A molecular analysis of the bacteria present within oral squamous cell carcinoma

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In order to characterize the bacterial microbiota present within oral cancerous lesions, tumorous and non-tumorous mucosal tissue specimens (approx. 1 cm^3) were harvested from ten oral squamous cell carcinoma (OSCC) patients at the time of surgery. Any microbial contamination on the surface of the specimens was eliminated by immersion in Betadine and washing with PBS. Bacteria were visualized within sections of the OSCC by performing fluorescent *in situ* hybridization with the universal oligonucleotide probe, EUB338. DNA was extracted from each aseptically macerated tissue specimen using a commercial kit. This was then used as template for PCR with three sets of primers, targeting the 16S rRNA genes of *Spirochaetes*, *Bacteroidetes* and the domain *Bacteria*. PCR products were differentiated by TA cloning and bacterial species were identified by partial sequencing of the 16S rRNA gene fragments. A total of 70 distinct taxa was detected: 52 different phylotypes isolated from the tumorous tissues, and 37 taxa from within the non-tumorous specimens. Differences between the composition of the microbiotas within the tumorous and non-tumorous mucosae were apparent, possibly indicating selective growth of bacteria within carcinoma tissue. Most taxa isolated from within the tumour tissue represented saccharolytic and aciduric species. Whether the presence of these bacteria within the mucosa has any bearing on the carcinogenic process is a concept worthy of further investigation.

INTRODUCTION

Oral cancer is one of the ten most prevalent cancers in the world (Reichart, 2001; Rosenquist *et al.*, 2005). The prognosis for oral cancers is notably poor, with the mean all-stage, 5-year survival rate being less than 50 % (Kujan *et al.*, 2005; Zakrzewska, 1999). Most worryingly, the incidence of cancer of the oral cavity appears to be increasing in many parts of the world, including the UK (Franceschi *et al.*, 2000; Hindle *et al.*, 2000; Llewellyn *et al.*, 2004; Reichart, 2001; Zakrzewska, 1999). More than 90 % of mouth neoplasms are squamous cell carcinomas (SCCs) originating from the oral mucosa (Chen & Myers, 2001).

It has been well established that most cases of oral SCC (OSCC) are associated with tobacco use and heavy alcohol consumption (Ogden, 2005; Warnakulasuriya *et al.*, 2005). However, these risk factors alone do not account for all cases and it is becoming apparent that other factors must play an important aetiological role, particularly in younger patients (Llewellyn *et al.*, 2004). Poor oral hygiene (Lissowska *et al.*, 2003; Rosenquist *et al.*, 2005; Velly *et al.*, 1998), periodontitis (Tezal *et al.*, 2005) and infection with viruses (Ha & Califano, 2004) or *Candida* species (Sitheeque & Samaranayake, 2003) have all been associated independently with oral cancer. Other possible risk factors may include infection with certain species of bacteria.

Interest in the possible relationships between bacteria and the different stages of cancer development has been increasing since the classification by the World Health Organization of *Helicobacter pylori* as a definite (class 1) carcinogen (Björkholm *et al.*, 2003). Various other bacterial infections have also been found to correlate with an increased risk of developing cancer, for instance, an increased risk of gallbladder carcinoma is associated with *Salmonella typhi* infection (Dutta *et al.*, 2000; Shukla *et al.*,

Abbreviations: FISH, fluorescent *in situ* hybridization; OSCC, oral squamous cell carcinoma; SCC, squamous cell carcinoma.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the novel phylotypes reported in this study are DQ093271–DQ093274.

2000) and there is a greater risk of developing colon cancer in *Streptococcus bovis*-infected patients (Ellmerich *et al.*, 2000; Waisberg & Matheus, 2002). Such epidemiological links have indirectly implied aetiological roles for these micro-organisms, which has prompted the study of potential mechanisms by which bacterial species may initiate or promote carcinogenesis. These include the induction of chronic inflammation (Coussens & Werb, 2002; Parsonnet, 1995), by interference, either directly or indirectly, with eukaryotic cell cycle and signalling pathways (Lax, 2005; Lax & Thomas, 2002), or via the metabolism of potentially carcinogenic substances (Knasmüller *et al.*, 2001; Salaspuro, 2003).

