

Myeloperoxidase Selectively Binds and Selectively Kills Microbes^{∇†}

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Myeloperoxidase (MPO) is reported to selectively bind to bacteria. The present study provides direct evidence of MPO binding selectivity and tests the relationship of selective binding to selective killing. The microbicidal effectiveness of H₂O₂ and of OCl⁻ was compared to that of MPO plus H₂O₂. Synergistic microbicidal action was investigated by combining *Streptococcus sanguinis*, a H₂O₂-producing microbe showing low MPO binding, with high-MPO-binding *Escherichia coli*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* without exogenous H₂O₂, with and without MPO, and with and without erythrocytes (red blood cells [RBCs]). Selectivity of MPO microbicidal action was conventionally measured as the MPO MIC and minimal bactericidal concentration (MBC) for 82 bacteria including *E. coli*, *P. aeruginosa*, *S. aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and viridans streptococci. Both H₂O₂ and OCl⁻ destroyed RBCs at submicrobicidal concentrations. Nanomolar concentrations of MPO increased H₂O₂ microbicidal action 1,000-fold. Streptococci plus MPO produced potent synergistic microbicidal action against all microbes tested, and RBCs caused only a small decrease in potency without erythrocyte damage. MPO directly killed H₂O₂-producing *S. pyogenes* but was ineffective against non-H₂O₂-producing *E. faecalis*. The MPO MICs and MBCs for *E. coli*, *P. aeruginosa*, and *S. aureus* were significantly lower than those for *E. faecalis*. The streptococcal studies showed much higher MIC/MBC results, but such testing required lysed horse blood-supplemented medium, thus preventing valid comparison of these results to those for the other microbes. *E. faecalis* MPO binding is reportedly weak compared to binding of *E. coli*, *P. aeruginosa*, and *S. aureus* but strong compared to binding of streptococci. Selective MPO binding results in selective killing.

The microbicidal activities of H₂O₂ and especially OCl⁻ are potent, but such activities are best achieved in the absence of competing substrates. The reactivities of these agents are indiscriminate and capable of damaging mechanisms of immune defense as well as destroying microbes. Alexander Fleming appreciated this relationship, stating that “leukocytes are more sensitive to the action of chemical antiseptics than are the bacteria, and, in view of this, it is unlikely that any of these antiseptics have the power of penetrating into the tissues and destroying the bacteria without first killing the tissues themselves” (16).

Healthy human adults produce about 100 billion neutrophil leukocytes per day. After a circulating lifetime of about 10 h, these neutrophils leave the blood to enter the body tissues (8). Myeloperoxidase (MPO), a 145-kDa dimeric alpha-heme haloperoxidase present in azurophilic granules, makes up approximately 5% of the dry weight of the neutrophil (33). If a neutrophil is assumed to have a cell volume of 450 fl, a specific gravity of 1.1, and a cell water content of 84%, healthy human adults synthesize about 2.8 μmol (i.e., 0.4 g) of MPO per day. Furthermore, the production of neutrophils and the MPO content per neutrophil are markedly increased in states of inflammation and with granulocyte colony-stimulating factor (G-CSF) treatment (7).

MPO exerts potent and broad-spectrum microbicidal action

against Gram-positive and Gram-negative bacteria, as well as yeast and fungi (23). Of the mammalian peroxidases, MPO is unique in its ability to catalyze the H₂O₂-dependent oxidation of Cl⁻ to OCl⁻. Such haloperoxidase activity is required for effective microbe killing (22). In addition to the requirement of H₂O₂ for OCl⁻ production, H₂O₂ also directly reacts with OCl⁻ to produce singlet molecular oxygen (¹O₂^{*}), a potent electrophilic oxygenating agent (21). The microbicidal action of MPO involves highly exergonic oxygenation reactions. These wet combustion reactions yield excited carbonyl products that relax with chemiluminescence (2, 3).

After leaving the blood, neutrophils migrate into cavities such as the mouth (38) and vagina (11). These spaces are characterized by acidic pH and the presence of flora rich in lactic acid bacteria (LAB). It is reasonable to assume that the neutrophils that have migrated into the acid milieu of the mouth or vagina eventually disintegrate, releasing their MPO content into these environments, and that this released MPO is available to interact with microbes of the resident flora.

MPO selectively binds to Gram-negative and many Gram-positive bacteria. However, streptococci, especially viridans streptococci, show little MPO binding (4, 6). Viridans streptococci are a major constituent of normal mouth flora (20). As members of the LAB, streptococci are cytochrome deficient and catalase negative and produce lactic acid and H₂O₂ as metabolic products (24). The rates of H₂O₂ production by *Streptococcus sanguinis* and *Streptococcus mitis* are reported to be in the range of 12 to 67 nanomoles/min/mg of dry weight (10). Another study of *Streptococcus oralis* and *S. sanguinis* reported a range of 1 to 5 nmol/min/10⁶ CFU (18). These rates of H₂O₂ production are adequate to drive MPO oxidation of Cl⁻ to OCl⁻ and for H₂O₂ reaction with OCl⁻ to produce

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$^1\text{O}_2^*$. At relatively low MPO concentrations, a H_2O_2 -producing LAB showing low MPO binding might escape damage. Furthermore, the streptococcus-generated H_2O_2 would be available to drive combustive reactions against competing microbes showing higher MPO binding.

The present report compares the microbicidal capacities of H_2O_2 , OCl^- , and nanomolar MPO plus low concentrations of H_2O_2 . The potency and selectivity of MPO microbicidal action are illustrated using either streptococci or glucose oxidase (GO) to generate H_2O_2 . The selectivity of microbicidal action was conventionally assessed by measuring the MIC and minimal bactericidal concentration (MBC) of MPO against a large spectrum of clinical bacterial isolates.

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MATERIALS AND METHODS

Reagents and enzymes. Stock H_2O_2 and NaOCl solutions were prepared and quantified. The concentration of the H_2O_2 stock solution was verified by measuring its UV absorbance spectrum using a 240-nm extinction coefficient (ϵ_{240}) of $43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and a 300-nm extinction coefficient (ϵ_{300}) of $1.0 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The concentration of the NaOCl stock solution was verified by measuring its absorbance spectrum using a 292-nm extinction coefficient (ϵ_{292}) of $350 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Porcine MPO, EC 1.11.1.7, was produced by ExOxEmis, Inc. The absorbance extinction coefficient at 430 nm (ϵ_{430}) for MPO is $178 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (1). The RZ (reinheitszahl or purity number) value, i.e., the ratio of 430-nm to 280-nm absorbance ($A_{430/280}$), estimates the purity of MPO relative to total protein. The $A_{430/280}$ RZ for the MPO was 0.7 in the initial binding studies and 0.8, i.e., crystalline purity, for the MIC/MBC studies. One picomole of MPO equals 0.145 μg . The guaiac activity of MPO was 380 guaiac units (GU)/mg of MPO. Spectrophotometric measurements were performed using a DW2000 UV-visible light spectrophotometer (SLM Instruments Co.).

Glucose oxidase from *Aspergillus niger*, EC 1.1.3.4, was used as a H_2O_2 generator for the MIC/MBC studies. High-purity GO with minimal catalase activity was prepared by ExOxEmis, Inc. One picomole of GO equals 0.16 μg . The activity of GO was 320 units/mg measured using horseradish peroxidase with *o*-dianisidine as a substrate. For the MIC/MBC studies, MPO and GO were used at a molar ratio of 4.4. The solution also contained 5.6 mM L-alanine, 7.2 mM L-proline, 7.2 mM glycine, and 300 mM D-glucose as a substrate.

Microbes. The initial descriptive investigation used six bacteria, i.e., *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11303, *Pseudomonas aeruginosa* ATCC 9027, viridans streptococcus, *Streptococcus pyogenes*, and *Enterococcus faecalis*, and a yeast, i.e., *Candida albicans* ATCC 10231. Viridans group streptococcus, *S. pyogenes*, and *E. faecalis* were clinical isolates. *S. pyogenes* produce H_2O_2 and show β -hemolysis (34). *E. faecalis* does not produce H_2O_2 and showed no hemolysis on blood agar (15). The viridans streptococcus used was an oropharyngeal isolate selected for its high alpha-hemolytic activity, i.e., viridans character. This alpha-hemolytic activity reflects H_2O_2 generation (10, 24). This viridans group *Streptococcus* isolate was further typed to be *S. sanguinis*.

