Formation of Trimethylamine from Dietary Choline by *Streptococcus sanguis I*, Which Colonizes the Mouth

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Choline is a component of the normal diet, and when humans ingest large amounts they excrete trimethylamine (which can impart a fishy body odor). In the presence of nitrite, trimethylamine can be converted to dimethylnitrosamine, a potent carcinogen. Bacteria in the large intestine metabolize choline to form trimethylamine. We determined that a bacterium normally present in the oral cavity also has this capacity. Mixed bacterial flora cultured from dental plaque and saliva converted choline to trimethylamine. The only organism with trimethylamine-forming capability isolated from these mixed cultures was identified as Streptococcus sanguis I *(a facultative anaerobe). The other products formed when choline was cleaved were ethanol and acetate. The formation of trimethylamine by* S. sanguis I *was enzyme-mediated. Activity was destroyed by heating at 100°C, and obeyed Michaelis-Menten kinetics ($K_{apparent}$ for choline = 184 ± 58 μM; $V_{max\,apparent}$ = 1.7 ± 0.1 μmol/mg protein/h). Activity was maximal at pH 7.5 to 8.5, was membrane-bound, and required a divalent metal cation (cobalt or iron). More trimethylamine was produced by bacteria incubated under a nitrogen than under an aerobic atmosphere. Activity was inhibited by deanol, betaine aldehyde, hemicholinium-3, iodoacetate, semicarbazide, and 2,4-dinitrophenol, and was enhanced by sulfhydryl-reducing agents (glutathione, 2-mercaptoethanol, DL-dithiothreitol) and sodium bisulfite. The enzyme activity that we describe in* S. sanguis I *is similar to that previously described in the anaerobic bacteria isolated from intestinal flora.*

Keywords: choline; trimethylamine; dimethylnitrosamine; oral bacteria; *Streptococcus sanguis I.*

Introduction

The formation of trimethylamine (TMA) within the oral cavity is important for two reasons: TMA can be nitrosated,[1,2] forming dimethylnitrosamine which is a potent carcinogen; and TMA imparts the characteristic odor of fish, which could contribute to mouth odor.

Dimethylnitrosamine is a potent carcinogen in a wide variety of animal species, including humans.[3-5] One precursor needed for dimethylnitrosamine synthesis, nitrite, is present in the oral cavity. Foods contain nitrate, which can be reduced to nitrite by bacteria in the mouth or in the hypochlorhydric or achlorhydric stomach.[6,7] Nitrite levels in saliva peak 2 to 4 hours after nitrate-rich food is ingested.[8]

Choline is a component of the normal diet, and bacteria in the large intestine form TMA and dimethylamine from choline.[9-11] This pathway has been studied in *Aerobacter aerogenes, Shigella alkalescens, Desulfovibrio desulfuricans, Clostridium* sp, and some *Proteus* species isolated from intestine, soil, or menstrual fluid.[12-17] The oral cavity is densely colonized with bacteria.[18] It is estimated that there may be over 200 different species that can be isolated from dental plaque.[19] In these studies, we determined whether any of the species of bacteria colonizing the oral cavity had the capacity to form TMA from choline.

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Materials and methods

Chemicals

Reagents were purchased from Sigma Chemicals Company (St. Louis, MO, USA) unless otherwise indicated. Choline chloride, potassium phosphate, and sodium phosphate were obtained from Fisher Scientific Corporation (Medfield, MA, USA). Dichloromethane was obtained from EM Science (Gibbstown, NJ, USA), sodium cyanide was from J. T. Baker Chemical Company (Phillipsburg, NJ, USA), and iodoacetic acid from Eastman Kodak Company (Rochester, NY, USA). Choline chloride and trimethylamine hydrochloride were recrystallized from methanol before use.

Sources of bacteria

Salivary isolates were obtained by the technique of repeated restreaking, on blood agar plates, of single colonies from cultures of human saliva. Reference strains of bacteria and bacteria isolated from subgingival dental plaque were kindly provided by Dr. Sigmund S. Socransky of the Forsyth Dental Center, Boston, MA, USA.

The plaque isolates screened for TMA-forming activity were: *Streptococcus sanguis I, Streptococcus sanguis II, Streptococcus morbillorum, Streptococcus intermedius, Streptococcus constellatus, Streptococcus mitis, Veillonella parvula, Bacteriodes asaccharolyticus, Bacteroides gingivalis, Bacteroides melaninogenicus, Bacteroides forsythus, Campylobactor concisus, Capnocytophaga ochracea, Capnocytophaga gingivalis, Eikenella corrodens, Fusobacterium nucleatum, Fusobacterium periodonticum, Wolinella recta,* and *Wolinella curva.*

