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Comparison of Killing of Gram-Negative and Gram-Positive Bacteria by Pure Singlet Oxygen†

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Gram-negative and gram-positive bacteria were found to display different sensitivities to pure singlet oxygen generated outside of cells. Killing curves for *Salmonella typhimurium* and *Escherichia coli* strains were indicative of multihit killing, whereas curves for *Sarcina lutea*, *Staphylococcus aureus*, *Streptococcus lactis*, and *Streptococcus faecalis* exhibited single-hit kinetics. The *S. typhimurium* deep rough strain TA1975, which lacks nearly all of the cell wall lipopolysaccharide coat and manifests concomitant enhancement of penetration by some exogenous substances, responded to singlet oxygen with initially faster inactivation than did the *S. typhimurium* wild-type strain, although the maximum rates of killing appeared to be quite similar. The structure of the cell wall thus plays an important role in susceptibility to singlet oxygen. The outer membrane-lipopolysaccharide portion of the gram-negative cell wall initially protects the bacteria from extracellular singlet oxygen, although it may also serve as a source for secondary reaction products which accentuate the rates of cell killing. *S. typhimurium* and *E. coli* strains lacking the cellular antioxidant, glutathione, showed no difference from strains containing glutathione in response to the toxic effects of singlet oxygen. Strains of *Sarcina lutea* and *Staphylococcus aureus* that contained carotenoids, however, were far more resistant to singlet oxygen lethality than were both carotenoidless mutants of the same species and other gram-positive species lacking high levels of protective carotenoids.

Singlet oxygen is an excited form of molecular oxygen and is capable of reacting with a variety of biologically important substrates. Singlet oxygen may be involved in a number of environmental and health effects as well as in the therapeutic effects of some drug and photochemotherapy regimens. We have recently described a system that allows the assessment of singlet oxygen effects in biological samples in the absence of other reactive species (6, 20). The system makes use of photosensitization, a very efficient means of generating singlet oxygen. Because the sensitizer is physically separated from the target, direct interaction with the target of the excited states of the sensitizer is not possible. Results seen with this system can be unambiguously attributed to singlet oxygen and confirmed by measurement of the lifetime of the reactive intermediate.

The gram-negative bacterial cell wall lipopolysaccharide coat (LPS) offers some protection from the toxic effects of exogenous agents (26). This capacity enables these bacteria to survive in what otherwise must be considered hostile environments, such as mammalian intestines. The LPS has previously been shown to present a physical or chemical barrier through which singlet oxygen generated outside of cells must pass to interact with a vital target, such as membrane or cytoplasmic components (6). As a result, some strains that fail to produce a large portion of the LPS have displayed greater sensitivity to exogenous singlet oxygen than do strains that retain this ability (6). Most gram-positive bacteria lack a protective structure analogous to the gram-negative LPS and the outer membrane in which it is anchored. In addition to possibly forming a structural barrier to penetration, this outer membrane may form a chemical trap for singlet oxygen; it is composed of unsaturated fatty acids and proteins, which are compounds known to react chemically with singlet oxygen (30). The outer membrane and LPS of gram-negative bacteria do not, however, represent vital targets for the lethal action of singlet oxygen, since these can be removed without killing the cells (spheroplast formation). Because the cell wall structure of gram-positive and gram-negative bacteria represents the fundamental difference between these cells, once the barrier is crossed by singlet oxygen, the targets and mechanisms for cell killing for both gram-positive and gram-negative bacteria may be expected to be similar or identical.

Carotenoid pigments are known to physically quench singlet oxygen (10) and to protect bacteria against the lethal effects of photosensitization, whether by endogenous or exogenous photosensitizers (16 and references cited therein). Mathews-Roth and co-workers (19) have correlated the protective effects of carotenoids against photosensitization and singlet oxygen lethality in bacteria. Carotenoids also have been found to protect *Sarcina lutea* from killing by leukocytes, presumably by quenching singlet oxygen (14). Administration of the carotenoid β-carotene has also been found to protect mice from lethal exposure to hematoporphyrin derivative and light and, in humans, to mitigate the photosensitivity associated with erythropoietic protoporphyria (17). We have included for study two different species of bacteria that produce high levels of carotenoid pigments in order to ascertain what protective effects the carotenoids may have against killing of these cells by exposure to pure exogenous singlet oxygen.