The bacteria were grown overnight (about 16 h) in Trypticase soy broth (TSB) at 35°C. The yeast was grown for about 16 h in Sabouraud's dextrose broth (SDB) at 35°C. The cultures were centrifuged at 3,000 rpm for 15 min, and the supernatants were discarded. The microbial pellets were resuspended and diluted with sterile 0.85% normal saline (NS) to an absorbance of 0.1 at a wavelength of 540 nm, i.e., about 10^8 bacteria CFU per ml and about 10^7 yeast CFU per ml.

Preparation and use of erythrocytes (RBCs). Blood was collected by venipuncture from a healthy human volunteer (R. C. Allen) using lithium heparin as an anticoagulant. The whole blood was centrifuged at 1,500 rpm for 15 min, and the plasma and leukocyte buffy coat were removed by aspiration, leaving the packed erythrocytes. These erythrocytes were suspended in NS, and following mixing, the red blood cells (RBCs) were again centrifuged, aspirated, and suspended in NS. The erythrocyte suspension was then passed through sterile cotton gauze to

remove remaining leukocytes. The centrifugation and aspiration steps were repeated, and the erythrocyte pellet was suspended and diluted with NS to a concentration of 10^8 RBCs per ml by hemocytometer count.

Erythrocytes (0.1 ml containing 10^7 RBCs) were added to the microbe suspensions where indicated. Human RBCs contain catalase, i.e., $\text{H}_2\text{O}_2:\text{H}_2\text{O}_2$ oxidoreductase, an enzyme that catalyzes the destruction of H_2O_2 producing H_2O and O_2 . The mechanism for removal of H_2O_2 in human erythrocytes is more than 90% dependent on catalase action (28). In addition to competitive destruction of H_2O_2 , erythrocytes can directly react with oxidizing agents and, thus, compete with microbes for available reactants. RBCs are susceptible to damage by high concentrations of H_2O_2 and lower concentrations of OCl^- . Introducing RBCs into the reaction mixture provides a means for assessing the specificity of the reactants for the microbe relative to the erythrocyte.

Following microbe-RBC incubation and centrifugation, the hemoglobin (Hgb) that remains in the pellet is proportional to the remaining intact erythrocytes, i.e., to RBC survival. Loss of Hgb from the pellet and its appearance in the supernatant indicate hemolysis, i.e., the destruction of RBC membrane integrity and release of cytoplasmic Hgb into the medium. The degree of hemolysis can be assessed by the distribution of Hgb in the pellet and supernatant. Complete hemolysis with Hgb destruction results in the disappearance of Hgb from both the pellet and supernatant. In addition to the oxidative reactants tested, microbial toxins can also produce hemolysis and Hgb destruction.

The concentrations of Hgb in the pellet and supernatant were measured by a derivative spectroscopic modification of the hemiglobincyanide (Drabkin) method (27). A suspension of 10^7 RBCs per ml yielded 0.2 to 0.3 mg of Hgb. The total Hgb of the supernatant and pellet was measured in the control and was expressed as unity, i.e., 1.0. The distribution of Hgb between the pellet and supernatant was expressed as a proportion of 1.0. This approach provides a rough but useful gauge of hemolytic activity. Small losses of Hgb from the pellet to the supernatant are an expected consequence of the preparation and experimental treatment, but large losses to the supernatant indicate active hemolysis. Complete destruction of Hgb is indicated by a total loss from the pellet and supernatant.

Measuring direct microbicidal action of H_2O_2 , OCl^- , H_2O_2 -MPO, and MPO alone. Using a sterile technique, 0.1 ml of the test microbe suspension (about 10^6 bacteria or 10^5 yeasts) and 0.1 ml of NS or RBC suspension were added to 12-by 75-mm polystyrene tubes. The reaction was initiated by adding the various dilutions of the H_2O_2 or OCl^- prepared in NS to a final volume 1 ml. The reaction suspension was mixed and incubated for 30 min at 23°C. After remixing to suspend the cellular components, a 0.1-ml aliquot of the suspension was added to 0.9 ml of NS, and this suspension was serially (10^4) diluted out to 10^{-3} . Then 0.1 ml of each dilution was uniformly spread on agar plates using the "glass hockey stick" technique. Trypticase soy agar (TSA) was used to culture the bacteria and Sabouraud's dextrose agar (SDA) was used to culture *C. albicans*. The plates were then incubated at 36°C for about 24 h. Microbe colonies were counted, and the number of CFU/test was derived by multiplying the number of colonies counted by the initial dilution factor plus the plate dilution factor.

***S. sanguinis*-MPO synergistic killing of *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*.** The bacteria, yeast, RBCs, and MPO were prepared and quantified as described above, and 0.1 ml of *S. sanguinis* (viridans group streptococcus) suspension (about 10^7 streptococci based on the optical density at 540 nm; 3.3×10^7 CFU), 0.1 ml of the target microbe suspension (about 10^6 microbes), 0.1 ml of NS or the MPO dilution indicated, 0.1 ml of glucose (1 mg), 0.5 ml of NS, and where indicated, either 0.1 ml of erythrocyte suspension (10^7 RBCs) or NS were added to each tube for a 1-ml final volume. No H_2O_2 was added. The contents were gently mixed, and the tubes were incubated undisturbed for 30 min at 23°C. Microbe killing was measured by the agar plate dilution technique as described above except that the colonies were grown for an additional day to increase the visibility of the small streptococcal colonies.

MPO is directly microbicidal against *S. pyogenes* but not against *E. faecalis*. Bacteria, RBCs, and MPO were prepared and quantified as described above. A 0.1-ml suspension (about 10^6 microbes) of either *S. pyogenes* (Lancefield group A) or *E. faecalis*, 0.1 ml of NS or the MPO dilution as indicated, 0.1 ml of glucose (1 mg), 0.1 ml of NS or erythrocyte suspension (10^7 RBCs), and 0.6 ml of NS were added to each tube for a final volume of 1.0 ml. No H_2O_2 was added. The contents were gently mixed, and the tubes were incubated for 30 min at 23°C. Microbe killing was measured by the agar plate dilution method.

MIC and minimum bactericidal concentration of MPO. In addition to the bacterial strains described above, a total of 78 clinical isolates were tested to determine sensitivity and consistency of MPO MICs and MPO MBCs. Eurofins Medinet Anti-Infective Services (Chantilly, VA) was contracted to perform these MIC and MBC studies on their collection of clinical isolates from diverse geographical regions within the United States. Fourteen *S. aureus* clinical isolates

were tested; six isolates were methicillin-sensitive (MSSA), six isolates were methicillin-resistant (MRSA), and two isolates were vancomycin-resistant (VRSA). Five clinical isolates of *E. coli* were tested; one *E. coli* isolate was ceftazidime sensitive (CEF-S), and four were ceftazidime resistant (CEF-R). Five clinical isolates of *P. aeruginosa* were tested; two *P. aeruginosa* isolate were CEF-S, and three were CEF-R. Seven clinical isolates of *E. faecalis* were tested; five of the *E. faecalis* isolates were vancomycin sensitive (VSE), and two were vancomycin resistant (VRE). Twenty clinical isolates of pathogenic Lancefield group A and B streptococci were tested; 10 isolates were *S. pyogenes* (group A), and 10 clinical isolates were *Streptococcus agalactiae* (group B). Twenty-seven isolates of viridans group streptococci were tested; nine were *S. mitis*, nine were *S. oralis*, and nine were *S. sanguinis*. In addition, *S. aureus* ATCC 29213 was used as the quality control strain to validate the modified Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (12, 29).

Conventional MIC and MBC testing is designed to measure the inhibitory effect of an antibiotic on bacterial protein synthesis or cell wall formation. Such testing is not well suited to measuring the oxidative microbicidal action of MPO. For example, the pH of cation-adjusted Mueller-Hinton broth (CAMHB) is mildly alkaline, pH 7.3, but the optimum pH for MPO action is in the range of 5 to 6, i.e., the pH of the neutrophil phagolysosomal space, the skin, the mouth, and the vaginal space. In addition to the nonoptimum pH imposed by CAMHB, supplementation of CAMHB with 5% lysed horse blood (CAMHB-5% LHB) is required for MIC and MBC testing of the streptococci. Lysed horse blood contains significant catalase activity that destroys H_2O_2 , thus removing the substrate required for MPO production of OCI^- and $^1O_2^*$. This lysed horse blood also contains Hgb and other molecular substrates that can reactively compete with microbes for available OCI^- and $^1O_2^*$. Lysed horse blood consumption of MPO-generated oxidants competitively inhibits microbicidal action.

