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Effects of a Chlorhexidine Gluconate-Containing Mouthwash on the Vitality and Antimicrobial Susceptibility of In Vitro Oral Bacterial Ecosystems

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Oral bacterial microcosms, established using saliva inocula from three individuals, were maintained under a feast-famine regime within constant-depth film fermenters. Steady-state communities were exposed four times daily, postfeeding, to a chlorhexidine (CHX) gluconate-containing mouthwash (CHXM) diluted to 0.06% (wt/vol) antimicrobial content. The microcosms were characterized by heterotrophic plate counts and PCR-denaturing gradient gel electrophoresis (DGGE). CHXM caused significant decreases in both total anaerobe and total aerobe/facultative anaerobe counts ($P < 0.05$), together with lesser decreases in gram-negative anaerobes. The degree of streptococcal and actinomycete inhibition varied considerably among individuals. DGGE showed that CHXM exposure caused considerable decreases in microbial diversity, including marked reductions in *Prevotella* sp. and *Selenomonas infelix*. Pure-culture studies of 10 oral bacteria (eight genera) showed that *Actinomyces naeslundii*, *Veillonella dispar*, *Prevotella nigrescens*, and the streptococci were highly susceptible to CHX, while *Lactobacillus rhamnosus*, *Fusobacterium nucleatum*, and *Neisseria subflava* were the least susceptible. Determination of the MICs of triclosan, CHX, erythromycin, penicillin V, vancomycin, and metronidazole for microcosm isolates, before and after 5 days of CHXM exposure, showed that CHXM exposure altered the distribution of isolates toward those that were less susceptible to CHX ($P < 0.05$). Changes in susceptibility distributions for the other test agents were not statistically significant. In conclusion, population changes in plaque microcosms following repeated exposure to CHXM represented an inhibition of the most susceptible flora with a clonal expansion of less susceptible species.

Chlorhexidine (CHX), a cationic bis-biguanide biocide with low mammalian toxicity and broad-spectrum antibacterial (6) activity, was first described in 1954 (5). The primary mechanism of action of this biocide is membrane disruption, causing concentration-dependent growth inhibition and cell death (18). Secondary interactions causing inhibition of proteolytic and glycosidic enzymes may also be significant (15). With respect to dental hygiene applications, the cationic nature of CHX enables it to bind to tooth surfaces and oral mucosa, reducing pellicle formation and increasing substantivity through controlled release of the agent (2). The efficacy of CHX in reducing oral bacterial viability (14, 36, 42), strongly inhibiting plaque regrowth, and preventing gingivitis (25) has been demonstrated in many studies (7). Relatively few investigations have considered longer-term effects of CHX use. An early study, however, demonstrated that oral treatment of human volunteers with CHX resulted in a 30 to 50% reduction in total bacterial counts with an associated reduction in counts of *Streptococcus mutans* (38).

Recent reports have demonstrated that the chlorinated diphenylether antibacterial triclosan (TCS) can select for mutants in the FabI gene of *Escherichia coli* at sublethal concentrations (23, 24, 31) that confer overt TCS resistance. Although the evidence is still ambiguous, assertions have been made that other antimicrobials might similarly select for resistance and that biocides in general could affect susceptibilities to chemically unrelated compounds (11, 24, 25, 40). With respect to CHX, use of this biocide has been associated with decreased susceptibility in hospital surveys (1, 21), while a recent report has associated a cation efflux pump with decreased CHX susceptibility in *Klebsiella pneumoniae* (8). Daily dental hygiene applications of CHX in human volunteers over 2 years, however, resulted in only a slight change in distribution toward those organisms which were less sensitive to CHX (38). Similar observations have also been made in animal studies (4).

Since the oral cavity represents an environment where chronic CHX exposure may occur, the aim of the present study was to investigate the impact of a CHX-containing mouthwash (CHXM) upon the microbial ecology and antimicrobial resistance properties of dental-plaque microcosms. These were grown in constant-depth film fermenters (CDFFs), which have previously been used to model complex (28, 35) and defined (19, 47) oral bacterial communities. Much of the microbial diversity of dental plaque is difficult to culture (22, 33), and many previous studies have used only viable-count procedures to characterize the ecological effects of CHX. In this study, culture-independent methods (denaturing gradient gel electrophoresis [DGGE]) (32, 48) were used to augment plate counts as a means of monitoring population dynamics in CHXM-exposed bacterial communities. In order to study the possible effects of CHXM exposure upon antimicrobial susceptibilities

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of exposed bacteria, the MICs of CHX, TCS, vancomycin (V), penicillin V (PV), metronidazole (MZ), and erythromycin (E) against the numerically dominant aerobic and facultative clones isolated both from baseline microcosms and following 5 days of CHX exposure were determined.

MATERIALS AND METHODS

MICs and MBCs. Stock solutions (4 mg/ml) of CHX, E, PV, MZ, and V were prepared in deionized, distilled water. TCS stock solutions were prepared in 25% ethanol. All solutions were sterilized by filtration through cellulose acetate filters (0.2-μm pore size; Millipore, Watford, Hertfordshire, United Kingdom) and stored at −70°C. MICs were determined by broth dilution endpoint using overnight cultures of reference strains or isolates from microcosms that were established using saliva from volunteer C. Test bacteria were grown in prereduced Wilkins-Chalgren broth within 96-well plates and then diluted to 10^5 CFU/ml in sterile prereduced broth within the anaerobic cabinet. In all cases, controls were run for the 25% ethanol TCS solvent. Following anaerobic incubation of MIC plates (37°C; 2 days), minimum bactericidal concentrations (MBCs) were determined by transferring 10 μl of broth from each well onto Wilkins-Chalgren agar within the anaerobic cabinet. Following incubation (37°C; 3 days), the MBCs were determined on the basis of the lowest concentration that prevented regrowth.

