MAJOR ARTICLE

Secretory IgA, Salivary Peroxidase, and Catalase-Mediated Microbicidal Activity during Hydrogen Peroxide Catabolism in Viridans Streptococci: Pathogen Coaggregation

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Viridans streptococci can kill methicillin-resistant *Staphylococcus aureus* (MRSA) through the production of hydrogen peroxide (H_2O_2). However, several hundred viridans streptococci cells are necessary to kill 1 cfu of MRSA. We analyzed the potency of bactericidal and fungicidal effector molecules induced by catabolism of H_2O_2 in the oral cavity. Secretory IgA (SIgA) and an unidentified salivary component bound *Streptococcus sanguinis,* a viridans streprococcus, and MRSA into coaggregates. In these coaggregates, salivary peroxidase and the MRSA catalase produced singlet molecular oxygen ($^1O_2$) from H_2O_2 produced by viridans streptococci. SIgA converted $^1O_2$ into ozone, which has potent bactericidal and fungicidal activity. We calculated that <10 cfu of *Streptococcus sanguinis* were necessary to kill 1 cfu of MRSA in the coaggregate. SIgA, *Aspergillus niger* catalase, and H_2O_2 in saliva killed *Candida albicans,* which is highly resistant to reagent H_2O_2. Together with indigenous bacteria and innate immunity, SIgA potentially constitutes a novel system that may sustain oral homeostasis.

Early colonization by viridans streptococci prevents the oral cavity of a newborn infant from being colonized by methicillin-resistant *Staphylococcus aureus* (MRSA) [1], suggesting that prevention of colonization relies on hydrogen peroxide (H_2O_2) produced by viridans streptococci. However, MRSA displays a strong catalase on its surface, and a single viridans streptococcus cell produces insufficient H_2O_2 to kill MRSA [2]; concentration or augmentation of the bactericidal activity of H_2O_2 in oral viridans streptococci is necessary for killing of MRSA.

Coaggregation between MRSA and viridans streptococci may compensate for the inadequate amount of H_2O_2. Secretory IgA (SIgA) is the most abundant immunoglobulin produced against both commensal microorganisms [3] and pathogens [4], even in the saliva of preterm infants [5, 6]. In addition to the 4 Fab binding sites [7, 8], glycan epitopes on the heavy chains and the secretory component provide SIgA with further bacteria-binding sites and possibly contribute to the formation of coaggregates.

Salivary peroxidase [9, 10] and bacterial catalase [11] may decompose the H_2O_2 produced by viridans streptococci into harmless final products. It has been suggested that, during its decomposition of H_2O_2, salivary peroxidase produces singlet molecular oxygen ($^1O_2$) as

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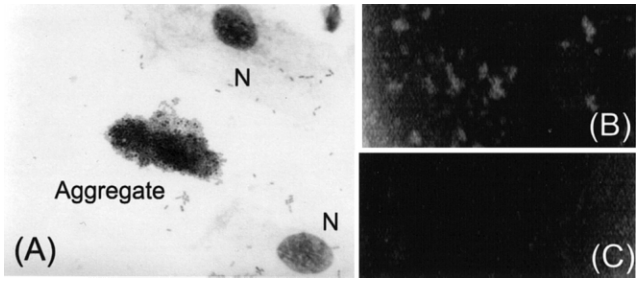

Figure 1. Bacterial aggregation in vivo and in vitro. *A,* Aggregation in the oral cavity of an infant. *B,* Aggregation after simple agitation of *Streptococcus sanguinis* in saliva (macroscopic field). *C,* Absence of aggregation in secretory IgA–depleted saliva. Aggregate, bacterial aggregation; N, epithelial-cell nucleus.

a short-lived by-product [12]. If so, we would hypothesize that $^1O_2$ is converted to ozone by the immunoglobulin-catalyzed water-oxidation reaction, as recently described by Wentworth et al. [13, 14], and that ozone has potent bactericidal activity. In the present study, we examined the coaggregation between MRSA and viridans streptococci and studied both (1) whether the H_2O_2 produced by viridans streptococci in saliva is converted to ozone and (2) whether it has bactericidal activity against MRSA and fungicidal activity against *Candida albicans*.

MATERIALS AND METHODS

Saliva. Samples of unstimulated whole saliva were obtained from 7 infants and from 5 healthy adult volunteers, by use of a method described elsewhere [15]. All infants were inpatients in the Neonatal Intensive Care Unit of Nagano Children's Hospital, and all were receiving mothers' milk. Before the study, formal permission was obtained from the hospital's ethics committee, and informed consent was obtained from the parents of each infant and from each adult volunteer.

Strains and growth conditions of microorganisms. The microorganisms used were *Streptococcus sanguinis* ATCC10556^T, *Streptococcus gordonii* ATCC10558^T, *Streptococcus oralis* NCTC11427^T, *Streptococcus mitis* NCTC12261^T, catalase-negative *Staphylococcus aureus* TW 4632, and clinical isolates of *Streptococcus sanguinis, C. albicans,* and MRSA. These strains were cultured on brain-heart infusion (BHI) broth (Nippon Becton Dickinson). Identification and susceptibility testing were accomplished by use of either MicroScan WalkAway (Dade) or a method described elsewhere [16].

Depletion of IgA or IgG from saliva. SIgA or secretory IgG was depleted from saliva by repeatedly passing the latter through a monoclonal anti-IgA–coupled (Hytest) or anti-IgG–coupled (Japan Biotest) affinity column (1 mL, HiTrap *N*-hydroxy-succinimide–activated high performance; Amersham Biosciences), until no residual antibodies were detected by ELISA. The concentration of salivary IgA was determined by use of a commercially available ELISA protein-detector kit (Kirkegaard & Perry Laboratories), according to the manufacturer's instructions.

