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Lactoperoxidase and Thiocyanate Protect Bacteria from Hydrogen Peroxide

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Lactoperoxidase and thiocyanate were shown to protect *Escherichia coli* and three oral streptococcal species from the bactericidal effect of hydrogen peroxide under aerobic conditions. Lactoperoxidase in the absence of thiocyanate was also protective for two of the bacterial species in a dilution medium but potentiated hydrogen peroxide toxicity for the other two under the same conditions. The products of the reaction between hydrogen peroxide and thiocyanate in the presence of lactoperoxidase were not bactericidal except in the case of *E. coli*, and then only under special conditions. The results demonstrate the effectiveness of lactoperoxidase and thiocyanate in protecting living cells from hydrogen peroxide toxicity. Although the effect on human cells was not examined in this study, extrapolation of these results to the cells of the oral mucosa would suggest an important protective role of lactoperoxidase and thiocyanate against the toxic effects of hydrogen peroxide in the oral cavity.

Hydrogen peroxide is capable of killing a variety of life forms, including bacteria and mammalian cells (1, 6, 7, 12, 14, 20, 26), even though these cells may have highly developed defense mechanisms against hydrogen peroxide (8). For this reason, free hydrogen peroxide may pose a threat to both human and bacterial cells living in the human body. Among the putative sites of hydrogen peroxide production in humans are the surfaces of the oral cavity, where hydrogen peroxide-forming bacteria constitute up to 60% of the facultatively anaerobic flora (18). Free hydrogen peroxide has not been detected in the saliva, however (18), presumably because it is quickly destroyed by the salivary enzymes lactoperoxidase and thiocyanate.

Hydrogen peroxide is a substrate of lactoperoxidase in oxidizing thiocyanate to hypothiocyanate (OSCN) (2, 15, 16). This product of the reaction oxidizes bacterial sulfhydryl groups (23, 29), damages bacterial cell membranes (19, 21), and causes a rapid bacteriostatic effect in both gram-positive and gram-negative bacteria (9, 17, 22, 24, 27; B. Reiter, A. Pickering, J. D. Oram, and G. S. Pope, J. Gen. Microbiol. **33:** xii, 1963). A slower bactericidal effect has been reported for some gram-negative bacteria, including *Escherichia coli* (5, 28). Lactoperoxidase and thiocyanate have also been shown to protect some oral bacteria from the effects of bactericidal concentrations of hydrogen peroxide under anaerobic conditions (6). This study examines the effects of lactoperoxidase and thiocyanate on the survival of bacteria treated with hydrogen peroxide under aerobic conditions.

MATERIALS AND METHODS

Microorganisms. *Streptococcus mutans* strain NCTC 10449, *S. salivarius* strain NCTC 9759, *S. sanguis* strain ATCC 10556, and *E. coli* K-12 strain AB1157 (3) were used as test strains.

Preparation of media. Trypticase-soy-yeast extract (TSY) broth (pH 7.3) contained, per liter, 17 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 3 g of phytone peptone (BBL), 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of NaCl, 2.5 g of K_2HPO_4, and 2.5 g of glucose. The broth was autoclaved at 121°C for 20 min. The dilution solution was prepared by dissolving 4.3 g of NaCl, 0.42 g of Na_2HPO_4, 1.0 g of K_2HPO_4, and 10 g of sodium β-glycerophosphate · $5H_2O$ in 500 ml of water, pH 7.0 (solution 1), and 0.24 g of $CaCl_2$ and 0.1 g of $MgCl_2 \cdot 6H_2O$ in 500 ml of water (solution 2) (25).

Solutions 1 and 2 were autoclaved separately, cooled, and pooled. Peptone-yeast extract-glucose (PYG) agar medium was prepared from two solutions. One solution contained, per liter, 20 g of tryptone (Difco), 10 g of yeast extract (Difco), and 20 g of NaCl. The other solution contained 28 g of agar (Difco) and 2 g of glucose. The two solutions were autoclaved separately, cooled to 50°C, mixed, and poured into plates.

The blood agar medium was prepared as described by Holdeman et al. (13) and contained defibrinated horse blood (GIBCO, Bio-Cult Ltd., Paisley, Scotland) hemolyzed by freeze-thawing.

Chemicals. Hydrogen peroxide (30%, wt/wt; Perhydrol) was from E. Merck AG, Darmstadt, Germany. Lactoperoxidase (L 2005; from milk) was from Sigma Chemical Co., St. Louis, Mo. Potassium thiocyanate was from Riedel-De Haen AG, Seelze-Hannover, Germany.