To date, there have been only a few investigations into the possible associations between bacterial species and oral carcinoma. In a study of intraoral carcinomas, Nagy *et al.* (1998) demonstrated increased numbers of certain members of the oral microbiota on the surface of tumours in comparison with control sites. More recently, it has been reported that patients with OSCC tend to possess significantly raised concentrations of certain bacteria in their saliva (Mager *et al.*, 2005). This apparent alteration of the oral microbiota in cases of OSCC is of particular interest because of its potential application as a diagnostic tool. Other groups have demonstrated the presence of specific bacterial species within tissues from tumours of the upper aerodigestive tract using culture-independent approaches. Species-specific molecular techniques have been used to show the presence of *Streptococcus anginosus* within tumour specimens from oropharyngeal, oesophageal and gastric carcinoma patients (Narikiyo *et al.*, 2004; Sasaki *et al.*, 1998; Shiga *et al.*, 2001; Tateda *et al.*, 2000). It has also been shown by PCR amplification that *Streptococcus mitis* and *Treponema denticola* are significantly more prevalent within oesophageal carcinoma tissues compared with non-tumorous tissue (Narikiyo *et al.*, 2004). However, at the time of writing, no previous studies have attempted to examine comprehensively the bacterial population within oral carcinoma tissue using a 'universal' molecular approach.

Techniques based on the gene encoding 16S rRNA are now a standard approach for characterizing the bacterial species present within polymicrobial populations (Amann *et al.*, 2001; Paster *et al.*, 2001; Spratt, 2004; Wade, 2004). The aims of this investigation were twofold: firstly, to determine the location of bacteria within OSCC using fluorescent *in situ* hybridization (FISH), and, secondly, to identify the species present within tumorous and non-tumorous mucosal tissue from OSCC patients, without the biases of culture, by PCR cloning and sequencing of the 16S rRNA gene.

METHODS

Collection of tissue specimens. Tissue specimens were obtained from nine male and one female OSCC patients, with a mean age of 65.3 ± 9.8 years. Ethical approval for the study was granted by the South Wales Local Research Ethics Committee and subjects agreed to participate with their informed consent. Tumours were removed surgically and specimens from the resected OSCC were harvested under aseptic conditions. 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 from within the tumorous mass without compromising the pathological margins. At the same time, control specimens consisting of non-tumorous tissue harvested at least 5 cm away from the primary tumour site were also obtained.

Specimens were aseptically transferred to the laboratory in separate vials of a reduced transport medium comprising tryptone (1 %, w/v), yeast extract (0.5 %, w/v), glucose (0.1 %, w/v), cysteine hydrochloride (0.1 %, w/v), sodium hydroxide (50 mM) and horse serum (2 %, v/v), sterilized using a 0.2 μm filter.

All subsequent handling of the specimens was carried out using aseptic technique on surfaces cleaned with hycolin phenolic disinfectant (2 %, v/v). Tissue specimens were placed in Betadine antiseptic solution (Seton Healthcare Group) for 3 min to disinfect the surface. Subsequently, tissues were vortexed in multiple 500 μl aliquots of PBS to encourage the removal of any bacteria on the tissue surface. Final washes were retained and analysed by both culture and culture-independent (PCR) methods to determine whether surface decontamination was successful. Standard clinical cultivation methods were used, culturing washes on non-specific media and under both aerobic and anaerobic conditions, as described previously (Hooper *et al.*, 2006). Specimens were stored in TE buffer at −80 °C prior to molecular analysis.