Continuous culture of in vitro oral bacterial ecosystems. CDFFs were used to grow dental bacteria under environmental conditions similar to those occurring in supragingival plaque (nutrient availability, presence of substrata, oxygen status, etc.). The apparatus consisted of a stainless steel rotor housing 15 removable polytetrafluoroethylene (PTFE) pans. Each PTFE pan holds five cylindrical pegs, which may be recessed to an accurate depth using calibrated rods. In operation, two spring-loaded PTFE blades constantly scrape the surface of the rotor and ensure that the microcosm can grow only to the depth to which the plugs have been recessed (34). The fermentation system was located in a sealed glass unit to prevent contamination and to enable control of the gaseous environment (35). Teflon substrata were used as previously described (50). The temperature (36°C) was maintained by locating the fermenters within a Perspex incubation chamber (Stuart Scientific, Redhill, Surrey, United Kingdom). The CDFF plugs were set to a depth of 200 μm, and the rotor speed was 3 rpm. A modified artificial-saliva medium was used (35, 41), containing (in grams per liter in distilled water) mucin (type II; porcine; gastric), 2.5; bacteriological peptone, 2.0; tryptone, 2.0; yeast extract, 1.0; NaCl, 0.35; KCl, 0.2; CaCl, 0.2; cysteine hydrochloride, 0.1; hemin, 0.001; and vitamin K_1, 0.0002. Saliva used for inoculation was obtained from three healthy adults (one female and two male) aged 24, 26, and 30 years and designated A, B, and C. These individuals had no history of periodontal disease and had used non-biocide-containing dentifrice exclusively for at least 5 months prior to saliva donation. Volunteers A, B, and C had taken no antibiotics for the previous 8 months and 2 and 5 years, respectively. Prior to inoculation, the CDFF plug surfaces were conditioned for 24 h with culture medium, which was continuously added to each fermenter by a peristaltic pump (9.6 ± 0.2 ml/h) (Minipuls 3; Gilson). The fermenters were inoculated with fresh saliva on three separate occasions (2.0 ± 0.5 ml/fermenter/inoculation) over a period of 24 h using fresh, pooled saliva from the donor. Anaerobiosis was maintained within the CDFFs by constant gassing with an anaerobic gas mixture (5:95 CO_2-N_2) at 1 liter/h. In order to simulate increased bacterial growth substrate conditions, which may occur in the mouth following a meal, plaque microcosms received an additional, electronically timed intermittent feeding (four times daily; 19 ml/h; 30-min duration), as previously described and validated (27, 28). The composition was (in grams per liter in distilled water) soluble starch, 5.0; casein, 3.0; bacteriological peptone, 3.0; sucrose, 2.0; yeast extract, 2.5; NaCl, 4.5; K_2HPO_4, 0.2; $CaCl_2$, 0.4; and $NaHCO_3$, 0.2. Once dynamic steady states were established (as evidenced by stability of the colony counts), CHXM, diluted to 0.06% (wt/vol) CHX content in sterile distilled water, was added over 5 days by peristaltic pump (8 ml/h for 5 min) immediately following each feeding. Throughout, samples were taken at regular intervals and processed in <30 min for bacteriology or archived at −80°C for subsequent DGGE analysis.

Differential bacteriological analysis. The selection of bacterial populations for use as markers of microcosm dynamics was based on numerical importance, together with ease of cultivation. For enumeration, samples of human saliva (1 ml) or dental microcosm (three sample plugs) were homogenized by mechanical shaking (0.5 min; 240 oscillations per min) in a bead beater (Griffin Scientific, London, United Kingdom). The maceration of microcosms grown on CDFF plugs was aided by the addition of 1.5 g of 3.5- to 5.5-mm-diameter sterile glass beads (BDH, Poole, United Kingdom). Samples were then serially diluted using prereduced, half-strength thioglycolate medium (USP). Appropriate dilutions (0.05 ml) were then plated in triplicate onto a variety of selective and nonselective media using a model CU spiral plater (Spiral Systems, Cincinnati, Ohio). These media were Wilkins-Chalgren agar (total anaerobes; confirmed during subculture); Wilkins-Chalgren agar with gram-negative supplements (total gram-negative anaerobes); cadmium, fluoride, acriflavin, tellurite agar (dental actinomycetes) (51); Rogosa agar (total lactobacilli); trypticase yeast extract, cysteine, sucrose agar (*Streptococcus* spp.) (46); and nutrient agar (total aerobes and facultative anaerobes). These agars were placed immediately in an anaerobic chamber (atmosphere, 10% H_2, 10% CO_2, 80% N_2) and maintained at 37°C for up to 5 days, except for nutrient agar, which was incubated aerobically in a standard incubator for 3 days. Morphologically distinct bacterial colonies were counted, subcultured, and archived at −80°C for subsequent identification.

Characterization of resistance properties. Isolated cell clones were randomly and exhaustively removed from isolation plates and archived (−80°C). MICs were then determined using the methods outlined above.