The reference strains screened for TMA-forming activity were: *S. sanguis I* (FDC strain), *V. parvula* (ATCC 10790), *Actinobacillus actinomycetemcomitans* (FDC Y 4) (FDC 650), *Bacteroides buccalis* (ATCC 35310), *Bacteroides denticola* (ATCC 33185), *B. forsythus* (ATCC 43037) (FDC 338), *B. gingivalis* (FDC 381), *Bacteroides gracilis* (ATCC 33236) (FDC 1084) (FDC 1083) (FDC 406) (FDC 402), *Bacteroides heparinolyticus* (ATCC 35895), *Bacteroides intermedius* (ATCC 25261), *Bacteroides oulorum* (ATCC 11871), *Bacteroides ureolyticus* (VPI 7814), *Bacteroides veroralis* (ATCC 33779), *Bacteroides zoogleoformans* (ATCC 33285), *C. concisus* (ATCC 33237) (FDC 484), *Campylobactor sputorum* (FDC 616), *E. corrodens* (FDC 373) (FDC 1073), *F. nucleatum* (FDC EM 48), (FDC 364), *F. periodonticum* (ATCC 33693), *Haemophilus aphrophilus* (FDC 626), *Kingella denitrificans, W. curva* (FDC 521) (FDC 640) (ATCC 33567) (VPI 10659), *W. recta* (ATCC 33238) (FDC 371), and *Wolinella succinogenes* (FDC IB 4).

Incubation conditions for screening for trimethylamine-forming activity

Bacteria from the above sources were restreaked on trypticase soy agar with 5% sheep blood (Baltimore Biological Laboratories, Cockeyesville, MD, USA) and incubated anaerobically (under 80% N_2, 10% H_2, 10% CO_2 in an anaerobic chamber, Vacuum/atmospheres Corporation, Los Angeles, CA, USA) at 35°C for 1 week. Bacteria were harvested from the agar surface using a sterile inoculating loop and placed into a tube containing cold 50 mM sodium or potassium phosphate buffer, pH 7.5. Cells were dispersed by vigorous mixing and, as indicated, were disrupted using sonication (on ice, setting 7 with 50% pulse duration; model W-225R, Heat Systems Ultrasonics Incorporated, Plainview, NY, USA). An aliquot of cell suspension (0.1 to 0.3 mg protein) was delivered into a 300-μl glass conical insert within a 4-ml glass vial which was sealed (Teflon-lined WISP septum, Waters Instruments, Milford, MA, USA). Choline chloride and 0.25 μCi [methyl-^{3}H]-choline chloride (80 Ci/mmol, New England Nuclear, Boston, MA, USA) were added to achieve a final concentration of 1 mM (final incubation volume, 260 μl). Vials were sealed and flushed with N_2 for 10 minutes before incubating at 37°C in a Dubnoff metabolic shaking incubator (speed 4; GCA/Precision Scientific). Trimethylamine formation from choline by *S. sanguis I* proceeded linearly for at least 1 hour. We therefore used a 30-minute incubation for most assays unless otherwise indicated. The reaction was terminated by adding HCl to achieve a final concentration of 0.1 M. Equal volumes of methanol and chloroform were added to precipitate protein. An aliquot of the aqueous layer was applied to a thin layer chromatography plate for separation of TMA as described below. A positive control (*D. desulfuricans* [ATCC 27774], which makes TMA from choline) and two negative controls (50 mM phosphate buffer; heat-killed *S. sanguis I*) were used in each assay.

Characterization of trimethylamine formation in S. sanguis I *isolated from dental plaque*

To determine whether TMA-forming activity was associated with the particulate fraction, bacteria were cultured for 24 hours, then disrupted using sonication as described above. Cell suspensions were centrifuged for 45 minutes at 4°C as 45,000 × *g* (model L7-55 W/R ultracentrifuge, rotor type: 50.2 Ti, Beckman Instruments, Palo Alto, CA). The supernatant was saved and used to assay for activity in the cytosol as described below. To remove unbroken cells, the pellet was resuspended in cold buffer (50 mM potassium phosphate, pH 7.5), centrifuged at 120 × *g* for 20 minutes (model DPR-6000, International Equipment Company, Needham Heights, MA, USA), and the supernatant saved. This resuspension and centrifugation at 120 × *g* of the pellet was performed twice. The resulting supernatants were combined and were then spun at 45,000 × *g* for 30 minutes at 4°C. The resulting pellet was then resuspended in cold buffer and centrifuged again (45,000 × *g*, 30 minutes), and the final pellet was resuspended in buffer for use in assays. The protein concentration of each fraction was adjusted with assay buffer (50 mM potassium phosphate, pH 7.5) so that

approximately equal amounts could be added to each incubation tube before assay.

In some experiments (as indicated), sonicated bacteria were dialyzed (Spectrapor membrane tubing with molecular weight cut-off of 6,000 to 8,000; Spectrum Medical Industries) against 1 L of 5 mM EDTA-50 mM potassium phosphate, pH 7.5, or against three changes of distilled water.

Intact bacteria were used in all other experiments. When additions were made to the incubation mixture (various potential inhibitors/enhancers of enzyme activity), they were dissolved in buffer (50 mM potassium phosphate) and were adjusted to pH 7.5 with HCl or NaOH before an aliquot (10 μl) was delivered into the incubation mixture. An equivalent aliquot of buffer was added to tubes without additives.

