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Nitrate-Reducing Bacteria on Rat Tongues

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Nitrite-producing bacteria (NPB) were isolated from tongues of laboratory rats. The most commonly found nitrite-producing organism was *Staphylococcus sciuri*, followed by *Staphylococcus intermedius*, *Pasteurella* spp., and finally *Streptococcus* spp. Both morphometric quantification of bacteria on tongue sections and enumeration of culturable bacteria (CFU) showed an increase in the density of bacteria towards the posterior tongue. Up to 65% of bacteria were located in the deep clefts on the posterior tongue. The proportion of culturable NPB in the total culturable microbial population increased from 6% (10^5 CFU cm^{-2}) on the anterior tongue to 65% (10^7 CFU cm^{-2}) on the posterior tongue. Different species compositions of NPB were found on different tongue sections with *S. intermedius* populations decreasing and *S. sciuri* and *Pasteurella* populations increasing towards the posterior tongue. Nitrite production was sensitive to oxygen, and significant nitrite production was only detected on the posterior tongue where the majority of bacteria are situated in deep clefts in the tongue surface. This study suggests the importance of bacteria in nitrite production, from nitrate, on the tongue. Nitrite produced on the tongue may subsequently form nitric oxide in the acidic environment of the stomach. Because of the antimicrobial properties of nitric oxide, a key role for nitrate-reducing tongue bacteria in host animal defense against food-borne pathogens is proposed.

Human nitrate and nitrite intake and metabolism have received considerable interest because nitrite formation in saliva is suspected to be involved in *N*-nitrosamine carcinogenesis in the acid environment of the stomach (18, 20, 22, 23). Dietary nitrate mainly originates from vegetables (24) and is actively concentrated by the salivary glands in most mammals. As a result, salivary nitrate concentrations are approximately 10 to 20 times those found in plasma (9, 10). Saliva collected directly from salivary ducts contains nitrate but no nitrite. However, saliva collected at other locations in the oral cavity also contains nitrite (9, 16, 20).

A number of investigations have indicated that the oral microflora play a major role in nitrate reduction (5, 10, 20). It has been demonstrated that the nitrite concentration increases after incubation of saliva at 37°C but not if the saliva is filter sterilized (9). Several nitrite-producing organisms in human saliva have been identified and include *Veillonella* spp., *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Nocardia* spp., a *Corynebacterium* sp. (probably *C. pseudodiphtheriticum*), and an anaerobic, filamentous organism tentatively identified as *Fusobacterium nucleatum* (20, 21).

By far the greatest nitrate reduction activity in the human oral cavity was demonstrated on the dorsum linguae (15). Studies with rats have shown that nitrate reduction occurs mainly on the posterior surface of the tongue (3) and could in principle be due to one or a combination of mechanisms (15). It could be caused by the action of (i) reducing substances in salivary secretions, (ii) reducing bacterial metabolites, (iii) mammalian nitrate reductase in the papilla linguae, and/or (iv) nitrate reductase enzymes of the microorganisms colonizing the tongue (15). Recent results have shown that nitrate reduction is virtually absent in germ-free rats and that nitrite production on the tongue of adult humans is greatly reduced after administration of broad-spectrum antibacterial agents (2, 3). These findings clearly indicate microbial nitrate reduction as the primary mechanism. However, no systematic studies on the population dynamics and the relative importance of individual species of nitrate-reducing tongue bacteria have so far been carried out because the ecological significance of salivary nitrate and its reduction to nitrite by oral bacteria was, until recently, unknown.

It has now become clear that salivary nitrite can greatly augment the antimicrobial activity of gastric acid against swallowed pathogenic microorganisms (1). Once swallowed, the acidic condition of the stomach protonates nitrite to form nitrous acid, which in turn dissociates to form oxides of nitrogen with strong antimicrobial activity (3). We have therefore postulated a novel host resistance mechanism which relies on a symbiotic relationship between mammals and nitrate-reducing bacteria which live mainly on the tongue (3).

In this study our aims were to (i) map and identify the nitrite-producing microorganisms on the tongue surface and (ii) compare nitrate reduction velocities on different tongue sections with the populations of nitrite-producing bacteria (NPB) and tongue morphology. Rats were chosen as a model system because their tongues can be divided into two morphologically different zones, the anterior rat tongue which has a smooth surface and the posterior tongue which has deep clefts which are filled with bacterial biofilms (3).

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MATERIALS AND METHODS

Enumeration of bacteria by morphometric quantification. To visualize the microbial population of the dorsal surface of the tongue, adult male Sprague-Dawley rats ($n = 5$) were anaesthetized in 100% CO_2 and killed by cervical dislocation. Tongues were excised aseptically as far as the larynx and cut into five coronal sections of equal thickness. Coronal slices (2 mm thick) were cut from each section and placed in 10% neutral-buffered formalin (4% formaldehyde) at room temperature for 24 h, dehydrated and processed by standard means to glycol methacrylate resin (JB4; Polysciences, Warrington, Pa.), sectioned at 2 μm, and stained for microorganisms by an adaptation of the Gram method (19). Sections were then examined with an Olympus CH2 optical microscope (Olympus Optical Co. Ltd., London, United Kingdom). In this study we classified the anterior three sections as "anterior" and the posterior two sections as "posterior" and pooled the results from the grouped sections. The microorganisms present were counted by superimposing an unbiased counting frame (7) on the histological section by using an Olympus drawing arm and the stage micrometer to enable morphometric quantification of bacterial cells.