DGGE analysis. Archived in vitro microcosm samples (three CDFF plugs) from microcosms established using saliva from volunteer C were mixed with 1 ml of sterile sodium phosphate buffer (0.12 M; pH 8.0), vortexed, and subjected to two cycles of freeze-heating (−60°C for 10 min; 60°C for 2 min). Samples were then transferred to a bead beater vial containing 0.3 g of sterile zirconia beads (0.1 mm diameter). Tris-equilibrated phenol (pH 8.0; 150 μl) was added, and the suspension was shaken three times for 80 s each time at maximum speed (Mini-Bead-Beater; Biospec Products, Bartlesville, Okla.). After 10 min of centrifugation at 13,000 × *g*, the supernatant was extracted three times with an equal volume of phenol-chloroform and once with chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated from the aqueous phase with 3 volumes of ethanol, air dried, and resuspended in 100 μl of deionized water. The amount and quality of DNA extracted were estimated by electrophoresis of 5-μl aliquots on a 0.8% agarose gel and in comparison to a molecular weight standard (stained using ethidium bromide). DNA extracts were stored at −80°C prior to analysis. The V2-V3 region of the 16S ribosomal DNA (rRNA gene; corresponding to positions 339 to 539 of *E. coli*) was amplified with the eubacterium-specific primers HDA1 (5′-GC CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) used by Walter et al. (48). The reactions were performed in 0.2-ml tubes using a Perkin-Elmer (Cambridge, United Kingdom) DNA thermal cycler (model 480) and Red *Taq* DNA polymerase ready mix (25 μl) (Sigma, Poole, Dorset, United Kingdom), the HDA primers (2 μl each; 5 mM), nanopure water (16 μl), and extracted community DNA (5 μl). Optimization studies, as described by Muyzer and Smalla (32), showed that the extracted community DNA required a minimum of 1:10 dilution to ensure reliable PCRs. The thermal program was as follows: 94°C (4 min), followed by 30 thermal cycles of 94 (30 s), 56 (30 s), and 68°C (60 s). The final cycle incorporated a 7-min chain elongation step (68°C). PCR products, derived from microcosm community samples, were resolved as follows. A D-code universal mutation detection system (Bio-Rad, Hemel Hempstead, United Kingdom) with 16- by 16-cm, 1-mm-deep polyacrylamide gels (8%), run with 1× TAE buffer diluted from 50× TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, and 1 mM EDTA), was used for these analyses. Initially, separation parameters were optimized by running PCR products from selected pure cultures of bacteria and PCR amplicons from extracted community DNA on gels with a 0 to 100% denaturation gradient perpendicular to the direction of electrophoresis (a 100% denaturing solution contained 40% [vol/vol] formamide and 7.0 M urea). Denaturing gradients were formed with two 8% acrylamide (acrylamide-bisacrylamide, 37.5:1) stock solutions (Sigma). On this basis, a denaturation gradient for parallel DGGE analysis ranging from 30 to 60% was selected. PCR amplicons from *Fusobacterium nucleatum* (ATCC 10953), *Lactobacillus rhamnosus* (AC413), *Neisseria subflava* (A1078), *Porphyromonas gingivalis* (W50), *Actinomyces naeslundii* (WVU627), and *Prevotella nigrescens* (T588) were run on a parallel gel in order to validate the separation conditions. For community analyses, the gels also contained a 30 to 60% denaturing gradient. Electrophoresis was carried out at 150 V and 60°C for ~4.5 h. All gels were stained with SYBR Gold stain (diluted to 10^{-4} in 1× TAE) [Molecular Probes (Europe), Leiden, The Netherlands] for 30 min. The gels were viewed and images were documented using a BioDocit system (UVP, Upland, Calif.).

Partial 16S ribosomal DNA gene sequencing of bacterial isolates and excised gel bands. All morphologically distinct colonies from each of the isolation media were subcultured on Wilkins-Chalgren agar. Bacterial colonies (two or three) were aseptically removed from the surface of the plate and homogenized in a reaction tube containing nanopure water (100 μl). The bacterial suspensions were heated to 100°C in a boiling water bath for 10 min and centrifuged for 10 min (10,000 × *g*). The supernatants were used as templates for PCR. Partial 16S

rRNA gene sequences were amplified using the primers 8FPL1 (5′-GAG TTT GAT CCT GGC TCA G-3′) and 806R (5′-GGA CTA CCA GGG TAT CTA AT-3′) at 5 μM each. Each PCR consisted of Red *Taq* DNA polymerase ready mix (25 μl of forward and reverse primers; 2 μl each; 5 μM), nanopure water (16 μl), and template DNA (5 μl). A Perkin-Elmer model 480 thermal DNA cycler was used to run 35 thermal cycles as follows: 94°C (1 min), 53°C (1 min), and 72°C (1 min). The final cycle incorporated a 15-min chain elongation step (72°C). For analysis of the major resolved DGGE amplicons, selected resolved bands were cut out of the polyacrylamide gels using a sterile scalpel under UV illumination and incubated at 4°C for 20 h together with 20 μl of nanopure water in nuclease-free universal bottles. Portions (5 μl) were removed and used as templates for a PCR identical to that outlined in "DGGE analysis" above. The reverse (non-GC clamp) primer (HDA2) was used in subsequent sequencing reactions. PCR products were purified using Qiaquick PCR purification kits (Qiagen Ltd., Crawley, West Sussex, United Kingdom) and sequenced. The sequencing cycles were 94°C (4 min), followed by 25 cycles of 96°C (30 s), 50°C (15 s), and 60°C (4 min). Once chain termination was complete, sequencing was done in a Perkin-Elmer ABI 377 sequencer. DNA sequences were compiled using Genetool Lite version 1.0 (http://www.biotools.com) to obtain consensus sequences or to check and edit unidirectional sequences. For excised DGGE band PCRs, the presence of a GC clamp upon sequence analysis confirmed that the correct target had been reamplified rather than an extraneous contaminant.

Sequence databases. BLAST (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST) searches were performed, with each compiled sequence matched against those in the EMBL prokaryote database.

Chemicals. Unless otherwise stated, chemicals and antimicrobial agents were obtained from Sigma. Formulated bacteriological media were purchased from Oxoid, Basingstoke, United Kingdom. TCS (DP300) was obtained from Oils and Soaps Ltd., Bradford, United Kingdom. The CHXM (Corsodyl; SmithKline Beecham) formulation contained 0.2% (wt/vol) CHX gluconate and 7% (vol/vol) ethanol, together with polyoxyethylene hydrogenated castor oil, sorbitol, and peppermint oil.