Glutathione is the major nonprotein thiol in many organisms ranging from bacteria to humans and may be a general antimutagenic and protective agent (23 and references cited therein). One of the functions of glutathione is to detoxify

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TABLE 1. Bacterial strains used

Species and strain	Relevant genotype or phenotype	Reference or source
Gram negative		
Salmonella typhimurium		
LT2	Wild type	15
TA1975	*rfa*; deep rough derivative of LT2	1
TA1534	*gsh*$^+$ parent strain	1
SB4312	TA1534 *gsh-6*	This study
SB4313	TA1534 *gsh-7*	This study
SB4314	*hisC3076* transduced into *TA1534 gsh-5*	This study
Escherichia coli		
SB3933	*gsh*$^+$; AB1157[a] derivative	2
SB3936	*gsh* derivative of SB3933	2
Gram positive		
Streptococcus faecalis	Wild type	P. C. Maloney, The Johns Hopkins University, Baltimore, Md.
Streptococcus lactis	Wild type	P. C. Maloney
Staphylococcus aureus		
ISP 773	Wild type strain 655 (pigmented)	24
ISP1704	655 *hisD36* (pigmented)	13
Colorless	Derivative (nonpigmented) of a pigmented strain	C. W. Payton, Morgan State University, Baltimore, Md.
Sarcina lutea		
ATCC 9341a	Wild type (pigmented)	16
Colorless	Derivative of 9341a (nonpigmented)	16

[a] See reference 4 for complete genotype of AB1157.

electrophilic reactive intermediates such as peroxides and free radicals (21). Singlet oxygen is electrophilic and may be capable of introducing into bacteria oxidative damage that a general reductant such as glutathione might quell. Glutathione (γ-L-glutamyl-L-cysteinylglycine) contains an amino acid, cysteine, that has been reported to react with singlet oxygen (29). These authors also report that small peptides react at essentially the same rate as do their constituent amino acids, which implies that glutathione should react with singlet oxygen. Rougee and co-workers (25) have demonstrated that the bimolecular rate constants for glutathione and cysteine reactions with singlet oxygen are in fact similar. Glutathione must therefore be considered a potential protective agent against killing of bacteria by singlet oxygen. Strains of both *E. coli* and *S. typhimurium* that lack glutathione have been included for study in comparison with their parent strains, which contain normal levels (low millimolar concentrations) of intracellular glutathione.

MATERIALS AND METHODS

Bacterial strains. Sources of bacterial strains used in this study are listed in Table 1.

Growth and collection of bacteria. *S. typhimurium*, *E. coli*, *Sarcina lutea*, and *Staphylococcus aureus* strains were grown in nutrient broth (Difco Laboratories, Detroit, Mich.), diluted with physiological saline (0.15 M NaCl), and deposited on membrane filters (Millipore Corp., Bedford, Mass.) in less than a monocellular layer as previously described (6). *Streptococcus faecalis* and *Streptococcus lactis* were cultured in broth containing 1% yeast extract (Difco), 1% tryptone (Difco), 0.4% KCl, 0.15% sodium acetate, 0.05% ascorbic acid, and 1% glucose. Dilution and deposition of bacteria on membrane filters were performed as for other strains.

Singlet oxygen exposures and scoring survival. Bacteria deposited on membrane filters were exposed to pure exogenous singlet oxygen as previously described (6). Singlet oxygen exposure was controlled by duration of steady-state singlet oxygen production. After exposure, filters were placed directly on plates of the culture media described above and containing 1.5% Bacto-Agar (Difco). Inoculated plates were incubated at 37°C to allow surviving cells to replicate and form countable colonies. Colonies were scored as previously described, and results were recorded as percent survival (6).