The actual epithelial surface was determined for microscope slides of the anterior and posterior tongue with a Leica Q500MC imaging package (Leica Ltd., Milton Keynes, United Kingdom). Average cleft dimensions on the posterior two tongue sections were determined by using a calibrated graticule, and the density of bacteria was determined for every fifth cleft as described above at depths of 0, 10 to 30, 57 to 77, and 133 to 153 μm (Fig. 1).

Microbial growth media used. The following media were used to isolate and enumerate microorganisms on rat tongues: nutrient broth (NB) (Oxoid CM1; Oxoid Ltd., Basingstoke, United Kingdom), nutrient broth with 10 g of KNO_3 per liter ($NBNO_3$) (BDH, Poole, United Kingdom), Columbia blood agar base (Oxoid CM331) with 5% laked horse blood (Oxoid SR48) (BA), Columbia blood agar base with 5% laked horse blood and *Streptococcus* selective supplement (Oxoid SR126E) (BAS), Columbia blood agar base with 5% laked horse blood and *Streptococcus*/*Staphylococcus* selective supplement (Oxoid SR 070E) (BASS), Columbia blood agar base with *Pseudomonas* selective supplement (Oxoid SR 103 E) (BAP), De Man, Rogosa & Sharp's agar (MRS) (Oxoid CM 3361), MacConkey agar (MC) (Oxoid CM115), Sabouraud dextrose agar (SDA) (Oxoid CM 41), nutrient agar (NA) (Oxoid CM3), and Schaedler anaerobe agar (AA) (Oxoid CM437).

Enumeration of bacterial and fungal CFU. Adult male Sprague-Dawley rats ($n = 6$) were killed as described above, and tongues were excised aseptically as far as the larynx and cut into five coronal slices of equal thickness. The macroscopic surface areas of the tongue sections were measured prior to homogenization of tongue sections in sterile 1/4-strength Ringer's solution (1/4 RS). The total number of bacteria and the number of NPB were determined for each section by (i) a most probable number (MPN) method (14, 17) and (ii) plating of diluted tongue homogenates onto general and semiselective agar-solidified microbial growth media.

For the MPN determinations, the tongue homogenate of each section was serially diluted in $NBNO_3$ in 10-ml test tubes with screw-top lids. Each homogenate was serially 10-fold diluted five times. Durham tubes were placed into test tubes to allow an assessment of gas production in the media. Test tubes were sealed with airtight screw tops, incubated for 72 h at 35°C, and then assessed for microbial growth, nitrite, and gas production in the different dilution steps. After serial dilution, tubes contained 9 ml of medium, leaving only 1 ml of headspace gas. This results in a rapid reduction in oxygen tension within the tube due to microbial respiration (17). Nitrite production was detected by NO_2-Merckoquant strips (detection limit, 5 ppm nitrite) (Merck, Darmstadt, Germany), and the absence of gas collecting in the Durham tubes was used as an indication that the tongue bacteria produce very little N_2O and N_2 (the gaseous end products of denitrification) from nitrate. The number of positive tubes in each dilution step was recorded and the MPNs were obtained by reference to Cochran's statistical table (14, 17).

For enumeration by plating, homogenates of each tongue section were serially diluted in 1/4 RS, and 10-μl aliquots of different dilution steps were transferred in triplicate onto the range of general and semiselective media described above. BAS, MRS, and AA plates were inoculated in duplicate. One plate was incubated aerobically and the other was incubated anaerobically by using an anaerobic jar (AMSTA, Stockholm, Sweden) and a gas generating kit (Oxoid Ltd.). The number of CFU appearing on aerobic incubating plates was counted after 16 and 28 h of incubation at 35°C. The number of CFU appearing on anaerobic incubating plates was counted after 28 h of incubation at 35°C.

Isolation and identification of denitrifying bacteria. Twenty-five well-separated colonies were selected from each type of general agar-solidified isolation medium that was used for enumeration of bacterial density by plating and subcultured onto BA plates. All isolates were tested for growth, gas, and nitrite production in $NBNO_3$ by the method described for MPN determination. One hundred forty strains of nitrite-producing organisms were then identified by conventional bacteriological methods (Gram stain, catalase, coagulase, motility, oxidase, shape, and heat resistance) and API test strips according to an identification scheme described previously (12).

Measurement of nitrite production on intact tongue sections. Tongues from an additional 10 rats from the same batch were cut into five coronal sections of equal thickness. From each section two circular plugs were removed with a number one cork borer (0.4-cm diameter) and quartered into four segments. For five tongues all manipulations were carried out under ambient oxygen conditions in a class 2 Envair flow cabinet (tongues were exposed for 30 min to ambient oxygen concentrations). The other five tongues were handled in an oxygen-free atmosphere in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, United Kingdom). Individual sections were placed into microwell plates containing 250 μl of 1/4 RS with potassium nitrate (KNO_3) to produce final concentrations of 100, 200, 400, 800, 1,000, 2,000, 10,000, and 20,000 μM NO_3^- and then incubated for 60 min at 37°C. For sections four and five, nitrite production was also measured after 15, 30, and 45 min to establish a time course of nitrite production. The nitrite concentration in the medium was then assayed colorimetrically by a microplate-based method (3), and data for the different nitrate substrate concentrations were used to calculate the K_m and the V_{max} of the nitrate reductase activity on tongue sections with different morphology (13, 14) (only the linear phase of nitrate reduction was used for K_m and V_{max} determinations).