Statistical analyses. Viable-count data at baseline and following 48 h of CHXM exposure for three separate saliva donors were subjected to *F* tests and then to two-sample Student *t* tests using Microsoft Excel. Individual MIC measurements for bacteria, randomly isolated before and after 5 days of CHXM exposure, were grouped as total bacteria or streptococci and arranged in distribution tables. These data were then subjected to χ^2 analysis using a Microsoft Excel macro.

Nucleotide sequence accession numbers. Sequences for isolated cell clones and sequenced DGGE amplicons were deposited in the EMBL sequence database. The accession numbers are as follows: (cell clones) *C. freundii* MBRG 8.2, AJ514240, and *Prevotella buccae* MBRG 8.1, AJ514252; (DNA amplicons) *Citrobacter* sp. strain CH1, AJ519803; uncultured *Variovorax* sp. strain B1, AJ519804; uncultured *Prevotella* sp. strain B2, AJ519805; uncultured beta proteobacterium species strain B3, AJ519806; uncultured alpha proteobacterium species strain B4, AJ519807; and *S. infelix* B5, AJ519808.

RESULTS

CHX susceptibility of selected oral type strains. Susceptibilities and MIC/MBC ratios varied considerably within genera (streptococci) and within gram-positive and gram-negative bacterial groups (Table 1). With respect to MIC data, *A. naeslundii* was the most susceptible bacterium, followed by the gram-negative anaerobes *Prevotella nigrescens*, *Porphyromonas gingivalis*, and *Veillonella dispar*. *S. mutans* and *Streptococcus sanguis* were also highly susceptible. *F. nucleatum* and *L. rhamnosus* were considerably less susceptible.

Bacteriological effects of CHXM. The data in Fig. 1 show culture-based enumeration of selected bacterial groups within the microcosms. Anaerobic counts of ca. 7 $\log_{10}$ CFU/mm^2 occurred in the fermenters, with lower numbers of aerobic and facultative species (ca. 6.5 $\log_{10}$ CFU/mm^2). Large numbers of streptococci and putative actinomycetes were also isolated. *Lactobacillus* counts ranged between <1.0 and ~4.0 $\log_{10}$ CFU/mm^2. Putative stability was attained in the fermenters within 3 days of inoculation and was maintained within baseline microcosms (data not shown). Addition of CHX caused statistically significant ($P < 0.05$) decreases in total anaerobes and total aerobes/facultative anaerobes (Student's *t* test), while interindividual variations were considerable for effects on gram-negative anaerobes, streptococci, and actinomycetes, so that the effects overall were not statistically significant (Fig. 1).

TABLE 1. MICs and MBCs of CHX against selected type strains of oral bacteria[a]

	MIC	MBC	Ratio
A. naeslundii WVU627	1.95	13 (3.7)	1:7
F. nucleatum ATCC 10953	15.6	20.8 (7.4)	1:1
L. rhamnosus AC413	10.4 (3.7)	13.0 (3.7)	1:1
N. subflava A1078	7.8	7.8	1:1
Prevotella nigrescens T588	3.3 (0.9)	20.8 (7.4)	1:6
Porphyromonas gingivalis W50	3.9	3.9 (2.7)	1:1
S. mutans NCTC10832	3.9	83.3 (29.5)	1:21
S. sanguis NCTC7863	3.9	13 (3.7)	1:3
Streptococcus oralis NCTC11427	7.8	7.8	1:1
V. dispar ATCC 17745	3.9	6.5 (1.8)	1:2

[a] Data were determined by broth dilution endpoint (doubling dilutions). The units are milligrams per liter. The data show means from replicate experiments ($n = 3$). Where data varied between replicates, standard deviations have been given in parentheses.

DGGE analysis. Based on viable-count data, samples from the microcosm showing the greatest dynamic change in response to CHXM (volunteer C) were subjected to PCR-DGGE in order to study dynamics among possible unculturable bacterial species. The presence of large numbers of bands (>20) on the gels at baseline (Fig. 2) indicated considerable

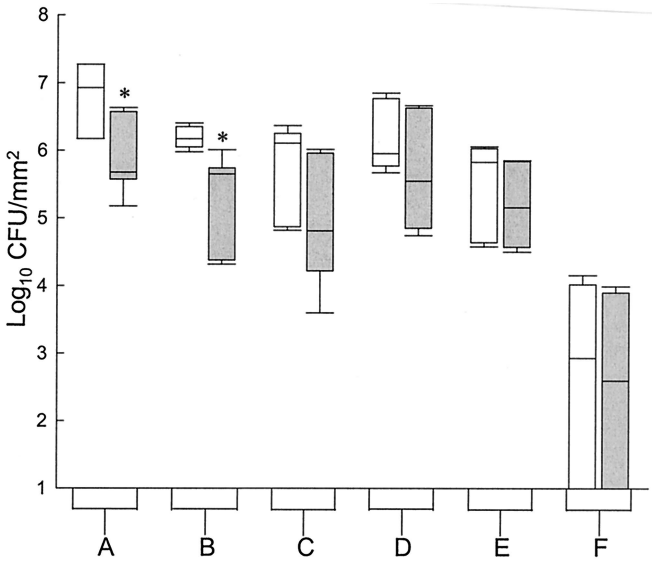

FIG. 1. Effect of 48-h exposure to pulsed CHXM (Corsodyl), adjusted to 0.06% (wt/vol) antimicrobial content, on groups of oral bacteria in dental-plaque microcosms. (A) Total strict anaerobes; (B) total aerobes and facultative anaerobes; (C) gram-negative anaerobes; (D) total streptococci; (E) actinomycetes; (F) total lactobacilli. The boxes represent distributions for three separate experiments (open, baseline samples; shaded, treated samples), each utilizing saliva from a different individual (volunteers A to C) as the inoculum. The horizontal bars within the boxes represent median values; the error bars indicate standard deviations. *, $P < 0.05$ (Student's *t* test).