***Salmonella* mutants lacking glutathione.** Glutathione-deficient mutants were isolated at The Johns Hopkins University by Ralph Cipriani. *S. typhimurium* TA1534 was mutagenized by using ICR-191 to induce tight frameshift mutations. Colonies were selected for resistance to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (28). Resistant cultures were plated on nutrient agar master plates. Colonies were replica plated onto minimal E medium (32) supplemented with L-histidine and biotin in Pyrex glass plates (Corning Glass Works, Corning, N.Y.). Glutathione was detected by using a variant of the histological mercury orange staining technique (3, 9). After incubation of replica plates, plates were inverted over glass lids containing a few drops of chloroform, the vapors of which helped permeabilize the cells. Plates were then set right side up without lids, and the colonies were submerged in 50 μM mercury orange dissolved in toluene. After 10 min, the mercury orange solution was poured off and the plates were rinsed with fresh toluene to remove any unbound mercury orange. Colonies showing white after this treatment were judged deficient in cellular glutathione. The original colonies from the master plates were picked into nutrient broth. Isolates were determined to be gram negative and P22 sensitive and to require histidine and biotin for growth. *uvrB* strains were tested for UV sensitivity. Total reduced and oxidized cellular glutathione was quantitated by the 5,5'-dithiobis(2-nitrobenzoic acid) recycling assay (31).

Reactivity of glutathione with pure singlet oxygen. The separated-surface-sensitizer pure singlet oxygen-generating

system (20) was used to test the ability of glutathione in solution to protect the target substrate, 2,5-bis(hydroxymethyl)furan (BHMF), against oxidation by singlet oxygen. BHMF oxidation reactions were set up as described elsewhere (7), with additions of glutathione as indicated. Reaction mixtures were separated on a liquid chromatograph (model 1090; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a 5-μm-particle-size C18 Nucleosil column (2.1 by 300 mm), with 0.01 M aqueous ammonium acetate–3% acetonitrile as the mobile phase. Eluent was monitored with a Hewlett-Packard 1090M diode array detector interfaced to a Hewlett-Packard series 9000 model 300 computer data station, which was used to calculate the areas of peaks corresponding to BHMF. The detector response and integration routine were calibrated by using BHMF solutions of known concentrations.

Extraction of carotenoids from bacteria. Pellets containing approximately 2×10^{12} cells of either *Streptococcus faecalis* or *Sarcina lutea* (pigmented) were saponified in 12% potassium hydroxide in methanol for 35 min at 65°C. Then 5 ml of distilled water was added to each sample, followed by 20 ml of hexane-diethylether (1:1) containing 0.125% butylated hydroxytoluene. The organic phase of each was removed, washed twice with distilled water, and made anhydrous with sodium sulfate. After the samples were decanted, the solvent was evaporated under a stream of N_2 and the deposits were redissolved in 1 ml of methanol each. Sample spectra were read with a Hewlett-Packard model 8452A diode array spectrophotometer. Extract components were separated with a Hewlett-Packard model 1090A liquid chromatograph equipped with an Alltech Econosphere C_{18} reverse-phase column. The mobile phase used was acetonitrile-methanol (85:15) for 15 min followed by acetonitrile–methanol–2-propanol (51:9:40). Eluents were monitored at 300, 375, and 425 nm with the diode array detector of the system.

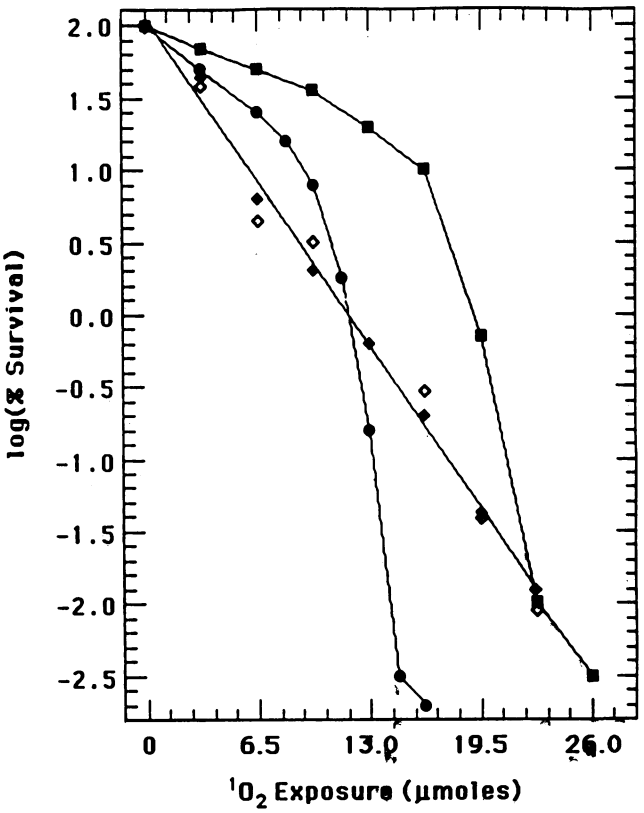

FIG. 1. Killing of gram-positive and gram-negative bacteria by pure exogenous singlet oxygen ($^1O_2$). Symbols: ■, *S. typhimurium* LT2 (wild type); ●, *S. typhimurium* TA1975 (deep rough); ◇, *Streptococcus lactis* wild type; ◆, *Streptococcus faecalis* wild type.