FISH. A portion of SCC tissue from one of the patients mentioned above (male, aged 59 years) was fixed overnight in 10 % formyl saline. This was processed, according to standard clinical pathology protocols (Cross, 2004), through graded alcohols and xylene, and embedded into paraffin wax. Sections of 4 μm thick were cut using a microtome and mounted onto SuperFrost glass slides (Menzel-Gläser). Prior to FISH, sections were pre-treated by submersion in xylene for 5 min. This was repeated a further two times and followed by two immersions in 96 % ethanol (5 min each) and then a single immersion in 70 % ethanol (5 min). Slides were rinsed with sterile PBS (1 min) and incubated in 50 mM TE buffer (pH 7.4) containing lysozyme (10 mg ml^{-1}) for 20 min at 37 °C to permeablize the cells to the oligonucleotide probe. Following another rinse with PBS, sections were again incubated in 50 mM TE buffer, this time containing proteinase K (7 μg ml^{-1}; 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), followed by 96 % ethanol (1 min). After air drying, slides were ready for FISH.

Sections were pre-incubated at 48 °C (20 min) in a hybridization buffer (300 μl) containing 0.9 M sodium chloride, 20 mM Tris/HCl (pH 7.4) and 0.5 % (w/v) SDS. Pre-warmed hybridization buffer (300 μl) containing 0.1 μM oligonucleotide probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′), which was 5′-end labelled with FITC (MWG Biotech), was carefully applied to the tissue sections. EUB338 is complementary to a portion of the 16S rRNA gene that is highly conserved within the domain *Bacteria* (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 counterstained with 0.025 % (w/v) concanavalin A–Alexa Fluor 594 conjugate (Molecular Probes) for 20 min. Each slide was again rinsed with water, dried (ending with partial air drying in the dark for 5 min) and subsequently mounted with FluoroSave (Calbiochem, Merck Bioscience). As a control, blank slides with no tissue sections were included to confirm that no bacteria were being introduced during the hybridization procedure.

Hybridized sections were viewed using a Provis AX70 microscope with a built-in incident light fluorescence illuminator (Olympus). Images were obtained using an attached Nikon DXM1200 digital camera and the associated ACT-1 software, version 2.63 (Nikon).

PCR cloning of 16S rRNA gene sequences. Tissue specimens were macerated aseptically and digested with proteinase K (2.5 μg ml^{-1}) overnight at 55 °C. DNA extracts were then prepared using a Puregene DNA isolation kit (Gentra Systems) and the 'DNA Isolation from Gram-positive Bacteria Culture Medium' protocol (http://www1.qiagen.com/HB/GentraPuregene_EN).

The DNA extracts were each used as the template for three separate PCRs using primers first described by Paster *et al.* (2001). The three reactions differed only in the reverse primer used, namely either C90 (5′-GTTACGACTTCACCCTCCT-3′, specific for *Spirochaetes*), F01 (5′-CCTTGTTACGACTTAGCCC-3′, specific for *Bacteroidetes*) or E94 (5′-GAAGGAGGTGWTCCARCCGCA-3′, specific for the domain *Bacteria*). All reactions used the universal D88 primer (5′-GAGAGTTTGATYMTGGCTCAG-3′) as the forward primer. Each reaction mixture comprised 0.5 μM of both forward and reverse primers, 200 μM each deoxynucleotide, 1.5 mM $MgCl_2$, the working concentration of magnesium-free buffer, 1.5 U *Taq* polymerase and 5 μl DNA extract (approx. 0.5 μg DNA). All PCRs were performed with 8 min of denaturation at 95 °C, followed by 30 cycles 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 for 10 min.

Fresh PCR products (4 μl) were cloned into *Escherichia coli* using the commercial vector pCR2.1 available in the TOPO TA cloning kit (Invitrogen Life Technologies). For each tissue specimen, a total of 30 successful clones, representing approximately 10 % of the total transformants (mean of 10.5 % of the successful clones from each specimen), was picked from the three cloning reactions. Overnight cultures of the transformed cells were inoculated into 5 ml Luria–Bertani broth containing kanamycin (50 μg ml^{-1}) and incubated at 37 °C for 18 h. Plasmids were purified from each culture using a GenElute Plasmid mini-prep kit (Sigma-Aldrich), following the manufacturer's instructions.

