ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2006, p. 949–954 Vol. 50, No. 3
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.3.949–954.2006

Involvement of Reactive Oxygen Species in the Action of Ciprofloxacin against *Escherichia coli*

M. Goswami, S. H. Mangoli, and N. Jawali*

Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India

Received 7 November 2005/Returned for modification 11 November 2005/Accepted 22 December 2005

Ciprofloxacin is an important and commonly used member of the fluoroquinolone group of antibiotics. Ciprofloxacin inhibits DNA topoisomerase II and DNA topoisomerase IV activities, eventually leading to bacterial cell death. In addition, an increase of reactive oxygen species in the bacterial cells in response to ciprofloxacin has been shown. We investigated the role of reactive oxygen species in the antibacterial action of ciprofloxacin by studying the effects of different antioxidant compounds on ciprofloxacin susceptibility of *Escherichia coli*. Among the antioxidants checked, glutathione and ascorbic acid provided substantial protection against ciprofloxacin. The involvement of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in the antibacterial action of ciprofloxacin was analyzed using superoxide dismutase, catalase, and alkyl hydroperoxide reductase knockout strains of *E. coli*. The effects of multicopy *sod* genes on ciprofloxacin susceptibility of *E. coli* were also analyzed. On the basis of our results, we conclude that O_2^- and H_2O_2 may be involved in antibacterial action of ciprofloxacin. Our findings that glutathione gave protection against other fluoroquinolones and not against nonfluoroquinolone antibiotics imply that reactive oxygen species may have a similar role in the antibacterial action of all these fluoroquinolones and that glutathione-mediated protection is not a general phenomenon but specific to fluoroquinolones. These observations are of significance, as fluoroquinolones are important antibiotics with immense therapeutic value, and the effectiveness of treatment by these drugs may be affected by dietary intake and cellular levels of these antioxidants.

The emergence of drug resistance against β-lactam and aminoglycoside antibiotics resulted in the discovery of newer classes of synthetic antibiotics, including quinolones. Since the discovery of the first quinolone, nalidixic acid, various structural and chemical modifications resulted in expanded-spectrum and broad-spectrum quinolones (reviewed in references 3 and 29). These drugs are well absorbed following oral administration, with moderate to excellent bioavailability, and they show rapid bactericidal effect against susceptible organisms and cover a broad antibacterial spectrum (29). Since these drugs consist of carboxyl and amine groups along with other chemical functionalities, their acid-base behavior is influenced by the physiochemical properties of the solvent and their antibacterial activity is pH dependent (30). The mechanism of antibacterial action of quinolones is not completely understood; however, it has been proposed that the initial event is the inhibition of DNA synthesis by interference with the nick sealing activity of DNA topoisomerase II (DNA gyrase) and DNA topoisomerase IV. In the presence of these antibiotics, the enzyme is trapped on the DNA, resulting in the formation of quinolone-enzyme-DNA complexes, and the subsequent release of DNA ends from this complex leads to the generation of "cellular poison" which ultimately leads to cell death (12, 14, 17).

Narrow-spectrum quinolones like nalidixic acid and oxolinic acid are used less often today because of their moderate gram-negative bacterial activity, minimal systemic distribution, and development of rapid resistance against them (29). Expanded-spectrum quinolones, such as norfloxacin and ciprofloxacin, marked the arrival of fluoroquinolones with better antibacterial coverage, since introduction of fluoro group at position 6 and piperazinyl side chain at position 7 of the quinolone ring expanded their gram-negative activity and broadened their spectrum to *Pseudomonas* (3). Further improvement in the chemical structure led to the development of fluoroquinolone derivatives that were more effective against gram-positive organisms and anaerobes as well (29).

Recently, a number of antibiotics, including ciprofloxacin, have been demonstrated to stimulate the production of reactive oxygen species (ROS) in bacterial cells (2, 6). Reactive oxygen species are reactive by-products formed by the partial reduction of molecular oxygen (32). Redox cycling of various chemical substances, including some antibiotics, affects the reactive oxygen species produced by cells during the oxidation process (10). Fluoroquinolones are known to induce the formation of singlet oxygen ($^1O_2$) and superoxide anion (O_2^-), which are responsible for the phototoxic effect of the fluoroquinolones (37). In addition, the two prominent side effects of aminoglycoside antibiotics, ototoxicity and nephrotoxicity, are also believed to involve ROS (9, 26). A number of diverse cellular processes that lead to cell death are also mediated through ROS (8, 11).