To determine the substrate specificity of bacterial enzymes, we examined whether compounds sharing structural features with choline could be metabolized to form TMA (or dimethylamine). Phosphocholine, phosphatidylcholine, dimethylaminoethanol (deanol), acetylcholine, betaine, and betaine aldehyde (at 1 mM final concentration) were incubated with bacteria as described above for 4 hours at 37°C in a shaking water bath. Choline was used as a control. The reaction was stopped by adding $HClO_4$ so that the final concentration of $HClO_4$ was 3%. Trimethylamine and dimethylamine were extracted and measured using gas chromatography. Some of the assays were also performed by adding radiolabeled choline analog, and TMA formation was determined using the thin layer chromatography method. In additional experiments, [methyl-^{14}C]- phosphocholine (40 mCi/mmol, New England Nuclear) or [methyl-^{14}C]-acetylcholine (40 mCi/mmol, ICN Radiochemicals, Irvine, CA, USA) were incubated with washed particulate fraction from sonicated *S. sanguis I*, and phosphatase or cholinesterase activity was assessed by measuring formation of ^{14}C-choline using the thin layer chromatography method.

Separation and quantitation of trimethylamine and dimethylamine using gas chromatography

Trimethylamine and dimethylamine were measured using a previously described gas chromatographic method.[20] An aliquot of the acidified sample was placed into a sealed vial (Teflon-lined WISP septum; Waters Instruments). 2-Propanol was added to each vial, along with an internal standard of isopropylamine. Potassium hydroxide (65%) was injected into the vial to bring the pH above 9. Vials were vigorously shaken, heated at 60°C for 30 minutes, then subjected to centrifugation at 1,000 × *g* for 5 minutes at −4°C. An aliquot was drawn into a syringe, followed by 1 μl of air and 1 μl of 30% ammonium hydroxide. This was injected into the glass-lined injection port (pretreated with potassium hydroxide to inactivate any binding sites for methylamines) of the gas chromatograph (Sigma 300; Perkin Elmer, Norwalk, CT, USA). We used a 2-m–long (2 mm i.d.) glass chromatography column packed with 60/80 CarbopackB/0.8% KOH on 4% Carbowax 20M (Supelco, Bellefonte, PA, USA), and eluted methylamines with helium (25 ml/min) and a temperature gradient (75°C for 2 minutes, then increasing to 200°C at 32°C/minute). Amines were detected with a nitrogen-phosphorus detector (NPD; Perkin Elmer), and peaks were integrated using a computing integrator (LCI-100; Perkin Elmer). During each sample run, we injected 20 μl of 30% ammonium hydroxide into the hot (150°C) column as it was heated to 200°C. This markedly reduced "ghosting" of methylamines on subsequent runs.

Separation and quantitation of trimethylamine using thin layer chromatography

An aliquot of the aqueous phase of the extract of the incubation mixture was applied to silica gel thin layer chromatography plate (250 μm; LK5D, Whatman Company, Clifton, NJ, USA) along with 400 nmol TMA hydrochloride as a carrier. Plates were developed with chloroform/methanol/0.1 M HCl (65:30:4, vol/vol). The bands which cochromatographed with authentic TMA standard were identified with iodine vapor, and were scraped off the plates and transferred to scintillation vials containing 1 ml 0.1 M HCl. Samples were mixed and 5 ml scintillation fluid (ScintiVerse E, Fisher Scientific) were added. Radioactivity (cpm) was determined by scintillation spectrophotometry (model 1211 MiniBetta, LKB Instruments, Gaithersburg, MD, USA) and disintegrations per minute were calculated using the external-standard channels ratio. In all studies, a blank (heat-killed bacteria) was used to correct calculations. This method had a coefficient of variation of 4% for TMA. Recovery of a standard of radiolabeled TMA (^{14}C-trimethylamine hydrochloride, 3 mCi/mmole, New England Nuclear) after thin layer chromatography was > 96%.

After some incubations, we determined whether betaine, betaine aldehyde, glycerophosphocholine, or phosphocholine contained radiolabel derived from choline using a method we previously reported.[21] An aliquot of the aqueous extract after an incubation was applied to a silica HPLC column (Pecosphere-3CSi, 5 μm, 4.6 × 83 mm; Perkin-Elmer) and choline and its metabolites were eluted using a binary nonlinear gradient of acetonitrile/ethanol/acetic acid/1 M ammonium acetate/water/0.1 M sodium phosphate (800:68:2:3:127:10 vol/vol changing to 400:68:44:88:400:10 vol/vol). Peaks were detected with an online radiometric detector.

To determine that choline was completely separated from TMA using thin layer chromatography, silica plates, after certain experiments, were covered with film (Kodak X-OMAT, XAR-5, 13 × 18 cm, Eastman Kodak Company) and stored in −90°C freezer for 1 week. [Methyl-^{14}C]-choline chloride (40 mCi/mmol, New England Nuclear) and [methyl-^{14}C]-trimethylamine hydrochloride (3 mCi/mmol, New England Nuclear) were also applied as external standards. The film was developed, and we determined

that bands containing choline were separated by at least 5 mm from bands containing TMA.

