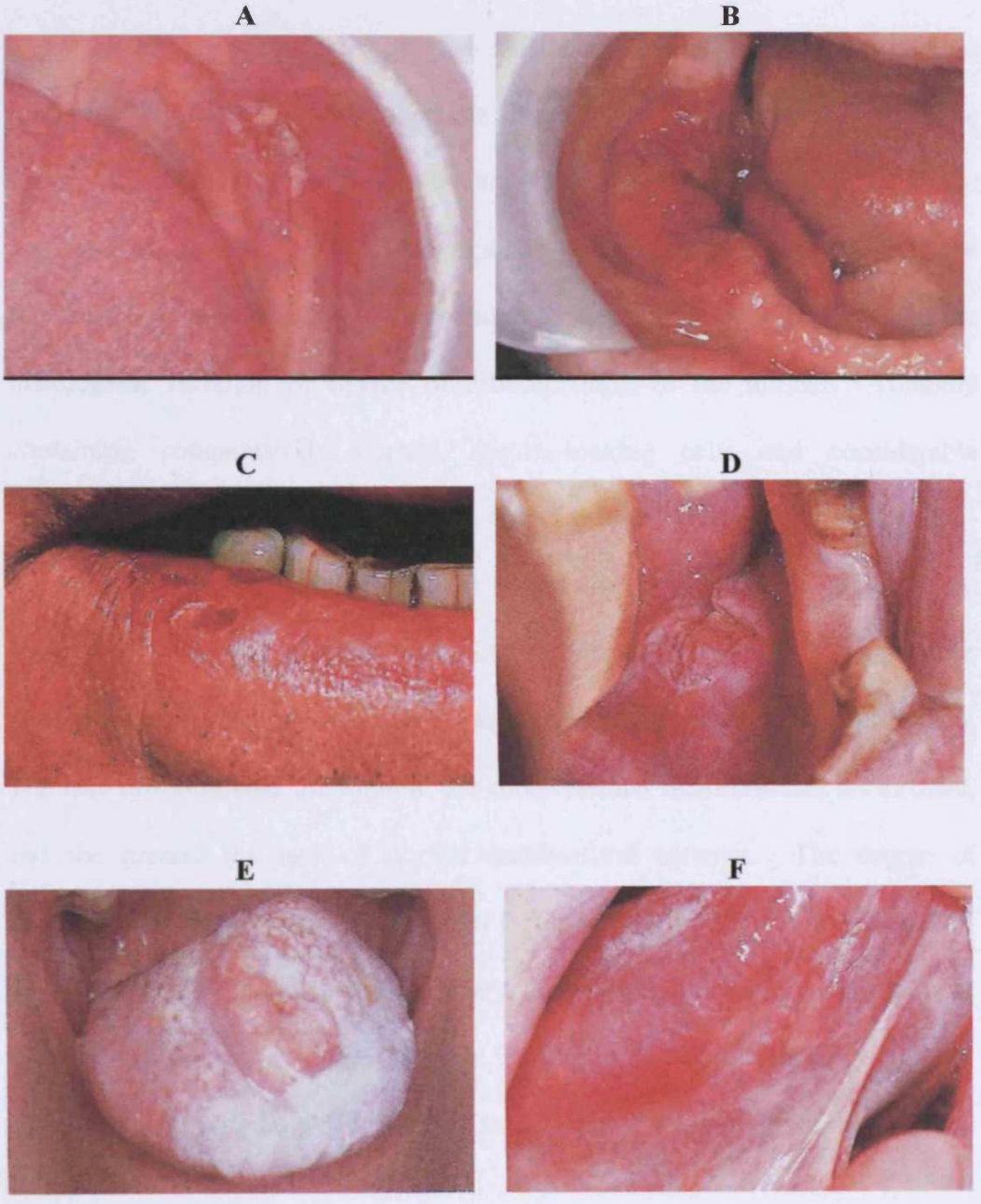
1.1.2.3 Oral squamous cell carcinoma (OSCC)

Carcinoma can present anywhere within the oral cavity (see Figure 1.4). However, the tongue is the most common site of OSCC with 25 – 40 percent of all cases occurring there (Chen & Myers, 2001; Gorsky *et al*, 2004; Neville & Day, 2002). Approximately 60 – 70 percent of cancers are found on the lateral border of the tongue, the floor of the mouth or the mandibular alveolus. This horseshoe-shaped area of the oral mucosa is at the greatest risk of cancer development, theoretically because it can act as a sump in which carcinogens can pool (Hyde & Hopper, 1999; Neville & Day, 2002; Sapp *et al*, 1997). As well as in the oral cavity proper, SCCs can develop on the lip vermilion and the oropharynx (Neville & Day, 2002).

Carcinomas appear similar to leukoplakias or erythroplakias in their earliest stages, namely as painless red, speckled or white patches. This similarity in appearance results from the processes of keratinisation and epithelial atrophy and do not necessarily indicate that malignancy has developed in a pre-existing lesion (Cawson & Odell, 1998). Generally, squamous cell carcinoma can be characterized as either exophytic, ulcerative or a combination of both. Ulcerative lesions are generally more common than the exophytic type, and can infiltrate the tissue deeply.

Figure 1.2 Oral squamous cell carcinoma

(A) Early SCC forming near the labial commissure; (B) Advanced ulcerative SCC, in which bony involvement was evident radiographically (A – B reproduced from Lamey & Lewis, 1997); (C) Ulcerative SCC affecting the lip; (D) Ulcerative SCC involving the floor of the mouth (E) Ulcerative SCC of the tongue (F) Exophytic SCC of the tongue (C – F reproduced from Langlais & Miller, 1998).



They can present as red or greyish ulcers with heaped up edges that rapidly undergo central necrosis. Exophytic lesions are rarer, slower-growing and less infiltrative. The appearance of exophytic tumours is often "frond-like" on the surface, and the tissue is friable and bleeds easily (Chen & Myers, 2001; Hyde & Hopper, 1999).

SCC is diagnosed by the histopathological examination of a representative biopsy of the cancerous tissue. In all SCCs, invasion into the underlying connective tissue is apparent. There is also an inherent risk that the malignant cells will erode the lymphatic and blood vessel walls and be transported to distant sites. This potential to metastasise is correlated with the histological variation or degree of differentiation of the tumour. Tumours containing comparatively normal, mature-looking cells and considerable amounts of keratin are described as being well-differentiated. Moderately-differentiated tumours produce little or no keratin yet still contain cells that are recognisable as being stratified squamous. Poorly differentiated SCCs produce no keratin and bear little resemblance to normal stratified squamous epithelium. The less differentiated the tumour, the more cellular abnormalities are present, and the greater the lack of normal architectural patterns. The degree of differentiation seems to be an important factor in determining the growth rate of the tumour and, generally, poorly differentiated carcinomas will infiltrate more widely and at an earlier stage, are more likely to metastasise, and carry a poorer prognosis (Cawson & Odell, 1998; Dimitroulis & Avery, 1998; Sapp *et al*, 1997).

Clinically, OSCC are described using the TNM staging system (see Table 1.1), which takes into account the staging of the primary tumour (T), the neck and condition of the lymph nodes (N), and an assessment for distant metastases (M). However, this system does not take into account the depth of invasion of the primary tumour and so is not always a suitable indicator of the prognosis for individual patients. Nevertheless, TNM classification remains the standard method of describing cancers and comparing the outcomes after treatment (Patel *et al*, 2003).

1.1.2.4 Tumour biology

Oral tumours, as with tumours found elsewhere in the human body, are a mass of clonogenic cells and supporting tissues such as connective tissues, blood vessels and nerves (Dimitroulis & Avery, 1998). Most known tumours, including human oral tumours, have a hypoxic and acidic microenvironment (Raghunand *et al*, 2003; Švastová *et al*, 2004). In head and neck squamous cell carcinomas, *in vitro* and *in vivo*, the expression of transcription factors such as VEGF and HIF-1α is increased under such hypoxic conditions, stimulating angiogenesis and promoting the development of solid tumours (Kyzas *et al*, 2005; Mohamed *et al*, 2004).

The tumour is typically surrounded by an abnormal stroma composed of fibroblast, vascular and local immune cells (Lewis *et al*, 2004; Mignogna *et al*, 2004a; Reichert *et al*, 2002). Cytokines and growth factors surround the neoplasia, influencing gene expression and the growth, death and differentiation of the cells. The cellular and protein milieu constituting a local tumour

Table 1.1 Clinical staging used in TNM classification of OSCC tumours

The clinical stagings used for the TNM classification of squamous cell carcinoma in the oral cavity. Tumours are assessed according to the staging of the primary tumour and the presence or absence of metastases. The information below is reproduced from Patel *et al* (2003).

Primary tumour (T)

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4 (lip)	Tumour invades adjacent structures, *e.g.* through cortical bone, inferior alveolar nerve, floor of mouth, skin of face
T4 (oral cavity)	Tumour invades adjacent structures, *e.g.* through cortical bone, into deep (extrinsic) muscle of tongue, maxillary sinus, skin.

Regional lymph nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension

Distant metastases (M)

MX	Presence of distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases

Stage grouping of oral cancer

Stage I	T1N0M0
Stage II	T2N0M0
Stage III	T3N0M0
	T3N1M0
Stage IV	T1-3N2M0
	Any T, N3, M0
	Any T, Any N, M1

microenvironment actively affects oral cavity tumour progression and the process of carcinogenesis (Knezevic *et al*, 2001; Mignogna *et al*, 2004a).

1.1.3 Aetiology of Oral Cancer

Oral cancer is a multifactorial disease where no single clearly recognisable cause has been found (Dimitroulis & Avery, 1998). Although several social habits and conditions have been associated with an increased risk of developing oral cancer, the precise role of any of the individual risk factors remains poorly understood. The major clinical risk factors are described here in further detail.

1.1.3.1 Tobacco

Tobacco use is the leading preventable cause of premature death worldwide (Warnakulasuriya *et al*, 2005). The predominant form of tobacco use in the West is smoking, either as factory-made cigarettes or as loose tobacco in pipes or hand-made cigarettes. Smoking is estimated to be the cause of 40 – 45 percent of all cancer deaths in industrialised countries, and 85 percent of oral cancer deaths (Johnson, 2001). However, a great deal of the world's tobacco is consumed without combustion, and all forms of tobacco use represent risk factors for oral cancer, not just smoking (Sham *et al*, 2003). There are two main types of smokeless tobacco, namely chewing tobacco and nasal snuff. In these cases, rather than via inhalation, the tobacco products are placed in the oral or nasal cavity where nicotine can be absorbed through the mucous membranes to obtain the pharmacological effect (Warnakulasuriya, 2004).

Over 300 carcinogenic chemicals have been identified in tobacco smoke or in the water-soluble components that will leach into saliva (Johnson, 2001). These carcinogens include metals and metal compounds, radio-isotopes, N-nitrosamines, aromatic amines, aldehydes, phenolic compounds, volatile hydrocarbons, and polynuclear aromatic hydrocarbons (PAH), which are contact carcinogens. PAHs include benzo-(a)pyrene, a powerful carcinogen which is metabolised by P450 isoenzymes, creating derivatives that can react with DNA to form adducts and initiate carcinogenesis.

Following the first exposure to tobacco, a delay of several decades is usually apparent before oral malignancies develop. Genetic polymorphisms may explain why some people are seemingly more susceptible to the carcinogenic effects of tobacco than others (Warnakulasuriya *et al*, 2005).

1.1.3.2 Alcohol consumption

Alcohol has long since been considered an important risk factor for oral cancer, despite the fact that, when examined in both animal studies and *in vitro,* pure ethanol shows no carcinogenic effects (Dimitroulis & Avery, 1998; Homann *et al*, 1997). Nevertheless, there is evidence that all forms of alcoholic drink are hazardous if heavily consumed, which would seem to suggest that it is the ethanol itself that is the aetiological factor (Johnson, 2001). The precise mechanism by which alcohol contributes to carcinogenesis remains unknown and its specific effects are difficult to see epidemiologically as most heavy drinkers are also tobacco users. It has been observed, however, that alcohol has a synergistic effect with tobacco and increases the risk of oral SCC super-multicatively (Hindle *et al,* 2000; Johnson, 2001; Lissowska *et al*, 2003). The

risk for smokers who are heavy drinkers is 6 to 15 times greater than that for non-smokers and non-drinkers (Reichart, 2001).

Several possible mechanisms have been suggested. For example, chronic consumption of alcohol may increase the permeability of the epithelium for tobacco-derived carcinogens (Ogden, 2005; Pöschl & Seitz, 2004). In an *in vitro* model, short-term exposure to 15 percent ethanol appeared to increase the permeability of the oral mucosa to tritiated water and also facilitated the passage of larger molecules such as albumin (Howie *et al*, 2001).

While ethanol is not itself carcinogenic, acetaldehyde, the first metabolite of ethanol, is carcinogenic in both animal models and *in vitro*. Acetaldehyde has been shown to produce mutagenic effects, such as DNA adducts, DNA cross-linking, aneuploidy, or chromosomal aberrations (Homann *et al*, 1997; Pöschl & Seitz, 2004). In humans, ethanol is converted to acetaldehyde by the enzymatic action of alcohol dehydrogenase (ADH) and eliminated by aldehyde dehydrogenase (ALDH). Further evidence of the role of acetaldehyde as the agent of alcohol-related carcinogenesis comes from studies of genetic polymorphisms. For instance, individuals carrying a heterozygous mutation of the gene encoding ALDH2 are at an increased risk of developing mucosal lesions and cancer of the upper aerodigestive tract (Muto *et al*, 2002; Pöschl & Seitz, 2004).

1.1.3.3 Areca nut in betel vine

The areca nut seed is used as the basic ingredient of several chewing products, used by approximately 200 – 400 million people throughout the world, especially in the Indo-Asian and Chinese populations (Warnakulasuriya, 2002).

Areca nut is often wrapped with tobacco in the leaf of the betel vine to form a quid for chewing. Slaked lime is also an ingredient in these betel quids, and is included to enhance the pharmacological effects by lowering the pH and accelerating the release of alkaloids from both the nut and the tobacco (Johnson, 2001).

That areca nut is most often chewed simultaneously with tobacco has complicated many studies to specifically see if betel quid chewing is an independent risk factor for oral cancer. Some evidence exists, however, to suggest that areca nut is separately correlated with an increased risk. Areca nut use has been positively associated with the development of precursor lesions such as oral submucous fibrosis, leukoplakia and verrucous lesions (Jacob *et al*, 2004; Shiu & Chen, 2004; Yang *et al*, 2005).

1.1.3.4 Viruses

Human papillomavirus (HPV) has been shown to be an important aetiological factor in cervical carcinogenesis (Motoyama *et al*, 2004), but its possible role in cancer of the oral cavity is still just hypothetical. DNA from the high-risk HPV types 16 and 18 has been detected in oral carcinomas and potentially malignant oral lesions, although its prevalence in such tissues varies considerably between reports (Giovannelli *et al*, 2002; Ha *et al*, 2002; Ha & Califano, 2004; Miller *et al*, 1994). The finding that oral keratinocytes can be immortalised with HPVs *in vitro* supports this circumstantial evidence of an association (Oda *et al*, 1996). However, the same HPVs have been found in normal oral mucosae and there is only slight evidence of HPV DNA being integrated into the host genome and of oncogene expression in OSCC. It has

been suggested, therefore, that HPVs contribute to only a small subset of oral cancers at most, and that they possibly only act in synergy with other carcinogens, such as alcohol, tobacco and betel quid (Ha & Califano, 2004; Sugerman & Shillitoe, 1997).

1.1.3.5 Fungal infections

Species of *Candida* are members of the oral microflora and are generally regarded as being commensals. However, they are capable of causing a range of opportunistic infections, referred to as candidoses, which are especially important in elderly, debilitated or immuno-compromised patients (Williams *et al*, 2001). Infection with *Candida* has been associated with malignant development in the oral cavity ever since it was found to cause candidal oral leukoplakias (Cawson, 1969; Sitheeque & Samaranayake, 2003) and correlate with oral epithelial dysplasia (McCullough *et al*, 2002). Candidal leukoplakia was observed in rats when their tongues were artificially inoculated with *Candida.* Long-term infection of the rat tongue resulted in hyperplasia and dysplasia of the epithelium (Sitheeque & Samaranayake, 2003). That epithelial dysplasia can improve following elimination of *Candida* from infected tissue also supports the idea of a causal link (Williams *et al,* 2001).

Candida-infected leukoplakias appear to have a higher rate of malignant transformation than other types (Reibel, 2003). Chronic hyperplastic candidosis, a form of candidosis characterised by hyphal invasion of the oral epithelium, is estimated to develop into a neoplasm in up to 10 percent of cases (Bartie *et al*, 2004). The exact role of *Candida* in malignant change is uncertain. However, nitrosamine compounds produced by *Candida* species may directly, or in concert

with other carcinogens, activate specific proto-oncogenes and thus initiate the development of a malignant lesion. N-nitroso-benzylmethylamine (NBMA) is a compound able to induce carcinoma of the oesophagus and the oral cavity in the rat (Fong *et al*, 1986). When strains of *Candida* were isolated from leukoplakia lesions and from normal mucosa, and then assessed for nitrosation potential, those with a relatively high potential for producing NBMA from salivary precursor molecules were comparatively more frequently isolated from lesions with more advanced precancerous changes (Krogh, 1990).

1.1.3.6 Intrinsic factors

Inherited genetic factors can increase people's susceptibilities to the effects of various chemical carcinogens. For instance, as mentioned previously, heterogeneity in the gene for ALDH appears to make a person more vulnerable to carcinogenic alcohol metabolites (Muto *et al*, 2002; Pöschl & Seitz, 2004). Recent studies have looked at genetic polymorphisms of specific carcinogen-metabolising enzymes (Chen & Myers, 2001). For example, enzymes in the cytochrome P450 (*CYP*) superfamily catalyse the oxidative metabolism of most endogenous and exogenous chemicals (Liu *et al*, 2005). Similarly, glutathione S-transferase (*GST*) enzymes are responsible for the detoxification of several metabolic intermediates, such as those that arise from the metabolism of tobacco carcinogens. Genetic polymorphisms in the gene encoding these enzymes, such as the *CYP*1A1 and *GST*M1-null genotypes, may predispose the lung and oral cavity of smokers to an even higher risk of DNA damage or carcinogenesis (Drummond *et al*, 2005; Liu *et al*, 2005; Sham *et al*, 2003; Sikdar *et al*, 2004).

1.1.3.7 Diet

The protective role against cancer of a healthy diet has been well-documented. Eating fruit, vegetables and fibre-rich food have all been shown to reduce the risk of developing cancers of the upper aerodigestive tract, including oral cancer (Rajkumar *et al*, 2003). Among fruit and vegetables the strongest protection seems to come from citrus fruits and tomatoes. More specifically, protection seems to come from antioxidants, such as vitamins A, C and E, which can scavenge the free radicals that could potentially damage cells (Calhoun *et al*, 1989; Lissowska *et al*, 2003; McLaughlin *et al*, 1988; Reichart, 2001).

The relationship between oral cancer and diets high in meat, either fresh or processed, is unclear. There is conflicting evidence from various studies, some showing a positive association (Rajkumar *et al*, 2003), and others showing an apparently protective effect (Lissowska *et al*, 2003).

1.1.3.8 Poor oral hygiene

Clinicians have long noticed an association between poor oral hygiene, poor dental status and oral cancer. Unfortunately, hard evidence of a correlation is difficult to come by as issues of socio-economic background, tobacco use, alcohol consumption, nutrition and other associates of cancer risk usually confound these factors (Johnson, 2001). Nevertheless, a few epidemiological studies have suggested that the number of teeth lost was an indicator of increased oral cancer risk (Lissowska *et al*, 2003). Furthermore, a lower risk was associated with increased teeth brushing and greater frequency of dental check-ups (Lissowska *et al*, 2003; Moreno-López, 2000; Velly *et al*, 1998). Additionally, at least one preliminary study has suggested an association

between periodontal disease and the presence of precancerous and neoplastic oral lesions. In an analysis of 13,798 subjects aged 20 years and older, clinical attachment loss was measured as a representation of the severity of periodontal disease and compared against three separate variables: the presence or absence of either a tumour, a precancerous lesion or another soft-tissue lesion in the oral cavity. Descriptive statistics suggested associations between periodontal disease and the risk for precancerous lesions and tumours (Tezal *et al*, 2005).

1.1.4 Carcinogenesis in the Oral Cavity

Research into the precise mechanisms that comprise the pathogenesis of OSCC suggests that cancer results from genetic damage, presumably caused by exogenous carcinogens. Damage to the DNA can lead to mutation, which can bring about a loss of function or aberrant expression of oncogenes and tumour-suppressor genes. An accumulation of dysregulated tumour-suppressor genes may release a stem cell from the normal cell cycle, allowing it to proliferate and prevent it from being killed by programmed cell death (Sugerman *et al*, 1995; Williams *et al*, 2000). Important tumour-suppressor genes linked to oral tumourigenesis include *p53*, *p16* or cyclin dependent kinase inhibitor 2/multiple tumour suppressor gene 1 (*CDKN2/MTS1*), *fas*, and the retinoblastoma (*Rb*) tumour suppressor gene (Nakahara *et al*, 2000; Pande *et al*, 1998; Tsai *et al*, 2001; Williams, 2000; Xu *et al*, 1998). For example, the reduced expression of *fas* seems to inhibit apoptosis of a malignant cell (Sugerman *et al*, 1995), while the deactivation of *Rb* seems to contribute to the hyperproliferation of oral keratinocytes (Niwa *et al*, 2001). Pathologically, the overexpression of *p53* in oral carcinoma, particularly in combination with high proliferative activity,

appears to be an important indicator of poor prognosis (Girod *et al*, 1998; Nylander *et al*, 2000). In the majority of cases where *p53* is overexpressed the gene contains a mutation that seemingly causes a loss of function of the P53 protein, altering its ability to effect apoptosis (Whyte *et al*, 2002).

Other key markers of oral carcinogenesis include the up-regulation of genes associated with inflammatory mediators such as cyclooxygenase (COX)-2 and tumour necrosis factor (TNF)-α, which is mediated at the molecular level through the activation of nuclear factor (NF)-κB (Fujiki *et al*, 2004; Wang, 2005).

1.1.5 Management Strategies for Patients with Oral Cancer

Patients with oral cancer require treatment from experienced physicians familiar with the ablative, reconstructive and rehabilitative principles intimately associated with the disease. Modern management places the onus on surgical skills, which embrace both tumour excision and functional reconstruction. Adjunctive radiotherapy is commonly required. In some cases it acts as the primary treatment. The rehabilitation of the patients involves a number of different specialties including cancer specialty nurses, dieticians, speech therapists, palliative physicians, physiologists, and care from family members as well as the primary medical and nursing teams in the hospital environment (Hyde & Hopper, 1999; Spencer *et al*, 2002). All this puts a heavy financial burden upon health authorities. However, there is a growing collection of evidence that these modern techniques have improved the quality of life experiences of those patients undergoing such treatment (Hammerlid & Taft, 2001; Hassanein *et al.*, 2001; Rogers *et al.*, 2001).

1.2 Bacteria and Cancer

1.2.1 The Presence of Bacteria Within Human Tumours

The presence of bacteria in human tumours has been recognised for more than 70 years. Since 1931, there have been countless reports of large numbers of viable bacteria having been found in various types of malignant tumour excised from patients (Broxmeyer, 2004; Cantwell & Kelso, 1981; Wainwright, 1999). More recently, using bacteria labelled with light-emitting proteins, several species have been shown to successfully survive and propagate within solid tumours in animal models (Yu *et al*, 2004). Strains of *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium,* and *Vibrio cholerae* were all intravenously injected into live mice and seen, in real time, to enter the tumour tissues and replicate within them. Furthermore, the survival of the bacteria was site-specific; despite the bacteria becoming well-distributed throughout the animals in the first few minutes following injection, within less than a week the labelled bacteria were replicating only inside the tumour tissue. Such selectivity was presumably because any bacteria were effectively shielded from the host immune system while within the solid tumour (Yu *et al*, 2004). It has been suggested that this "tumour-finding" ability of the bacteria may offer new methods to detect cancer and to deliver gene therapy treatments specifically to tumour locations (Lemmon *et al,* 1997; Yu *et al*, 2004).

The possible significances of bacteria living within malignant or premalignant tumours are unclear. Nevertheless, it is of interest to know what microorganisms are carried by cancer patients as the possibility of infection is an important consideration. Patients undergoing treatment for cancer often have

impaired cellular immunity and so are particularly vulnerable to opportunistic infections, including septicaemia, pulmonary infection and infection of the central nervous system (Klastersky & Aoun, 2004).

More recently, bacteria have been implicated as having possible roles in different aspects of cancer development. If bacteria are suspected of being capable of causing cancer, then it is surely necessary to identify those species that can be found within tumours.

1.2.2 The Role of Bacteria in the Development of Cancer

Carcinogenesis is a multi-step process (Lax & Thomas, 2002; Warren *et al*, 1993) and hence there are many stages at which the development of cancer can be promoted or inhibited. Considerable evidence exists to suggest that there are several species of bacteria capable of encouraging the initiation, promotion or progression of human malignancies. Some examples of how bacteria may be associated with carcinogenesis in humans are described below.

1.2.2.1 Epidemiology of bacterial infections and cancer

Much of the evidence linking specific species of bacteria to carcinogenesis is epidemiological in nature. Perhaps the most famous example is that of the common pathogenic bacterium *Helicobacter pylori* and its association with gastric cancer. Gastric cancer arises when the normal mucosa develops into gastritis, which can then lead to atrophy, intestinal metaplasia, dysplasia, and adenocarcinoma. Infection with *H. pylori* was found to correlate with the incidence of gastritis, indicating its involvement in the initiation and promotion of gastric adenocarcinoma in a subset of patients. This hypothesis

was subsequently supported by numerous clinical and animal studies and, in 1994, *H. pylori* became the first species of bacteria to be recognised by the World Health Organization International Agency for Research on Cancer (IARC) as a definite cause of cancer in humans (Björkholm *et al*, 2003; Peek & Blaser, 2002).

Since then an increasing number of possible associations between different types of bacteria and cancer have been reported. For instance, a prior cervical infection with *Chlamydia trachomatis* has been associated with an increased risk for the development of invasive cervical cancer (Wallin *et al*, 2002). *Chlamydophila* (formerly *Chlamydia*) *pneumoniae* infections have been linked with both malignant lymphoma and male lung cancer patients (Anttila *et al*, 1998; Kocazeybek, 2003). Patients with *Streptococcus bovis*-caused infectious endocarditis seemingly have a greater risk of developing colonic carcinoma (Ellmerich *et al*, 2000; Waisberg & Matheus, 2002). In addition, there is a significantly increased risk of developing carcinoma of the gallbladder in patients infected with *Salmonella typhi* (Dutta *et al*, 2000; Shukla *et al*, 2000).

1.2.2.2 Infection, inflammation and carcinogenesis

A critical discovery from recent molecular investigations into carcinogenesis has been the importance of inflammation to the entire process. The inflammatory microenvironment surrounding the tumour is now known to actively participate in the induction, selection and expansion of neoplastic cells (Mignogna *et al*, 2004a). These findings have led to a better understanding of the observed links between several chronic inflammatory disorders and the development of cancer. At the clinical level several such associations have been

known for a long time. For example, inflammation appears to increase the risk of cancer at many different body sites, including the pancreas (Whitcomb, 2004), stomach (Zavros *et al*, 2004), colon (Itzkowitz & Yio, 2004), liver (Rogers & Fox, 2004), bladder (Rosin *et al*, 1994), prostate (Palapattu *et al*, 2004) and ovaries (Ness & Cottreau, 1999). Epithelial inflammation is also a factor in oral carcinogenesis, and it is thought that inflammation is the mechanism that links the use of smokeless tobacco products and areca nut extract to an increased risk of cancer (Jeng *et al*, 2003; Vishwanatha *et al*, 2003).

Inflammation can have several effects on cancer. Acute inflammation has been said to counteract cancer, while, on the other hand, chronic inflammation has been seen to promote cancer development (Philip *et al*, 2004). Whereas acute inflammation is characterised mainly by vascular changes, chronic inflammation is recognised as a process of tissue repair and destruction and by the damaged tissue becoming infused with mononuclear cells such as lymphocytes, plasma cells and macrophages. Chronic inflammation is often as such from the outset, but may also develop from an acute inflammatory response if the causal agent persists. Any persistent stimulus of the immune system, including chemical irritants such as asbestos and silica or an infection by a viral or bacterial pathogen, is capable of causing chronic inflammation that can often last for years (Christen *et al*, 1999; Macarthur *et al*, 2004).

Once activated, macrophages release into the vicinity of the inflamed tissue a great number of bioactive molecules, which form part of the body's defence against injury and invasion. Such inflammatory mediators include enzymes, complement proteins, coagulation factors, growth factors, cytokines, reactive metabolites of oxygen and nitric oxide. In the short term these mediator

macromolecules and oxidants offer protection by destroying invading pathogens, but their continuing presence can also be harmful to the host. Cytokines and growth factors can have the knock-on effects of inhibiting apoptosis and enhancing cell proliferation, both of which, can promote mutation and carcinogenesis (see section 1.2.2.3) (Christen *et al*, 1999; Coussens & Werb, 2002; Macarthur *et al*, 2004).

Reactive oxygen metabolism is an important aspect of inflammation-related carcinogenesis. The activation of phagocytes results in the induction of key enzymes such as NADPH oxidase and nitric oxide synthase (NOS), which then catalyse the production of large amounts of oxygen metabolites, including superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Conner & Grisham, 1996; Maeda & Akaike, 1998; Ohshima *et al*, 2003). Such reactive molecules can cause damage to a number of biological macromolecules, for instance DNA-strand breakage, alteration of the structure of lipids and the nitration of proteins and nucleotides. Damage on this level is not only cytotoxic but can cause DNA mutation, and can potentially turn a benign tumour into a malignant one (Okada, 2002). Accumulation of such mutations can promote the development of invasive tumours and carcinogenesis, especially if the DNA damage affects key oncogenes such as the p53 tumour suppressor gene or the endogenous DNA repair systems (Christen *et al*, 1999; Itzkowitz & Yio, 2004; Jaiswal *et al*, 2001; Maeda & Akaike, 1998). Reactive oxygen metabolites also appear to upregulate the production of pro-inflammatory enzymes and cytokines, thereby promoting a cycle of inflammatory mediators and amplifying the inflammation response (Conner & Grisham, 1996).

One of the most prominent mediators of inflammation is the cyclooxygenase pathway. Cyclooxygenase is a rate-limiting enzyme in the biosynthesis of prostaglandins and thromboxanes, a reaction that also generates reactive oxygen species. Prostaglandins are important for the initiation, promotion and progression of carcinogenesis, and can also suppress the immune action responsible for killing the malignant cancer cells (Jeng *et al*, 2003). In humans it is found in two isoforms, namely COX-1 and COX-2. Whereas the gene for COX-1 is expressed ubiquitously, COX-2 is normally undetectable in most tissues but is inducible by a variety of stimuli including mitogens, growth factors and cytokines (Hussain *et al*, 2003; Pathak *et al*, 2005). COX-2 is up-regulated in inflamed tissues (Morton & Dongari-Bagtzoglou, 2001) and also in most cancers of the body sites (Hussain *et al*, 2003). Increased expression of COX-2 is also observed in cancer and premalignant lesions of the oral cavity (Wang, 2005). Recent studies have demonstrated that overexpression of COX-2 is sufficient to induce breast tumours in transgenic mice, providing more evidence that inflammation can act as a promoter of cancer development (Liu *et al*, 2001).

Further evidence of the significance of inflammation during the progression of carcinogenesis comes from the observation that the use of anti-inflammatory drugs can reduce the likelihood of developing cancer. For instance, long-term use of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) appears to reduce the risk of colon cancer by 40-50% (Coussens & Werb, 2002). NSAIDs and other inhibitors of cyclooxygenase provide a possible opportunity for anti-cancer therapy (Evans & Kargman, 2004).

Recent estimates indicate that inflammation related to chronic infection is responsible for 21% of new cancers in the developing world and 9% in the developed world (Christen *et al*, 1999). This figure includes infection with viruses, *e.g.* hepatitis B and C viruses causing liver cancer (Rogers & Fox, 2004), and parasitic infection by organisms like schistosomes or liver flukes (Christen *et al*, 1999), as well as bacterial infection such as is seen in *Helicobacter pylori*-related gastric cancer.

The most recent research indicates that it is the mechanisms of chronic inflammation that are responsible for the observed link between *H. pylori* and carcinogenesis. Infections with *H. pylori*, especially with strains positive for the cagA virulence factor, predictably cause an inflammatory response, which includes the induction of COX-2 expression (Sun *et al,* 2004) and the invasion of the local tissue by neutrophils and phagocytes, accompanied by the production of proinflammatory cytokines (Björkholm *et al*, 2003; Peek & Blaser, 2002). Peptides from *H. pylori* have also been shown to induce the activation of NADPH oxidase and produce oxygen radicals (Betten *et al*, 2001).

Other bacteria can also cause inflammation in the stomach and so may also play a role in gastric carcinogenesis. Infection of the stomach with *Acinetobacter lwoffi*, for example, can also result in chronic gastritis independently of *H. pylori* (Rathinavelu *et al*, 2003).

Several other associations between certain bacteria and cancers are based on inflammatory mechanisms. For instance, it has been suggested that *Propionibacterium acnes* infection may possibly be linked with the development of prostate cancer. *Propionibacterium acnes* was positively associated with a higher degree of prostatic inflammation, a condition which has in turn been

implicated with carcinogenesis (Cohen *et al,* 2005). *Propionibacterium acnes* is known to stimulate the production of inflammatory mediators (Graham *et al,* 2004; Nagy *et al,* 2005).

The induction of inflammation may also explain the observed links between infections with *Chlamydophila pneumoniae* and *Streptococcus bovis* and an increased risk of lung and colon cancer, respectively. *Chlamydophila pneumoniae* can infect human lung epithelial cells and induce the expression of pro-inflammatory cytokines, including IL-8, IFN-γ and TNF-α (Yang *et al,* 2003). Similarly, *Str. bovis* releases proteins that are able to stimulate intestinal cells to produce inflammatory mediators such as IL-8 and PGE_2. This has also been seen to promote the progression of preneoplastic lesions in the colonic mucosa of rats (Ellmerich *et al,* 2000; Biarc *et al,* 2004).

The multitudes of bacteria that reside in the human oral cavity do so without necessarily causing inflammation. However, as our knowledge of periodontal disease shows, given the correct circumstances some species of oral bacteria can initiate inflammation in their host (Delima *et al,* 2002; Tlaskalová-Hogenová *et al,* 2004). For example, *Porphyromonas gingivalis* can induce COX-2 expression (Kuramitsu *et al,* 2002) and bring about an increased production of proinflammatory mediators such as tumour necrosis factor alpha and cytokines including interleukins 6, 8 and 1β (Andrian *et al,* 2004). Likewise, the periodontopathic species *Eikenella corrodens* is able to stimulate human oral epithelial cells to produce various mediators including interleukins 6 and 8, and prostaglandin E2, seemingly via the secretion of soluble proteins (Yumoto *et al,* 2001). In addition, periodontitis patients testing positive for salivary *Streptococcus anginosus* also exhibit significantly higher levels of 8-

hydroxy-deoxyguanosine (8-OHdG). 8-OHdG is a product of oxidative DNA damage, arising from photodynamic action or the actions of singlet oxygen or hydroxyl radicals, and is a commonly used marker for evaluating inflammation and oxidative damage. Increases in 8-OHdG levels have previously been associated with human pre-malignant lesions and cancerous tissues (Sugano *et al*, 2003).

