Distinct Bacteriocin Groups Correlate with Different Groups of *Streptococcus mutans* Plasmids

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A correlation between the presence of 5.6-kilobase plasmids and bacteriocin activity was found in human-derived strains of *Streptococcus mutans*. Compared with bacteriocin activity of randomly selected clinical isolates of plasmid-negative strains, bacteriocin activity of plasmid-positive strains significantly inhibited not only two laboratory strains of *S. mutans*, OMZ176 and AHT (P < 0.0001 and P = 0.038, respectively), but most plasmid-negative clinical isolates as well (P = 0.0005). Comparisons of inhibition between pairs of plasmid-positive strains revealed two groups, group I and group II, that produced distinct bacteriocins we designated as mutacin I and mutacin II, respectively. Within each group, a strain produced inhibitory activity against all the members of the other group but against no members of its own group. Plasmid DNA from plasmid-positive strains of each group was isolated and analyzed by restriction endonuclease digestion. Plasmids from the two groups that were apparently identical in size differed in digestion patterns for EcoRI, HaeIII, and TaqI, even though six TaqI fragments appeared to be common to all. Based on bacteriocin profiles and restriction enzyme digests, two distinct groups of plasmid-positive *S. mutans* strains emerged. Although the bacteriocin activity of plasmid-positive strain LM7 (serotype e) placed it in group I, clear differences in restriction digests distinguished it from the other plasmid-containing strains.

Plasmids are found in virtually every species of the genus *Streptococcus* and mediate a wide variety of biological activities, including antibiotic or heavy-metal resistance, bacteriocin production and immunity, and various sexual functions (4). Other streptococcal plasmids have unknown functions and are designated cryptic. These include the 5.6-kilobase (kb) plasmid found within strains of the species *Streptococcus mutans* and first described in 1973 (7). A plasmid (or plasmids) having this size appears in *S. mutans* isolated from clinical populations at a prevalence of 5 to 13% (3, 14). With the exception of the serotype e strain LM7, all known plasmid-containing strains of *S. mutans* isolated from humans are biotype I (serotypes c/f) (3, 13, 14). Accordingly, the observations that plasmids isolated from human-derived *S. mutans* appear identical in size, are almost exclusively of one biotype, and exhibit similarities in restriction enzyme patterns suggested that a single disseminated plasmid was common to all strains (13, 14).

Previously, we used plasmid DNA as an epidemiological marker for denoting homology of *S. mutans* strains within families (3). In addition, we investigated several bacteriocin-typing methods (2, 8, 11), also used for ascertaining homology of strains. In doing this, we observed a relationship between the presence of plasmids and bacteriocin activity. (The preliminary results of this investigation have been reported [P. W. Caffield, N. Childers, J. B. Hansen, Y. Wannemuehler, and H. Austin, Abstr. Ann. Meet. Am. Soc. Microbiol. 1983, D89, p. 73].)

**MATERIALS AND METHODS**

Study population; collection and cultivation of samples; plasmid screening. Samples of whole, unstimulated saliva were collected from approximately 315 healthy children, aged 2 to 12 years, scheduled to receive routine dental care at the University of Alabama School of Dentistry. Isolates of *S. mutans* (usually four per child) were obtained and biotyped by methods previously described (3). Clinical isolates of *S. mutans* were screened for the presence of plasmid DNA by the method of Caffield et al. (3).

**Bacterial strains.** In addition to strains UA37, UA140, UA109, and UA405 (plasmid-positive representative clinical isolates selected for large-scale plasmid isolation and restriction enzyme characterization) (Table 1), 13 plasmid-positive and 28 plasmid-negative clinical isolates (not listed in Table 1) were used in bacteriocin activity testing. Laboratory strains of *S. mutans* representing the major serotypes were used in the bacteriocin activity assay; strains LM7 and V318 were also included as prototypes containing a 5.6-kb plasmid (14) (Table 1). Clinical isolates and laboratory strains used in experiments were stored frozen at −70°C.