Choline assay

The bacterial cell suspension was extracted using 1 M formic acid/acetone (3:17, vol/vol), and choline was measured using gas chromatography/mass spectrometry as previously described.[21] Choline concentrations were used to calculate the specific activity of radiolabeled choline during incubations.

Ethanol assay

Ethanol was determined enzymatically using a spectrophotometric method.[22]

Acetate assay

Acetate was determined after ether extraction by gas chromatography using a flame ionization detector.[23] Samples were acidified with sulfuric acid, sodium chloride was added, and acetate was extracted into ethyl ether. An aliquot of the ether layer was injected onto a glass packed column (2 mm i.d., 6 ft, GP 15% SP-1,200/1% H_3PO_4 on 100/120 Chromosorb WAW; Supelco) on a Hewlett Packard 5880A Series Gas Chromatograph (Avondate, PA, USA) and data were integrated using a 5880A Series GC terminal. Oven temperature was 140°C, injector temperature was 140°C, and detector temperature was 240°C. The flow rate of nitrogen carrier gas was 30 ml/minute.

Protein assay

Protein was measured using the colorimetric assay of Bradford.[24]

Statistical methods

Student's *t* test was used for simple comparison of hypothesized positive and negative groups studied. One-way analysis of variance (ANOVA) and Dunnett's test were used for comparing experimental group means with the control group. Either one-way ANOVA and Scheffe's test or one-way ANOVA and Duncan's multiple-range test were used for making all possible comparisons between groups.[25]

Results

Mixed cultures of bacteria from dental plaque and saliva were capable of forming TMA from 1 mM choline (plaque bacteria formed 567 pmol TMA/mg wet wt/hr ± 16 SEM; salivary bacteria formed 18 nmol TMA/mg protein/hr ± 1 SE). TMA formation by *S. sanguis I* was similar when measured as mass of TMA formed from choline (using gas chromatography) or when assessed as radiolabel derived from choline (using the thin layer chromatography method). Trimethylamine was only formed by bacteria isolated from dental plaque and saliva which were identified as *S. sanguis I*. (These organisms were gram-positive cocci found in bunches or short chains. Colonies were pleomorphic in shape, varying from rough to smooth, shiny when grown anaerobically on trypticase soy agar with 5% sheep blood, and hard when grown on Mitis Salivarius agar. The organisms were facultative anaerobes, caused α-hemolysis on blood agar, were negative for nitrate reductase activity but positive for arginine hydrolase, and failed to ferment either mannitol or sorbitol.) When these bacteria were identified using these data and a probability matrix[26] or the Rapid STREP system (Analytab Products, Plainview, NY, USA), they were identified as *S. sanguis I* with a probability exceeding 0.99. Of the reference strains known to colonize the mouth that we tested, only *S. sanguis I* formed TMA.

Intact *S. sanguis I* used choline and, to a lesser extent, phosphocholine and acetylcholine as substrates for the formation of TMA (*Figure 1*). Phosphatidylcholine, betaine, and betaine aldehyde were not substrates; dimethylaminoethanol was not degraded to form dimethylamine (*Figure 1*; $P < 0.01$ different from choline by one-way ANOVA and Dunnett's test). For each mole of TMA formed from choline by *S. sanguis I*, 0.5 mole of ethanol and 0.5 mole of acetate were formed. Many oral bacteria grow best *in vitro* at approximately pH 7.0, which is the approximate pH of saliva. However, the pH of dental plaque can drop to below 5.0 during eating, when sugars are metabolized. Trimethylamine formation was maximal at pH 7.5 to 8.5, and was essentially nonexistent when the pH dropped to 6.5 or 5.5 (433 nmol/mg protein/hr ± 33 at pH 8.5; 322 nmol/mg protein/hr ± 73 at pH 7.5; 5 nmol/mg protein/hr ± 5 at pH 6.5; and 5 nmol/mg protein/hr ± 3 at pH 5.5; $P <$

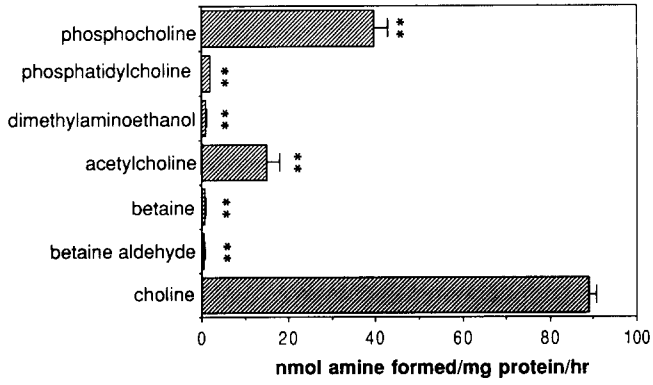

Figure 1 Formation of TMA from choline, choline metabolites, and choline analogs by *Streptococcus sanguis I*. *Streptococcus sanguis I* (120 μg protein) in 50 mM sodium phosphate buffer, pH 7.5, was incubated with the indicated compounds (at 1 mM final concentration) in a shaking water bath for 4 hours at 37°C. The reaction was stopped by adding $HClO_4$. Dimethylamine and TMA were extracted and measured using gas chromatography. Data are expressed as mean nanomoles TMA formed per milligram protein per hour ± SD (n = 2/point), except for dimethylaminoethanol, in which data are expressed as mean nanomole dimethylamine formed per milligram protein per hour ± SD. All values were calculated by subtracting the dimethylamine or TMA (as appropriate) present in a sample in which heat-killed *S. sanguis I* were incubated with the respective analog. $^{**}P < 0.01$ different from choline by one-way ANOVA and Dunnett's test.