Aggregation and coaggregation. Viridans streptococci, MRSA (unstained or stained with ethidium bromide), or both were mixed, on a glass slide for several minutes, with either intact saliva, IgA- or IgG-depleted saliva, preadsorbed saliva (saliva adsorbed with MRSA), or colostral SIgA (Cappel), and the aggregates were observed macro- and microscopically. For study of the formation of bacterial aggregates in vivo, oral cavities were swabbed and were stained with May-Grünwald-Giemsa solution.

Flow cytometry. MRSA (1×10^7 cfu) was incubated, for 30 min at room temperature, in either 0.5 mL of 50% saliva in

Table 1. IgA concentration in saliva, and ability to aggregate *Streptococcus sanguinis.*

Source (age)	Colonization[a]		Initial isolation		Aggregation[b]	IgA in saliva, μg/mL	Coaggregation[c]	Anti-MRSA IgA[d]
	MRSA	VS	MRSA	VS				
Infant 1 (276 days)	+	−	day 170		+	45	−	+
Infant 2 (84 days)	+	−	day 4		+	88	−	−
Infant 3 (90 days)	+	−	day 2		+	24	−	−
Infant 4 (49 days)	−	+		day 15	+	26	+	−
Infant 5 (6 days)	−	+		day 3	+	12	−	−
Infant 6 (133 days)	+	+	day 113	day 133	+	4	−	−
Adults [e]					+	78.2 ± 90.2[f]	+	+

NOTE. MRSA, methicillin-resistant *Staphylococcus aureus;* VS, viridans streptococci.

[a] First day of isolation during active surveillance.
[b] Ability of saliva to form VS aggregates.
[c] Ability of saliva to form coaggregates of viridans streptococci and MRSA.
[d] Analyzed by flow cytometry.
[e] 5 Adult volunteers.
[f] Mean ± SD (not significantly different [$P = .406$] from that in the infants [33.0 ± 30.4]).

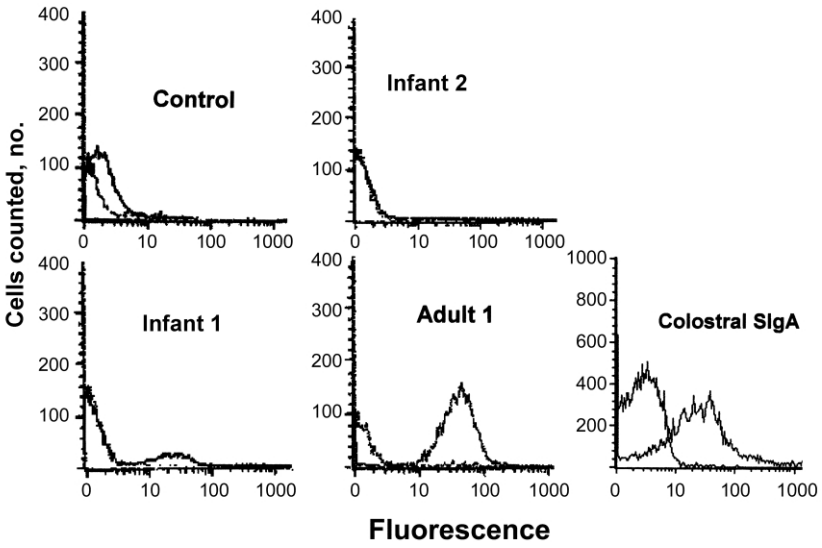

Figure 2. Representative samples of fluorescence-activated–cell-sorter analyses of secretory IgA directed against methicillin-resistant *Staphylococcus aureus* (anti-MRSA SIgA) in saliva. Except for that of infant 1, none of the saliva samples from infants contained anti-MRSA SIgA, although the overall concentrations of SIgA were not significantly different from those in adults (see table 1). Infant 1 (9.2 months old) was colonized by MRSA throughout the last 100 days of hospitalization and had very small amounts of anti-MRSA SIgA. Colostral SIgA contained anti-MRSA.

PBS, 2 mg of colostral SIgA, or PBS. After being washed in PBS, MRSA was stained with rabbit $F(ab)'_2$ anti–human IgA conjugated to fluorescein isothiocyanate (Dako) and were fixed with 2% paraformaldehyde. A total of ~10^4 cells were counted in a gated region comprising unclamped cells, by use of a FACScan (Becton Dickinson).

2-Dimensional gel electrophoresis, and analysis by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) analysis. Proteins were analyzed according to the method described by Yao et al. [17]. A 2-mL aliquot of *Streptococcus sanguinis* cell sediments (a result of centrifugation at 1300 *g* for 30 min at 4°C) was incubated, for 60 min at room temperature, with 10 mL of saliva and was washed 3 times with PBS. Salivary components bound to the cell surface were extracted with 4 mol/L NaCl. *Streptococcus sanguinis* proteins were extracted with a mixture of 8 mol/L urea, 2 mol/L thiourea, and 4% 3-[(cholamidopropyl)dimethylammonio]-1-propane sulfonate [18, 19]. The solvents of these extracted proteins were replaced with either PBS or 8 mol/L urea, by use of a centrifugal filter device (Millipore). *Streptococcus sanguinis* proteins bound to SIgA were prepared, by use of a colostral SIgA–coupled affinity column. These proteins were analyzed by 2-dimensional gel electrophoresis and MALDI TOF MS (Applied Biosystems). Peptide-mass fingerprints were used to screen tryptic-fragment libraries, by use of Mascot (Matrix Sciences) and Protein Prospector (University of California, San Francisco). All experiments were performed 3 times.