**Demonstration of bacteriocin-like activity.** Production of and sensitivity to bacteriocin-like inhibitory substances were assayed by a modification of the deferred antagonism technique (8, 11). Strains used as producers were initiated from frozen culture into Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 24 h in an anaerobic atmosphere containing 85% N₂, 10% H₂, and 5% CO₂ at 37°C. Plates of Trypticase soy agar (BBL) supplemented with 2% yeast extract (Difco Laboratories, Detroit, Mich.) (Trypticase soy agar-yeast) were inoculated with a multiple-needle inoculator with the broth cultures of the producer strains. After 24 h of anaerobic incubation, stab plates were overlaid with 4.0 ml of molten Trypticase soy broth-yeast extract-soft agar (Trypticase soy broth with 0.75% agar and 2% yeast added) containing 0.5 ml of an overnight culture of an indicator strain, 2.5 mg of mannitol per ml, and 0.4 mg of 2,3,5-triphenyl tetrazolium chloride (TTC; Sigma Chemical Co., St. Louis, Mo.) per ml. The addition of mannitol and TTC enhanced the reading of zones of inhibition. Plates were scored for zones of inhibition by a single individual in a standardized manner. Before inoculation of plates, all producer strains were coded and then
TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>S. mutans strain</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>AHT</td>
<td>Serotype a</td>
<td>T. Shiota&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BHT</td>
<td>Serotype b</td>
<td>T. Shiota</td>
</tr>
<tr>
<td>10449</td>
<td>Serotype c</td>
<td>ATCC&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OMZ176</td>
<td>Serotype d</td>
<td>B. Guggenheim&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6715</td>
<td>Serotype e</td>
<td>J. Navia&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>LM7</td>
<td>Serotype f</td>
<td>T. Shiota</td>
</tr>
<tr>
<td>V318</td>
<td>Serotype g</td>
<td>This paper</td>
</tr>
<tr>
<td>UA37</td>
<td>Biotype c/f, contains plasmid pLM7</td>
<td>This paper</td>
</tr>
<tr>
<td>UA140</td>
<td>Biotype c/f, contains group I plasmid</td>
<td>This paper</td>
</tr>
<tr>
<td>UA109</td>
<td>Biotype c/f, contains group II plasmid</td>
<td>This paper</td>
</tr>
<tr>
<td>UA 405</td>
<td>Biotype c/f, contains group II plasmid</td>
<td>This paper</td>
</tr>
</tbody>
</table>

<sup>a</sup> Department of Microbiology, University of Alabama at Birmingham, Birmingham, Ala.
<sup>b</sup> American Type Culture Collection, Rockville, Md.
<sup>c</sup> University of Zurich, Switzerland.
<sup>d</sup> School of Public Health, University of Alabama at Birmingham, Birmingham, Ala.
<sup>e</sup> Department of Biology, Washington University, St. Louis, Mo.

randomly assigned positions on the agar plates to eliminate examiner bias during the assessment of zones of inhibition. Colonies exhibiting clear, lytic-like zones greater than 10 mm in diameter after anaerobic incubation for 24 h at 37°C were scored positive for bacteriocin-like inhibition (see Fig. 1).

Survey of strains for bacteriocin-like activity or sensitivity. Fifteen clinical isolates of plasmid-containing S. mutans strains, a random selection of 18 plasmid-negative S. mutans strains, and strains V318 and LM7 (Table 1) were used as producers and tested against the following S. mutans indicator strains for bacteriocin-like activity as described above: AHT, BHT, 10449, OMZ176, LM7, and 6715 (Table 1). Data from this experiment were analyzed by the Fisher Exact test.

All 15 plasmid-positive clinical isolates, strains V318 and LM7, and 16 randomly selected plasmid-negative clinical strains were also tested against 11 randomly selected clinical S. mutans strains used as indicators. Data from this experiment were analyzed by the Sign test.

The 15 plasmid-positive clinical isolates and strains LM7 and V318 were further tested for bacteriocin-like activity among themselves. Each of the 17 plasmid-positive strains was used both as producer and as indicator.