RESULTS

Singlet oxygen toxicity. Both gram-positive and gram-negative bacteria died quickly when exposed to singlet oxygen. Figure 1 shows the results of exposure of *S. typhimurium* wild-type and deep rough strains to singlet oxygen, along with results for wild-type isolates of *Streptococcus faecalis* and *Streptococcus lactis* for comparison. Both *Salmonella* strains displayed survival curves indicative of multihit killing by exposure to singlet oxygen. The deep rough strain, however, showed faster initial killing by this toxicant than did the wild-type strain, whereas the maximum rates of killing, demonstrated by the late portions of these curves, were indistinguishable. In contrast, the gram-positive *Streptococcus* wild-type isolates display single-hit kinetics. The two *Streptococcus* species died at the same rate, which was initially faster than the rate of either of the *Salmonella* strains but slower than the maximum rate of inactivation seen in the gram-negative species.

Protection by carotenoids. Data are shown in Fig. 2 for the exposure of two species of bacteria, *Staphylococcus aureus* and *Sarcina lutea*, which contain high levels of carotenoid pigments. Also shown are the responses of colorless mutants of these two species to singlet oxygen exposure. Shown for comparison is the survival curve of *Streptococcus faecalis* from Fig. 1. The two carotenoid-containing strains displayed mutually identical survival curves and much greater resistance than did the colorless mutants, whose survival patterns were identical to each other. The *Streptococcus* strain exhibited sensitivity results intermediate to those for these two groups. This strain was extracted and found to contain trace amounts of three carotenoids, with absorption maxima at 394, 417, and 443 nm, 398, 419, and 445 nm, and 413, 437, and 465 nm, respectively. The spectra of the first two indicated compounds with eight conjugated double bonds, whereas the third spectrum indicated a carotenoid chromophore composed of nine conjugated acyclic double bonds (17).

Relative rates of inactivation. Figure 3 shows the initial slopes for all of these bacterial species, which were calculated relative to the slope for the *Salmonella* wild-type strain (Table 2). The slopes for the two pigmented species were greater than the initial slope of the *Salmonella* wild-type strain and very nearly identical to that of the *Salmonella* deep rough strain. One other pigmented *Staphylococcus aureus* strain, ISP1704, by a one-point rate determination for inactivation at exposure to 6.5 μmol of singlet oxygen, gave a slope of −0.06, identical to the slope of the deep rough *Salmonella* strain, which was killed initially twice as fast as was the wild-type strain. The *Streptococcus* species were killed by singlet oxygen at nearly four times the initial rate of the *Salmonella* wild-type strain and approximately twice the rate of the pigmented gram-positive species. Those strains that were derived from originally pigmented wild types but had lost pigmentation were killed at a rate five times faster than that of the colored wild type and nearly eight times faster than that of the *Salmonella* wild-type strain.

Glutathione-deficient strains. We also tested the ability of another putative protective agent, glutathione, to mitigate

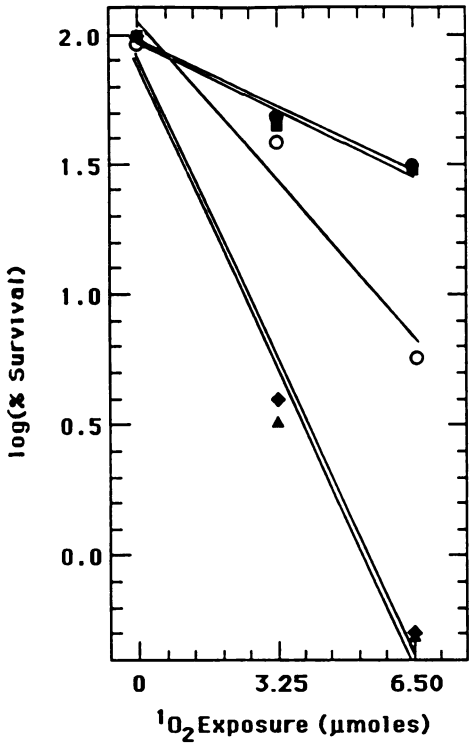

FIG. 2. Killing of gram-positive pigmented and nonpigmented bacteria by singlet oxygen ($^1O_2$). Symbols: ■, *Sarcina lutea* wild type (pigmented); ●, *Staphylococcus aureus* ISP773 (pigmented); ○, *Streptococcus faecalis* wild type; ◆, *Sarcina lutea* colorless mutant (nonpigmented); ▲, *Staphylococcus aureus* colorless derivative (nonpigmented).