Statistical analysis. Direct counting of bacterial cells was carried out double blind and analyzed by analysis of variance with repeated measures by using the Statview statistical software package (Abacus Concepts, Inc., California).

All other statistical analyses were carried out using the Minitab software

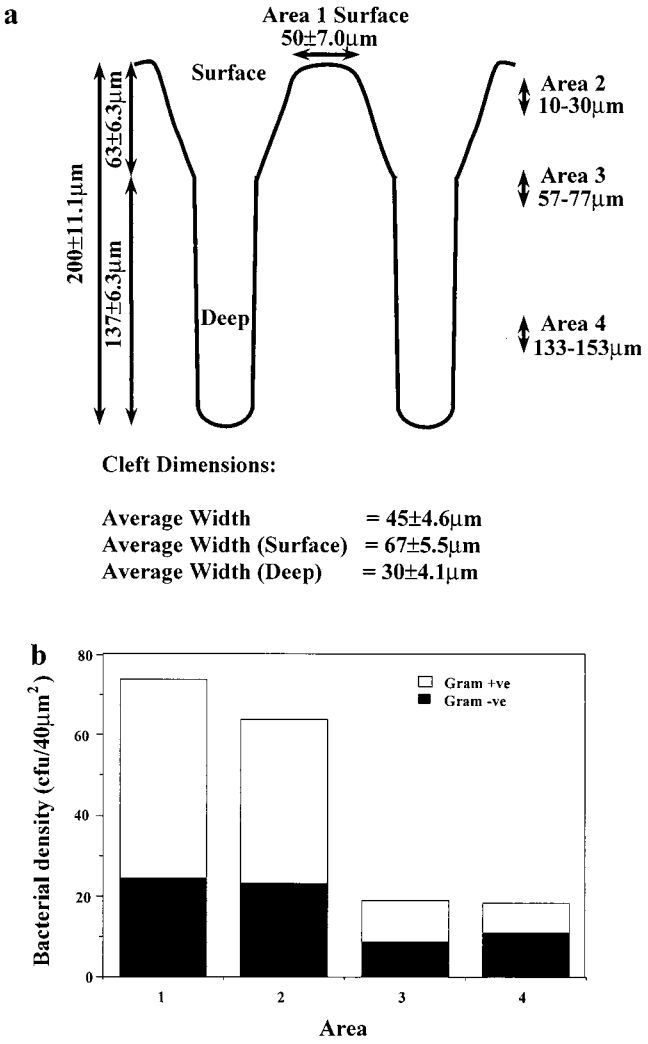

FIG. 1. (a) Diagram of the dimensions of tongue clefts found on the posterior third of the rat tongue (the anterior two thirds had a much smoother surface). Areas 1 to 4 represent defined depths where a direct count of the bacterial populations was made. (b) Bacterial density (cells/40 μm^2) found at each of the areas measured (panel a). Solid areas of the bars represent gram-negative bacteria and open areas of the bars represent gram-positive bacteria. Analysis of variance of gram-positive and -negative populations formed at different areas showed a significant difference between areas ($P < 0.001$) and a significant interaction between bacterial group (gram positive or negative) and area. Values are expressed as means of five determinations.

TABLE 1. Bacterial populations (log 10 CFU/cm^2) on tongues of laboratory rats as determined by direct microscopic counts, dilution and plating onto general and semiselective growth media, and MPN methods

Microbial group	Enumeration method[a]	Bacterial population on tongue section (log 10 CFU/cm^2)[b]				
		1	2	3	4	5
Gram positive	DC	7.4ab	6.8a	7.7b	8.5c	8.4c
Gram negative	DC	7.2ab	6.5a	7.4b	8.4c	8.1bc
Total bacteria						
Total	DC	7.6ab	7.0a	7.9b	8.8c	8.4bc
NA	PC	6.5a	6.9a	6.9a	7.2b	7.5b
BA	PC	6.4a	6.8a	6.8a	7.3ab	7.5b
BA		6.3a	6.8ab	7.1b	7.5bc	7.7c
BAS	PC	6.4a	6.7a	6.6a	7.3b	7.6b
BASS	PC	6.6a	6.8a	6.8a	7.3ab	7.6b
AA	PC	6.5a	6.7a	6.7a	7.4b	7.6b
$NBNO_3$	MPN	6.6a	6.6a	7.0a	7.7b	7.8b
% Culturable[c]	MPN	2%	8%	3%	2%	5%
NPB[d]						
$NBNO_3$	MPN	5.4a	5.6a	6.3b	6.8b	7.6c
% NPB		7%	9%	20%	12%	63%

[a] DC, direct microscopic counts; PC, dilution and plating onto agar-solidified general and semiselective media.

[b] Analysis of variance generally showed a significant difference between tongue sections ($P < 0.001$). Values followed by the same letter are not significantly different according to least significant difference ($P < 0.05$).

[c] Percentage of total number of bacteria counted microscopically.

[d] Values are proportionate to the total number of culturable bacteria determined by MPN method.

(Minitab Ltd.). Data from the MPN determinations and those from colony counts on agar-solidified isolation media (BA, BAS, BASS, AA, and NA) were log transformed and compared for different tongue sections by analysis of variance and by calculating the least significant difference. Colony counts for AA, BAS, and BASS were also analyzed for differences between aerobic and anaerobic incubation. Regression analyses were carried out for the proportions of *Staphylococcus sciuri*, *Staphylococcus intermedius*, *Streptococcus* spp. and gram-negative species in the total population of NPB on different tongue sections. Regression analysis was also carried out between the calculated maximum reaction velocity of nitrite production and the density of culturable NPB on different tongue sections.