Preliminary characterization of bacteriocin-like activity. Sensitivity to pronase and trypsin of zones of inhibition produced by the bacteriocin-like substance of plasmid-positive strains was tested as previously described (8). Strain OMZ176 was used as the indicator.

Four plasmid-positive strains were tested for formation of bacteriophage-like plaques as a test of infectivity by the method described by Berkowitz and Jordan (2).

Seven plasmid-positive and two plasmid-negative clinical S. mutans strains were used as producers in a modification of the UV induction technique described by Hamada and Ooshima (8).

Six plasmid-positive and three plasmid-negative clinical S. mutans strains were tested for mitomycin C-induced bacteriocin-like activity. The nine test strains were inoculated onto duplicate Trypticase soy agar-yeast plates without puncturing the agar surface and then overlaid with a control Trypticase soy broth-yeast-soft agar suspension containing a sublethal concentration of mitomycin C (Sigma) (0.6 μg/ml). The producer colonies were then incubated overnight and overlaid with indicator strain OMZ176. After incubation, zones of inhibition were compared between control and mitomycin C plates.

The bacteriocin-like activity against OMZ176 produced by 17 plasmid-positive strains was tested for sensitivity to pH. Producer strains were stabbed onto Trypticase soy agar-yeast plates buffered with appropriate mixtures of 0.2 M Na₂HPO₄ and NaH₂PO₄ solutions to yield pH values of 6.0, 7.0, and 8.0. After overnight incubation, the plates were overlaid with the indicator strains in similarly buffered Trypticase soy broth-yeast-soft agar suspensions. Zones of inhibition and growth of the indicator strain were observed after overnight incubation.

Isolation of plasmid DNA. Plasmid DNA for restriction endonuclease digests was isolated from S. mutans strains V318, LM7, UA37, UA140, UA109, and UA405 (Table 1) by a protocol derived from several published methods (6, 13, 16). One-liter portions of Todd-Hewitt broth (BBL) were inoculated with overnight cultures and incubated aerobically at 37°C for approximately 12 h to yield an optical density of approximately 0.4 at 620 nm. After the addition of 15 g of glycine and incubation for 45 min at 37°C with continuous shaking, cells were harvested by centrifugation, washed in 25 ml of 25% glucose in TE buffer (50 mM Tris, 20 mM EDTA, pH 8.0), pelleted by centrifugation, suspended in 5 ml of 25% glucose in TE buffer—1.25 ml of lysozyme solution (6 mg/ml in TE buffer), and incubated for 2 h at 37°C. A protease solution (protease K, type XI; Sigma) (6 mg/ml in TE buffer; preincubated for 1 h at 37°C) was then added, and incubation continued for another 30 min. After the addition of 5 ml of 20% sodium dodecyl sulfate in TE buffer and 20 min of incubation at room temperature, a series of separate solutions were added and mixed with gentle inversion: (i) 1 ml of freshly made 3 N NaOH; (ii) 2 ml of 2 M Tris (pH 7.0); and (iii) 6.5 ml of 5 M NaCl. After overnight refrigeration at 4°C, the supernatant obtained by centrifugation at 17,300 × g for 30 min in a Sorvall SS34 rotor was mixed with 2 parts absolute ethanol and 0.1 part 2 M sodium acetate (pH 5.3). DNA was precipitated by incubation overnight at −20°C or for 15 min in a dry ice-ethanol bath and then centrifugation for 15 min in a Sorvall SS34 rotor. After the precipitate was dissolved in 5 ml of TE buffer, plasmid DNA was purified by one or two cycles of CsCl-ethidium bromide density equilibration centrifugation (6).