Exposure to lactoperoxidase-thiocyanate-hydrogen peroxide. The strains were grown at 37°C in the broth

under aerobic conditions. In exponential growth, they were diluted in aerated dilution solution to a density of about 3 × 10^4 organisms per ml. At this dilution of the growing culture, no components of the broth interfered with hydrogen peroxide toxicity or substituted for thiocyanate in its reaction with lactoperoxidase. A 0.2-ml sample of bacterial suspension was added to 1.8 ml of the dilution solution containing various combinations of lactoperoxidase and thiocyanate. Hydrogen peroxide was added to the bacteria 2 min after the start of the dilution procedure. Samples (0.1 ml) were taken at regular time intervals from the reaction mixtures and were spread over the surface of agar plates. When the plates were incubated anaerobically, they were stored at 37°C in an anaerobic box with an atmosphere of 10% H_2 and 5% CO_2 in nitrogen (31). The numbers of viable cells were determined.

In one series of experiments, a 0.2-ml sample of strain AB1157 in exponential growth in broth was added to 1.8 ml of aerated dilution solution giving about 10^7 organisms per ml. All other conditions were the same as above. Only under these conditions could a bactericidal effect of the hydrogen peroxide-lactoperoxidase-thiocyanate combination be observed.

RESULTS

When *E. coli* at a density of about 3 × 10^3 cells per ml of dilution solution was exposed to 1 mM hydrogen peroxide, lactoperoxidase protected from the bactericidal effect of hydrogen peroxide in both the presence and absence of thiocyanate (Fig. 1). Similar results were obtained with *S. salivarius*. When *S. mutans* was tested under similar conditions, lactoperoxidase protected only in the presence of thiocyanate. In the absence of thiocyanate, lactoperoxidase potentiated the bactericidal effect of hydrogen peroxide on *S. mutans* (Fig. 2). Results with *S. sanguis* were similar to those obtained with *S. mutans*.

A bactericidal effect of lactoperoxidase-thiocyanate-hydrogen peroxide greater than that of hydrogen peroxide itself was demonstrated only in *E. coli* and then only under very specific conditions (Fig. 3). An exponentially growing culture of *E. coli* K-12 strain AB1157 in broth was diluted 10-fold in dilution solution giving a density of about 10^7 organisms per ml and exposed for more than 1 h to lactoperoxidase-thiocyanate-hydrogen peroxide. Many of the cells were damaged to such an extent that they could not recover when incubated on PYG agar or blood agar aerobically. When incubated anaerobically on blood agar, however, most of the cells recovered even after a 3-h exposure. A pronounced bacteriostatic effect of lactoperoxidase-thiocyanate-hydrogen peroxide on *E. coli* was found under these conditions (Fig. 3), and readily visible colonies did not usually develop on the agar plates until after more than 24 h of incubation. The surviving cells under all other conditions (Fig. 3) formed large colonies within 24 h. Lactoperoxidase in the absence of thiocyanate under these conditions (Fig. 3) did not protect from hydrogen peroxide toxicity, as in the experiments illustrated in Fig. 1. However, cells exposed to hydrogen peroxide in the presence of lactoperoxidase did recover much faster than cells exposed to hydrogen peroxide in the absence of lactoperoxidase. The former cells started to grow rapidly within 1 h, whereas the others did not (Fig. 3).

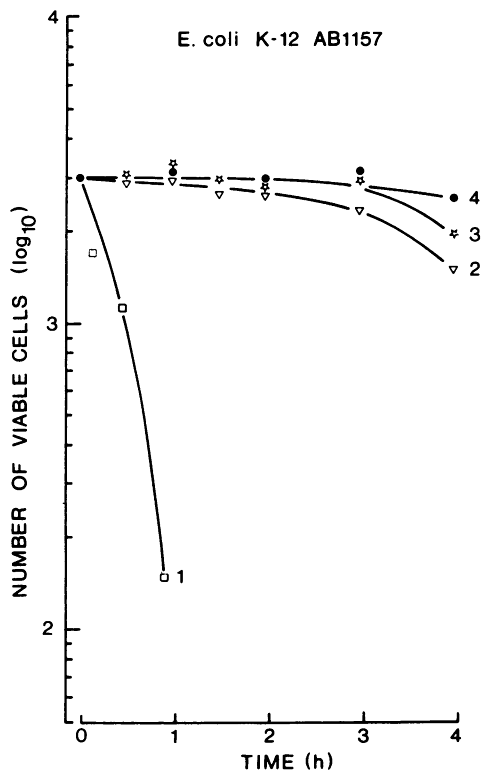

FIG. 1. Killing of *E. coli* in dilution solution at 37°C when exposed under aerobic conditions to various combinations of lactoperoxidase, thiocyanate, and hydrogen peroxide. The number of viable cells determined on blood agar incubated anaerobically after various time intervals in the presence of (1) 1 mM H_2O_2; (2) 1 mM H_2O_2 plus lactoperoxidase (25 μg) plus 2 mM thiocyanate; (3) 1 mM H_2O_2 plus lactoperoxidase (25 μg/ml); (4) no addition. Similar viable counts were obtained on PYG agar incubated aerobically.