The enzymatic defense system against ROS comprises of specific enzymes, like superoxide dismutase, catalase, and peroxidase, which decrease the steady-state level of reactive oxygen (11, 15). *Escherichia coli* has three different superoxide dismutase (SOD) enzymes encoded by *sodA*, *sodB*, and *sodC* that metabolize O_2^-. *sodA* codes for an inducible cytosolic Mn-SOD (21, 36), *sodB* codes for Fe-SOD, which is constitutively expressed at basal levels during normal metabolic pro-

* Corresponding author. Mailing address: Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India. Phone: (91) 22-25595078. Fax: (91) 22-25505326. E-mail: enjay@apsara.barc.ernet.in.

TABLE 1. List of the bacterial strains and plasmids used in the study

Strain or plasmid	Relevant genotype	Source or reference
E. coli K-12 strains		
MG1655	F$^-$ λ$^-$ *rph-1*	*E. coli* Genetic Stock Center
AB1157	F$^-$ λ$^-$ *thi-1 thr-1 leuB6* Δ(*gpt-proA*)*62 his-4 argE3 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 kdgK51 tsx-33 supE44*	*E. coli* Genetic Stock Center
JI130	AB1157 *sodA* Cam^r	James Imlay (19)
JI131	AB1157 *sodB* Kan^r	James Imlay (19)
AS393	AB1157 *sodC*::Spec	James Imlay (19)
JI374	MG1655 *katG17*::Tn*10* Δ(*katG17*::Tn*10*)*1* (Tet^s) Δ*ahpCF′ kan*::*′ahpF*	James Imlay (35)
JI377	MG1655 *katG17*::Tn*10* Δ(*katG17*::Tn*10*)*1 katE12*::Tn*10* Δ*ahpCF′ kan*::*′ahpF*	James Imlay (35)
NJ01	MG1655 *sodA* Cam^r	This study
NJ02	MG1655 *sodB* Kan^r	This study
Plasmids		
pDT1.5	*sodA*-expressing plasmid of pHC79 cosmid library, Amp^r	James Imlay
pFeSOD	*sodB*-expressing plasmid of pHC79 cosmid library, Tet^r	James Imlay
pSodC2.3	pBR329 expressing *sodC*, Amp^r	James Imlay

cesses inside the cell (33, 38), and *sodC* codes for a periplasmic Cu-Zn-SOD, which takes care of the periplasmic and extracellular O_2^- (7, 18, 20). The product of the dismutation reaction of O_2^- is H_2O_2 (24), an important entity with a highly reactive nature and capable of damaging critical biomolecules. *E. coli* has two catalases, hydroperoxidase I (HPI) and HPII, involved in detoxification of intracellular H_2O_2 (23). HPI is encoded by *katG*, which is present during aerobic growth and transcriptionally controlled at different levels, and HPII is encoded by *katE*, which is induced during stationary phase (11). In addition, alkyl hydroperoxide reductase (*ahpCF*) provides an additional mechanism for scavenging H_2O_2 (35).

Even though ROS are reported to be induced by fluoroquinolones (1, 6), their role in the antibacterial action of these antibiotics is not clearly understood. Antioxidant-mediated reduction in antibiotic sensitivity would be an indication of the involvement of ROS in this process. Since dietary supplements, such as vitamin C (ascorbic acid) and vitamin E (α-tocopherol), which have antioxidant properties, are sometimes prescribed along with antibiotics during the course of treatment of an infection, it is important to understand the effects of these antioxidants on the antibacterial action of these antibiotics. The aim of the present study was to investigate the role of ROS in the antibacterial action of fluoroquinolones. This was undertaken by supplementing the growth medium with antioxidants and by introducing mutations in genes whose products are known to reduce the steady-state levels of ROS in the cell. We examined the effects of antioxidant compounds, such as ascorbic acid, glutathione, histidine, mannitol, and sodium pyruvate, on the ciprofloxacin sensitivity of *E. coli* cells. Further, we studied the effects of mutations in oxidative stress defense genes, viz., superoxide dismutase (*sodA*, *sodB*, and *sodC*), catalase (*katE* and *katG*), and alkyl hydroperoxide reductase (*ahpCF*) on the ciprofloxacin sensitivity of *E. coli* cells. The effects of multicopy *sod* genes on ciprofloxacin sensitivity were also examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown in Luria broth (LB) or plated in Luria agar. The *sod* mutants of strain MG1655 were constructed by P1-mediated transduction as previously described (25). Strains NJ01 and NJ02 were generated by transduction of *sodA* and *sodB* from strains JI130 and JI131, respectively, to MG1655. Plasmid transformations were performed using the $CaCl_2$ method as described by Sambrook et al. (34).