Restriction enzymes. The restriction endonucleases HindIII, EcoRI, HaeIII, PstI, and TaqI were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used with buffers as described previously (6). All endonuclease digestions were for 30 min at 37°C, except for PstI (30°C) and TaqI (65°C).

agarase and agarase-acrylamide gel electrophoresis. The products of endonuclease digestion were electrophoresed on 0.7% agarose horizontal slab gels or agarase-acrylamide vertical slab gels (2% acrylamide, 0.1% bisacrylamide, 0.7% agarose) (10). The running buffer was 40 mM Tris hydrochloride—5 mM sodium acetate—1 mM EDTA, adjusted to pH 7.6 with acetic acid. For loading, DNA was mixed with 0.5% glycerol-0.15% bromophenol blue-0.3% sodium dodecyl sulfate; with agarase-acrylamide gels, the sodium dodecyl sulfate was omitted. After electrophoresis, gels were stained in a solution of 0.2 μg of ethidium bromide...
per ml and then photographed with UV light, Polaroid type 107 film, and a red filter.

Molecular weight standards for supercoiled plasmid DNA were plasmids from Escherichia coli V517 (12). Molecular weight standards for linear DNA fragments were bacteriophage lambda DNA digested with HindIII or double-stranded φX174 DNA digested with HaeIII (Bethesda Research Laboratories). Standard curves were drawn as plots of molecular weight versus relative mobility.

RESULTS

Plasmid-containing strains of S. mutans. Extending our previous work (3), we found a plasmid similar in size to the 5.64-kb (3.6-megadalton) plasmid described by Macrina et al. (13, 14) in 26 of 315 children screened (8% prevalence of plasmid-containing strains). All of the plasmid-positive and 98% of the plasmid-negative S. mutans clinical isolates were biotype 1 (serotype c/f). We selected 15 plasmid-positive strains for further study. For reference, we also included two laboratory strains with partially characterized 5.6-kb plasmids: LM7 (serotype e) and V318 (serotype c) (1, 7, 9, 13, 14).

Inhibition of representative serotypes of S. mutans laboratory strains by plasmid-positive strains. We examined bacteriocin-like properties of both plasmid-positive and plasmid-negative strains against six different indicator strains representing S. mutans serotypes a, b, c, d, e, and g (Table 1). Only two strains showed statistically significant differences between patterns of inhibition produced by plasmid-positive and plasmid-negative strains. Of 17 plasmid-positive strains (including strains LM7 and V318), 15 produced zones of inhibition against S. mutans OMZ176; none of 18 plasmid-negative strains tested inhibited this strain (Table 2). A typical stab plate showing positively scored zones of inhibition against OMZ176 is shown in Fig. 1. Relative frequencies of inhibition versus noninhibition revealed a statistically significant relationship (P < 0.0001, Fisher Exact test) between the presence of the plasmid and the zones of inhibition against indicator strain OMZ176. Although strain AHT showed a significant association between zones of inhibition and presence of a plasmid (P = 0.038), OMZ176 was more selective and therefore was used in subsequent experiments.

Susceptibility of plasmid-negative and plasmid-positive clinical isolates to bacteriocins produced by plasmid-positive strains. The 17 plasmid-positive strains were next tested for their ability to produce inhibitory zones against randomly selected plasmid-negative clinical isolates (Table 3). Plasmid-positive strains of S. mutans inhibited plasmid-negative strains in 76% of all possible tests of pairs, whereas plasmid-negative strains used as producers showed inhibition in only 12% of the tests. The difference between these frequencies was significant (P = 0.0005, Sign test). Although all 17 plasmid-positive strains tested contained a 5.6-kb plasmid and 15 of 17 produced zones of inhibition against indicator strain OMZ176, we found that when they were tested for inhibitory action against each other, the 17 strains could be divided into two distinct groups: 7 strains (including LM7) inhibited the remaining 10 strains (including V318). Conversely, these 10 strains when tested as producers inhibited the remaining 7 strains. None of the 7 strains in the first group or the 10 strains in the second group inhibited members within its own group. On the basis of these patterns of inhibition, we designated the seven strains that included LM7 as group I and the 10 strains that included V318 as group II.

Properties of bacteriocins from group 1 and 2 plasmid-positive strains. The effects of various treatments on zones of inhibition against strain OMZ176 were tested. Solid agar was used because inhibitory activity against sensitive strains of S. mutans was not apparent in liquid cultures.