1.2.2.3 Cellular microbiology and cancer

The relatively-new discipline of cellular microbiology focuses on the myriad of interactions between bacteria and the cells of their host. It is these interactions, or disruptions of the normal two-way communications, that can cause bacterial diseases (Henderson, 2002). Several species of bacteria been discovered to directly interfere with eukaryotic cellular signalling in a way that is characteristic of tumour promoters (Lax & Thomas, 2002). Examples of these are discussed in more detail below.

1.2.2.3.1 Increased proliferation of eukaryotic cells

Cellular proliferation has a pivotal role in carcinogenesis. Mutations in DNA regularly arise from exposure to exogenous or endogenous mutagens. Hyperproliferation simultaneously reduces the time available to repair any of these mutations and also increases the risk of spontaneous mutation due to errors in DNA replication. The likelihood that mutations get transmitted into the next generation is thereby significantly increased (Butterworth & Goldsworthy, 1991).

Several, phylogenetically unrelated species of bacteria are known to increase the proliferation of eukaryotic cells, directly via the secretion of virulence factors. For instance, *Pasteurella multocida*, a commensal of the digestive and respiratory tracts of many warm-blooded animals, releases a protein referred to as PMT (*Pasteurella multocida* toxin). This toxin shows a high degree of homology to proteins secreted by other species of bacteria such as the cytotoxic necrotising factor (CNF) from *Escherichia coli* (Pullinger *et al*, 2001). PMT has been described as the most potent eukaryotic mitogen ever reported (Henderson *et al*, 1998). It is the main virulence factor in the porcine disease atrophic rhinitis and can induce the proliferation of quiescent cells at picomolar concentrations. Human growth factors, such as platelet derived growth factor (PDGF), require more than 300 times the concentration of PMT to achieve the same level of effect (Lax & Grigoriadis, 2001). In addition, PMT displays anti-apoptotic action (see section 1.2.2.3.2).

Bartonella henselae, formerly known as *Rochalimaea henselae*, is a Gram-negative rod responsible for zoonotic infections in animals as well as humans. *Bartonella* species are more famous in humans as the cause of cat-scratch fever and can induce the proliferation and migration of vascular endothelial cells via the release of soluble protein factors. As a result large vasoproliferative tumours, termed bacillary angiomatosis, develop (Dehio, 1999; Maeno *et al*, 1999).

It is thought that one of the mechanisms by which *Helicobacter pylori* is able to cause gastric cancer is by over-stimulating the proliferation of the gastric epithelium. This pathogen induces its host to over-produce gastrin, a hormone

which increases epithelial cell propagation, promoting tumourigenesis (Peek & Blaser, 2002).

As well as *Helicobacter pylori* colonisation, several other bacterial infections are known to cause hyperplasia in the gastrointestinal epithelium of mammals. *Lawsonia intracellularis* is the causative agent of proliferative enteropathy, prevalent in pigs, and has been implicated in the aetiology of ulcerative colitis in humans (McCluskey *et al*, 2002). *Citrobacter rodentium* is a naturally occurring pathogen of laboratory rats and has been shown to be capable of inducing the proliferation and promotion of colonic adenomas initiated by chemical carcinogens in epithelial cell populations. Although not itself a pathogen of humans *C. rodentium* has a hyperproliferative effect similar to that seen in human inflammatory bowel diseases, which are known to lead to a higher risk of colorectal cancer (Higgins *et al*, 1999; Luperchio & Schauer, 2001; Newman *et al*, 2001). However, unlike *H. pylori*, both *L. intracellularis* and *C. rodentium* infect intestinal epithelia with only a minimal, if any, inflammatory response (Lax & Thomas, 2002). This would seem to indicate that bacterial infection-mediated hyperproliferation is not just a by-product of inflammation and is an independent factor in cancer promotion.

It appears that common members of the oral cavity microflora can also promote the proliferation of host cells. *Porphyromonas gingivalis*, a notorious pathogen associated with periodontal disease, contains proteins and lipopolysaccharides on its outer surface that have been shown to stimulate human fibroblasts to proliferate *in vitro* (Mihara *et al*, 1993; Putnins *et al*, 2002; Takemura *et al*, 1998).

1.2.2.3.2 Suppression of programmed cell death

Apoptosis, or programmed cell death, is the mechanism by which multicellular organisms dispose of damaged and atypical cells in response to physiological and pathologic stresses. When a cell is transformed and becomes such that homeostasis and the intricate system of cell signalling is disrupted, apoptosis can be triggered, destroying the cell and preventing it from developing into a malignant tumour. Thus, any agent that can impede apoptosis promotes the atypical build-up of cancerous cells (Dixon *et al*, 1997; Lowe & Lin, 2000). There have been several examples of bacteria suppressing apoptosis and potentially promoting carcinogenesis in this way.

Escherichia coli releases a range of virulence factors. One of these, cytotoxic necrotizing factor type 1 (CNF1), prevents apoptosis in epithelial cells ostensibly by activating a cascade of cell signalling involving the Rho GTP-binding protein (Rippere-Lampe *et al*, 2001). This has the ultimate affect of promoting the expression of anti-apoptotic members of the Bcl-2 gene family (Fiorentini *et al*, 1998).

Chlamydophila pneumoniae-infected epithelial cells are resistant to apoptosis induced by chemicals or death receptors. This is thought to be due in part to its ability to induce the expression of IL-10, which can down-regulate the expression of major histocompatibility complex class I molecules (Yang *et al*, 2003).

Species of *Mycoplasma* such as *M. fermentans* and *M. penetrans* have been shown to prevent apoptosis *in vitro*. Cultures of the murine myeloid cell line 32D ordinarily undergo apoptosis upon withdrawal of interleukin 3 (IL-3) from the culture medium. However, this phenomenon does not occur when 32D

is cultured in the presence of either live or heat-killed *Mycoplasma* cells. Furthermore, when infected with live *Mycoplasma* for periods of 4 to 5 weeks, 32D cells underwent malignant transformation, after which they required neither IL-3 supplements nor *Mycoplasma* to survive (Feng *et al*, 1999). *M. fermentans* has also been shown to inhibit apoptosis in a human cell line, the myelomonocytic U937 cell line, by affecting the TNF-α signalling pathway (Gerlic *et al*, 2004). A number of mycoplasmal species, including *M. fermentans*, have been detected as members of the normal oral microflora in saliva, on the mucosal surfaces and in plaque (Chingbingyong & Hughes, 1996; Paster *et al*., 2001; Shibata *et al*, 1999). However, at the time of writing, there is no evidence to link *Mycoplasma*-related suppression of apoptosis with carcinogenesis of the oral cavity.

1.2.2.4 The activation of carcinogens via bacterial metabolism

The majority of known chemical carcinogens need to be metabolised before they are able to interact with cellular macromolecules and initiate carcinogenesis in humans. This process of activation can potentially be performed by the hosts' own cells, via the action of xenobiotic-metabolising enzymes (Raunio *et al*, 1995), or by local bacterial species.

Laboratory and epidemiological studies have indicated an association between colon cancer and the bacteria that reside within the human intestinal tract, and it has been suggested that this relationship may depend on such a mechanism (Gorbach & Goldin, 1990). Heterocyclic aromatic amines (HAs) and other pro-carcinogenic chemicals, which are found in common foodstuffs such as cooked meats and fish, are frequently ingested. Much work on the

effects of different dietary mixtures on the health of laboratory animals has implicated HAs as initiators of carcinogenesis and shown them to be capable of producing tumours at multiple organ sites (Felton *et al*, 1997; Knasmüller *et al*, 2001). Furthermore, studies have indicated that intestinal bacteria have a strong impact on the genotoxicity of at least one of these carcinogenic HAs: DNA damage caused by the HA chemical 2-amino-3-methylimidazo[4,5,f]quinoline (IQ) was 3 to 5-fold greater in rats containing a natural microflora as opposed to germ-free specimens (Kassie *et al*, 2001). It should be noted however, that this effect is dependent on the species comprising the microflora of the individual. For example, whereas *Bacteroides fragilis* can cause a distinct increase in mutagenicity in the presence of HAs, lactobacilli species result in a decrease (Knasmüller *et al*, 2001).

Similarly, non-carcinogenic ethanol can be converted into the carcinogenic derivative acetaldehyde. This reaction can be catalysed by alcohol dehydrogenase enzymes in the epithelium (Homann *et al*, 1997; Pöschl & Seitz, 2004), and also by microorganisms. Gram-positive aerobic bacteria, *Neisseria* species and yeasts have all been seen to produce acetaldehyde, increasing the risk of cancer of the aerodigestive tract (Homann *et al*, 2000; Muto *et al*, 2000; Salaspuro, 2003).

1.3 The Oral Microflora and Carcinogenesis in the Oral Cavity

1.3.1 Microorganisms Associated with Head and Neck Cancer

The oral cavity has a diverse natural microflora, which for the most part exists harmoniously with its host. An estimated 500 – 600 bacterial species

reside on the oral surfaces of humans (de Lillo *et al,* 2004; Paster *et al*, 2002), making the oral cavity an obvious target for bacteria-induced pathogenesis. Considering that poor oral hygiene and periodontitis may be independent risk factors for head and neck cancer (Lissowska *et al*, 2003; Moreno-López *et al*, 2000; Tezal *et al*, 2005; Velly *et al*, 1998), it is perhaps especially surprising to realise that the relationship between oral bacteria and the development of cancer has not yet been extensively investigated.

Several reports have observed that significant numbers of patients with intraoral cancer have had "abnormal" bacterial flora, containing noticeable numbers of potential pathogens, both before and after treatment (Buckley *et al*, 1998; Gill & Rice, 1975; Rice & Gill, 1976). Furthermore, at least one study has observed that oral cancer patients who maintain a normal flora have a better prognosis than those who do not (Rice & Weimert, 1978). Since these original observations, only a few investigations into the relationship between oral cancer patients, or patients with a high risk of developing oral cancer, and their intraoral microflora have been reported. The findings of these studies are described below.

1.3.1.1 Changes in oral microflora associated with cancer risk factors

The observation that current smokers were about four times more likely to have periodontitis than non-smokers (Johnson & Slach, 2001) has prompted the theory that tobacco use may alter the levels of certain pathogenic bacteria. The idea of periodontal pathogens being a possible link between smoking and the aetiology of oral cancer is further supported by the apparent correlation between the occurrences of periodontitis and oral neoplasms (Tezal *et al*, 2005).

However, different studies into the composition of the subgingival microflora in smoking and non-smoking periodontitis patients report conflicting findings. For instance, in some groups of patients statistically higher risks of infection with such pathogenic species as *Treponema denticola, Tannerella forsythensis* (formerly *Bacteroides forsythus*), *Prevotella intermedia, Porphyromonas gingivalis, Peptostreptococcus micros, Fusobacterium nucleatum, Eikenella corrodens, Campylobacter rectus* and *Actinobacillus actinomycetemcomitans* were found in smokers than non-smokers (Shiloah *et al*, 2000; van Winkelhoff *et al*, 2001). Yet, in other, molecular-based comparisons of the prevalence of these species plus *Prevotella nigrescens, Selenomonas noxia* and *Streptococcus intermedius*, no significant differences were found between smoking and non-smoking cohorts (Apatzidou *et al*, 2005; Darby *et al*, 2000; Natto *et al*, 2005). Recent evidence suggests that, rather than the presence or absence of specific microorganisms, it is the host response to bacterial challenge that determines susceptibility to periodontitis (Van Dyke & Sheilesh, 2005).

Nevertheless, there is some evidence that cigarette smoking can lead to the selective growth of more carcinogenic strains, if not species, of bacteria. For example, in one study all but a few oral strains of *Staphylococcus aureus* did not grow in the presence of cigarette-smoke condensates. At least one of these tar-resistant isolates was found to be able to induce inflammatory mediators, such as TNF-α, *in vitro* (Fujiki *et al*, 2004). The increased production of TNF-α is an important part of inflammation and has been heavily implicated in oral carcinogenesis (Jeng *et al*, 2003), suggesting that the tar-resistant *S. aureus* may be carcinogenic. The reasons behind any possible correlation between

Additionally, PCR has been used to detect *Streptococcus mitis* and *Treponema denticola* in esophageal carcinoma tissues. These species were found in 60 out of 69 (87%) and 27 out of 69 (39%) such specimens, respectively, possibly indicating their preferential infection of cancerous tissues (Narikiyo *et al*, 2004).

1.3.2 Bacteria from the Oral Cavity Implicated in Carcinogenesis

Comparatively little research has been done to specifically investigate how bacteria in the oral cavity may promote carcinogenesis. However, two theoretical mechanisms have been putatively studied *in vitro* and are worthy of note. These are described below.

1.3.2.1 Salivary microbes as a source of acetaldehyde

Heavy consumption of alcohol is an important risk factor for cancers of the upper gastrointestinal tract, including oral carcinoma. As mentioned previously, ethanol by itself is not carcinogenic but can be converted to the carcinogenic derivative acetaldehyde by the action of ADH enzymes in the epithelium (Homann *et al*, 1997; Pöschl & Seitz, 2004). Following ingestion of alcohol, acetaldehyde may also be formed in high concentrations in saliva by the action of the oral microflora. To date primarily Gram-positive aerobic bacteria and yeasts have been associated with acetaldehyde production (Homann *et al*, 2000; Salaspuro, 2003). Additionally, *Neisseria* species, traditionally regarded as non-pathogenic residents of the oral cavity, have been reported to exhibit extremely high levels of ADH activity and produce significant amounts of acetaldehyde in the presence of ethanol (Muto *et al*, 2000). Moreover, salivary

analysis shows that increases in microbial acetaldehyde production correlate with smoking and heavy alcohol consumption. This discovery is of particular interest given its obvious links with the known risk factors for oral cancer as it offers a feasible mechanism for the pathogenesis of alcohol and tobacco-related carcinogenesis. Despite these implications, higher acetaldehyde production, microbial-based or otherwise, has not been demonstrated in patients with oral cancer (Homann *et al*, 2000).

1.3.2.2 Oral streptococci as a cause of inflammation

Species of *Streptococcus* isolated from carcinoma tissues (see section 1.3.1.3) have been found to be capable of promoting an inflammatory response. *S. anginosus* and *S. mitis* were observed to induce the production of inflammatory cytokines in human esophageal epithelial cell lines (Narikiyo *et al*, 2004). Similarly, supernatants from cultures of *S. anginosus* strain NCTC 10713 contained an antigen which was found to induce nitric oxide synthesis as well as produce inflammatory cytokines in murine peritoneal exudate cells (Sasaki *et al*, 2001). Periodontitis patients whose saliva tested PCR-positive for *S. anginosus* have also been found to exhibit significantly higher levels of 8-hydroxy-deoxyguanosine (8-OHdG), a commonly used marker for evaluating inflammatory cell infiltration and oxidative DNA damage, than patients negative for the bacterium. Although the salivary levels of *Str. anginosus* were relatively low, there was a correlation between the level of *Str. anginosus* and 8-OHdG (Sugano *et al*, 2003). It has been hypothesised that *Str. anginosus* in particular may play a significant role in many cases of esophageal cancer by causing inflammation and promoting the carcinogenic process (see section 1.2.2.2).

Eradication of these streptococci may decrease the risk of recurrence of oesophageal cancer (Narikiyo *et al*, 2004).

1.4 Aims

In summary, oral cancer is a lethal disease with an increasing incidence that cannot be wholly explained by the traditional risk factors. There is increasing interest in the relationship between bacteria and the different stages of cancer development, yet the association of bacteria with cancer of the oral cavity has yet to be adequately examined. To date there have only been a few investigations into the microflora present within oral neoplasms and these have been screening for specific species. Therefore, with this is mind, the broad objectives of the work in this thesis were:

(i) To design protocols with which any microorganisms present within tissue from oral cancers could be detected. In order to theoretically detect all species present, a combination of culture and molecular approaches were to be used.

(ii) To identify any bacterial isolates to species-level, wherever possible, in order to facilitate the observation of trends and correlations.

(iii) To similarly characterise the bacteria within both tumourous and non-tumourous tissue, so as to enable comparison between the microbiota in both cancerous and 'normal' mucosa.

CHAPTER TWO

CULTIVATION OF THE MICROFLORA FROM WITHIN TISSUE FROM ORAL SQUAMOUS CELL CARCINOMA

2.1 Introduction

It has been demonstrated that the microflora associated with the surface of intra-oral squamous cell carcinomas (SCC) differs in comparison to control sites. *Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces, Clostridium, Haemophilus*, *Enterobacteriaceae*, *Streptococcus* and *Candida* species have all been cultured in increased numbers from the biofilms present at the SCC tumour sites (Nagy *et al*, 1998). It has been suggested that these changes in the surface microflora may be important when considering the risk of local and systemic infections, which may complicate the morbidity of the patient. However, at the time of writing, no attempts to culture the microorganisms present within such oral tumours have been previously reported. Since the development of solid culture media in the 19th century, microorganisms have routinely been isolated from human disease sites and physically separated into pure subcultures (Wade, 2002). In the last 150 years the nutritional and physiological requirements of thousands of species have been elucidated and replicated *in vitro*. Most of these microbes have been extensively characterised as a consequence of being able to cultivate them, allowing relationships between microbes and diseases to be recognised. In addition, the cultivation of microorganisms has allowed the development of other, now commonplace, medical diagnostic techniques and non-cultural assays (Wilson *et al*, 1997). Although much research now uses culture-independent techniques, cultural isolation remains a vital part of current detection and identification protocols.

Correctly identifying microbial isolates is an important practice in clinical microbiology. Knowing the correct designation of microorganisms

associated with both the health and disease states of the body can give valuable insight into the aetiological agents of infectious diseases (Clarridge, 2004). Traditionally, prokaryotic organisms have been characterised, classified and identified by such classic taxonomic methods as morphology and carbon source utilisation (Kolbert & Persing, 1999). However, these conventional methods can sometimes fall short when used to identify clinical isolates of bacterial pathogens. Atypical isolates, organisms with little or no unique metabolic reactivity, or strains that exhibit unusual phenotypic profiles may not be accurately identified (Drancourt *et al*, 2004; Kiratisin *et al*, 2003; Kolbert & Persing, 1999). As a way of overcoming this, an increasing number of diagnostic laboratories are using 16S ribosomal RNA (rRNA) gene sequence analysis as the preferred method of isolate identification.

Sequencing the small subunit (16S) rRNA gene is a ubiquitous method for identifying bacteria and, for many years, has been used by bacterial taxonomists to assess the relative positions of different organisms in the evolutionary order (Kolbert & Persing, 1999; Patel, 2001). Several features of the 16S gene make it a useful target for both clinical identification and phylogenetic analysis. Firstly, as 16S rRNA is a critical component of cell function, the gene has been well-conserved throughout evolution and is present in all bacteria. Secondly, the gene is composed of both constant and variable regions. Whilst the variable regions are known to contain enough interspecies polymorphisms to be distinctive, the constant regions are sufficiently conserved between species to provide ideal targets for universal oligonucleotide primers. Finally, at approximately 1500 base pairs (bp) in length, the 16S rRNA gene is both long enough to contain adequate sequence information to be statistically

significant and short enough to make sequencing a fairly straightforward process. In fact, there is enough sequence variability in the first 500 bp to differentiate between most species and so, for practical reasons, most identifications are made using 500 bp sequences (Clarridge, 2004; Lane *et al,* 1985; Patel, 2001; Wade, 2002).

The 16S rRNA gene sequence data has been ascertained for a large number of strains. At the time of writing there are around 100,000 16S rRNA gene sequences available in public databanks (Cole *et al*, 2005; Clarridge, 2004), meaning there are many reference sequences to compare the sequence of an unknown strain against. With the introduction of rapid and relatively low-cost DNA amplification and sequencing technologies, this method is likely to become the principal method for routine identification of microorganisms in clinical laboratories (Kiratisin *et al*, 2003; Kolbert & Persing, 1999).

2.2 Aims

The aim of this study was to determine whether viable microorganisms are present within tumourous and non-tumourous tissue from patients with oral squamous cell carcinoma (OSCC). Any organisms cultivated were identified as closely to species-level as possible using current 16S rRNA gene sequence analysis methodologies.

2.3 Materials and Methods

2.3.1 Acquisition of tissue specimens

Ethical approval for the study was granted by the South Wales LREC and subjects agreed to participate by informed consent. All tumours were removed surgically and, under the same aseptic conditions, specimens from the resected tumour were harvested. The technique involved the surgeon rescrubbing and placing the specimen on a separate sterile surface. With a new blade for each cut, a 1 cm^3 specimen was removed without compromising the pathological margins. This specimen was further divided into a 'deep tissue' specimen (consisting entirely of tissue from within the tumour mass) and a 'superficial' specimen (consisting of tissue from within and from the surface of the tumour). Whenever possible, a control specimen consisting of non-tumourous tissue harvested at least 5 cm away from the primary tumour site was also obtained. Details of patients from whom tissue was harvested are shown in Table 2.1. . There were 16 male and 4 females patients with an average age of 66.9 (± 12.7).

Specimens were aseptically transferred to the laboratory in separate vials of reduced transport media (RTM), used to maintain the viability of any bacterial cells present. RTM comprised of tryptone (1% w/v, Oxoid), yeast extract (0.5% w/v, Oxoid), glucose (0.1% w/v, BDH Ltd.), cysteine hydrochloride (0.1% w/v, BDH Ltd.), sodium hydroxide (50 mM, Sigma), and horse serum (2% v/v, TCS Ltd.), and was sterilised by filtration using a 0.2 μm filter.

Table 2.1 Details of patients from whom tissue specimens were acquired in this study

Some details of the OSCC patients from which specimens were obtained. The type of specimen, *i.e.* comprising of either just tumourous ("deep tumour"), tumour and overlying mucosal ("superficial"), or entirely non-tumourous tissue, successfully harvested and surface decontaminated from each patient is also shown. Tumours were classified clinically using the TNM system, details of which can be seen in Table 1.1.

Patient No.	Sex	Age at time of surgery (years)	Specimens obtained			Tumour classification		
			Deep tumour	Superficial	Non-tumourous	T	N	M
1	M	54	+	+	-	2	1	0
2	M	56	+	+	-	2	1	0
3	M	66	+	+	-	2	0	0
4	F	95	+	+	-	3	2	0
5	F	72	+	+	-	2	0	0
6	M	71	+	+	-	2	0	0
7	M	48	+	-	-	2	0	0
8	F	56	+	+	+	2	0	0
9	M	86	+	+	-	2	1	0
10	M	81	+	+	+	3	2	0
11	M	65	+	+	+	2	1	0
12	M	64	+	+	+	2	0	0
13	M	63	+	+	+	2	1	0
14	M	54	+	+	+	3	1	0
15	M	71	+	+	+	2	0	0
16	F	80	+	+	+	2	1	0
17	M	78	+	+	+	3	1	0
18	M	49	+	+	+	2	1	0
19	M	59	+	+	+	3	2	0
20	M	70	+	+	+	2	0	0

2.3.2 Surface decontamination of specimens

All subsequent handling of the specimens was performed using aseptic technique on surfaces cleaned with hycolin phenolic disinfectant (2% v/v, Bilaurand Labs Ltd., Bridlington, UK). Tissue specimens of all types were placed in Betadine® antiseptic solution (Seton Healthcare Group plc., Oldham, UK) for approximately 3 min to disinfect the surface. This was followed by vortexing the tissues in multiple 500 µl aliquots of phosphate buffered saline (PBS) to remove any bacteria on the tissue surface. Final washes were retained in order to determine whether surface decontamination was successful. This was ascertained by analysing the washes by the culture and PCR methods described below (sections 2.3.3 and 2.35 respectively).

Specimens were aseptically bisected. Half was placed in Tris-EDTA (TE) buffer and stored at -80°C for molecular analysis (see Chapter 3). The remainder was subjected to immediate cultural analysis.

2.3.3 Cultural isolation of microflora

Tissue specimens were aseptically macerated with disposable scalpels, vortexed for 30 s in PBS (500 µl), and the neat suspensions used to make tenfold (10^{-1}) and one-hundredfold (10^{-2}) dilutions. Neat suspensions, dilutions and final PBS washes of the specimens (see above) (50 µl) were each spread onto Blood Agar (BA), Fastidious Anaerobe Agar (FAA), and Sabouraud's agar (Sab) (Lab M™, International Diagnostics Group plc, Bury, UK; BA and FAA supplemented with 5% defibrinated sheep blood, TCS Biosciences Ltd., Buckingham, UK) (see Figure 2.1). BA and Sab plates were incubated aerobically at 37°C for approximately 48 h. FAA plates were incubated in an

anaerobic environment (10% v/v CO_2, 20% v/v H_2, 70% v/v N_2) at 36 – 37°C for approximately 96 h.

Following incubation, all agar plates were examined for microbial growth. All distinct colony types were sub-cultured to purity using the same conditions as before. Purity of the sub-cultures was determined by macroscopic examination of colonies and microscopic examination of the bacteria after standard Gram staining. To Gram stain isolates a wet film of bacteria was fixed by heat onto a glass slide and stained with 1% (w/v) crystal violet (Sigma; 2 min). Slides were rinsed with tap water and stained with 1 x Lugol's iodine (see Appendix II; 1 min). After another flushing with tap water, slides were rinsed with pure acetone (Fisher, Loughborough, UK; approx. 20 s) and washed with water again. This was followed by counterstaining with 1 x Carbol fuchsin Appendix II; 1 min) and a final washing with tap water. Stained cells were examined under oil immersion with 100 x objective lens magnification. Gram-positive organisms appeared blue and Gram-negative were red.

Pure isolates were cryogenically stored at -80°C using Microbank™ cryovials (Pro-lab Diagnostics, Neston, Wirral, UK).

2.3.4 Identification of *Candida* isolates

Isolates suspected of being yeasts on the basis of colony and cell appearance were subcultured onto CHROMagar™ Candida (M-Tech Diagnostics Ltd., Warrington, UK). After growth for 48 h at 37°C, isolates of *Candida albicans*, *Candida tropicalis* and *Candida krusei* could be distinguished from other yeasts by their distinctive colouration (Beighton *et al*, 1995) (see Figure 2.2).

Figure 2.1 Primary microbial growth plates

Microbial growth plates resulting from the serial dilution of a suspension of a macerated tissue specimen, in this example the deep tumour specimen from patient 14. Neat suspensions and dilutions (10^{-1} and 10^{-2}) were cultured on Blood Agar and Sabouraud's agar for aerobic growth, and Fastidious Anaerobic Agar for anaerobic growth. Isolates were picked from each distinct colony type and subcultured (see section 2.3.3).

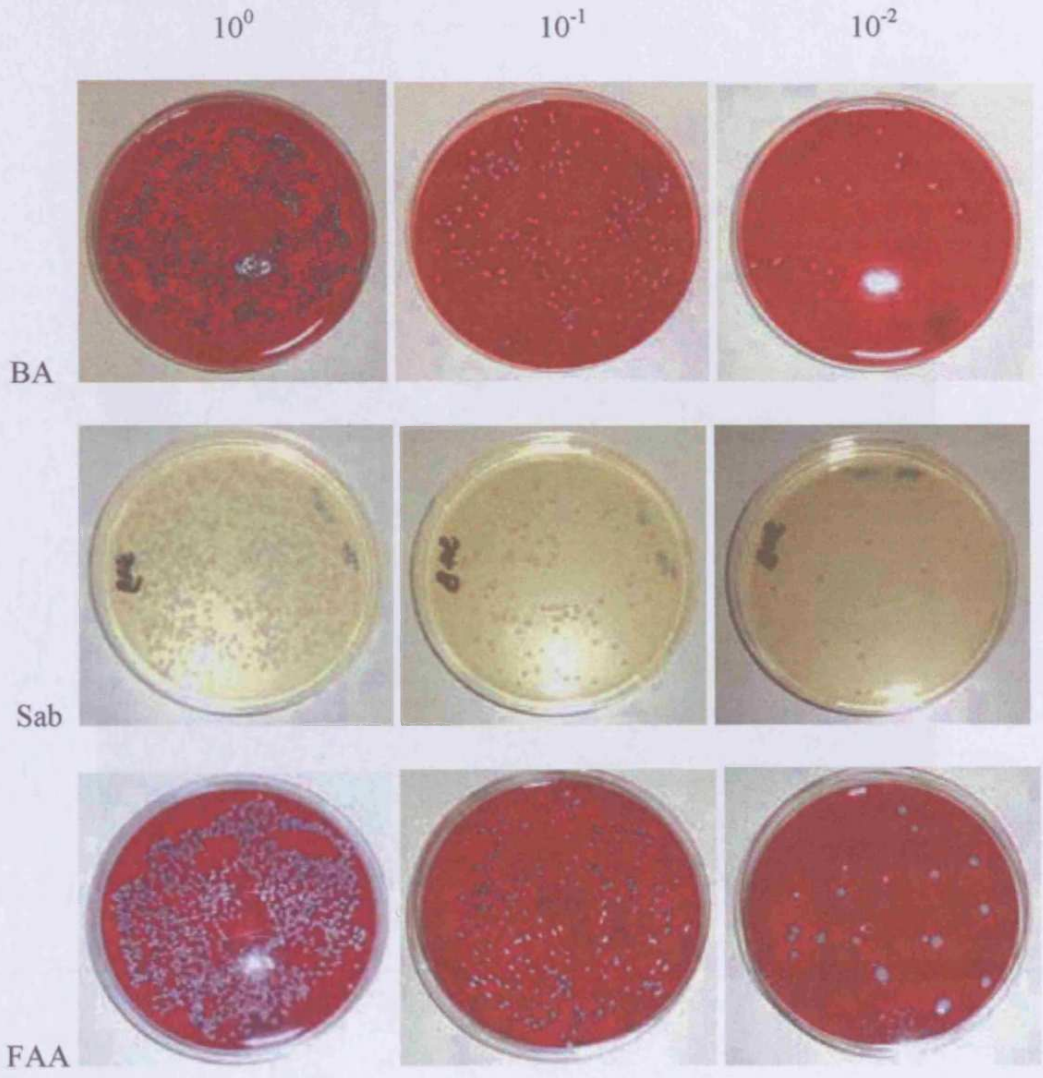


Figure 2.2 Growth of *Candida* isolates on a CHROMagar plate

An example of a CHROMagar™ growth plate showing the various colours that different species of *Candida* can appear. As in the manufacturer's instructions, *C. albicans* can be identified by its characteristic green colouration, *C. tropicalis* produces a dark blue colour, and *C. krusei* appears pink (Odds & Bernaerts, 1994).

2.3.5 Amplification of 16S rRNA genes from bacterial isolates

DNA was extracted from suspensions of each pure microbial isolate (1 – 5 colonies in 1 ml PBS) using a Gentra Puregene® DNA isolation kit (Flowgen, Nottingham, UK) and following the "DNA Purification From 1 ml Gram-positive Bacteria Culture Medium" protocol (see Appendix IV). The protocol worked efficiently with Gram-positive, Gram-negative and yeast isolates. On average, this method gave stock DNA extracts of approximately 200μg/ml.

16S rRNA genes were PCR amplified using the 27F/1492R primer pair (0.5 μM of each; oligonucleotides synthesised by Invitrogen Ltd., Paisley, UK), both of which target all known members of the domain *Bacteria* (Dymock *et al*, 1996) and were also found to amplify the corresponding 23S rRNA gene sequence in eukaryotic *Saccharomyces* species (for primer sequences see Appendix I). PCR was performed in a total volume of 50 μl and used 1.5 U of *Taq* DNA polymerase, 200 μM of each deoxynucleotide, and the working concentration of the appropriate magnesium-free buffer (Promega, Southampton, UK). Additionally, 2.0 mM of $MgCl_2$ (Promega) was used, with 5 μl of DNA extract as template. A touchdown protocol was used whereby, in the first cycle, denaturation was performed at 94°C for 6 min, primer annealing was performed at 65°C for 1 min, and extension was performed at 72°C for 2min. In subsequent cycles denaturation was performed for 1min and the annealing temperature was decreased by 2°C each cycle for 11 cycles, after which 25 cycles were carried out under the same conditions. In the final cycle, extension was performed for 12 min. PCR was carried out using a PTC-200 thermal cycler (MJ Research Ltd., GRI, Braintree, UK).

The PCR products were purified by precipitation and washing with ethanol. Firstly, 15 µl of 5 M NaCl (Sigma-Aldrich, Steinheim, Germany) and 15 µl of 40% polyethylene glycol (Mol. Wt. 8000; Sigma) was added to each PCR reaction volume. This was centrifuged (16,000 g, 15 min), and the supernatant aspirated and replaced with 200 µl 70% ethanol (v/v; Fisher Scientific, Loughborough, UK). Centrifugation, aspiration and ethanol washing steps were repeated. Following another centrifugation step the PCR products were dried, either in a vacuum (approx. 30 min) or under a fume hood (EDF1200, Envair, Rossendale, UK) (overnight), and re-suspended with nuclease-free water (30 µl). Products could then be stored at -20°C as necessary.

2.3.6 **Sequencing of 16S rRNA gene isolates**

PCR products were efficiently sequenced using ABI Prism BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems, Warrington, UK). A total reaction volume of 20 µl was used and contained the working concentrations of BigDye Sequencing Buffer and Ready Reaction Premix (Applied Biosystems), 0.5 µM of an oligonucleotide primer (see below; synthesised by Invitrogen), and 6 µl of purified PCR product (approx. 5 – 20 ng). Reactions were performed in a PTC-200 thermal cycler (MJ Research Ltd.) and comprised of 1 min of denaturation at 94°C followed by 30 cycles of denaturation at 94°C (15 s), annealing at 50°C (30 s) and extension at 60°C (5 min).

Extension products were purified, firstly by precipitating via the addition of 1 µl sodium acetate (3 M; Sigma) and 1 µl EDTA (0.5 M; Sigma), followed by 80 µl chilled absolute ethanol, to each reaction mix. The supernatant was

aspirated following centrifugation (16,000 g, 15 min), and replaced with chilled 70% ethanol (200 µl). This was repeated and after a final centrifugation and drying under vacuum, products were re-suspended in formamide (30 µl) and run on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyser; Applied Biosystems). This produced chromatograms that gave reliable sequences of at least 500 nucleotides length that could be compared to sequences in the public databases (see section 2.3.7). An example of a sequencing gel chromatogram is shown in Appendix V.

For all isolates sequencing was performed using the universal primer 357F (Lane, 1991). For sequences where it was impossible to obtain a conclusive identification, namely from those isolates suspected of being novel sequences or from unnamed organisms, PCR products were further sequenced using the 27F and 1492R primers to give a sequence of at least 1200 nucleotides in length.

2.3.7 **Identification of bacterial isolates by 16S rRNA gene sequence analysis**

Sequences obtained in this study were identified by comparison to the GenBank DNA sequence database (Benson *et al*, 2004) using the FASTA sequence homology search program (http://www.ebi.ac.uk/services/index.html; Pearson, 1990).

