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 aerobic/facultative anaerobe counts ($P < 0.05$), together with lesser decreases in gram-negative anaerobes. The degree of streptococcal and actinomycte 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 more 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 due to 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...
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 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. CDFEs 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 repositioned 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. The fermentation system was located in a sealed glass unit to prevent contamination and to enable control of the gaseous environment (35). Teflon substrates 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, 1.3; NaCl, 0.5; KCl, 0.2; CaCl2·2H2O; 0.2; cystine hydrochloride, 0.1; hemin, 0.001; and vitamin K, 0.0002. Saliva used for inoculation was obtained from healthy individuals (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 donation. A, B, and C had taken no antibiotics for the previous 8 months and 5 years, respectively. Prior to inoculation, the CDFF plugs 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 CDFEs by constant gassing with an anaerobic gas mixture (595 CO2:N2) at 1 l/hr. 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; 1 min each feeding; 19 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, Los Angeles, Calif.). The maceration CDFF plugs were added to 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 subsequent subculture with Wilkins-Chalgren medium); gram-negative supravital stain; facultative anaerobes; cadmium, fluoride, acriflavin, tellurite agar (dental actinomyces (51); Rogosa agar (total lactobacilli); trypticase yeast extract, cysteine, sucrose agar (Streptococcus spp.) (46); and nutrient agar (total anaerobes and facultative anaerobes). These agar plates were incubated in an anaerobic chamber (at 37°C for 3 days). Following incubation (37°C; 3 days), the 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.

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 microcosms (three CDFF plugs) from microcosms established using saliva from volunteer C were mixed with 1 ml of sterile sodium phosphate buffer (pH 8.0, 800 mOsM/L) and subjected to two cycles of freeze-thawing (−60°C for 10 min; 40°C for 2 min). Samples were then transferred to a bead beater vial containing 0.5 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 30 s each time in a Minit Bead-Beater (BioSpec Products, Bartsville, 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-isooamyl alcohol (24:1 [vol/vol]). The RNA 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 eubacterial-specific primers HDA1 (5′-CGC CGG CCG CGG GCC GCC GCG GGC GCG GCG GGG GCA GGG GGC GCG GCC GGG GCG GGC GGG GCG GGC GCG GCC GGG GGG AGC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CCG CTG CTC GTA C3′) used by Walter et al. (48). The reactions were performed in 0.2-ml tubes using a Perkin-Elmer (Cetus, Norwalk, Conn.) 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 μM), nanopure water (16 μl), and extracted community DNA (5 μl). Optimization studies, as described by Mayuer 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 (30 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 DNA sequencing gel (Biorad, 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 (AH178), Porphyromonas gingivalis (W50), Actinomyces naeslundii (WWU627), and Prevotella nigrescens (TS88) 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× 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 1 ml of sterile saline (100 μl). These 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
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 CHX. The data in Fig. 1 show culture-based enumeration of selected bacterial groups within the microcosms. Anaerobic counts of ca. \(7 \times 10^{10} \text{ CFU/mm}^2\) occurred in the fermenters, with lower numbers of aerobic and facultative species (ca. \(6.5 \times 10^{10} \text{ CFU/mm}^2\)). Large numbers of streptococci and putative actinomycetes were also isolated. Lactobacillus counts ranged between \(1.0 \text{ and } 4.0 \times 10^{5} \text{ CFU/mm}^2\). Putative stability was attained in the fermenters within 3 days of inoculation and was maintained within base-line 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).

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
cubacterial 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 cubacterial 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. χ² 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-

![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.](http://aem.asm.org/)

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

<table>
<thead>
<tr>
<th>Time</th>
<th>DNA amplicon or cell clone</th>
<th>Ambiguity</th>
<th>Closest relative (% sequence similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bp</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>B1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>170</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>B2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>174</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>B3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>174</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>B4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>177</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>MBRG 8.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>763</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>B5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>261</td>
<td>3.8</td>
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<tr>
<td>After CHXM</td>
<td>CH1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>181</td>
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</tr>
<tr>
<td></td>
<td>MBRG 8.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>763</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amplicon derived from DGGE gel. See Fig. 1 for origin of PCR DGGE amplicons.

<sup>b</sup> Amplicon derived from isolated cell clones.

<sup>c</sup> Identities based on BLAST database.
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 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 CHX would tend to increase the amplitude of antimicrobial effects, since actively growing bacteria are generally more 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

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**Table 3.** Distribution of MICs in percentages of total streptococcal clones

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>No. of clones tested</th>
<th>% of clones in MIC range (mg/liter) of:</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.01–0.1</td>
<td>0.11–1.0</td>
<td>1.1–10.0</td>
</tr>
<tr>
<td>CHX</td>
<td>23/7</td>
<td>46/26</td>
<td>7/12</td>
<td>2/19</td>
</tr>
<tr>
<td>TCS</td>
<td>23/7</td>
<td>0/0</td>
<td>2/2</td>
<td>3/28</td>
</tr>
<tr>
<td>E</td>
<td>23/7</td>
<td>32/14</td>
<td>7/0</td>
<td>0/0</td>
</tr>
<tr>
<td>PV</td>
<td>23/7</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>V</td>
<td>23/7</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>MZ</td>
<td>10/4</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

* 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).

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**Table 4.** Distribution of MICs as percentages of total bacterial clones

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>No. of clones tested</th>
<th>% of clones in MIC range (mg/liter) of:</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.01–0.1</td>
<td>0.11–1.0</td>
<td>1.1–10.0</td>
</tr>
<tr>
<td>CHX</td>
<td>45/29</td>
<td>46/26</td>
<td>12/53</td>
<td>19/7</td>
</tr>
<tr>
<td>TCS</td>
<td>48/31</td>
<td>0/0</td>
<td>26/16</td>
<td>28/7</td>
</tr>
<tr>
<td>E</td>
<td>42/29</td>
<td>11/8</td>
<td>11/3</td>
<td>0/2</td>
</tr>
<tr>
<td>PV</td>
<td>43/29</td>
<td>0/0</td>
<td>5/4</td>
<td>17/10</td>
</tr>
<tr>
<td>V</td>
<td>48/30</td>
<td>13/0</td>
<td>18/7</td>
<td>0/3</td>
</tr>
<tr>
<td>MZ</td>
<td>15/8</td>
<td>0/0</td>
<td>0/0</td>
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</tr>
</tbody>
</table>

* See legend to Table 3.

b *P* < 0.05 (χ²).
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. Further, 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 CHX exposure, a significant change in distribution toward reduced susceptibility occurred for total bacteria against CHX.

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