Results with buffered media indicated that the zones of inhibition were not caused by acid production as measured by pH: the indicator strain OMZ176 grew equally well outside the zones on all the buffered plates, and intact zones of equal size were present on media buffered to pH 7.0, 6.5, and 6.0. (Zones were markedly reduced in size, however, on media buffered to pH 8.0.) Inhibitory zones were not caused by hydrogen peroxide since inhibitory activity was expressed in an anaerobic environment. Zones of inhibition were markedly reduced or eliminated, however, by incubation with trypsin or pronase. Inhibitory activity was not inducible with either UV light or mitomycin C, and excised zones of inhibition were not infective when placed on lawns.

<table>
<thead>
<tr>
<th>TABLE 2. Correlation between presence of plasmids and bacteriocin-like activity in S. mutans strains</th>
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<tr>
<td>Activity against OMZ176</td>
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<tr>
<td>-------------------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Inhibition</td>
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<tr>
<td>No inhibition</td>
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* P+, Plasmid positive; P−, plasmid negative. Differences between groups are statistically significant (P < 0.0001, Fisher exact test).

FIG. 1. Zones of inhibition around producer colonies. Nine potential producer colonies were grown on agar-solidified medium and overlaid with indicator strain OMZ176. Large, positive zones of inhibition are seen around six of the colonies, while the three other colonies (at one o'clock, eight o'clock, and ten o'clock) show negligible inhibition.

<table>
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<th>TABLE 3. Bacteriocin inhibition profiles of plasmid-positive and plasmid-negative S. mutans strains against randomly selected plasmid-negative strains</th>
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<tbody>
<tr>
<td>Producer strains (no.)</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Plasmid positive (17)</td>
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<tr>
<td>Plasmid negative (16)</td>
</tr>
</tbody>
</table>

* 17 plasmid-positive and 16 plasmid-negative isolates were used as producer strains with 11 randomly chosen plasmid-negative strains for a total of 187 and 176 pair tests, respectively, for inhibition. Number of tests showing inhibition/total number of tests (%). Difference between frequency of plasmid-negative strains inhibited by plasmid-positive versus plasmid-negative strains is statistically significant (P = 0.0005, Sign test).
of sensitive bacteria, suggesting that bacteriophage were not responsible for zones of inhibition.

Consistent with criteria established by other investigators (2, 8, 11), we concluded that the inhibitory substances formed by group I and group II plasmid-positive strains were bacteriocins. We tentatively designated the group I and group II bacteriocins as mutacin I and mutacin II, respectively.

**Restriction enzyme analysis of plasmids from group I and II strains.** Data from the immunity experiments (above) led us to conclude that at least two distinct bacteriocins were produced by strains of *S. mutans* harboring a 5.6-kb plasmid. We hypothesized that the difference in bacteriocin type is a function of different plasmid genomes, despite apparent identical sizes and previously published data of similar restriction endonuclease digest patterns (14). We isolated the plasmid DNA of three plasmid-positive strains from each of the two bacteriocin groups. Strains from group I included LM7, UA37, and UA140. Strains from group II included V318, UA109, and UA405 (Table 1).

Digestion of plasmids from both groups with the restriction endonuclease *Hind*III, *Pst*I, or *Hpa*I yielded one, one, or two fragments, respectively, similar to data previously reported (1, 9, 14). However, restriction endonuclease digestion with *Eco*RI, *Hae*III, and *Taq*I revealed differences between the plasmids of group I and group II strains. Hereafter, we will refer to plasmids from the two groups as group I and group II plasmids.

*Eco*RI cut each group I plasmid (pLM7 once; pUA37 and pUA140 twice) (Fig. 2, lanes D through F) but cut no group II plasmid (lanes G through I). Controls of undigested plasmids pLM7 and pVA318 are shown in lanes B and C. *Hae*III cut each group II plasmid once (Fig. 3, lanes E through G) but did not cleave group I plasmids pUA37 and pUA140 (lanes C and D). The other group I plasmid, pLM7, was cut twice; one fragment was almost full-sized (Fig. 3, lane B). The second, 270-base-pair fragment was seen by agarose-polyacrylamide gel electrophoresis (data not shown).