MPO activity was tested by mixing a solution containing MPO and GO with a solution containing glucose. The enzyme and substrate solutions were mixed together in various proportions just prior to microbe exposure to produce the desired concentration of MPO. The molar ratio of MPO to GO was 4.4. Thus, GO production of H_2O_2 was in proportion to the available MPO. Broth MIC and MBC determinations were performed according to CLSI procedure specifications with modifications to accommodate the rapid *in vitro* activity of MPO. The enzyme solution was diluted in double-strength CAMHB and dispensed in microdilution trays. All streptococcal species were tested in double-strength CAMHB supplemented with 5% lysed horse blood (CAMHB-5% LHB) according to CLSI procedural specifications. Isolates were prepared by suspending colonies from an overnight culture on Trypticase soy agar with 5% sheep blood into sterile saline, and the bacterial suspension density was adjusted to a 0.5 McFarland standard ($\sim 10^8$ CFU/ml). Standardized bacterial suspensions were further diluted in double-strength substrate solution so that approximately 5×10^5 CFU/ml were mixed with serial MPO-GO dilutions. Contact with glucose activated the enzyme system. The microdilution trays were incubated in ambient air at 35°C for 18 to 24 h.

The MIC was the lowest concentration of MPO observed to completely inhibit microbe growth. The MBC was determined using the same modified broth microdilution method. The last MPO well on the microdilution tray showing visible growth and each of the clear wells were sampled by removing a 10- μ l sample per well that was plated onto TSA with 5% sheep blood and incubated in ambient air for 24 h before examination for growth and colony counts. The MBC was determined as the lowest antimicrobial concentration demonstrating a >99.9% reduction in the number of CFU relative to the starting inoculum. The inhibitory effect of 5% lysed horse blood was documented by simultaneously retesting the MPO-dependent MIC and MBC activities of the same seven strains of *E. faecalis* in CAMHB and in CAMHB-5% LHB.

SPSS, version 17.0, and SigmaPlot, version 11, software were used for exploratory data analysis (37), one-way analysis of variance (ANOVA), an independent *t* test, and graphic presentation.

RESULTS

Antimicrobial action of H_2O_2 in the absence and presence of MPO. The direct microbicidal action of H_2O_2 and the protective effect of human erythrocytes (RBCs) were measured using *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*. The results are presented in Table 1.

Erythrocytes provide a means for assessing the relative specificity of oxidative action. In the absence of RBCs, 700 mM H_2O_2 was effective in destroying all bacteria tested but was

only partially effective against *C. albicans*. However, the microbicidal capacity of 700 mM H_2O_2 was markedly inhibited by the presence of RBCs. Only *P. aeruginosa* bacteria were completely killed. Note that 700 mM H_2O_2 produced complete erythrocyte destruction and complete Hgb destruction; i.e., Hgb completely disappeared from the pellets and the supernatants. As such, H_2O_2 activity was nonspecific. The RBCs served as a competitive substrate and were destroyed in the process.

Without RBCs, H_2O_2 in the range of 7.0 to 70 mM showed bactericidal action but was unable to kill *C. albicans*. In the presence of RBCs, H_2O_2 was essentially ineffective as a microbicidal agent at concentrations of 70 mM or lower. At 70 mM H_2O_2 , hemolysis and Hgb destruction were present but incomplete. No significant hemolysis or Hgb destruction was detected at or below H_2O_2 concentrations of 7.0 mM. For all microbes tested, the H_2O_2 concentration required for microbicidal action was greater than the H_2O_2 concentration producing complete RBC destruction. Erythrocytes are more susceptible to destruction by H_2O_2 than are microbes.

The data of Table 2 illustrate the microbicidal action of MPO at H_2O_2 concentrations incapable of directly damaging RBCs or microbes. In the absence of exogenous H_2O_2 , 5 nM (~ 0.7 μ g/ml) MPO did not show significant microbicidal action against these catalase-positive microbes. However, when MPO was presented with H_2O_2 concentrations several orders of magnitude lower than required for direct H_2O_2 microbicidal action, all microbes tested were successfully killed. MPO-dependent microbe killing was effective at H_2O_2 concentrations below 100 μ M.

Microbicidal action of hypochlorite and the protective effect of RBCs. The microbicidal potency of hypochlorite is well established (9, 14, 17, 25, 26). This set of experiments examined the microbicidal action of OCI^- and the protective effect of RBCs. As for the previous H_2O_2 experiments, *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans* were tested, and the results presented in Table 3.

In the absence of RBCs, 63 μ M OCI^- completely destroyed all microbes tested, and 6.3 μ M OCI^- was microbicidal for all bacteria tested. Compared to findings reported in Table 1, the microbicidal action of OCI^- is 1,000-fold more potent than that of H_2O_2 on a molar basis. These findings are consistent with the range of 2 to 2.0 ppm, i.e., 4 to 40 μ M OCI^- , previously reported to show effective bactericidal action (9).

The presence of RBCs completely inhibited the microbicidal action of 630 μ M OCI^- for all microbes tested, except *S. aureus*, which was inhibited at 63 μ M OCI^- . Note that 6.3 mM (i.e., 6,300 μ M) OCI^- completely destroyed all RBCs and Hgb; i.e., no Hgb was detected in the pellets or supernatants. At 630 μ M, OCI^- caused RBC hemolysis, liberating Hgb into the supernatant with partial destruction of Hgb. At 63 μ M, OCI^- caused hemolysis and release of Hgb into the supernatant for all microbes except *P. aeruginosa*. As such, OCI^- oxidative activity was nonspecific; i.e., the RBCs served as a competitive substrate and were destroyed. As previously described for H_2O_2 , OCI^- is a potent microbicidal agent, but it is more effective at destroying host cells, e.g., RBCs, than killing microbes (14, 16).

Selectivity of MPO binding. Selectivity of MPO binding is illustrated in Fig. 1. Exposure of bacterial suspensions to MPO

TABLE 1. Direct microbicidal action of hydrogen peroxide in the absence and presence of human erythrocytes

Microbe	H ₂ O ₂ concn (mM)	No. of CFU/ml ^a		Hemoglobin content ^{a,b}	
		Without RBCs	With RBCs (10 ⁷)	Supernatant	Pellet
<i>E. coli</i>	0.0000	1,600,000	1,300,000	0.0	0.9
	700.0000	0	10,000	0.0	0.0
	70.0000	0	1,100,000	0.0	0.4
	7.0000	670,000	1,300,000	0.1	0.9
	0.7000	1,600,000	1,200,000	0.1	0.9
	0.0700	1,300,000	1,700,000	0.1	0.9
	0.0070	1,600,000	1,200,000	0.1	0.9
	0.0007	1,200,000	1,300,000	0.0	1.0
<i>S. aureus</i>	0.0000	1,200,000	1,300,000	0.0	0.9
	700.0000	0	280,000	0.0	0.0
	70.0000	0	1,600,000	0.0	0.4
	7.0000	0	1,600,000	0.1	0.9
	0.7000	880,000	1,500,000	0.1	0.9
	0.0700	1,400,000	1,400,000	0.1	0.9
	0.0070	1,400,000	1,200,000	0.1	0.9
	0.0007	1,400,000	1,200,000	0.0	1.0
<i>P. aeruginosa</i>	0.0000	2,000,000	2,000,000	0.0	0.9
	700.0000	0	0	0.0	0.0
	70.0000	0	2,200,000	0.1	0.2
	7.0000	4,500	1,700,000	0.1	0.9
	0.7000	2,000,000	1,800,000	0.1	0.9
	0.0700	1,600,000	1,700,000	0.1	0.9
	0.0070	1,200,000	2,000,000	0.1	0.9
	0.0007	1,400,000	1,700,000	0.1	0.9
<i>C. albicans</i>	0.0000	190,000	350,000	0.0	1.0
	700.0000	78,000	150,000	0.0	0.0
	70.0000	230,000	300,000	0.0	1.0
	7.0000	150,000	380,000	0.0	1.0
	0.7000	180,000	330,000	0.0	1.0
	0.0700	170,000	280,000	0.0	0.9
	0.0070	160,000	290,000	0.0	0.9
	0.0007	270,000	320,000	0.0	1.0

^a Values in boldface are less than 3 SDs from the expected mean and therefore indicate significant killing or significant hemolysis.

