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## Viable Bacteria Present within Oral Squamous Cell Carcinoma Tissue

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Despite increasing interest in the possible relationships between bacteria and the different stages of cancer development, the association of bacteria with cancer of the oral cavity has yet to be adequately examined. With that in mind, the primary objective of this study was to identify any bacterial species within oral squamous cell carcinoma tissue using a standard microbiological culture approach. At the time of surgery, a 1-cm<sup>3</sup> portion of tissue was harvested from deep within the tumor mass using a fresh blade for each cut. Whenever possible, “superficial” portions from the mucosa overlying the tumor and nontumorous control specimens from at least 5 cm away from the primary tumor site were also obtained. Surface contamination was eliminated by immersion in Betadine and washing with phosphate-buffered saline. Each specimen was aseptically macerated and cultured on nonselective media under both aerobic and anaerobic conditions. Isolates were identified by 16S rRNA gene sequencing. Twenty deep-tissue specimens, 19 with corresponding superficial tissues and 12 with control tissues, were successfully processed. A diversity of bacterial taxa were isolated and identified, including several putatively novel species. Most isolates were found to be saccharolytic and acid-tolerant species. Notably, some species were isolated only from either the tumorous or nontumorous tissue type, indicating a degree of restriction. Successful surface decontamination of the specimens indicates that the bacteria detected were from within the tissue. A diversity of bacterial groups have been isolated from within oral squamous cell carcinoma tissue. The significance of these bacteria within the tumor warrants further study.

Oral cancer is the sixth most common malignancy worldwide (32) and is particularly prevalent in developing countries, such as in Southeast Asia, where up to 40 percent of all malignancies are located within the oral cavity (25). More than 90% of cancers in the mouth are squamous cell carcinomas (SCCs) originating from the oral mucosa (5). With an average all-stage, 5-year survival rate for oral cancer of less than 50%, the annual mortality figures are comparable to those of carcinoma of the cervix and malignant melanoma (3, 40). There is evidence that the incidence of cancer of the oral cavity is increasing in many parts of the world, including the United Kingdom (8, 15, 16). In the past, oral cancer predominantly affected men in their sixth or seventh decade. However, more recently the male-to-female ratio has reduced dramatically and there has been a striking increase in the number of cases in those under the age of 45 (15, 38).

It has been reported that the majority of cases of head and neck cancer can be related to tobacco use and heavy alcohol consumption (10). Other possible risk factors include viral infections (29), poor oral hygiene (14), and infection with *Candida* species. The latter microorganisms are associated with some forms of leukoplakic lesions, the presence of which has long been recognized as an independent risk factor for carcinoma (4).

The involvement of other microorganisms, particularly bac-

teria, has not been studied to any great extent, although recently there has been increasing evidence to suggest that closer study of this issue may be warranted. A number of bacterial species have been associated with different cancers following either epidemiological or laboratory-based studies. For example, *Chlamydia trachomatis* infection has been associated with an increased risk for the development of invasive cervical carcinoma (37). Bacteremia and endocarditis due to *Streptococcus bovis* have likewise been linked with malignancies in the colon (7), and *Helicobacter pylori* infection has long since been considered a causative agent of both gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphomas (1, 35). Moreover, several mechanisms by which different bacteria may play a role in cancer development have been proposed, for instance, through the induction of chronic inflammation, by interference, either directly or indirectly, with eukaryotic cell cycle and signaling pathways (13), or via the metabolism of potentially carcinogenic substances. The latter mechanism is of relevance in the oral cavity, where the local microflora may promote carcinogenesis by converting ethanol into its carcinogenic derivative, acetaldehyde. Following the ingestion of alcohol, salivary bacteria have been shown to produce levels of acetaldehyde that can induce DNA damage, mutagenesis, and secondary hyperproliferation of the epithelium (23, 27). Interestingly, microbial acetaldehyde production is increased in heavy drinkers and smokers, offering a possible explanation for these risk factors (9).