A >99% homology to the 16S rRNA gene sequence of the type strain, or other suitable reference strain, was the criterion used to identify an isolate to the species level. Where more than one reference species exhibited >99% sequence homology, the match with the greatest homology was taken as the identity,

wherever the sequence was shown to be reproducible and reliable. If there were no significant matches to known reference strains the identity of the isolate was based upon the results of the indiscriminate GenBank search.

2.3.8 Statistical analysis of species isolated

The comparatively low number of specimens and isolates precluded the use of most statistical analyses. With that in mind the incidences of the species isolated in each tissue specimen type were expressed as percentages and analysed, in total and grouped by familial association, using the ANOVA technique (Altman & Bland, 1996). Additionally, the significance of the varying occurrences of each phylotype in the different types of tissue was tested using a two-sided Fisher's exact test. Two-sided p-values, as opposed to one-sided (or one-tailed), were used as neither a positive or negative association between phylotypes and the tissue types could be assumed. The results are generally more accurate with this method when analyzing relatively small total sample sizes than with a chi-squared test (Agresti, 1992).

2.3 Results

Bacteria were isolated from all specimens; 20 deep tissue tumour specimens, 19 corresponding superficial specimens and the non-tumourous control tissues, which were obtained from 12 of the 20 patients.

The species identities are summarised in Table 2.2. The results of PCR and both aerobic and prolonged anaerobic culture of surface washings were

routinely negative, indicating that the chosen surface decontamination method was successful.

Taxa isolated in this study are shown according to specimen in Table 2.3. On average 6 isolates were cultured from each specimen giving a total of 90 distinct species or phylotypes, representing a wide range of bacteria and including several seemingly novel taxa. The isolates were mostly bacterial, but also included isolates of *Candida albicans* from 3 patients and a single isolate of a *Saccharomyces* species. A greater number of different taxa were isolated from the superficial samples (n=78, from 19 samples) in comparison to the deep tissues (n=54, from 20 samples). For all patients studied, the superficial tissues yielded exactly the same isolates as the corresponding deep tissue specimens plus, in most cases, additional species. Thirty-eight different taxa were isolated from the 12 non-tumourous control samples.

Nucleotide accession numbers for the 16S rRNA gene sequences of the un-named isolates, obtained in this study and deposited with GenBank, are also given in Table 2.2. In addition, the most commonly isolated taxa are summarised separately in Table 2.5.

The results of the Fisher's exact tests are shown in Table 2.2 and the analysis of variance tests performed on the groups of isolates are shown in Table 2.4. All but four of the differences were not statistically significant ($p > 0.05$).

Single factor ANOVA was also performed using the proportions of all 90 species found in the different specimen types. A significant difference was observed when comparing the proportions of isolates from the 3 tissue categories (p-value = 0.016). Breaking this down, significance was observed between the tumourous and superficial specimens ($p = 0.0007$), but not between tumourous

and non-tumourous tissues, or between superficial and non-tumourous ($p = 0.203$, $p = 0.159$ respectively).

Table 2.2 Microorganisms cultivated from tissue specimens, grouped according to phylum

Multiple names are given wherever partial 16S rRNA gene sequences from isolates exhibited a significant (>99%) match to more than one species. Accession numbers for all type or reference strains used in the identification of the bacterial isolates are shown. Where the 16S rRNA gene sequence was novel or homologous only to a phylotype from an unrecognized species the accession numbers of the appropriate GenBank entries are given below. The significances of the differences in occurrence of each taxon between the various tissue types were calculated by 2-sided Fisher's exact test.

Species	No. of tissue specimens cultured from			Significance of difference in occurrence (2-sided p-values)		
	Deep tumour (n=20)	Tumour and overlying mucosa ("Superficial") (n=19)	Non-tumourous control (n=12)	Between deep tumour and superficial tissues	Between deep tumour and non-tumourous tissues	Between superficial and non-tumourous tissues
Proteobacteria						
Acinetobacter lwoffi; X81665	1	1		1.000	1.000	1.000
Citrobacter koseri; AF025372	1	1		1.000	1.000	1.000
Eikenella corrodens; AF320620	1	1		1.000	1.000	1.000
Moraxella osloensis; AY730714			2	1.000	0.133	0.142
Neisseria elongata; L06171/AJ247252		1		0.487	1.000	1.000
Neisseria perflava; AJ239295 / *N. subflava*; AJ239291	1	1		1.000	1.000	1.000
Pseudomonas aeruginosa; Z76672	1	1		1.000	1.000	1.000
Pseudomonas monteilii; AF064458			1	1.000	0.375	0.387
Serratia marcescens subsp. *sakuensis*; AB061685	1	1	1	1.000	1.000	1.000
Unknown *Moraxella*-like sp.[1]		1		0.487	1.000	1.000
Actinobacteria						
Actinomyces graevenitzii; AJ540309			1	1.000	0.375	0.387
Actinomyces odontolyticus; AJ234040		1	2	0.487	0.540	0.543
Actinomyces viscosus; X82453			1	1.000	0.375	0.387
Atopobium parvulum; AF292372	1	2	3	0.605	0.136	0.350

Atopobium rimae; AF292371			1	1.000	0.375	0.387
Bifidobacterium longum; M58739/M84781			1	1.000	0.375	0.387
Bifidobacterium sp. (oral strain H6-M4 phylotype)[2]		2		0.231	1.000	0.510
Brachybacterium rhamnosum; AJ415376			1	1.000	0.375	0.387
Corynebacterium singulare; Y10999	1			1.000	1.000	1.000
Corynebacterium striatum; X84442		1		0.487	1.000	1.000
Corynebacterium tuberculostearicum; AJ438050	2	3	1	0.661	1.000	1.000
Dermabacter hominis; X76728	1	1		1.000	1.000	1.000
Dietzia psychralcaliphila; AB049630		1		0.487	1.000	1.000
Gordonia sputi; X80634		1		0.487	1.000	1.000
Micrococcus luteus; AJ536198	5	6		1.000	0.130	0.059
Micrococcus lylae; X80750	1	1		1.000	1.000	1.000
Olsenella uli; AY005814		1		0.487	1.000	1.000
Propionibacterium acnes; AB042288	6	7	6	0.741	0.288	0.710
Propionibacterium avidum; AJ003055	1	1		1.000	1.000	1.000
Propionibacterium granulosum; AJ003057		1		0.487	1.000	1.000
Rothia amarae; AY043359		1		0.487	1.000	1.000
Rothia mucilaginosa; X87758	1	1	2	1.000	0.540	0.543
Scardovia genosp. C1[3]	1	1		1.000	1.000	1.000
Streptomyces aureus; AY094368 / *S. griseorubiginosus*; AJ399488		1		0.487	1.000	1.000
Unknown *Corynebacterium*-like sp. phylotype i[4]		1		0.487	1.000	1.000
Unknown *Corynebacterium*-like sp. phylotype ii[5]	1	1		1.000	1.000	1.000
Unknown *Georgenia*-like sp.[6]	1	1		1.000	1.000	1.000
Unknown *Olsenella*-like sp.[7]		1	1	0.487	0.375	1.000
Firmicutes						
Bacillus licheniformis; X68416	1	1		1.000	1.000	1.000
Bacillus mycoides; AB021192 / *B. weihenstephanensis*; AB021199	1	1		1.000	1.000	1.000
Bacillus psychrodurans; AJ277983	1	1		1.000	1.000	1.000
Dialister invisus; AY162469		1		0.487	1.000	1.000
Dialister pneumosintes; X82500	1	1		1.000	1.000	1.000
Enterococcus faecalis; AB012212		1		0.487	1.000	1.000
Eubacterium yurii subsp. *yurii*; L34629		1		0.487	1.000	1.000
Exiguobacterium oxidotolerans; AB105164	2	2		1.000	0.516	0.510

Finegoldia magna; AF542227	1	2	1	0.605	1.000	1.000
Gemella haemolysans; L14326		2		0.231	1.000	0.510
Gemella morbillorum; L14327	1	1	1	1.000	1.000	1.000
Granulicatella adiacens; D50540	3	5	3	0.451	0.647	1.000
Lactobacillus casei; D86517 / *L. zeae*; D86516	1	1		1.000	1.000	1.000
Lactobacillus crispatus; Y17362	1	1		1.000	1.000	1.000
Lactobacillus fermentum; M58819/AB017345	2	2	1	1.000	1.000	1.000
Lactobacillus gasseri; AF519171	3	3	1	1.000	1.000	1.000
Lactobacillus rhamnosus; AF243146	1	1	1	1.000	1.000	1.000
Lactobacillus salivarius subsp. *salivarius*; AF089108		1		0.487	1.000	1.000
Megasphaera micronuciformis; AF473834		1	1	0.487	0.375	1.000
Mogibacterium neglectum; AB037875	1	1		1.000	1.000	1.000
Peptostreptococcus micros; AY323523	4	4	2	1.000	1.000	1.000
Selenomonas dianae; AF287801		1		0.487	1.000	1.000
Selenomonas sputigena; AF287793		1		0.487	1.000	1.000
Solobacterium moorei; AY044916			1	1.000	0.375	0.387
Staphylococcus aureus subsp. *anaerobius*; D83355	2	2		1.000	0.516	0.510
Staphylococcus capitis subsp. *urealyticus*; AB009937 / *S. caprae*; Y12593 / *S. epidermidis*; D83363	2	5	2	0.235	0.620	0.676
Staphylococcus cohnii subsp. *cohnii*; D83361	1	1		1.000	1.000	1.000
Staphylococcus hominis subsp. *hominis*; X66101	1	1		1.000	1.000	1.000
Staphylococcus saprophyticus; L37602	1	1		1.000	1.000	1.000
Staphylococcus warneri; L37603	2	3	1	0.661	1.000	1.000
Streptococcus agalactiae; AB002479	1	1		1.000	1.000	1.000
Streptococcus anginosus; AF145245	1	4	1	0.182	1.000	0.624
Streptococcus constellatus; AF104676	2	4	2	0.407	0.620	1.000
Streptococcus cristatus; AY104676	1	1		1.000	1.000	1.000
Streptococcus gordonii; AY485606	1	1		1.000	1.000	1.000
Streptococcus mitis; AY485601 / *Str. oralis*;AY485602	2	5	5	0.235	0.073	0.447
Streptococcus parasanguinis; AY485605	3	6	5	0.273	0.116	0.705
Streptococcus salivarius; AY188352	2	3	3	0.661	0.338	0.653
Streptococcus sp. (oral strain T4-E3 phylotype)[8]		1		0.487	1.000	1.000
Unknown Gram-positive anaerobic coccoid sp.[9]			1	1.000	0.375	0.387

Veillonella atypica; AF439641		1	1	0.487	0.375	1.000
Veillonella dispar; AF439639	1	1	3	1.000	0.136	0.272
Veillonella parvula; X84005	2	2		1.000	0.516	0.510
Bacteroidetes						
Prevotella intermedia; X73965		1		0.487	1.000	1.000
Prevotella melaninogenica; AY323525	2	3		0.661	0.516	0.265
Prevotella veroralis; AY836507			2	1.000	0.133	0.142
Prevotella sp. (oral clone BE073 phylotype) [10]	1	1	2	1.000	0.540	0.543
Unknown *Capnocytophaga*-like sp.[11]		1		0.487	1.000	1.000
Fusobacteria						
Fusobacterium naviforme; AJ006965	1	3		0.342	1.000	0.265
Fusobacterium nucleatum subsp. nucleatum; AJ133496	1	2	1	0.605	1.000	1.000
YEASTS						
Candida albicans	2	3	1	0.661	1.000	1.000
Saccharomyces sp.			1	1.000	0.375	0.387
Total number of taxa:	**58**	**78**	**38**			

[1] Novel *Moraxella* sp. isolate S12-08; AY880059. Closest sequence homology to *Moraxella osloensis*; AY730714 (94.1% match).
[2] Unknown *Bifidobacterium* sp.; AY880048, AY880049. Also homologous to oral strain H6-M4 phylotype (AF385524) and the proposed species "*Bifidobacterium urinalis,*" which does not currently have standing in nomenclature (strains CCUG 26938 and CCUG 344441, AJ278694 and AJ278695 respectively).
[3] *Scardovia* sp. isolate; AY880045. Sequence match to *Scardovia* genomospecies C1 (AY278626).
[4] Novel *Corynebacterium* sp. isolate S18-03; AY880057. Closest sequence homology to *Corynebacterium suicordis*; AJ504424 (98.1% match).
[5] Novel *Corynebacterium* sp. isolate T13-01; AY880058. Closest sequence homology to *Corynebacterium appendicis*; AJ314919 (95.0% match).
[6] Novel *Georgenia* sp. isolate T04-04; AY880044. Closest sequence homology to *Georgenia muralis*; X94155 (96.7% match).
[7] Novel *Olsenella* sp. isolates S13-10, N13-17; AY880047, AY880046. Closest sequence homology to *Olsenella profusa*; AF292374 (98.1% match).
[8] Unknown *Streptococcus* sp. isolates S16-08, S16-11; AY880050, AY880051. Sequence match to oral strain T4-E3 phylotype (AF385526).
[9] Novel bacterium isolate N14-24; AY880043. Closest sequence homology to *Clostridium* sp. BN II; X75909 (86.6% match).
[10] Unknown *Prevotella* sp. isolates N12-20, N19-22, N19-31, T05-04; AY880052 – AY880055 respectively. Sequence match to oral clone BE073 phylotype (AF385551).
[11] Novel *Capnocytophaga* sp. isolate S12-14; AY880056. Closest sequence homology to *Capnocytophaga ochracea*; U41350 (95.3% match).

Table 2.3 A summary of the microorganisms cultured from tissue specimens, grouped according to patient
Results have been aligned to compare the species found in each specimen type.

Specimen	Species from deep tumour tissue	Species from "superficial" tissue	Species from non-tumourous tissue
1		*Actinomyces odontolyticus*	n/a
	Lactobacillus gasseri	*Lactobacillus gasseri*	
	Prevotella melaninogenica	*Prevotella melaninogenica*	
	Propionibacterium acnes	*Propionibacterium acnes*	
	Propionibacterium avidum	*Propionibacterium avidum*	
		Propionibacterium granulosum	
	Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	
	Scardovia genosp. C1[3]	*Scardovia* genosp. C1[3]	
		Streptococcus anginosus	
2		*Granulicatella adiacens*	n/a
	Micrococcus luteus	*Micrococcus luteus*	
	Propionibacterium acnes	*Propionibacterium acnes*	
	Rothia mucilaginosa	*Rothia mucilaginosa*	
	Streptococcus parasanguinis	*Streptococcus parasanguinis*	
3		*Corynebacterium striatum*	n/a
		Enterococcus faecalis	
		Finegoldia magna	
	Lactobacillus casei/zeae	*Lactobacillus casei/zeae*	
	Lactobacilus crispatus	*Lactobacilus crispatus*	
	Lactobacillus fermentum	*Lactobacillus fermentum*	
	Staphylococcus aureus	*Staphylococcus aureus*	
	Staphylococcus cohnii	*Staphylococcus cohnii*	
	Staphylococcus saprophyticus	*Staphylococcus saprophyticus*	
	Streptococcus agalactiae	*Streptococcus agalactiae*	
		Veillonella atypica	

4	*Acinetobacter lwoffi* *Corynebacterium tuberculostearicum* *Dermabacter hominis* *Exiguobacterium oxidotolerans* *Micrococcus luteus* *Micrococcus lylae* *Propionibacterium acnes* *Staphylococcus capitis/caprae/epidermidis* Unknown *Georgenia*-like sp.[6]	*Acinetobacter lwoffi* *Corynebacterium tuberculostearicum* *Dermabacter hominis* *Dietzia psychroalcaliphila* *Exiguobacterium oxidotolerans* *Gordonia sputi* *Micrococcus luteus* *Micrococcus lylae* *Propionibacterium acnes* *Staphylococcus capitis/caprae/epidermidis* Unknown *Georgenia*-like sp.[6]	n/a
5	*Citrobacter koseri* *Fusobacterium naviforme* *Prevotella melaninogenica* *Prevotella* sp. (oral clone BE073 phylotype)[10]	*Citrobacter koseri* *Fusobacterium naviforme* *Prevotella melaninogenica* *Prevotella* sp. (oral clone BE073 phylotype)[10]	n/a
6	*Granulicatella adiacens* *Peptostreptococcus micros* *Streptococcus constellatus* *Streptococcus gordonii* *Streptococcus parasanguinis*	*Granulicatella adiacens* *Peptostreptococcus micros* *Staphylococcus capitis/caprae/epidermidis* *Streptococcus constellatus* *Streptococcus gordonii* *Streptococcus parasanguinis*	n/a
7	*Corynebacterium singulare* *Propionibacterium acnes*	n/a	n/a

8	*Fusobacterium nucleatum subsp. nucleatum* *Micrococcus luteus* *Peptostreptococcus micros* *Staphylococcus warneri* *Streptococcus salivarius*	*Corynebacterium tuberculostearicum* *Fusobacterium naviforme* *Fusobacterium nucleatum subsp. nucleatum* *Micrococcus luteus* *Peptostreptococcus micros* *Staphylococcus warneri* *Streptococcus constellatus* *Streptococcus salivarius*	*Actinomyces graevenitzii* *Prevotella veroralis* *Propionibacterium acnes* *Staphylococcus warneri* *Streptococcus salivarius* *Veillonella dispar*
9	*Bacillus psychrodurans* *Propionibacterium acnes*	*Bacillus psychrodurans* *Propionibacterium acnes*	n/a
10	*Propionibacterium acnes*	*Propionibacterium acnes* *Rothia amarae* *Staphylococcus capitis/caprae/epidermidis* *Staphylococcus warneri*	*Propionibacterium acnes*
11	*Bacillus licheniformis* *Lactobacillus fermentum* *Lactobacillus gasseri* *Staphylococcus aureus* *Staphylococcus capitis/caprae/epidermidis*	*Bacillus licheniformis* *Lactobacillus fermentum* *Lactobacillus gasseri* *Staphylococcus aureus* *Staphylococcus capitis/caprae/epidermidis*	*Corynebacterium tuberculostearicum* *Lactobacillus fermentum* *Lactobacillus gasseri* *Moraxella osloensis* *Saccharomyces* sp.

12	*Bacillus mycoides/weihenstephanensis* *Exiguobacterium oxidotolerans*	*Bacillus mycoides/weihenstephanensis* *Eubacterium yurii subsp. schtitka* *Exiguobacterium oxidotolerans* *Gemella haemolysans* *Neisseria elongata* *Prevotella melaninogenica* *Selenomonas dianae* *Selenomonas sputigena* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* Unknown *Capnocytophaga*-like sp.[11] Unknown *Moraxella*-like sp.[1]	 *Megasphaera micronuciformis* *Prevotella* sp. (oral clone BE073 phylotype)[10] *Rothia mucilaginosa* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Veillonella dispar*
13	 *Corynebacterium tuberculostearicum* *Staphylococcus hominis* *Streptococcus cristatus* Unknown *Corynebacterium*-like sp. phylotype ii[5]	*Atopobium parvulum* *Bifidobacterium* sp. (oral strain H6-M4 phylotype)[2] *Corynebacterium tuberculostearicum* *Dialister invisus* *Olsenella uli* *Prevotella intermedia* *Propionibacterium acnes* *Staphylococcus hominis* *Streptococcus anginosus* *Streptococcus cristatus* *Streptomyces aureus/griseorubiginosus* Unknown *Corynebacterium*-like sp. phylotype ii[5] Unknown *Olsenella*-like sp.[7]	*Atopobium parvulum* *Atopobium rimae* *Propionibacterium acnes* Unknown *Olsenella*-like sp.[7]

14	*Atopobium parvulum* *Eikenella corrodens* *Granulicatella adiacens* *Lactobacillus rhamnosus* *Mogibacterium neglectum* *Peptostreptococcus micros* *Serratia marcescens subsp. sakuensis* *Streptococcus constellatus* *Veillonella parvula*	*Atopobium parvulum* *Eikenella corrodens* *Granulicatella adiacens* *Lactobacillus rhamnosus* *Mogibacterium neglectum* *Peptostreptococcus micros* *Serratia marcescens subsp. sakuensis* *Streptococcus constellatus* *Veillonella parvula*	*Actinomyces odontolyticus* *Atopobium parvulum* *Bifidobacterium longum* *Lactobacillus rhamnosus* *Peptostreptococcus micros* *Serratia marcescens subsp. sakuensis* *Staphylococcus capitis/caprae/epidermidis* *Streptococcus constellatus* Unknown Gram-positive anaerobic coccoid sp.[9]
15	*Dialister pneumosintes* *Gemella morbillorum* *Peptostreptococcus micros*	*Dialister pneumosintes* *Fusobacterium naviforme* *Fusobacterium nucleatum subsp. nucleatum* *Gemella morbillorum* *Micrococcus luteus* *Peptostreptococcus micros* *Streptococcus anginosus* *Streptococcus constellatus* *Streptococcus mitis/oralis*	 *Fusobacterium nucleatum subsp. nucleatum* *Gemella morbillorum* *Granulicatella adiacens* *Peptostreptococcus micros* *Solobacterium moorei* *Streptococcus constellatus* *Streptococcus mitis/oralis*
16	 *Micrococcus luteus* *Streptococcus salivarius*	*Gemella haemolysans* *Granulicatella adiacens* *Micrococcus luteus* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Streptococcus salivarius* *Streptococcus* sp. (oral strain T4-E3 phylotype)[8]	 *Granulicatella adiacens* *Propionibacterium acnes* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Streptococcus salivarius*

17	*Granulicatella adiacens* *Micrococcus luteus* *Staphylococcus aureus* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Veillonella dispar*	*Granulicatella adiacens* *Micrococcus luteus* *Propionibacterium acnes* *Staphylococcus aureus* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Veillonella dispar*	*Granulicatella adiacens* *Propionibacterium acnes* *Pseudomonas monteilii* *Streptococcus mitis/oralis* *Streptococcus parasanguinis*
18	 *Lactobacillus gasseri*	*Bifidobacterium* sp. (oral strain H6-M4 phylotype)[2] *Lactobacillus gasseri* *Lactobacillus salivarius subsp. salivarius* *Streptococcus salivarius* Unknown *Corynebacterium*-like sp. phylotype i[4]	*Atopobium parvulum* *Brachybacterium rhamnosum* *Moraxella osloensis* *Streptococcus parasanguinis* *Streptococcus salivarius* *Veillonella atypica*
19	 *Finegoldia magna* *Streptococcus anginosus* *Streptococcus mitis/oralis* *Veillonella parvula*	 *Finegoldia magna* *Megasphaera micronuciformis* *Streptococcus anginosus* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Veillonella parvula*	*Actinomyces odontolyticus* *Actinomyces viscosus* *Finegoldia magna* *Prevotella* sp. (oral clone BE073 phylotype)[10] *Prevotella veroralis* *Propionibacterium acnes* *Rothia mucilaginosa* *Staphylococcus capitis/caprae/epidermidis* *Streptococcus anginosus* *Streptococcus mitis/oralis* *Streptococcus parasanguinis* *Veillonella dispar*

20	*Neisseria perflava/subflava*	*Neisseria perflava/subflava* *Staphylococcus capitis/caprae/epidermidis*	none

[1] Novel *Moraxella* sp. isolate S12-08; AY880059
[2] Unknown *Bifidobacterium* sp.; AY880048, AY880049. Also homologous to oral strain H6-M4 phylotype (AF385524) and the proposed species "*Bifidobacterium urinalis,*" which does not currently have standing in nomenclature (strains CCUG 26938 and CCUG 344441, AJ278694 and AJ278695 respectively).
[3] *Scardovia* sp. isolate; AY880045. Sequence match to *Scardovia* genomospecies C1 (AY278626).
[4] Novel *Corynebacterium* sp. isolate S18-03; AY880057.
[5] Novel *Corynebacterium* sp. isolate T13-01; AY880058.
[6] Novel *Georgenia* sp. isolate T04-04; AY880044.
[7] Novel *Olsenella* sp. isolates S13-10, N13-17; AY880047, AY880046
[8] Unknown *Streptococcus* sp. isolates S16-08, S16-11; AY880050, AY880051. Sequence match to oral strain T4-E3 phylotype (AF385526).
[9] Novel bacterium isolate N14-24; AY880043.
[10] Unknown *Prevotella* sp. isolates N12-20, N19-22, N19-31, T05-04; AY880052 – AY880055 respectively. Sequence match to oral clone BE073 phylotype (AF385551).
[11] Novel *Capnocytophaga* sp. isolate S12-14; AY880056.

Table 2.4 Frequencies of the various taxa isolated from the 3 different tissue specimen types, analysed by ANOVA.

Isolates were grouped into families and the differences in proportion analysed by ANOVA. 'ND' indicates a p-value result could not be determined by this method. Significant results ($p \leq 0.05$) are shown in blue.

Family of taxa isolated	Taxa	Proportion of tissue specimens isolated from (%)			P-values	
		Tumour	Non-tumourous	Superficial	Difference between tumour & non-tumourous tissues	Difference between superficial and non-tumourous tissues
Acidaminococcaceae	*D. invisus*	0.00	0.00	5.26	0.439	0.827
	D. pneumosintes	5.00	0.00	5.26		
	M. micronuciformis	0.00	8.33	5.26		
	S. dianae	0.00	0.00	5.26		
	S. sputigena	0.00	0.00	5.26		
	V. atypica	0.00	8.33	5.26		
	V. dispar	5.00	25.00	5.26		
	V. parvula	10.00	0.00	10.53		
Actinomycetaceae	*A. graevenitzii*	0.00	8.33	0.00	0.016	0.046
	A. odontolyticus	0.00	16.67	5.26		
	A. viscosus	0.00	8.33	0.00		
Bacteriaceae	*B. licheniformis*	5.00	0.00	5.26	0.002	0.002
	B. mycoides / weihenstephanensis	5.00	0.00	5.26		
	B. psychrodurans	5.00	0.00	5.26		
	E. oxidotolerans	10.00	0.00	10.53		
Bifidobacteriaceae	*B. longum*	0.00	8.33	0.00	0.749	0.579
	Bifidobacterium sp. strain H6-M4	0.00	0.00	10.53		
	Scardovia genosp. C1	5.00	0.00	5.26		
Coriobacteriaceae	*A. parvulum*	5.00	25.00	10.53	0.140	0.398
	A. rimae	0.00	8.33	0.00		
	O. uli	0.00	0.00	5.26		
	Unknown *Olsenella* sp.	0.00	8.33	5.26		

Corynebacteriaceae	*C. singulare*	5.00	0.00	0.00	0.379	0.168
	C. striatum	0.00	0.00	5.26		
	C. tuberculostearicum	10.00	8.33	15.79		
	Unknown *Corynebacterium* type I	0.00	0.00	5.26		
	Unknown *Corynebacterium* type II	5.00	0.00	5.26		
Dermabacteriaceae	*B. rhamnosum*	0.00	8.33	0.00	0.764	0.785
	D. hominis	5.00	0.00	5.26		
Enterobacteriaceae	*C. koseri*	5.00	0.00	5.26	0.860	0.817
	S. marcescens	5.00	8.33	5.26		
Eubacteriaceae	*E. yurii*	0.00	0.00	5.26	0.423	ND
	M. neglectum	5.00	0.00	5.26		
Fusobacteriaceae	*F. naviforme*	5.00	0.00	15.79	0.860	0.210
	F. nucleatum	5.00	8.33	10.53		
Lactobacillaceae	*L. casei / zeae*	5.00	0.00	5.26	0.395	0.180
	L. crispatus	5.00	0.00	5.26		
	L. fermentum	10.00	8.33	10.53		
	L. gasseri	15.00	8.33	15.79		
	L. rhamnosus	5.00	8.33	5.26		
	L. salivarius	0.00	0.00	5.26		
Micrococcaceae	*M. luteus*	25.00	0.00	31.58	0.533	0.362
	M. lylae	5.00	0.00	5.26		
	R. amarae	0.00	0.00	5.26		
	R. mucilaginosa	5.00	16.67	5.26		
Moraxellaceae	*A. lwoffi*	5.00	0.00	5.26	0.539	0.743
	M. osloensis	0.00	16.67	0.00		
	Unknown *Moraxella* sp.	0.00	0.00	5.26		
Neisseriaceae	*E. corrodens*	5.00	0.00	5.26	0.116	ND
	N. elongata	0.00	0.00	5.26		
	N. perflava / subflava	5.00	0.00	5.26		
Peptostreptococcaceae	*F. magna*	5.00	8.33	10.53	0.696	0.934
	P. micros	20.00	16.67	21.05		
	Unknown GPAC sp.	0.00	8.33	0.00		
Propionibacteriaceae	*P. acnes*	30.00	50.00	36.84	0.806	0.967
	P. avidum	5.00	0.00	5.26		
	P.granulosum	0.00	0.00	5.26		
Prevotellaceae	*P. intermedia*	0.00	0.00	5.26	0.426	0.774
	P. melaninogenica	10.00	0.00	15.79		

	P. veroralis	0.00	16.67	0.00		
	Prevotella sp. BE073	5.00	16.67	5.26		
Pseudomonadaceae	*P. aeruginosa*	5.00	0.00	5.26	0.764	0.785
	P. monteilii	0.00	8.33	0.00		
Staphylococcaceae	*G. haemolysins*	0.00	0.00	10.53	0.428	0.086
	G. morbillorum	5.00	8.33	5.26		
	S. aureus	10.00	0.00	10.53		
	S. capitis / caprae / epidermidis	10.00	16.67	26.32		
	S. cohnii	5.00	0.00	5.26		
	S. hominis	5.00	0.00	5.26		
	S. saprophyticus	5.00	0.00	5.26		
	S. warneri	10.00	8.33	15.79		
Streptococcaceae	*S. agalactiae*	5.00	0.00	5.26	0.227	0.955
	S. anginosus	5.00	8.33	21.05		
	S. constellatus	10.00	16.67	21.05		
	S. cristatus	5.00	0.00	5.26		
	S. gordonii	5.00	0.00	5.26		
	S. mitis / oralis	10.00	41.67	26.32		
	S. parasanguinis	15.00	41.67	31.58		
	S. salivarius	10.00	25.00	15.79		
	Streptococcus sp. T4-E3	0.00	0.00	5.26		
Yeasts	*C. albicans*	10.00	8.33	15.79	0.574	0.961
	Saccharomyces sp.	0.00	8.33	0.00		

Table 2.5 The taxa most commonly isolated from the tissue specimens by cultivation

The species detected within samples from at least 20% of the patients are listed below. The relative proportions of the different tissue sample types that were positive for each species are also shown.

Taxa	Patients isolated from (n=20)	Proportion of tissue samples positive for taxa (%)		
		Deep tumour	Superficial	Non-tumourous
Granulicatella adiacens	6	15	26.32	25
Micrococcus luteus	6	25	31.58	0
Peptostreptococcus micros	4	20	21.05	16.67
Propionibacterium acnes	11	30	36.84	50
Staphylococcus capitis/caprae/epidermidis	7	10	26.32	16.67
Streptococcus anginosus	4	5	21.05	8.33
Streptococcus constellatus	4	10	21.05	16.67
Streptococcus mitis/oralis	5	10	26.32	41.67
Streptococcus parasanguinis	6	15	31.58	41.67

2.5 Discussion

At the time of writing, and to our best knowledge, this is the only time that viable bacteria have been isolated from within oral squamous cell carcinoma tissue. Immersion in Betadine® and PBS washing of the specimens was used to successfully eliminate any viable bacteria on the surface that may have been present due to salivary or instrument contamination during surgery.

Cultural analysis remains a valuable tool for establishing the presence of live bacteria in clinical samples, but it is important to be aware of the limitations of the technique. In this study, as in common clinical practice, agar plates were examined and each of the different colony types seen were sub-cultured for purity and identified by 16 rRNA gene sequencing. For a fully comprehensive study every colony grown would have had to have been picked, sub-cultured and identified, which, unfortunately, was impractical in this case. Therefore, although every care was taken to avoid it, there exists the possibility that some culturable species were missed from each specimen. The risk of overlooking the presence of some species is particularly apparent for those organisms with very similar looking macroscopic colony appearance. For instance some α-haemolytic Gram-positive cocci, such as various species of *Streptococcus, Enterococcus, Staphylococcus* and *Aerococcus*, have been observed to have similar appearances (Zhang *et al*, 2000).

Partial sequencing of the 16S rRNA gene proved to be a suitably effective method for the identification of the bacterial isolates. The *Bacteria*-specific primers used were degenerate enough to work on every strain examined. As seen in previous studies (Drancourt *et al,* 2004; Kiratisin *et al,* 2003), this

technique was able to identify several unusual bacteria, *i.e.* bacteria not previously or only infrequently isolated from clinical samples. Such species are not well characterised and so would have been very difficult to identify by phenotypic means. For example, the Gram-positive, rod-shaped bacterium *Mogibacterium neglectum*, isolated from the tumourous and "superficial" specimens from one patient, was first described only recently and is inert in most conventional biochemical tests. This species would have been difficult, if not impossible, to distinguish from other *Mogibacterium* species or asaccharolytic *Eubacterium* species without DNA sequence analysis (Nakazawa *et al*, 2002).

As seen in other investigations, for most isolates a single 500 bp sequence, derived using the universal sequencing primer 357F, is optimal for species-level identification (Clarridge, 2004; Munson *et al*, 2004). However, 6 taxa seen in this study showed equal homology to more than one species type strain, and so their identity could not be so precise. Species for which this region of the 16S rRNA gene was insufficiently discriminatory to allow distinction between the type strains include *Bacillus mycoides* and *B. weihenstephanensis*, *Lactobacillus casei* and *L. zeae*, *Neisseria perflava* and *N. subflava*, *Streptococcus mitis* and *Str. oralis, Streptomyces aureus* and *S. griseorubiginosus,* and *Staphylococcus caprae, S. epidermidis* and *S. capitis* subsp. *urealyticus*. With the exception of the *Streptomyces* strains, the particular similarity of the 16S gene sequences from these species has been reported previously (Lechner *et al*, 1998; Kawamura *et al,* 1995; Mori *et al*, 1997; Munson *et al*, 2004; Poyart *et al*, 2001; Spratt, 2004). For the purposes of this investigation, this level of identification was sufficient. Nevertheless, sequencing of the entire 16S rRNA gene could probably be applied successfully

to further identify these isolates, should the precise species designation of these isolates be needed for future investigations. Alternatively, PCR amplification and sequence analysis of other genes could also be used, such as the RNA polymerase beta subunit gene (*rpoB*) for identifying *Bacillus* species (Blackwood *et al*, 2004), or the manganese-dependent superoxide dismutase (*sodA*) gene for both *Streptococcus* and *Staphylococcus* isolates (Poyart *et al*, 1998, 2001). These methods are potentially informative, but were not used here as they are not as well-established as standard 16S rRNA sequencing and little sequence data is available in the public databases.

The successful identification of isolates is limited by the accuracy of the sequence generated. However, the technology for producing and reading such sequences has become so precise that with a robust protocol, such as was used here, errors only occur at an estimated rate of 1 in 5000 to 1 in 10,000 bp (Clarridge, 2004). This is too small a difference to significantly affect the species designation, so it was not considered a problem in this study.