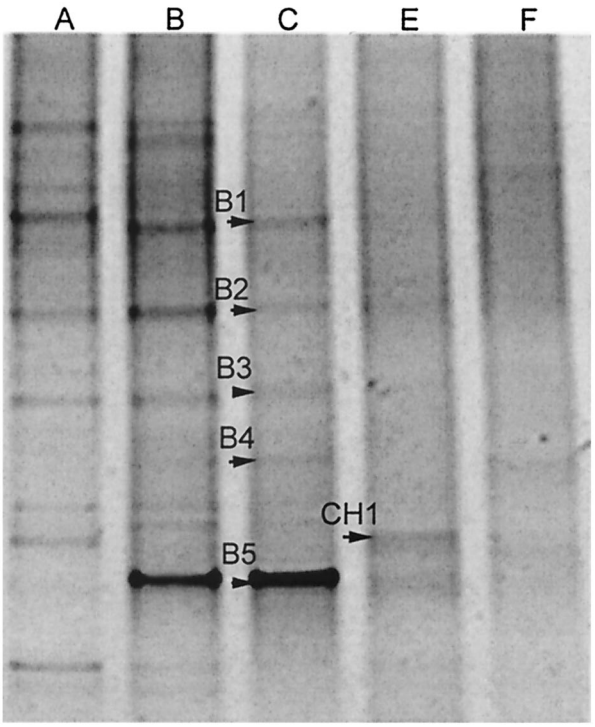

FIG. 2. Negative image of a parallel DGGE gel showing microcosm samples removed on days 3 (A), 5 (B), and 11 (C) (baseline), together with days 15 (E) and 17 (F) (after 3 and 5 days of CHXM addition, respectively). Bands B1 to B5 and CH1 are indicated.

eubacterial diversity within the microcosms, although only a few species (<6) dominated. These dominant organisms were related to the gram-negative anaerobic oral bacteria *Prevotella* sp. (B2 in Fig. 2) and *Selenomonas infelix* (B5) (20) and to unidentified alpha (B4) and beta (B3) proteobacteria (Table 2). A bacterium with homology to the freshwater genus *Variovorax* (B1) was also detected at baseline. Dynamic changes were apparent within the microcosms prior to the addition of CHXM. For example, *S. infelix* became detectable between days 3 and 5, although the majority of species appeared to be under putative stability, as evidenced by the stable maintenance of the majority of bands, including the major bands B1 to B4. CHXM exposure caused massive decreases in eubacterial diversity, as evidenced by a reduction in the total number of DGGE bands, together with major decreases in the abundance of amplicons B1 to B4. Following CHXM treatment, a bacterium with homology to the enteric bacterium *Citrobacter freundii* (band CH1) became clonally expanded within the microcosm, as indicated by the appearance of band CH1 (Fig. 2).

Effect of CHXM on microcosm drug susceptibilities. Tables 3 and 4 show distributions of MICs for streptococci and total bacterial clones, respectively, expressed as percentages of total numbers of randomly selected clones. χ^2 analysis of these data demonstrated that the only statistically significant shift in the susceptibility distribution occurred for the susceptibility of total bacteria to CHX.

DISCUSSION

Relatively few studies have used culture-independent techniques to measure bacteriological effects of biocides in dental microcosms; most rely on selective isolation. The possible effect of biocides on antimicrobial susceptibility in dental plaque has also received little research attention. The aims of this study were, therefore, to combine culture with DGGE to investigate dynamic changes within dental-plaque microcosms caused by 5 days of exposure to CHXM. A secondary objective was to evaluate such dynamic changes in terms of the community susceptibility profile. Microcosms were grown in CDFFs under steady-state conditions using a previously validated feast-famine regimen (27, 28).

CHX susceptibilities (MICs and MBCs) were determined for 10 dental bacteria, comprising a consortium of organisms used extensively for studies of mixed cultures of plaque bacteria (3, 26, 30). These data are shown in Table 1. High MIC/MBC ratios suggest that growth inhibition and lethality are related to interaction with different targets. This appears to be particularly relevant for *Prevotella nigrescens*, *S. mutans*, and *A. naeslundii* (Table 1). Interestingly, defined community studies using bacteria similar to those tested in this study support the susceptibility profiles reported here. Addition of CHX caused the loss of *A. naeslundii* from in vitro CDFF plaques, while *Porphyromonas gingivalis* was markedly inhibited (19). Similarly, defined community chemostat studies confirmed the non-

TABLE 2. Characterization of dynamic changes in microcosms from sequences of dominant PCR amplicons at baseline and following 5 days of exposure to CHXM

Time	DNA amplicon or cell clone	Ambiguity		Closest relative (% sequence similarity)[c]
		bp	%	
Baseline	B1[a]	170	14	*Variovorax paradoxus* AY127900 (93)
	B2[a]	174	14	Uncultured *Prevotella* sp. strain AJ514233 (100)
	B3[a]	174	24	Uncultured beta proteobacterium species strain AY144221 (83)
	B4[a]	177	23	Uncultured alpha proteobacterium species strain AF446310 (89)
	MBRG 8.1[b]	763	0.4	*Prevotella buccae* L16478 (99)
	B5[a]	261	3.8	*S. infelix* AJ514231 (100)
After CHXM	CH[a]	181	11.6	*C. freundii* AJ233408 (87)
	MBRG 8.2[b]	763	0	*C. freundii* AF25365 (99)

[a] Amplicon derived from DGGE gel. See Fig. 1 for origin of PCR DGGE amplicons.
[b] Amplicon derived from isolated cell clones.
[c] Identities based on BLAST database.