Assay of catalase activity. MRSA or *C. albicans* (100 μL) was incubated in 2.9 mL of substrate solution (0.1 mL of 30% H_2O_2 in 50 mL of 0.05 mol/L PBS [pH 7.0]), and the decrease in absorbance at 240 nm at 25°C was measured 2 times. One catalase unit was defined as the amount that results in decomposition at a rate of 1.0 μmol of H_2O_2/min by 1×10^8 cfu, at pH 7.0 and 25°C [2].

Indigo carmine–oxidation reaction, in PBS and in saliva. The method described by Wentworth et al. [14] was used to

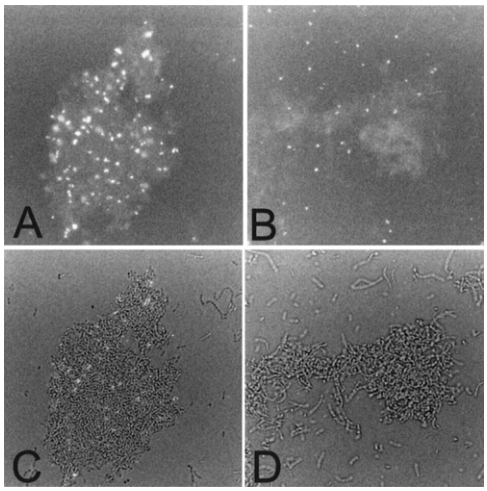

Figure 3. Coaggregation between *Streptococcus sanguinis* cells and methicillin-resistant *Staphylococcus aureus* (MRSA) cells. MRSA cells stained by ethidium bromide emit fluorescence in the coaggregate (*A* and *C*). Preadsorption of intact saliva with MRSA cells inhibited coaggregation, although the ability of intact saliva to form *S. sanguinis* aggregates remained (*B* and *D*). Results of both fluorescent microscopy *(A* and *B)* and light microscopy (*C* and *D*) are shown.

Table 2. Mass-spectrometry data used for protein assignment, and proteins' ability to bind to secretory IgA (SIgA) or *Streptococcus sanguinis.*

Protein assigned	Molecular weight, daltons (isoelectric point)		Species with homologous protein	Peptide matched	Coverage, %	Bound to IgA-coupled affinity column
	Observed	Theoretical				
Translation elongation factor G	76,000 (4.8)	76,783 (4.86)	*S. pneumoniae*	14	23	–
dnaK protein	65,000 (4.3)	64,802 (4.6)	*S. pneumoniae*	15	18	–
Enolase	46,000 (4.4)	47,074 (4.70)	*S. pneumoniae*	11	24	–
Phosphoglycerate kinase	43,000 (4.8)	41,973 (4.86)	*S. pneumoniae*	16	42	–
Glyceraldehyde-3-phosphate dehydrogenase	39,000 (5.5)	38,739 (5.78)	*S. pneumoniae*	10	23	–
30S ribosomal protein S2	32,000 (4.9)	28,252 (5.11)	*S. pyogenes*	11	48	–
Fructose-bisphosphate aldolase	30,000 (5.0)	31,382 (5.00)	*S. pneumoniae*	11	26	+[a]
Manganase-dependent dismutase	24,000 (4.8)	15,727 (4.81)	*S. sanguinis*	6	46	+ (a few)
Hepatocellular carcinoma–associated protein TB6	85,000 (6.0)	83,232 (5.58)	Human	22	29	Bound to *S. sanguinis* cell surface
Immunoglobulin heavy-chain variant	62,000 (5.3)	44,758 (5.75)	Human	10	27	Bound to *S. sanguinis* cell surface

[a] The most abundant protein that bound to the SIgA-coupled–affinity column.

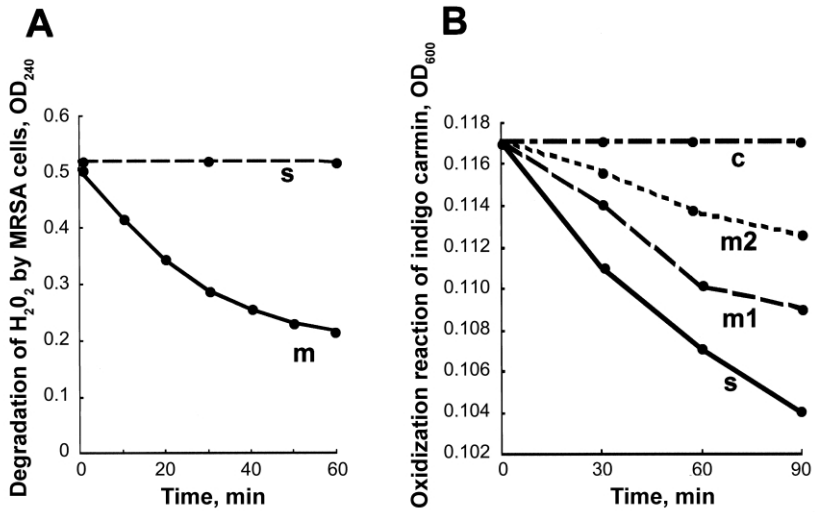

Figure 4. Decomposition of hydrogen peroxide (H_2O_2) in PBS. *A,* Decomposition by methicillin-resistant *Staphylococcus aureus* (MRSA) catalase (line m) and spontaneous degradation when MRSA cells are absent (line s). *B,* Spontaneous degradation in the presence of indigo carmine (reactive to singlet molecular oxygen [i.e., $^1O_2$], O_3, and H_2O_3). Very small but detectable amounts of oxidants were formed, some of which oxidized indigo carmine (line s). MRSA catalase inhibited this oxidant formation, in a dose-dependent manner (line m1, 2.5×10^5 cfu of MRSA [equivalent to the activity with 1.4 mU of catalase]; line m2, 1.0×10^6 cfu of MRSA [equivalent to the activity with 5.5 mU of catalase]). Overall, the comparative oxidant formation in these 3 situations, expressed in terms of the lines s, m1, and m2, was s > m1 ($P < .02$) and m1 > m2 ($P < .04$). *Aspergillus niger* catalase (30 mU) also inhibited oxidant formation (line c).