Antioxidants and antibiotics. Antioxidants were freshly prepared as mentioned below before use. Stock solutions (250 mM) of glutathione and ascorbic acid were prepared in sterile distilled water and 0.5 N NaOH, respectively, followed by filter sterilization through 0.22-μm membrane (Millipore) and added to the media prior to pouring the plates. Stock solutions of histidine, mannitol, and sodium pyruvate were prepared in sterile distilled water and were added to Luria agar media prior to autoclaving. The pH of the media in general was adjusted to 7.4 before sterilization. Wherever required, Luria agar was supplemented with antibiotics and antioxidants at the concentration indicated.

Growth conditions. Strains were grown at 37°C for 12 to 18 h in Luria broth (Bacto tryptone, 10 g/liter; yeast extract, 5 g/liter; and NaCl, 10 g/liter) medium. Overnight cultures were inoculated into fresh medium. Cells in midexponential phase (optical density at 600 nm of ~0.5 to 0.6) were used for the determination of antibiotic sensitivity. Wherever required, chloramphenicol (Cam), kanamycin (Kan), spectinomycin (Spec), and tetracycline (Tet) were added to a final concentration of 12.5, 30, 120, and 12.5 μg/ml, respectively. For the maintenance of plasmids pDT1.5 and pSodC2.3, ampicillin (Amp) was used at a final concentration of 100 and 300 μg/ml, respectively.

Sensitivity to antibiotics. Three methods were used for measuring antibiotic sensitivity.

(i) Antibiotic disk diffusion method. The antibiotic disk diffusion method was used as a qualitative measure to appreciate the differences in antibiotic sensitivities due to various treatments. Overnight *E. coli* cultures were diluted (1:100) in LB and grown afresh at 37°C. Mid-exponential-phase cultures were used to prepare the lawns of cells by the Kirby-Bauer method (5). A known amount of antibiotic solution was spotted on 5.5-mm-diameter Whatman filter disks placed on the bacterial lawn (no closer than 30 mm from the center of the disk), and the plates were incubated overnight at 37°C.

After overnight incubation, plates were scanned at a resolution of 300 × 300 dots per inch against a black background using a flat-bed scanner. The diameter of the zone of complete inhibition (as judged by the unaided eye) was measured using ImageJ software (http://rsb.info.nih.gov/ij). All the experiments were repeated more than two times to check the reproducibility of the results. The data of one representative experiment are presented here. The mean values of three measurements of the diameter taken at different angles were reported. For the antibiotic concentration that showed no visible zone of inhibition, a diameter of 5.5 mm was recorded (since the diameter of the zone of inhibition includes the diameter of disk spotted). As we have used various antibiotic concentrations, all the diameters of zone of inhibition were not within the quality control range for the diameter of the zone of inhibition (8 to 12 mm).

(ii) MIC. Ciprofloxacin MIC was determined by the agar dilution method as outlined by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards [NCCLS]) (27, 28). An inoculum of approximately 10^4 to 10^5 CFU (simultaneously determined by plating) per spot was applied to the agar by a micropipette delivering 10 μl per spot. The MIC was the lowest concentration of antimicrobial agent that prevented visible growth after 20 h of incubation at 37°C. A slight haze of growth was ignored.

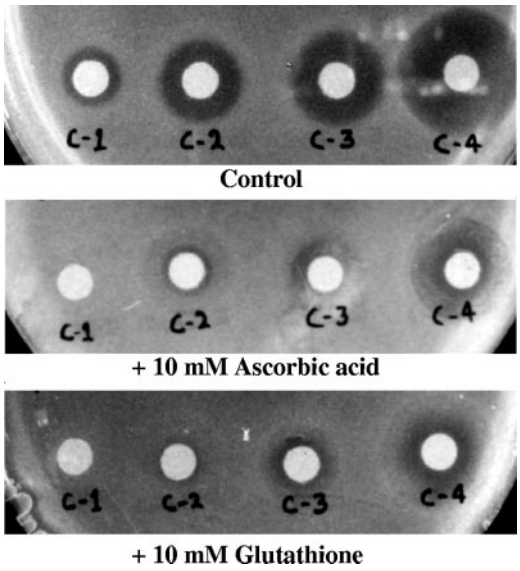

FIG. 1. Decreased sensitivity of *E. coli* MG1655 against ciprofloxacin in the presence of 10 mM glutathione or ascorbic acid. C-1, C-2, C-3, and C-4 correspond to 40, 200, 400, and 2,000 ng of ciprofloxacin, respectively, spotted on the Whatman disk.