RESULTS

Morphometric measurement of surface area, cleft dimensions, and bacterial number. The epithelial surface area of a macroscopic square centimeter on the anterior tongue was found to be approximately 1.6 cm^2, having a relatively smooth surface. The posterior tongue, however, had a surface with regular clefts of 200 μm in depth and 45 μm in width (Fig. 1), giving a macroscopic square centimeter of tongue of approximately 3.3 cm^2. Microbial densities are expressed on a macroscopic surface area basis throughout. Significantly more bacterial cells ($P < 0.002$) were detected on 2-μm Gram-stained sections from the back of the tongue than were found near its tip (posterior, 4.4×10^8 cells cm^{-2}; anterior, 4.2×10^7 cells cm^{-2}). Populations consisted nearly entirely of gram-positive cocci and gram-negative rods at a ratio of 1.6:1 on the anterior tongue and 1.3:1 on the posterior. On the posterior tongue 58% of bacteria were located in clefts within the tongue surface and 21% were in the deep narrow part (Fig. 1). There was a significantly greater proportion of gram-negative rods towards the base of the clefts.

Enumeration of culturable bacteria (CFU). There was also a greater-than-30-fold increase in the density of culturable bacteria between the anterior and posterior end of the tongue ($P < 0.05$), but there were no significant differences among the numbers found on different isolation media (NA, BA, BASS, BAS, and AA) on the same section (Table 1). There were no significant differences in culturable bacterial numbers between aerobically and anaerobically incubated isolation media (AA, BAS, and BASS). Numbers of CFU on SDA (used to estimate the numbers of filamentous fungi and yeasts on the tongue) remained at or below the detection limit of the dilution method used (5×10^1 CFU cm^{-2}). No colonies developed on MRS (selective for *Lactobacillus* spp.), MCA (selective for coliform bacteria), and BAP (selective for *Pseudomonas* spp.). The proportion of culturable bacterial cells to cells counted microscopically in thin sections of tongue was 4% (± 1.2) (Table 1).

Nitrate and nitrite reduction from intact tongue sections. Nitrite production and nitrite metabolism were virtually absent (below 7.8 nmol cm^{-2} h^{-1}) from the anterior three sections of the tongue and increased very little with increasing nitrate or nitrite substrate concentrations. On the two posterior tongue sections, nitrite production increased with increasing nitrate substrate levels to between 400 and 800 nmol cm^{-2} h^{-1} at the highest (10 and 20 mM) nitrate concentrations (Fig. 2). When nitrite production was measured in 15-min intervals after transfer of tongue sections into nitrate solutions, a clear lag phase of approximately 20 min was observed with all nitrate concentrations when tongues had been exposed to ambient oxygen. In tongue sections handled under anaerobic conditions, only a short 5- to 10-min lag phase was observed (Fig. 3). The kinetics of nitrate reduction (K_m, 830 to 1,100 μM NO_3^-; V_{max}, 600 to 1,000 nmol cm^{-2} h^{-1} NO_2^-) after the initial lag phase were not significantly different in tongues exposed to oxygen and tongues kept under anaerobic conditions (Fig. 2 and 3).

Nitrite metabolism was detected and increased with increas-

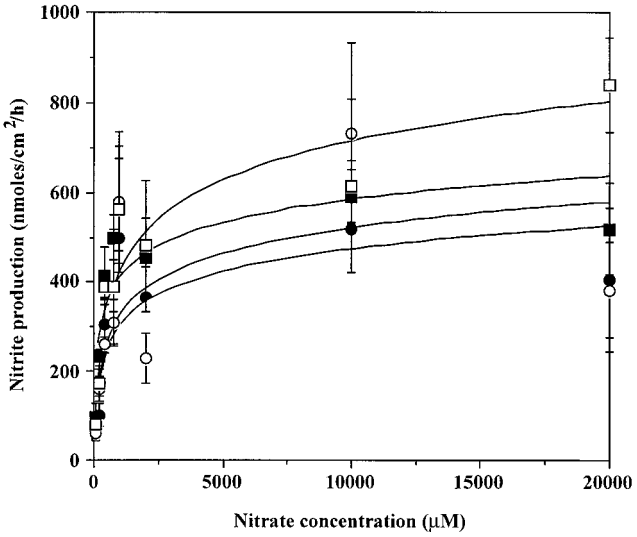

FIG. 2. Nitrite production (nmol cm^{-2} h^{-1}) from intact tongue sections incubated in increasing concentrations of potassium nitrate (100 to 20,000 μM). Results are for posterior sections 4 (closed circle, incubated aerobically; open circle, incubated anaerobically) and 5 (closed square, incubated aerobically; open square, incubated anaerobically). Sections 1 to 3 (anterior tongue) showed nitrite production below the detection level of the assay used. Analysis of variance showed no difference between tongues exposed to oxygen and those handled under anaerobic conditions, but there was a significant difference between sections 4 and 5. Values are means of 10 tongue sections and are for the linear phase of nitrite production only (see Fig. 3).