DISCUSSION

In the presence of thiocyanate, lactoperoxidase has been shown to protect microorganisms from the bactericidal effect of hydrogen peroxide under anaerobic conditions (6). The present study demonstrated that this protection against hydrogen peroxide toxicity was also effective under aerobic conditions. For a variety of organ-

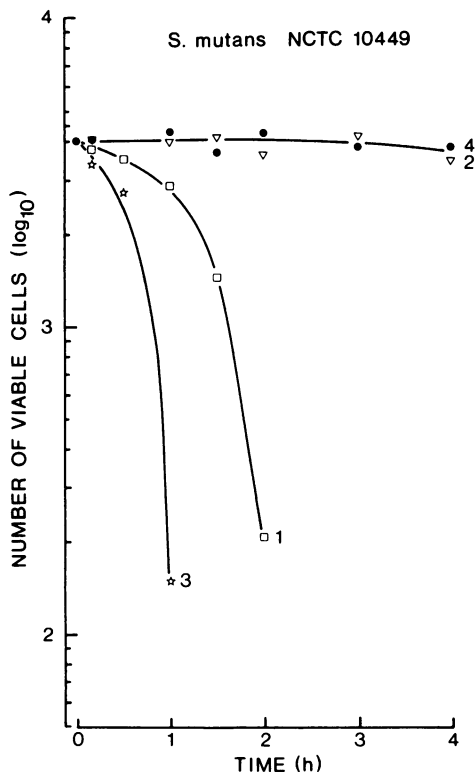

FIG. 2. Killing of *S. mutans* in dilution solution at 37°C when exposed under aerobic conditions to various combinations of lactoperoxidase, thiocyanate, and hydrogen peroxide. See legend to Fig. 1. Similar viable counts were obtained on blood agar incubated anaerobically and on PYG agar incubated aerobically.

isms over a wide range of hydrogen peroxide concentrations, lactoperoxidase and thiocyanate offered protection (6). Even *E. coli*, for which a bactericidal effect of lactoperoxidase-thiocyanate-hydrogen peroxide has been demonstrated (4, 5, 21, 28; Reiter et al., J. Gen. Microbiol. 33:xii, 1963), was protected. The present study showed that this bactericidal effect was expressed only when the cells were cultured under aerobic conditions after exposure to lactoperoxidase-thiocyanate-hydrogen peroxide. Most of the cells incubated under anaerobic conditions on blood agar after the exposure proved to be viable and apparently had the capacity to repair any lesions that may have been formed during aerobic exposure to the products of the lactoperoxidase reaction.

In the absence of thiocyanate, lactoperoxidase potentiated the toxic effect of hydrogen peroxide in some strains and prevented it in others. This effect was strain specific, but was also influenced by other conditions during the exposure to hydrogen peroxide in the presence of lactoperoxidase. In dilution solution, lacto-