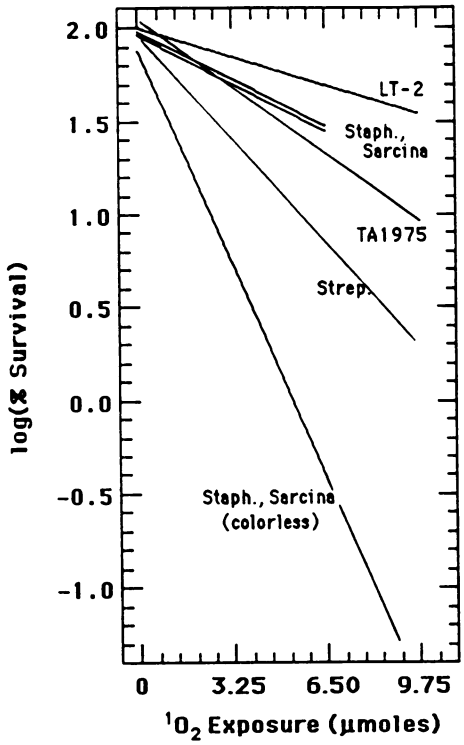

FIG. 3. Initial slopes for bacterial killing curves by singlet oxygen ($^1O_2$).

the lethal effects of exogenous singlet oxygen. Table 3 shows the results for *Salmonella* and *E. coli* strains that contain either normal levels (low millimolar concentrations) of internal glutathione or less than 3% of these levels. In each case, the rate of killing of cells was independent of glutathione concentration. Since the *E. coli gsh* mutant lacks the first enzyme in the pathway for biosynthesis of glutathione, γ-glutamyl-cysteinyl transferase, there is no accumulation of this initial peptide which could account for an alternative source of glutathionelike protection. Although none of the *Salmonella gsh* mutants were mapped genetically, preliminary evaluation by paper chromatography of extracts indicated no detectable levels of soluble thiols, which suggested that they probably also carry mutations in their *gshA* genes.

The results for the glutathione-deficient strains raised the question of whether the glutathione is inherently unable to protect against singlet oxygen damage or fails to protect because it is compartmentalized in a site where it is unable to quench singlet oxygen. We therefore tested the ability of glutathione in solution to protect a target substrate, BHMF, against oxidation by singlet oxygen. The rate of singlet oxygen reaction with the furan derivative was unchanged in the presence of 1 or 10 mM glutathione (relative rates of 0.95 ± 0.03 and 0.96 ± 0.04, respectively). Glutathione at 10 mM represents a level several times higher than is found in the bacterial cells. Therefore, the concentrations of glutathione found in these cells do not quench singlet oxygen efficiently enough to exert a protective effect. For comparison, 1 to 3 mM histidine and carnosine, a histidine-containing dipeptide, each protect BHMF from oxidation by singlet oxygen under these conditions (7) and protect bacteria from the lethal effects of externally generated singlet oxygen (6).

DISCUSSION

Nonpigmented derivatives of *Staphylococcus aureus* arise in stored cultures of pigmented wild-type isolates (5). These white subcultures often show greater susceptibility to inactivation by agents encountered in vivo than do pigmented strains (12). Carotenoids protect a number of organisms, from bacteria to humans, against the harmful effects of

TABLE 2. Initial slopes for singlet oxygen killing of bacteria

Organism	Slope	
	Initial	Relative to slope of *S. typhimurium* LT2
Gram negative		
Salmonella typhimurium		
LT2 (wild type)	−0.03	1.00
TA1975 (deep rough)	−0.06	2.00
Gram positive		
Streptococcus faecalis	−0.11	3.67
Streptococcus lactis	−0.11	3.67
Staphylococcus aureus		
Pigmented[a]	−0.05	1.67
Nonpigmented	−0.23	7.67
Sarcina lutea		
Pigmented[b]	−0.05	1.67
Nonpigmented	−0.23	7.67

[a] Contains as many as nine different C_{30} carotenoids (11, 27).
[b] Contains sarcinene, sarcinaxanthin (a C_{50} carotenoid), and derivatives of sarcinaxanthin (11).