The association of bacteria with oral tumors is of increasing interest. In a study of intraoral carcinomas, Nagy et al. (20) demonstrated a difference in the microflora associated with the

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surface of tumors in comparison to control sites. More recently it has also been reported that patients with oral SCC (OSCC) tend to possess significantly raised concentrations of certain bacteria in their saliva. This apparent alteration of the oral microflora in OSCC cases is of particular interest because of its potential application as a diagnostic tool to predict oral cancer (17). In order to demonstrate a role for bacteria in the development of oral cancer, the first step must be to identify such organisms within tumor specimens. Furthermore, sufficient attention must be given to the elimination from any tissues tested of the microbes that occur naturally on the surfaces of the tumors. In addition, salivary contamination of the sample must be prevented during subsequent handling. The presence of *Streptococcus anginosus* DNA in oropharyngeal tumors has been reported following studies using specific PCR primers (18, 31). However, this molecular approach was limited to a single group of bacteria, and no inferences can be made regarding the viability and therefore potential activity of the species detected.

The aim of this investigation was to determine whether viable bacteria are present within the tissue of oral squamous cell carcinoma. A prerequisite of this was the development of a robust method for the elimination of surface microbial contamination from specimens of tumor tissue.

#### MATERIALS AND METHODS

**Sample collection.** Tissue specimens were obtained from 16 male and 4 female OSCC patients with an average age of 66.9 years ( $\pm 12.7$ ). Ethical approval for the study was granted by the South Wales Local Ethics Research Committee, and subjects agreed to participate with their informed consent. Tumors 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<sup>3</sup> 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 tumor mass) and a "superficial" specimen (consisting of tissue from within and from the surface of the tumor). Whenever possible, a control specimen consisting of nontumorous tissue harvested at least 5 cm away from the primary tumor site was also obtained.

Specimens were aseptically transferred to the laboratory in separate vials of transport medium, used to maintain the viability of any bacterial cells present. Transport medium was comprised of tryptone (1% wt/vol; Oxoid), yeast extract (0.5% wt/vol; Oxoid), glucose (0.1% wt/vol; BDH Ltd.), cysteine hydrochloride (0.1% wt/vol; BDH Ltd.), sodium hydroxide (50 mM; Sigma), and horse serum (2% vol/vol; TCS Ltd.) and was sterilized by filtration using a 0.2- $\mu$ m filter.

All subsequent handling of the specimens was carried out using aseptic technique on surfaces cleaned with Hycolin phenolic disinfectant (2% vol/vol; Billastrand Labs Ltd.). Tissue specimens were placed in Betadine antiseptic solution (Seton Healthcare Group plc) for 3 min to disinfect the surface of each. Subsequently, tissues were vortexed in multiple 500- $\mu$ l aliquots of phosphate-buffered saline (PBS) to encourage the removal of any bacteria on the tissue surface. Final washes were retained and analyzed by both culture-dependent and culture-independent techniques to determine whether surface decontamination was successful.

Specimens were aseptically bisected. Half of each was placed in Tris-EDTA buffer (pH 7.4) and stored at  $-80^{\circ}\text{C}$  for molecular analysis. The remainder was subjected to immediate culture-dependent analysis.

**Bacterial culture.** Tissue specimens were aseptically macerated with disposable scalpels and vortexed for 30 seconds in PBS (500  $\mu$ l), and the neat suspensions were used to make 10-fold ( $10^{-1}$ ) and 100-fold ( $10^{-2}$ ) dilutions. Neat suspensions (50  $\mu$ l) were each spread onto blood agar (BA), fastidious anaerobe agar (FAA) (BA and FAA supplemented with 5% defibrinated sheep blood; TCS Biosciences Ltd.), and Sabouraud's agar (Lab MTM; International Diagnostics Group plc). The dilutions and final PBS washes of the specimens (see above) were spread onto BA and FAA. BA and Sabouraud's agar plates were incubated aerobically at  $37^{\circ}\text{C}$  for 48 h. FAA plates were incubated in an anaerobic cabinet at  $36^{\circ}\text{C}$  for 96 h.