0.01 for difference of pH 5.5 or pH 6.5 compared with pH 7.5 or pH 8.5 by one-way ANOVA and Duncan's test). More TMA was produced when vials were incubated under N_2 (1,476 nmol/mg protein/hr ± 53) than when vials were incubated under air (1,091 nmol/mg protein/hr ± 23; $P < 0.01$ different from N_2 by one-way ANOVA and Dunnett's test). Trimethylamine formation increased as the incubation (under N_2) temperature was elevated (from 15 nmol/mg protein/hr ± 8 at 4°C; 200 nmol/mg protein/hr ± 13 at 25°C; 1,476 nmol/mg protein/hr ± 53 at 37°C; and 2,027 nmol/mg protein/hr ± 102 at 50°C; $P < 0.01$ activity at 50°C greater than at 37°C and at 37°C greater than at 25°C by one-way ANOVA and Dunnett's test). Activity was markedly diminished by incubation at 100°C (76 nmol/mg protein/hr ± 9; $P < 0.01$ different from 37°C by one-way ANOVA and Dunnett's test; this probably reflects activity before the sample's temperature reached 100°C, as no activity was present in bacteria heated for 10 minutes at 100°C before choline was added).

Trimethylamine-forming activity in *S. sanguis I* was similar in whole cells and in sonicated cell preparations (*Figure 2*). The capacity to convert choline to TMA was associated with the particulate fraction (*Figure 2*). Washed particulate fraction of *S. sanguis I* formed choline and TMA from phosphocholine and acetylcholine (data not shown). Activity in each fraction could be destroyed by heating at 100°C for 10 minutes. Trimethylamine formation from choline by *S. sanguis I* was saturable (*Figure 3*). We used nonlinear regression analysis (Fitfunction on a Digital VAX computer; BBN Research Systems, Cambridge, MA, USA) with the following equation:

$$v = \frac{[S]\ Vmax}{[S] + Km}$$

in which v = velocity of TMA production and [S] = choline concentration. The best fit ($r^2 = 0.9913$) estimates for the kinetic constants of the saturation curve were $K_{apparent}$ = 184 ± 58 μM and $V_{max\ apparent}$ = 1,683 ± 140 nmol/mg protein/hr.

Sodium azide and arsenate did not inhibit TMA formation from choline (*Table 1*). 2,4-Dinitrophenol, hemicholinium-3, dimethylaminoethanol, betaine aldehyde, iodoacetate, and semicarbazide inhibited TMA formation (*Table 1*). The addition of sodium cyanide greatly increased the variability of, and tended to decrease, TMA formation (*Table 1*). D,L-Dithiothreitol and 2-mercaptoethanol reduced glutathione, and sodium bisulfite enhanced TMA formation (*Table 1*). When 1 mM betaine aldehyde was added to incubation mixtures, most radiolabel remained in the form of choline. The amount of label incorporated into TMA was markedly diminished (*Table 1* and *Figure 4*), and no radiolabel accumulated as betaine aldehyde (assessed using high-pressure liquid chromatography).

When sonicated *S. sanguis I* organisms were extensively dialyzed against water or against 5 mM EDTA, TMA formation was completely inhibited (0% control; $P < 0.01$ different from control by one-way ANOVA and Dunnett's test). Activity could not be recovered by adding 20 mM KCl, 10 mM Fe^{2+}, 10 mM Co^{2+}, or 1 mM Mg^{2+}, or by adding undialyzed supernatant to dialyzed pellet. When extracts of *S. sanguis I* were preincubated with 5 mM EDTA for 2 hours, TMA formation was diminished to 21% of control ± 4 ($P < 0.01$ different from control by one-way ANOVA and Dunnett's test). We also noted that the rate of TMA formation