^b RBC damage was assessed by measuring the hemoglobin (Hgb) retained in the pelleted intact RBCs, lost to the medium as a consequence of hemolysis, or completely destroyed by oxidation. The yield of 10⁷ RBCs is 0.25 to 0.34 mg of Hgb. The total Hgb distributed in the pellet and supernatant is expressed as proportions of unity (i.e., 1.0).

resulted in dark coloration of most but not all of the bacterial pellets formed when the suspensions were centrifuged. Note that the pellets of *E. coli*, *P. aeruginosa*, and *S. aureus* show dark MPO coloration, but that the pellet of the viridans streptococcus *S. sanguinis* does not.

In the picture, the center tube contains 2 mg/ml MPO, the amount of MPO added to each bacterial suspension. The suspensions were then centrifuged to concentrate the pellets, as shown to the right in Fig. 1. For comparison, the untreated bacteria pellets are shown in the tubes to the left in the photograph. Visual comparison of the pellets shows the degree of MPO binding, i.e., MPO staining. Except for viridans group streptococci, all bacteria tested showed MPO binding.

Synergistic *S. sanguinis*-MPO microbicidal action and effect of RBCs. The rate of H₂O₂ production by *S. sanguinis* and other viridans streptococci is reported to be in the range of 12 to 67 nmol/min/mg of dry weight (10) and 1 to 5 nmol/min/10⁶ CFU (18). As such, *S. sanguinis* produces sufficient H₂O₂ to drive MPO oxidation of Cl⁻ to OCl⁻ and to drive H₂O₂ reaction with OCl⁻ to produce ¹O₂*. Singlet oxygen is a reactive oxygenating agent with a finite reactive lifetime. It is an elec-

tronically excited molecule with a microsecond half-life that restricts reactivity to the proximity of its generation (4, 30, 35). As such, combustive microbicidal activity is essentially confined to sites of MPO binding. Presenting a small quantity of MPO to a mixture of two bacteria, i.e., a low-MPO-binding microbe, such as *S. sanguinis*, and any of the high-MPO-binding microbes previously described, provides a model for testing the relationship of selective MPO binding to selective MPO killing. The results of such testing, presented in Table 4, illustrate the synergistic *S. sanguinis*-MPO killing of *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*.

Figure 2 is a photograph of the actual 10⁻³ CFU dilution petri plates of the *E. coli* study. The top row of plates presents the findings in the absence of RBCs; reading from left to right, the final MPO concentrations were 0, 1.9, 5.6, and 50 nM. Note that in the absence of MPO, only the large *E. coli* colonies are observed; there is no evidence of the small *S. sanguinis* colonies. MPO at a concentration of 1.9 nM, i.e., 0.28 µg/ml, killed more than 99% of the *E. coli* and allowed the emergence of numerous small *S. sanguinis* colonies that are visible in Fig. 2. At 5.6 nM MPO, killing of *E. coli* was complete, and *S. san-*

TABLE 2. Microbicidal action of hydrogen peroxide in the absence and presence of MPO

Microbe	H ₂ O concn (mM)	No. of CFU/ml ^a	
		Without MPO	With 5 nM MPO
<i>E. coli</i>	0.0000	3,200,000	2,500,000
	0.5600	2,800,000	0
	0.1120	3,200,000	0
	0.0045	2,100,000	0
	0.0009	3,000,000	19,000
<i>S. aureus</i>	0.0000	2,400,000	1,700,000
	0.5600	1,600,000	0
	0.1120	2,000,000	0
	0.0045	2,700,000	30,000
	0.0009	2,300,000	2,300,000
<i>P. aeruginosa</i>	0.0000	2,100,000	2,500,000
	0.5600	1,800,000	0
	0.1120	2,700,000	0
	0.0045	1,800,000	220,000
	0.0009	2,600,000	2,500,000
<i>C. albicans</i>	0.0000	620,000	760,000
	0.5600	460,000	0
	0.1120	420,000	0
	0.0045	440,000	180,000
	0.0009	420,000	520,000

^a Values in boldface are less than 3 SDs from the expected mean and therefore indicate significant killing.

guinis was completely spared. Note the numerous small streptococci colonies, i.e., $\sim 2 \times 10^7$ *S. sanguinis* CFU/ml, visible on the 1.9 and 5.6 nM MPO plates.

At 50 nM, MPO killing of *S. sanguinis* was also observed; the *S. sanguinis* colony count dropped to $\sim 80,000$ CFU, a decrease of more than 100-fold. At a relatively high MPO concentration, streptococcal H₂O₂ was directed against the streptococci. Such observations strongly support the proposition that low concentrations of MPO provide selective advantage to the viridans streptococci in their competition with MPO-binding microbes such as *E. coli* for dominance in the mouth flora. Thus, in addition to its role in neutrophil antimicrobial action, MPO can serve the host by providing a selective advantage to beneficial flora and by controlling the population density of such flora.

Erythrocytes show little or no MPO binding (4). At relatively low H₂O₂ concentrations, the presence of erythrocytes allows assessment of the relative specificity of MPO-dependent oxidative activity. When oxidative activity is nonspecific, the RBCs serve as a competitive substrate and are damaged or destroyed in the process. If the oxidative activity is specifically directed, erythrocyte catalase can decrease the concentration of H₂O₂ with no or minimal damage to the RBCs. The bottom plates in Fig. 2 show the effect of RBCs on *S. sanguinis*-MPO synergistic microbicidal action. Reading from left to right, the final MPO concentrations were 0, 1.9, 5.6, and 50 nM. In the presence on 10^7 RBCs, 1.9 nM MPO produced modest microbicidal action, killing about two-thirds of the *E. coli* and allowing the emergence of very small streptococcus colonies that are visible on the plate. Despite the presence of erythrocytes at an RBC/*E. coli* ratio of about 5:1, MPO at a concentration of 5.6 nM killed

more than 99% of the *E. coli* bacteria while sparing *S. sanguinis*. With RBCs present, 50 nM MPO produced complete killing of *E. coli* and spared *S. sanguinis* (about 10^7 CFU). There was no evidence of RBC hemolysis and, therefore, no significant erythrocyte damage at any concentration of MPO tested. MPO oxidative activity was specific. Erythrocyte catalase is expected to decrease H₂O₂ and protect streptococci. There was no evidence of hemolysis or Hgb destruction. The consumption of the streptococcal H₂O₂ by RBC catalase was apparently sufficient to protect both the erythrocytes and the streptococci from bystander injury associated with unbound MPO.

Comparison of the data of Tables 1, 3, and 4 confirms that the inhibition of *S. sanguinis*-MPO synergistic microbicidal action by RBCs was minuscule relative to the inhibition of H₂O₂ and OCl⁻ microbicidal action by RBCs. No significant hemolysis or Hgb destruction was observed in the *S. sanguinis*-MPO synergistic microbicidal studies. These findings are consistent with selective MPO microbe binding, the short reactive lifetime of ¹O₂^{*}, and the competitive consumption of H₂O₂ by erythrocyte catalase. Confining oxygenation activity to the site of MPO binding concentrates combusive action and limits bystander injury.

The photograph in Fig. 3 shows CFU dilution plates of the *S. sanguinis*-MPO and *S. aureus* study. The top row of 10^{-3} dilution plates presents the results of testing in the absence of RBCs. Reading from left to right, the final MPO concentrations were 0, 5.6, 16.7 and 50 nM.

In the absence of MPO, both the larger yellow *S. aureus* colonies and the smaller *S. sanguinis* (viridans group) colonies are visible. An MPO concentration of 16.7 nM produced complete killing of *S. aureus* and spared the viridans streptococcus; i.e., the *S. sanguinis* count was $\sim 10^7$ CFU. At a concentration of 50 nM, MPO produced complete killing of *S. aureus* but also decreased the viridans streptococcus count by 10-fold; i.e., the *S. sanguinis* count was $\sim 10^6$ CFU. Low concentrations of MPO provided a selective advantage to *S. sanguinis* in its competition with MPO-binding *S. aureus*.

The dishes on the bottom row of Fig. 3 show the *S. aureus* findings in the presence of RBCs. Reading from left to right, the final MPO concentrations were 0, 5.6, 16.7, and 50 nM. The plates are shown at the 10^{-3} dilution except for the 50 nM plate, which is shown at the 10^{-2} dilution; no *S. aureus* colony was observed on the 10^{-3} plate. The presence of RBCs provided somewhat more protection to *S. aureus* than previously observed with *E. coli*. In the presence of RBCs, greater than 99.5% of the *S. aureus* bacteria were killed with 50 nM MPO, but this concentration of MPO also decreased the *S. sanguinis* count. Erythrocyte inhibition of *S. sanguinis*-MPO synergistic microbicidal action against *S. aureus* is consistent with, but slightly less impressive than, that observed for *E. coli*. No significant hemolysis or Hgb destruction was observed.