The cloned 16S rRNA gene sequences from each plasmid preparation were amplified using M13 primers, as described by the manufacturer. Products were analysed by standard agarose gel electrophoresis and visualized under UV light. PCR products of approximately 1500 bp 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.

Identification of cloned isolates. Amplified cloned inserts were purified by precipitation and washing with ethanol. First, 15 μl 5 M NaCl and 15 μl 40 % polyethylene glycol (molecular mass 8000 Da) were added to each PCR reaction. This was centrifuged (16 000 ***g***, 15 min), and the supernatant aspirated and replaced with 200 μl 70 % ethanol. The centrifugation, aspiration and ethanol washing steps were repeated. Following another centrifugation step, the PCR products were air dried and resuspended in nuclease-free water (30 μl).

The 16S rDNA PCR products were partially sequenced using primer 357F (5′-CTCCTACGGGAGGCAGCAG-3′; Lane, 1991), ABI Prism BigDye terminator cycle sequencing ready reaction kits and an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems). This gave reliable sequences of at least 500 nt, which were compared to all GenBank DNA sequence entries using the FASTA sequence homology search program (http://www.ebi.ac.uk/services/index.html; Pearson, 1990). Whenever this sequence was insufficient to provide a conclusive identification, PCR products were further sequenced using primers 27F (5′-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CACGGATCCTACGGGTACCTTGTTACGACTT-3′) (Lane, 1991) to give a sequence of at least 1200 nt.

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 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 recognized nomenclature, the database 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 on the results of the indiscriminate GenBank search.

Possible chimeric sequences were detected 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).

The null hypothesis that the probability of each phylotype occurring is the same for both types of tissue was tested using two-sided Fisher's exact tests (Agresti, 1992).

RESULTS AND DISCUSSION

Surface decontamination of tissue specimens

Surface decontamination of the samples was achieved by immersion in Betadine and washing with PBS, a protocol similar to that used to decontaminate samples in reported work (Morita *et al.*, 2003). This was critical in order to eliminate any organisms occurring naturally on the surface of the tumours or there as a result of salivary or instrument contamination during surgery. The method was validated by PCR, and both aerobic and prolonged anaerobic culture of surface washings, which were found to be negative for the tumorous and non-tumorous control specimens from all ten patients.

FISH

OSCC tissue was successfully processed and stained with the fluorescent Alexa 594 conjugate. 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 Fig. 1. Bacteria were observed spread throughout the sections among the cells and fibres of the tissue, not just at the tissue border. The bacteria 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.

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. In future studies, it may be of interest to examine the stained sections by confocal laser