The other limitation of 16S rRNA gene sequence analysis is the information available in the databanks in the public domain. Unknown bacteria may not be identified because their 16S rRNA gene sequences have not been previously reported and deposited in a public database. Additionally, some of the existing data in databases such as GenBank is questionable and it is worthwhile to examine the quality of sequences before using them (Clarridge, 2004). Some of the sequences available, particularly those older entries derived using less dependable equipment and reagents, are not very accurate or may contain a number of ambiguous bases, making matches unreliable. For example, the original entry for the type strain of *Actinomyces graevenitzii* (GenBank

accession number Y09589) contains 5% ambiguous bases, entered as N's, in the first 500 bp (Clarridge, 2004). In this case it is possible to use a more recent entry for the type strain (accession number AJ540309) for comparison. It was also observed in the course of this work that there are no suitable entries in GenBank for the 16S rRNA gene sequences of the type strains of *Eikenella corrodens*, *Lactobacillus rhamnosus* or *Moraxella osloensis*. There was, however, reliable 16S rRNA sequence data for other, closely related strains that could be used instead, *i.e. Eikenella corrodens* isolate 1664276 (AF320620), *Lactobacillus rhamnosus* MCRF-412 (AY299488) and *Moraxella osloensis* strain 170804JB8 (AY730714).

An added complication was encountered when identifying isolates belonging to the "*Streptococcus milleri*" group. This group is currently organised into three distinct species based upon DNA hybridisation studies, namely *Streptococcus anginosus, S. constellatus* and *S. intermedius* (Daley *et al*, 2005). However, members of each species are heterogeneous with regard to their 16S gene sequences. *S. constellatus* has been found to comprise at least 2 ribogroups (Schouls *et al*, 2003), while *S. anginosus* comprises 5 distinct ribogroups (Jacobs *et al*, 2000). This is not a problem *per se* for investigations such as this, but it is important to be aware of it and necessary to compare the sequences from each isolate with data from each ribogroup. All of the *S. anginosus* isolates found in this study had the closest 16S rRNA gene sequence identity to the so-called "motility" ribogroup, which, in the region sequenced here, is more homologous to the type strain of *S. intermedius* (ATCC 27335, GenBank accession number AF104671) than that of *S. anginosus* (ATCC 33397, accession number AF104678). There is evidence that this 16S sequence

heterogeneity is due to recombination and horizontal transfer of segments of the gene between different members of the group (Schouls *et al*, 2003). No phenotypic characteristics have been found to be consistently associated with any of the ribogroups, although trends of phenotypic difference are evident. For instance, the vast majority of motility ribogroup *S. anginosus* strains examined have been isolated from the urogenital tract and can ferment mannitol and raffinose, reactions which most other strains of *S. anginosus* can not catalyse (Jacobs *et al*, 2000).

A diversity of species was detected from the all three types of specimen (see Table 2.2). The majority of the taxa found have previously been isolated from the oral cavity (Chhour *et al*, 2005; Johnson *et al*, 1999; Kazor *et al*, 2003; Marchandin *et al*, 2003; Marsh & Martin, 1999; Munson *et al*, 2004; Paster *et al*, 2001, 2002; Senpuku *et al*, 2003; Szczerba & Krzeminski, 2002; Tada *et al*, 2004; Tanner *et al*, 1994), or from clinical specimens from other human body sites (Agrawal & Mahapatra, 2005; Bell & Friedman, 1994; Blue *et al*, 1995; Elomari *et al*, 1997; Feurer *et al*, 2004; Lee *et al*, 2005; Mättö *et al*, 2004; Rathinavelu *et al*, 2003; Riegel *et al*, 1996; Riegel *et al*, 1997). However, there were a small number of exceptions. For example, some species previously only reported (at the time of writing) as isolates from environmental sources were detected, such as *Bacillus psychrodurans, Dietzia psychralcaliphila, Exiguobacterium oxidotolerans* and *Rothia amarae*. However, all these species were only first described in the last three years (Abd El-Rahman *et al*, 2002; Fan *et al*, 2002; Yumoto *et al*, 2002, 2004), so it is not necessarily surprising that there be few reports of their isolation. It is probably worth noting that closely

related species have previously been isolated from human sources. For example, other psychrotolerant species of *Bacillus* are well-known pathogenic food contaminants and have been cultivated from cases of gastroenteritis and systemic infection (Ginsburg *et al*, 2003; Latsios *et al*, 2003; Stenfors & Granum, 2001); *Dietzia maris* has been isolated from an immunocompromised patient with bacteraemia (Bemer-Melchior *et al,* 1999) and a bone biopsy of a man undergoing total hip prosthesis replacement (Pidoux *et al,* 2001); *Exiguobacterium aurantiacum* has been detected within the subgingival pockets of patients with periodontal disease (Zijnge *et al*, 2003); *Rothia dentocariosa,* a member of the normal oral microflora, has been found in patients with dental caries (Munson *et al*, 2004), endocarditis (Boudewijns *et al*, 2003) and septicaemia (Shin *et al*, 2004).

Other taxa from this study that have not previously been isolated from clinical sources include the apparently novel species cultivated here. Seven distinct taxa were cultured that had 16S rRNA gene sequences with no significant matches (<99% homology) to anything in the public databases at that time. In addition, four taxa were isolated that only matched GenBank entries from uncharacterised, uncultured species. Four specimens contained a *Prevotella* species with a significant sequence match to "Oral clone BE073," an uncultured putative species detected in purified crevicular epithelial cells (Kazor *et al*, 2003). One tumour tissue specimen yielded an isolate matching "*Scardovia* genomospecies C1," a taxon recently detected in the microflora of dental caries (Munson *et al*, 2004). A *Streptococcus* species was detected from one superficial specimen that matched the "*Streptococcus* sp. oral strain T4-E3" phylotype, previously detected by molecular means on the surface of the tongue

dorsa (Kazor *et al*, 2003). Isolates were cultivated from 2 superficial tissues that sequence-matched "*Bifidobacterium* sp. oral strain H6-M4," another taxon cloned from the tongue dorsa microflora (Kazor *et al*, 2003). The 16S sequence for these isolates also matched the proposed species "*Bifidobacterium urinalis*," although this nomenclature is as yet unpublished and has no current standing. The 16S data for each of these previously-uncultured taxa has been submitted to GenBank and it will be of great interest to see if more matching isolates are reported from other investigations. Should more isolates of these taxa be cultivated, it will be interesting to characterise these isolates further, both genotypically and phenotypically, and determine definitively if they are representative of novel species. Recent proposals suggest a minimum 5 strains isolated from separate sources need to be described before an emerging species is officially recognised (Drancourt *et al*, 2004). So far none of the "novel" isolates found in this study have been isolated in sufficient quantity.

The great diversity of species isolated together with the relatively low number of patients and specimens used in this study makes it difficult to apply statistical analyses to draw conclusions regarding bacterial specificity. With that in mind, when ANOVA and Fisher's exact tests were performed to compare the species isolated from the tumourous and "superficial" specimens with those from the non-tumourous control tissues, it was not unexpected to see that most of the results showed no significance (p-value > 0.05; see Table 2.4). Only two findings exhibit statistical significance by the ANOVA method. Firstly, that members of the *Bacteriaceae* group, namely the three *Bacillus* taxa and closely-related *Exiguobacterium* species, were only isolated from tumourous tissue-containing specimens. Secondly, that *Actinomycetaceae* were detected within

non-tumourous tissues, but not at all from tumourous specimens and from only one "superficial" specimen.

Statistical considerations aside, a number of interesting trends are apparent from the results. Several species detected in the non-tumourous control tissue were not detected in the tumour tissues, and vice versa. For instance, in addition to the *Bacteriaceae* species, *Micrococcus* spp., *Prevotella melaninogenica, Staphylococcus aureus* and *Veillonella parvula* were only isolated from tumourous or superficial specimens, and not at all from non-tumourous ones. Conversely, *Moraxella osloensis* and *Prevotella veroralis* were only grown from non-tumourous tissues. These observations could feasibly be used to form hypotheses for future research.

In all cases, the superficial tissues yielded exactly the same isolates as the corresponding deep tissue specimens plus, in most cases, additional species. The greater number of different taxa isolated from the superficial samples (n=78, from 19 samples) in comparison to the deep tissues (n=54, from 20 samples) appears to be significant ($p = 0.0007$). This may indicate a degree of restriction of bacteria in the deeper tumour tissue in comparison to the overlying mucosal sites. Perhaps not all species coming from the oral cavity and invading the mucosa can invade or survive in the tumourous tissue.

The most commonly isolated taxa from the OSCC patients' tissue specimens were *Granulicatella* (formerly *Abiotrophia*) *adiacens, Micrococcus luteus, Peptostreptococcus micros, Propionibacterium acnes, Staphylococcus capitis/caprae/epidermidis, Streptococcus anginosus, Streptococcus constellatus, Streptococcus mitis/oralis* and *Streptococcus parasanguinis* (see Table 2.5), all of which have previously been detected within human oral

cavities (Marsh & Martin, 1999; Tanner *et al,* 1994). Furthermore, all have been associated with infection and disease (Araki *et al*, 2004; Debelian *et al*, 1992; Gomes *et al*, 2004; Jacobs *et al*, 2003; Lang *et al*, 1999; Rôças & Siqueira, 2005; von Eiff *et al*, 1996). *Peptostreptococcus micros* and *S. epidermidis* have been seen to adhere to epithelial cells (Almeida & Oliver, 2001; Kremer *et al*, 1999). *S. constellatus* can invade and survive within cells from human epithelium (Eick & Pfister, 2004). Most relevantly, *Str. anginosus*, cultivated from 4 patients here, has previously been detected in oral SCC specimens (Tateda *et al*, 2000; Morita *et al*, 2003). As discussed earlier (see section 1.3.1.3), the species has been implicated in the process of carcinogenesis due to both its association with tumours and its ability to induce inflammation (Sasaki *et al*, 2001; Sugano *et al*, 2003). *Propionibacterium acnes*, isolated from 11 patients, also has a longstanding association with inflammation. More specifically it has been associated as a cause of inflammatory acne (Farrar & Ingham, 2004; Graham *et al,* 2004) and, more recently, with inflammation of the prostate gland. Prostatic inflammation has been implicated in the development of prostate cancer (Cohen *et al,* 2005). At the time of writing, the other most commonly isolated taxa have not been associated with any aspect of carcinogenesis.

It is particularly interesting to note that the majority of species isolated were acid-tolerant. Whereas the pH of all parts of the healthy human mouth is generally neutral, between 6.75 and 7.25 (Marsh, 2003), the microenvironment within solid tumours is known to be hypoxic and with an extracellular pH that is characteristically low (Raghunand *et al*, 2003). This acidity is believed to exist within tumours as a result of the accumulation of lactic acid and also via the local conversion of CO_2 to bicarbonate plus a hydrogen ion (Švastová *et al*,

2004). As many bacterial species have a relatively narrow pH range in which they grow (Marsh, 2003), it is to be expected therefore that not all members of the typical human mouth microbiota could survive within an oral cavity tumour. Only species with the capacity to survive acidic environments are likely to be found in a viable state within an OSCC, and this is indeed what was seen with this cultural analysis. The vast majority of species isolated have previously been discovered within dental caries lesions (Chhour *et al*, 2005; Munson *et al*, 2004), the resting pH of which can typically fall as low as pH 4.0. Most of the isolates were from saccharolytic groups such as actinomycetes, bifidobacteria, lactobacilli, streptococci, prevotellae, veillonellae and yeasts – species which convert carbohydrates into short chain organic acids and are known to grow at low pH (Bradshaw & Marsh, 1998; Brailsford *et al*, 2001; Shah & Gharbia, 1989; Svensäter *et al*, 2003; Takahashi *et al*, 2004). The asaccharolytic species *Fusobacterium nucleatum,* also detected in this study, has been shown to grow successfully at the relatively low pH 5.0 *in vitro* (Takahashi, 2003). Species of β- and γ-Proteobacteria, similar to some of the isolates here, are also aciduric (Curtis *et al*, 2002).

The implications of the presence of a diversity of viable bacteria deep within the tissue of squamous cell carcinoma are unclear. The fact that bacteria are inside the tumour tissue may indicate that the infection began before or in the early stages of carcinogenesis, although it has been shown in animal models that bacteria injected intravenously may seed to tumour tissue (Yu *et al*, 2004). The types of bacteria isolated and the fact that the composition of the deep tissue microflora was similar to, but less species-rich than, the overlying mucosa seems

to imply a local origin for the bacteria detected within the tumour. The apparent differences between the microflora of the tumour and control tissues suggests a degree of bacterial specificity that merits further study. The particular species found in the mucosal and tumourous tissues of OSCC patients, and whether these viable bacteria were present before the instigation of cancer or represent later colonisers are concepts worthy of further investigation.

CHAPTER THREE

ANALYSIS OF THE BACTERIA WITHIN TUMOUR TISSUES BY DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

3.1 Introduction

The work in Chapter 2 described the analysis of bacteria present within oral squamous cell carcinoma (OSCC) tissues by culture. However, not all bacteria can be grown in the laboratory. Indeed, following the application of molecular biological techniques to bacterial communities, it has been estimated that less than 2% of all species are recoverable in standard culture media (Wade, 2002). The main reason for the "unculturability" of many bacteria is probably the current lack of knowledge of the conditions under which they grow in their natural habitat, and the inherent difficulty in accurately re-creating these conditions *in vitro* (Spratt, 2004; Wade, 2002). This is further complicated by the fact that most bacteria grow in mixed communities and many species may have evolved to be dependent on others. Isolating individual species during cultivation can separate them from the bacterial cytokine networks that they normally exist within, possibly disrupting the signalling systems that govern the growth of each cell (Kell & Young, 2000). Additionally, a number of bacteria, particularly Gram-negative species, exist as "viable but non-culturable" (VBNC) forms, not replicating yet metabolically active and maintaining their pathogenic characteristics. This is thought to be a survival strategy to allow the cells to endure through unfavourable environmental conditions (Kell & Young, 2000; Lleo *et al*, 2005; Oliver, 2005).

Approximately half of the oral microflora is unculturable (Wade, 2004). Therefore, when investigating the diversity and composition of any oral communities of bacteria it is necessary to use additional, culture-independent approaches.

The use of molecular approaches has allowed investigators to bypass the cultivation step when studying microbial populations. In particular, amplification of the 16S rRNA gene by PCR using "universal" primers, which will theoretically target sequences present in all known species of bacteria (Baker *et al*, 2003; Relman, 1993; Spratt, 2004), has provided a suitably efficient means by which to explore bacterial diversity. The ubiquity and uniqueness of the 16S rRNA gene makes it an extremely useful method for identifying bacteria (Clarridge, 2004). This approach has been used in conjunction with genetic profiling procedures to develop rapid and relatively easy techniques with which to analyse microbial communities (Gafan *et al*, 2005; Spratt, 2004).

Genetic fingerprinting techniques work on the basis of the physical separation of unique nucleic acid molecules and can be used to generate profiles of the diversity of species present in complex communities (Muyzer, 1999). One such technique is denaturing gradient gel electrophoresis (DGGE). DGGE was originally developed as a means of detecting single mutations in genomic DNA, but has more recently been adapted by microbial ecologists for analysing whole bacterial communities (Li *et al*, 2005). In DGGE, fragments of DNA are separated in a sequence-dependent manner. Double-stranded DNA molecules with different sequences will exhibit different melting behaviour and so, when progressed by electrophoresis through a polyacrylamide gel containing a linear gradient of DNA denaturants, will stop migrating at different positions in the gel (Muyzer, 1999). The separation and detection of fragments with little difference in sequence, such as single-base changes, can be improved by preventing complete disassociation of the two DNA strands and slowing down their migration through the gel. This is usually achieved by amplifying the target

sequences using a PCR primer with a GC-clamp, a G+C-rich sequence attached to the 5'-end (Sheffield *et al*, 1989; Wu *et al*, 1998).

PCR-DGGE of 16S rRNA gene fragments has been used to visualise the bacterial microflora from many different environmental sources, for instance from soil (Arias *et al*, 2005; Bossio, *et al*, 2005), hot springs (Ferris *et al*, 1997), underwater hydrothermal vents (Sievert *et al*, 2000), and ancient wall paintings (Rölleke *et al*, 1996). More recently, the technique has been used to detect microbes in food (Ercolini, 2004) and to assess the microbial diversity in human clinical specimens, such as biopsies of chronic venous leg ulcers (Davies *et al*, 2004), swabs from ocular infections (Schabereiter-Gurtner *et al*, 2001), and faecal samples from patients undergoing antimicrobial therapy (Donskey *et al*, 2003). 16S rRNA gene PCR-DGGE has also been used to profile the bacterial communities within the human oral cavity in both health (Li *et al*, 2005) and disease states. For instance, the technique has been used to demonstrate the reduction in diversity of the subgingival microflora immediately following periodontitis treatment (Zijnge *et al*, 2003). Similarly, it has also been used to establish that microbial communities are more complex and species-rich in deep periodontal pockets compared to shallow ones (Fujimoto *et al*, 2003). Furthermore, the technique has also been used to monitor the microflora associated with caries (Li *et al*, 2005) and endodontic infections (Rôças *et al*, 2004; Siqueira *et al*, 2005). At the time of writing, however, the microflora present within human OSCC has not previously been analysed by this technique.

3.2 Aims

The aim of this study was to develop a method for the PCR-DGGE analysis of 16S rRNA gene sequences in order to visualise and compare the richness of bacterial species present within oral squamous cell carcinoma (OSCC) tissue and non-tumourous tissue from the same patients.

3.3 Materials and Methods

3.3.1 Extraction of DNA from tissue specimens

Total DNA was extracted from tissue specimens that were obtained from patients 1 – 20 (Table 2.1) and surface decontaminated, as described previously (see sections 2.3.1 and 2.3.2). Tissues were initially digested by incubating in proteinase K (2.5 μg/ml; Promega) at 55°C overnight. DNA was isolated from the resultant suspension by the use of a Puregene kit (Gentra Systems) and following the "DNA Isolation From Gram-positive Bacteria Culture Medium" protocol (Appendix IV).

3.3.2 PCR amplification of bacterial 16S rRNA genes

In order to produce a high enough concentration of PCR product to be visualised by polyacrylamide gel electrophoresis a nested PCR protocol was used. Two-round nested PCR is known to be significantly more sensitive than single-round PCR (Fukano, 2004; Stärk *et al*, 1998). The first round of PCR used the primers D88 and E94 (0.5 μM of each; synthesised by Invitrogen), which are both specific to the domain *Bacteria* and were first described by

Paster *et al* (2001) (see Appendix I), to produce a fragment approximately 1500-bp in size. Each reaction mixture had a total volume of 20 µl and also contained 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, the working concentration of magnesium-free buffer, 1.5 U of *Taq* polymerase (all from Promega) and 5 µl of DNA extract (approximately 0.5 µg) as template. The protocol started with 8 min of denaturation at 95°C and was followed by 30 cycles consisting of denaturation at 95°C (45 s), annealing at 60°C (60 s) and extension at 72°C (105 s, increasing by 5 s each cycle). A final extension step at 72°C (10 min) was included.

Product from the first round (0.5 µl) was used as the template for a second round of PCR.

A 194-bp fragment of the 16S ribosomal RNA gene was amplified using the primers 341f and 534r (0.5 µM of each), which cover the V3 variable region of the gene and were first described by Muyzer *et al* (1993) (Appendix I). The forward primer contained at its 5' end a 40-base GC clamp (Rölleke *et al*, 1996). Reaction mixtures were a total of 50 µl and contained 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, the working concentration of magnesium-free buffer and 1.25 U of *Taq* polymerase (Promega) in a total volume of 50 µl. A touchdown protocol was used whereby, following an initial 5 min denaturation step at 95°C, there were 20 cycles of denaturation at 94°C (1 min), annealing at 65°C (1 min) and extension at 72°C (1 min) with the annealing temperature being reduced by 0.5°C each cycle. Another 10 cycles were then performed with the annealing temperature at 55°C, and the protocol was finished with 10 min of extension at 72°C. Touchdown protocols are known to increase the sensitivity and specificity of PCR (Duckworth & Rule, 2003; Fukano, 2004).

The numbers of bands in each profile, as estimated by the Quality One® band detection software, are listed in Table 3.1. The average numbers of bands for each of the 3 types of tissue are also shown. Examples of band detection, as performed on the tumourous tissue specimens, are shown in Figure 3.6.

Once profiles from each specimen were aligned, the Quality One® band-matching program was used to estimate in how many of the specimens distinct band-types were present. A total of 54 different relative positions or band types were observed in the various profiles. The occurrence of many of the bands in the 3 specimen types and the statistical significance of the differences in proportions are listed in Table 3.2. Bands were observed that were present in only one type of specimen, tumourous, superficial or non-tumourous. In addition, bands were detected in all three types (for example, the bands designated as 1, 16 and 36; see Table 3.2), although no single band type was detected in every profile. According to the Fisher's exact tests (Table 3.2), there were 12 bands (designated 6, 13, 14, 19, 22, 25, 39, 47, 48, 50, 53 & 54) present in significantly more tumourous specimens than superficial ($p < 0.05$) and, conversely, 8 bands (2, 3, 7, 9, 12, 18, 27 & 29) that were in significantly fewer. Likewise, there were 3 bands (6, 13 & 25) observed in a significantly higher number of the tumourous than non-tumourous specimens, and 7 bands (3, 11, 14, 18, 21, 27, & 34) in significantly more non-tumourous than tumourous. There were 5 bands (9, 12, 33, 37 & 41) present in a significantly higher proportion of tumour-containing superficial specimens than non-tumourous ones. On the other hand, there were 9 bands (11, 14, 19, 34, 39, 45, 49, 52 & 54) in significantly fewer superficial than non-tumourous tissues.

Figure 3.1 A typical gel picture of 16S rRNA gene nested PCR products from 5 tissue specimens showing products suitable for DGGE analysis

Some typical products from the nested PCR of DNA extracts from five specimens, in this case the deep tumour tissues from patients 01 – 05. Products have been electrophoresed alongside a 100-bp molecular marker (Promega). Products suitable for DGGE analysis were approximately 260 bp in size.

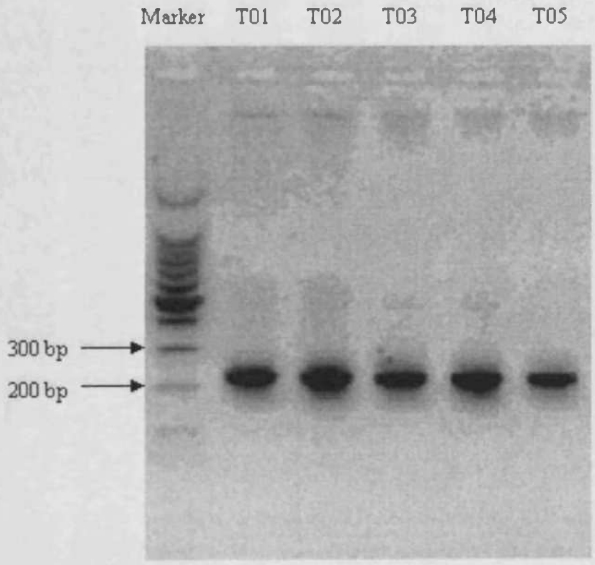


Figure 3.2 DGGE profiles representing the bacterial diversity in the deep tumour tissue specimens from patients 1 – 10

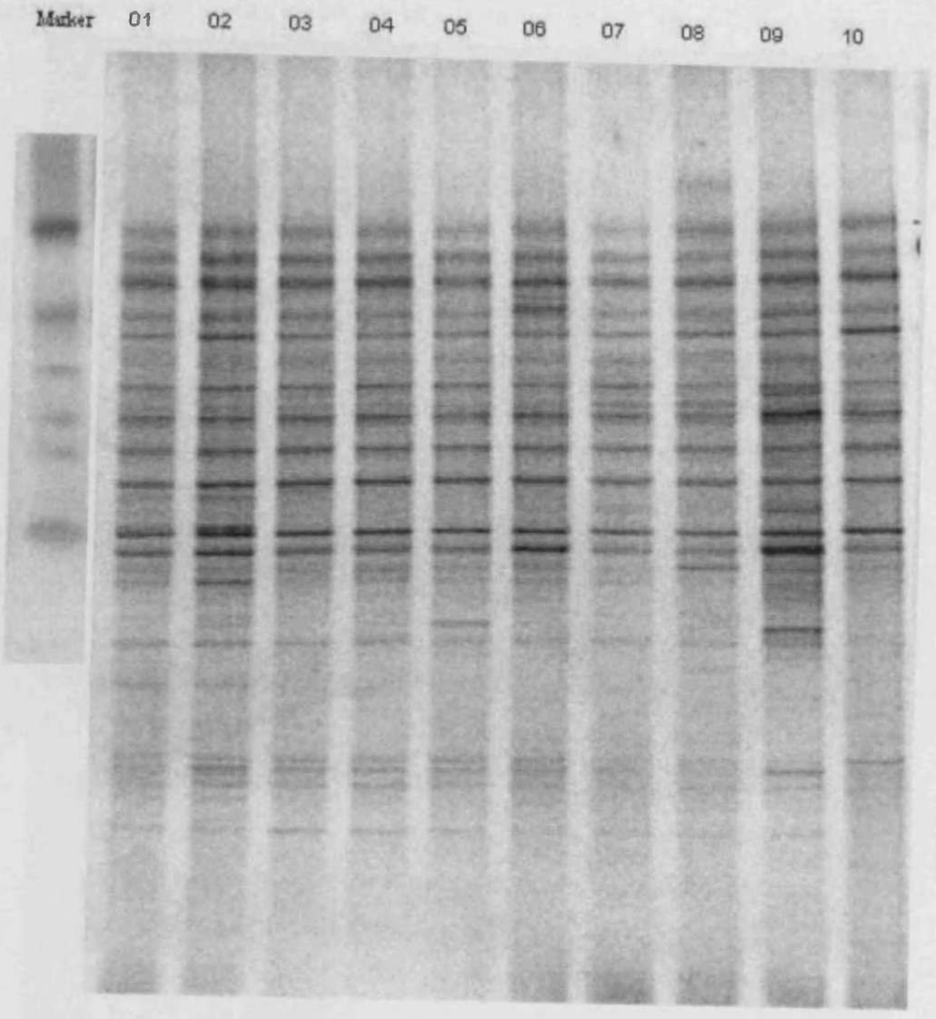


Figure 3.3 **DGGE profiles representing the bacterial diversity in the deep tumour tissue specimens from patients 11 – 20**

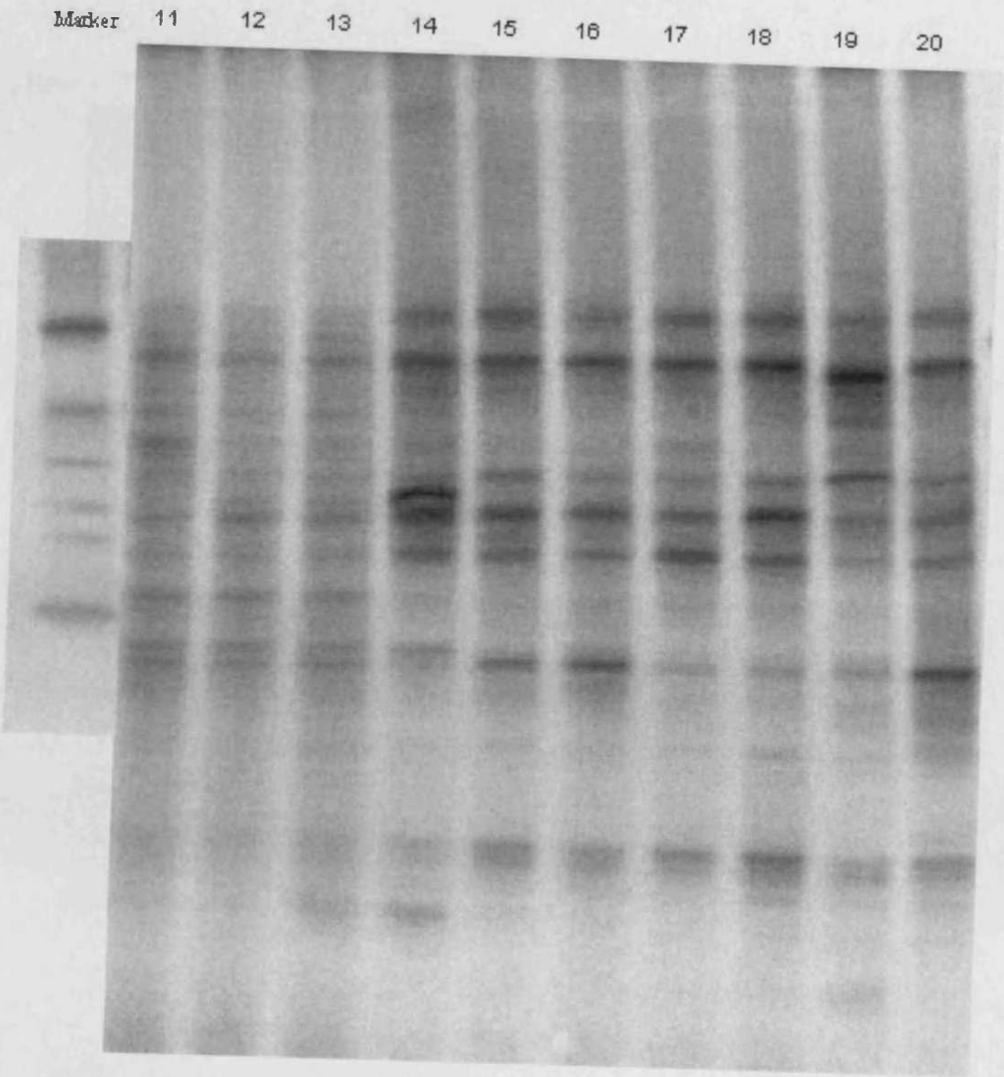


Figure 3.4 **DGGE profiles representing the bacterial diversity in the 19 "superficial" specimens, obtained from patients 01 – 06 and 08 – 20**

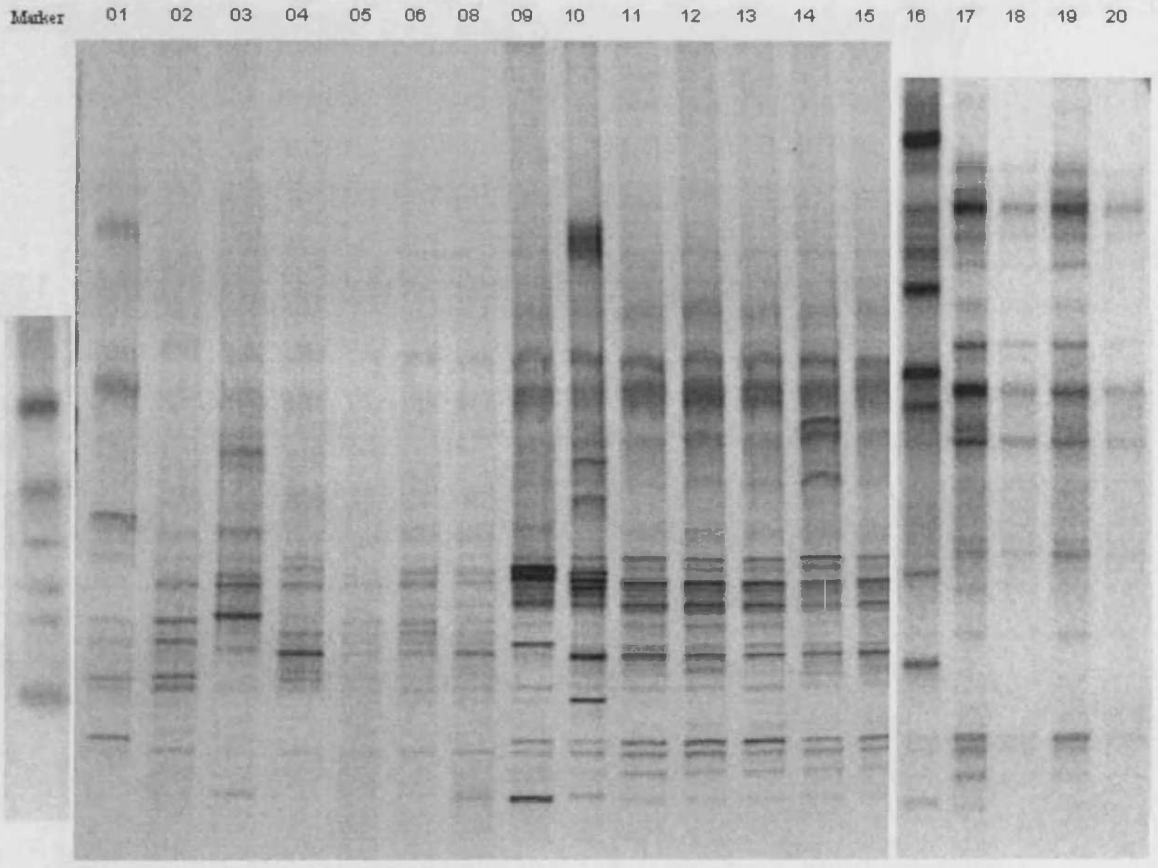


Figure 3.5 DGGE profiles representing the bacterial diversity in the 12 non-tumourous, control tissue specimens from patients 08 and 10 – 20

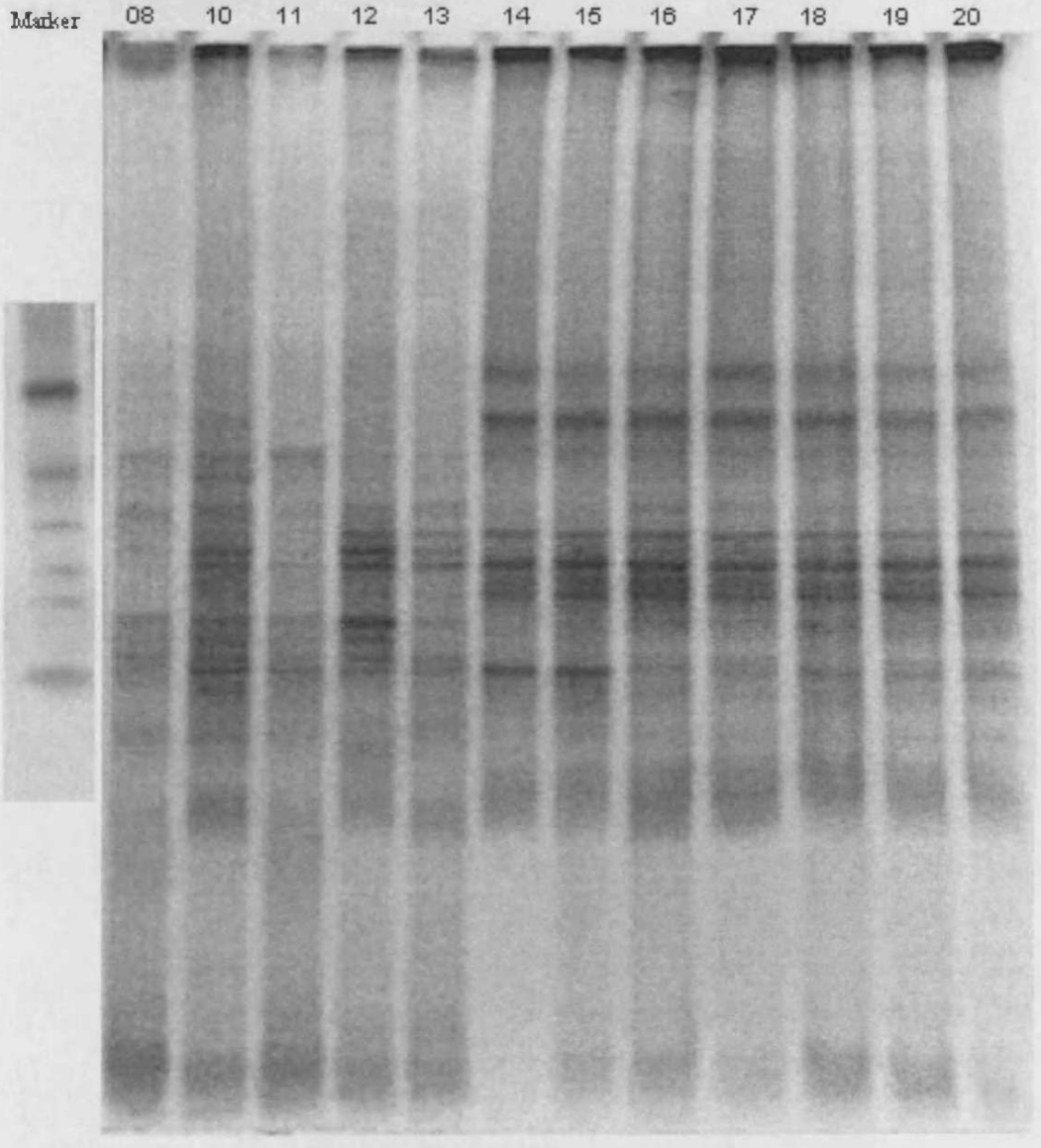


Figure 3.6 Examples of band richness counts by the Quantity One quantitation software
These two images show the bands recognised and counted for the 20 tumourous tissue specimens.