Synergistic microbicidal action of MPO-viridans group *Streptococcus* against *P. aeruginosa* was consistent with the *E. coli* results. In the absence of RBCs, MPO exerted potent microbicidal action against *P. aeruginosa* at all concentrations tested. In the presence of RBCs, killing of *P. aeruginosa* was extensive but incomplete. In the presence of RBCs, 1.9 nM MPO was sufficient to kill 99.9% of the *P. aeruginosa* bacteria. No hemolysis or Hgb destruction was observed.

TABLE 3. Direct microbicidal action of hypochlorite in the absence and presence of human erythrocytes

Microbe	OCl ⁻ concn (μM)	No. of CFU/ml ^a		Hemoglobin content ^{a,b}	
		Without RBCs	With RBCs (10 ⁷)	Supernatant	Pellet
<i>E. coli</i>	0.0000	1,700,000	1,500,000	0.0	0.9
	6,300.0000	0	0	0.0	0.0
	630.0000	0	1,400,000	0.3	0.1
	63.0000	0	1,400,000	0.7	0.0
	6.3000	0	1,700,000	0.0	1.0
	0.6300	1,600,000	1,500,000	0.0	0.9
	0.0630	1,500,000	1,500,000	0.0	1.0
	0.0063	1,500,000	1,600,000	0.0	1.0
	0.0006	1,400,000	1,600,000	0.0	1.0
<i>S. aureus</i>	0.0000	1,500,000	1,400,000	0.0	1.0
	6,300.0000	0	0	0.0	0.0
	630.0000	0	71,000	0.3	0.1
	63.0000	0	1,200,000	1.0	0.0
	6.3000	0	1,500,000	0.0	1.0
	0.6300	1,300,000	1,400,000	0.0	0.9
	0.0630	1,400,000	1,500,000	0.0	1.0
	0.0063	1,300,000	1,200,000	0.0	1.0
	0.0006	1,500,000	1,300,000	0.0	1.0
<i>P. aeruginosa</i>	0.0000	1,800,000	2,700,000	0.1	0.9
	6,300.0000	0	0	0.0	0.0
	630.0000	0	2,000,000	1.0	0.0
	63.0000	0	2,300,000	0.0	1.0
	6.3000	400	2,100,000	0.0	1.0
	0.6300	5,000	2,300,000	0.1	0.9
	0.0630	2,100	2,300,000	0.0	1.0
	0.0063	1,200	2,900,000	0.1	0.9
	0.0006	2,100,000	2,500,000	0.0	0.9
<i>C. albicans</i>	0.0000	360,000	340,000	0.1	1.0
	6,300.0000	0	0	0.0	0.0
	630.0000	0	350,000	0.2	0.0
	63.0000	0	350,000	0.9	0.1
	6.3000	240,000	250,000	0.1	0.9
	0.6300	250,000	310,000	0.1	0.7
	0.0630	280,000	360,000	0.1	0.8
	0.0063	240,000	340,000	0.1	0.9
	0.0006	290,000	290,000	0.1	0.9

^a Values in boldface are less than 3 SDs from the expected mean and therefore indicate significant killing or significant hemolysis.

^b RBC damage was assessed by measuring the hemoglobin (Hgb) retained in the pelleted intact RBCs, lost to the medium as a consequence of hemolysis, or completely destroyed by oxidation. Hgb was calculated as described in footnote *b* of Table 1.

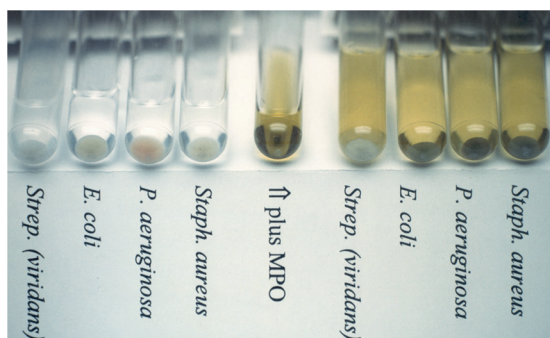


FIG. 1. Photographic demonstration of MPO binding and coloration of *S. aureus*, *E. coli*, and *P. aeruginosa*. Note the absence of MPO binding to *S. sanguinis* (viridans group). The four tubes to the left show the centrifuged microbe pellets in the absence of MPO exposure. The center tube contains 2 mg/ml MPO without microbes. The four tubes to the right show the centrifuged microbe pellets exposed to MPO.

In the absence of RBCs, the *S. sanguinis*-MPO synergistic microbicidal action against *C. albicans* was consistent with the *P. aeruginosa* results, but the presence of RBCs provided strong protection against MPO microbicidal action. In the presence of RBCs, 50 nM MPO killed 98% of the *C. albicans*, but no significant microbicidal action was observed at the lower MPO concentrations. The binding of MPO to *C. albicans* is considerably less than that observed for the bacteria tested, but MPO binding to *C. albicans* is greater than MPO binding to *S. sanguinis* (4, 6). There was no evidence of significant bystander damage to RBCs.

MPO without exogenous H₂O₂ kills *S. pyogenes* but not *E. faecalis*. Table 5 presents the results of MPO microbicidal action against *S. pyogenes* and *E. faecalis* in the absence of H₂O₂ and in absence and presence of RBCs. In the absence of the protective effect of RBCs, *S. pyogenes* bacteria were directly killed by MPO without exogenous H₂O₂. Erythrocyte catalase protected *S. pyogenes* from its metabolic product, H₂O₂, thus limiting MPO action. Previous binding studies have

TABLE 4. Viridans group *S. sanguinis*-myeloperoxidase synergistic microbicidal action in the absence and presence of human erythrocytes

Microbe added to <i>S. sanguinis</i> culture ^a	MPO concn (nM)	No. of CFU/ml ^b		Hemoglobin content ^c	
		Without RBCs	With RBCs (10 ⁷)	Supernatant	Pellet
<i>E. coli</i>	0.0	2,000,000	2,600,000	0.0	1.0
	50.0	0	0	0.0	1.0
	16.7	0	0	0.0	1.0
	5.6	0	10,000	0.0	1.0
	1.9	10,000	930,000	0.0	1.1
	0.6	650,000	2,600,000	0.0	1.0
	0.2	1,900,000	2,500,000	0.0	1.0
<i>S. aureus</i>	0.0	1,600,000	1,800,000	0.0	1.0
	50.0	0	8,000	0.0	0.9
	16.7	0	740,000	0.0	1.0
	5.6	820,000	930,000	0.0	1.0
	1.9	430,000	1,900,000	0.0	1.0
	0.6	1,600,000	1,700,000	0.1	1.0
	0.2	1,700,000	1,900,000	0.0	1.0
<i>P. aeruginosa</i>	0.0	2,000,000	3,400,000	0.0	1.0
	50.0	0	0	0.1	1.0
	16.7	0	10,000	0.0	0.9
	5.6	0	7,200	0.0	0.9
	1.9	0	6,200	0.0	1.0
	0.6	0	520,000	0.1	1.0
	0.2	10,000	4,500,000	0.0	1.0
<i>C. albicans</i>	0.0	200,000	240,000	0.0	1.0
	50.0	0	4,000	0.0	0.8
	16.7	0	250,000	0.0	0.9
	5.6	0	180,000	0.0	1.0
	1.9	0	240,000	0.0	1.0
	0.6	6,000	250,000	0.0	1.0
	0.2	160,000	210,000	0.0	1.0

^a Each suspension contained approximately 10⁷ *S. sanguinis* bacteria plus the additional microbe, as indicated. Microbe concentration was estimated by the optical density measurement at 540 nm. No H₂O₂ was added.

^b Values in boldface are less than 3 SDs from the expected mean and therefore indicate significant killing.

^c RBC damage was assessed by measuring the hemoglobin (Hgb) retained in the pelleted intact RBCs, lost to the medium as a consequence of hemolysis, or completely destroyed by oxidation as described in footnote b of Table 1.

reported that *E. faecalis* showed stronger MPO binding than *S. pyogenes* (group A) or *S. agalactiae* (group B) and that *S. pyogenes* and *S. agalactiae* showed stronger MPO binding than *S. sanguinis* (4).