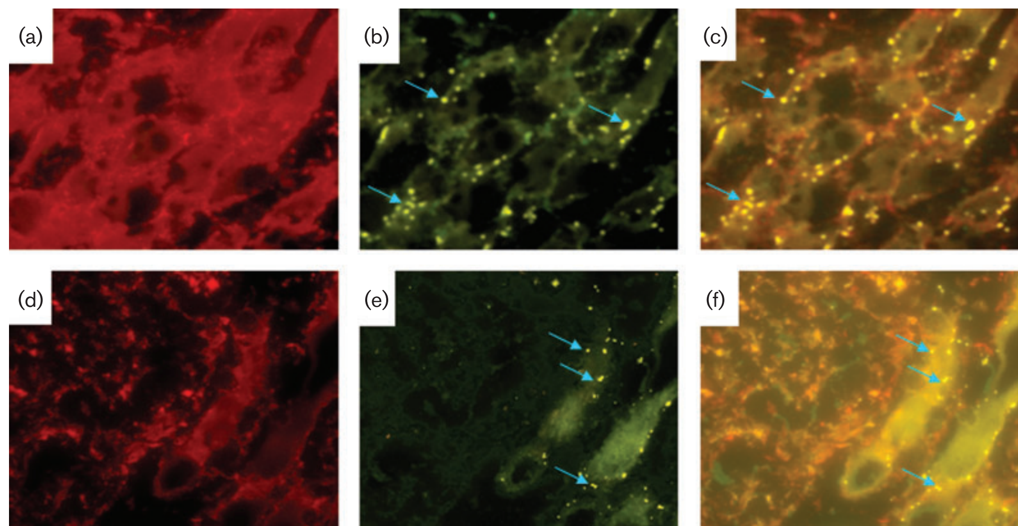

Fig. 1. Images from two different sections of OSCC tumour after staining with both the all-tissue counterstain Alexa Fluor 594 conjugate and the eubacterial probe EUB338–FITC. Section 1 is represented in (a–c) and section 2 in (d–f). Images are shown of the view through a red filter (Alexa Fluor 594) (a, d), the view through a green filter (EUB338–FITC) (b, e) and the composite view of both stains (c, f). Individual bacterial cells hybridized with EUB338–FITC could be observed using the green filter and fluoresced brightly throughout the tissue in all sections. Some examples of bacterial cells are indicated on the photomicrographs by arrows. Magnification ×600.

scanning microscopy, which may well provide significantly more information regarding the location of the bacteria (Thurnheer *et al.*, 2004) and allow us to quantify the bacteria present. However, it should be noted that biases in hybridization and from differing ribosome contents per cell are problems inherent to FISH that limit quantification (Moter & Göbel, 2000). Additionally, although it has been somewhat validated by its use in numerous studies, there is some evidence that the probe EUB338 will not hybridize to all bacteria in a sample with equal efficiency (Vaahtovuo *et al.*, 2005). Nevertheless, the distribution of bacteria in the sections suggested that these micro-organisms are found throughout the tumour tissue and do not represent contaminants present solely on the tissue surface.

Identification of bacteria by 16S rRNA gene cloning and sequencing

Species-specific PCR primers have been used in other studies to detect individual bacterial groups in tissue from OSCC patients (Morita *et al.*, 2003; Narikiyo *et al.*, 2004; Shiga *et al.*, 2001; Tateda *et al.*, 2000). However, to the best of our knowledge, this is the first time that PCR with non-specific, universal primers has been used to characterize 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. It is important to remember, however, that the technique relies on the assumption that the gene sequences of all bacteria present in the sample are complementary to the universal primers used. Recent discoveries of new taxa have indicated that several standard 'universal' 16S rRNA gene primers do not recognize all species of bacteria, and that current 16S rRNA gene libraries are not representative of true prokaryotic biodiversity (Baker *et al.*, 2003). However, the three primer sets chosen for this study have been well validated through their use in the characterization 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 (Aas *et al.*, 2005; Becker *et al.*, 2002; Kazor *et al.*, 2003; Paster *et al.*, 2001, 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 E90. 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. This was surprising given the previously reported presence of *Treponema denticola* in similar SCC tissues from oesophageal cancers (Narikiyo *et al.*, 2004). This apparent redundancy between primer sets meant that the results of the cloning were pooled and a single library of sequences constructed (Table 1).

A diversity of bacteria was detected from within both tumorous and non-tumorous tissue specimens. Of the 600 clones screened, a total of 526 16S rRNA sequences was

Table 1. Phylotypes isolated from tissue specimens by PCR cloning and sequencing of 16S rRNA genes