determine the indigo carmine–oxidation reaction. In each well of a 96-well microtiter plate, 3 μL of indigo carmine (1 mmol/L), which reacts with O_3, $^1O_2$, and H_2O_3, was mixed with a solution of PBS (pH 7.4), 5 μL of MRSA in PBS at various concentrations (2.5×10^5, 1.0×10^6, 2.0×10^6, and 4.0×10^6), 1 μL of 1% H_2O_2, and 91 μL of either PBS or saliva and was incubated at 37°C. At various time points, the decrease in absorbance at 600 nm was measured, 3 times, by use of a microtiter plate reader (Toso). In some experiments, various doses (0.03–300 mU) of either *Aspergillus niger* catalase (Sigma-Aldrich) or *Staphylococcus aureus* TW4632 (catalase-negative strain) were used instead of MRSA. To measure SIgA interference with the reaction, 0, 0.5, or 2.0 mg of human colostral SIgA was added to the reaction mixture comprising indigo carmine, MRSA (2.5×10^5 cfu), H_2O_2, and PBS. Each experiment was performed 5 times.

Assay of 4-vinyl benzoic acid oxidation. The method described by Wentworth et al. [14] was used to measure 4-vinyl benzoic acid oxidation. Saliva (150 μL) was mixed with a solution of 1 mmol/L of 4-vinyl benzoic acid, which reacts with O_3, in 150 μL of PBS (pH 7.4), 30 μL of 1% H_2O_2, and 1.1 mg of human colostral SIgA in 20 μL of PBS and was incubated for 3 h at room temperature. Aliquots (20 μL) were removed and diluted 1:3 in an acetonitrile:water (1:1) mixture. The product composition was determined by reversed-phase high-performance liquid chromatography performed by use of a Jasco LC-800 apparatus (Nihon Bunko) with a Shim-Pack CLC-ODS column. Products were detected by UV light at 254 nm (retention time [RT] for 4-vinyl benzoic acid, 11.47 min; RT for 4-carboxybenzaldehyde, 3.83 min; RT for 4-oxiranyl-benzoic acid, 4.31 min). In the control assay, 4-vinyl benzoic acid was oxidized by irradiation on a transilluminator (312 nm, 0.8 mW/cm^2), at room temperature in the presence of SIgA, and the composition of the products was determined by a similar method.

***Killing of MRSA by* Streptococcus sanguinis.** Killing of MRSA by *Streptococcus sanguinis* was measured by use of 2 small (0.4 cm^3) chambers separated by a dialyzing-cellulose membrane (Wako Pure Chemical) made with silicon-rubber sheets, between 2 glass slides. To 1 of these chambers, 1×10^4 cfu of MRSA in BHI broth was added; to the other chamber, 5×10^6 cfu of *Streptococcus sanguinis* was added; and both chambers were cultured for 16 h at 37°C. Cells in each chamber were recovered and were stained with 0.01% acridine orange in PBS for 45 s and then were viewed under a fluorescence microscope [20, 21]. In the control study, the same doses of MRSA were cultured in a similar device but in only 1 chamber. SIgA's augmentation of the killing of MRSA by *Streptococcus sanguinis* was measured. MRSA (25 μL, 230 cfu) was mixed with various doses (200–3200 cfu) of *Streptococcus sanguinis* in intact, IgA- or IgG-depleted saliva (125 μL), and the solution was incubated for 4 h at 37°C. MRSA viability was measured on the basis of the rate of recovery in the number of colony-forming units in orthophthaldialdehyde/*Staphylococcus* medium (Nippon Becton Dickinson). These experiments were performed 3 times.

Killing of MRSA by* Streptococcus sanguinis *in 50% saliva–agarose plates. MRSA (3 mL, 1×10^8 cfu) in PBS was mixed

