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Susceptibility of *Escherichia coli* to Bactericidal Action of Lactoperoxidase, Peroxide, and Iodide or Thiocyanate

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The bactericidal action that results from lactoperoxidase-catalyzed oxidation of iodide or thiocyanate was studied, using *Escherichia coli* as the test organism. The susceptibility of intact cells to bactericidal action was compared with that of cells with altered cell envelopes. Exposure to ethylenediaminetetraacetic acid, to lysozyme and ethylenediaminetetraacetic acid, or to osmotic shock were used to alter the cell envelope. Bactericidal action was greatly increased when the cells were exposed to the lactoperoxidase-peroxide-iodide system at low temperatures, low cell density, or after alteration of the cell envelope. When thiocyanate was substituted for iodide, bactericidal activity was observed only at low cell density or after osmotic shock. Low temperature and low cell density lowered the rate of destruction of peroxide by the bacteria. Therefore, competition for peroxide between the bacteria and lactoperoxidase may influence the extent of bactericidal action. Alteration of the cell envelope had only a small effect on the rate of destruction of peroxide. Instead, the increased susceptibility of these altered cells suggested that bactericidal action required permeation of a reagent through the cell envelope. In addition to altering the cell envelope, these procedures partly depleted cells of oxidizable substrates and sulfhydryl components. Adding an oxidizable substrate did not decrease the susceptibility of the altered cells. On the other hand, mild reducing agents such as sulfhydryl compounds did partly reverse bactericidal action when added after exposure of cells to the peroxidase systems. These studies indicate that alteration of the metabolism, structure, or composition of bacterial cells can greatly increase their susceptibility to peroxidase bactericidal action.

Myeloperoxidase, H_2O_2, and Cl^- or I^- provide an antimicrobial system within phagocytic vesicles of polymorphonuclear leukocytes (12), whereas lactoperoxidase, H_2O_2, and I^- or SCN^- provide an antimicrobial system in milk (1) and saliva (6, 18, 25). Other antimicrobial agents may enhance the action of these peroxidase systems. Before phagocytosis, bacteria are exposed to antibodies and other antimicrobial serum components. Within phagocytic vesicles, bacteria are exposed to lysozyme, "permeability-inducing factor" (24), and other antimicrobial proteins either before or at the same time as myeloperoxidase (11). Milk and saliva contain lysozyme, antibodies to bacterial surface components, and other antimicrobial proteins as well as lactoperoxidase (4, 5). Many of these agents can alter the structure and permeability of the bacterial cell envelope. Lysozyme, ethylenediaminetetraacetic acid (EDTA), or antibodies to bacterial surface components increase the activity of many antibiotics by permitting their entry through the cell envelope (14).

The aim of this study was to determine whether alterations of the cell envelope could increase the susceptibility of *Escherichia coli* to peroxidase bactericidal action. The action of the lactoperoxidase, H_2O_2, and I^- or SCN^- systems was measured using intact cells and cells subjected to procedures that cause well-defined alterations of the cell envelope of gram-negative bacteria. The procedures selected for use in this study do not in themselves cause loss of viability of *E. coli*.

MATERIALS AND METHODS

Lactoperoxidase, isolated from bovine milk (16), was provided by M. Morrison. Horseradish peroxidase (type VI) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co., St. Louis, Mo., and lysozyme was obtained from Worthington Biochemicals Corp., Freehold, N.J. Crystal violet (Allied Chemical, New York, N.Y.) was dissolved in water (0.5 mg/ml), reduced with excess sodium borohydride to yield leuko-crystal violet, made 40 mM in hydrochloric acid, and filtered.