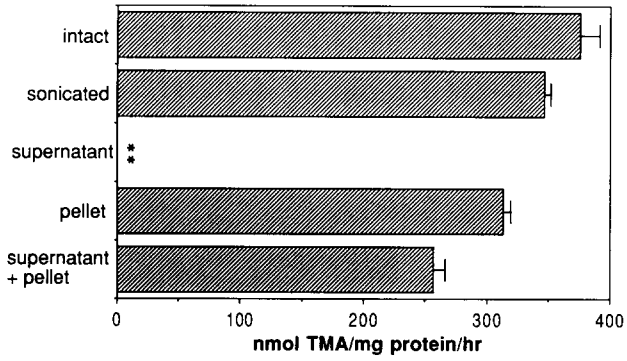

Figure 2 Breakdown of choline by whole cells and cell-free fractions. *Streptococcus sanguis I* were suspended in 50 mM potassium phosphate buffer, pH 7.5, and sonicated. The broken cell suspension was centrifuged for 45 minutes at 45,000 × *g*. The pellet was resuspended in cold potassium phosphate buffer and centrifuged at low speed (120 × *g*, 20 minutes) to remove unbroken cells. This pellet washing procedure was performed twice. The supernatant was then centrifuged for 30 minutes at 45,000 × *g*, washed with cold buffer, and recentrifuged (45,000 × *g*, 30 minutes). The pellet was resuspended in buffer. The protein concentration of each fraction was adjusted with assay buffer (50 mM potassium phosphate, pH 7.5) so that approximately equal amounts (0.3 mg protein) could be added to each incubation tube before assay. They were then incubated with 1 mM choline and 0.25 μCi [methyl-^{3}H] choline chloride in a shaking water bath for 4 hours at 37°C. The reaction was stopped, and TMA was measured using the thin layer chromatography method. Data are expressed as mean nanomoles TMA per milligram protein per hour ± SEM (n = 3/point). $^{**}P < 0.01$ different from sonicated bacteria by one-way ANOVA and Scheffe's test.

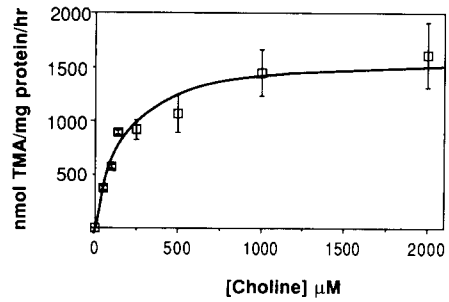

Figure 3 Trimethylamine formation from choline by *S. sanguis I*: Michaelis-Menten plot. *Streptococcus sanguis I* (20 μg protein) in 50 mM potassium phosphate buffer, pH 7.5, was incubated with choline and 0.25 μCi [methyl-^{3}H] choline chloride in a shaking water bath for 30 minutes at 37°C. Choline concentrations were varied as indicated. The reaction was stopped by adding HCl so that the final concentration of HCl was 0.1 N, followed by extraction into HCl/methanol/chloroform (1:1:1, by vol). Trimethylamine was measured using the thin layer chromatography method. Nonlinear regression analysis using the equation v = [S] Vmax/[S] + Km was used to construct the fitted curve that is shown ($r^2 = 0.9913$). Data are expressed as mean nanomoles TMA per milligram protein per hour ± SEM (n = 3/point).

was diminished when incubations were carried out for more than 3 hours, even when no EDTA was added (rate decreased to 41% of control ± 3, $P < 0.01$ by Student's t test different from rate measured during a 1-hour incubation period). Fe^{2+} or Co^{2+} (10 mM), when added to incubation mixtures, reversed the inhibitory effects of EDTA or of prolonged preincubation of tissues (with Fe^{2+} = 166% control ± 1; with Co^{2+} = 131% control ± 7).

Table 1 Effect of various additives on trimethylamine formation by *Streptococcus sanguis I*

Agent	TMA formation (% control ± SEM)
Electron transport blockers	
10 mM sodium cyanide	48 ± 17
1 mM sodium azide	107 ± 7
Uncouplers	
1 mM 2,4-dinitrophenol	12 ± 6[a]
1 mM arsenate	79 ± 14
Analogs of choline	
0.5 mM hemicholinium-3	41 ± 11[a]
1 mM hemicholinium-3	12 ± 2[a]
1 mM dimethylaminoethanol	24 ± 9[a]
0.5 mM betaine aldehyde	3 ± 3[a]
1 mM betaine aldehyde	0 ± 0[a]
Sulfhydryl-reducing agents	
20 mM DL-dithiothreitol	938 ± 150[a]
20 mM reduced glutathione	730 ± 88[a]
20 mM 2-mercaptoethanol	852 ± 170[b]
Other	
1 mM iodoacetate	20 ± 4[b]
1 mM sodium bisulfite	1057 ± 3[a]
20 mM semicarbazide	0 ± 0[a]

TMA formation by *streptococcus sanguis I* was measured after adding 1 mM [methyl-^{3}H]choline chloride and the indicated additive. Incubation conditions were as described in the Materials and Methods section. Samples were incubated for 30 minutes at 37°C, and radiolabel in TMA was determined using thin layer chromatography and scintillation spectrophotometry as described. Data are present as mean percent control ± SEM, n = 3/point.

[a] $P < 0.01$ different from control by one-way ANOVA and Dunnett's test.

[b] $P < 0.05$ different from control by one-way ANOVA and Dunnett's test.