TABLE 3. Relative initial rates of singlet oxygen killing of *Salmonella* and *E. coli* strains with or without internal glutathione

Strain	Relevant genotype and phenotype	% Glutathione	Relative initial rate[a] of killing
Salmonella typhimurium			
TA1534	*gsh*$^+$ parent strain	100	1.00 ± 0.09
SB4312	TA1534 *gsh-6*	3	0.95 ± 0.06
SB4313	TA1534 *gsh-7*	<3[b]	0.97 ± 0.12
SB4314	TA1534 *gsh-5*	<3[b]	0.99 ± 0.08
Escherichia coli			
SB3933	*gsh*$^+$ parent strain	100	1.00 ± 0.07
SB3936	SB3933 *gshA2*	<3[b]	1.00 ± 0.07

[a] Rates are for *gsh* mutants relative to their *gsh*$^+$ parent strains only. The initial rates of killing of these *E. coli* strains, and of strain AB1157 from which they are derived, are 0.28 ± 0.06 relative to these *S. typhimurium* strains and strain LT2, from which they are derived (6).

[b] Contained no detectable glutathione in the recycling assay. A conservative assessment of the limits of detection for this assay is 3%.

photosensitization and singlet oxygen generation (see introduction). The *Sarcina lutea* pigments, like those in *Staphylococcus aureus*, are carotenoids and therefore may serve a protective role against damages the bacteria encounter in vivo. The *Sarcina lutea* colorless strain used in these studies was originally isolated to assess the protective function of carotenoids (16). Our experiments (Table 2) show that the carotenoid pigments found in *Sarcina lutea* and *Staphylococcus aureus* substantially protect these bacteria from the lethal effects of singlet oxygen generated outside the cells. The pigments are located in the cytoplasmic membranes of these cells (5, 16, 27). It has been suggested that the cytoplasmic membrane is the site of lethal damage to bacteria via photosensitization and singlet oxygen (16). Cytoplasmic membrane damage, particularly via singlet oxygen-mediated mechanisms, also appears to predominate when eucaryotic cells are exposed to photosensitizers (reviewed in reference 32).

Because the two *Streptococcus* species used in this study are not pigmented, it is not surprising that they display greater sensitivity to singlet oxygen toxicity than do species that contain high levels of protective carotenoids. Since some streptococcal species have been reported to contain carotenoids (11), the *Streptococcus faecalis* strain used in our toxicity studies was extracted for the presence of carotenoid pigments. It was found to contain trace amounts of three carotenoids, only one of which displayed an absorption spectrum indicative of a compound with nine conjugated double bonds. Only those carotenoids with nine or more conjugated double bonds protect against exogenous photosensitization and singlet oxygen damage (18, 19). Therefore, the presence of this compound, although not sufficient to color the cells, might offer the cells some protection against singlet oxygen. The strength of their resistance relative to the resistance of strains lacking protective carotenoids may indicate that they have, in addition to a trace of protective carotenoid pigment, some other defense against singlet oxygen damage which the visibly pigmented bacteria lack. Thus, although the colored cells are better protected than the streptococcal species, without these pigments they are more susceptible to singlet oxygen than are strains that naturally lack high levels of this potent defensive mechanism.

Mutants of *S. typhimurium* and *E. coli* that lack glutathione are no more sensitive to exogenous singlet oxygen killing than are their parental strains containing glutathione (Table 3). The reaction of glutathione with singlet oxygen will protect the bacteria from singlet oxygen only if glutathione is sufficiently reactive and present at high enough concentrations to effectively compete with the critical targets for reaction with singlet oxygen. The cellular location of glutathione could be a factor in its ability to protect cells against damage. Glutathione in gram-negative bacteria may be located throughout both the cytoplasm and periplasmic space that separates the inner (cytoplasmic) and outer membranes. *S. typhimurium* TA1534 (Table 3) contains approximately 2 mM internal glutathione and exports about 30% of the total it makes (23). Despite containing a reactive cysteine residue, glutathione did not protect an alternate target, BHMF, at these concentrations (see Results). Therefore, glutathione at cellular concentrations does not appear sufficiently reactive with singlet oxygen to afford the cells protection, regardless of its location. This observation is consistent with the bimolecular rate constants observed for the reaction of glutathione with singlet oxygen (25). Furthermore, glutathione in bacteria did not inhibit the lethal effects of any secondary reaction products that might arise from singlet oxygen oxidation of cellular components.