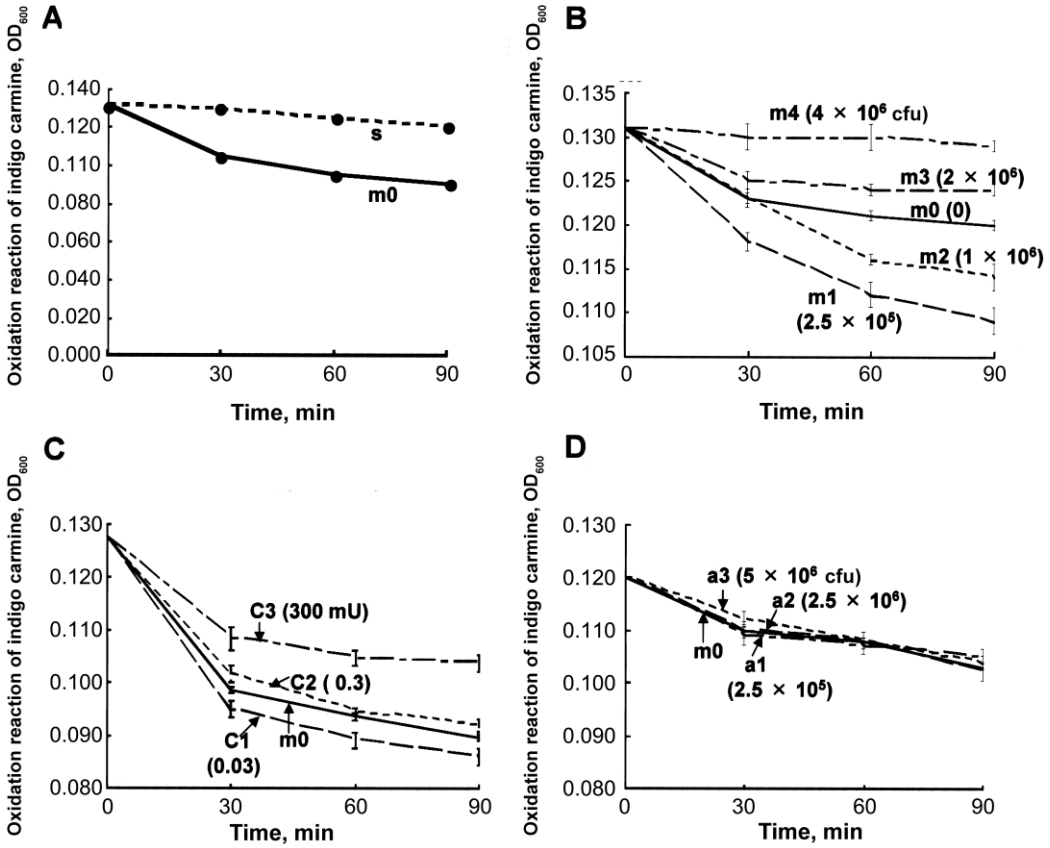

Figure 5. Decomposition of hydrogen peroxide (H_2O_2) in saliva. *A,* Decomposition by salivary peroxidase, which produced more indigo carmine–reactive oxidant (line m0) than was produced in the spontaneous degradation (line s) ($P < .001$). *B,* Decomposition by methicillin-resistant *Staphylococcus aureus* (MRSA). At smaller doses (2.5×10^5 cfu [line m1] and 1.0×10^6 cfu [line m2) of MRSA, MRSA catalase increased the oxidant-producing activity of salivary peroxidase (line m0) (for m0 vs. m1, $P < .02$); at larger doses (2×10^6 cfu, equivalent to the activity with 11.0 mU of catalase [line m3] and 4×10^6 cfu, equivalent to the activity with 22.0 mU of catalase [line m4]), this activity was inhibited (for m0 vs. m4, $P < .05$). *C,* Decomposition by *Aspergillus niger.* At 0.03 mU (line c1), *A. niger* catalase increased the oxidant-producing activity of salivary peroxidase (line m0) (for m0 vs. c1, $P < .003$); at 0.3 mU (line c2), it had no effect; at 300 mU (line c3), it inhibited this activity (for m0 vs. c3, $P < .001$). *D, Staphylococcus aureus* (catalase-negative strain TW4632) in saliva, which had no effect on the oxidant-producing activity of salivary peroxidase (line m0), at any of the 3 doses used (2.5×10^5 cfu [line a1], 2.5×10^6 cfu [line a2], and 5×10^6 cfu [line a3]).

with 9 mL of BHI broth (preincubated for 10 min at 50°C) containing 50 mg of low-melting-point agarose (Takara) and 6 mL of saliva, and the solution was poured onto a plastic plate. After the solution solidified, 3.3 mg of colostral SIgA (20 μL) was spotted onto 3 points on the plate and was allowed to soak into the gel, after which 1×10^8, 1×10^7, or 1×10^6 cfu of *Streptococcus sanguinis* (ATCC10556^T) (10 μL) were each spotted onto 1 of these SIgA-soaked points; the same dose of *Streptococcus sanguinis* solution, but without SIgA, was similarly spotted onto 3 points on the plate. The plates were cultured overnight at 35°C in 5% CO_2. This experiment was performed 2 times.

Killing of* C. albicans *by H_2O_2 in 50% saliva–agarose plates. *C. albicans* (1×10^8 cfu) was embedded in saliva/BHI-broth–agarose plates by use of the method described above, and colostral SIgA was soaked into the gel. *A. niger* catalase (0.3 or 5.0 U) was spotted onto the points soaked with SIgA and onto 2 points not soaked with SIgA. Then, 1% H_2O_2 (10 μL) was spotted onto the points soaked with SIgA, catalase, or both and onto a point not soaked with either SIgA or catalase. The plate was cultured overnight at 35°C in 5% CO_2. This experiment was performed 2 times.

Statistical analysis. Student's paired *t* test was used to compare, at the 3 time points (30, 60, and 90 min), differences in either the growth of MRSA cocultured with *Streptococcus sanguinis* or the absorbance in the indigo carmine–oxidation reaction. $P < .05$ was considered to be significant.

RESULTS

Aggregation and coaggregation. Figure 1*A* shows a representative sample of bacterial aggregate in the oral cavity of an