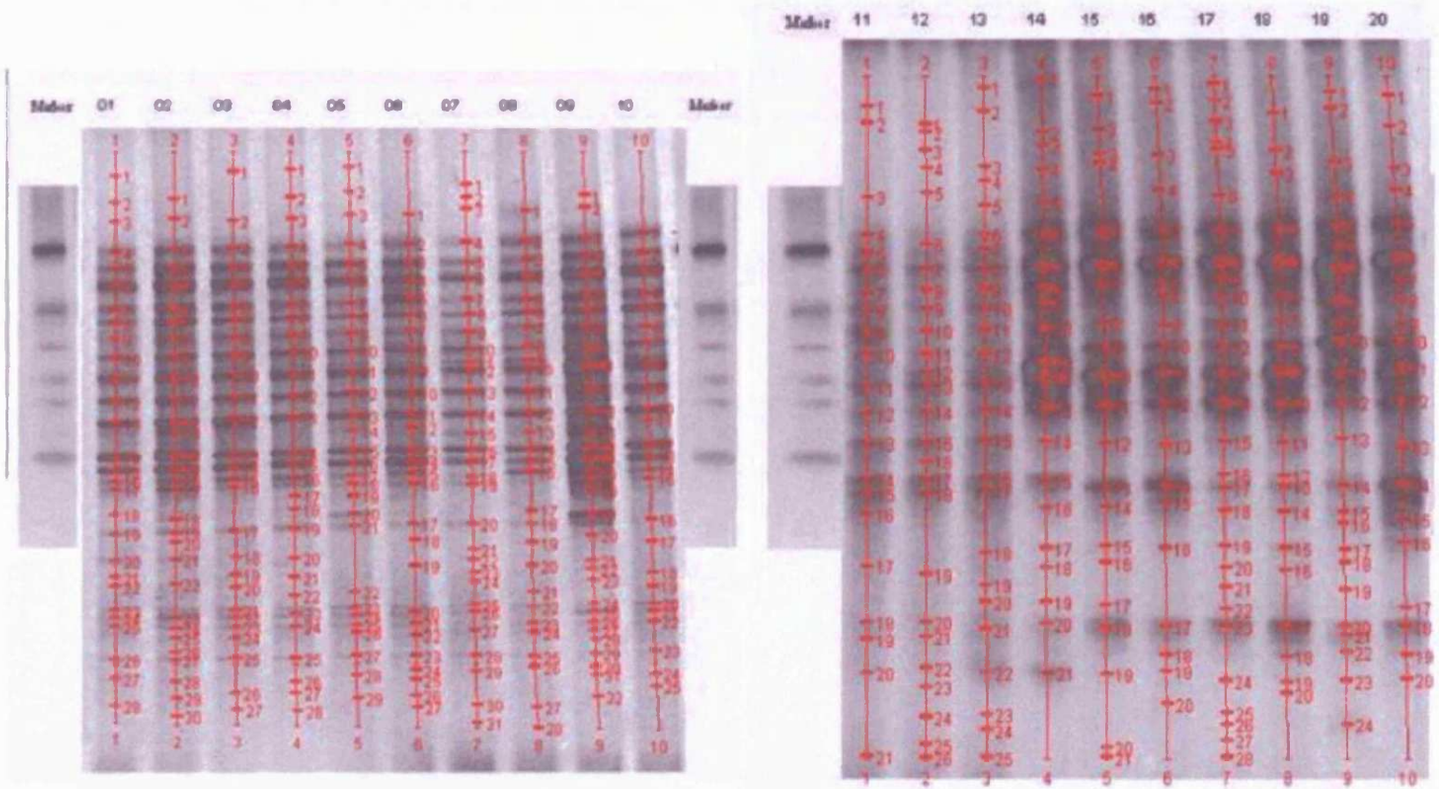


Table 3.1 The number of bands observed in the DGGE profiles for each tissue specimen

Band richness was measured using the Quantity One quantitation software.

Patient number	Specimen type		
	Tumourous tissue	Tumourous and overlying mucosal tissue	Non-tumourous tissue
01	28	18	n/a
02	30	24	n/a
03	27	17	n/a
04	28	19	n/a
05	29	23	n/a
06	27	30	n/a
07	31	n/a	n/a
08	28	26	24
09	32	22	n/a
10	25	21	27
11	21	22	27
12	26	23	31
13	25	25	29
14	21	23	28
15	21	23	20
16	20	21	28
17	28	24	23
18	20	25	27
19	24	22	26
20	20	27	23
Average	25.55	22.89	26.08

Table 3.2 Correlations between the presences of some bands within gel patterns from the different specimen types

The significances of the differences in occurrence were calculated by the use of 2-tailed Fisher's exact tests. P-values are shown to 3 significant figures. Significant results ($p < 0.05$) are shown in blue. The band designations are the arbitrary ones supplied by the band-matching software and are not the same designations shown in Figure 3.6.

Band designation	Specimen type detected within			Significance of difference in occurrence (p-value)		
	Tumourous tissue (n = 20)	Tumourous and overlying mucosal tissue (n = 19)	Non-tumourous tissue (n = 12)	Between tumourous and superficial specimens	Between tumourous and non-tumourous specimens	Between superficial and non-tumourous specimens
1	**9**	**9**	**5**	1.00	1.00	1.00
2	**1**	**7**	**4**	0.0196	0.0531	1.00
3	**3**	**13**	**10**	0.00106	0.000223	0.433
4	**13**	**11**	**3**	0.748	0.0659	0.138
5	**5**	**5**	**2**	1.00	0.683	0.676
6	**10**	**1**	**1**	0.00334	0.0232	1.00
7	**2**	**14**	**5**	0.0000686	0.0735	0.130
8	**7**	**2**	**2**	0.127	0.422	0.630
9	**4**	**15**	**2**	0.000360	1.00	0.00105
10	**3**		**2**	0.231	1.00	0.142
11	**4**	**2**	**8**	0.661	0.0213	0.00200

12	**8**	**15**	**1**	0.0225	0.103	0.000171
13	**8**	**1**		0.0197	0.0135	1.00
14	**20**	**11**	**12**	0.00123	1.00	0.0116
15	**1**	**5**	**8**	0.0915	0.000361	0.0596
16	**8**	**8**	**4**	1.00	1.00	1.00
17	**5**	**4**		1.00	0.130	0.139
18	**5**	**12**	**9**	0.0248	0.0100	0.697
19	**11**	**1**	**7**	0.00125	1.00	0.00197
20	**7**	**9**	**3**	0.523	0.703	0.274
21	**5**	**11**	**9**	0.0536	0.0100	0.452
22	**5**			0.0471	0.130	1.00
23	**4**	**2**		0.661	0.271	0.510
24	**9**	**5**	**5**	0.320	1.00	0.447
25	**12**	**3**	**2**	0.00791	0.0276	1.00
26	**7**	**6**	**3**	1.00	0.703	0.785
27	**3**	**10**	**6**	0.0187	0.0493	1.00
28	**3**			0.231	0.274	1.00
29		**5**		0.0202	1.00	0.128
30	**10**	**7**	**8**	0.523	0.471	0.149
31	**5**	**1**	**4**	0.182	0.696	0.0600
32	**3**	**3**	**1**	1.00	1.00	1.00
33	**15**	**17**	**5**	0.407	0.130	0.0118

34			**5**	1.00	0.00393	0.00466
35	**4**	**1**		0.342	0.271	1.00
36	**3**	**3**	**1**	1.00	1.00	1.00
37	**4**	**9**	**1**	0.0958	0.626	0.0464
38	**2**	**5**	**2**	0.235	0.620	0.676
39	**7**	**1**	**5**	0.0436	0.724	0.0217
40	**7**	**5**	**5**	0.731	0.723	0.447
41	**6**	**10**		0.200	0.0613	0.00409
42	**4**	**1**		0.342	0.271	1.00
43	**2**	**6**	**1**	0.127	1.00	0.201
44	**4**	**2**	**2**	0.661	1.00	0.630
45	**4**		**5**	0.106	0.240	0.00466
46	**2**			0.487	0.516	1.00
47	**9**	**2**	**5**	0.0310	1.00	0.0776
48	**8**	**1**	**2**	0.0197	0.248	0.543
49	**1**		**4**	1.00	0.0531	0.0157
50	**5**		**1**	0.0471	0.370	0.387
51	**3**		**1**	0.231	1.00	0.387
52	**2**	**1**	**5**	1.00	0.0735	0.0217
53	**6**		**2**	0.0202	0.676	0.142
54	**7**	**1**	**9**	0.0436	0.0659	0.0000957

3.5 Discussion

The total number of bands in each profile is related to the number of dominant phylotypes. This 'band richness' can be used to compare the populations from the different specimens (Fromin *et al*, 2002). The different average numbers of bands in the three specimen categories may, therefore, indicate different numbers of phylotypes in the various tissue types. However, it is surprising that there be less richness, on average, in the "superficial" specimens comprising tumour and overlying mucosa than in the other tissues, particularly given that cultural analysis of the same samples indicated that there were generally increased numbers of taxa in these specimens (Chapter 2).

It is interesting to note that there are bands that are present in the majority of specimens. For instance, the band at designated position 14 was observed in each of the deep tumour and non-tumourous, and 11 of the 19 "superficial" specimens. This may be an indicator that there was at least one species that was present in every OSCC patient who formed part of this study. Conversely, there are bands that were apparently only present in a few profiles, such as those labelled 32 and 36, demonstrating that there was variety in microbial compositions of the different patients.

Fisher's exact tests revealed that certain bands occurred in the various specimen types at significantly different frequencies (see Table 3.2). This gives an indication that there are significant differences in the species present in tumourous and non-tumourous mucosa.

Many reports that have used PCR-DGGE also analyse the profiles by measuring the intensities of the bands and comparing between the different communities. Band intensity can be assumed to be related to the relative proportion of each dominant phylotype in the sample. Furthermore, it can be computationally combined with band richness measurements to create refined results for the statistical comparison of banding patterns (Fromin *et al*, 2002; McCaig *et al*, 2001). This approach was not taken with this study due to the problem of bias. Quantification of 16S rRNA gene sequences in molecular analyses may be impaired by biases introduced during both the extraction and PCR amplification of the bacterial DNA (McCaig *et al*, 2001). For instance, it can only ever be assumed that every bacterium in a sample has been successfully lysed and that the recovery of DNA has been proportionally equal from both Gram-positive and Gram-negative cells (Wade, 2004). Additionally, the main drawback of "universal" PCR is that the non-specific primers used can be biased towards sequences from certain species. The differential amplification of 16S rRNA gene sequences in multi-template PCR has been observed in several studies (Ishii & Fukui, 2001; Kurata *et al*, 2004; Polz & Cavanaugh, 1998). When amplifying mixed templates from a polymicrobial source, some universal primers appear to selectively amplify the sequences with a low G+C content. This may be because GC-rich DNA templates disassociate relatively poorly (Ishii & Fukui, 2001; Polz & Cavanaugh, 1998) and also because polymerases will work comparatively inefficiently with such sequences, presumably due to the formation of hairpin-like secondary structures (Mytelka & Chamberlin, 1996). Further bias can come from the different copy numbers of 16S rRNA genes and sizes of the genomes of the various species present in the template

mixture. The number of 16S rRNA genes present in the genome ranges between 1 to 14, depending on the species (Farrelly *et al*, 1995).

Bias from PCR was particularly problematic in this study due to the use of a nested PCR protocol. The use of 2 sets of universal primers in 2 rounds of PCR unavoidably increases any effects of bias exponentially, making the measurement of any band intensities unreliable. This was observed when repetition of each PCR-DGGE showed that, although the number of bands seen for each specimen was reproducible, the relative intensity of the bands was variable. Unfortunately, the nested PCR approach was necessary to provide enough DNA to be seen on the gel. Despite the sensitivity of the protocol with the DGGE primers 341f and 534r (Rölleke *et al*, 1996), 1-round PCR using these primers could not provide enough product to be detectable by agarose gel electrophoresis. Presumably this is due to the low numbers of bacteria and low quantities of 16S rRNA gene DNA in the tissue extracts. An alternative to nested PCR would have been to perform multiple single-round reactions for each specimen and pool the results until there was enough DNA to be visualised under UV light. This approach was not used here as it would require more DNA extract to use as the PCR template, but only a small and finite amount was available.

As well providing a visual fingerprint of a bacterial community, PCR-DGGE of the 16S rRNA gene has been used in previous studies to identify the species present. Bands, particularly those representing taxa not detected from the same sources by culture methods, have been excised from DGGE gels and the DNA fragments sequenced and characterised (Davies *et al*, 2004; Rölleke *et*

al, 1996; Siqueira *et al*, 2005). Theoretically this method could have been applied to the tissue specimens examined here to potentially detect uncultivated species. However, using DGGE as a technique for species identification does have severe limitations. It has been observed, especially in more complex mixed populations, that DGGE can fail to separate 16S rRNA gene fragments despite their multiple base differences. Sequences from different species can co-migrate to the same position in the gel, possibly misrepresenting the number of species present (Jackson *et al*, 2000). This problem was observed during the course of this investigation. For instance, although a reproducible banding pattern was obtained when PCR products from the 4 NCTC marker strains were run in the same lane, each band seen was found to contain 16S rRNA gene fragments from more than one of the bacteria (data not shown). On a practical level, such co-migration can add an extra complication to the characterisation of each band because every fragment excised from the gel must be singularised before it can be sequenced. This can involve either another gradient gel electrophoresis step or a time-consuming cloning process.

Nevertheless, even though the PCR-DGGE technique was not used to quantify or identify the species present in the tissue specimens, a suitable method for visualising bacterial richness in OSCC tissues was developed. The profiles obtained reveal that there are significant differences in the composition of the microbiota from the 3 types of tissue from OSCC patients. The hypothesis that a significantly differing diversity of bacterial species is present within OSCC tumours compared to the healthy mucosa from the same patients has been supported. It is of interest to characterise the composition of the

microflora, identify the bacteria present and highlight the potential differences in diversity. Future studies on greater numbers of specimens may confirm whether specific bands or species correlate significantly with different tissues types, and also whether there are any associations between particular bands and different types of tumour. For instance, it is entirely possible that the microflora present with the OSCC tissue is age-dependent and that it changes as the disease progresses, and so it may be interesting to examine specimens from carcinomas from more patients of different age groups and with tumours of varying stages of progression.

CHAPTER FOUR

ANALYSIS OF THE BACTERIA WITHIN TISSUES FROM ORAL SQUAMOUS CELL CARCINOMA BY CLONING AND SEQUENCING OF 16S RIBOSOMAL RNA GENES

4.1 Introduction

When considering the bacteria from all environments, less than 2% are culturable *in vitro* using artificial media. In the case of the human oral cavity it has been estimated that only 50% of the bacterial species present can be cultured in the laboratory (Wade, 2002). The "unculturability" of some species *in vitro* is, in most cases, probably due to our lack of knowledge about their nutritional or atmospheric requirements. Current culture media may be lacking in an essential nutrient, or may contain a substance toxic or inhibitory to the growth of the uncharacterised species. Alternatively, some species may appear unculturable because, having evolved to exist in a mixed biofilm, their growth is dependent on the presence of another species (Wade, 2002; Wilson *et al*, 1997). Additionally, some species appear to exist in a "viable but unculturable" state, seemingly as a survival mechanism for adverse conditions (Wade, 2004). Whatever the reasons behind our inability to grow these microorganisms *in vitro*, it is evident that examining specimens containing mixed populations of bacteria using culture techniques alone is insufficient for a complete analysis of the microflora present.

The use of molecular, and specifically 16S ribosomal RNA-based, approaches has allowed researchers to bypass the cultivation step when studying microbial populations. Possibly the most powerful culture-independent tool currently used to analyse bacterial diversity is PCR amplification, cloning and sequencing of 16S rRNA genes. PCR primers have been designed based on sequences known to occur in all previously studied bacterial genomes. These so-called "universal" primers will theoretically bind to complementary targets

within the 16S rRNA gene of any member of the *Bacteria* domain, including previously uncharacterised taxa (Baker *et al*, 2003; Relman, 1993; Spratt, 2004).

Once amplified by universal PCR, the 16S rRNA sequences from a mixed bacterial population can be singularised by cloning the amplicons into *Escherichia coli*. Sequencing the cloned 16S rRNA gene inserts and comparing the sequences to those in the public databanks can then be performed to identify the species present in the original specimen (Munson *et al,* 2004; Paster *et al*, 2001; Wade, 2002). This technique has proved its usefulness amongst environmental microbiologists (Gray & Herwig, 1996; Liles *et al*, 2003) and has become a well-established method of identifying putative pathogens in clinical samples. For example, the bacterial species associated with many different disease states, including brain abscesses (Tsai *et al*, 2004) and inflamed maxillary sinuses (Paju *et al*, 2003), have been elucidated using this molecular approach. 16S rRNA gene sequencing has already been used to reveal the diversity of the human oral microbiota. Bacteria connected to several oral conditions has been characterised, including the microflora associated with plaque (Paster *et al*, 2001), caries (Becker *et al*, 2002; Chhour *et al*, 2005; Munson *et al,* 2004), halitosis (Kazor *et al*, 2003), endodontic infections (Munson *et al*, 2002; Rolph *et al*, 2001), dentoalveolar abscesses (Dymock *et al*, 1996) and advanced noma lesions (Paster *et al*, 2002).

PCR has been used previously to determine the presence of several species of bacteria within tissues from squamous cell carcinoma (SCC) of the upper aerodigestive tract. *Streptococcus anginosus, Streptococcus mitis* and *Treponema denticola* have all been detected within such cancers by PCR using primers specific for these species (Narikiyo *et al*, 2004; Shiga *et al*, 2001; Tateda

et al, 2000). However, at the time of writing, no previous studies have specifically examined the total microflora in OSCC using a molecular approach.

4.2 Aims

The aim of this study was to use a molecular approach (16S ribosomal RNA gene cloning and sequencing) to further identify species of bacteria present within tumourous tissue from oral squamous cell carcinoma (OSCC) and non-tumourous mucosal tissue from the same patients without the biases of culture.

4.3 Materials and Methods

4.3.1 Amplification of 16S rRNA gene sequences

Tumourous 'deep tissue' specimens and corresponding non-tumourous control tissues from patients 11 – 20 (see Table 2.1) were obtained and surface decontaminated as described previously (see sections 2.3.1 and 2.3.2). DNA extracts had been previously prepared by digesting the tissue overnight with proteinase K (2.5 μg/ml; Promega) at 55°C and subsequently using a Puregene® DNA isolation kit (Gentra Systems) and the "DNA Isolation From Gram-positive Bacteria Culture Medium" protocol (Appendix IV).

The DNA extracts were each used as the template for 3 separate PCRs. The three reactions differed only in the reverse primer used, namely either C90 (specific for *Spirochaetes*), F01 (specific for *Bacteroidetes*), or E94 (specific for the domain *Bacteria*). All reactions used the universal D88 as the forward

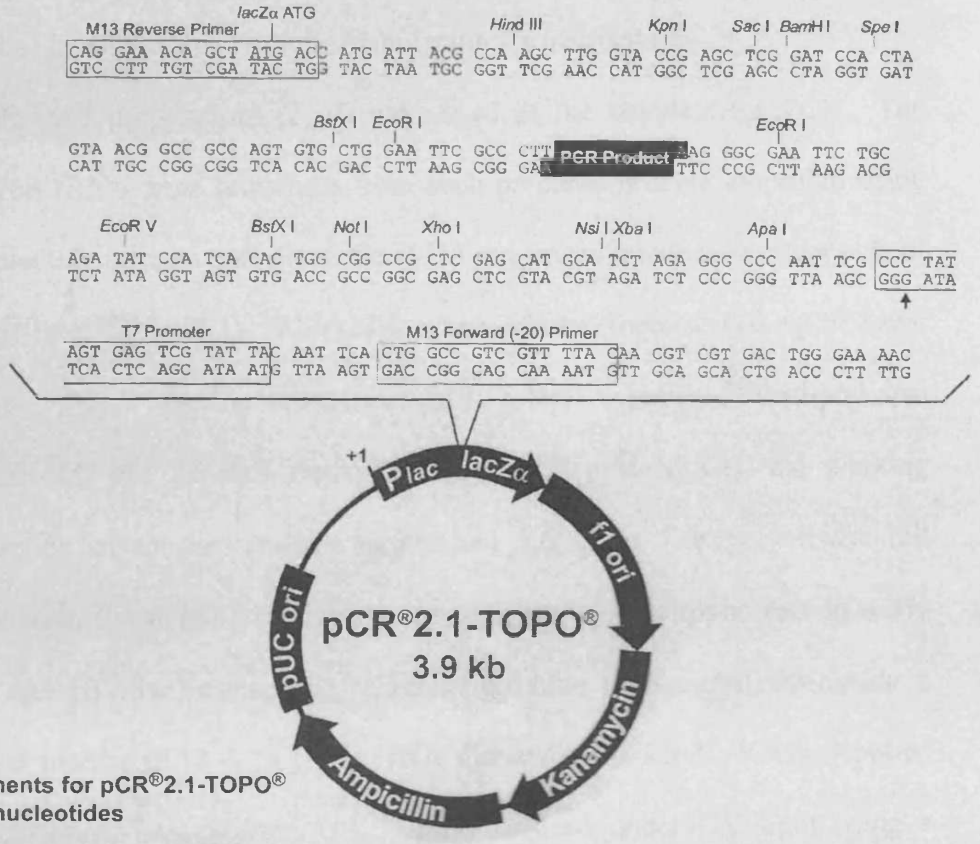
primer. These primers were first described by Paster *et al* (2001). Each reaction mixture comprised 0.5 μM of both forward and reverse primer (synthesised by Invitrogen), 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, the working concentration of magnesium-free buffer, 1.5 U of *Taq* polymerase (all reagents from Promega), and 5 μl DNA extract (approximately 0.5 μg of DNA). As a negative control nuclease-free water (5 μl) was used instead of a DNA extract template. All PCR reactions were performed with a total reaction volume of 50 μl, in a PTC-200 thermal cycler (MJ Research Ltd.) and started with 8 min of denaturation at 95°C. This was followed by 30 cycles consisting of denaturation at 95°C (45 s), annealing at 60°C (60 s), and extension at 72°C (105 s, increasing by 5 s each cycle), and a final 72°C extension step (10 min).

4.3.2 **Cloning of 16S rRNA gene PCR products**

Fresh PCR products (4 μl) were cloned into TOP10 competent *Escherichia coli* using the commercial vector pCR®2.1 (see Figure 4.1) available in the TOPO TA cloning kit (Invitrogen). Transformed cells were cultured overnight at 37°C on Luria-Bertani (LB) agar containing kanamycin (50 μg/ml, Sigma) and X-Gal (40 mg/ml; Bioline). Successful clones could be selected and sub-cultured based on their white appearance after 18 h growth. For each tissue specimen a total of 30 white colonies were picked from the 3 cloning reactions.

Figure 4.1 Plasmid map of the pCR®2.1 vector

Plasmid map showing the features of the commercial vector (produced by Invitrogen) used in the cloning of the 16S rRNA gene fragments. Restriction sites and the sequence surrounding the TOPO® cloning site are shown. Image reproduced from the TOPO® TA cloning Instructional Manual (Invitrogen).



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

4.3.3 Screening transformants for positive clones

Each transformed colony was inoculated into 5 ml of LB broth containing kanamycin (50 μg/ml, Sigma) and incubated at 37°C for 18 h. Plasmids were purified from each culture using a GenElute Plasmid mini-prep kit (Sigma) in accordance with the manufacturer's instructions.

Plasmid preparations (3 μl) were used as the template for PCR. The cloned 16S rRNA gene sequences from each preparation were amplified using oligonucleotide primers specific to the 'M13 sequences' present on either side of the insert (see Figure 4.1), M13(-20)forward and M13reverse (10 ng of each; Invitrogen). Each reaction was performed in a total volume of 50 μl and also contained 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, the working concentration of magnesium-free buffer and 1.5 U of *Taq* polymerase (all reagents from Promega). Products were analysed by electrophoresis in a 1% agarose gel (Bioline) containing ethidium bromide (125 ng/ml) alongside a molecular marker (0.12 – 23.1 kbp DNA molecular marker II; Roche Applied Science, Lewes, East Sussex, U.K.) and visualised under UV light using a GelDoc system (BioRad). PCR products of approximately 1500 bp size were considered to be positive results. If the amplified insert sequences were of any other size they were assumed to be the result of the formation of chimeric molecules or some other PCR error and were not subjected to sequence analysis.

4.3.4 Sequencing of cloned 16S rRNA gene fragments

Amplified cloned inserts were purified by precipitation and washing with ethanol. Firstly, 15 µl of 5 M NaCl (Sigma) and 15 µl of 40% polyethylene glycol (Mol. Wt. 8000; Sigma) were added to each PCR reaction volume. This was centrifuged (16,000 *g*, 15 min), and the supernatant aspirated and replaced with 200 µl 70% ethanol (v/v; Fisher). Centrifugation, aspiration and ethanol washing steps were repeated. Following another centrifugation step the PCR products were dried, either under vacuum (approx. 30 min) or overnight under an EDF1200 fume hood (Envair), and re-suspended with nuclease-free water (30 µl).

Once purified, the cloned inserts were sequenced using ABI Prism BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems), as described in section 2.3.6. Extension products were purified, firstly by precipitating via the addition of 1 µl sodium acetate (3 M; Sigma) and 1 µl EDTA (0.5 M; Sigma), followed by 80 µl chilled absolute ethanol, to each reaction mix. The supernatant was aspirated following centrifugation (16,000 *g*, 15 min) and replaced with chilled 70% ethanol (200 µl). This was repeated and after a final centrifugation and drying under vacuum, products were re-suspended in formamide (30 µl) and run on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyser; Applied Biosystems). This system yielded reliable sequences of at least 500 nucleotides length that could be compared to sequences in the public databases (see section 4.3.5), an example of which is shown in Appendix VI.

For all isolates, sequencing was performed using the universal primer 357F (Lane, 1991). For sequences where it was impossible to obtain a

conclusive identification, namely from those isolates suspected of having novel sequences or from unnamed organisms, PCR products were further sequenced using the 27F and 1492R primers to give a sequence of at least 1200 nucleotides in length.

4.3.5 **Analysis of 16S rRNA gene sequences**

Cloned sequences obtained in this study were identified by comparison to those retained in the GenBank DNA sequence database (Benson *et al*, 2004) using the FASTA sequence homology search program (http://www.ebi.ac.uk/services/index.html; Pearson, 1990).

A >99% homology to the 16S rRNA gene sequence of the type strain, or other suitable reference strain, was the criterion used to identify an isolate to the species level. Where more than one reference species exhibited >99% sequence homology, the match with the greatest homology was taken as the identity, wherever the sequence was shown to be reproducible and reliable. If there were no significant matches to known strains with currently recognised nomenclature, as confirmed by the online "List of Prokaryotic Names with Standing in Nomenclature" (http://www.bacterio.net; Euzeby, 1997), the databank entry from the uncultured or cloned 16S rRNA gene sequence with the greatest (>99%) homology was used as the identity. If there were no significant matches to any existing entries the isolate was named based upon the results of the indiscriminate GenBank search.

Whether sequences were chimeric or not was inferred by examination with both the online Bellerophon program (http://foo.maths.uq.edu.au/%7Ehuber/ bellerophon.pl; Huber *et al*, 2004) and the Ribosomal Database

Project's chimera check function (http://35.8.164.52/cgis/chimera.cgi?su=SSU; Cole *et al*, 2003).

4.3.6 Statistical analysis of taxa isolated

The null hypothesis that the probability of each phylotype occurring is the same for both types of tissue was tested using a two-sided Fisher's exact test. Two-sided p-values, as opposed to one-sided (or one-tailed), were used as neither a positive or negative association between phylotypes and the two tissue types could be assumed. The results are generally more accurate with this method when analysing relatively small total sample sizes than with a chi-squared test (Agresti, 1992).

4.4 Results

The specimens used came from 9 male and 1 female patients, with an average age of 65.3 (± 9.8).

Of the 600 clones produced a total of 526 16S sequences were sequenced, 277 from the tumourous specimens and 249 from the non-tumourous control tissues. The remaining 74 clones (12.3% of the total) produced M13 PCR products of sizes other than approximately 1500 bp and so were assumed to be chimeric molecules and not analysed further. Of the sequenced clones 25 (4.75% of the total) had no matches to any existing entries in the databases and were found from only one specimen. It was impossible to prove whether these were chimeras or not, and so they were also discarded.

Overall, 70 distinct phylotypes were detected. These are listed in Table 4.1 and arranged according to patient in Table 4.2. From the tumourous tissues, 52 phylotypes were identified. Fewer phylotypes were isolated from the non-tumourous specimens, a total of 37 distinct taxa.

All but 4 of the phylotypes matched previous entries in GenBank, either from known species with currently-standing nomenclature or sequences that originated from other culture-independent studies. The 4 with no significant matches to any previously existing entries in the public database were each isolated from more than one specimen, so it seems unlikely that they arose from the creation of chimeric molecules. These 4 phylotypes and the sequences to which they showed the greatest homology are shown in Table 4.3.

Two-sided Fisher's exact tests for each of the phylotypes revealed no significant differences ($p < 0.05$) between the proportions detected in the two types of specimen.

The PCR-cloning and sequencing of 16S rRNA genes revealed different taxa in each specimen than were previously detected by the cultural analysis (see Chapter 2). A summary of these differences is shown in Table 4.4. From the deep tumour specimens a total of 87 distinct taxa were isolated by the combination of cultivation and PCR-cloning. In comparison, only 61 taxa were detected within the non-tumourous control specimens using the same combined methods. A total of 33 of the 70 taxa seen by PCR-cloning represented species also isolated by culture in the same specimens. In many cases the two methods detected the taxa in different proportions of the two specimen types. The taxa and their relative differences in proportion are listed in Table 4.5.

Table 4.1 The phylotypes isolated from the tissue specimens by PCR-cloning and sequencing of 16S rRNA genes, grouped according to phylum

Values of significance (2-tailed p-values) for the difference in occurrence of each phylotype in the two specimen types were calculated using a Fisher's exact test.