Bacteria. *E. coli* ML 308-225 was grown aerobically at 37°C in medium A (2) modified by addition of 5 mM tris(hydroxymethyl)aminomethane (Tris)-chloride, pH 6.6, with 1% disodium succinate as carbon

source. Cells were harvested in late logarithmic phase of growth by centrifugation, at 25°C for 10 min, at 5,300 × *g*, then suspended at 25°C in 0.1 M potassium phosphate buffer, pH 6.6, with 1 mM magnesium sulfate, to yield "intact cells." Cells were extracted with EDTA by the procedure of Leive (13) to remove lipopolysaccharide from the outer membrane, then centrifuged and suspended as above to yield "EDTA-extracted cells." The release of lipopolysaccharide was confirmed by the spectrophotometric assay for 2-keto-3-deoxyoctonate (21, 22). Cells were subjected to the osmotic shock procedure of Neu and Heppel (20) to remove the soluble periplasmic proteins from the space between the outer and inner membranes. The release of proteins was confirmed by gel electrophoresis in the presence of sodium dodecyl sulfate (23). The cells were centrifuged at 0 to 5°C and then suspended as above to yield "osmotically shocked cells." To degrade the peptidoglycan layer of the cell envelope, cells were suspended in 30 mM Tris-chloride, pH 8.0, with 20% (wt/vol) sucrose, followed by addition of lysozyme (0.5 mg/ml) and potassium EDTA (10 mM) (8). The suspension was incubated at 25°C for 1 h without agitation and then centrifuged at 25°C. The cells were suspended in the 0.1 M phosphate buffer containing 10 mM magnesium sulfate and 20% sucrose, to yield "spheroplasts."

Rate of respiration. Consumption of O_2 was measured at 25°C with an O_2 electrode (Yellow Springs Instrument Co.). Relative rates of O_2 consumption were determined from the linear portion of the continuous recording of O_2 consumption over 3 to 10 min. When the addition of an oxidizable substrate was desired, suspensions were made 0.5% in disodium succinate immediately before measuring O_2 consumption.

Sulfhydryl content of cells. Sulfhydryl content was measured by the reaction of the disulfide compound DTNB with 1 mol of sulfhydryl to yield the mixed disulfide and 1 mol of 5-thio-2-nitrobenzoic acid (3). Fractions of 2 ml of suspensions containing 6×10^8 cells per ml were diluted with 4 ml of a solution containing 0.1 M Tris base, 10 mM EDTA, and hydrochloric acid to adjust to pH 8.0. Portions of 0.1 ml of 10 mM DTNB in 0.1 M phosphate buffer, pH 7.0, and 0.5 ml of 10% (wt/vol) sodium dodecyl sulfate were added. The mixtures were incubated at 37°C for 1 h and then cooled to 0 to 5°C and centrifuged at 18,000 × *g* for 10 min. Absorbance of the supernatants was measured at 412 nm. A molar extinction coefficient of 13,600 was assumed for 5-thio-2-nitrobenzoic acid (3), and one of 230 was assumed for DTNB.

Rate of destruction of H_2O_2. Fractions of 2 ml of suspensions containing 6×10^7 to 6×10^8 cells per ml were incubated with 0.25 mM H_2O_2 for 15 s and then 1 ml of 3.2 M acetic acid was added. The pH was adjusted to 4.5 with 1 ml of 20% (wt/vol) sodium acetate, and then 0.6 ml of leuko-crystal violet (0.5 mg/ml), 1.4 ml of water, and 10 nmol of horseradish peroxidase were added. After 15 min, the mixtures were centrifuged at 6,300 × *g* for 10 min. Supernatants were removed, and their absorbance was measured at 596 nm. Rate of destruction of H_2O_2 was determined from the linear portion of the plot of H_2O_2 concentration versus cell density, assuming a molar extinction coefficient of 75,000 for crystal violet (19).

Peroxidase systems. Portions of 2 ml of cell suspensions containing 6×10^8 cells per ml were incubated with 0.1 μM lactoperoxidase and 10 μM KI or 1 mM KSCN. At 3-min intervals, H_2O_2 was added with each addition sufficient to give a concentration of 30 μM. A total of 10 additions were made, and incubation was continued for 15 min after the last addition.