TABLE 3. Distribution of MICs in percentages of total streptococcal clones

Antimicrobial	No. of clones tested		% of clones in MIC range (mg/liter) of[a]:											
			0.01–0.1		0.11–1.0		1.1–10.0		10.1–100		100.1–200		>200	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CHX	23	7	43	29	9	43	35	28	13	0	0	0	0	0
TCS	23	7	0	0	39	29	26	0	35	57	0	24	0	0
E	23	7	32	14	5	0	0	0	21	0	5	0	37	86
PV	23	7	35	14	0	0	5	14	30	29	17	14	13	29
V	23	7	0	0	14	15	14	14	0	0	0	0	72	57
MZ	10	4	0	0	0	0	0	0	83	50	17	50	0	0

[a] The data show MICs as determined by broth dilution (replicate determinations for each isolate; $n = 4$) for randomly isolated cell clones at baseline (Before) and following 5 days of CHXM exposure (After).

susceptibility of *Lactobacillus casei* (29), suggesting that hierarchies of susceptibility in pure culture can be extrapolated to community effects.

In a previous study, large variations in the composition of microcosm plaques in replicate fermenters were demonstrated using the same pooled saliva as inocula (28). Therefore, in order to maximize the validity of these experiments, separate microcosms were established using saliva from three human volunteers. The efficacy of this approach is supported by the large interindividual variation in count data shown in Fig. 1, both at baseline and with respect to variations in the magnitudes of antimicrobial effects, which would not have been revealed using pooled inocula. This variation is presumably due to interindividual variation in bacterial carriage, represented in the microcosms. Comparable specificity and variation in effects have been shown in many previous studies, including an investigation using a novel supragingival-plaque model (13), in human volunteer studies (38), and in a defined bacterial ecosystem (3, 30). Considerable variation in the extent of effects is also apparent between laboratories and is probably due to variation in experimental parameters or among individual donors.

In this study, the use of discontinuous feeding prior to each addition of CHXM would tend to increase the amplitude of antimicrobial effects, since actively growing bacteria are generally most susceptible to antimicrobial effects (12) and feeding may have relieved nutrient limitation and enhanced susceptibility. This approach is arguably more representative of reality than more reductive approaches, since dental-plaque communities may frequently be exposed to biocidal products following a meal and plaque communities are normally subjected to feast-famine conditions in situ (28).

The presence and clonal expansion of *C. freundii* during CHX addition is interesting. This organism, although not a classic oral bacterium, was a resident species in the mouth of the volunteer and was comparatively nonsusceptible to CHX (data not shown), conferring a selective advantage during dosing. With respect to unculturable species within the fermenters, of the six dominant phylotypes identified by DGGE, only *Prevotella buccae* (MBRG 8.1) and *C. freundii* (MBRG 8.2) had been isolated by exhaustive culture procedures, demonstrating the importance of adopting culture-independent methods. In fact, the proportion of yet-to-be-cultivated bacteria in subgingival plaque-type ecosystems has been estimated at >50% (22).

The detection of nontypical oral species in the microcosms reinforces the utility of DGGE over hybridization methods and real-time PCR, since there is no experimental bias toward typical resident oral species. DGGE will theoretically identify any amplifiable target sequence above detection thresholds (32), whereas hybridization techniques measure the abundance of a finite number of species (17, 43, 44). The use of DGGE to monitor dynamic changes in microbial ecosystems may be complicated by the detection of nonviable organisms, since valid real-time analysis depends on rapid turnover of dead cells and the degradation of associated DNA within the test community. In this respect, the considerable proteolytic activities detected in plaque ecosystems (49) are likely to rapidly degrade dead cells, while the half-life of target DNA is likely to be low, since many streptococci and other plaque microorganisms produce

TABLE 4. Distribution of MICs as percentages of total bacterial clones

Antimicrobial	No. of clones tested		% of clones in MIC range (mg/liter) of[a]:											
			0.01–0.1		0.11–1.0		1.1–10.0		10.1–100		100.1–200		>200	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CHX[b]	45	29	46	26	12	53	19	7	28	14	0	0	0	0
TCS	48	31	0	0	26	16	28	7	35	45	11	26	0	6
E	42	29	11	8	11	3	0	0	20	0	11	8	48	81
PV	43	29	9	0	5	4	17	10	29	7	12	34	28	45
V	48	30	13	0	18	7	0	3	0	0	2	3	67	87
MZ	15	8	0	0	0	0	0	0	67	37	38	13	13	50

[a] See legend to Table 3.
[b] $P < 0.05$ (χ^2).

nucleases (9). Validation studies in our laboratory using samples taken at 12-h time intervals suggested that turnover was considerably faster than 12 h (data not shown).

Care should be taken when making phylogenetic inferences from sequenced DGGE bands, since derived sequences are short and may be of variable quality (Table 2). Such ambiguities probably arise from amplification of different phylotypes with similar or identical electrophoretic mobilities. The relatively short sequences derived from DGGE also reduce the refinement of phylogenetic determination. Despite these concerns, DGGE is currently one of the only techniques that allows reproducible visual comparisons of profiles from microbial communities to be derived and has been successfully applied to a wide variety of microbial ecosystems (37, 45, 48).

When a bacterial community is subjected to an inimical treatment, the antimicrobial agent may (i) select for resistant mutants of susceptible bacteria or (ii) alter the susceptibility distribution toward those organisms which were originally less susceptible. It appears that in this study the latter scenario occurred. Furthermore, statistically significant alterations in susceptibility to antimicrobials that were chemically unrelated to CHX were not observed (Tables 3 and 4).