Phylotype	No. of deep tissue specimens detections (n=10)	No. of 'normal' tissue specimens detections (n=10)	Significance of different occurrence between specimen types (P-value)	No. of clones	
				From tumour tissue specimens	From non-tumourous tissue specimens
PROTEOBACTERIA					
Acinetobacter calcoaceticus LMG 1046; AJ633631	1		0.999	2	
Bradyrhizobium japonicum DSM 30131; X87272		1	0.999		1
Citrobacter koseri strain CDC 3613-63; AF025372	1		0.999	1	
Delftia acidovorans IFO 13582; AB020186	1		0.999	1	
Eikenella corrodens isolate 1664276; AF320620	1		0.999	1	
Escherichia coli ATCC 11775 / *Shigella flexneri* ATCC 29903; X80725/X96963*		1	0.999		1
Moraxella osloensis strain 170804JB8; AY730714	1		0.999	1	
Moraxella sp. isolate S12-08; AY880059†	1		0.999	4	
Neisseria elongata ATCC 25295; L06171/AJ247252	1	1	1.000	2	1
Ralstonia insidiosa strain AU2944; AF488779	4		0.087	9	
Ralstonia pickettii ATCC 27511; AY741342	1		0.999	1	
Ralstonia solanacearum GMI1000; AL646064	1		0.999	1	
Rhizobium giardinii strain H152; U86344		1	0.999		1
Schlegelella sp. KB1a; AY538706		1	0.999		2
Sphingomonas sp. PC5.28; X89909	3	8	0.070	5	25
Tepidimonas aquatica strain CLN-1; AY324139	1		0.999	1	

Thermus scotoductus strain Se - 1; AF032127	**1**		0.999	1	
Uncultured alpha-proteobacterium (larval intestine clone D); AJ459874	**1**	**2**	1.000	1	2
Uncultured beta-proteobacterium HJ12; AY237409	**1**		0.999	1	
Uncululred gamma-proteobacterium phylotype T12HS05; DQ093273‡	**2**		0.473	2	
ACTINOBACTERIA					
Atopobium parvulum ATCC 22793; AF292372	**6**	**6**	1.000	24	51
Atopobium rimae ATCC 49626; AF292371	**1**		0.999	4	
Clavibacter michiganensis subsp. *tessellarius* ATCC 33566; U30254	**3**		0.211	4	
Curtobacterium flaccumfaciens LMG 3645; AJ312209	**1**		0.999	1	
Olsenella uli ATCC 49627; AY005814	**1**		0.999	2	
Olsenella sp. isolate S13-10; AY880047†	**1**		0.999	1	
Plantibacter flavus DSM 14012; AJ310417	**1**		0.999	1	
Propionibacterium acnes ATCC 6919; AB042288	**3**	**2**	0.999	6	3
Rhodococcus erythropolis DSM 43066; X79289	**1**		0.999	1	
Rothia mucilaginosa DSM 20746; X87758	**1**		0.999	1	
Uncultured *Atopobium* phylotype T15FO04; DQ093271‡	**2**	**2**	1.000	2	2
FIRMICUTES					
Bacillus circulans ATCC 4513; AY724690		**1**	0.999		1
Bacillus mycoides ATCC 6462 / *B. weihenstephanensis* DSM 11821; AB021192, AB021199*	**1**		0.999	1	
Bacillus thermoamylovorans strain R-19047; AJ586361		**1**	0.999		3
"*Bacillus silvestris*" strain SAFN-010; AY167818•		**1**	0.999		1
Bacillus sp. R-7413; AY422985		**1**	0.999		2
Enterococcus faecalis JCM 5803; AB012212	**1**		0.999	1	
Finegoldia magna CCUG 17636; AF542227		**1**	0.999		1
Granulicatella adiacens ATCC 49175; D50540	**5**	**9**	0.141	18	30
Lachnospiraceae-like sp. isolate Adhufec020khh; AY471655	**1**		0.999	1	
Lactobacillus gasseri ATCC 33323; AF519171	**1**		0.999	1	
Megasphaera micronuciformis strain AIP 412.00; AF473834	**1**		0.999	1	
Paenibacillus sp. SAFN-016; AY167814		**1**	0.999		2
Peptostreptococcus micros ATCC 33270; AY323523	**2**		0.473	9	

Selenomonas sp. oral clone DY027; AF385492		1	0.999		1
Staphylococcus capitis ATCC 49326 / *S. caprae* DSM 20608 / *S. epidermidis* ATCC 14990; AB009937/Y12593/D83363*	1	1	1.000	1	1
Streptococcus anginosus ATCC 33397; AF104678	6	6	1.000	25	21
Streptococcus constellatus ATCC 27823; AF104676	1		0.999	11	
Streptococcus cristatus ATCC 51100; AY188347		1	0.999		1
Streptococcus gordonii ATCC 10558; AY485606		1	0.999		1
Streptococcus intermedius ATCC 27335; AF104671		1	0.999		1
Streptococcus mitis ATCC 49456 / *S. oralis* ATCC 35037; Y485601/AY485602*		3	0.211		3
Streptococcus parasanguinis ATCC 15912; AY485605	6	5	1.000	8	5
Streptococcus salivarius ATCC 7073; AY188352		1	0.999		2
Veillonella atypica ATCC 17744; AF439641	6	5	1.000	26	14
Veillonella dispar ATCC 17748; AF439639	2	1	1.000	7	17
BACTEROIDES					
Capnocytophaga sp. isolate S12-14; AY880056†	1		0.999	1	
Capnocytophaga sp. oral strain S3; AY005073	5	3	0.650	18	10
Porphyromonas gingivalis ATCC 33277; AF414809		4	0.087		7
Prevotella intermedia ATCC 25611; X73965	1		0.999	1	
Prevotella melaninogenica ATCC 25845; AY323525	1	3	0.582	3	3
Prevotella nigrescens ATCC 33563; AF414833	3	2	0.999	3	5
Prevotella veroralis ATCC 33779; AY836507	2	1	1.000	3	1
Prevotella sp. oral clone BE073; AF385551†	5	1	0.141	9	1
Uncultured *Capnocytophaga* phylotype N17LB09; DQ093272‡	1	2	1.000	1	3
Uncultured eubacterium E1-K12; AJ289169		1	0.999		2
FUSOBACTERIA					
Fusobacterium canifelinum RMA 1036 / *F. nucleatum* subsp. *nucleatum* ATCC 25586; AY162221/AJ133496*	1		0.999	16	
Fusobacterium naviforme NCTC 11464; AJ006965	4		0.087	4	
Leptotrichia shahii strain LB37; AY029806	1		0.999	1	
Unculutured *Leptotrichia* phylotype N16LA25; DQ093274‡	3	5	0.650	11	9

Total:	**52**	**37**		262	237

* These cloned phylotypes exhibited equally significant homology to the type strains from multiple species.

‡ Novel phylotypes detected in this study. For closest sequence matches see Table 4.3.

• The only named strain for which the cloned sequence is a significant match (*Bacillus silvestris* SAFN-010; AY167818) seems distinct from any other strain of the same species. This strain of *B. silvestris* and the corresponding phylotype isolated in this study both exhibit only an approximately 96% match to the 16S rRNA gene sequences in the public databases from other *B. silvestris* strains, including the type strain (AJ006086).

† Cultivated in the prior cultural analysis of the same tissue specimens (see Chapter 2), but seemingly not in any previous studies.

Table 4.2 Phylotypes cloned from tissue specimens, grouped according to patient and aligned to compare the taxa found in each specimen type

Patient specimen number	Taxa isolated from deep tumour tissue	Taxa isolated from non-tumourous tissue
11	*Acinetobacter calcoaceticus*	
		Bacillus circulans
		"Bacillus silvestris" •
		Bacillus sp. R-7413
		Bacillus thermoamylovorans
		Bradyrhizobium japonicum
	Citrobacter koseri	
	Clavibacter michiganensis ssp. *tessellarius*	
	Delftia acidovorans	
	Enterococcus faecalis	
		Granulicatella adiacens
	Moraxella osloensis	
		Paenibacillus sp. SAFN-016
		Porphyromonas gingivalis
	Prevotella sp. oral clone BE073†	
	Propionibacterium acnes	*Propionibacterium acnes*
	Ralstonia insidiosa	
		Rhizobium giardinii
	Sphingomonas sp. PC5.28	*Sphingomonas* sp. PC5.28
		*Staphylococcus capitis / S. caprae / S. epidermidis**
	Streptococcus parasanguinis	
		Uncultured alpha-proteobacterium clone D

12		*Atopobium parvulum*
	*Bacillus mycoides / B. weihenstephanensis**	
	Capnocytophaga sp. isolate S12-14†	
	Clavibacter michiganensis ssp. *tessellarius*	
		Granulicatella adiacens
	Megasphaera micronuciformis	
	Moraxella sp. isolate S12-08†	
	Neisseria elongata	
	Novel gamma-proteobacterium phylotype T12HS05‡	
	Plantibacter flavus	
		Prevotella melaninogenica
	Prevotella sp. oral clone BE073†	
	Propionibacterium acnes	
	Ralstonia solanacearum	
		Schlegelella sp. KB1a
		Selenomonas sp. oral clone DY027
	Sphingomonas sp. PC5.28	*Sphingomonas* sp. PC5.28
		*Streptococcus mitis / Str. oralis**
	Streptococcus parasanguinis	
	Veillonella dispar	*Veillonella dispar*

13	*Atopobium parvulum* *Atopobium rimae* *Clavibacter michiganensis* ssp. *tessellarius* *Curtobacterium flaccumfaciens* *Olsenella* sp. isolate S13-10† *Prevotella intermedia* *Ralstonia insidiosa* *Ralstonia pickettii* *Rothia mucilaginosa* *Tepidimonas aquatica* *Thermus scotoductus* Uncultured alpha-proteobacterium clone D Uncultured beta-proteobacterium HJ12	*Atopobium parvulum* *Capnocytophaga* sp. oral strain S3 *Escherichia coli / Shigella flexneri** *Granulicatella adiacens* *Neisseria elongata* *Propionibacterium acnes* *Streptococcus anginosus* *Streptococcus mitis / Str. oralis**
14	*Atopobium parvulum* *Capnocytophaga* sp. oral strain S3 *Peptostreptococcus micros* *Prevotella* sp. oral clone BE073† *Streptococcus constellatus* *Streptococcus parasanguinis*	*Atopobium parvulum* *Granulicatella adiacens* Novel *Atopobium* phylotype T15FO04‡ Novel *Leptotrichia* phylotype N16LA25‡ *Porphyromonas gingivalis* *Sphingomonas* sp. PC5.28 *Streptococcus anginosus* *Streptococcus parasanguinis* *Veillonella atypica*

15	*Atopobium parvulum* *Fusobacterium naviforme* *Granulicatella adiacens* *Lactobacillus gasseri* Novel *Atopobium* phylotype T15FO04‡ *Olsenella uli* *Prevotella nigrescens* *Propionibacterium acnes* *Ralstonia insidiosa* *Staphylococcus capitis / S. caprae / S. epidermidis** *Streptococcus anginosus* *Veillonella atypica*	*Atopobium parvulum* *Capnocytophaga* sp. oral strain S3 Novel *Atopobium* phylotype T15FO04‡ Novel *Capnocytophaga* phylotype N17LB09‡ *Prevotella melaninogenica* *Prevotella nigrescens* Uncultured eubacterium E1-K12
16	*Atopobium parvulum* *Capnocytophaga* sp. oral strain S3 *Eikenella corrodens* *Fusobacterium naviforme* *Granulicatella adiacens* Novel *Atopobium* phylotype T15FO04‡ Novel *Leptotrichia* phylotype N16LA25‡ *Ralstonia insidiosa* *Rhodococcus erythropolis* *Streptococcus anginosus* *Streptococcus parasanguinis* *Veillonella atypica*	*Atopobium parvulum* *Granulicatella adiacens* Novel *Leptotrichia* phylotype N16LA25‡ *Porphyromonas gingivalis* *Sphingomonas* sp. PC5.28 *Streptococcus anginosus* *Streptococcus gordonii* *Streptococcus parasanguinis* *Streptococcus salivarius* *Veillonella atypica*

17	*Atopobium parvulum* *Capnocytophaga* sp. oral strain S3 *Fusobacterium naviforme* *Granulicatella adiacens* Novel *Capnocytophaga* phylotype N17LB09‡ *Prevotella veroralis* *Sphingomonas* sp. PC5.28 *Streptococcus anginosus* *Streptococcus parasanguinis* Uncultured bacterium clone Adhufec020khh *Veillonella atypica* *Veillonella dispar*	*Capnocytophaga* sp. oral strain S3 *Finegoldia magna* *Granulicatella adiacens* Novel *Capnocytophaga* phylotype N17LB09‡ Novel *Leptotrichia* phylotype N16LA25‡ *Porphyromonas gingivalis* *Prevotella melaninogenica* *Prevotella nigrescens* *Prevotella* sp. oral clone BE073† *Prevotella veroralis* *Sphingomonas* sp. PC5.28 *Streptococcus anginosus* *Streptococcus parasanguinis* *Veillonella atypica*
18	 *Capnocytophaga* sp. oral strain S3 *Fusobacterium naviforme* *Granulicatella adiacens* Novel *Leptotrichia* phylotype N16LA25‡ *Peptostreptococcus micros* *Prevotella nigrescens* *Prevotella* sp. oral clone BE073† *Streptococcus anginosus* *Veillonella atypica*	*Atopobium parvulum* *Granulicatella adiacens* Novel *Leptotrichia* phylotype N16LA25‡ *Sphingomonas* sp. PC5.28 *Streptococcus anginosus* *Streptococcus cristatus* *Streptococcus intermedius* *Streptococcus parasanguinis* *Veillonella atypica*

19	*Fusobacterium canifelinum / F. nucleatum* ssp. *nucleatum** *Leptotrichia shahii* *Streptococcus anginosus* *Veillonella atypica*	*Granulicatella adiacens* *Sphingomonas* sp. PC5.28
20	*Atopobium parvulum* *Capnocytophaga* sp. oral strain S3 *Granulicatella adiacens* Novel *Leptotrichia* phylotype N16LA25‡ *Prevotella nigrescens* *Prevotella* sp. oral clone BE073† *Prevotella veroralis* *Streptococcus anginosus* *Streptococcus parasanguinis* *Veillonella atypica*	*Granulicatella adiacens* Novel *Leptotrichia* phylotype N16LA25‡ *Sphingomonas* sp. PC5.28 *Streptococcus anginosus* *Streptococcus mitis / Str. oralis** *Streptococcus parasanguinis* Uncultured alpha-proteobacterium clone D *Veillonella atypica*

* These cloned phylotypes exhibited equally significant homology to the type strains from multiple species.

‡ Novel phylotypes detected in this study.

• The only named strain for which the cloned sequence is a significant match (*Bacillus silvestris* SAFN-010; AY167818) seems distinct from any other strain of the same species. This strain of *B. silvestris* and the corresponding phylotype isolated in this study both exhibit only an approximately 96% match to the 16S rRNA gene sequences in the public databases from other *B. silvestris* strains, including the type strain (AJ006086).

† Cultivated in the prior cultural analysis of the same tissue specimens (see Chapter 2), but seemingly not in any previous studies.

Table 4.3 Novel phylotypes isolated by PCR-cloning of 16S rRNA genes

The four sequences that appear to represent novel phylotypes, listed alongside the pre-existing GenBank entries with the greatest homology. Although these 4 are quite close in sequence to known taxa, each was isolated several times and from several sources and, as the sequencing was reproducible in each case, were assumed to represent novel phylotypes. Each was submitted to GenBank accordingly and the accession numbers are shown below.

Phylotype	Accession number	Closest match	Identity (%)
N16LA25	DQ093274	*Leptotrichia wadeii* strain LB16; AY029802	98.7
N17LB09	DQ093272	*Capnocytophaga gingivalis* strain LMG 12118; U41346	98.7
T12HS05	DQ093273	Bacterium SG-3; AF548381*	95.1
T15FO04	DQ093271	*Atopobium parvulum* ATCC 22793; AF292372	98.4

* This unnamed *Proteobacterium* is a member of the *Xanthomonadaceae* family

Table 4.4 Comparison of taxa isolated by PCR-cloning and culture techniques

A summary of the results of the PCR-cloning of 16S rRNA genes compared to the results of cultural analysis (see Chapter 2). Species that were detected in the tissue specimens by both methods are shown below.

Patient / Specimen number	Tumourous tissues			Non-tumourous tissues		
	Number of taxa isolated by culture	Number of taxa isolated by PCR-cloning	Common taxa	Number of taxa isolated by culture	Number of taxa isolated by PCR-cloning	Common taxa
11	5	11	No common species	5	13	No common species
12	2	14	*B. mycoides/weihenstephanensis*	6	8	*Veillonella dispar*
13	4	13	No common species	4	8	*Atopobium parvulum* *Propionibacterium acnes*
14	9	6	*Atopobium parvulum* *Peptostreptococcus micros* *Streptococcus constellatus*	9	9	*Atopobium parvulum*
15	3	12	No common species	7	7	No common species

16	2	12	No common species	5	10	*Granulicatella adiacens* *Streptococcus parasanguinis* *Streptococcus salivarius*
17	6	12	*Granulicatella adiacens* *Streptococcus parasanguinis* *Veillonella dispar*	5	14	*Granulicatella adiacens* *Streptococcus parasanguinis*
18	1	9	No common species	6	9	*Atopobium parvulum* *Streptococcus parasanguinis* *Veillonella atypica*
19	4	4	*Streptococcus anginosus*	12	2	No common species
20	1	10	No common species	0	8	No common species

Table 4.5 A summary of the species detected at differing frequencies by the culture and PCR-cloning methodologies

Taxa for where a difference ≥30% in proportion between the 2 approaches was evident for either the deep tumour or non-tumourous specimens are highlighted in **bold.**

Taxa	Proportion of specimens positive for taxon (%)					
	Deep tumour tissues			Non-tumourous tissues		
	Isolated by culture	Isolated by PCR-cloning	Difference	Isolated by culture	Isolated by PCR-cloning	Difference
Atopobium parvulum	**5.0**	**60.0**	**55.0**	**25.0**	**60.0**	**35.0**
Atopobium rimae	0.0	10.0	10.0	8.3	0.0	-8.3
Bacillus mycoides/weihenstephanensis	5.0	10.0	5.0	0.0	0.0	0.0
Capnocytophaga-like sp. isolate S12-14	0.0	10.0	10.0	0.0	0.0	0.0
Citrobacter koseri	5.0	10.0	5.0	0.0	0.0	0.0
Eikenella corrodens	5.0	10.0	5.0	0.0	0.0	0.0
Enterococcus faecalis	0.0	10.0	10.0	0.0	0.0	0.0
Finegoldia magna	5.0	0.0	-5.0	8.3	10.0	1.7
Fusobacterium canifelinum / nucleatum subsp. *nucleatum*	5.0	10.0	5.0	8.3	0.0	-8.3
Fusobacterium naviforme	**5.0**	**40.0**	**35.0**	0.0	0.0	0.0
Granulicatella adiacens	**15.0**	**50.0**	**35.0**	**25.0**	**90.0**	**65.0**
Lactobacillus gasseri	15.0	10.0	-5.0	8.3	0.0	-8.3
Megasphaera micronuciformis	0.0	10.0	10.0	8.3	0.0	-8.3
Moraxella osloensis	0.0	10.0	10.0	16.7	0.0	-16.7
Moraxella-like sp. isolate S12-08	0.0	10.0	10.0	0.0	0.0	0.0
Neisseria elongata	0.0	10.0	10.0	0.0	10.0	10.0
Olsenella uli	0.0	10.0	10.0	0.0	0.0	0.0
Olsenella-like sp. isolate S13-10	0.0	10.0	10.0	8.3	0.0	-8.3
Peptostreptococcus micros	20.0	20.0	0.0	16.7	0.0	-16.7

Prevotella intermedia	0.0	10.0	10.0	0.0	0.0	0.0
Prevotella melaninogenica	10.0	10.0	0.0	**0.0**	**30.0**	**30.0**
***Prevotella* sp. (oral clone BE073 phylotype)**	**10.0**	**50.0**	**40.0**	16.7	10.0	-6.7
Prevotella veroralis	0.0	20.0	20.0	16.7	10.0	-6.7
Propionibacterium acnes	30.0	30.0	0.0	**50.0**	**20.0**	**-30.0**
Staphylococcus capitis subsp. *urealyticus / caprae / epidermidis*	10.0	10.0	0.0	16.7	10.0	-6.7
Streptococcus anginosus	**5.0**	**60.0**	**55.0**	**8.3**	**60.0**	**51.7**
Streptococcus constellatus	10.0	10.0	0.0	16.7	0.0	-16.7
Streptococcus gordonii	5.0	0.0	-5.0	0.0	10.0	10.0
Streptococcus mitis/oralis	10.0	0.0	-10.0	41.7	30.0	-11.7
Streptococcus parasanguinis	**15.0**	**60.0**	**45.0**	41.7	50.0	9.3
Streptococcus salivarius	10.0	0.0	-10.0	25.0	10.0	-15.0
Veillonella atypica	**0.0**	**60.0**	**60.0**	**10.0**	**50.0**	**40.0**
Veillonella dispar	5.0	20.0	15.0	25.0	10.0	-5.0

4.5 Discussion

Although PCR using primers specific for several species has been applied to detect bacteria in tissue from oral squamous cell carcinoma patients before, to our knowledge this is the first time PCR with non-specific, universal primers has been used to characterise the range of bacteria present in OSCC.

PCR amplification and sequence analysis of 16S rRNA genes is a versatile technique and has been used extensively to assess microbial diversity. The technique relies on the assumption that the gene sequences of all the bacteria present in the sample are complementary to the universal primers used. Recent discoveries of new taxa, though, have indicated that several standard "universal" 16S rRNA gene primers do not recognise all species of bacteria, and that current 16S rRNA gene libraries are not representative of true prokaryotic biodiversity (Becker *et al*, 2003). However, the three primer sets chosen for this study had been well-validated through their use in the characterisation of the species present in several oral bacterial communities. These primers have, in the past, been successful in detecting bacteria from 11 distinct phyla, including *Acidobacteria, Actinobacteria, Bacteroidetes, Deferribacteres, Deinococcus, Fusobacteria, Firmicutes, Proteobacteria, Spirochaetes* and two phyla with no currently known cultivable representatives, namely TM7 and Obsidian Pool OB11 (Becker *et al*, 2002; Kazor *et al*, 2003; Paster *et al*, 2001; Paster *et al*, 2002). That said, in this study taxa from just five phyla were detected, and these were all amplified by the universal primer pair, D88 and E94. PCR with the *Bacteroidetes*-specific F01 primer resulted in relatively few clones, all of which were also represented by the universal pair. In this study, the *Spirochaetes*-specific primer (C90) resulted in no detectable PCR product and no successful

clones. The scarcity of clones produced by 2 of the 3 primer pairs used is why clones from all three reactions were ultimately pooled for each specimen and 30 clones picked from the total. In any future studies on OSCC specimens, C90 and F01 may not be required. The use of other *Spirochaetes*-specific primers may however be advisable, particularly given the previously-reported presence of *Treponema denticola* in oesophageal SCC (Narikiyo *et al*, 2004).

One limitation of this study was that not all the clones produced, and hence not all amplicons from the extracts, were sequenced. As the investigation was not comprehensive, it is entirely possible there were phylotypes present in each specimen that were not detected. This may be the reason that this technique apparently failed to detect several taxa recovered by culture from the same specimens (Table 4.4), such as *Micrococcus luteus* (cultured but not cloned from deep tumour specimens 16 and 17; see Table 2.3) and *Corynebacterium tuberculostearicum* (from deep tumour specimen 13 and non-tumourous specimen 11). Similarly, some species detected from the specimens by both cultural and molecular analysis were isolated at noticeably different frequencies by the two methods (see Table 4.5). For instance, *Propionibacterium acnes* was detected in more non-tumourous specimens by culture (6 out of 12, or 50.0%) than by PCR-cloning (2 of 10, or 20% of specimens).

The absence and under-representation of some taxa from the molecular analysis may also be caused by primer bias. Although the "universal" primers used appear capable of targeting all members of the *Bacteria* domain, differential amplification of 16S rRNA gene sequences in multi-template PCR is a recognised problem (Farrelly *et al*, 1995; Ishii & Fukui, 2001; Kurata *et al*, 2004; Polz & Cavanaugh, 1998). One possible reason for this occurrence is the

different G+C content of the sequences from different taxa. GC-rich DNA templates are known to disassociate relatively poorly (Ishii & Fukui, 2001; Polz & Cavanaugh, 1998). Furthermore, *Taq* polymerase appears to work comparatively inefficiently with such sequences, presumably due to the formation of secondary structures (Wade, 2004). Using high concentrations of template and performing fewer cycles can reduce PCR bias (Polz & Cavanaugh, 1998). Both of these considerations were taken into account when designing the used protocol. Replicating each reaction and mixing the products can also be used to reduce PCR bias (Polz & Cavanaugh, 1998), which should probably be considered in any future PCR-cloning studies. This approach was not used in this investigation in order to minimise the amount of DNA extract used, as only a finite quantity was available for each specimen.

Bias towards certain taxa in molecular analysis may also be caused by the different copy numbers of the rRNA gene on the chromosomes of different species (Farrelly *et al*, 1995) or by the differential efficiencies of DNA extraction from various species. Gram-positive species tend to have thicker cell walls and hence can be more difficult to lyse during the initial stages of DNA extraction (Wade, 2004). A bias against the *Actinobacteria* phyla, members of which are both high in G+C content and Gram-positive and include *C. tuberculostearicum, M. luteus and P. acnes,* has been reported previously (Munson *et al*, 2002). Another limitation of this technique is that PCR-cloning of the 16S rRNA gene detects nucleic acid from both living and dead bacteria (Wilson *et al*, 1997). Therefore, unlike cultural analysis, this approach does not indicate whether these species are viable within the tumourous or mucosal tissues. One possible way to overcome this shortcoming is to extract the total

RNA, instead of the DNA, and use it as the template for reverse transcriptase (RT)-PCR in order to analyse the extracts. As RNA is labile and has a relatively short half-life, it potentially provides a more representative indication of the active constituents of a bacterial population. RT-PCR has been used in this way to identify bacterial 16S rRNA sequences in food and environmental samples (Botero *et al*, 2005; Miskin *et al*, 1999; Randazzo *et al*, 2002) and also the bacteria present in clinical samples of synovial fluid (Cox *et al*, 2003). However, RNA is generally more difficult to isolate than DNA due to its rapid degradation by RNases (Miskin *et al*, 1999). Extraction of bacterial RNA from tissues would require specimens to either have been processed for extraction immediately following acquisition from surgery or kept in a suitable storage solution, such as RNA*later* (Sigma).

A total of 25 clones had to be discarded as suspect chimeras. The formation of chimeric or recombinant sequences is another recognised limitation of culture-independent techniques, particularly those using broad-specificity primers. Chimeras are thought to form during PCR amplification when a prematurely terminated amplicon re-anneals to another DNA strand and is copied to completion in the subsequent cycles. The result is a sequence composed from two or more phylogenetically distinct parent sequences. When this occurs with 16S rRNA gene sequences it can create a product that suggests the presence of a non-existent organism (Hugenholtz & Huber, 2003; Wang & Wang, 1996). It is, therefore, vitally important to check all 16S rRNA gene sequences produced by PCR-cloning using the available tools (Cole *et al*, 2003; Huber *et al*, 2004) and reject any suspected of being of chimeric origin.

As seen previously with the identification of the cultural isolates (Chapter 2) and reported in other investigations (Clarridge, 2004; Munson *et al*, 2004), partial sequencing of the 16S rRNA gene fragments was sufficient to characterise most isolates to species-level. As with identifying cultured isolates by 16S rRNA gene sequence analysis, the success of identification is limited by the accuracy of the sequence generated. Errors during the production and reading of such sequences occur at an estimated rate of 1 in 5000 to 1 in 10,000 bp (Clarridge, 2004). These error rates are too small to significantly affect the species designation and were not therefore considered a problem in this study.

The majority of phylotypes detected (41 out of 70) represented known, culturable species and were relatively easily identified, with the exception of 5 phylotypes that showed equal homology to two or more known type strains. These were *Bacillus mycoides* and *B. weihenstephanensis*, *Escherichia coli* and *Shigella flexneri*, *Fusobacterium canifelinum* and *F. nucleatum* subsp. *nucleatum*, *Streptococcus mitis* and *S. oralis,* and *Staphylococcus caprae, S. epidermidis* and *S. capitis* subsp. *urealyticus*. As with the cultural analysis, this level of identification was sufficient for the purposes of this investigation. Nevertheless, sequencing of the entire 16S rRNA gene could probably be applied successfully to further identify these isolates, should the precise species designation of these isolates be needed for future investigations.

Four phylotypes detected here were homologous to some of the putatively novel, previously uncultured species isolated in the cultural analysis. These taxa were the unknown *Capnocytophaga* sp. (isolate S12-14; AY880056), the unknown *Moraxella*-like sp. (isolate S12-08; AY880059), the unknown

Olsenella-like sp. (isolate S13-10; AY880047) and the *Prevotella* sp. homologous to oral clone BE073 (AF385551).

Several unusual bacteria, not usually associated with human clinical samples, were detected in this study. Aside from the novel phylotypes, these included, from the tumourous tissues, *Clavibacter michiganensis, Plantibacter flavus, Tepidimonas aquatica* and *Thermus scotoductus*, and from the non-tumourous tissues, *Bacillus thermoamylovorans, Bradyrhizobium japonicum*, and *Rhizobium giardinii*. All of these have, to the best of our knowledge, only previously been isolated from non-clinical sources: *Bradyrhizobium japonicum, Clavibacter michiganensis, Plantibacter flavus* and *Rhizobium giardinii* from plants (Amarger *et al*, 1997; Behrendt *et al*. 2002; Ruan & Peters, 1992), *Bacillus thermoamylovorans* from contaminated foodstuffs (Combet-Blanc *et al*, 1995; De Clerck *et al*, 2004), *Tepidimonas aquatica* from domestic water supplies (Freitas *et al*, 2003), and *Thermus scotoductus* from thermal springs and soil samples (Balkwill *et al*, 2004; Welch *et al*, 1998). Each was only detected in relatively small amounts, namely single clones from single specimens, and none were also isolated by culture, suggesting that they may not have been present in the tissues as living cells.

These unusual, environmental bacteria, present in low quantities, may be the result of contamination. DNA from environmental species of bacteria has previously been reported as a possible contaminant of DNA extraction solutions (Tanner *et al*, 1998). Similarly, DNA from both *Escherichia coli* and *Thermus* species, single clones of which were isolated here, and other unidentified species have reportedly been found contaminating PCR reagents (Böttger, 1990; Hughes *et al*, 1994; Newsome *et al*, 2004; Rand & Houck, 1990; Schmidt *et al*, 1991).

Such issues of contaminants in the commercial reagents are known and general sterile techniques were used to minimise further contamination and reduce the risk of "false positive" PCR products. A degree of contamination seems, unfortunately, to be an unavoidable limitation of the technique due to the broad range and extremely sensitive level of detection the PCR used is capable of (Kwok & Higuchi, 1989). However, should this technique be used in future studies it may be worth trying additional approaches to further reduce the amounts of possible contaminant DNA, such as filtration of the DNA extraction kit reagents through a DNA-isolating column (Mohammadi *et al*, 2005), and treating the plasticware with 8-methoxypsoralen and long-wavelength (366 nm) UV light (Hughes *et al*, 1994).

Statistical analysis showed that none of the phylotypes occurred significantly more or less often in either tissue type. However, the lack of statistical significance is unsurprising due to the relatively low total numbers of specimens examined. Also, the incidences of each phylotype may be skewed due to the fact that it was not possible to sequence every clone. Nevertheless, several interesting trends can be observed.

As demonstrated in Chapter 2, the majority of species found are saccharolytic and aciduric, perhaps reflecting the selective nature of the acidic and hypoxic microenvironment found within the tumour (Raghunand *et al*, 2003; Švastová *et al*, 2004). *Proteobacteria* and members of the genera *Streptococcus, Prevotella,* and *Veillonella*, are known to be aciduric (Curtis *et al*, 2002; de Soet *et al*, 2000; Marchant *et al*, 2001; Švastová *et al*, 2004). Indeed, most of the taxa isolated from the tumourous tissues in this study have previously been detected in acidic dental caries lesions (Chhour *et al*, 2005; Munson *et al*, 2004).

Asaccharolytic *Fusobacterium* species, only isolated here from tumourous tissue specimens, have also been shown to grow successfully at a relatively low pH *in vitro* (Takahashi, 2003). Similarly, *Ralstonia* species, particularly *R. insidiosa,* were also only detected in the tumourous specimens. Members such as these of the *Ralstonia pickettii* lineage are acid-producing species (Vaneechoutte *et al*, 2004), so their presence within an acidic microenvironment is not extraordinary.

Conversely, *Porphyromonas gingivalis* was detected only within non-tumourous tissues. This species is known to grow in a relatively narrow pH range (pH 6.5-7.0) (Takahashi, 2003), so it is not surprising that it was not detected within acidic tumour tissue.

The most commonly detected species (*i.e.* seen in 40% or more specimens) from the tumourous tissues were *Atopobium parvulum, Capnocytophaga* oral strain S3, *Fusobacterium naviforme, Granulicatella adiacens, Prevotella* sp. oral clone BE073, *Ralstonia insidiosa, Streptococcus anginosus, Streptococcus parasanguinis* and *Veillonella atypica*, all of which have previously been isolated from human oral cavities (Marsh & Martin, 1999; Munson *et al*, 2002; Paster *et al*, 2001; Tanner *et al,* 1994). Furthermore, all except *R. insidiosa* and the *Prevotella* strain have been associated with infections and diseases of the oral cavity (Bancescu *et al*, 1999; Becker *et al*, 2002; Downes *et al*, 2001; George *et al*, 1981; Gomes *et al*, 2004; Munson *et al*, 2004; Paster *et al*, 2001; Rôças & Siqueira, 2005). *Prevotella* sp. oral strain BE073 is a relatively unknown species, seemingly uncultured until the culture analysis of these tissues (Chapter 2), but has previously been detected in purified crevicular epithelial cells (Kazor *et al*, 2003). *Ralstonia insidiosa* is a relatively

recently proposed species but has already been isolated from the sputum of several immunocompromised patients (Coenye *et al*, 2003). Also, it has possibly been detected previously within the subgingival plaque of a refractory periodontitis patient, as the 16S sequence is significantly homologous to "*Burkholderia* sp. oral clone AK168" (AY005032) (Paster *et al*, 2001). Of particular interest is that *Streptococcus anginosus* was isolated from 6 patients in this present study, as this species has previously been detected in oral SCC specimens (Tateda *et al*, 2000; Morita *et al*, 2003). As discussed previously (see section 1.3.2.2), *S. anginosus* has been implicated in the process of carcinogenesis due to both its association with tumours and its ability to induce inflammation (Sasaki *et al*, 2001; Sugano *et al*, 2003).

Some of the species identified were also commonly detected within the non-tumourous tissues, namely *A. parvulum, G. adiacens, Str. anginosus, Str. parasanguinis* and *V. atypica.* Other species commonly detected in the non-tumourous mucosa were *Porphyromonas gingivalis*, *Sphingomonas* sp. PC5.28 and the novel *Leptotrichia* phylotype, referred to as N17LA25. *Porphyromonas gingivalis* has long been associated with infection and periodontal disease (Hosogi & Duncan, 2005) and is known to be capable of invading human epithelium and inducing inflammation (Andrian *et al*, 2004; Kuramitsu *et al*, 2002). The taxa referred to as *Sphingomonas* sp. PC5.28 is also homologous to "*Sphingomonas* oral clone AV069," which was isolated from oral noma lesions (Paster *et al*, 2002).

The noticeable differences between the microflora of the tumour and non-tumourous tissues suggest a degree of bacterial specificity that merits further study. The presence of species in the mucosal and tumourous tissues of

5.1 Introduction

Previous chapters in this thesis have demonstrated the isolation of bacteria from oral squamous cell carcinoma tissue. Although it has been shown that these organisms do not represent surface contaminants, it is still necessary to visualise where in the tissue they reside.

The morphological details of most bacteria remain largely unknown. Traditional microscopic analyses and bacterial stains, like Gram or Ziehl-Neelsen, are useful but limited as they rely on the prior cultivation of the bacteria. Cultivation techniques will only work for a minority of species, and so the development of alternative methods for the visualisation of bacteria has become a necessity (Amann *et al*, 2001; Moter & Göbel, 2000).

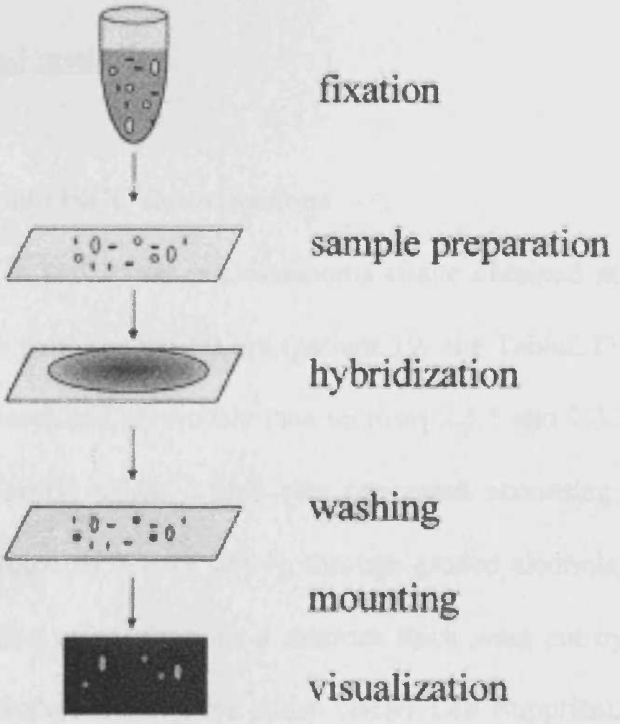
A common, culture-independent approach for detecting and visualising microorganisms is fluorescence *in situ* hybridisation (FISH). Hybridisation works by using oligonucleotide probes to target nucleic acids, rather than antigens (Amann *et al*, 2001). Most often, probes are used that target ribosomal RNA molecules as these are functionally conserved and present in all organisms. The small subunit (16S) rRNA molecules are ideal targets for bacterial detection because they are stable and the high copy numbers of molecules per cell (usually more than 10,000) means that probing can be highly sensitive (Amann, 1995; Moter & Göbel, 2000). As 16S rRNA sequence data is readily available for a wide variety of species, probes can be designed either to target signature sequences of specific species or chosen groups, or to hybridise with sequences conserved in all known bacteria (Amann, 1995; Amann *et al*, 2001). The probes used in FISH are typically between 15 and 30 nucleotides in length and covalently linked at the 5'-end to a single fluorescent dye molecule (Wagner *et*

al, 2003). Suitable fluorophors include carbocyanide dyes, tetramethylrhodamine and fluorescein (Amann *et al*, 2001).