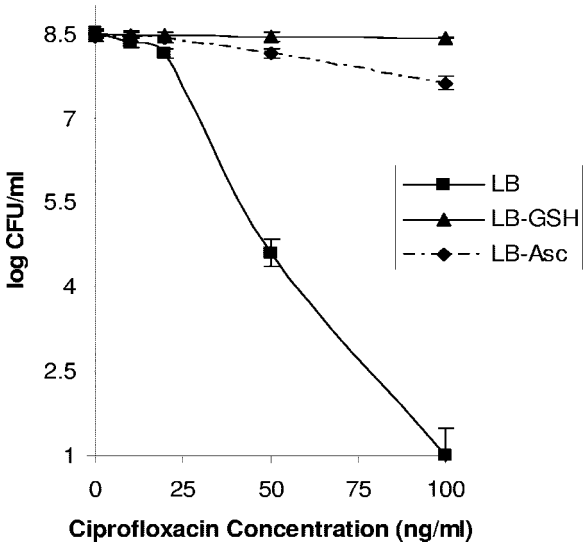

FIG. 2. Effects of glutathione and ascorbic acid on the viable counts of strain MG1655 in the presence of various concentrations of ciprofloxacin. Ascorbic acid (Asc) (10 mM) and glutathione (GSH) (10 mM) were added to LB. LB alone was used as a control. Since a zero value cannot be plotted on a log scale, a numerical value of 1 was used whenever no CFU was obtained.

(iii) Survival curves. Overnight cultures were diluted (1:100) in LB medium and grown at 37°C. Cells from mid-exponential phase of growth were serially diluted and plated in duplicate on LB agar containing various concentrations of antibiotic with or without antioxidants. The number of colonies formed was counted and recorded after the agar plates were incubated overnight at 37°C. The count (log CFU/ml) was used as an estimate of bacterial viability. However, when the difference between two strains or treatments was less than 1 log unit, we represented the data in terms of % survival. In these cases, counts corresponding to the number of bacteria on LB agar without any antibiotics were taken as 100% survival for the respective culture.

Statistical analysis. The data reported are the average values from minimum of three experiments. Differences between two bacterial strains or treatments were analyzed by Student's *t* test. A *P* value of 0.05 was used as the cutoff for statistical significance.

RESULTS

Effects of antioxidants on ciprofloxacin sensitivity of *E. coli* MG1655. The presence of 10 mM glutathione or ascorbic acid in the growth medium rendered MG1655 cells less susceptible to ciprofloxacin (Fig. 1) as seen by the antibiotic disk diffusion method. However, other antioxidants, such as histidine, mannitol, and sodium pyruvate, did not alter the ciprofloxacin sensitivity of MG1655 even at 25 mM concentration (data not shown). These results suggest that protection against the antibacterial action of ciprofloxacin is restricted to only a few antioxidants.

Quantification of antioxidant-mediated protection against ciprofloxacin sensitivity. Survival curves of strain MG1655 in the presence of various concentrations of ciprofloxacin were generated with and without the addition of a 10 mM concentration of either glutathione or ascorbic acid in the medium and were used to quantify the antioxidant-mediated protection. Our data that 50 and 100 ng/ml of ciprofloxacin decrease the number of CFU by more than 3 and 7 log units, respectively, indicated that MG1655 is highly sensitive to ciprofloxacin. Addition of glutathione to the medium gave complete protection up to 100 ng/ml of ciprofloxacin (Fig. 2). The survival was ~90% in the presence of glutathione even at a 150-ng/ml concentration of ciprofloxacin (data not shown). In comparison, ascorbic acid gave ~95% survival up to 25 ng/ml of ciprofloxacin and showed partial protection at higher concentrations (i.e., ~51% survival at 50 ng/ml and 15% survival at 100 ng/ml). These data showed that the protective effect against ciprofloxacin is more pronounced with glutathione than for ascorbic acid.