Following incubation, all colony types on the plates were subcultured for purity, the cellular morphologies of Gram-stained smears were recorded, and isolates were stored at  $-80^{\circ}\text{C}$  using Microbank cryovials (Pro-Lab Diagnostics) prior to identification by 16S rRNA gene sequence analysis.

**Molecular identification of isolates.** Genomic DNA was extracted from suspensions of each of the bacterial isolates using a commercial kit (Puregene; Gentra Systems) according to the manufacturer's instructions (extraction protocol for 1 ml gram-positive bacteria; <http://www.gentra.com/pdf/01120.pdf>).

PCR amplification of 16S rRNA genes was performed in a reaction volume of 50  $\mu$ l, consisting of 0.2 mM each deoxynucleoside triphosphate, 2.0 mM MgCl<sub>2</sub>, 1.5 U *Taq* DNA polymerase, 5  $\mu$ l 10 $\times$  PCR buffer (all reagents from Promega), 5  $\mu$ l (between 50 fg and 50 ng) of extracted DNA as template, and 0.5  $\mu$ M each primer. The primer pair used were 27F (5'-GTGCTGCAGAGAGTTTGTATCC TGGCTCAG-3') and 1492R (5'-CACGGATCTACGGGTACCTTGTTACG ACTT-3'), specific for the domain *Bacteria* (6) (synthesized by Invitrogen). A touchdown protocol was used whereby in the first cycle, denaturation was performed at  $94^{\circ}\text{C}$  for 6 min, primer annealing was performed at  $65^{\circ}\text{C}$  for 1 min, and extension was performed at  $72^{\circ}\text{C}$  for 2 min. In subsequent cycles, denaturation was performed for 1 min and the annealing temperature was decreased by  $2^{\circ}\text{C}$  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. Contamination controls (tissue surface washes) were also subjected to PCR amplification; negative results indicated successful decontamination.

The 16S rRNA gene PCR products were partially sequenced using the 357F primer (5'-CTCCTACGGGAGGCAGCAG-3') (12), ABI Prism BigDye Terminator cycle sequencing ready reaction kits (Perkin-Elmer), and an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems). This gave reliable sequences of at least 500 nucleotides in length, which were compared to all GenBank DNA sequence entries using the FASTA sequence homology search program (<http://www.ebi.ac.uk/services/index.html>) (22).

Whenever this sequence was insufficient to provide a conclusive identification, PCR products were further sequenced using the 27F and 1492R primers to give a sequence of at least 1,200 nucleotides in length.

**Nucleotide sequence accession numbers.** Sequences AY880043 through AY880059 were deposited into GenBank.

#### RESULTS

The results of the PCR and both aerobic and prolonged anaerobic cultures of surface washings were routinely negative, indicating that the surface decontamination protocol used was successful. Bacteria were isolated from all specimens: 20 deep-tissue tumor specimens, 19 corresponding superficial specimens, and the nontumorous control tissues that were obtained from 12 of the 20 patients.

The species identities are summarized in Table 1. An average of 6 isolates was cultured from each specimen with a total of 90 distinct species or phylotypes. The isolates were mostly bacterial, but also included isolates of *Candida albicans* from three patients and a single isolate of a *Saccharomyces* species. A wide range of bacteria was seen, including a number of taxa with low 16S rRNA gene sequence homology to anything previously existing in the public databanks. Complete 16S rRNA gene sequences were obtained for these seemingly novel taxa and deposited with GenBank; nucleotide accession numbers are given in Table 1.

For all patients studied, the superficial tissues yielded the same isolates found in the corresponding deep-tissue specimens, with additional species in most cases. Comparison of the proportions of each sample type positive for each species revealed 17 organisms for which the difference in proportion between tumorous and nontumorous tissues was at least 10%, where "tumorous" tissues represented both the deep tumor and superficial samples combined (Table 2).