FISH procedures generally comprise 5 steps (Figure 5.1). Specimens must first be fixated and then pre-treated, usually involving a permeablisation step in order to facilitate entry of the probe into the cells. Subsequently, hybridisation of the target sequence and the fluorescent probe is followed by washes to remove unbound oligonucleotides. Hybridised samples can then be mounted and labelled cells detected by microscopy or flow cytometry (Amann *et al*, 2001; Moter & Göbel, 2000).

FISH targeted to 16S rRNA sequences has been applied to many diverse bacterial systems, often in conjunction with other molecular-based profiling approaches such as denaturing gradient gel electrophoresis or gene sequencing. The first uses of the technique were in the detection and identification of bacteria in environmental specimens, including activated sludge granules (Sekiguchi *et al*, 1999) and marine sediments (Amann *et al*, 2001; Rossello-Mora *et al*, 1999). FISH has also been applied to the study of potentially medically-important bacteria in clinical samples, such as human faecal samples (Jansen *et al*, 1999; Vaahtovuo *et al*, 2005) and polymicrobial specimens from the oral cavity. For instance, using FISH bacteria have been detected within samples of human carious dentine (Banerjee *et al*, 2002), dental plaque (Thurnheer *et al*, 2001; Wecke *et al*, 2000), periapical endodontic lesions (Sunde *et al*, 2003) and buccal epithelial cells (Rudney *et al*, 2001, 2005). However, at the time of writing, this approach has not yet been applied to specimens of tissue from oral squamous cell carcinoma (OSCC).

Figure 5.1 A flow chart summarising the typical steps of a FISH protocol
Image reproduced from Moter & Göbel (2000).



5.2 Aims

The aim of this study was to detect and visualise any bacteria present within sections of tissue from oral squamous cell carcinoma using *Bacteria*-specific fluorescence *in situ* hybridisation.

5.3 Materials and methods

5.3.1 Preparation of OSCC tissue sections

A specimen of squamous cell carcinoma tissue obtained at the time of surgery from a male patient aged 59 yrs (patient 19, see Table2.1) and surface decontaminated as described previously (see sections 2.3.1 and 2.3.2) was fixed overnight in 10% formyl saline. This was processed according to standard clinical pathology protocols (Cross, 2004), through graded alcohols, xylene and embedded into paraffin wax. Sections 4 microns thick were cut by microtome and mounted onto SuperFrost® glass slides (BDH Lab Supplies). One such section was stained by the routine haematoxylin and eosin (H and E) procedure (Cross, 2004).

Prior to fluorescence *in situ* hybridisation, sections were pre-treated by submersion in xylene (Fisher) for 5 minutes. This was repeated a further 2 times, followed by 2 immersions in 96% ethanol (5 min each; Sigma) and then a single immersion in 70% ethanol (5 min). Slides were rinsed with sterile phosphate buffered saline (1 min) and then, in order to permeabilise the cells to the oligonucleotide probe, incubated in 50 mM Tris-EDTA (TE; Sigma) buffer

containing lysozyme (10 mg/ml; Sigma) for 20 min at 37°C. Following another rinse with PBS, sections were again incubated in 50 mM TE buffer, this time containing proteinase K (7 μg/ml; Promega), at 37°C (20 min). Subsequent to this incubation, slides were rinsed thoroughly with double-distilled water and immersed in 70% ethanol (1 min), and then in 96% ethanol (1 min). After air-drying, slides were ready for FISH.

5.3.2 **Fluorescence *in situ* hybridisation (FISH)**

Sections were pre-incubated at 48°C (20 min) in a hybridisation buffer (300 μl) containing 0.9 M sodium chloride, 20 mM Tris-HCl (pH 7.4), and 0.5% SDS (all reagents from Sigma). Pre-warmed hybridisation buffer (300 μl) containing 0.1 μM of oligonucleotide probe, EUB338 (Amann *et al*, 1990; see Appendix I), which was synthesised commercially (MWG Biotech, Ebersberg, Germany) and 5' end-labelled with fluorescein isothiocyanate (FITC), was carefully applied to the tissue sections. Probe EUB338 is complementary to a portion of the 16S rRNA gene conserved in the domain *Bacteria* (corresponding to positions 338 to 355 in the *E. coli* 16S rRNA gene) and can, in principle, be used to visualise entire bacterial populations (Amann *et al*, 1990; Banerjee *et al*, 2002; Sunde *et al*, 2003). Following incubation for 3.5 h in a dark humid chamber at 46°C, each slide was rinsed thoroughly with sterile double-distilled water and air-dried in the dark. Sections were counter-stained with 0.025% (w/v) concanavalin A-Alexa Fluor® 594 conjugate (Molecular Probes, Invitrogen, Paisley, U.K.) for 20 minutes. The Alexa dye conjugate is capable of fluorescently labelling all the protein in the section and so is suitable for most histological applications (Panchuk-Voloshina *et al*, 1999). Each slide was again

rinsed with water, air-dried in the dark and subsequently mounted with FluoroSave™ (Calbiochem, Merck Biosciences Ltd., Nottingham, U.K.).

5.3.3 Epifluorescent microscopy

Hybridised sections were viewed using an Olympus Provis AX70 microscope with build-in incident light fluorescence illuminator (Olympus UK Ltd., Southall, Middlesex, U.K.). Images were obtained using an attached Nikon DXM1200 digital camera and the associated ACT-1 software, version 2.63 (Nikon UK Ltd., Kingston upon Thames, Surrey, UK).

5.4 Results

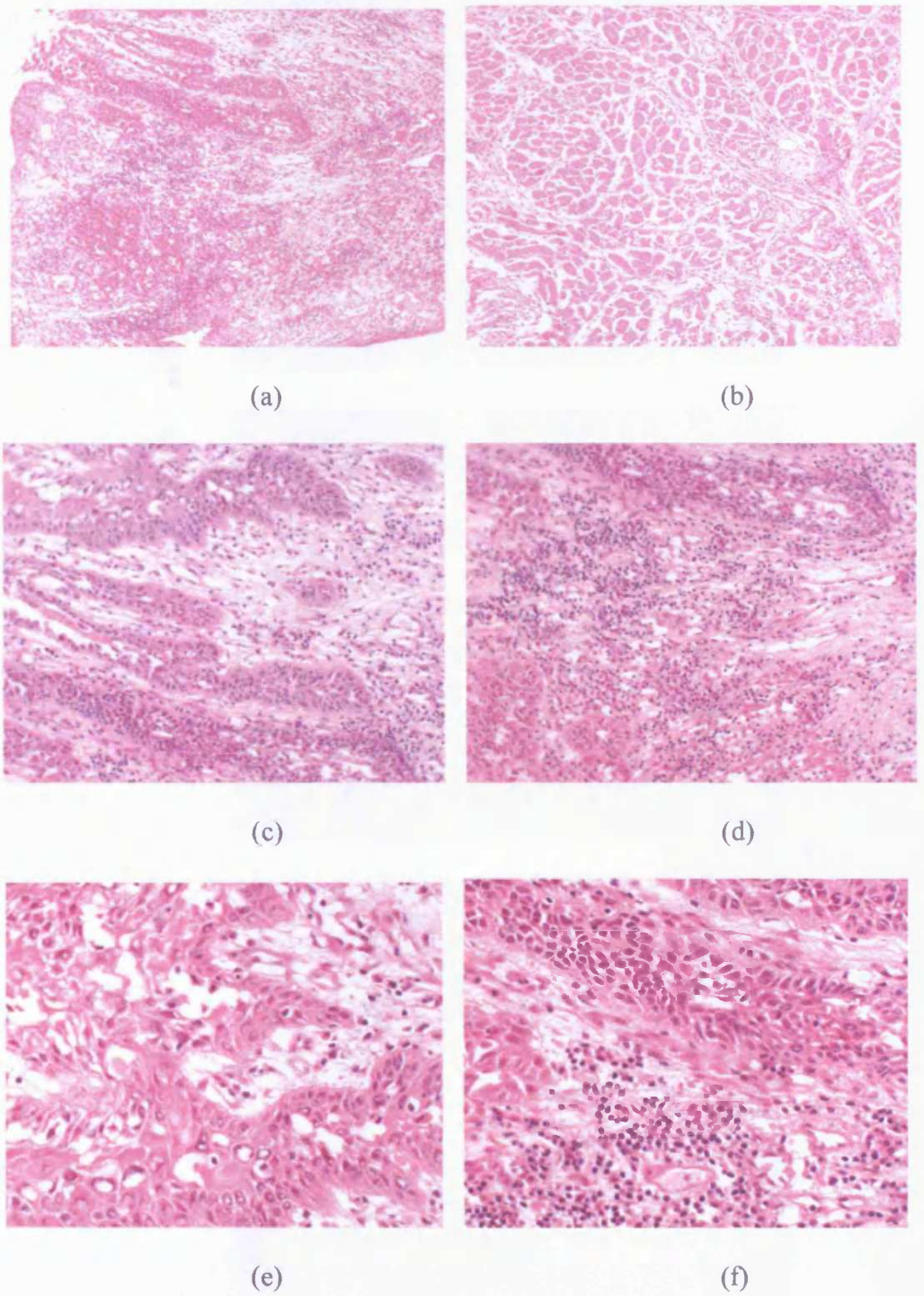
Tissue from a specimen of oral squamous cell carcinoma was successfully processed and stained by both standard H and E staining (see Figure 5.2) and with the fluorescent Alexa 594 stain (see Figure 5.3).

Using the universal eubacterial probe EUB338-FITC, bacteria could be detected by FISH in all sections of OSCC tissue examined. Examples of sections containing fluorescently-labelled bacteria are shown in Figures 5.4 – 5.6. Bacteria were observed spread throughout the sections among the cells and fibres of the tissue, not just at the tissue border. The bacteria seen appeared to be present as both individual cells and in larger clumps.

At this level of magnification it was difficult to make out the precise morphologies of the bacteria observed, but most individual cells appeared to be cocci or coccobacilli.

Figure 5.2 Sections of the tumour specimen after standard H and E staining, viewed under microscope

Photomicrographs (a), (c) and (e) are from one section, (b), (d) and (f) from another. (a) – (b) are at x100 magnification, (c) – (d) are at x200, and (e) – (f) are at x400. The specimen was from a well differentiated squamous cell carcinoma, which had previously been clinically classified as being T3N2M0 (see Table 2.1).



(a) (b)

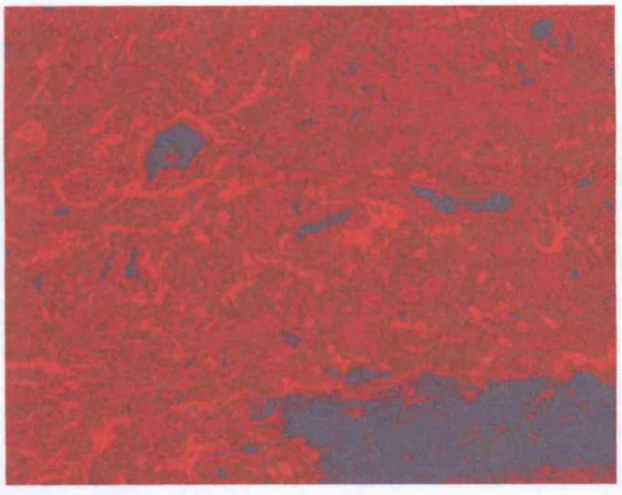
(c) (d)

(e) (f)

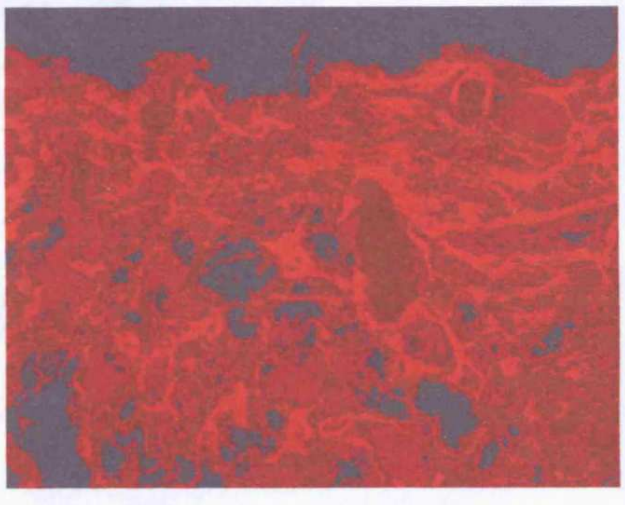
Figure 5.3 Sections of the OSCC tumour specimen after staining with the concanavalin A-Alexa Fluor® 594 conjugate

Three photomicrographs from a single section of the specimen, viewed under the microscope at x400 magnification.

(a)



(b)



(c)

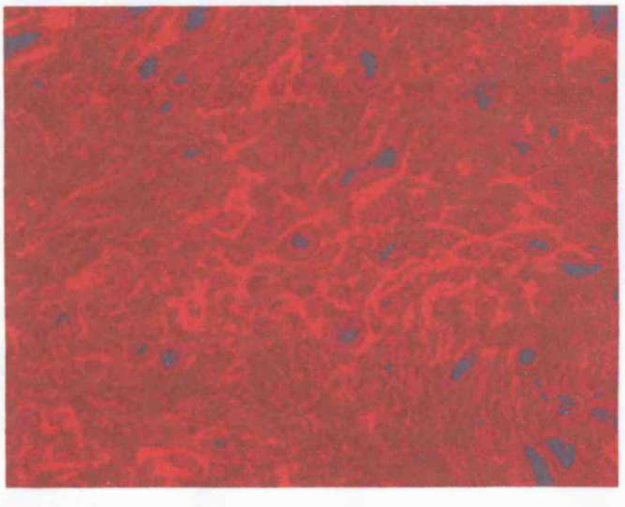
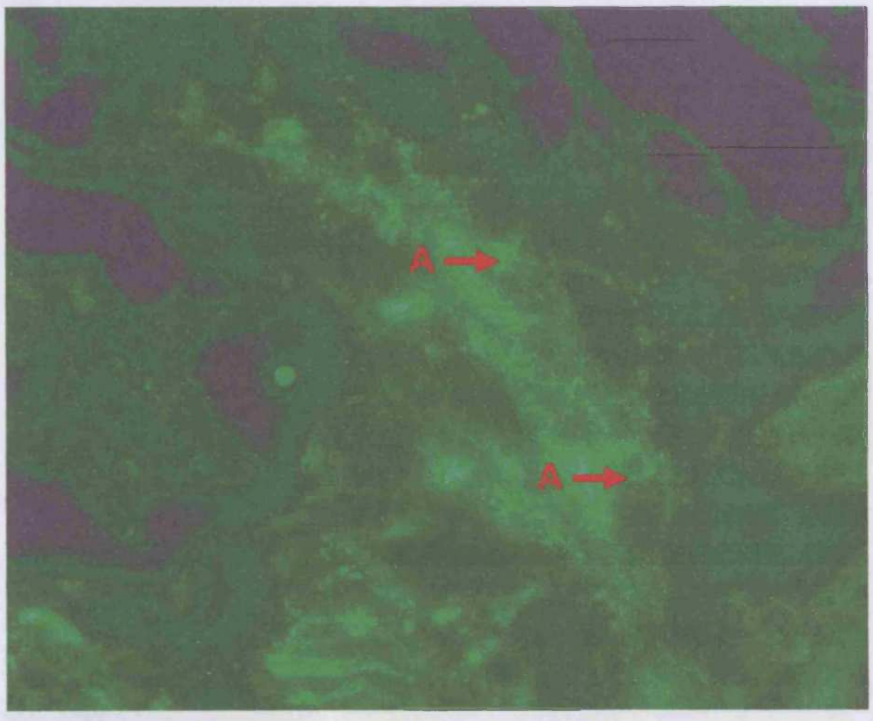


Figure 5.4 Photomicrographs of OSCC sections after hybridisation with the eubacterial probe EUB338-FITC

Images from 2 different sections, viewed at x600 magnification. Bacterial cells were seen individually as well as in highly fluorescent clumps, examples of which are indicated by the arrows labelled A.

(a)



(b)

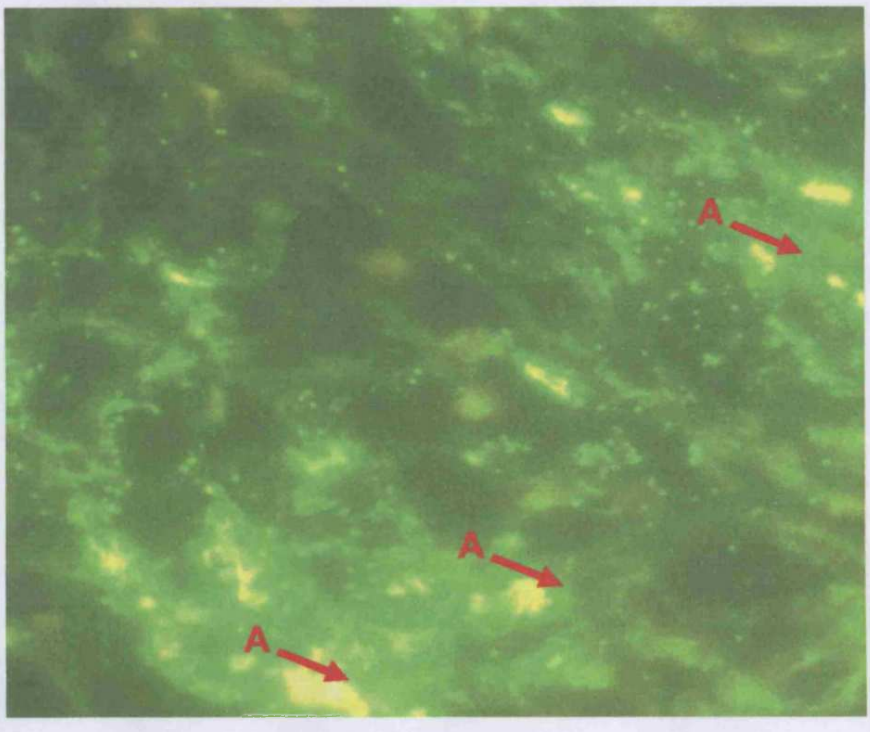
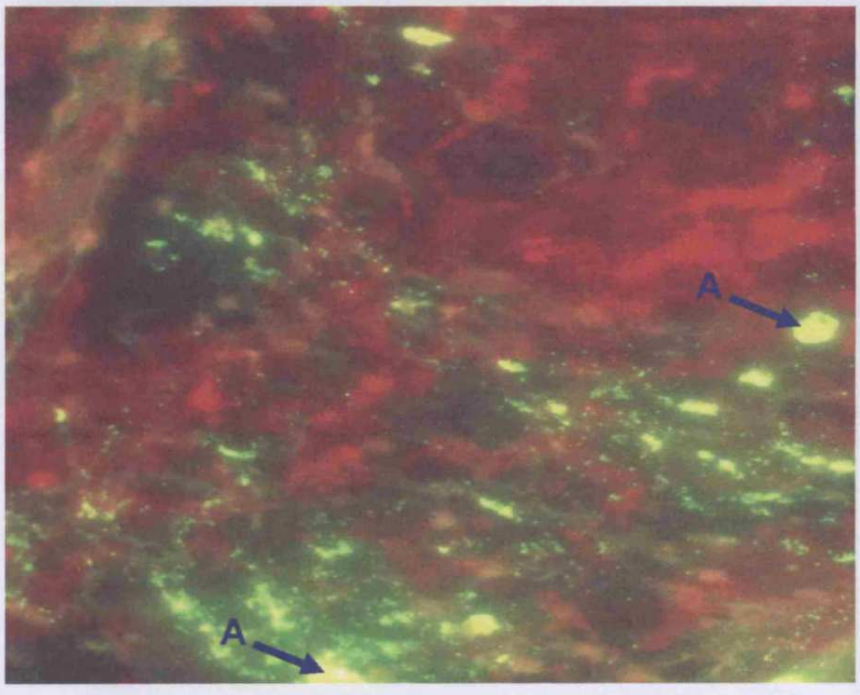


Figure 5.5 Composite photomicrographs showing both the EUB-FITC-labelled bacteria (green) and A-Alexa Fluor594® (red) fluorescence

Images from 2 separate sections of the tissue, viewed at a magnification of x400. Bacterial cells were seen throughout the sections, individually as well as in highly fluorescent clumps, examples of which are indicated by the arrows labelled A.

(a)



(b)

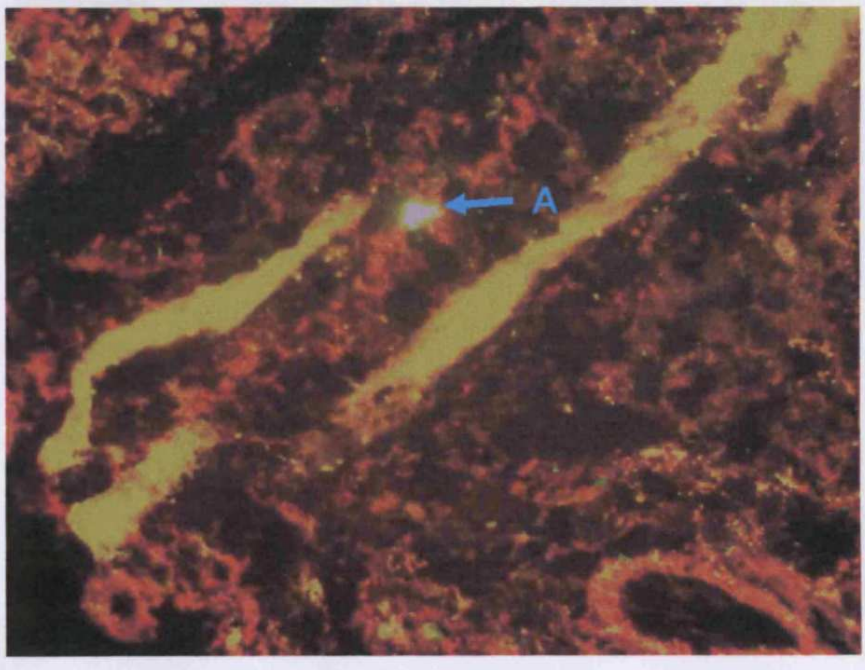
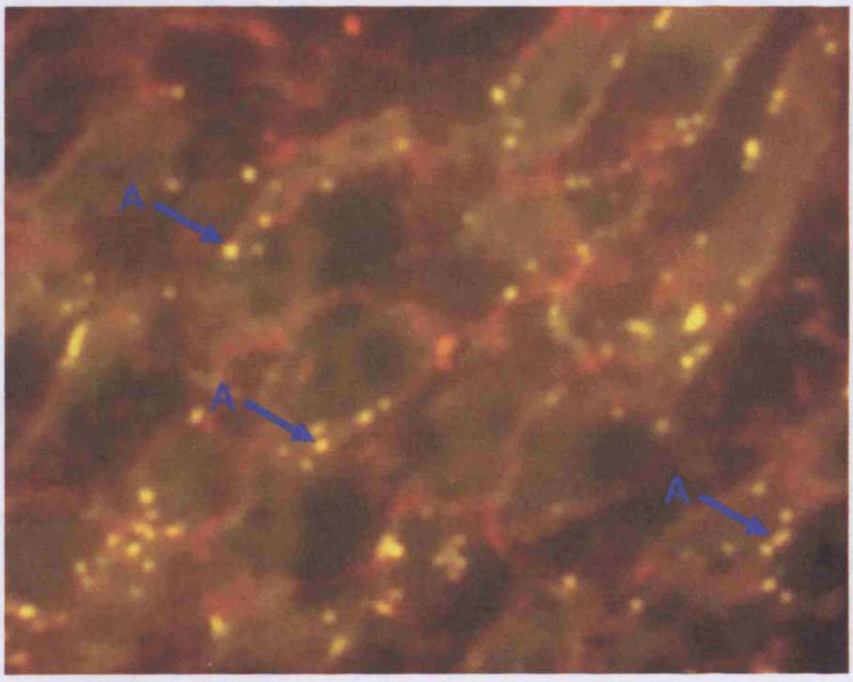


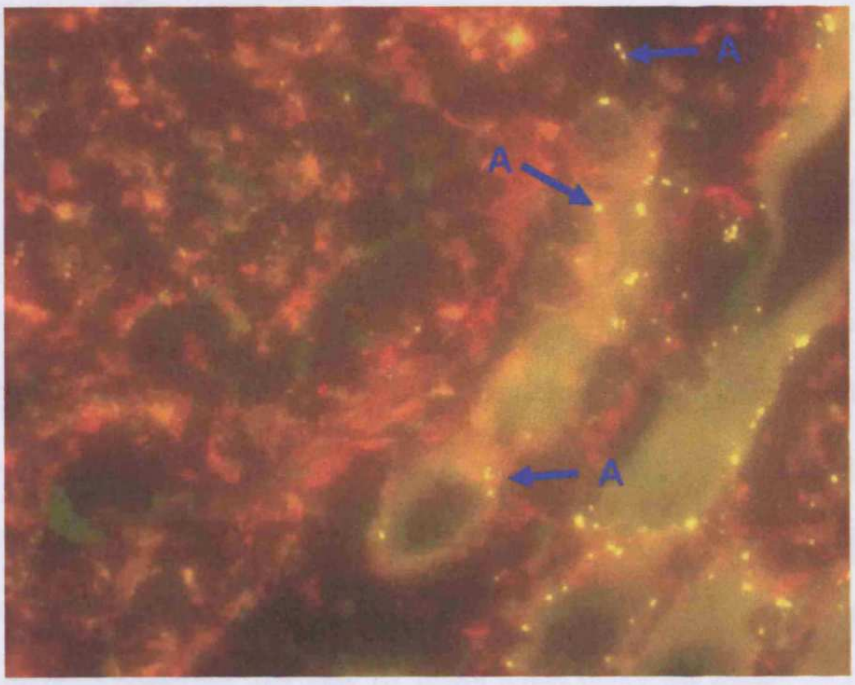
Figure 5.6 Composite photomicrographs showing both the EUB-FITC-labelled bacteria (green) and A-Alexa Fluor594® (red) fluorescence

Images from 2 separate sections of the tissue, viewed at a magnification of x600. Individual bacterial cells, examples of which are labelled A, could be observed throughout the tissue in all sections.

(a)



(b)



5.5 Discussion

To the best of our knowledge, this is the first investigation where FISH has been used to demonstrate the presence of bacteria within OSCC tissue. The distribution of the bacteria in the sections suggests that these microorganisms are found throughout the tumour tissue.

Although it was not carried out in this study, several studies have used FISH for enumerating the bacteria in samples (Banerjee *et al*, 2002; Oliveira *et al*, 2003). There are several limitations which can preclude the use of this technique for detecting and enumerating bacteria within specimens. For instance, bias in the hybridisation can result in misrepresentation in the counts. Although it has been somewhat validated by its use in numerous studies (Banerjee *et al*, 2002; Jansen *et al*, 1999; Pernthaler *et al*, 2002; Thiel & Blaut, 2005; Wecke *et al*, 2000), there is some evidence that the non-specific probe EUB338, supposedly able to bind to all members of the domain *Bacteria*, will not hybridise all bacteria in a sample with equal efficiency (Vaahtovuo *et al*, 2005). This preferential binding could result in bacterial cells being overlooked, which would mean any attempts at quantification could be erroneous.

Additional biases can come from differing ribosome contents per cell. Although normally abundant, the amount of rRNA depends on the physiological state of the cell and can also vary significantly between species (Moter & Göbel, 2000). A relatively low amount of rRNA in a cell may result in a low fluorescent response and the cell being undetectable (Yilmaz & Noguera, 2004). However, there are several possible methods of minimising the risk of low signal intensities or false-negatives that may be worth considering for future studies. For instance, it is possible to label the oligonucleotide probe with additional

fluorescent dye molecules. A second fluorophor can be enzymatically attached to the 3'-end using terminal transferase, theoretically doubling the signal obtainable from each bacterium. Furthermore, sensitivity of detection can be improved by simultaneously using more than one oligonucleotide probe, each selective for the same species and labelled with the same fluorescent dye. Multiple labelling would theoretically increase the number of fluorescent molecules present within each cell, improving the signal intensity. This approach is restricted by the availability of suitable target sites (Moter & Göbel, 2000).

Low signal intensity may also be the result of inadequate infiltration of the probe into the bacterial cells (Moter & Göbel, 2000). Not all bacterial cells can be effectively permeabilised using standard fixation procedures (Wagner *et al*, 2003), particularly Gram-positive *Actinobacteria* (Carr *et al*, 2005; Sekar *et al*, 2003). Enzymatic treatment of the sections in this study with lysozyme and proteinase K was used to counter this problem. However, combining enzyme treatments with a step of mild acid hydrolysis has been shown to be even more effective at permeabilising *Actinobacteria* (Carr *et al*, 2005) and so may be worth considering in any future investigations.

Additionally, in future studies it would be valuable to include some negative controls in order to confirm that the fluorescent signal seen was not just produced by the probe reacting non-specifically with material on the slide and that it was bacterial RNA being detected. One way to control non-specific binding, as reported in previous studies, is to use a complimentary anti-sense probe (NON338) simultaneously with EUB338 (Glöckner *et al*, 1999; Sunde *et al*, 2003). Another useful control is to treat a section with RNase prior to

incubation with the probe. RNA digestion should render the probe unable to hybridise with the section, abolishing any potential fluorescent signal; if the signal is not abolished the probe is binding non-specifically with cell components other than the RNA, indicating a likelihood of false positive results.

Further detail may come from examining FISH-stained slides using a confocal laser scanning microscope (CLSM), rather than standard epifluorescent microscopy. CLSM has been established as a valuable tool for obtaining high-resolution images and three-dimensional reconstructions of a variety of fluorescently-labelled biological samples (Lopez *et al*, 2005; Rudney *et al*, 2005; Sunde *et al*, 2003; Wagner *et al*, 2003). Theoretically CLSM could be applied to the hybridised sections prepared in this or future studies, allowing higher resolution images to be obtained and more details of the morphologies and relative spatial arrangements of individual cells to be determined. However, this may not be as successful as using CLSM to examine, for example, a stained slide of a bacterial biofilm (Thurnheer *et al*, 2004) given the relatively low numbers of bacterial cells seen here. Epifluorescence microscopy has the advantage of being comparatively more straightforward and more readily available (Lopez *et al*, 2005).

Now that a basic protocol for using FISH to examine OSCC sections has been successfully developed, the technique can feasibly be used in future studies to screen sections from additional tissues, including archived paraffin wax sections, for the presence of bacteria. In addition, it would be of interest to perform FISH using probes designed to hybridise with specific species or groups of species. For example, probes have been designed for and successfully used to

detect many oral cavity microorganisms, such as *Actinobacillus actinomycetemcomitans, Actinomyces naeslundii, Candida albicans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythensis, Veillonella dispar, Veillonella parvula*, and species of *Bifidobacterium, Fusobacterium, Streptococcus,* and *Treponema* (Jansen *et al,* 1999; Rudney *et al*, 2005; Sunde *et al*, 2003; Thurnheer *et al*, 2001, 2004; Wecke *et al*, 2000). Many of these species or genera have been isolated from OSCC specimens by previous culture or PCR-cloning analysis (see Chapters 2 and 4), so it would be particularly interesting to corroborate those findings by visualising those specific organisms within the tissue by FISH. This could also provide information as to how the various species present are arranged and interact with each other. In addition, this approach could also be used to compare the species present within OSCC with those in non-tumourous control tissues, both from the same patients and from other age-matched patients without cancer.

CHAPTER SIX

GENERAL DISCUSSION

6. Discussion

Interest in the possible relationships between bacteria and the different stages of cancer development has been increasing since the classification by the W.H.O. of *Helicobacter pylori* as a definite (class 1) carcinogen (Björkholm *et al*, 2003). Numerous mechanisms by which different species of bacteria may initiate or promote carcinogenesis have been proposed and are currently under investigation. These include the induction of chronic inflammation (Christen *et al*, 1999) by interference, either directly or indirectly, with eukaryotic cell cycle and signalling pathways (Lax & Thomas, 2002), or via the metabolism of potentially carcinogenic substances (Salaspuro, 2003).

Despite this, the association of bacteria with carcinogenesis in the oral cavity has not yet been studied to any great extent. It has been reported that a high proportion of cases of head and neck cancer can be related to tobacco use and heavy alcohol consumption (Johnson, 2001). Other possible risk factors include poor oral hygiene (Lissowska *et al*, 2003) and infections with viruses (Scully, 2002) or *Candida* species (Sitheeque & Samaranayake, 2003). The incidence of cancer of the oral cavity appears to be increasing in many parts of the world, including the UK, in a manner that it is difficult to explain within the context of traditional risk factors alone (Hindle *et al,* 2000; Llewellyn *et al*, 2001).

In a study of intraoral carcinomas, Nagy *et al.* (1998) demonstrated increased numbers of certain members of the oral microflora on the surface of tumours in comparison to control sites. However, when investigating the possible role of bacteria in the development of oral cancer it is also of interest to

identify the organisms within the tumour specimens. Only a handful of species have previously been detected, using PCR, within tissue from carcinoma of the upper aerodigestive tract, including *Streptococcus anginosus, Streptococcus mitis* and *Treponema denticola* (Morita *et al*, 2003; Narikiyo *et al*, 2004; Shiga *et al*, 2001; Tateda *et al*, 2000). Therefore, the primary objective of this research was to identify whether bacteria exist within OSCC tissue using a combination of standard culture and molecular techniques that could detect nearly all species present.

When studying the microbes within tissue specimens, it is critical to remove any organisms that occur naturally on the surface of the tumours that are there as a result of salivary or instrument contamination during surgery. Surface decontamination was achieved by immersion in Betadine® and washing with PBS, a similar protocol to that used to decontaminate samples in previously reported work (Morita *et al*, 2003). The method was validated using both cultural and molecular techniques.

A diversity of bacterial species was identified from within all the specimens analysed by the combination of cultural and PCR-cloning methods (see Tables 2.2 and 4.1). The different biases and limitations meant that some species were isolated by culture but not detected by the culture-independent molecular method, and *vice versa.* This concurs with previous studies of complex microfloras (Davies *et al*, 2004; Munson *et al*, 2002; Spratt, 2004; Wilson *et al*, 1997), reinforcing the idea that a combined approach is advantageous.

