H$_2$O$_2$ Produced by Viridans Group Streptococci May Contribute to Inhibition of Methicillin-Resistant Staphylococcus aureus Colonization of Oral Cavities in Newborns

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In an accompanying report, we showed that viridans group streptococci may prevent methicillin-resistant Staphylococcus aureus (MRSA) colonization of the oral cavities of newborns. In the present study, we investigated the mechanism of prevention in vitro. Most viridans group streptococci had bacteriocin-like activity and killed MRSA, Burkholderia cepacia, Enterobacter aerogenes, and Pseudomonas aeruginosa; however, Escherichia coli, Enterobacter cloacae, and Candida albicans were resistant. The activity was induced only by H$_2$O$_2$-secreting strains and was inhibited by horseradish peroxidase or catalase in a dose-dependent manner. The mean concentration of H$_2$O$_2$ produced by 18 strains of viridans group streptococci (1 $\times$ 10$^8$ cfu in 200 $\mu$L of culture medium ± standard deviation was 1.24 ± 0.60 mmol. Viridans group streptococci inhibited MRSA growth in saliva as well as in culture media. These results indicate that H$_2$O$_2$ produced by viridans group streptococci may inhibit MRSA colonization of oral cavities in newborns.

Despite extensive prevention efforts, infection or colonization by methicillin-resistant Staphylococcus aureus (MRSA) is a serious problem in neonatal intensive care units [1–3]. Moreover, emergence of strains resistant to mupirocin [4, 5] and vancomycin [6] makes the situation more complicated.

The role of normal flora in preventing infectious diseases caused by many pathogenic microorganisms has been a fascinating subject for investigators, although only a few investigations were made in the 1990s. However, in recent years, as the importance of limiting antibiotic use has been understood, investigators have been motivated to study the subject again [7–10], and some of them (including our group) have reported specific examples [9, 11].

H$_2$O$_2$ and H$_2$O$_2$-producing normal flora are expected to protect against infectious diseases caused by many pathogens. Lactobacillus species producing H$_2$O$_2$ are believed to protect pregnant women against vaginosis [7, 10], and viridans group streptococci inhabiting oral cavities also have an antagonistic effect on many bacterial species [8, 12–14]. In an accompanying report [15], we showed that viridans group streptococci colonizing oral cavities in newborns inhibit MRSA colonization. In this study, we analyze whether H$_2$O$_2$ produced by viridans group streptococci really inhibits MRSA growth in vitro.
**MATERIALS AND METHODS**

**Microbiological testing.** All swab specimens obtained from newborns in Neonatal Intensive Care at Nagano Children’s Hospital (Toyoshina, Japan) during the 26-month study period, from April 1995 through May 1997, were inoculated onto plates with 5% sheep blood agar, chocolate agar, modified Drigalsky agar, or OPA *Staphylococcus* agar (all plates were purchased from BBL Becton Dickinson Microbiology Systems), and the plates were incubated for 24 h at 37°C in 5% CO₂ in air. Bacterial identification and antibiotic susceptibility testing were performed by autoSCAN-4 (Dade Behring) and the conventional method as described elsewhere [16, 17]. MRSA was defined as *S. aureus* for which the oxacillin MIC was ≥4 µg/mL.

**Assay of the bacteriocin-like activity of viridans group streptococci.** *S. aureus* (1 × 10⁶ cfu of MRSA) in 100 µL of PBS was mixed with 10 mL of melting (at 37°C) brain-heart infusion broth medium (Nissui) with 1.5% low-melting-point agar (Eastman Kodak) and poured into a 15 × 100-mm plastic dish (BBL Becton Dickinson Microbiology Systems). After solidification, the mixture was overlaid with 10 mL of the same medium without MRSA, to prevent direct contact between MRSA and viridans group streptococci, and the overlay was solidified (this overlay was omitted in some of the experiments). Suspensions of bacteria (various species of viridans group streptococci, *Enterococcus faecalis, or Enterococcus epidermidis; 1 × 10⁶ cfu) in 10 µL of PBS were spotted on the agar plate and incubated overnight at 37°C. In the control group, 10 µL of various dilutions of reagent H₂O₂ was similarly spotted. After incubation, 10 mL of low-melting-point agar was overlaid and stained with 0.04% crystal violet in both groups. Diameters of the zones of inhibition were measured. Five MRSA strains (N803, N810, N815, N822, and N830) were targeted. The activity of viridans group streptococci against *S. epidermidis* was measured in a similar manner.