TABLE 1. A summary of the microorganisms cultured from tissue specimens, grouped according to phylum<sup>a</sup>

Species	No. of tissue specimens cultured, by source		
	Deep tumor (n = 20)	Tumor and overlying mucosa ("superficial") (n = 19)	Nontumorous control (n = 12)
<b>Proteobacteria</b>			
<i>Acinetobacter lwoffii</i>	1	1	
<i>Citrobacter koseri</i>	1	1	
<i>Eikenella corrodens</i>	1	1	
<i>Moraxella osloensis</i>			2
<i>Neisseria elongata</i>		1	
<i>Neisseria perflava/subflava</i>	1	1	
<i>Pseudomonas aeruginosa</i>	1	1	
<i>Pseudomonas monteilii</i>			1
<i>Serratia marcescens</i>	1	1	1
Unknown <i>Moraxella</i> -like sp. <sup>b</sup>		1	
<b>Actinobacteria</b>			
<i>Actinomyces graevenitzi</i>			1
<i>Actinomyces odontolyticus</i>		1	2
<i>Actinomyces viscosus</i>			1
<i>Atopobium parvulum</i>	1	2	3
<i>Atopobium rimae</i>			1
<i>Bifidobacterium longum</i>			1
<i>Bifidobacterium</i> sp. (oral strain H6-M4 phylotype) <sup>c</sup>		2	
<i>Brachybacterium rhamnorum</i>			1
<i>Corynebacterium singulare</i>	1		
<i>Corynebacterium striatum</i>		1	
<i>Corynebacterium tuberculostearicum</i>		3	1
<i>Dermabacter hominis</i>	1	1	
<i>Dietzia psychrhalcaliphila</i>		1	
<i>Gordonia sputi</i>		1	
<i>Micrococcus luteus</i>	5	6	
<i>Micrococcus lysae</i>	1	1	
<i>Olsenella uli</i>		1	
<i>Propionibacterium acnes</i>	6	7	6
<i>Propionibacterium avidum</i>	1	1	
<i>Propionibacterium granulosum</i>		1	
<i>Rothia amarae</i>		1	
<i>Rothia mucilaginosus</i>	1	1	2
<i>Scardovia</i> genosp. C1 <sup>d</sup>	1	1	
<i>Streptomyces aureus/griseorubiginosus</i>		1	
Unknown <i>Corynebacterium</i> -like sp. phylotype i <sup>e</sup>		1	
Unknown <i>Corynebacterium</i> -like sp. phylotype ii <sup>f</sup>	1	1	
Unknown <i>Georgenia</i> -like sp. <sup>g</sup>	1	1	
Unknown <i>Olsenella</i> -like sp. <sup>h</sup>		1	1
<b>Firmicutes</b>			
<i>Bacillus licheniformis</i>	1	1	
<i>Bacillus mycoides/weihenstephanensis</i>	1	1	
<i>Bacillus psychrodurans</i>	1	1	
<i>Dialister invisus</i>		1	
<i>Dialister pneumosintes</i>	1	1	
<i>Enterococcus faecalis</i>		1	
<i>Eubacterium yurii</i>		1	
<i>Exiguobacterium oxidotolerans</i>	2	2	
<i>Fingoldia magna</i>	1	2	1
<i>Gemella haemolysans</i>		2	
<i>Gemella morbillorum</i>	1	1	1
<i>Granulicatella adiacens</i>	3	5	3
<i>Lactobacillus casei/zeae</i>	1	1	
<i>Lactobacillus crispatus</i>	1	1	
<i>Lactobacillus fermentum</i>	2	2	1
<i>Lactobacillus gasseri</i>	3	3	1
<i>Lactobacillus rhamnosus</i>	1	1	1
<i>Lactobacillus salivarius</i>		1	
<i>Megasphaera micronuciformis</i>		1	1
<i>Mogibacterium neglectum</i>	1	1	
<i>Peptostreptococcus micros</i>	4	4	2
<i>Selenomonas diana</i>		1	