Phylotype	No. of tissue samples positive for species (n=10 patients)	
	Tumorous	Non-tumorous control
Proteobacteria		
Acinetobacter calcoaceticus LMG 1046^T; AJ633631	1	
Bradyrhizobium japonicum DSM 30131^T; X87272		1
Citrobacter koseri strain CDC 3613-63; AF025372	1	
Delftia acidovorans IFO 13582; AB020186	1	
Eikenella corrodens isolate 1664276; AF320620	1	
Escherichia coli ATCC 11775^T; X80725		1
Moraxella osloensis strain 170804JB8; AY730714	1	
Moraxella sp. isolate S12-08; AY880059	1	
Neisseria elongata ATCC 25295^T; L06171/AJ247252	1	1
Novel gamma-proteobacterium phylotype T12HS05; DQ093273*	2	
Ralstonia insidiosa strain AU2944^T; AF488779	4	
Ralstonia pickettii ATCC 27511^T; AY741342	1	
Ralstonia solanacearum GMI1000; AL646052	1	
Rhizobium giardinii strain H152^T; U86344		1
Schlegelella sp. KB1a; AY538706		1
Sphingomonas sp. PC5.28; X89909	3	8
Tepidimonas aquatica strain CLN-1^T; AY324139	1	
Thermus scotoductus strain Se-1^T; AF032127	1	
Uncultured alpha-proteobacterium (larval intestine clone D); AJ459874	1	2
Uncultured beta-proteobacterium HJ12; AY237409	1	
Actinobacteria		
Atopobium parvulum ATCC 33793^T; AF292372	6	6
Atopobium rimae ATCC 49626^T; AF292371	1	
Clavibacter michiganensis subsp. *tessellarius* ATCC 33566^T; U30254	3	
Curtobacterium flaccumfaciens LMG 3645^T; AJ312209	1	
Novel *Atopobium* phylotype T15FO04; DQ093271*	2	2
Olsenella uli ATCC 49627^T; AY005814	1	
Olsenella sp. isolate S13-10; AY880047	1	
Plantibacter flavus DSM 14012^T; AJ310417	1	
Propionibacterium acnes ATCC 6919^T; AB042288	3	2
Rhodococcus erythropolis DSM 43066^T; X79289	1	
Rothia mucilaginosa DSM 20746^T; X87758	1	
Firmicutes		
Bacillus circulans ATCC 4513^T; AY724690		1
Bacillus mycoides ATCC 6462^T/*Bacillus weihenstephanensis* DSM 11821^T; AB021192, AB021199†	1	
Bacillus thermoamylovorans strain R-19047; AJ586361		1
Bacillus silvestris strain SAFN-010; AY167818‡		1
Bacillus sp. R-7413; AY422985		1
Enterococcus faecalis JCM 5803^T; AB012212	1	
Finegoldia magna CCUG 17636^T; AF542227		1
Granulicatella adiacens ATCC 49175^T; D50540	5	9
Lachnospiraceae-like sp. isolate Adhufec020khh; AY471655	1	
Lactobacillus gasseri ATCC 33323^T; AF519171	1	
Megasphaera micronuciformis strain AIP 412.00^T; AF473834	1	
Paenibacillus sp. SAFN-016; AY167814		1
Peptostreptococcus micros ATCC 33270^T; AY323523	2	
Selenomonas sp. oral clone DY027; AF385492		1
Staphylococcus capitis subsp. *urealyticus* ATCC 49326^T/*Staphylococcus caprae* DSM 20608^T/*Staphylococcus epidermidis* ATCC 14990^T; AB009937/Y12593/D83363†	1	1
Streptococcus anginosus ATCC 33397^T; AF104678	6	6
Streptococcus constellatus ATCC 27823^T; AF104676	1	
Streptococcus cristatus ATCC 51100^T; AY188347		1

Table 1. cont.