**Bactericidal activity of viridans group streptococci against MRSA.** A 96-well micro–test plate. Various amounts of viridans group streptococci (1 × 10⁶, 1 × 10⁵, 1 × 10⁴, and 1 × 10³ cfu) were cocultured overnight at 37°C in 200 µL of brain-heart infusion broth medium with 2 × 10³ cfu of MRSA. After incubation, MRSA organisms surviving in the coculture were examined by use of OPA staphylococcus agar. *Streptococcus parasanguinis* American Type Culture Collection (ATCC) 15912, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, *Streptococcus crista* National Collection of Type Cultures (NCTC) 12479, *Streptococcus mitis* NCTC 12261, *Streptococcus oralis* NCTC 11427, and *Streptococcus pneumoniae* NCTC 7465 were also examined as control strains.

**Inhibition of bacteriocin-like activity by horseradish peroxidase or catalase.** The inhibitory activity of horseradish peroxidase against the bacteriocin-like activity of viridans group streptococci was measured by the agar plate method described above, with a minor modification. Brain-heart infusion broth medium with low-melting-point agar containing various amounts (0, 16, 80, 400, and 2000 µg/mL) of horseradish peroxidase was used as an overlay on solidified MRSA. The inhibitory activity of horseradish peroxidase against culture products of 4 different strains of viridans group streptococci isolated from newborns (*S. gordonii, S. mitis, S. sanguinis, and S. oralis*) was assayed. The inhibitory activity of catalase (from *Aspergillus niger; Sigma*) was assayed in a similar manner.

**Determination of H₂O₂ concentration in supernatant fluid of bacterial culture.** Suspensions of bacteria (absorbance at 620 nm, 0.78) in 0.9% NaCl were prepared from overnight cultures of viridans group streptococci (20 strains) or enterococci (2 strains). In a 1.5-mL micro–test tube (Sarstedt), an aliquot of the suspension (100 µL aliquots, each containing ~ 1 × 10⁸ cfu) was mixed with 100 µL of brain-heart infusion broth medium and cultured at 37°C for 9 h. After incubation, 20 µL of supernatant fluid or a 1:2 serial dilution of supernatant fluid with PBS was mixed with 20 µL of horseradish peroxidase (0.5 mg/mL of PBS [pH 7.0]) and 50 µL of a solution of 0.1 mg of 3,3′,5,5′-tetramethylbenzidine/mL of 0.05 M phosphate citrate buffer (pH 5.0) (Sigma) and incubated at room temperature for 1 min. Then, the enzymatic reaction was stopped by the addition of 100 µL of 2-M H₂SO₄, and color intensity was measured at 420 nm by the ELISA processor II (Dade Behring). Simultaneously, the bacteriocin-like activity of 10 µL of bacterial suspension (~ 1 × 10⁸ cfu) was measured by the agar plate method.

**Catalase activity.** Catalase activity on the cell surface was measured as follows: 0.1 mL of bacterial suspension was added to 2.9 mL of substrate solution (0.1 mL of 30% H₂O₂ to 50 µL of 30% H₂O₂).
Figure 2. Squares of the diameters of zones of inhibition made by 18 strains of H 2O2-producing viridans group streptococci, Streptococcus salivarius, and enterococci were compared with squares of the diameters of zones of inhibition created by H 2O2 produced by the same bacterial strains, in a study of the contribution of H 2O2 produced by viridans group streptococci to inhibition of methicillin-resistant Staphylococcus aureus colonization of oral cavities in newborns. The square of the diameter was directly proportional to the concentration of H2O2 secreted (r = .962).

Figure 3. Horseradish peroxidase inhibition of the bacteriocin-like activity of viridans group streptococci (Streptococcus gordonii, Streptococcus mitis, and Streptococcus oralis) in a study of the contribution of H 2O2 produced by viridans group streptococci to inhibition of methicillin-resistant Staphylococcus aureus (MRSA) colonization of oral cavities in newborns. Brain-heart infusion broth medium with low-melting-point agar containing horseradish peroxidase was poured on solidified MRSA, and an overlay was made; viridans group streptococci were then spotted on it, and culture was performed. Viridans group streptococci killed MRSA and created zones of inhibition (0 μg of horseradish peroxidase/plate). However, horseradish peroxidase neutralized the activity and reduced the diameter of the zone of inhibition in a dose-dependent manner.

RESULTS

Bacteriocin-like activity of viridans group streptococci. Eighteen strains of viridans group streptococci that secreted H 2O2 and 2 strains each of S. salivarius, E. faecalis, and S. epidermidis were examined for their ability to inhibit MRSA growth. Because almost the same results were found for all the MRSA targets (5 strains tested), data for representative samples are shown. All viridans group streptococci secreting H 2O2 inhibited MRSA growth on brain-heart infusion agar and created zones of inhibition. The mean diameter of the zones of inhibition ± SD was 13.7 ± 4.3 mm. However, S. salivarius, E. faecalis, and S. epidermidis, which lack the ability to secrete H 2O2, did not have such activity. Three representative samples of zones of inhibition are shown in figure 1. The diameter of the zone of inhibition for reagent H 2O2 (10-μL spot of a 1:100 dilution of 30% H 2O2) was approximately the same as those for viridans group streptococci. Growth of S. epidermidis was inhibited by H 2O2-secreting viridans group streptococci as effectively as was that of MRSA (data not shown).