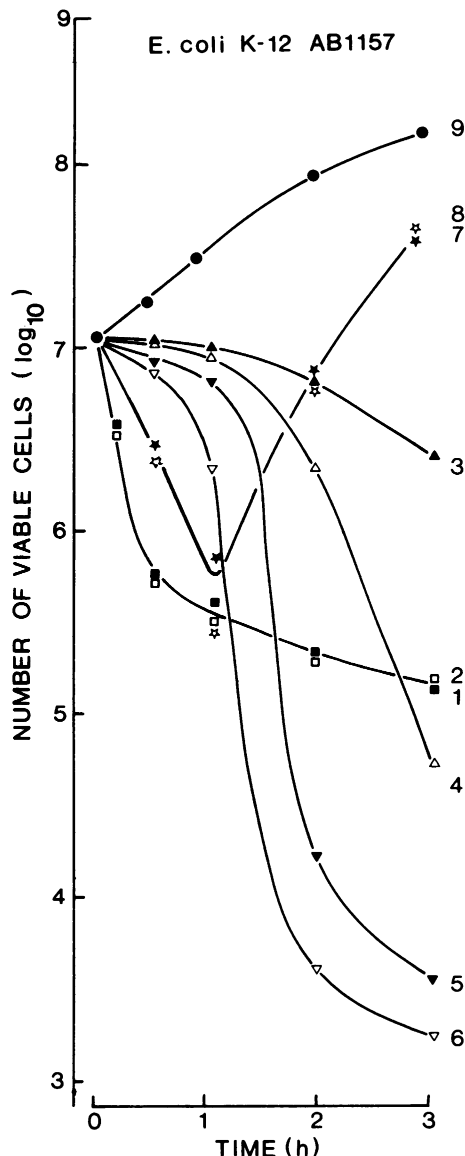

FIG. 3. Killing of *E. coli* K-12 when an exponentially growing culture in broth was diluted 10-fold in aerobic dilution solution and exposed at 37°C to various combinations of 25 μg of lactoperoxidase (LPO) per ml, 2 mM thiocyanate (SCN^-), and 1 mM H_2O_2. After various time intervals, 0.1-ml samples of the reaction mixtures were diluted and subcultured on the surface of blood agar (BA) or PYG agar plates under aerobic or anaerobic conditions. The number of viable cells was determined after various time intervals in the presence of the following additions and incubation conditions: (1) H_2O_2, BA, anaerobic; (2) H_2O_2, PYG, aerobic; (3) H_2O_2 plus LPO plus SCN^-, BA, anaerobic; (4) H_2O_2 plus LPO plus SCN^-, PYG, anaerobic; (5) H_2O_2 plus LPO plus SCN^-, BA, aerobic; (6) H_2O_2 plus LPO plus SCN^-, PYG, aerobic; (7) H_2O_2 plus LPO, BA, anaerobic; (8) H_2O_2 plus LPO, PYG, aerobic; (9) no addition, BA, anaerobic.

peroxidase protected *E. coli* K-12 strain AB1157 (Fig. 1), but it did not significantly influence cell killing when a growing culture of this strain was exposed to hydrogen peroxide in dilution solution containing 10% TSY broth (Fig. 3). Lactoperoxidase potentiated the peroxide-mediated killing of *S. sanguis* strain ATCC 10556 in dilution solution, but did not influence the killing of a growing culture in TSY broth (6). It is possible that various components of the cell and the broth could function as a substrate in reactions catalyzed by lactoperoxidase in the presence of hydrogen peroxide. In some organisms this could be lethal for the cell, whereas in others it protected the cell from the toxic effect of hydrogen peroxide.

The toxicity of hydrogen peroxide is not limited to bacteria. Human cells can be much more sensitive to hydrogen peroxide toxicity than many bacteria. Human cells in culture can be killed at concentrations of hydrogen peroxide lower than 10 μM (12, 26). Primary lesions in both human cells and bacteria are single-stranded breaks in DNA (1, 12). Most mammalian cells and aerobic bacteria have highly developed defense mechanisms against hydrogen peroxide and other intermediates of oxygen reduction, such as superoxide and hydroxyl radicals (8, 11). The concerted action of the enzymes superoxide dismutase, catalase, and peroxidase keep the intracellular concentrations of the oxygen intermediates at nontoxic levels. If a cell actually becomes injured, there are systems for the repair of the lesions (1, 7, 12). The cells of the mucous membranes of the oral cavity may be particularly exposed to oxygen intermediates because they are colonized by bacteria in the presence of significant levels of oxygen (10). Many oral bacteria, especially streptococci, form hydrogen peroxide (14, 18, 29), and an obvious function of lactoperoxidase and thiocyanate in salivary secretions could be to protect the cells of the mucous membrane against hydrogen peroxide toxicity. Lactoperoxidase converts the highly toxic hydrogen peroxide formed by oral streptococci into the less toxic product $OSCN^-$, and this product actually inhibits the formation of hydrogen peroxide by the streptococci (24).

Verification of such a protective role on the part of salivary lactoperoxidase and thiocyanate for the human oral mucosa awaits the direct demonstration of protection in vitro of human epithelial cells and studies on the oral conditions in persons who may be lacking salivary lactoperoxidase. In the absence of lactoperoxidase, the hydrogen peroxide concentration in the oral cavity may reach significant levels. The existence of hydrogen peroxide in saliva has not been directly demonstrated (18), presumably because it is quickly converted to $OSCN^-$ by salivary lactoperoxidase and thiocyanate. However, it is possible to estimate the concentration of hydrogen peroxide which would exist in the absence of lactoperoxidase. In human saliva, the average measured $OSCN^-$ concentration is approximately 40 μM (K. M. Pruitt, J. Tenovuo, W. Fleming, and M. Adamson, Caries Res., in press). Therefore, in the absence of lactoperoxidase, the average hydrogen peroxide concentration should be at least 40 μM. Catalase in whole saliva is not efficient in destroying hydrogen peroxide in this concentration range (18). Since $OSCN^-$ may inhibit hydrogen peroxide-producing bacteria (24), the rate of production of hydrogen peroxide might increase in the absence of lactoperoxidase. In addition, because a bacterium is essentially a point source of hydrogen peroxide, the local hydrogen peroxide concentration on the colonized epithelial cell surfaces could be much higher than that extrapolated from whole-saliva assays of $OSCN^-$.

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