Since carbohydrates do not react readily with singlet oxygen (30), reduced numbers of LPS sugar residues per se in deep rough mutants probably cannot account for the increased susceptibility of these strains to singlet oxygen toxicity. In addition to lacking the extensive oligosaccharide chain of their outer cell walls, deep rough mutants of *Salmonella* display altered membrane permeabilities, possibly as a result of changes in the lateral associations of the phospholipids in this membrane (22). Alternatively, they may have a periplasm of abnormal composition. Differences in the relative permeabilities of the outer membranes or periplasmic constituents may account for the greater initial rate of killing seen with TA1975 and two other independently isolated deep rough strains (6) relative to the rate for the wild type. This greater killing rate was observed only for strains with the LPS reduced within the core galactose residues; *rfc*, *rfb*, and *gal* strains grown without galactose all exhibit wild-type inactivation rates (6) despite having shortened LPS sugar chains. A very large amount of oligosaccharide can be lost, therefore, before any changes in sensitivity to singlet oxygen are observed. Despite different initial rates of killing, however, the maximum rates of killing for the deep rough and wild-type strains, as shown by the late portions of the curves, are indistinguishable. The mechanism of killing is probably not different for these various strains, therefore, once the initial barrier is passed.

Figure 4 illustrates a model of the effect of the cell wall structure on singlet oxygen toxicity. Although this model depicts the cytoplasmic membrane as the vital target for singlet oxygen interaction, the model would still serve for any vital target inside this location for singlet oxygen arriving from outside the cells. The gram-negative cell wall is represented schematically in Fig. 4A. Singlet oxygen reaching the outer membrane-LPS portion of the cell wall (large arrow) may either collide without penetration or reaction (broken-line arrow), react with the components of the outer membrane, or penetrate through the various layers to the vital target (small arrow). Reaction with the outer membrane components could lead to generation of reactive secondary products, such as peroxy radicals, which may in turn be able to cause lethal damage to the vital target (lower arrow). The total toxicity, then, would be the sum of the singlet oxygen reaching the inner membrane and the lethal effects of secondary reaction products from the outer membrane. Figure 4B shows a similar schematic for deep rough mutants.