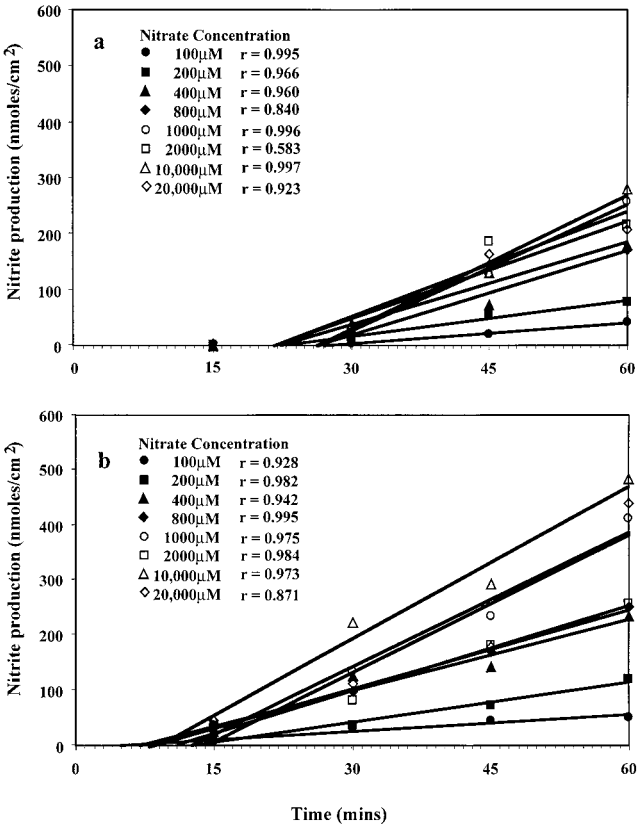

FIG. 3. Nitrite production (nmol cm^{-2}), measured at four time points (15 to 60 min), from intact tongue sections (4 and 5) incubated in increasing concentrations of potassium nitrate (100 to 20,000 μM). Tongue sections were incubated aerobically (a) and anaerobically (b). Linear regressions are plotted through the linear proportions of each graph, the *x* intercept indicating the approximate starting point of nitrite production. Values are means from 10 determinations.

ing nitrite substrate concentrations but remained at only 20% of the velocity measured for nitrate reduction at all concentrations measured. Boiling of tongues removed the ability to metabolize nitrite.

Density of NPB and nitrite production on tongue sections. There was a significant ($P < 0.001$) increase in the density of culturable NPB (Table 1) and in nitrite production (Fig. 2) between the anterior and posterior end of the tongue and a significant correlation ($r = 0.93$; $P < 0.05$) between the density of culturable NPB and the maximum velocity of nitrite production on tongue sections. The proportion of nitrite-producing CFU in the total culturable bacterial population increased from 6.5% on the anterior tongue to 65% on the posterior section of the tongue (Table 1).

Identification of NPB. From a total of 1,275 strains isolated from different isolation media and tongue sections, 147 isolates produced nitrite. The main nitrite-producing genera isolated from rat tongues were *Staphylococcus* (65% of strains isolated), *Pasteurella* (20%), *Streptococcus* (10%), and *Listonella* (5%) (Tables 2 to 4).

The most frequently isolated bacterial species were *S. sciuri* (40% of strains isolated) and *S. intermedius* (25% of strains). Strains from both species showed virtually identical carbohydrate utilization patterns, differing only in their ability to utilize *N*-acetyl-glucosamine and lactose. Populations of gram-negative bacteria and *Streptococcus* consisted of a wider range of species. Gram-negative bacteria included three different species of *Pasteurella* (*P. pneumotropica*, *P. multocida*, and *P. haemolytica*) and *Listonella demsela*, and *Streptococcus* spp. included *Enterococcus faecalis*, *Aerococcus viridans*, and 5 strains which could not be identified by the API computer software (Tables 2 to 4). *Streptococcus* and gram-negative isolates showed a much more limited carbohydrate utilization pattern than the *Staphylococcus* isolates (Tables 2 to 4).

There were significant positive linear relationships between the proportions of *S. sciuri* and gram-negative bacteria in the total populations of denitrifying bacteria and distance from the anterior tip of the tongue (Fig. 4). Conversely, there was a significant negative relationship between this parameter and the proportion of *S. intermedius* and no relationship for *Streptococcus* spp. (Fig. 4).

DISCUSSION

The finding of similar numbers of bacteria on the general growth media (BA and NA) and blood agar containing *Streptococcus* or *Streptococcus*/*Staphylococcus* selective supplements indicates that *Staphylococcus* and *Streptococcus* species are a main component of the microbial flora on the rat tongue. The relative importance of *Staphylococcus* and *Streptococcus* spp. as tongue inhabitants, however, could not be determined since the blood agar medium containing the *Streptococcus* selective supplement was not sufficiently selective towards *Streptococcus*

TABLE 2. Characteristics of nitrate-reducing *Staphylococcus* species isolated from rat tongues[a]

Characteristic	*S. intermedius*	*S. sciuri*
No. of strains	37	59
Gram stain	+	+
Cocci	+	+
Motility	−	−
Catalase	+	+
Acid from glucose[b]	+	+
Reduction of NO_3	+	+
Alkaline phosphate	+	+
Acetoin[c]	+	+
Arginine dehydrolase	+ (62)	−
Urease	+ (93)	−
Coagulase	+[d]	−
Oxidase	ND[e]	+
Acid from:		
D-Glucose	+	+
D-Frucose	+	+
D-Mannose	+	+
Maltose	+	+
Lactose	− (52)	+
D-Trehalose	+	+
D-Mannitol	+	+
Xylitol	−	−
D-Melibiose	−	−
Raffinose	−	−
Xylose	−	−
Sucrose	+	+
α-Methyl-D-glucoside	−	−
N-Acetylglucosamine	+ (93)	+ (75)

[a] Numbers in parentheses indicate percentages of isolates with respective characteristics.
[b] Acid production detected from glucose, in anaerobic conditions, by the method of Hugh and Leifson.
[c] Acetoin detected by the method of Voges-Proskauer.
[d] Only determined when required for species identification.
[e] ND, not done.