Phylotype	No. of tissue samples positive for species (n=10 patients)	
	Tumorous	Non-tumorous control
Streptococcus gordonii ATCC 10558^T; AY485606		1
Streptococcus intermedius ATCC 27335^T; AF104671		1
Streptococcus mitis ATCC 49456/*Streptococcus oralis* ATCC 35037^T; AY485601/ AY485602†		3
Streptococcus parasanguinis ATCC 15912^T; AY485605	6	5
Streptococcus salivarius ATCC 7073^T; AY188352		1
Veillonella atypica ATCC 17744^T; AF439641	6	5
Veillonella dispar ATCC 17748^T; AF439639	2	1
Bacteroides		
Capnocytophaga sp. isolate S12-14; AY880056	1	
Capnocytophaga sp. oral strain S3; AY005073	5	3
Novel *Capnocytophaga* phylotype N17LB09; DQ093272*	1	2
Porphyromonas gingivalis ATCC 33277^T; AF414809		4
Prevotella intermedia ATCC 25611^T; X73965	1	
Prevotella melaninogenica ATCC 25845^T; AY323525	1	3
Prevotella nigrescens ATCC 33563^T; AF414833	3	2
Prevotella veroralis ATCC 33779^T; AY836507	2	1
Prevotella sp. oral clone BE073; AF385551	5	1
Uncultured eubacterium E1-K12; AJ289169		1
Fusobacteria		
Fusobacterium canifelinum RMA 1036^T/ *Fusobacterium nucleatum* ssp. *nucleatum* ATCC 25586^T; AY162221/AJ133496†	1	
Fusobacterium naviforme NCTC 11464; AJ006965	4	
Leptotrichia shahii strain LB 37^T; AY029806	1	
Novel *Leptotrichia* phylotype N16LA25; DQ093274*	3	5
Total	52	37

T, Type strain.
*Novel phylotypes detected in this study.
†These cloned phylotypes exhibited equivalent homology to the multiple type strains documented.
‡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 *Bacillus silvestris* and the corresponding phylotype isolated in this study both exhibited only an approximately 96 % match to the 16S rRNA gene sequences in the public databases from other *B. silvestris* strains, including the type strain (GenBank no. AJ006086).

determined, 277 from the tumorous specimens and 249 from the non-tumorous 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 were 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 determine whether these represented chimeric molecules and so they were not included in further analyses.

Of the sequenced phylotypes, all but four matched previous entries in GenBank that originated from other culture-independent studies. The four 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 chimeras. These four phylotypes included phylotype T15FO04 (98.4 % homology to the type strain of *Atopobium parvulum*; GenBank accession no. AF292372), phylotype N17LB09 (98.7 % sequence similarity to *Capnocytophaga gingivalis* strain LMG 12118; GenBank accession no. U41346), phylotype N16LA25 (98.7 % match to the type strain of *Leptotrichia wadei*; GenBank accession no. AY029802) and phylotype T12HS05 (95.1 % match to the *Xanthomonadaceae* 'Bacterium SG-3'; GenBank accession no. AF548381). The first three of these in particular were very close in sequence to known species but, as they were isolated here several times from several sources and the sequencing was reproducible in each case, they appeared to represent novel phylotypes and accordingly were submitted to GenBank (accession numbers DQ093271, DQ093272, DQ093274 and DQ093273, respectively).

Several unusual bacteria, not usually associated with human clinical samples, were detected in this study. In addition to the novel phylotypes, these included, from the

tumorous tissues, *Clavibacter michiganensis*, *Plantibacter flavus*, *Tepidimonas aquatica* and *Thermus scotoductus*, and from the non-tumorous 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. Each was only detected in relatively small amounts, namely single clones from single specimens, and none was also isolated by culture (Hooper *et al.*, 2006), suggesting that they may have been transients and were not present in the tissues as living cells. Alternatively, these unusual environmental bacteria, present in low quantities, may have been the result of contamination. DNA from environmental species of bacteria has 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 (Hughes *et al.*, 1994). Such problems of contaminants in commercial reagents are known, and standard sterile techniques were used to minimize further contamination and reduce the risk of 'false-positive' PCR products. It is interesting to note that Mohammadi *et al.* (2005) recently reported some success in eliminating contaminant DNA by filtering reagents through a DNA-isolating column prior to use. It may be worth incorporating this step into any future work in order to reduce contamination further and possibly to elucidate the origin of the suspect bacterial DNA.