Assay of viability. Serial 1:10 dilutions were prepared from 1-ml samples of cell suspensions in sterile, modified medium A without a carbon source. When exposure to reducing agents was desired, 10 mM solutions of these agents in modified medium A without a carbon source were sterilized by filtration and used to prepare the 1:10 and 1:100 dilutions. One-milliliter portions of appropriate dilutions were plated onto solid media containing modified medium A, 2% (wt/vol) agar (Difco), and 1% glucose. Spheroplasts were diluted into 0.5% sodium chloride, with 20% sucrose and 10 mM magensium sulfate, and plated onto media containing modified medium A, 20% sucrose, 10 mM magnesium sulfate, 2% agar (Difco), and 1% glucose. Plates were incubated at 25°C, and the number of colonies was recorded after 2 to 3 days.

RESULTS

Characterization of the altered cells. Extraction with EDTA partly depleted the cells of lipopolysaccharide and certain outer membrane peptides. Osmotic shock removed the periplasmic proteins, as well as those components removed by EDTA. Spheroplasts were depleted of the components removed by osmotic shock. Although the spheroplasts retained the rodlike shape of intact cells, these spheroplasts were osmotically sensitive, as indicated by their rapid lysis when diluted into water. Because the osmotic shock procedure required incubation of cells at 0 to 5°C, the effect of low temperature alone was examined. No macromolecular components were released from cells during a 15-min incubation at 0 to 5°C.

Exposure of cells to low temperature, EDTA extraction, osmotic shock, or spheroplast formation did not cause loss of viability but did cause 17, 95, 97, and 77% inhibition, respectively, of their endogenous rate of O_2 consumption measured at 25°C. This inhibition could be reduced to 9, 14, 48, and 3%, respectively, by addition of succinate prior to measurement of O_2 consumption. Therefore, this inhibition appeared due in part to depletion of oxidizable substrates.

As shown in Table 1, incubation of cells at 0 to 5°C had no effect on their sulfhydryl content, whereas the other procedures partly depleted cells of sulfhydryl components. Osmotically shocked cells had the lowest sulfhydryl content. After the EDTA extraction, osmotic shock, and spheroplast formation procedures were complete, the cells were incubated for 15 min and then centrifuged to determine what proportion of sulfhydryl components had been released into

the medium. Most of the sulfhydryl content of the cell suspensions was retained within the cells. However, osmotically shocked cells continued to lose a large fraction of their sulfhydryl components. This loss was especially high at 0 to 5°C.

As shown in Table 2, intact *E. coli* cells destroyed H_2O_2 rapidly, and the rate was twice as fast at 25°C as at 0 to 5°C. Extraction with EDTA, osmotic shock, or spheroplast formation had little effect on the rate of destruction of H_2O_2. Less than 1% of the H_2O_2-destroying activity was released from cells by any of these procedures. The rate of destruction of H_2O_2 was proportional to cell density over a wide range. Although the cells could destroy H_2O_2, they did not alter the concentration of I^- or SCN^-. Intact or altered cells did not bind, transport, or accumulate significant amounts of I^- or SCN^-.

TABLE 1. *Sulfhydryl content of cell suspensions*[a]

Bacterial cells and temp (°C)	Sulfhydryl content (nmol/ml)		
	Before centrifugation	After centrifugation	
		Cells	Supernatant
Intact cells, 25	51	49	1
Intact cells, 0–5	51	48	2
EDTA-extracted cells, 25	40	37	2
Osmotically shocked cells, 25	34	29	5
Osmotically shocked cells, 0–5	35	25	11
Spheroplasts, 25	39	36	2

[a] Sulfhydryl content of cell suspensions (6×10^8 cells per ml) was measured after 15 min of incubation at the indicated temperature (before centrifugation) and again after cells were collected by centrifugation at the indicated temperature and suspended to the original cell density. All the cell suspensions were prepared from the same culture. Results shown are average values from determinations using three different cultures.

Bactericidal action. No loss of viability resulted from exposure of intact or altered cells to lactoperoxidase, H_2O_2, I^- or SCN^- individually or the combination of any two. Table 3 shows the effect of the complete lactoperoxidase, H_2O_2, and I^- or SCN^- systems on intact cells and on cells subjected to the procedures that alter the cell envelope. Also shown are results obtained when the sulfhydryl compound, dithiothreitol (DTT), was added after exposure of the cells to the peroxidase systems. Results are presented as the logarithm of the ratio of the number of viable cells in an untreated suspension to that in an equal portion exposed to the peroxidase system. A value of 0 indicates a ratio of 1, or no loss of viability. A value of 4 indicates that only 1 cell in 10,000 remained viable.