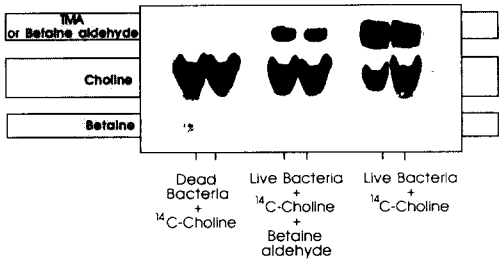

Figure 4 Betaine aldehyde inhibits the formation of TMA from choline. *Streptococcus sanguis I* (156 μg protein) in 50 mM potassium phosphate buffer, pH 7.5, was incubated with 1 mM choline and 0.25 μCi [methyl-^{14}C] choline chloride in a shaking water bath for 30 minutes at 37°C. In some tubes, 1 mM betaine aldehyde was also added as indicated. In some tubes, heat-killed bacteria were used instead of live bacteria as indicated. The reaction was stopped and extracted as described in the Materials and methods section. An aliquot of the aqueous phase was applied to a silica gel plate and developed in chloroform/methanol/0.1N HCl (65:30:4, vol/vol). An autoradiogram of the plate was prepared as described. The gray bars on the left indicate the distances migrated by authentic standards. Note that betaine aldehyde co-chromatographs with TMA.

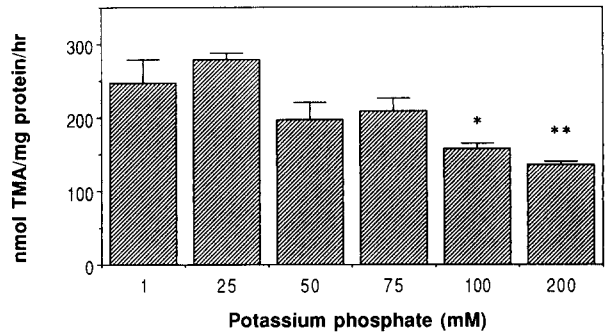

Figure 5 Effect of varying ionic strength on TMA production. *Streptococcus sanguis I* were grown on agar submersed in potassium phosphate buffer, pH 7.5, at the concentrations indicated for 24 hours. Sonicated preparations of *S. sanguis I* (50 to 65 μg protein) were then prepared and incubated in potassium phosphate (at the indicated concentrations) buffer, pH 7.5, containing 1 mM choline and 0.25 μCi [methyl-^{3}H] choline chloride for 30 minutes at 37°C. The reaction was stopped, and TMA was measured using the thin layer chromatography method. Data are expressed as mean nanomoles TMA per milligram protein per hour ± SEM (n = 3/point).

Streptococcus sanguis I cells were grown on agar for 1 day; we then overlaid potassium phosphate buffer (pH 7.5) of varying osmotic strength for 24 hours. Bacteria were harvested and TMA formation measured using the radiolabel-thin layer chromatography method. We found that TMA formation decreased slightly as the osmotic strength of the buffer increased (*Figure 5*; $P < 0.01$ for difference of 200 mM versus 25 mM; $P < 0.05$ for difference of 200 mM versus 1 mM and 100 mM versus 25 mM by one-way ANOVA and Scheffe's test).

Discussion

We isolated TMA-forming bacteria from cultures of saliva or dental plaque, and screened libraries of reference strains known to colonize the mouth. We repeatedly identified only one species of bacteria which could form TMA: *S. sanguis I*. This is the first time that a bacterium with this capacity has been identified in the oral cavity. The ability to form TMA from choline has previously been identified in bacteria which colonize the lower intestine, menstrual fluid, and soil, including *Acinetobacter calcoaceticus, A. aerogenes, S. alkalescens, Proteus* sp. (*rettgeri, ichthyosmius, vulgaris, mirabilis*), *Clostridium, D. desulfuricans, Serratia* sp, *and Pseudomonas* sp.[12-16,27-30] *Streptococcus sanguis I* colonization of the oral cavity increases when dental hygiene is poor or in the presence of oral pathology.[31-33] Choline is present in the oral cavity when food is eaten (the adult human ingests 300 to 1000 mg of choline per day).[34,35]

Trimethylamine-forming activity in *S. sanguis I* was destroyed by heat and was saturable. Choline, phosphocholine, and acetylcholine could be used as substrates for TMA formation by *S. sanguis I* (*Figure 1*). Because we observed the formation of choline from phosphocholine or acetylcholine by the particulate

fraction of *S. sanguis I*, it is possible that TMA was formed from choline rather than directly from these choline esters. Phosphatase activity has been previously described in *S. sanguis I*.[36] We did not observe dimethylamine formation from dimethylaminoethanol; in *Proteus* sp, significant production of ethanol and acetaldehyde from less methylated analogs of choline has been observed.[30] Trimethylamine-forming activity was inhibited by hemicholinium-3 and dimethylethanolamine, competitive inhibitors of choline uptake and metabolism in many tissues.[37-43] Choline dehydrogenase activity in liver forms betaine aldehyde from choline.[44] Under certain conditions, betaine aldehyde can chemically degrade to form TMA (unpublished observation). We observed that betaine aldehyde was a potent inhibitor of ^{14}C-TMA formation from ^{14}C-choline (*Table 1; Figure 4*). One possible explanation might have been that the TMA-forming reaction proceeded as ^{14}C-choline → ^{14}C-betaine aldehyde → ^{14}C-TMA, and that the addition of unlabeled betaine aldehyde prevented the label accumulated within betaine aldehyde from being converted to TMA. However, this was not the case, as we found that after addition of betaine aldehyde, the radiolabel remained in choline and did not accumulate as betaine aldehyde (*Figure 4*). In addition, we found that betaine aldehyde itself was not a substrate for the TMA-forming enzyme of *S. sanguis I* (*Figure 1*). For these reasons, we believe that betaine aldehyde was not an intermediate in this pathway.