Like *S. sanguinis*, beta-hemolytic *S. pyogenes* is an LAB and produces the H₂O₂ (34) required for MPO haloperoxidase activity. Consequently, *S. pyogenes* is susceptible to MPO microbicidal action in the absence of RBCs. Consistent with

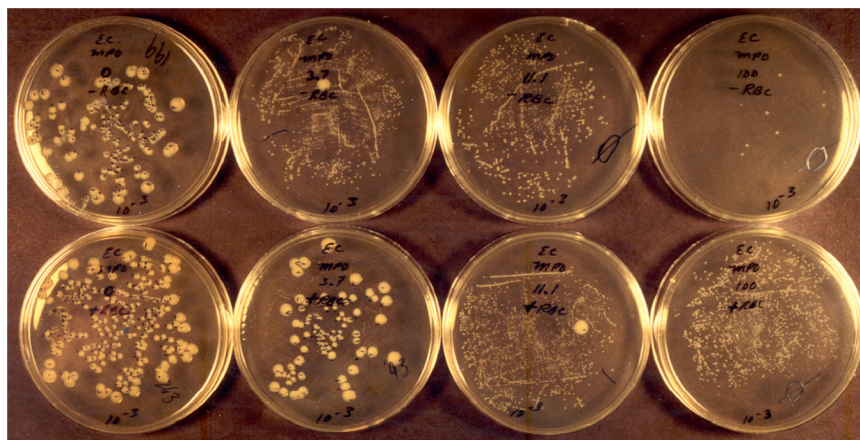


FIG. 2. Photograph of petri plates at the 10⁻³ CFU dilution used to measure *S. sanguinis*-MPO synergistic action against *E. coli*. The top and bottom rows of plates present the findings in the absence and presence of RBCs, respectively. From left to right, the final MPO concentrations were 0, 1.9, 5.6, and 50 nM. The plates were incubated for about 48 h to better visualize the smaller streptococcal colonies.

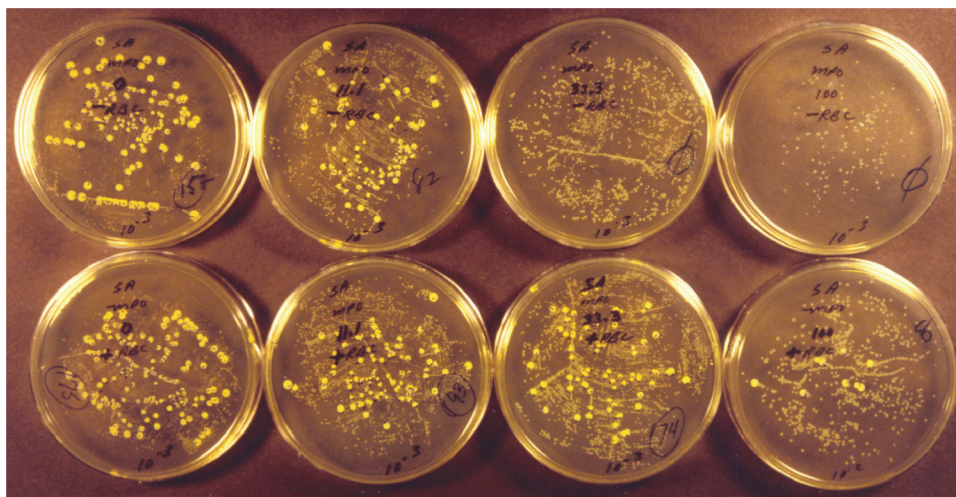


FIG. 3. Photograph of petri plates at the 10^{-3} CFU dilution (top four and bottom left three plates) and 10^{-2} CFU dilution (bottom far right plate) used to measure *S. sanguinis*-MPO synergistic action against *S. aureus*. The top and bottom rows of plates present the findings in the absence and presence of RBCs, respectively. From left to right, the final MPO concentrations were 0, 5.6, 16.7, and 50 nM. The plates were incubated for about 48 h to better visualize the smaller streptococcal colonies.

MPO binding, MPO killing of *S. pyogenes* was greater than that observed against *S. sanguinis* in the previous experiments. In the absence of RBCs, killing of *S. sanguinis* was insignificant below 50 nM MPO.

E. faecalis does not excrete appreciable amounts of H_2O_2 and shows no hemolysis on blood agar. *E. faecalis* contains NADH peroxidase, i.e., NADH: H_2O_2 oxidoreductase. This nonheme flavoprotein catalyzes the direct reduction of H_2O_2 to H_2O , which is a pseudo-catalase activity (15). Consequently, any H_2O_2 generated by *E. faecalis* metabolism is destroyed by reduction, thus depriving MPO of a substrate. Despite significant MPO binding (4), no microbicidal action was observed at any of the MPO concentrations tested in the absence or in the

presence of RBCs. As shown in the MIC and MBC results, *E. faecalis* is highly susceptible to MPO when a source of H_2O_2 is provided.

MIC and MBC of MPO against diverse bacteria. MPO selectively binds to bacteria (4, 6), and when a source of H_2O_2 is present, selective MPO binding results in selective microbe killing. The following conventional MIC and MBC studies were conducted to expand the range of observations in order to better quantify and statistically analyze the selectivity of MPO microbicidal action across a broad selection of bacteria. The previously described ATCC strains plus the control *S. aureus* ATCC 29213 and an additional 78 clinical isolates from diverse geographic areas of the United States were tested to establish

TABLE 5. Direct myeloperoxidase microbicidal action against streptococcal species in the absence and presence of human erythrocytes

Microbe ^a	MPO concn (nM)	No. of CFU/ml ^b		Hemoglobin content ^c	
		Without RBC's	With RBCs (10^7)	Supernatant	Pellet
<i>S. pyogenes</i> beta-hemolytic group A	0.00	2,500,000	970,000	0.1	0.9
	50.00	0	290,000	0.1	0.9
	16.67	0	280,000	0.1	0.9
	5.56	0	260,000	0.3	0.7
	1.85	0	300,000	0.1	0.9
	0.62	0	130,000	0.2	0.8
	0.21	600	750,000	0.1	0.9
	0.07	1,300,000	970,000	0.0	1.0
<i>E. faecalis</i> nonhemolytic group D	0.00	1,900,000	1,700,000	0.2	0.8
	50.00	1,600,000	1,500,000	0.2	0.8
	16.67	1,900,000	1,300,000	0.4	0.6
	5.56	1,700,000	1,500,000	0.2	0.8
	1.85	1,500,000	1,400,000	0.1	0.9
	0.62	1,500,000	1,600,000	0.1	0.9
	0.21	1,700,000	1,700,000	0.2	0.8
	0.07	1,800,000	1,500,000	0.2	0.8

^a Each suspension contained either *S. pyogenes* (Lancefield group A) or *E. faecalis* (group D) alone. No H_2O_2 was added.

^b Values in boldface are less than 3 SDs from the expected mean and therefore indicate significant killing.

^c RBC damage was assessed by measuring the hemoglobin (Hgb) retained in the pelleted intact RBCs, lost to the medium as a consequence of hemolysis, or completely destroyed by oxidation, as described in footnote b of Table 1.

TABLE 6. MIC and MBC of MPO against a spectrum of bacteria

Medium and microbe ^a	Resistance phenotype ^b	No. of isolates	MPO MIC (nM) ^c			MPO MBC (nM) ^c		
			Mean	SD	Median	Mean	SD	Median
CAMHB								
<i>E. coli</i>	CEF-S	2	1.276	0.634	1.276	1.276	0.634	1.276
	CEF-R	4	1.500	0.448	1.724	2.793	2.768	1.724
<i>P. aeruginosa</i>	CEF-S	3	0.345	0.010	0.414	0.690	0.239	0.828
	CEF-R	3	0.345	0.010	0.414	1.862	1.522	1.724
<i>S. aureus</i>	MSSA	8	0.129	0.048	0.104	0.142	0.054	0.104
	MRSA	6	0.129	0.048	0.104	0.142	0.054	0.104
	VISA/VRSA	2	0.155	0.073	0.155	0.155	0.073	0.155
<i>E. faecalis</i> (group D)	VSE	5	2.759	0.944	3.449	6.207	4.496	3.449
	VRE	2	1.276	0.634	1.276	5.173	2.439	5.173
CAMHB—5% LHB								
<i>S. pyogenes</i> (group A)	NA	10	2.497	1.038	2.586	4.656	1.999	3.449
<i>S. agalactiae</i> (group B)	NA	10	1.973	1.091	1.724	2.842	1.731	2.586
<i>S. mitis</i> (viridans group)	NA	9	8.813	3.898	6.897	13.028	8.754	6.897
<i>S. oralis</i> (viridans group)	NA	9	12.261	6.703	13.794	15.327	7.538	13.794
<i>S. sanguinis</i> (viridans group)	NA	9	6.131	3.351	6.897	9.579	4.145	6.897

^a The bacteria included ATCC stains of *E. coli* (one), *P. aeruginosa* (one), and *S. aureus* (two) and 78 clinical isolates collected from diverse geographical areas in the United States. CAMHB, cation-adjusted Mueller-Hinton broth; LHB, lysed horse blood.