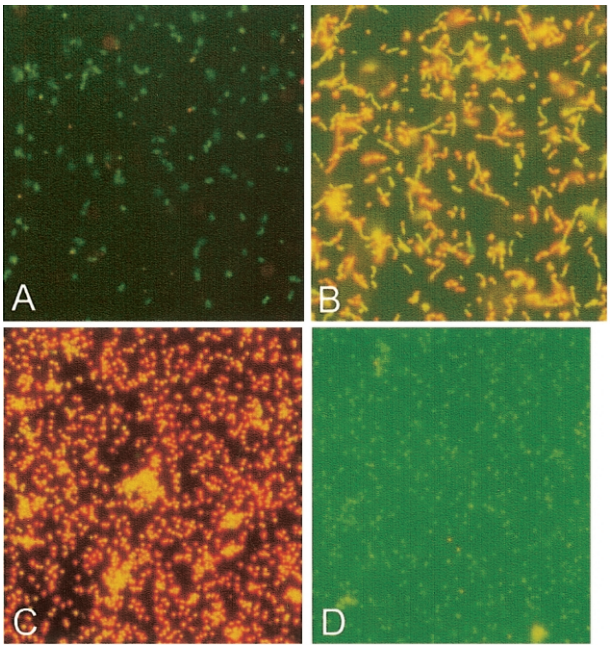

Figure 6. Killing of methicillin-resistant *Staphylococcus aureus* (MRSA) by *Streptococcus sanguinis.* Two small (0.4 cm^3) chambers separated by a dialyzing-cellulose membrane were constructed between 2 glass slides. To 1 of these chambers, 1×10^4 cfu of MRSA in brain-heart infusion broth was added; to the other chamber, 5×10^6 cfu of *Streptococcus sanguinis* was added; and both chambers were cultured for 16 h at 37°C. Staining with acridine orange showed that the effector molecule(s) produced by *Streptococcus sanguinis* passed through the cellulose membrane and killed MRSA cells in the neighboring chamber *(A [no fluorescence]),* whereas *Streptococcus sanguinis (B [orange fluorescence]),* and MRSA in a single-chamber device *(C)* survived; and MRSA cells killed by reagent H_2O_2 did not emit fluorescence *(D).*

infant. Although the bacterial composition of this aggregate was not determined, similar aggregates were easily formed in vitro by simple agitation of viridans streptococci, either in saliva (figure 1*B*) or in colostral SIgA (table 1). However, depletion of IgA (figure 1*C*), but not of IgG, from saliva almost completely abolished aggregation. These data indicate that both SIgA and viridans streptococci are important contributors to bacterial aggregation in vivo. The concentration of IgA in the 7 infants' saliva (33.0 ± 30.4 μg/mL) was not significantly different from that in the 5 adults' saliva (78.2 ± 90.2 μg/mL) ($P = .406$) (see table 1); in contrast, none of the saliva and none of the colostral SIgA formed MRSA aggregates, although both saliva from the 5 adults and from 1 infant (infant 1) and colostral SIgA contained anti-MRSA SIgA (figure 2). Coaggregation between MRSA and *Streptococcus sanguinis* occurred in the saliva of all 5 adults (figure 3*A* and 3*C*) and in that of 1 infant (infant 4, without anti-MRSA SIgA). The saliva of infant 1 contained anti-MRSA SIgA but did not form coaggregates (see table 1). Preadsorption of the 5 adults' saliva with MRSA successfully inhibited coaggregation but not *Streptococcus sanguinis* aggregation (figure 3*B* and 3*D*), which suggests that a salivary component other than anti-MRSA SIgA is necessary to bind viridans streptococci aggregates to MRSA.

***Binding of salivary proteins to the* Streptococcus sanguinis *cell surface and binding of colostral SIgA to* S. sanguinis.** Salivary IgA and β-catenin bound to the cell surface of *Streptococcus sanguinis;* of the cell-surface proteins of *Streptococcus sanguinis,* fructose-bisphosphate aldolase and some manganese-dependent dismutase bound to the SIgA-coupled affinity column (table 2).

Decomposition of H_2O_2 by bacterial cells, in PBS and in saliva. The average catalase activity of 5 strains of MRSA was 0.43 ± 0.24 U/1×10^8 cfu; that of *C. albicans* was 0.03 ± 0.01 U/1×10^8 cfu. In PBS, MRSA catalase decomposed H_2O_2 (figure 4*A,* line m), whereas reagent H_2O_2 remained stable (figure 4*A,* line s). Very small but detectable amounts of oxidants were produced during the spontaneous degradation of H_2O_2 in the presence of indigo carmine (figure 4*B,* line s), and the velocity of the reaction was proportional to the H_2O_2 concentration (data not shown); MRSA catalase inhibited this oxidant formation, in a dose-dependent manner (as shown by figure 4*B,* lines m1 [2.5×10^5 cfu of MRSA, equivalent to the activity with 1.4 mU of catalase] and m2 [1.0×10^6 cfu of MRSA, equivalent to the activity with 5.5 mU of catalase]; overall, the comparative oxidant formation in these 3 situations, expressed in terms of the lines in figure 4*B,* was s > m1 [$P < .02$] and m1 > m2 [$P < .04$]). *A. niger* catalase (30 mU) also inhibited this oxidant formation (figure 4*B,* line c). These data show that,

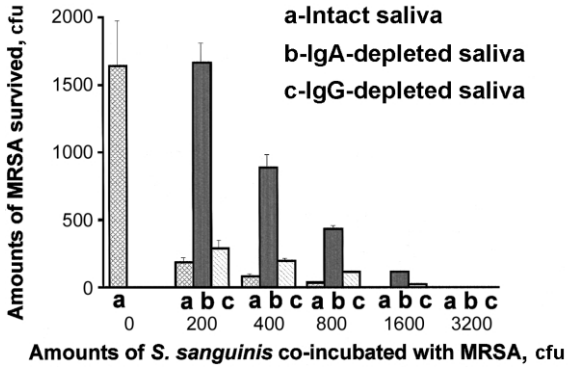

Figure 7. Survival of methicillin-resistant *Staphylococcus aureus* (MRSA), in coaggregation between MRSA and *Streptococcus sanguinis,* after incubation for 4 h. In the coaggregates formed in intact saliva, incubation with *S. sanguinis* significantly decreased the survival of MRSA, in a dose-dependent manner, compared with incubation of MRSA alone ($P < .001$). Initially, complete killing of 230 cfu of MRSA needed >7 times (≥1600 cfu) the number of *S. sanguinis.* Although *S. sanguinis* decreased the survival of MRSA similarly in IgA-depleted saliva, the recovery in the rate of survival differed significantly ($P < .001$) in secretory IgA–depleted saliva vs. either intact or IgG-depleted saliva.