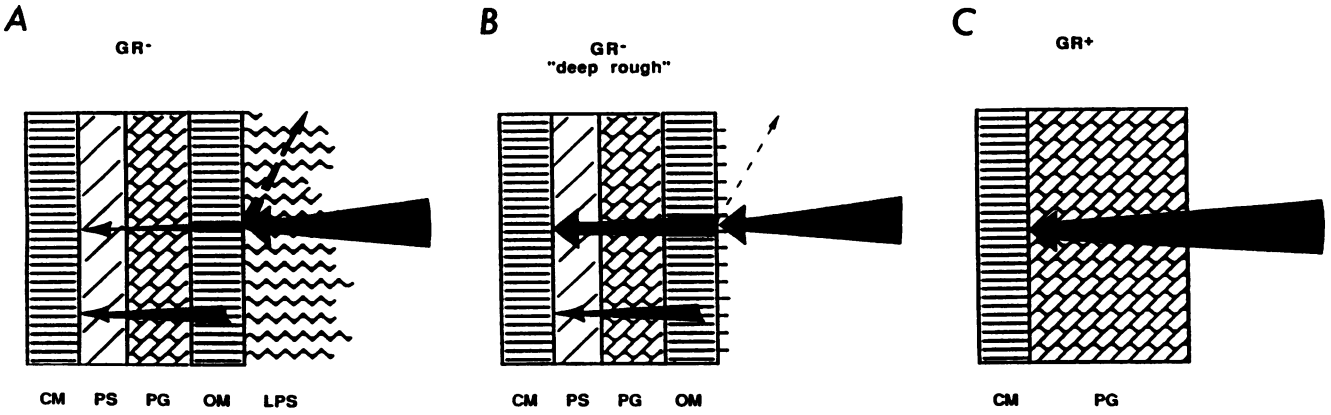

FIG. 4. Schematic diagrams of singlet oxygen interactions with gram-positive (GR^+) and gram-negative (GR^-) cell walls (not drawn to scale). CM, Cytoplasmic membrane; PS, periplasmic space; PG, peptidoglycan; OM, outer membrane; LPS, lipopolysaccharide. (A) Events in gram-negative wild type. Singlet oxygen impinging on the cell (heavy arrow) may be deflected (broken-line arrow) or penetrate the outer membrane barrier. Some of the singlet oxygen that penetrates will reach the cytoplasmic membrane (thin arrow), while some may react with unsaturated fatty acids and proteins in the outer membrane. Reaction products of singlet oxygen with outer membrane components may be capable of undergoing reaction to cause cell death (lower arrow). Changes in permeability that result from reaction of singlet oxygen at the outer membrane would also enhance penetration of singlet oxygen to the cytoplasmic membrane at greater exposures. (B) Events in gram-negative deep rough strains; as in panel A, except that LPS is shown reduced to the first few sugar residues and greater permeability of the outer membrane may reduce the physical barrier capacity, as shown by small deflected and larger penetration arrows. (C) Events in gram-positive bacteria. Singlet oxygen reaches the cytoplasmic membrane unimpeded and unreacted with peptidoglycan of the cell wall. Since these bacteria lack outer membranes, secondary reaction products would be formed at a lower rate in gram-positive than in gram-negative bacteria; this would also account for the greater final inactivation slope (maximum rate of killing) of gram-negative bacteria. Quenchers such as carotenoids which are located in the cytoplasmic membrane protect by reducing the effective dose of singlet oxygen but probably do not alter the mechanism of toxicity.

Because of the greater permeability of the outer membrane in these strains, more singlet oxygen may penetrate this barrier, as depicted by the smaller (than in Fig. 4A) broken-line arrow representing exclusion from the cell surface and the larger arrow representing singlet oxygen penetration. Reaction of singlet oxygen in the outer membrane may generate reactive species (lower arrow). The greater initial killing of the deep rough strains is thus envisioned as due to the greater penetration of singlet oxygen. As exposure continues, the influence of the secondary reaction products may become greater, either (or both) as a source of additional toxic species or by permeabilizing the membrane to greater singlet oxygen penetration.

Figure 4C illustrates the gram-positive cell wall. Singlet oxygen probably diffuses readily through the relatively open structure of the peptidoglycan layer of the cell wall to react with the vital target. The rate of killing should depend then only on direct singlet oxygen-vital target interactions, without any need to invoke secondary reaction mechanisms such as in the gram-negative outer membrane. In fact, single-hit kinetics for singlet oxygen toxicity are observed in the gram-positive species. Yet a large number of singlet oxygen collisions are required on average to inactivate a cell (10^{10} to 10^{11}). From earlier dosimetry measurements (6), we estimate that 1.3×10^{-5} mol of singlet oxygen reaches a 1.5-cm-diameter surface in 20 min, for a singlet oxygen exposure of 3.7×10^{-3} mol/m^2 per min. Taking a typical *S. typhimurium* cell size as 1.0 by 0.7 μm gives a projected surface area for exposure of 7×10^{-13} m^2 per bacterium. This yields an average exposure of 2.6×10^{-15} mol per bacterium per min, or 1.6×10^9 molecules per bacterium per min. From the curves presented in this paper, LT2 requires on average 4×10^{10} molecules of singlet oxygen per bacterium to inactivate 90% of exposed cells and 5×10^{10} molecules per bacterium to inactivate 99%. Similarly, TA1975 and the streptococcal strains require on average 3×10^{10} molecules per cell for 99% killing; colorless derivatives of *Staphylococcus aureus* and *Sarcina lutea* require only about 6×10^9 molecules per cell. This finding suggests a low probability of vital target-singlet oxygen interaction, so that most collisions do not result in cell death. However, the probability that the inactivating collision will occur never changes. A single reaction of singlet oxygen could conceivably have devastatingly global effects, such as initiating lipid peroxidation and subsequent radical-mediated chain reactions. Single-hit kinetics in this case are not likely due to lethal DNA damage, as singlet oxygen does not react very readily with DNA in bacteria (6, 8).

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