With respect to the possible selection for resistance in normally susceptible bacteria, previous attempts to train CHX-resistant *E. coli*, streptococci, and *Staphylococcus aureus* in pure culture by repeated sublethal exposure have been largely unsuccessful (10, 16). This has been borne out in situ by clinical experience and in long-term human volunteer studies (39). To our knowledge, there have been few published studies that have examined susceptibility effects of CHX in complex ecosystems, and fewer still have studied possible changes in susceptibility to chemically unrelated antimicrobial compounds. The data presented in this paper not only support previous observations that CHX exposure alters the susceptibility distribution (39), they also provide evidence that this susceptibility change does not result in significant alterations in the distribution of sensitivity to chemically unrelated biocides and antibiotics.

Conclusions. In these investigations, we have used isolation techniques to demonstrate the broad-spectrum activity of CHX in dental-plaque microcosms. DGGE corroborated these observations, gave an indication of the stability of the baseline in vitro plaques, and enabled phylogenetic information about the major community phylotypes that altered in abundance during CHXM exposure to be obtained. Importantly, we have shown that susceptibility to a range of antibacterial compounds varied widely in microcosm isolates and that dynamic changes within these communities during CHX exposure included clonal expansion of less-susceptible bacterial strains. In terms of alterations in antimicrobial susceptibility distributions following CHXM exposure, a significant change in distribution toward reduced susceptibility occurred for total bacteria against CHX.

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REFERENCES

1. **Block, C., and M. Furman.** 2002. Association between intensity of chlorhexidine use and microorganisms of reduced susceptibility in a hospital environment. J. Hosp. Infect. **51:**201–206.
2. **Bonesvoll, P., P. Lokken, G. Rölla, and P. N. Paus.** 1974 Retention of chlorhexidine in the human oral cavity after mouthrinses. Arch. Oral Biol. **19**:1209–1212.
3. **Bradshaw, D. J., P. D. Marsh, G. K. Watson, and D. Cummins.** 1993. The effects of triclosan and zinc citrate, alone and in combination, on a community of oral bacteria grown *in-vitro.* J. Dent. Res. **72:**25–30.
4. **Briner, W. W., and J. A. Wunder.** 1977. Sensitivity of dog plaque microorganisms to chlorhexidine during longitudinal studies. J. Periodontal Res. **12:**135–139.
5. **Davies, G. E., J. Francis, A. R. Martin, F. L. Rose, and G. Swain.** 1954. 1:6-Di 4-chlorophenyldiguanidohexane ("Hibitane"). Laboratory investigation of a new antibacterial agent of high potency. Br. J. Pharmacol. **9:**192–196.
6. **Denton, G. W.** 1991. Chlorhexidine, p. 274–289. *In* S. S. Block (ed.), Disinfection, sterilization and preservation, 4th ed. Lea and Febiger, Philadelphia, Pa.
7. **Emilson, C. G.** 1994. Potential efficacy of chlorhexidine against mutans streptococci and human dental caries. J. Dent. Res. **73:**682–691.
8. **Fang, C. T., H. C. Chen, Y. P. Chuang, S. C. Chang, and J. T. Wang.** 2002. Cloning of a cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella pneumoniae.* Antimicrob. Agents Chemother. **46:**2024–2028.
9. **Ferreira, B. T., L. C. Benchetrit, A. C. De Castro, T. G. Batista, and L. Barrucand.** 1992. Extracellular deoxyribonucleases of streptococci: a comparison of their occurrence and levels of production among beta-hemolytic strains of various serological groups. Zentbl. Bakteriol. **277:**493–503.
10. **Fitzgerald, K. A., A. Davies, and A. D. Russel.** 1992. Sensitivity and resistance of *Escherichia coli* and *Staphylococcus aureus* to chlorhexidine. Lett. Appl. Microbiol. **14:**33–36.
11. **Fraise, A. P.** 2002. Biocide abuse and antimicrobial resistance—a cause for concern? J. Antimicrob. Chemother. **49:**11–12.
12. **Gilbert, P., P. J. Collier, and M. R. Brown.** 1990. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrob. Agents Chemother. **34:**1865–1868.
13. **Guggenheim, B., W. Giertsen, P. Schupbach, and S. Shapiro.** 2001. Validation of an in-vitro biofilm model of supragingival plaque. J. Dent. Res. **80:**363–370.
14. **Hase, J. C., S. Edwardsson, J. Rundegren, R. Attstrom, and E. Kelty.** 1998. Six-month use of 0.2% delmopinol hydrochloride in comparison with 0.2% chlorhexidine digluconate and placebo. II. Effect on plaque and salivary microflora. J. Clin. Periodontol. **25:**841–849.
15. **Hastings, D. C.** 2000. Non-antibiotic plaque chemotherapy, p. 523–548. *In* H. N. Newman and M. Wilson (ed.). Dental plaque revisited: oral biofilms in health and disease. Bioline Press, Cardiff, United Kingdom.
16. **Hennessey, T. S.** 1973. Some antibacterial properties of chlorhexidine. J. Periodont. Res. Suppl. **12:**61–67.
17. **Hopkins, M. J., R. Sharp, and G. T. Macfarlane.** 2001. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut **48:**198–205.
18. **Hugo, W. B., and A. R. Longworth.** 1966. The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus.* J. Pharm. Pharmacol. **18:**569–578.
19. **Kinniment, S. L., J. W. Wimpenny, D. Adams, and P. D. Marsh.** 1996. The effect of chlorhexidine on defined, mixed culture oral biofilms grown in a novel model system. J. Appl. Bacteriol. **81**:120–125.
20. **Kolenbrander, P. E., R. N. Andersen, and L. V. Moore.** 1989. Coaggregation of *Fusobacterium nucleatum*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia*, and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. Infect. Immun. **57:**3194–3203.
21. **Koljalg, S., P. Naaber, and M. Mikelsaar.** 2002. Antibiotic resistance as an indicator of bacterial chlorhexidine susceptibility. J. Hosp. Infect. **51:**106–113.
22. **Kroes, I., P. W. Lepp, and D. A. Relman.** 1999. Bacterial diversity within the human subgingival crevice. Proc. Natl. Acad. Sci. USA **96:**14547–14552.
23. **Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice, and J. B. Rafferty.** 1999. Molecular basis of triclosan activity. Nature **398:**383–384.
24. **Levy, S. B.** 2000. Antibiotic and antiseptic resistance: impact on public health. Pediatr. Infect. Dis. J. **19:**S120–S122.
25. **Löe, H., and C. R. Schiött.** 1970. The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. J. Peridontal Res. **5**:79–83.
26. **Marsh, P. D., J. R. Hunter, G. H. Bowden, I. R. Hamilton, A. S. McKee, J. M. Hardie, and D. C. Ellwood.** 1983. The influence of growth rate and nutrient

limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. J. Gen. Microbiol. **129:**755–770.
27. **McBain, A. J., R. G. Bartolo, C. E. Catrenich, D. Charbonneau, R. G. Ledder, A. H. Rickard, S. A. Symmons, and P. Gilbert.** 2003. Microbial characterization of biofilm in domestic drains and the establishment of stable biofilm microcosms. Appl. Environ. Microbiol. **69:**177–185.
28. **McBain, A. J., R. G. Ledder, and P. Gilbert.** 2003. Growth and molecular characterization of dental plaque microcosms. J. Appl. Microbiol. **94:**655–664.
29. **McDermid, A. S., A. S. McKee, and P. D. Marsh.** 1987. A mixed-culture chemostat system to predict the effect of anti-microbial agents on the oral flora: preliminary studies using chlorhexidine. J. Dent. Res. **66:**1315–1320.
30. **McKee, A. S., A. S. McDermid, D. C. Ellwood, and P. D. Marsh.** 1985. The establishment of reproducible, complex communities of oral bacteria in the chemostat using defined inocula. J. Appl. Bacteriol. **59:**263–275.
31. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Triclosan targets lipid synthesis. Nature **394:**531–532.
32. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek **73:**127–141.
33. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst.** 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. **183:**3770–3783.
34. **Peters, A. C., and J. W. T. Wimpenny.** 1988. A constant-depth laboratory model film fermenter. Biotechnol. Bioeng. **32:**263–270.
35. **Pratten, J., and M. Wilson.** 1999. Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. Antimicrob. Agents Chemother. **43:**1595–1599.
36. **Rosin, M., A. Welk, T. Kocher, A. Majic-Todt, A. Kramer, and F. A. Pitten.** 2002. The effect of a polyhexamethylene biguanide mouth rinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque re-growth. J. Clin. Periodontol. **29:**392–399.
37. **Satokari, R. M., E. E. Vaughan, A. D. Akkermans, M. Saarela, and W. M. de Vos.** 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67**:504–513.
38. **Schiött, C. R., W. W. Briner, J. J. Kirkland, and H. Löe.** 1976. Two year oral use of chlorhexidine in man. II. The effect on the salivary bacterial flora. J. Peridontal Res. **11:**145–152.
39. **Schiött, C. R., W. W. Briner, J. J. Kirkland, and H. Löe.** 1976. Two year oral use of chlorhexidine in man. III. Changes in sensitivity of the salivary flora. J. Peridontal Res. **11:**153–157.
40. **Schweizer, H. P.** 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol. Lett. **202:**1–7.
41. **Shah, H. N., R. A. D. Williams, G. H. Bowden, and J. M. Hardie.** 1976. Comparison of the biochemical properties of *Bacteroides melinogenicus* from human dental plaque and other sites. J. Appl. Bacteriol. **41:**473–492.
42. **Shapiro, S., E. Giertsen, and B. Guggenheim.** 2002. An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouthrinses. Caries Res. **36:**93–100.
43. **Socransky, S. S., C. Smith, and A. D. Haffajee.** 2002. Subgingival microbial profiles in refractory periodontal disease. J. Clin. Periodontol. **29:**260–268.
44. **Socransky, S. S., C. Smith, L. Martin, B. L. Paster, F. E. Dewhirst, and A. E. Levin.** 1994. "Checkerboard" DNA-DNA hybridization. BioTechniques **17:**788–792.
45. **Tresse, O., M. J. Lorrain, and D. Rho.** 2002. Population dynamics of free-floating and attached bacteria in a styrene-degrading biotrickling filter analyzed by denaturing gradient gel electrophoresis. Appl. Microbiol. Biotechnol. **59:**585–590.
46. **Van Palenstein, W. H., M. Ijsseldijk, and J. H. J. Huis In 'T Veld.** 1983. A selective medium for the two major subgroups of the bacterium *Streptococcus mutans* isolated from human dental plaque and saliva. Arch. Oral Biol. **28**:599–603.
47. **Vroom, J. M., K. J. De Grauw, H. C. Gerritsen, D. J. Bradshaw, P. D. Marsh, G. K. Watson, J. J. Birmingham, and C. Allison.** 1999. Depth penetration and detection of pH gradients in biofilms by two-photon excitation microscopy. Appl. Environ. Microbiol. **65:**3502–3511.
48. **Walter, J., G. W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D. M. Loach, K. Munro, and T. Alatossava.** 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. **66:**297–303.
49. **Wei, G. X., J. S. van der Hoeven, J. W. Smalley, F. H. Mikx, and M. W. Fan.** 1999. Proteolysis and utilization of albumin by enrichment cultures of subgingival microbiota. Oral Microbiol. Immunol. **14:**348–351.
50. **Wilson, M.** 1999. Use of constant depth film fermenter in studies of biofilms of oral bacteria. Methods Enzymol. **310:**264–279.
51. **Zybler, L. K., and H. V. Jordan.** 1981. Development of a selective medium for detection and enumeration of *Actinomyces viscosus* and *Actinomyces naseslundii* in dental plaque. J. Clin. Microbiol. **15:**253–259.