Overall, 70 distinct taxa were detected (see Table 1). From the tumorous tissues, 52 phylotypes were identified, a mean of 10.3 taxa per sample. Slightly fewer phylotypes were isolated from the non-tumorous specimens, a total of 37 distinct taxa (a mean of 8.8 taxa per sample). The use of a paired-sample *t*-test indicated that this difference was not significant ($P=0.173$). Two-sided Fisher's exact tests for each of the phylotypes revealed no significant differences ($P>0.05$ for all taxa) between the numbers of positive tissue samples detected in the two types of specimen (Table 1). However, the lack of statistical significance is not surprising given the relatively low total numbers of specimens and the high number of taxa isolated. Nevertheless, several interesting trends were observed. For instance, when considering the relative numbers of samples positive for each bacterium, four taxa were isolated in $\geqslant 30\%$ more of the control specimens than in the tumorous specimens, namely *Granulicatella adiacens*, *Porphyromonas gingivalis*, *Sphingomonas* sp. PC5.28 and *Streptococcus mitis/oralis*. Similarly, *Clavibacter michiganensis* subsp. *tessellarius, Fusobacterium naviforme* and *Ralstonia insidiosa* were all detected in $\geqslant 30\%$ more of the tumorous than non-tumorous samples. These differences could be indicative of real changes in the microbiota present within the mucosal tissue of these patients.

The majority of species found within the tumorous tissues were saccharolytic and aciduric, reflecting, perhaps, the selective nature of the acidic and hypoxic microenvironment found within tumours (Raghunand *et al.*, 2003). For instance, *Proteobacteria*, and members of the genera *Fusobacterium*, *Streptococcus*, *Prevotella* and *Veillonella*, are all known to be capable of surviving at relatively low pH (Curtis *et al.*, 2002; Marchant *et al.*, 2001; Svensäter *et al.*, 1997; Takahashi, 2003). Indeed, most of the taxa isolated from the tumorous samples in this study have previously been detected in acidic dental caries lesions (Chhour *et al.*, 2005; Munson *et al.*, 2004). A similar trend was apparent when these tissue specimens were analysed previously by culture analysis (Hooper *et al.*, 2006). Many of the species detected by PCR cloning were also cultivated from OSCC tumour and control samples, indicating that the bacteria found to be present within the tissue in this study could well have been viable *in vivo*. The cultural analysis also found a slightly higher number of taxa in the tumorous tissues than in the non-tumorous specimens, a trend that was repeated in this study (a total of 52 separate phylotypes were isolated from the tumorous compared with the 37 taxa from the non-tumorous tissues). That different taxa were isolated by the culture and culture-independent approaches reflects the different specificities and species-dependent biases of the two methods, as has been reported previously (Davies *et al.*, 2004; Munson *et al.*, 2002; Wilson *et al.*, 1997). It is worth remembering that all of the steps involved in isolating bacterial taxa from a polymicrobial source by PCR cloning may have some biases, which in turn may skew the results (Spratt, 2004).

Of particular interest was the isolation of *Streptococcus anginosus* from six patients in this present study. This species has been detected in OSCC specimens in the past (Tateda *et al.*, 2000; Morita *et al.*, 2003), and has been implicated in the process of carcinogenesis due both to its association with tumours and to its ability to induce inflammation (Sasaki *et al.*, 2001; Sugano *et al.*, 2003).

The implications of the presence of a diversity of viable bacteria deep within the tissue of SCC are unclear. As most of the taxa detected here are known members of the oral microbiota, it would seem that the bacteria within the tumour and mucosal tissue originated from the oral cavity. This would support reported studies that have suggested that oral bacteria are capable of translocating from the mucosal surfaces of oral cancer patients into the regional lymph nodes, presumably via the cancerous lesion (Sakamoto *et al.*, 1999). However, it is also worth noting that, in animal models, bacteria present in the blood have been shown to seed preferentially to tumours (Yu *et al.*, 2004).

The apparent differences between the microbiota of the tumour and control tissues suggests a degree of bacterial specificity that merits further study. Furthermore, as evidence that bacteria are involved in the development of many different cancers increases, it is interesting to speculate that the species found in this study may play a role in the processes of carcinogenesis. Whether these

bacteria simply represent later secondary colonizers or whether bacterial–host interactions have significant effects on carcinogenesis are ideas worthy of further investigation.

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