Continued on following page

TABLE 1—Continued

Species	No. of tissue specimens cultured, by source		
	Deep tumor (n = 20)	Tumor and overlying mucosa ("superficial") (n = 19)	Nontumorous control (n = 12)
<i>Selenomonas sputigena</i>		1	
<i>Solobacterium moorei</i>			1
<i>Staphylococcus aureus</i>	2	2	
<i>Staphylococcus capitis/caprae/epidermidis</i>	2	5	2
<i>Staphylococcus cohnii</i>	1	1	
<i>Staphylococcus hominis</i>	1	1	
<i>Staphylococcus saprophyticus</i>	1	1	
<i>Staphylococcus warneri</i>	2	3	1
<i>Streptococcus agalactiae</i>	1	1	
<i>Streptococcus anginosus</i>	1	4	1
<i>Streptococcus constellatus</i>	2	4	2
<i>Streptococcus cristatus</i>	1	1	
<i>Streptococcus gordonii</i>	1	1	
<i>Streptococcus mitis/oralis</i>	2	5	5
<i>Streptococcus parasanguinis</i>	3	6	5
<i>Streptococcus salivarius</i>	2	3	3
<i>Streptococcus</i> sp. (oral strain T4-E3 phylotype) <sup>j</sup>		1	
Unknown gram-positive anaerobic coccoid species <sup>f</sup>			1
<i>Veillonella atypica</i>		1	1
<i>Veillonella dispar</i>	1	1	3
<i>Veillonella parvula</i>	2	2	
<b>Bacteroidetes</b>			
<i>Prevotella intermedia</i>		1	
<i>Prevotella melaninogenica</i>	2	3	
<i>Prevotella veroralis</i>			2
<i>Prevotella</i> sp. (oral clone BE073 phylotype) <sup>k</sup>	1	1	2
Unknown <i>Capnocytophaga</i> -like sp. <sup>l</sup>		1	
<b>Fusobacteria</b>			
<i>Fusobacterium naviforme</i>	1	3	
<i>Fusobacterium nucleatum</i> subsp. <i>nucleatum</i>	1	2	1
<b>Eukaryotes</b>			
<i>Candida albicans</i>	2	3	1
<i>Saccharomyces</i> sp.			1

<sup>a</sup> Multiple names are given wherever partial 16S sequences from isolates exhibited a significant (>99%) match to more than one species. Where the sequence was novel or homologous only to a phylotype from an unrecognized species, the accession numbers of the appropriate GenBank entries are given below.

<sup>b</sup> Novel *Moraxella*-like sp. isolate S12-08; accession no. AY880059.

<sup>c</sup> Unknown *Bifidobacterium* sp.; accession no. AY880048 and AY880049. Also homologous to oral strain H6-M4 phylotype (accession no. AF385524) and the proposed species "*Bifidobacterium urinalis*," which does not currently have standing in nomenclature (strains CCUG 26938 and CCUG 344441, accession no. AJ278694 and AJ278695, respectively).

<sup>d</sup> *Scardovia*-like sp. isolate; accession no. AY880045. Sequence match to *Scardovia* genomospecies C1 (accession no. AY278626).

<sup>e</sup> Novel *Corynebacterium*-like sp. isolate S18-03; accession no. AY880057.

<sup>f</sup> Novel *Corynebacterium*-like sp. isolate T13-01; accession no. AY880058.

<sup>g</sup> Novel *Georgenia*-like sp. isolate T04-04; accession no. AY880044.

<sup>h</sup> Novel *Olsenella*-like sp. isolates S13-10 and N13-17; accession no. AY880047 and AY880046.

<sup>i</sup> Unknown *Streptococcus* sp. isolates S16-08 and S16-11; accession no. AY880050 and AY880051. Sequence match to oral strain T4-E3 phylotype (accession no. AF385526).

<sup>j</sup> Novel bacterium isolate N14-24; accession no. AY880043.

<sup>k</sup> Unknown *Prevotella* sp. isolates N12-20, N19-22, N19-31, T05-04; accession no. AY880052 to AY880055, respectively. Sequence match to oral clone BE073 phylotype (accession no. AF385551).

<sup>l</sup> Novel *Capnocytophaga*-like sp. isolate S12-14; accession no. AY880056.