^b CEF-S, ceftazidime sensitive; CEF-R, ceftazidime resistant; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*; VISA/VRSA, vancomycin intermediate/resistant *S. aureus*; VSE, vancomycin-sensitive *E. faecalis*; VRE vancomycin-resistant *E. faecalis*; NA, not applicable.

^c Glucose oxidase (GO) generated the H₂O₂ required for MPO action. The molar ratio of MPO to GO was 4.4.

the MICs and MBCs of MPO. In these studies MPO haloperoxidase activity was driven using glucose plus glucose oxidase (GO) as the H₂O₂ generator. The molar ratio of MPO to GO was 4.4 to 1. The range of MPO tested was 0.028 to 55 nM, or 0.004 to 8.0 µg MPO/ml. The MIC/MBC studies followed CLSI guidelines (12, 29). Such testing is designed to measure conventional antibiotics that inhibit bacterial protein synthesis or cell wall formation and is far from optimum for measuring MPO activity. The cation-adjusted Mueller-Hinton broth (CAMHB) used has a pH of 7.3. This hydrogen ion concentration is 10-fold to 100-fold less than optimum for MPO haloperoxidase activity (3). However, despite this limitation, the GO-MPO system showed good microbicidal action, as measured by MIC and MBC.

As described in Table 6, the lowest MIC and MBCs for MPO were observed for *S. aureus*. Methicillin-sensitive *S. aureus* (MSSA) showed no significant difference from methicillin-resistant *S. aureus* (MRSA) or vancomycin-intermediate/resistant *S. aureus* (VISA/VRSA) with regard to either MIC or MBC. Consistent with the results of the MPO binding studies (4, 6) and the empirical findings presented above, the MIC and MBC for Gram-negative bacteria were also relatively low, although higher than observed for *S. aureus*. With regard to ceftazidime sensitivity, *E. coli* and *P. aeruginosa* showed no significant differences in either MICs or MBCs. The MIC and especially the MBC of MPO were higher for *E. faecalis* than for the other bacteria tested in CAMHB. Vancomycin-sensitive and vancomycin-resistant *E. faecalis* (VSE and VRE, respectively) showed no significant difference in MIC and MBC results.

The CLSI guidelines for MIC/MBC testing of streptococcal species required the use of cation-adjusted Mueller-Hinton

broth supplemented with 5% lysed horse blood (CAMHB-5% LHB) for growth (12). In addition to the nonoptimal pH of CAMHB, the 5% LHB contained abundant catalase activity capable of consuming the H₂O₂ required as a substrate for MPO generation of OCl⁻ and ¹O₂*. This lysed horse blood also presents Hgb and additional molecular substrates capable of reacting with the MPO-generated oxidants and competitively inhibiting microbicidal action.

The effect of 5% lysed horse blood on the MICs and MBCs of GO-driven MPO was investigated by retesting the same seven *E. faecalis* isolates in both CAMHB and CAMHB-5% LHB. The mean MIC ± standard deviation (SD) was 1.4357 ± 0.4772 nM in CAMHB and 45.3264 ± 13.7190 nM in CAMHB-5% LHB. The mean MBC ± SD was 3.4493 ± 1.1730 nM in CAMHB and 53.2093 ± 7.3737 nM in CAMHB-5% LHB. Legitimate comparison of the MIC/MBC results requires that the condition of testing be essentially the same. Thus, the results based on CAMHB and CAMHB-5% LHB were considered separately. The left portion of Fig. 4 presents the Tukey box plots of MPO MIC and MBC results for the CAMHB testing groups, i.e., Gram-negative *E. coli* and *P. aeruginosa*, *S. aureus*, and *E. faecalis*. The right portion of Fig. 4 presents plots for the CAMHB-5% LHB testing groups, i.e., the Lancefield group A *S. pyogenes* and group B *S. agalactiae* and the viridans group streptococci *S. mitis*, *S. oralis*, and *S. sanguinis*.

Independent *t* tests were used to compare the results for the independent bacterial groups tested with CAMHB and separately for those tested with CAMHB-5% LHB. For the first two CAMHB groups, i.e., Gram-negative bacteria group (mean MIC ± SD of 0.9024 ± 0.6317 nM and mean MBC ± SD of 1.7817 ± 1.8176 nM) and *S. aureus* (MIC of 0.1229 ±

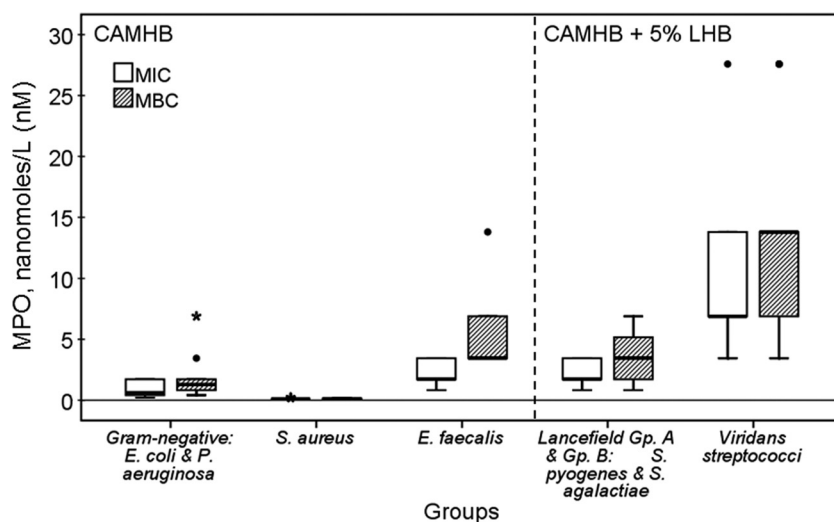


FIG. 4. The composite results of MPO MIC and MBC testing with the data presented as Tukey box plots. The results are grouped by the medium used, i.e., CAMHB and CAMHB-5% LHB. Five groups of bacteria were tested. As indicated on the x axis, the groups (number of isolates) are as follows: Gram-negative *E. coli* ($n = 6$) and *P. aeruginosa* ($n = 6$), *S. aureus* ($n = 16$), *E. faecalis* ($n = 7$), Lancefield group A *S. pyogenes* ($n = 10$) and group B *S. agalactiae* ($n = 10$), and viridans group streptococci ($n = 27$). The bottom and top portions (hinges) of each box are the lower and upper quartiles, respectively, and the hinge or H -spread (i.e., the interquartile range) is the distance between the bottom and top of the box. The heavy horizontal band shows the median. The whiskers are the lines drawn from the upper hinge to the upper adjacent value and from the lower hinge to the lower adjacent value within $1.5 \times$ the H -spread. An outlier is marked with a circular dot if it is between the inner fences (i.e., $1.5 \times$ the H -spread) and outer fence (i.e., $3 \times$ the H -spread). An extreme outlier is marked by an asterisk if it is beyond the outer fences.

0.0417 nM and MBC of 0.1293 ± 0.0463 nM), the t value with 11 df [$t(11)$] was 4.267 with a P value of 0.0013 for the MIC, and $t(11)$ was 3.148 with a P value of 0.0093 for the MBC. Analysis of Gram-negative bacteria and *E. faecalis* (MIC of 2.3351 ± 1.0886 nM and MBC of 5.9117 ± 3.8371 nM) gave a $t(8)$ of -3.183 with a P value of 0.0123 for the MIC and a $t(17)$ of -3.207 with a P value of 0.0052 for the MBC*. The asterisk indicates that this MBC analysis had a Levene's test for equality of variances of >0.05 , and, as such, equal variance was assumed. The Levene's test was <0.05 for all other analyses, and consequently, equal variance was not assumed. Analysis of *S. aureus* and *E. faecalis* gave a $t(6)$ of -5.375 with a P value of 0.0017 for MIC and $t(6)$ of -3.987 with a P value of 0.0072 for the MBC.