Table 3. Killing of methicillin-resistant *Staphylococcus aureus* (MRSA) by *Streptococcus sanguinis,* and killing of *Candida albicans* by hydrogen peroxide in 50% saliva–agarose plate with or without exogenous colostral secretory IgA (SIgA).

	A. Killing of MRSA		
	S. sanguinis inoculated on plate		
	1×10^8 cfu	1×10^7 cfu	1×10^6 cfu
With SIgA (3.3 mg)[a]	57.8 ± 3.1	49.9 ± 1.6	13.5 ± 2.1
Without SIgA	50.9 ± 1.6	20.9 ± 3.0	0
	B. Killing of *C. albicans*[b]		
	Aspergillus niger catalase added to plate		
	0 units	0.3 units	5.0 units
With SIgA (3.3 mg)[a]	22.6 ± 1.9	47.9 ± 5.2	7.8 ± 0.8
Without SIgA	0	0	0

NOTE. Data are mean ± SD square millimeters of radii of clear zones in which MRSA or *C. albicans* did not grow; around the clear zones, MRSA or *C. albicans* culture produced confluent colonies.

[a] Exogenously added.

[b] Reagent hydrogen peroxide (10 μL, 1%), instead of *S. sanguinis,* was used.

in PBS, both *A. niger* catalase and MRSA catalase reduce the bactericidal potency of H_2O_2.

Salivary peroxidase decomposed H_2O_2 and produced more indigo carmine–reactive oxidant (figure 5*A,* line m0) than was produced in the spontaneous decomposition of H_2O_2 (figure 5*A,* line s) ($P < .001$). However, in contrast to the oxidation-reaction outcome in PBS, MRSA in saliva did not always inhibit the oxidant formation by salivary peroxidase: at smaller doses (2.5×10^5 cfu [figure 5*B,* line m1] and 1.0×10^6 cfu [figure 5*B,* line m2) of MRSA, MRSA catalase increased the oxidant-producing activity of salivary peroxidase (figure 5*B,* line m0) (for m0 vs. m1, $P < .02$); at larger doses (2×10^6 cfu [equivalent to the activity with 11.0 mU of catalase] [figure 5*B,* line m3] and 4×10^6 cfu [equivalent to the activity with 22.0 mU of catalase] [figure 5*B,* line m4]) of MRSA, this activity was inhibited (for m0 vs. m4, $P < .05$). For *A. niger* in saliva, the results were similar: 0.03 mU (figure 5*C,* line c1) of *A. niger* catalase increased the oxidant-producing activity of salivary peroxidase (figure 5*C,* line m0) ($P < .003$), 0.3 mU of this catalase (figure 5*C,* line c2) had no effect on this activity, and 300 mU of this catalase (figure 5*C,* line c3) inhibited this activity ($P < .001$). *Staphylococcus aureus* (catalase-negative strain TW4632) in saliva had no effect on the oxidant-producing activity of salivary peroxidase, at any of the 3 doses used (2.5×10^5 cfu [figure 5*D,* line a1], 2.5×10^6 cfu [figure 5*D,* line a2], and 5×10^6 cfu [figure 5*D,* line a3).

SIgA-mediated interference with the indigo carmine–oxidation reaction. After 180 min, exogenous SIgA (0.5 or 2.0 mg) decreased both the rate of the indigo carmine–oxidation reaction by salivary peroxidase and the rate of the MRSA catalase–catalyzed H_2O_2-decomposition reaction, by 11.9% and 28.6% ($P < .001$), respectively (data not shown). This result suggests that SIgA mediates the production of an indigo carmine–nonreactive product, presumably the reformation of H_2O_2. If so, then the indigo carmine–reactive components—ozone and $^1O_2$—are both products of the reaction. Next, we tried to ascertain whether the reaction products included ozone.

Assay of 4-vinyl benzoic acid oxidation. Ozonolysis of 4-vinyl benzoic acid was measured by reversed-phase high-performance liquid chromatography. In the presence of SIgA and saliva, H_2O_2 oxidized 4-vinyl benzoic acid (RT, 11.47 min) and produced 4-carboxybenzaldehyde (RT, 3.83 min) and 4-oxiranyl-benzoic acid (RT, 4.31 min), whereas neither H_2O_2 in PBS nor SIgA and saliva without H_2O_2 oxidized it; and 4-vinyl benzoic acid was similarly oxidized by ozone produced by UV irradiation (data not shown). These data show that H_2O_2 is decomposed by saliva and is converted to ozone by the catalytic activity of SIgA.

***Killing of MRSA by* Streptococcus sanguinis.** Staining with acridine orange showed that the effector molecule(s) produced by *Streptococcus sanguinis* passed through the cellulose membrane and killed MRSA in the neighboring chamber (figure 6*A,* no fluorescence), whereas *Streptococcus sanguinis* (figure 6*B,* orange fluorescence) and MRSA in a single-chamber device (figure 6*C*) survived. MRSA killed by reagent H_2O_2 did not emit fluorescence (figure 6*D*). SIgA augmented this bactericidal activity of *Streptococcus sanguinis.* In the coaggregate (intact saliva), *Streptococcus sanguinis* decreased the survival rate of MRSA in a dose-dependent manner, compared with the rate when MRSA was incubated alone ($P < .001$). Initially, complete killing of 230 cfu of MRSA required >7 times more (≥1600 cfu of) *Streptococcus sanguinis* (figure 7). However, *Streptococcus sanguinis* decreased the survival rate of MRSA similarly in IgA-depleted saliva; the survival rate of MRSA recovered completely in SIgA-depleted saliva, whereas it did not recover in either intact or IgG-depleted saliva ($P < .001$).