TABLE 3. Characteristics of nitrate-reducing *Streptococcus* species isolated from rat tongues

Characteristic	*E. faecalis*	*A. viridans*	Metabolic profiles of unidentifiable *Streptococcus* spp.[a]			
			1	2	3	4
No. of strains	3	6	2	1	1	2
Gram stain	+	+	+	+	+	+
Chains of cocci	+	+	+	+	+	+
Motility	−	−	−	−	−	−
Catalase	−	−	−	−	−	−
Reduction of NO_3	+	+	+	+	+	+
Acetoin[b]	+	+	+	−	−	+
Arginine dihydrolase	+	−	−	+	+	−
Hydrolysis	+	+	−	−	−	+
β-Glucosidase	+	+	+	−	+	+
Pyrrolidonylarylamidase	+	+	−	−	−	+
α-Galactosidase	−	−	+	−	−	−
β-Glucuronidase	−	−	+	−	−	−
β-Galactosidase	−	−	−	−	−	−
Alkaline phosphatase	−	−	+	−	−	−
Leucine arylamidase	+	−	+	+	+	−
Acid from:						
Ribose	+	−	+	−	−	+
L-Arabinose	−	−	+	−	−	−
Mannitol	+	−	+	−	−	+
Sorbitol	+	−	−	−	−	+
Lactose	+	−	+	−	−	+
Trehalose	+	+	+	−	−	+
Inulin	−	−	−	+	−	−
Raffinose	−	−	+	−	−	−
Starch	+	−	−	−	−	+
Glycogen	−	−	−	−	−	−

[a] Chain-forming gram-positive cocci with metabolic profiles that could not be identified by the APILAB software (BioMérieux, Marcy l'Etoile, France).
[b] Acetoin detected by the method of Voges-Proskauer.

spp. (*Staphylococcus* species were frequently isolated from this medium). Fungi, *Pseudomonas* spp., *Lactobacillus* spp., and coliform bacteria appear not to be important as tongue inhabitants, since they were only present in low numbers or below the detection limit of the methods used. These results are similar to those found in previous surveys on the microbial flora of human tongues. For example, Gordon and Gibbons (6) found that 70% of human tongue isolates were gram positive (mainly *Staphylococcus*, *Micrococcus*, and *Streptococcus* species) and 30% were gram negative.

The finding of similar numbers of CFU on aerobically and anaerobically incubated media indicates that the tongue microflora comprise few obligately aerobic species and that the majority of tongue bacteria are facultative anaerobes. Although standard methods were used for the isolation of anaerobes, their number may still have been underestimated due to the difficulty in recovering anaerobes on solid growth media. However, previous surveys on bacterial populations on human tongues have also described anaerobic populations to be relatively small (below 15% of isolates) (6, 13).

The populations of nitrate-reducing bacteria inhabiting animal tongues have to date received very little interest. In this study, a large proportion of culturable bacteria (up to 65% of strains on the posterior tongue) was found to reduce nitrate to nitrite and nitrite metabolism was also evident. The sensitivity of nitrate reductase activity on the tongue sections to oxygen indicates that respiratory nitrate reductase enzymes are responsible for nitrate reduction, since the respiratory electron flow is known to be diverted to oxygen in oxygen-rich atmospheres. Assimilatory nitrate reductase enzymes on the other hand were not previously described to be sensitive to oxygen (11). Approximately 20% of nitrite was found to be further metabolized, but there was no evidence for significant N_2O and N_2 generation (indicating low levels or absence of complete denitrification). This is somewhat surprising since complete denitrification is common in other environments (e.g., the rhizosphere of plants and soil) which are inhabited by nitrate-reducing bacteria (11). However, tongues are colonized by different bacterial communities (*Staphylococcus*, *Streptococcus*, and *Pasteurella* spp.) compared to soil environments where *Pseudomonas*, *Enterobacter*, and *Bacillus* spp. are the main nitrate-reducing organisms (11). Future investigations should, therefore, determine the exact nitrate reduction pathways in the different NPB species found on tongues.

Nitrate reduction is rare in the genus *Enterococcus* and has not previously been reported for *Aerococcus* spp. (8). These strains could represent novel previously unknown groups of streptococci adapted to the tongue environment. The failure of most *Streptococcus* isolates to match known streptococcus profiles which form the basis for the API computer could also indicate a previously uncharacterized grouping. However, a more detailed identification of strains involving fatty acid profiling and/or DNA analysis would be required to characterize them in sufficient detail to assign them to or separate them from known species.