Eighty-seven distinct taxa were isolated from the deep tumour specimens by the combination of cultivation and PCR-cloning. Only 61 taxa were detected

within the non-tumourous control specimens using the same methods. The observation that the bacterial populations within tumourous and non-tumourous tissues are dissimilar was confirmed by the variation in DGGE profiles (Chapter 3). By both the DGGE and culture methods, a microflora with a different composition to that of the tumour tissues was also seen in the "superficial" specimens, consisting of both tumourous and overlying mucosal tissue. The cultural isolation techniques indicated that there were more microbial species in this overlying tissue than in the pure tumour tissue. Furthermore, in all cases, the superficial tissues yielded exactly the same isolates as the corresponding deep tissue specimens plus, in most cases, additional species. The variation in bacterial composition within the different specimen types would seem to suggest a degree of specificity possibly induced by the tumour microenvironment. This restriction does not appear to be merely due to the ability of anaerobic species to survive better in the deeper, less well oxygenated tissue. As described previously, tumour tissue presents with a different environment in comparison to healthy tissues (see section 1.1.2.4) and it seems likely that this milieu may select for aciduric strains and species of bacteria (see sections 2.5 and 4.5).

It would be of great interest, in possible future investigations, to apply the culture and PCR-cloning protocols established here to specimens from additional patients. A greater number of tissues would increase the significance of any statistical analyses and perhaps reveal if the occurrences of certain taxa or phylotypes really are clinically relevant. Furthermore, the use of a larger cohort of patients could possibly uncover correlations between the presence or absence of certain taxa and clinical details, such as traditional risk factors already associated with the patient or the staging and variety of the tumour. However,

the number of OSCC and other tissue specimens acquired is, of course, subject to availability. Even when consenting OSCC patients are available it is not always possible to obtain specimens at the time of surgery without compromising the pathological margins of the resected tissue.

Similarly, if possible, it would useful to sequence additional, if not all, cloned 16S rRNA gene isolates from the PCR-amplified tissue extracts. Also, given the observed bias of "universal" 16S rRNA gene primers (Kurata *et al*, 2004; Polz & Cavanaugh, 1998), it could be beneficial to repeat both the PCR-cloning and PCR-DGGE techniques using other sets of primers. Many other primers specific for the domain *Bacteria* have been previously reported, and the pooling of PCR reactions utilising different primers can seemingly reduce bias (Baker *et al*, 2003). This would theoretically make the investigation more comprehensive, increasing the likelihood of detecting every bacterial taxon present and arguably making any statistical observations more reliable.

In addition, it would be interesting to apply the PCR-cloning of 16S rRNA genes methodology to the superficial specimens. This could confirm if the same variety in diversity is observed as was seen with the cultural work, potentially providing further evidence of the aforementioned tumour-specificity trend.

It would also be extremely interesting to apply the same approaches employed here to non-tumourous mucosal tissues from patients without cancer. Specimens could be harvested from, for example, patients having wisdom teeth removed or undergoing some other minor maxillofacial surgery. Providing the patients were age-matched to the cases used in this study, this would provide an excellent control for testing the hypothesis that people with OSCC have an

altered microflora. If certain species are found in significantly greater proportions of cancer patients than non-cancer patients, this may be indirect evidence of a possible role in the carcinogenic process. Furthermore, at the time of writing, there have been no cultural or culture-independent investigations of the microbiota within healthy human oral mucosa, although several species have been shown capable of penetrating the epithelium or existing within epithelial cells *in vitro* (Dorn *et al*, 1998; Drago *et al*, 2000; Eick & Pfister, 2004; Lamont & Jenkinson, 2000). Additionally, if particular microbial species are found to be significantly associated with OSCC lesions, this could potentially be used as a diagnostic indicator. Hypothetically, patients testing positive for the presence of such species, following simple screening procedures, could be at a higher risk of developing cancer. As described in 1.3.1.2, a similar approach has recently been suggested based on the detection of raised counts of certain bacterial species in the saliva of OSCC patients.

The bacteria within OSCC tumours may be there because they are causative carcinogenic agents or promoting the development of the tumour. On the other hand they may well have no role in tumourigenesis and are merely secondary colonisers, present simply because of a favourable microenvironment. It is even possible that they play a probiotic role, inhibiting the formation of the tumour by promoting the involvement of elements of the host immune system that can limit cancer progression, such as cytotoxic T lymphocytes and IL-2 activated natural killer cells (Chikamatsu *et al*, 1999; Knutson & Disis, 2005). Therefore, the next stage of investigation must surely look at the interactions between the bacteria associated with the tumours and human epithelial cells. The most commonly found taxa in this study, and hence possible candidate

species to study with regards to epithelial cell interactions, include *Atopobium parvulum, Capnocytophaga* oral strain S3, *Fusobacterium naviforme, Granulicatella adiacens, Micrococcus luteus, Peptostreptococcus micros, Prevotella* sp. oral clone BE073, *Propionibacterium acnes, Ralstonia insidiosa, Staphylococcus capitis/caprae/epidermidis, Streptococcus anginosus, Streptococcus constellatus, Streptococcus parasanguinis* and *Veillonella atypica.* Potential lines of exploration include studying the capability of the bacteria to invade or adhere to the epithelium by using *in vitro* cell lines, similar to what has previously been performed with *Peptostreptococcus micros* (Kremer *et al*, 1999), *Streptococcus constellatus* (Eick & Pfister, 2004) and other oral pathogens including *Actinobacillus actinomycetemcomitans* (Gasparetto *et al*, 2001) and *Porphyromonas gingivalis* (Dorn *et al*, 2000). The penetrative ability of microorganisms can also be looked at using reconstituted human epithelium models, such as has been done with *Candida albicans* (Bartie *et al*, 2004; Jayatilake *et al*, 2005). Also, the effects on cultured human cells of bacterial supernatants from species related to carcinomas could be studied as part of an investigation of their potential virulence (Stephens *et al*, 2003). Carcinogenic potential could also be considered by measuring the species' ability to induce the expression of inflammation mediators (Fujiki *et al*, 2004; Israel *et al*, 2001; Narikiyo *et al*, 2004). All investigations could be performed using both reference strains and strains isolated from OSCC patients.

To our knowledge this is the first time that viable bacteria have been detected within the tissue of oral squamous cell carcinoma. The implications of the presence of a diversity of bacteria deep within the tissue of OSCC are

unclear. It has been shown in animal models that bacteria injected intravenously may seed to and replicate within tumour tissue (Yu *et al*, 2004). However, the types of bacteria isolated in this study, and the fact that the composition of the deep tissue microflora was similar to but less species-rich than the overlying mucosa, tends to imply a local origin for the bacteria detected within the tumour. The apparent differences between the microflora of the tumour and control tissues suggests a degree of bacterial specificity which merits further investigation. Moreover, the appreciation that bacteria may be involved in the carcinogenic process via a number of mechanisms reinforces the need for continued study of this subject.

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APPENDICES

APPENDIX I

Sequence data for the oligonucleotide primers and probes used in this study

Primer name	Nucleotide sequence	Reference
27F	5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3'	Dymock *et al* (1996)
341f (including GC clamp)	5'-CGCCCGCCGCGCGCGGCGGGCGGGCGGG GGCACGGGGGGCCTACGGGAGGCAGCAG-3'	Rölleke *et al* (1996)
357F	5'-CTCCTACGGGAGGCAGCAG-3'	Lane (1991)
534r	5'-ATTACCGCGGCTGCTGG-3'	Rölleke *et al* (1996)
1492R	5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3'	Dymock *et al* (1996)
C90	5'-GTTACGACTTCACCCTCCT-3'	Paster *et al* (2001)
D88	5'-GAGAGTTTGATYMTGGCTCAG-3'	Paster *et al* (2001)
E94	5'-GAAGGAGGTGWTCCARCCGCA-3'	Paster *et al* (2001)
EUB338	5'-GCTGCCTCCCGTAGGAGT-3'	Amann *et al* (1990)
F01	5'-CCTTGTTACGACTTAGCCC-3'	Paster *et al* (2001)
M13 (-20) forward	5'-GTAAAACGACGGCCAGTG-3'	Invitrogen Ltd., Paisley, U.K.
M13 reverse	5'-CAGGAAACAGCTATGAC-3'	Invitrogen Ltd., Paisley, U.K.

APPENDIX II

Recipes for buffers and media used in this study

Once mixed as described each was sterilised by autoclaving for 20 min at 15 psi, with the exception of the Reduced Transport Medium, which was sterilized by filtration using a 0.2 μm filter.

Buffer name	Reagent	Amount	Supplier
Hybridisation buffer	5 % SDS (w/v)	10 ml	Sigma
	5 M Sodium chloride	18 ml	Sigma
	1 M Tris-HCl (pH 7.4)	2 ml	Sigma
	Double distilled water	Up to 100 ml	
Luria-Bertani (LB) agar	LB broth	1 L	See below
	Agar	15 g	Sigma
Luria-Bertani (LB) broth	Tryptone	10 g	Oxoid
	Yeast extract	5 g	Oxoid
	Sodium chloride	10 g	Sigma
	Double distilled water	Up to 1 L	
Reduced Transport Media (RTM)	Tryptone	1 g	Oxoid
	Yeast extract	0.5 g	Oxoid
	Glucose	0.1 g	BDH Ltd.
	Cysteine hydrochloride	0.1 g	BDH Ltd.
	1 M Sodium hydroxide	0.5 ml	Sigma
	Horse serum	2 ml	TCS Ltd.
	Double distilled water	Up to 100 ml	
Tris Acetate EDTA (TAE) buffer (50x)	Tris base	242 g	Sigma
	Glacial acetic acid	57.1 ml	Fisher
	EDTA	18.6 g	Sigma
	Double distilled water	Up to 1 L	
Tris-Borate-EDTA (TBE) buffer	5 x TBE	As provided	Sigma
	Double distilled water	Up to 1L	
Tris-EDTA (TE) buffer	1 M Tris-HCl (pH 7.4)	10 ml	Sigma
	0.5 M EDTA	2 ml	Sigma
	Double distilled water	Up to 1L	

APPENDIX III

Recipes for stains used in this study

Stain name	Reagent	Amount	Supplier
Carbol fuchsin (10x)	Basic fuchsin	1 g	Sigma
	95% Ethanol	10 mL	Fisher
	Phenol	5 g	Sigma
	Double distilled water	Up to 100 mL	
Lugol's iodine (5x)	Iodine	5 g	BDH Ltd.
	Potassium iodide	10 g	Sigma
	Double distilled water	100 mL	

APPENDIX IV

The Puregene® "DNA Purification From 1 ml Gram-Positive Bacteria Culture Medium" protocol

The DNA isolation protocol used in this study to extract microbial DNA from cultures and digested tissue suspensions. Reproduced from http://www.gentra.com/pdf/01120.pdf.

PUREGENE®

Protocol

Gentra SYSTEMS

PUREGENE® DNA Purification Kit

DNA Purification From 1 ml Gram-Positive Bacteria Culture Medium

Expected Yield 6-60 µg DNA

Cell Lysis

1. Add 1.0 ml cell suspension (*e.g.*, overnight culture containing approximately 1-3 billion cells) to a 1.5 ml tube on ice.
2. Centrifuge at 13,000-16,000 x g for 5 seconds to pellet cells. For some species centrifugation for up to 60 seconds may be required to obtain a tight cell pellet. Remove as much supernatant as possible using a pipet.
3. Add 600 µl **Cell Suspension Solution** to cell pellet and gently pipet up and down until cells are suspended.
4. Add 3.0 µl **Lytic Enzyme Solution** and invert tube 25 times to mix.
5. Incubate at 37°C for 30 minutes to digest cell walls. Invert sample occasionally during the incubation.
6. Centrifuge at 13,000-16,000 x g for 1 minute to pellet the cells. Remove supernatant.
7. Add 600 µl **Cell Lysis Solution** to the cell pellet and gently pipet up and down to lyse the cells.
8. For some species heating the sample to 80°C for five minutes may be required to complete cell lysis.

RNase Treatment

1. Add 3.0 µl **RNase A Solution** to the cell lysate.
2. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15-60 minutes.

Protein Precipitation

1. Cool sample to room temperature.
2. Add 200 µl **Protein Precipitation Solution** to the cell lysate.
3. Vortex vigorously at high speed for 20 seconds to mix the **Protein Precipitation Solution** uniformly with the cell lysate. For species with a high polysaccharide content, placing the sample on ice for 15-60 minutes may be required.
4. Centrifuge at 13,000-16,000 x g for 3 minutes. The precipitated proteins will form a tight white pellet. If the protein pellet is not tight, repeat Step 3 followed by incubation on ice for 5 minutes, then repeat Step 4.

DNA Precipitation

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5 ml microfuge tube containing 600 µl **100% Isopropanol** (2-propanol).
2. Mix the sample by inverting gently 50 times.
3. Centrifuge at 13,000-16,000 x g for 1 minute; the DNA should be visible as a small white pellet.
4. Pour off supernatant and drain tube briefly on clean absorbent paper. Add 600 µl **70% Ethanol** and invert tube several times to wash the DNA pellet.
5. Centrifuge at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol.
6. Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 minutes.

DNA Hydration

1. Add 200 µl **DNA Hydration Solution** (200 µl will give a concentration of 100 µg/ml if the yield is 20 µg DNA).
2. Rehydrate DNA by incubating sample 1 hour at 65°C and/or overnight at room temperature. If possible, tap tube periodically to aid in dispersing the DNA.
3. Store DNA at 4°C. For long-term storage, store at -20°C or -80°C.

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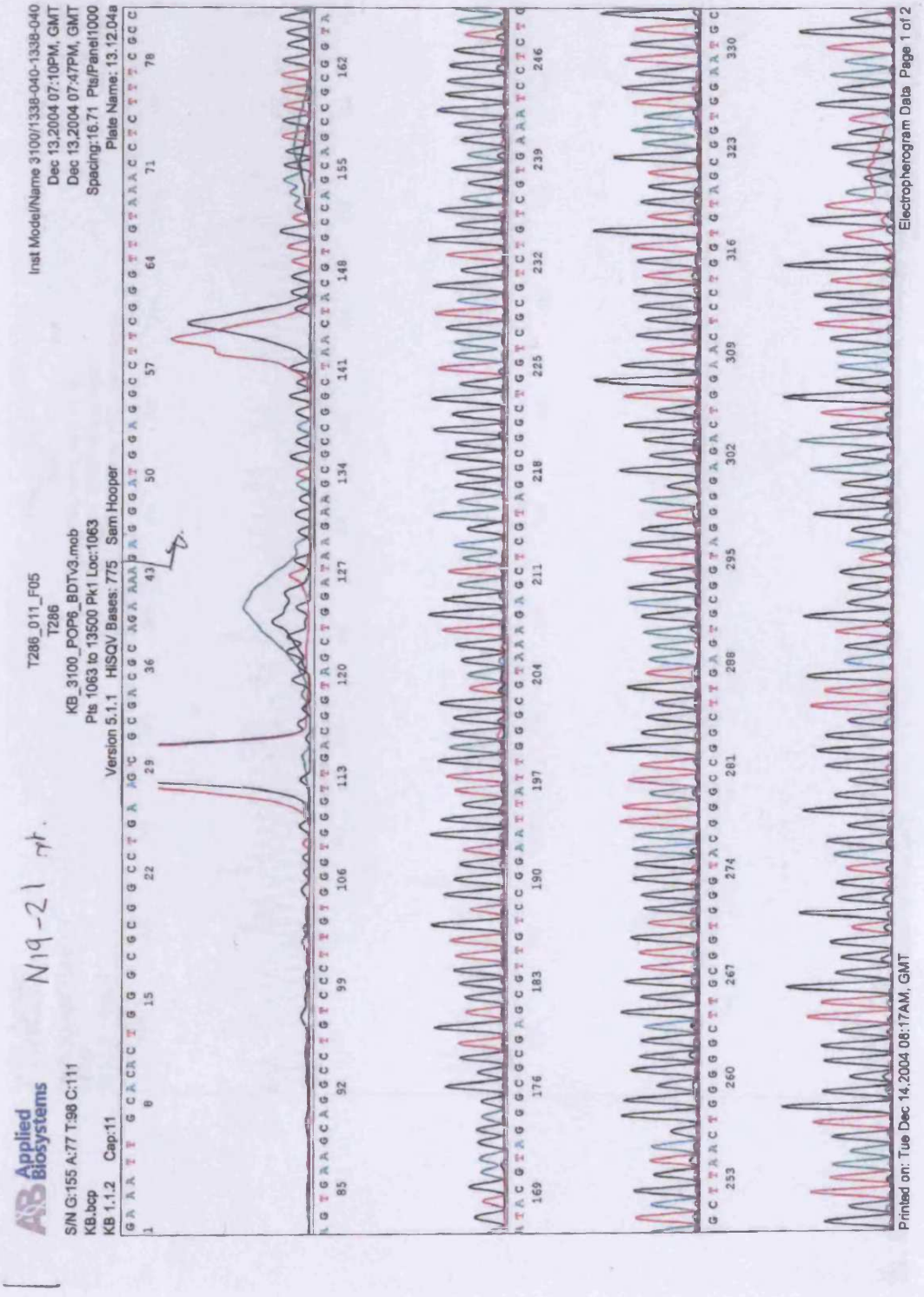
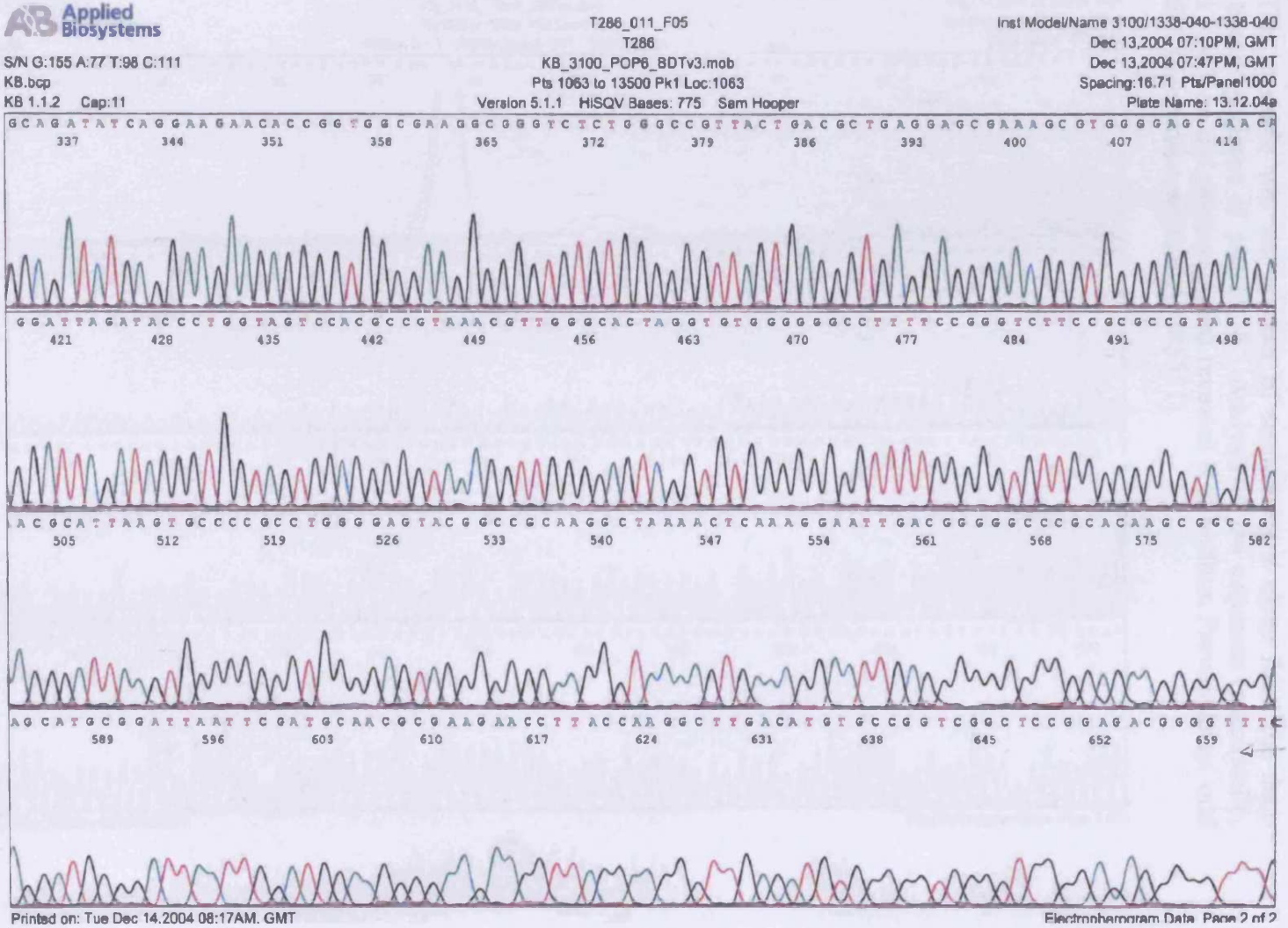
APPENDIX V

A sequencing gel chromatogram produced from a cultivated bacterial isolate

This example was the product of sequencing with primer 357F the PCR-amplified 16S rRNA gene fragment of an bacterium cultured from patients 19's non-tumourous tissue specimen. Analysis of the sequence (specifically, bases 44 – 662 on the chromatogram) revealed the isolate to be *Actinomyces viscosus*.

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S/N G:155 A:77 T:98 C:111
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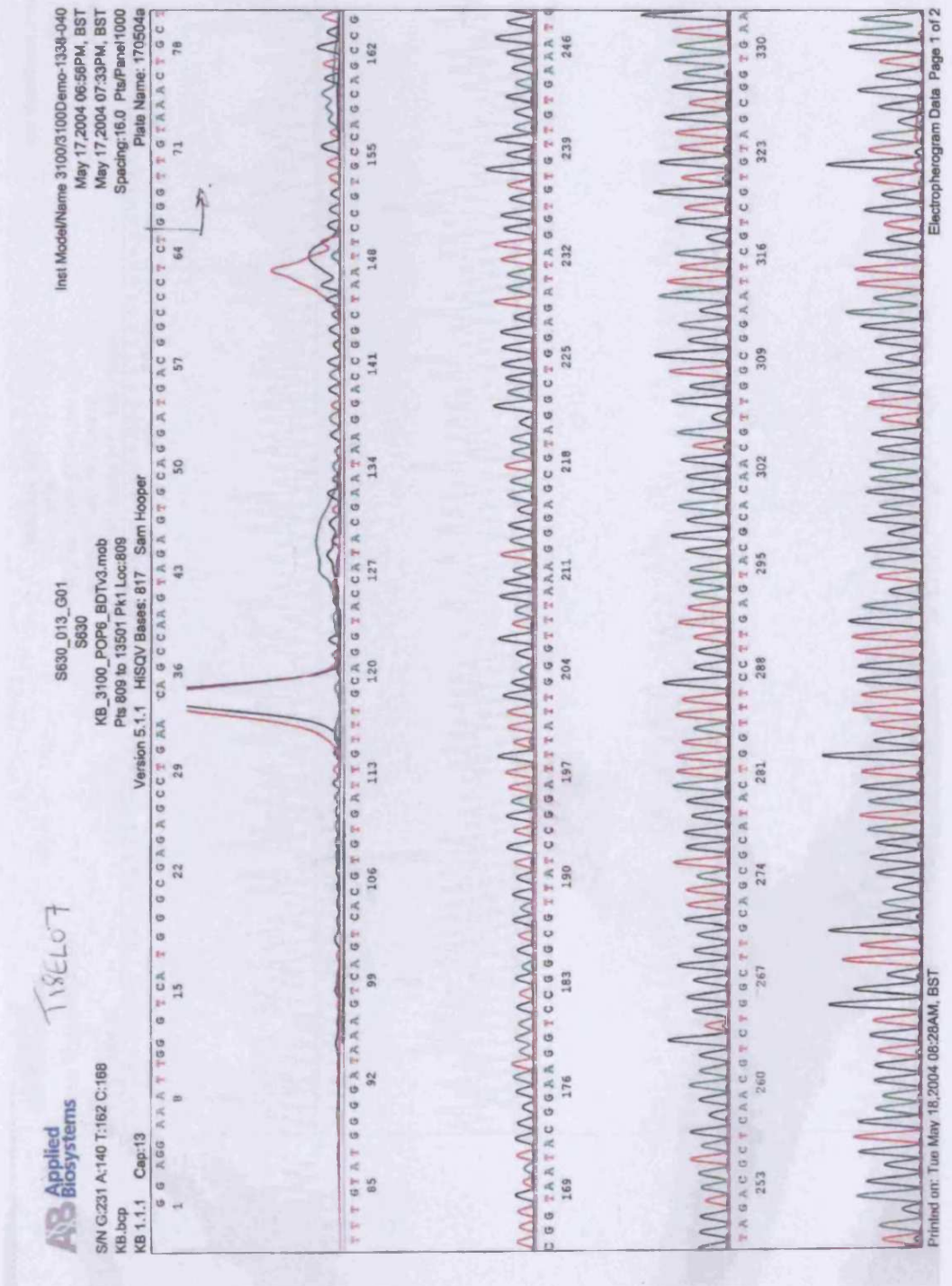
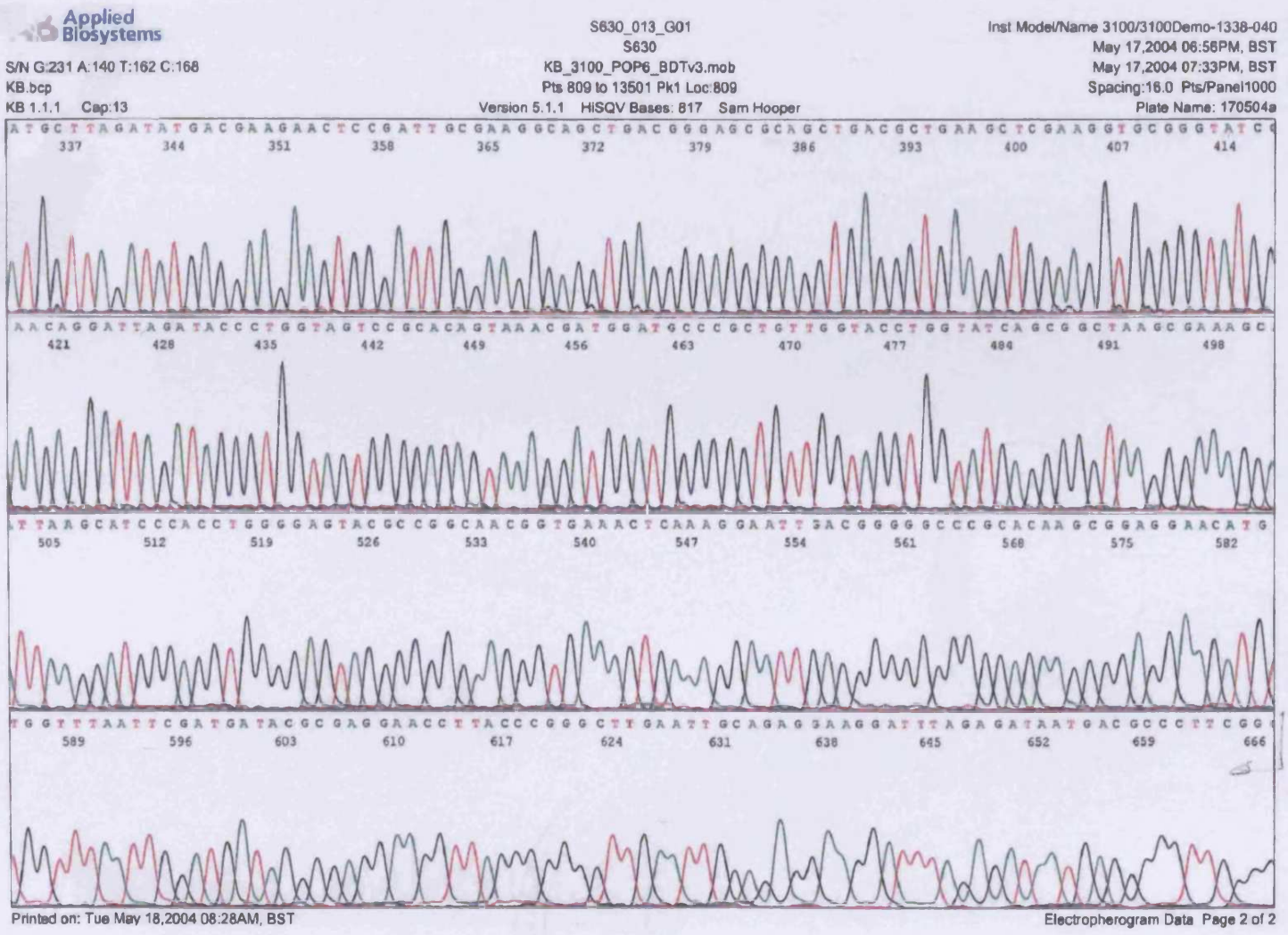
Electropherogram Data Page 2 of 2

APPENDIX VI

A sequencing gel chromatogram produced from the PCR-cloning of 16S rRNA gene sequences

This example was the product of sequencing a clone from the deep tumour tissue specimen of patient 18. Analysis of the sequence (specifically, bases 66 – 667 on the chromatogram) revealed it to be from *Prevotella* sp. oral clone BE073 (accession number AF385551).

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KB 1.1.1 Cap:13
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Electropherogram Data Page 2 of 2

carcinogenic potential and resistance to cigarette smoke in certain bacterial strains are unclear.

1.3.1.2 Changes in salivary microflora associated with OSCC

It has been reported that patients with OSCC tend to possess notably raised concentrations of certain bacteria in their saliva compared with OSCC-free individuals. Using checkerboard DNA-DNA hybridization, counts of *Capnocytophaga gingivalis, Prevotella melaninogenica* and *Streptococcus mitis* were all found to be elevated in the subjects with OSCC (Mager *et al,* 2005). This apparent alteration of the salivary microflora in the presence of OSCC lesions is of particular interest because of its potential application as a diagnostic tool for predicting oral cancer.

1.3.1.3 Human oral carcinoma surface biofilms

Biofilms from the surface of human oral squamous cell carcinoma have been shown by basic cultural analysis to harbour increased number of bacteria compared to healthy control sites. The bacteria detected in this study were a range of aerobes and anaerobes, including *Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces, Clostridium, Haemophilus, Enterobacteriaceae* and *Streptococcus* species. *Candida albicans* was also found to be present in these biofilms in contrast to the control sites (Nagy *et al,* 1998). All of these species are recognised members of the normal flora (Tanner *et al,* 1994) and there is no direct evidence of a causal link. However, it is suggested that the increased numbers in the surface microflora may increase the

risk of local and systemic infections, which may complicate the morbidity of the patient (Nagy *et al*, 1998).

1.3.1.4 Bacteria detected within upper aerodigestive tract carcinoma by molecular-based techniques

Several recent studies have linked the species *Streptococcus anginosus*, a member of the *Streptococcus milleri* species-group commonly found as part of the oral cavity microflora (Hirai *et al*, 2005), to carcinomas of the upper aerodigestive tract. Using polymerase chain reaction (PCR) with species-specific primers, *Str. anginosus* ribosomal DNA was detected within surgical specimens from oesophageal and gastric cancer patients (22 of 33, or 67%, and 18 of 43, or 43% of specimens, respectively), as well as in dysplasia of the oesophagus (4 of 6, or 67% of specimens) (Morita *et al*, 2003; Sasaki *et al*, 1998) and head and neck squamous cell carcinomas (all 221 specimens analysed) (Shiga *et al*, 2001; Tateda *et al*, 2000). It has also been identified within cancer tissue from some oral cavity carcinomas (5 of 38, or 13% of samples). In the same study only 1 of 13 (8%) of the non-cancerous tissues tested contained a detectable amount of *Str. anginosus* DNA (Morita *et al*, 2003). *Str. anginosus* is typically present at low levels in saliva. However, the average level of *Str. anginosus* has been shown to increase with age by quantitative real-time PCR of samples from systemically healthy patients aged 25-70 (Morita *et al*, 2004). These data would appear to indicate a strong association between this prevalent oral species and esophageal cancer, and implicate *Str. anginosus* in the carcinogenesis of head and neck squamous cell carcinoma.

For positive controls, and to provide a standard marker for the DGGE, the second round of PCR was also performed using genomic DNA from known bacterial strains as template. Genomic DNA was extracted as before (section 3.3.1) from *Enterococcus faecalis* NCTC 775, *Escherichia coli* NCTC 10418, *Porphyromonas gingivalis* NCTC 11834 and *Streptococcus anginosus* NCTC 10713 (Health Protection Agency, London, UK).

All PCRs were performed with a PTC-200 thermal cycler (MJ Research Ltd.). The success of each reaction was assessed by electrophoresis on a 1% agarose (Bioline, London, U.K.) gel containing 125 ng/ml ethidium bromide (Sigma). PCR products were visualised in the gel under UV light using a GelDoc system (Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K.).

3.3.3 **Preparation of parallel denaturing gradient gels**

Polyacrylamide gels were cast using a model 385 gradient former (Bio-Rad). All gels comprised of 1 x Tris-acetate-EDTA (TAE) buffer (see Appendix II) with 10% (w/v) acrylamide, 0.1% (v/v) TEMED, and 0.1% (w/v) ammonium persulphate (all materials from Sigma). Gels also contained a parallel 30-60% gradient of denaturants (where 100% denaturant concentration was equal to 7 M urea and 40% [v/v] deionised formamide; Sigma).

3.3.4 **Separation of PCR products by electrophoresis**

Parallel DGGE was performed using the Bio-Rad D-Code system. Products from tissue extract PCRs were run alongside a marker comprising of the PCR products from the known bacterial stains. The use of a marker allowed standardisation of the gels.

DGGE was run in 1 x TAE buffer at 56°C with a current of 70 V for 20 min, followed by 170 V for 4 h.

3.3.5 Analysis of gels

DGGE gels were stained by submersion in the (1 x) working concentration of SYBR® Green I nucleic acid gel stain (Sigma) at approximately 4°C for 30 min. Banding patterns were visualised under UV light using the GelDoc system (Bio-Rad). Gels were aligned using the markers. Quantity One® quantitation software (Bio-Rad) was used to standardise the gels, detect the bands present and estimate the number present in each lane. The presence of individual bands in each profile was assessed using the same software, set to a 1% tolerance of band matching. The significance of the varying occurrences of each band was estimated using a 2-tailed Fisher's exact test. Because the total sample size is relatively small it is preferable to use an exact test rather than a Chi-squared test as the probability results are likely to be more accurate (Agresti, 1992).

3.4 Results

The products of the nested PCR of each tissue specimen DNA extract (Figure 3.1) were successfully separated by DGGE. The gel profiles from the 20 deep tumour specimens are shown in Figures 3.2 and 3.3. Profiles from the 19 "superficial" specimens, comprising of tumourous and overlying mucosal tissue, are in Figure 3.4. The non-tumourous control tissue profiles are shown in Figure 3.5.

OSCC patients could be used to form the basic hypotheses of future investigations.

CHAPTER FIVE

VISUALISATION OF THE BACTERIA WITHIN ORAL SQUAMOUS CELL CARCINOMA TISSUE BY FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

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