For the two CAMHB-5% LHB groups, i.e., Lancefield group A *S. pyogenes* and group B *S. agalactiae* (MIC of 2.2346 ± 1.0709 nM and MBC of 3.7485 ± 2.0442 nM) and viridans group streptococci (MIC of 9.0683 ± 5.3380 nM and MBC of 12.6445 ± 7.2210 nM), the $t(28)$ was -6.478 with a P value of <0.0001 for the MIC and the $t(31)$ was -6.081 with a P value of <0.0001 for the MBC. The composite MIC and MBC results are consistent with, and conclusively document, the mixed culture results described earlier.

DISCUSSION

Members of the lactic acid family of bacteria (LAB) are common to the normal flora of humans. Viridans group streptococci, such as *S. sanguinis*, *S. mitis*, and *S. oralis*, are indigenous flora of the healthy human mouth (20). These LAB do not synthesize heme and, consequently, lack respiratory cytochromes and catalase. As such, streptococcal redox metabolism is dependent on flavoenzymes generating lactic acid and,

in many cases, H_2O_2 , as metabolic products (10, 18, 24). As described by Rosebury, "Wherever in nature two or more species of microorganisms grow in intimate association, each will interact with the others; and if the microorganisms grow upon a host organism, then the host will be in some way influenced by the interaction" (31). The status of LAB in the normal flora of humans suggests the probability of a symbiotic host-microbe interaction. According to Sanders, "The interaction of man's indigenous microflora and exogenously acquired pathogens has been the subject of sporadic investigation and continuous speculation for more than 5 decades. However, only recently has it been demonstrated conclusively that antagonistic interactions may enhance man's capacity to resist infection" (32).

Microbial antagonism was demonstrated by Colebrook in his 1915 report that pneumococcus and viridans group streptococcus kill meningococcus and other Gram-negative bacteria (13). The importance of streptococci in suppressing the growth of potential pathogens is also implied by the phenomenon of superinfection following antibiotic therapy. The significance of viridans group streptococci in this regard was described by Sprunt et al. "Members of the viridans group of streptococci, the predominant strains of the oropharyngeal flora in most individuals, can inhibit the growth of enteric Gram-negative bacilli, the organisms that commonly overgrow at this site following therapy with massive doses of penicillin. It was proposed that suppression (or elimination) of these streptococci by massive doses of antibiotics suppresses (or eliminates) their inhibitory action and permits multiplication of the previously inhibited (or newly introduced) bacilli" (36).

The results presented herein support the conclusion that low MPO binding to viridans streptococci provides these LAB with a competitive advantage over high-MPO-binding microbes.

LAB-generated H_2O_2 is the substrate for the MPO-catalyzed oxidation of Cl^- to OCl^- and for the reaction of H_2O_2 with OCl^- to produce singlet oxygen, $^1O_2^*$ (2, 3, 19). When H_2O_2 is limiting, OCl^- can directly participate in dehydrogenations and chlorination of amines, but when sufficient H_2O_2 is available, reaction with OCl^- produces $^1O_2^*$ (19). The microsecond reactive lifetime of $^1O_2^*$ restricts combustive oxygenation activity to within a radius of about 0.2 μm of its point of generation (4, 30, 35). Thus, combustive oxygenations are focused to the site of MPO binding with relatively little bystander injury to microbes or erythrocytes showing no or low MPO binding.

The migration of neutrophil leukocytes from the blood to body spaces such as the mouth and vagina (11, 38) guarantees delivery of MPO to these sites and the possibility for MPO interaction with the microbes present. In the competition among microbes for a place within the flora, the presence of low concentrations of MPO favors non-MPO-binding LAB. The role of MPO in such competition is demonstrated by *S. sanguinis*-MPO synergistic microbicidal action against *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*.

When oxidative activity is nonspecific, RBCs serve as a competitive substrate and are destroyed. When oxidative activity is specific and focused and the concentration of H_2O_2 is relatively low, erythrocyte catalase can effectively consume excess H_2O_2 with little or no erythrocyte hemolysis. RBCs show essentially no MPO binding (4).

The results for the H_2O_2 and OCl^- studies demonstrate nonspecific oxidative action. RBCs competitively inhibit antimicrobial activity and are destroyed in the process. RBCs are consumed before microbicidal action can begin; i.e., erythrocytes are more susceptible than microbes to H_2O_2 and to OCl^- . The *S. sanguinis*-MPO synergistic microbicidal model demonstrates that MPO-dependent killing is focused and specific and without bystander damage to the RBCs. Erythrocyte catalase, by consuming streptococcal H_2O_2 , prevents significant erythrocyte injury and minimizes damage to the low-MPO-binding streptococci that are the source of H_2O_2 .

MPO binding to *S. pyogenes* is stronger than binding to *S. sanguinis* (4, 6). In the absence of RBCs, *S. pyogenes* and, to a lesser extent, *S. sanguinis* are directly killed by MPO. Both streptococci generate H_2O_2 metabolically, and, as such, exogenous H_2O_2 is not necessary for killing. Conventional MIC and MBC testing confirms that *S. pyogenes* and *S. agalactiae* are significantly (*P* values of less than 0.00001) more susceptible to the microbicidal action of the GO-driven MPO system than are the viridans group streptococci.

Although weaker than for *E. coli*, *P. aeruginosa*, and *S. aureus*, MPO binding to *E. faecalis* is relatively stronger than MPO binding to *S. pyogenes* and *S. agalactiae* (4, 6), but in the absence of added H_2O_2 , MPO is not directly microbicidal. *E. faecalis* contains a pseudo-catalase, i.e., NADH peroxidase, that prevents metabolically generated H_2O_2 from accumulating. This NADH peroxidase uses NADH to reduce H_2O_2 to H_2O (15). As demonstrated in the MPO MIC and MBC studies, *E. faecalis* is susceptible to MPO microbicidal action when GO is present as a H_2O_2 generator. Consistent with MPO binding activities, the MPO concentrations required for MIC and MBC action against *E. faecalis* were significantly higher than those for *E. coli*, *P. aeruginosa*, and *S. aureus*. The MIC

and MBC studies further demonstrate that selective MPO binding results in selective microbicidal action.

The relationship of bacterial metabolism to MPO microbicidal action is clinically illustrated by chronic granulomatous disease (CGD), a disorder resulting from defective neutrophil NADPH oxidase function and characterized by severe and recurrent staphylococcal, coliform, and fungal infections (5). The neutrophils of CGD patients are capable of phagocytosis and azurophilic granule fusion producing an MPO-rich phagolysosomal space surrounding the phagocytized microbe. However, the NADPH oxidase of CGD neutrophils is defective and incapable of H_2O_2 production. Consequently, there is no H_2O_2 to drive MPO generation of OCl^- and $^1O_2^*$ as required for combustive microbicidal action and chemiluminescence. Pertinent to this discussion, streptococcal infections are not increased in CGD patients. Phagocytosis of viable streptococci by CGD neutrophils results in microbicidal action and chemiluminescence (5).

The process of neutrophil phagocytosis obviates the necessity for selective MPO binding. Phagocytosis, phagosome formation, and fusion of the MPO-rich azurophilic granules producing the phagolysosome guarantee that MPO is confined to the proximity to the phagocytized microbe. There is no apparent necessity for MPO binding selectivity within the phagolysosomal space. However, the evidence presented herein suggests that specificity of microbe binding can affect flora composition when microbes contact MPO released from the neutrophils that have migrated into a body space such as the mouth.

In summary, MPO plus streptococci exert a synergistic microbicidal action against a range of MPO-binding microbes, suggesting a mechanistic explanation for the dominance of viridans group streptococci in the normal mouth flora. Streptococci belong to the heme-deficient lactic acid family of bacteria and generate lactic acid and H_2O_2 . These metabolic products satisfy the pH and substrate requirements for MPO oxidation of chloride to hypochlorite and for reaction of H_2O_2 with hypochlorite to produce $^1O_2^*$, a potent oxygenating agent with a microsecond half-life. The short lifetime of $^1O_2^*$ restricts reactivity to within a radius of about 0.2 μm from its point of generation. As such, no or low MPO binding serves to protect H_2O_2 -producing viridans group streptococci and erythrocytes from MPO-generated OCl^- and $^1O_2^*$. Microbes with strong MPO binding are targeted for MPO-dependent OCl^- and $^1O_2^*$ generation when H_2O_2 is available. Combustive destruction is concentrated on the MPO-bound microbes. MPO MIC and MBC studies provide statistical evidence that high MPO binding is related to low MPO MIC and MBC values. Selective MPO binding results in selective MPO killing. The composite findings suggest a role for MPO in the establishment and maintenance of the normal floras of the human mouth.

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