There was a significant positive correlation between the density of culturable NPB and nitrite production, which again

TABLE 4. Characteristics of gram-positive nitrate-reducing bacteria isolated from rat tongues

Characteristic	*P. pneumotropica*	*P. multocida*	*P. haemolytica*	*L. demsela*	Unidentified[a]
No. of strains	3	17	8	7	1
Gram stain	−	−	−	−	−
Rod	+	+	+	+	+
Catalase	+	+	+	+	+
Reduction of NO_3	+	+	+	+	+
Indole production	−	+	−	−	+
Acidification of glucose	−	−	−	−	−
Arginine dihydrolase	−	−	−	+	+
Urease	+	−	−	+	+
β-Glucosidase	−	−	−	−	−
Protease	−	−	−	−	−
β-Galactosidase	−	−	−	−	−
Cytochrome oxidase	ND[b]	+	ND	ND	ND
Assimilation from:					
Glucose	−	−	−	+	+
Arabinose	−	−	−	−	−
Mannose	−	−	−	−	−
Mannitol	−	−	−	−	−
N-Acetylglucosamine	−	−	−	−	−
Maltose	−	−	−	+	+
Gluconate	−	−	−	−	−
Caprate	−	−	−	−	−
Adipate	−	−	−	−	−
Malate	−	−	−	−	−
Citrate	−	−	−	−	−
Phenyl-acetate	−	−	−	−	−

[a] Gram-negative rods with metabolic profiles that could not be identified by the APILAB software (BioMérieux).
[b] ND, not done.

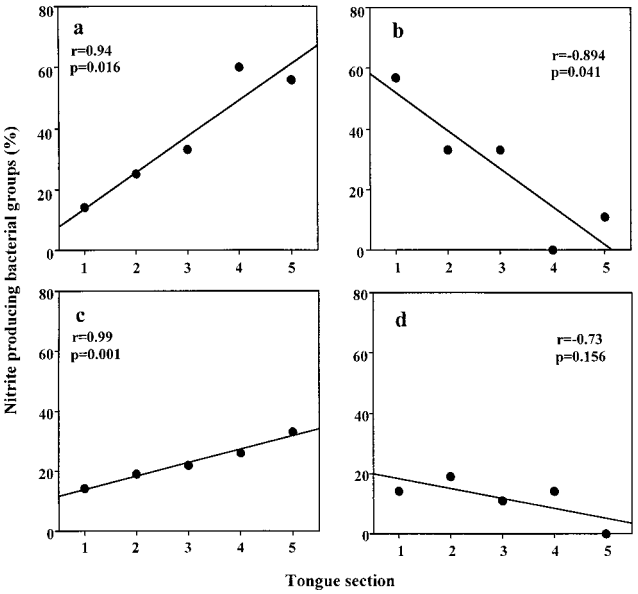

FIG. 4. Distribution of different bacterial species and groups on rat tongue sections. (a) *S. sciuri*. (b) *S. intermedius*. (c) Gram-negative species. (d) *Streptococcus* spp. Values are means from 8 tongues (6 tongues for gram-negative species).

supports the hypothesis that microbial nitrate reduction is responsible for nitrite production.

A prominent feature of many animal tongues (including the two posterior sections of the rat tongue) is the presence of deep clefts which are filled with bacteria (3). At the posterior tongue 58% of bacteria are present in these clefts (21% in the deep part of the cleft), which are constantly filled with saliva in situ. Their depths and the density of facultative anaerobic bacteria present are likely to result in a steep gradient in oxygen tension due to bacterial respiration. Many bacteria which can use nitrate as an alternative electron acceptor are known to produce respiratory nitrate reductase enzymes only under low oxygen tensions (11). Given the apparent sensitivity of nitrate reduction on the tongue to oxygen, it is therefore reasonable to hypothesize that the increase in nitrite production on the posterior tongue is due to increased expression of respiratory nitrate reductase enzymes in conditions of reduced oxygen tension in the deep clefts on the posterior tongue. However, due to the difficulties in obtaining realistic oxygen tension measurements from live animals and the likely changes in oxygen tensions in anaesthetized rats or in excised tongues, it is difficult to determine the degree of anaerobiosis in various areas of the oral cavity. Previous studies into the oxygen tensions in the oral cavity have usually been restricted to surface measurements (4, 13). Future work will therefore include in vitro studies into (i) the activity of nitrate reduction by different NPB at different oxygen tensions and (ii) spatial expression of nitrate reductase in the tongue matrix by immunological and molecular methods.

Staphylococcus, *Pasteurella*, and *Streptococcus* spp. were the dominant nitrite-producing microorganisms of the rat tongue, and one species, *S. sciuri*, clearly dominated (60% of strains isolated) on the posterior tongue where most of the nitrite was produced. A large proportion of the nitrite production might therefore be due to just one bacterial species. However, additional investigations into the kinetics of the nitrate reductase activity on the tongue surface and of nitrate reductase enzymes produced by different tongue bacteria would have to be carried out to identify the genera, species, or strains of bacteria which contribute most towards nitrite production.

Direct counting of bacterial numbers on the tongue surfaces showed the presence of high numbers of both gram-positive cocci and gram-negative rods and an increase in bacterial numbers from the anterior to the posterior tongue, thus confirming the general trends of population studies and identification results based on cultural bacterial populations. However, enumeration of culturable bacteria by plate counts and MPN methods resulted in much lower population estimates. Such discrepancies are common, and the difference between culturable and microscopically counted bacterial populations in other environments (e.g., soil) may be considerably higher than that found in the study presented here (11). It is currently not possible to determine to what extent differences in population estimates were due to dead and/or viable nonculturable bacterial cells being present on the tongue surface. Population estimates therefore carry a degree of error both quantitatively and qualitatively. In our opinion, only the development of molecular probes which can demonstrate the expression of species-specific nitrate reductase enzymes in situ may overcome this limitation. Based on the isolates obtained in this study we have started to develop such probes.

The nitrate concentration allowing maximum velocity of nitrite production on the posterior tongue (600 to 1,000 nmol cm^{-2} h^{-1}) matched the nitrite levels commonly found in human saliva after consumption of high-nitrate diets (approximately 1,000 nmol/ml) (3). This indicates that changes in dietary nitrate intake can greatly affect nitrite production on the tongue and consequent stomach NO-mediated resistance of animals against food-borne diseases.