Exposure of the intact cells to the I^- system at 25°C resulted in only a small bactericidal effect. Lowering the temperature at which cells were exposed to the I^- system caused a striking increase in bactericidal action. The cells were not irreversibly altered by incubation at low temperatures. When cells were cooled to 0 to 5°C for 10 min and then warmed and exposed to the I^- system, the same results were obtained as with cells that had not been exposed to low temperatures. In contrast to the I^- system, the SCN^- system had no effect at 25 and had only a slight effect at 0 to 5°C.

Both extraction with EDTA and spheroplast

TABLE 2. *Destruction of H_2O_2*[a]

Bacterial cells	Rate of destruction of H_2O_2 (μmol/min per 10^8 cells)	
	25°C	0–5°C
Intact cells	1.2	0.6
EDTA-extracted cells	1.1	0.5
Osmotically shocked cells	0.9	0.4
Spheroplasts	1.0	0.5

[a] Results are the average of determinations using cell suspensions from three cultures.

TABLE 3. *Enhancement and reversal of bactericidal action*[a]

Bacterial cells and temp (°C)	Log (untreated/treated)			
	I^- system		SCN^- system	
	Without DTT	With DTT	Without DTT	With DTT
Intact cells, 25	0.5	0.2	0.0	0.0
Intact cells, 0–5	2.9	1.3	0.3	0.0
EDTA-extracted cells, 25	3.4	1.4	0.0	0.0
Osmotically shocked cells, 25	4.2	4.1	2.7	1.2
Osmotically shocked cells, 0–5	4.7	4.4	1.8	0.6
Spheroplasts, 25	3.3	1.0	0.2	0.0

[a] Cells were incubated with lactoperoxidase, H_2O_2, and either KI (I^- system) or KSCN (SCN^- system) at the indicated temperature. Viability was measured in the absence or presence of DTT. Results shown are average values from determinations on suspensions from at least three cultures.

formation increased the susceptibility of cells to the bactericidal action of the I^- system but not of the SCN^- system. Results obtained with EDTA-extracted cells or spheroplasts at 25°C were similar to those obtained with intact cells at 0 to 5°C. Osmotic shock increased the susceptibility to the bactericidal action of both the I^- and SCN^- systems. The effects of low temperature and osmotic shock on susceptibility to the SCN^- system were not cumulative. Instead, osmotically shocked cells were more susceptible at 25 than at 0 to 5°C.

Table 3 also shows that a greater yield of viable cells was obtained when DTT was added after exposure of cells to the I^- system. Similarly, DTT partly reversed bactericidal action of the SCN^- system against osmotically shocked cells.

Table 4 shows the relative ability of sulfhydryl compounds and other mild reducing agents to reverse bactericidal action. Sulfhydryl compounds were the most effective of the compounds tested. The relative ability of each compound to reverse bactericidal action was about the same when tested with either the I^- or SCN^- system. In contrast to these results, addition of an oxidizable substrate such as succinate either before or after exposure to the peroxidase systems did not alter susceptibility to bactericidal action.

Bactericidal action was not increased by raising the lactoperoxidase concentration or by adding more H_2O_2. However, when the I^- concentration was increased from 10 μM to 1 mM, complete loss of viability was obtained ([log (untreated/treated) > 8]). Because the loss of viability was complete, there was no apparent difference in susceptibility at 25 or 0 to 5°C or between intact and altered cells. In contrast, no bactericidal action was observed with 10 μM SCN^-, and bactericidal action against osmotically shocked cells was not complete regardless of the SCN^- concentration.

Figure 1 shows that the difference in susceptibility of intact and osmotically shocked cells was nearly abolished when the cells were exposed to the peroxidase systems at low cell densities. The striking difference between susceptibility of intact and osmotically shocked cells was observed only at high cell densities. The cell density was varied by dilution, not by selecting cells from different phases of growth. Lactoperoxidase, H_2O_2, I^- or SCN^- individually, or the combination of any two, did not cause loss of viability when added to cells at a density of 6×10^7 cells per ml.