H 2O2 secretion by viridans group streptococci. The 18 strains of viridans group streptococci that were tested secreted H 2O2 into the culture supernatant; the mean concentration of H 2O2 ± SD was 1.24 ± 0.60 mmol. However, 2 strains each of S. salivarius and E. faecalis did not produce H 2O2. The amount of H 2O2 secreted was directly proportional to the square of the diameter of the zones of inhibition on the agar plate (figure 2). This activity was bactericidal, since 2 × 10^5 cfu of MRSA cocultured with 1 × 10^6 cfu of viridans group streptococci (4 different strains were tested) on the micro–test plate was completely killed, as assessed by culture on OPA staphylococcus agar. However, when the amount of viridans group streptococci was reduced by one-tenth (1 × 10^5 cfu), MRSA survived. The mL of 0.05-M PBS, pH 7.0), and the time required for absorbance at 240 nm to decrease from 0.450 to 0.400 at 25°C was measured by the spectrophotometer CL 1200 (Shimadzu). One unit was defined as the activity decomposing 1.0 μmol of H2O2/min by cfu of bacteria at pH 7.0 at 25°C.

Statistical analysis. Group data are expressed as mean ± SD, and differences between groups were analyzed by the paired Student’s t test or the χ^2 test.
amount of viridans group streptococci necessary to completely kill 1 cfu of MRSA was >500 cfu.

**Inhibition of bacteriocin-like activity by horseradish peroxidase or catalase.** Bacteriocin-like activity was inhibited by horseradish peroxidase or a catalase layer laid between viridans group streptococci and MRSA. The amount of horseradish peroxidase necessary for complete inhibition of the bacteriocin-like activity was directly proportional to the diameter of the zone of inhibition (figure 3). The concentration of horseradish peroxidase necessary for complete inhibition of the bacteriocin-like activity of viridans group streptococci was 400 μg per plate, and even at this concentration the growth of MRSA and viridans group streptococci in the control cultures was never inhibited. Similarly, bacteriocin-like activity of viridans group streptococci and reagent H₂O₂ was inhibited by catalase, and diameters of zones of inhibition were directly proportional to the catalase activity (figure 4). On figure 4, the line representing viridans group streptococci demonstrates the average of data for 4 different species (S. oralis, S. sanguinis, S. mitis, and S. gordonii). For complete inhibition of bacteriocin-like activity of viridans group streptococci, an extremely large amount of catalase was required (20 U/plate).

**Catalase activity on cell surfaces and resistance to reagent H₂O₂.** Several bacteria isolated from oral cavities of newborns were tested for their ability to resist reagent H₂O₂, and the relationship between resistance to H₂O₂ and catalase activity on cell surfaces was studied. According to the mean diameter of the zones of inhibition ± SD, isolates were divided into 2 groups: those susceptible to H₂O₂ and those resistant to H₂O₂. Susceptible bacteria included MRSA, S. salivarius, Enterobacter aerogenes, Burkholderia cepacia, and Pseudomonas aeruginosa (mean diameter of the zones of inhibition ± SD, 15.8 ± 3.6 mm), and resistant bacteria included S. oralis, S. mitis, S. sanguinis, Escherichia coli, Enterobacter cloacae, and Candida albicans (mean diameter of the zones of inhibition ± SD, 2 ± 2.2 mm). Contrary to our expectations, except for E. cloacae, the mean catalase activity of isolates resistant to H₂O₂ ± SD (46 ± 74 mU) was lower than that of susceptible bacteria (359.2 ± 458 mU) (table 1).

**Production of bacteriocin-like activity in saliva.** All 4 species of viridans group streptococci had bacteriocin-like activity during 15-h cultures of saliva specimens. In the control culture, MRSA was grown, and its cell number increased from 50 to 1.78 × 10⁸ cfu, whereas growth of MRSA cocultured with viridans group streptococci was effectively inhibited. For example, the cell number of MRSA cocultured with 5 × 10⁹ cfu of S. sanguinis (ratio of the cell number of MRSA to that of viridans group streptococci, 1:100) increased, within 15 h, from 50 to only 4.8 × 10⁵ cfu (figure 5).