S. sciuri and most of the other nitrite-producing bacterial species identified are known commensal inhabitants of animals. If similar organisms were present on tongues of domesticated animals, such as the pig, they may provide the basis for probiotic therapy in animals susceptible to gastroenteritis (e.g., neonatal animals which lack NPB and adult animals after antibiotic therapy which has greatly reduced tongue populations of bacteria) (2, 3). An in-depth understanding of this new animal defense mechanism may not only reduce one of the most important problems in intensive animal husbandry but, since the mechanism relies on nitrate intake by animals, may also change the currently negative perception of high nitrate and/or nitrite levels in foods such as green vegetables, cured meats, and drinking water.

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REFERENCES

1. **Benjamin, N., F. O'Driscoll, H. Dougall, C. Duncan, L. Smith, M. Golden, and H. McKenzie.** 1994. Stomach NO production. Nature **368:**502.
2. **Dougall, H. T., L. Smith, C. Duncan, and N. Benjamin.** 1995. The effect of amoxycillin on salivary nitrite concentrations and important mechanism of adverse reactions. Br. J. Clin. Pharm. **39:**460–462.
3. **Duncan, C., H. Dougall, P. Johnston, S. Green, R. Brogan, C. Leifert, L. Smith, M. Golden, and N. Benjamin.** 1995. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat. Med. **1:**546–551.
4. **Globerman, D. Y., and I. Kleinberg.** 1979. Interoral pO_2 and its relation to bacterial accumulation on the oral tissues, p. 275–291. *In* I. Kleinberg, S. A. Ellison, and I. D. Mandel (ed.), Saliva and dental caries. IRL Press, London, United Kingdom.
5. **Goaz, P. W., and H. A. Biswell.** 1961. Nitrite reduction in whole saliva. J. Dent. Res. **40:**355–365.
6. **Gordon, R. J., and R. J. Gibbons.** 1966. Studies of the predominant cultivable micro-organisms from the human tongue. Arch. Oral Biol. **11:**627–635.
7. **Gundersen, J. G.** 1977. Notes on the estimation of the numerical density of arbitrary profiles. J. Microsc. **111:**219–223.
8. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergey's manual of determinative bacteriology, 9th ed. Williams & Wilkins, Baltimore, Md.

9. **Ishiwata, H., P. Boriboon, M. Harada, A. Tanimura, and M. Ishidate.** 1975. Changes of nitrite and nitrate concentration in incubated human saliva. J. Food Hyg. Soc. Jpn. **16:**93–98.
10. **Ishiwata, H., A. Tanimura, and M. Ishidate.** 1975. Nitrite and nitrate concentrations in human saliva collected from salivary ducts. J. Food Hyg. Soc. Jpn. **16:**89–92.
11. **Killham, K.** 1994. Soil ecology. Cambridge University Press, Cambridge, United Kingdom.
12. **Leifert, C., W. M. Waites, and J. R. Nicholas.** 1989. Bacterial contaminations of micropropagated plant cultures. J. Appl. Bacteriol. **67:**353–361.
13. **Marsh, P., and M. Martin.** 1992. Oral microbiology. Chapman & Hall, London.
14. **Page, A. L., R. H. Miller, and D. R. Keeney.** 1982. Chemical and microbiological properties—agronomy monograph no. 9, 2nd ed., p. 815–820. Soil Science Society of America, Madison, Wis.
15. **Sasaki, T., and K. Matano.** 1979. Formation of nitrite from nitrate at the dorsum linguae. J. Food Hyg. Soc. Jpn. **20:**363–369.
16. **Savostianov, G. M.** 1937. On the question of nitrites in saliva. Fiziol. Zh. **23:**159–164.
17. **Schinner, F., R. Öhlinger, E. Kandeler, and R. Margesin.** 1993. Bodenbiologische Arbeitsmethoden. 2. auflage. Springer-Verlag, Berlin, Germany.
18. **Sen, N. P., D. C. Smith, and L. Schwinghamer.** 1969. Formation of N-nitrosamines from secondary amines and nitrite in human and animal gastric juice. Food Cosmet. Toxicol. **7:**301–307.
19. **Stevens, A.** 1990. Gram method, p. 290–293. *In* J. D. Bancroft and A. Stevens (ed.), Theory and practice of histological techniques, 3rd ed. Churchill Livingstone Inc., New York.
20. **Tannenbaum, S. R., A. J. Sinskey, M. Weisman, and W. Bishop.** 1974. Nitrite in human saliva. Its possible relationship to nitrosamine formation. J. Natl. Cancer Inst. **53:**79–84.
21. **Tannenbaum, S. R., M. Weisman, and D. Fett.** 1976. The effect of nitrate on nitrite formation in human saliva. Food Cosmet. Toxicol. **14:**549–552.
22. **Tannenbaum, S. R., D. Fett, V. R. Young, P. D. Land, and W. R. Bruce.** 1978. Nitrite and nitrate are formed by endogenous synthesis in the human intestine. Science **200:**1487–1489.
23. **Tannenbaum, S. R., S. W. John, and D. L. Cynthia.** 1991. Inhibition of nitrosamine formation by ascorbic acid. Am. J. Clin. Nutr. **53:**247–250.
24. **White, J. W., Jr.** 1975. Relative significance of dietary sources of nitrate and nitrite. J. Agric. Food Chem. **23:**886–891.