After *Taq*I digestion, all three group II plasmids had identical patterns of eight fragments each (Fig. 4a, lanes E through G). Two group I plasmids had identical patterns showing 10 fragments, but pLM7 had one *Taq*I site fewer (lanes B through D). All plasmids in both groups had six *Taq*I fragments of identical size (Fig. 4a and b).

In all restriction endonuclease digestion experiments, the sizes of the fragments resulting from endonuclease digestion were calculated by comparison with linear size standards. The sum of fragments from any individual digestion was within 5% of the expected 5.64-kb plasmid contour length as measured by Hansen et al. (9).

Because the group I plasmid pLM7 differed from group I plasmids pUA37 and pUA140, we isolated plasmid DNA from two additional group I strains (UA174 and UA728). They showed *Taq*I, *Eco*RI, and *Hae*III digestion patterns of fragments that were identical to those of both pUA37 and pUA140 (data not shown).

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**FIG. 2.** Agarose gel electrophoresis of group I and group II *S. mutans* plasmids. Lanes B and C contain intact plasmids pLM7 and pVA318, respectively. Plasmid DNAs in lanes D through I were all reacted with restriction endonuclease *Eco*RI: D, pLM7; E, pUA37; F, pUA140; G, pVA318; H, pUA109; I, pUA405. Lanes A and J contain linear size-standard DNA with the sizes (in kilobases) of four fragments indicated. See the text for additional details.

**FIG. 3.** Agarose gel electrophoresis of group I and group II *S. mutans* plasmids. Plasmid DNAs in lanes B through G were all reacted with restriction endonuclease *Hae*III: B, pLM7; C, pUA37; D, pUA140; E, pVA318; F, pUA109; G, pUA405. Lanes A and H contain linear size-standard DNA with the sizes (in kilobases) of three fragments indicated. See the text for additional details.

**FIG. 4.** (a) Agarose gel electrophoresis of group I and group II *S. mutans* plasmids. Plasmid DNAs in lanes B through G were all reacted with restriction endonuclease *Taq*I: B, pLM7; C, pUA37; D, pUA140; E, pVA318; F, pUA109; G, pUA405. Lanes A and H contain linear size-standard DNA with the sizes (in kilobases) of six fragments indicated. See the text for additional details. (b) Graphic representation of *S. mutans* plasmids of pLM7 and of groups I and II after *Taq*I digestion and agarose gel electrophoresis. Positions of size-standard DNAs (kilobases) are indicated to the right.
DISCUSSION

We showed that (i) the presence of 5.6-kb plasmids in strains of \textit{S. mutans} was correlated with bacteriocin production and immunity, (ii) plasmid-positive strains of \textit{S. mutans} could be divided into at least two distinct bacteriocin groups, and (iii) plasmids from the two bacteriocin groups also composed two groups, based on differences in restriction endonuclease digest patterns.

The significant correlation between the presence of the 5.6-kb plasmid and bacteriocin activity against two representative serotypes of \textit{S. mutans} (OMZ176 and AHT) and against clinical biotype I (serotypes c/f) plasmid-negative strains does not prove that the plasmid produces or regulates the bacteriocin. Neither is there proof in the fact that we found differences in plasmids only after discovering the different bacteriocin groups. Transformation of the plasmid into plasmid-negative strains or removal of the plasmid from plasmid-positive strains may help test the possible role of the plasmid in bacteriocin production and immunity. In fact, transformation of \textit{S. mutans} with both plasmid and chromosomal DNA has been accomplished with the aid of antibiotic markers (15), but selecting transformants having bacteriocin immunity or production awaits development of a sensitive screening technique. We are currently exploring methods whereby immunity to group I or II bacteriocin can be used for selecting transformants. Plasmid-negative strains transformed with a recombinant plasmid derivative of pVA318 containing erythromycin resistance and a pBR322 fragment did not exhibit bacteriocin activity (J. B. Hansen, H. H. Murchison, P. W. Caufield, D. Allen, N. Childers, and R. Curtiss, unpublished data). We now speculate that insertion of foreign DNA at the HindIII site in pVA318 may interfere with expression of plasmid genes responsible for bacteriocin activity. Also, our attempts to generate plasmid-negative derivatives by exposure of strains to acridine orange or mitomycin C or by growth at elevated temperature have been unsuccessful (data not shown).