## DISCUSSION

To our knowledge this is the first time that viable bacteria have been detected within the tissue of oral squamous cell carcinoma. Immersion in Betadine was required to eliminate any viable bacteria on the surface that may have been present due to salivary or instrument contamination during surgery.

A diversity of species were isolated. However, it is important to remember that this is not a comprehensive analysis of the microflora; it has been well established that approximately

50% of oral bacteria are not cultivable by standard culture techniques as used in this study (36). Nevertheless, cultivation of bacteria remains a useful diagnostic tool for the detection and identification of viable organisms, including many known pathogenic species.

The majority of the taxa detected have previously been isolated from the oral cavity. However, there were a small number of exceptions. For example, some species previously only reported as isolates from environmental sources were detected, including *Dietzia psychrhalcaliphila* and *Gordonia sputi*.

TABLE 2. The relative proportions of the different tissue sample types that were positive for certain species of bacteria

Species	Proportion of tissue samples positive for species (%) <sup>a</sup>				
	Deep tumor	Superficial	Tumorous	Nontumorous control	Difference
<i>Micrococcus luteus</i>	25.0	31.6	28.2	0.0	28.2
<i>Prevotella melaninogenica</i>	10.0	15.8	12.8	0.0	12.8
<i>Exiguobacterium oxidotolerans</i>	10.0	10.5	10.3	0.0	10.3
<i>Fusobacterium naviforme</i>	5.0	15.8	10.3	0.0	10.3
<i>Staphylococcus aureus</i>	10.0	10.5	10.3	0.0	10.3
<i>Veillonella parvula</i>	10.0	10.5	10.3	0.0	10.3
<i>Prevotella</i> sp. (oral clone BE073 phylotype)	5.0	5.3	5.1	16.7	-11.5
<i>Rothia mucilaginosa</i>	5.0	5.3	5.1	16.7	-11.5
<i>Streptococcus salivarius</i>	10.0	15.8	12.8	25.0	-12.2
<i>Actinomyces odontolyticus</i>	0.0	5.3	2.6	16.7	-14.1
<i>Moraxella osloensis</i>	0.0	0.0	0.0	16.7	-16.7
<i>Prevotella veroralis</i>	0.0	0.0	0.0	16.7	-16.7
<i>Propionibacterium acnes</i>	30.0	36.8	33.3	50.0	-16.7
<i>Atopobium parvulum</i>	5.0	10.5	7.7	25.0	-17.3
<i>Streptococcus parasanguinis</i>	15.0	31.6	23.1	41.7	-18.6
<i>Veillonella dispar</i>	5.0	5.3	5.1	25.0	-19.9
<i>Streptococcus mitis/oralis</i>	10.0	26.3	17.9	41.7	-23.7

<sup>a</sup> "Tumorous" represents the combined proportions of both superficial and deep samples. The difference in detected proportions between tumorous and nontumorous control tissues is shown. Only the species for which there was at least a 10% difference in proportions are listed.

A number of isolates were detected which, although not typically regarded as common members of the oral microflora, have been isolated from the oral cavity previously and/or are known to be human pathogens, for example, those belonging to the genera *Micrococcus*, *Propionibacterium*, *Streptomyces*, *Bacillus*, *Enterococcus*, *Exiguobacterium*, *Staphylococcus*, and *Pseudomonas*.

As the successful surface decontamination indicates that the bacteria isolated from the samples were not carried over from other sources, such as saliva, it is interesting to note that so many species, including ones not commonly regarded as pathogenic, were present in the tumor tissue. This may support the emerging findings that many different bacterial species are capable of infecting oral tissue and invading epithelial cells. For instance, it is of some interest to note that recent reports find streptococci to be a major component of this epithelial intracellular microflora (26), as several members of this genus were isolated from within both tumorous and nontumorous samples. The detection of *Streptococcus anginosus* in these tissues is also particularly noteworthy because it supports the findings from previous studies in which *S. anginosus* DNA was detected in head and neck carcinoma by PCR (18, 28, 31). Additionally, the presence of *Streptococcus mitis*/*Streptococcus oralis* in both the nontumorous and tumorous samples is consistent with the previously reported detection of these microorganisms in esophageal carcinoma and control tissues by molecular means (21).