Type strains S. oralis, S. mitis, S. gordonii, and S. sanguinis similarly produced H₂O₂ and killed MRSA on the agar plates.
Table 1. Resistance to reagent H$_2$O$_2$ and catalase activity on cell surfaces in a study of the contribution of H$_2$O$_2$ produced by viridans group streptococci to inhibition of methicillin-resistant Staphylococcus aureus (MRSA) colonization of oral cavities in newborns.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Average diameter of zones of inhibition, mm</th>
<th>Catalase activity of 1 × 10$^8$ cfu of organism on cell surface, mU$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus oralis M801</td>
<td>3.8</td>
<td>24</td>
</tr>
<tr>
<td>Streptococcus mitis M701</td>
<td>4.2</td>
<td>12</td>
</tr>
<tr>
<td>Streptococcus sanguinis M601</td>
<td>4.0</td>
<td>4</td>
</tr>
<tr>
<td>Escherichia coli O101</td>
<td>0 (slightly killed)</td>
<td>12</td>
</tr>
<tr>
<td>Enterobacter cloacae P501</td>
<td>0 (slightly killed)</td>
<td>196</td>
</tr>
<tr>
<td>Candida albicans C101</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.0 ± 2.2</td>
<td>46.0 ± 74.0</td>
</tr>
<tr>
<td>MRSA N803</td>
<td>20.2</td>
<td>304</td>
</tr>
<tr>
<td>Burkholderia cepacia R601</td>
<td>18.8</td>
<td>88</td>
</tr>
<tr>
<td>Enterobacter aerogenes Q501</td>
<td>14.7</td>
<td>192</td>
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<tr>
<td>Pseudomonas aeruginosa S501</td>
<td>13.0</td>
<td>1160</td>
</tr>
<tr>
<td>Streptococcus salivarius M501</td>
<td>12.1</td>
<td>52</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>15.8 ± 3.6</td>
<td>359.2 ± 458</td>
</tr>
</tbody>
</table>

NOTE. Ten microliters of a 1:100 dilution of 30% H$_2$O$_2$ was added to a 3-mm (diameter) well on a plate containing 1 × 10$^8$ cfu of bacteria.

$^a$ All bacteria used were clinical isolates from the oral cavities of newborns.

$^b$ One unit was defined as the activity decomposing 1.0 μmol of H$_2$O$_2$/min at pH 7.0 at 25°C. All results are the average value for triplicate tests.

On the basis of these results, we conclude that the bactericidal activity of viridans group streptococci is due to H$_2$O$_2$.

**DISCUSSION**

Beneficial and harmful effects of H$_2$O$_2$ produced by several bacteria because of alteration of human physiology have been the subject of special investigation. The α-hemolysin of viridans group streptococci, a potential virulence factor, was identified as H$_2$O$_2$ [18], and streptolysin S and H$_2$O$_2$ synergistically injure vascular endothelial cells [19]; however, H$_2$O$_2$ produced by lactobacilli may protect pregnant women from vaginosis [7].

As shown in an accompanying report [15], viridans group streptococci may prevent MRSA colonization of oral cavities in newborns, and, in the present report, we indicate that the bacteriocin-like activity of viridans group streptococci is the probable cause for such protection. This bacteriocin-like activity was abrogated by the addition of horseradish peroxidase or catalase, and we conclude that the substance possessing the bacteriocin-like activity is H$_2$O$_2$.

The precise role of H$_2$O$_2$ produced by viridans group streptococci in oral cavities is still under investigation. Ryan and Kleinberg [20] denied that H$_2$O$_2$ has an antagonistic effect on pathogenic bacteria, because H$_2$O$_2$ produced by viridans group streptococci was rapidly degraded by bacteria in the oral cavity, including Neisseria sicca, Haemophilus segnis, Haemophilus parainfluenzae, Actinomyces viscosus, and S. epidermidis (which are all species that use H$_2$O$_2$). If so, fortunately, the bacterial composition of oral cavities in newborns (especially very young
newborns such as those included in our study and in the accompanying investigation [15]) is simple compared with that of the oral cavities of adults. Although small amounts of S. epidermidis coexisted with viridans group streptococci, Neisseria species and Haemophilus species usually did not colonize (colonization with Actinomyces was not tested). These situations may prolong H$_2$O$_2$ retention in the microenvironment of the oral cavity. Moreover, it is interesting that bacteria with high levels of catalase activity were not always highly resistant to H$_2$O$_2$. Catalase activity, with a few exceptions, tends to be inversely proportional to resistance. Exogenously added catalase protected MRSA from the antagonistic effects of H$_2$O$_2$, but the amounts required were extremely large compared with the amounts of catalase normally associated with MRSA cells. According to these results, studies of the unknown mechanisms controlling susceptibility of H$_2$O$_2$ to decomposition seem to be more important than studies of the mechanisms of activity of decomposition. Some investigators believed in the importance of an antagonistic effect of H$_2$O$_2$ and performed basic investigations on decomposition. In our study, viridans group streptococci successfully inhibited MRSA growth even in saliva. In the present report, we show that H$_2$O$_2$ produced by viridans group streptococci kills MRSA in vitro. However, further studies should be performed to prove that H$_2$O$_2$ kills MRSA in vivo.

Acknowledgments

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References