TABLE 4. *Restoration of viability by reducing agents*[a]

Reducing agent	Log (untreated/treated)	
	I^- system	SCN^- system
None	2.0	2.7
Cysteine	0.9	1.4
Reduced glutathione	1.0	—[b]
DTT	1.1	1.4
Sodium thiosulfate	1.5	1.6
β-Mercaptoethanol	1.6	1.8
Sodium bisulfite	1.8	1.9
Sodium ascorbate	1.8	1.8

[a] Intact cells were incubated with lactoperoxidase, H_2O_2, and KI at 0 to 5°C. Osmotically shocked cells were incubated with lactoperoxidase, H_2O_2, and KSCN at 25°C. Cells were subsequently exposed to the reducing agents indicated, and viability was determined as in the text.

[b] —, Not determined.

DISCUSSION

Procedures that alter the cell envelope increased the susceptibility of *E. coli* to peroxidase bactericidal action. This result indicates that the target of bactericidal action lies toward the cell interior, within the protection of the permeability barrier of outer cell layers. Alteration of the cell envelope could increase its permeability to reactive chemical species produced by the peroxidase system and result in increased modification of essential intracellular components.

Intact cells were susceptible to bactericidal action at low cell densities or high I^- concentrations. Therefore, outer cell layers did not provide an absolute barrier to peroxidase bactericidal action. However, the permeability of the cell envelope may have been increased by exposure to the peroxidase system itself. Exposure of mitochondria to lactoperoxidase and high concentrations of H_2O_2 and I^- causes increased permeability and ultimate lysis (7).

At least two factors other than permeability

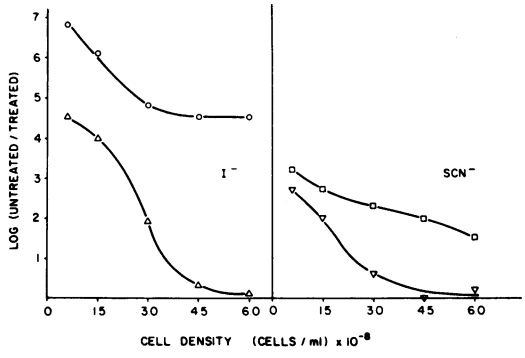

FIG. 1. *Loss of viability as a function of cell density. Intact cells* (△, ▽) *and osmotically shocked cells* (○, □) *at the indicated cell densities were incubated at 25°C with lactoperoxidase, H_2O_2, and either KI (left;* ○, △) *or KSCN (right,* □, ▽). *Viability was measured as in the text.*

of the cell envelope may have contributed to increased bactericidal action. First, lowering the temperature or the cell density lowered the rate of destruction of H_2O_2 by the cells. The ability to destroy H_2O_2 would be significant when the rate of peroxidase-catalyzed oxidation of I^- or SCN^- limits antimicrobial activity. Second, the procedures used to alter the cell envelope also partly depleted cells of sulfhydryl components. The loss of sulfhydryls may increase susceptibility, as indicated by the ability of added sulfhydryls to reverse bactericidal action.

Reversal of the effects of the peroxidase systems by reducing agents suggests that a substantial portion of antimicrobial action results from oxidative damage to cell components. The role of sulfhydryl components is likely to be complex. Intracellular sulfhydryl components or other easily oxidized components could serve as competitive substrates, protecting essential cell components from chemical modification. Some sulfhydryl-containing cell components, such as proteins, may be essential to viability. In addition, certain sulfhydryl compounds can inhibit peroxidase-catalyzed reactions (17). The presence of sulfhydryl compounds during exposure of bacteria to peroxidase, H_2O_2, and halide ions or SCN^- has been reported to prevent antimicrobial action (9, 10, 15).

Results presented here suggest that agents present in leukocytes, milk, and saliva could increase the susceptibility of gram-negative bacteria to peroxidase antimicrobial action as a direct or indirect consequence of altering the bacterial cell envelope. This increase of susceptibility may result from inhibition of cell functions, loss of cell components, and increased access of reactive chemical species to essential cell components.

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