The great diversity of species isolated together with the relatively low number of patients and specimens used in this study make it difficult to apply statistical analyses to draw conclusions regarding bacterial specificity. However, a number of interesting trends are apparent from the results. In all cases, the superficial tissues yielded exactly the same isolates as the corresponding deep-tissue specimens plus, in most cases, additional species. This may indicate a degree of restriction of bacteria in the deeper tumor 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 tumorous tissue.

Several species detected in the nontumorous control tissue were not detected in the tumor tissues, and vice versa. For instance, *Exiguobacterium oxidotolerans*, *Prevotella melaninogenica*, *Staphylococcus aureus*, *Veillonella parvula*, and species of *Bacteria* and *Micrococcus* were isolated only from tumorous specimens and not at all from nontumorous ones. Conversely, *Moraxella osloensis*, *Prevotella veroralis*, and species of *Actinomyces* were grown only from nontumorous tissues. This could indicate that, while bacteria are present within all the oral mucosal tissues, there are potentially significant differences between the microfloras within tumorous compared to nontumorous mucosae. Although we were unable to apply full hypothesis-testing statistics to support this finding, the observed alteration in microflora composition may perhaps be similar to the reported differences in the proportions of salivary bacteria that can theoretically be used as a predictor for OSCC (17). The simple descriptive parameters used in this study suggest six candidate species, isolated from over 10% more of the amalgamated tumorous than nontumorous samples, that may prove to be significantly associated with OSCC tissue (Table 2). Similarly, there were 11 taxa that were in proportionally at least 10% more of the control samples than the tumorous tissues, which may represent the species that are most likely not to be associated with OSCC.

It is interesting to note that the majority of species isolated were saccharolytic and acid tolerant. For instance, yeasts, actinomycetes, bifidobacteria, lactobacilli, streptococci, and veillonellae, all of which were detected in this study, are known to produce short-chain organic acids from carbohydrates and consequently to lower the pH of their local environment (2, 30, 33). Asaccharolytic or weakly fermentative species isolated from these specimens, such as *Fusobacterium* and *Prevotella* species, have also been shown to be capable of survival at relatively low pHs (34). The microenvironment of solid tumors is typically hypoxic, with an acidic extracellular pH (24), and so

it is not surprising that there might be a degree of selectivity in favor of acid tolerance. To what degree the presence of acid-producing bacteria within tumors contributes to the acidic microenvironment is as yet unknown.

Based on the 16S rRNA gene sequence data, several of the cultivated isolates appear to represent species either not previously characterized or without standing in the current nomenclature. There were 7 different phylotypes (from a total of 10 isolates) that failed to demonstrate significant homology (<99% sequence match) to any GenBank entry. Given the current wide-ranging nature of the sequence databases for known bacteria, it is possible that these represent "novel" species. Interestingly, an additional nine isolates showed significant sequence matches to existing sequences representing taxa that are unnamed and have seemingly not been cultured or characterized before. 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 (11). One tumor tissue specimen yielded an isolate matching "*Scardovia* genomospecies C1," a taxon recently detected in the microflora of dental caries (19). 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 (11). Isolates were cultivated from two superficial tissues that sequence-matched "*Bifidobacterium* sp. oral strain H6-M4," another taxon cloned from the tongue dorsa microflora (11). 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. It will be of great interest to characterize these isolates further.

The implications of the presence of a diversity of viable bacteria deep within the tissue of squamous cell carcinoma are unclear. Although it has been shown in animal models that bacteria injected intravenously may seed to tumor tissue (39), 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 tend to imply a local origin for the bacteria detected within the tumor. The apparent differences between the microflora of the tumor and control tissues suggest a degree of bacterial specificity that merits further study. For instance, it would be useful in future studies to include an assessment of microbial load to determine the numerical predominance of selected species. As evidence that bacteria are involved in the development of many different cancers increases, it is interesting to speculate that the species isolated from the tumor tissue may play a role in the carcinogenic process, a concept worthy of further investigation.

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