Killing of MRSA by* Streptococcus sanguinis *and killing of* C. albicans *by H_2O_2, in a 50% saliva–agarose plate. MRSA cultured on a 50% saliva–agarose plate produced confluent opaque colonies. Around the *Streptococcus sanguinis* colonies without exogenous SIgA, MRSA was killed by 1×10^8 cfu of *Streptococcus sanguinis* and by 1×10^7 cfu of *Streptococcus sanguinis,* and the plate remained clear; however, MRSA was not killed by 1×10^6 cfu of *Streptococcus sanguinis.* To assess the level of killing, we compared the squares of the radii of the clear zones. The addition of 3.3 mg of colostral SIgA, which contained anti-MRSA SIgA (see figure 2), augmented the killing of MRSA, and larger areas remained clear around *Streptococcus sanguinis* at 1×10^8 cfu, 1×10^7 cfu ($P < .001$), and 1×10^6 cfu ($P < .004$) (table 3*A*).

The data in table 3*B* show that very few *C. albicans* cells, even those just below the spot of reagent H_2O_2 (10 μL, 1%),

were killed in the absence of SIgA, even though the plate contained saliva and *A. niger* catalase—and that the addition of SIgA significantly augmented the killing. Large doses (5 U) of *A. niger* catalase inhibited the killing of *C. albicans,* even in the presence of SIgA, compared with the killing in cultures without this catalase; however, large doses of this catalase did not completely eliminate production of $^1O_2$, because the augmentation in the killing of *C. albicans* in the presence of SIgA, compared with the killing in the absence of SIgA, indicates that $^1O_2$ is present; and small doses (0.3 U) of the catalase augmented the killing of *C. albicans* in the presence of SIgA, compared with the level of killing in the absence of the catalase ($P < .002$).

DISCUSSION

The data in the present study demonstrate a previously unrecognized process that may help to maintain homeostasis in the oral cavity and thereby defend the oral environment against pathogens. Coaggregation between viridans streptococci and pathogens, in conjunction with the catabolism of H_2O_2 in the aggregates to produce ozone by SIgA, seems to be the key event in this process. SIgA is responsible for mucosal defense, not only by immune exclusion [22], but also by direct killing of pathogens.

SIgA contains various bacteria-binding sites in its glycans, in addition to its 4 Fab binding sites [7, 8], and it binds to bacterial surface proteins, such as fructose bisphosphate aldolase of *Streptococcus pneumoniae* [23]. In fact, the present study found that SIgA binds to viridans streptococci and forms aggregates. However, such aggregation was not found when MRSA was used, presumably because of the latter's significant ζ potential (i.e., the electrical potential of the interface between the bacterial surface and the aqueous environment) [24], a characteristic that is an advantage in the development of an MRSA-killing process: even in the presence of anti-MRSA SIgA, MRSA cells invading the oral cavity may remain unaggregated until they associate with numerous viridans streptococci cells [25] already aggregated to various extents. Additional salivary component(s) that can reduce ζ potential may be necessary for coaggregation; however, if such coaggregation occurs, ζ potential may augment bactericidal activity, even in infants' saliva without anti-MRSA SIgA.

Salivary peroxidase decomposes H_2O_2 into an indigo carmine–reactive oxidant, presumably either $^1O_2$, O_3, or H_2O_3 [12, 14]. However, this oxidant is not strong enough to kill some resistant microorganisms, such as *C. albicans* [2]. In the present study, the addition of SIgA and small doses of *A. niger* catalase significantly augmented this oxidant's bactericidal and fungicidal effects, and more *C. albicans* cells were killed by the newly synthesized oxidant. Ozone may be produced by the SIgA-catalyzed water-oxidation reaction [14]. If so, then the oxidant produced before the addition of SIgA is $^1O_2$, one of the starting materials of the reaction [12], and the products of 4-vinyl benzoic acid oxidation strongly suggest that ozone is present [14]. In this situation, MRSA catalase may potentiate not the survival but, instead, the suicide of MRSA.

None of the viridans streptococci tested in our earlier study were low-H_2O_2–producing strains [2]; and the present study used physiological amounts of SIgA, *A. niger* catalase, and reagent H_2O_2. In the present study, the concentration of SIgA that was soaked into the plates was 5 times greater than that in intact saliva, and this concentration may be achieved by the coaggregation formation even in infants. Similarly, the concentrations of H_2O_2 and *A. niger* catalase were equivalent to the amounts produced by 1×10^8 cfu of either viridans streptococci [25] or MRSA and may be achievable in the oral cavity [2]. Ozone may be toxic to oral epithelia [26]; however, because it is short lived [14], tissue damage is limited to a narrow region around the coaggregates. In BHI broth, >500 viridans streptococci cells are necessary to kill 1 MRSA cell [2], whereas the results of the present study show that only 7 viridans streptococci cells are necessary to kill each MRSA cell in the coaggregates. Therefore, the killing of MRSA and of *C. albicans* may actually occur in the oral cavity. Mucosal surfaces other than the oral cavity, such as the vaginal mucosa, which harbors H_2O_2-producing lactobacilli, may have a similar system to destroy pathogens [27].

In conclusion, the results of the present study show that, in cooperation with H_2O_2-producing viridans streptococci and salivary components, SIgA can kill MRSA and *C. albicans*—and that this killing potential is augmented by bacterial catalase.

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