It is likely that the TMA-forming enzyme of *S. sanguis I* cleaves the C-N bond of choline to make TMA in the same manner as has been previously described for *P. mirabilis*.[30] In *P. mirabilis*, the choline-cleaving activity was coupled to the dismutation of acetaldehyde and required factors in the supernatant fraction and particulate fractions.[30] In *S. sanguis I*, TMA-forming activity was only present in the particulate fraction (*Figure 2*). Assuming that the overall pathway in *S. sanguis I* was similar to that of *Proteus* sp, the system for dismutation of acetaldehyde must have been present in the particulate fraction or dismutation must not have been coupled to choline cleavage. Acetaldehyde was also an intermediate formed when the C-N bond of choline was cleaved by other bacteria.[15,30,45] Presumably, the dismutation of acetaldehyde was catalyzed by alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (EC 1.2.1.10), and acetokinase (EC 2.7.2.1), as has been suggested for *Proteus* sp.[30] Semicarbazide reacts with carbonyl groups of ketones or aldehydes to form the more stable semicarbazone. The complete inhibition of TMA formation by semicarbazide (*Table 1*) suggests the possible presence of an intermediate with carbonyl group (e.g., acetaldehyde) when choline gets cleaved. Trimethylamine production and the dismutation of acetaldehyde were decreased by semicarbazide in *P. mirabilis*.[30] Sodium azide (1 mM) did not inhibit TMA production in *S. sanguis I* (*Table 1*), which is in agreement with the data for *D. desulfuricans*[14] and for *P. mirabilis*.[30] 2,4-Dinitrophenol inhibited TMA formation by *S. sanguis I* (*Table 1*). The mechanism for inhibition by 2,4-dinitrophenol is not known. Although this effect may be due to uncoupling of oxidative phosphorylation, 2,4-dinitrophenol inhibition of TMA formation from choline has also been reported in *D. desulfuricans*,[14] in *Clostridium* sp,[46] and in *P. mirabilis*[30] even under anaerobic conditions. In the *Proteus* sp system, 2,4-dinitrophenol was a much more effective inhibitor of TMA production than of dismutation.[30]

The activity we describe has several other similarities to the activities previously described in other bacteria. Trimethylamine-forming activity in *D. desulfuricans* and in *P. mirabilis* required ferrous iron.[15,30] The TMA-forming enzyme of *S. sanguis I* requires a divalent cation which could be Co^{2+} or Fe^{2+}. Although activity lost after treatment with EDTA could be restored by Co^{2+} or Fe^{2+}, our data do not prove that there is an absolute requirement for these ions. Less tightly bound metal ions may have been displaced from EDTA by the twofold excess of Co^{2+} and Fe^{2+} that we added. The TMA-forming enzyme of *S. sanguis I* was inhibited by iodoacetate. This has also been reported for TMA formation in aerobic *P. mirabilis*, and in anaerobic *D. desulfuricans*.[14,30] The enhancing effect of sulfhydryl-reducing agents (glutathione, 2-mercaptoethanol, DL-dithiothreitol) on TMA formation by *S. sanguis I* (*Table 1*) may be secondary to the protection of such a sulfhydryl group. In *P. mirabilis*, only glutathione, and not other sulfhydryl-reducing agents, enhanced choline degradation.[30] We found that sodium bisulfite stimulated the rate of TMA formation by *S. sanguis I* (*Table 1*). Bradbeer[45] also noted this effect when studying TMA formation in *Clostridia* sp. Sodium bisulfite can reduce disulfide bonds. In *Proteus* sp, bisulfite inhibits TMA formation by reacting with the presumed intermediate acetaldehyde.[30]

We were interested in determining whether TMA production increased as an adjustment to exposure to increased ionic strength (osmolarity) in the environment. Small molecules, including choline and betaine, play an important role in cell volume homeostasis as organic osmolytes in *Escherichia coli*.[47] We observed a decrease in TMA production when cells were exposed to increasing ionic strength (*Figure 5*), which suggests that TMA was not being formed for use as an osmoprotectant.

In summary, we identified a bacteria within the oral cavity that can form TMA from dietary choline. Once formed, TMA can be demethylated by bacterial species to form dimethylamine.[11,29,48] Nitrites are also present in the oral cavity. It is possible that dimethylnitrosamine is formed within the mouth from these precursors. In addition, the acidic conditions existing in the stomach favor the formation of nitrous anhydride and nitrosyl compounds which nitrosate dimethylamine and TMA to form dimethylnitrosamine.[49]

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