The plasmid-associated inhibitory substances described in this paper seemed to be bacteriocins: they were proteinaceous, active against members of their own species, and not autoinhibitory to the cells making bacteriocins or to members of the same bacteriocin group. Tests for inducibility or infectivity failed to show bacteriophage involvement in the inhibition. Although pHs below neutrality enhanced inhibitory zone formation, low pH was not itself responsible for inhibitory action because inhibition was present on buffered plates. Because zones formed equally well in both reduced O\textsubscript{2} and anaerobic environments, inhibition was not due to hydrogen peroxide production. We adopted the term mutacin from Hamada and Ooshima (8) to denote bacteriocins produced by \textit{S. mutans}.

Bacteriocin production and immunity have been linked to plasmids in several genera of bacteria, including \textit{Streptococcus} (4, 5), but not in \textit{S. mutans} (9, 13, 14). This is likely due to the limited number of plasmid-positive strains available for testing as well as to differences in methods for demonstrating bacteriocin activity. For example, Macrina and co-workers (13) observed differences in bacteriocin-like inhibition patterns among three plasmid-containing strains (LM7, V310 and V318) but concluded that they were due to different host chromosomal genes, not different plasmids.

Of the 17 plasmid-positive strains, two failed to produce zones of inhibition against indicator strain OMZ176. The reason for the lack of detectable inhibition against OMZ176 is not clear. Because scoring of zones is not only a function of the presence of bacteriocins but also of the quantity produced, we cannot say definitively that these strains produced no bacteriocin. Rather, within the limits of the assay used, we could not detect inhibitory activity. These same two strains (UA31 and UA37) did, however, produce bacteriocins against members of group II and against randomly selected plasmid-negative strains. Consistent with bacteriocin grouping, restriction digest patterns clearly placed both strains into group I.

Of six representative serotype strains, serotype \textit{d/g} strain OMZ176 was the most useful indicator for plasmid-associated bacteriocin activity. Interestingly, Hamada and Ooshima (8) found strain OMZ176 unique among streptococci they tested; as a producer it did not inhibit any of their other strains, and as an indicator it was insensitive to 24 of 25 \textit{S. mutans} strains. We tested two other biotype III (serotypes \textit{d/g}) clinical isolates, but neither could differentiate plasmid-positive from plasmid-negative strains as did strain OMZ176 (data not shown). Most plasmid-negative clinical isolates of biotype I (serotypes \textit{c/f}) were sensitive to bacteriocin produced by both group I and group II strains. In fact, several clinical isolates have also been found useful as indicators for plasmid-associated bacteriocin (data not shown).

Our results contrasted with those of some published work. We found more \textit{TaqI} sites in the \textit{S. mutans} 5.6-kb plasmids than previously reported (14), as well as a difference between plasmids pLM7 and pVA318 not previously detected (14). In our work, the sum of all the \textit{TaqI} fragments from any individual digestion was always within 5% of the size of the entire plasmid, and the patterns were repeatable (data not shown). Thus, we evidently obtained complete digests (which may include some yet-undetected fragments smaller than 270 base pairs).

Restriction endonuclease data suggested that plasmids of groups I and II were at least 70% homologous (based on the sum sizes of the six apparently common \textit{TaqI} fragments). Our present working hypothesis is that the regions of the plasmids responsible for the observed restriction fragment length polymorphisms are also involved with bacteriocin production or immunity, or both. We conclude that instead of a single disseminated plasmid among strains of \textit{S. mutans} as originally proposed (14), there appear to be at least two distinct, although related, disseminated 5.6-kb plasmids in \textit{S. mutans}. Plasmid pLM7 is the sole example of a possible third type.

ACKNOWLEDGMENTS

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LITERATURE CITED