Further quantitative estimates of the protection offered by glutathione and ascorbic acid against ciprofloxacin were made by measuring the MIC of ciprofloxacin for strain MG1655 in the presence and absence of either antioxidant. The MICs increased threefold (from 30 ng/ml to 90 ng/ml) in the presence of ascorbic acid and 10-fold (from 30 ng/ml to 300 ng/ml) in the presence of glutathione compared to the controls (data not shown).

Effects of glutathione on strain MG1655 sensitivity to other fluoroquinolone and nonfluoroquinolone antibiotics. Glutathione-mediated protection to MG1655 cells against some other fluoroquinolones, viz., norfloxacin, ofloxacin, and gatifloxacin, and nonfluoroquinolone antibiotics, such as ampicillin, chloramphenicol, and tetracycline, was investigated by the disk diffusion method. The diameters of the zone of inhibition were determined as described in Materials and Methods. For all the fluoroquinolones, the diameters of the zone of inhibition in the presence of 10 mM glutathione were lower than those of the corresponding controls (Table 2), suggesting that glutathione interfered with a step that is common among fluoroquinolones to bring about their antibacterial action. However, for all the nonfluoroquinolone antibiotics, the diameters of the zone of inhibition in the presence of 10 mM glutathione were not statistically different from the values for the corresponding controls (Table 3), suggesting that glutathione-mediated protection is not a general phenomenon but is specific to fluoroquinolones.

TABLE 2. Effects of antioxidants on the diameters of the zone of inhibition produced by fluoroquinolone antibiotics

Antibiotic spotted on the disk	Concn (ng)	Diam of zone of inhibition (mm) (mean ± SD)	
		Control	+Glutathione (10 mM)
Gatifloxacin	2,000	12.51 ± 0.41	8.56 ± 0.48[a]
	400	10.82 ± 0.10	7.58 ± 0.16[a]
	200	7.99 ± 0.10	5.5 ± 0[a]
	40	5.5 ± 0	5.5 ± 0
Ofloxacin	2,000	15.84 ± 0.38	10.62 ± 0.34[a]
	400	11.68 ± 0.29	6.63 ± 0.42[a]
	200	6.34 ± 0.17	5.5 ± 0[a]
	40	5.5 ± 0	5.5 ± 0
Norfloxacin	2,000	8.21 ± 0.19	5.5 ± 0[a]
	400	5.5 ± 0	5.5 ± 0
	200	5.5 ± 0	5.5 ± 0
	40	5.5 ± 0	5.5 ± 0

[a] Numerical values that are significantly different from the values for the corresponding controls ($P = 0.05$).

Roles of *katE*, *katG*, and *ahpCF* in ciprofloxacin sensitivity of strain MG1655. H_2O_2 is an important molecule with a highly reactive nature and potential to react with various biomolecules inside the cell. We analyzed the effects of mutations in genes encoding enzymes that metabolize H_2O_2, i.e., *katE*, *katG*, and *ahpCF*, on the ciprofloxacin sensitivity of MG1655 cells. Mutating any of these genes independently did not alter the ciprofloxacin sensitivity of the MG1655 strain (data not shown). We examined all possible combinations of multiple mutations for these three genes. *katG ahpCF* double mutant (JI374) and *katE katG ahpCF* triple mutant (JI377) strains showed significant changes in ciprofloxacin sensitivity levels (Fig. 3). Except for these strains, all other combinations did not produce an observable effect on the ciprofloxacin sensitivity level (data not shown). Both JI374 and JI377 exhibited increased ciprofloxacin sensitivity than their parent strain MG1655 (e.g., the number of CFU had decreased by about 3 log units at 30-ng/ml ciprofloxacin concentration) with JI377 showing relatively higher sensitivity (Fig. 3). Both these mutants also showed a twofold decrease in the MIC for ciprofloxacin compared to their parent strain MG1655.

TABLE 3. Effects of antioxidants on the diameters of the zone of inhibition produced by nonfluoroquinolone antibiotics

Antibiotic spotted on the disk	Concn (ng)	Diam of zone of inhibition (mm) (mean ± SD)[a]	
		Control	+Glutathione (10 mM)
Ampicillin	2,000	8.54 ± 0.53	8.54 ± 0.42
	1,000	6.22 ± 0.14	6.08 ± 0.15
Chloramphenicol	2,000	14.26 ± 1.02	14.51 ± 0.17
	1,000	12.13 ± 0.14	12.38 ± 0.13
Tetracycline	2,000	15.02 ± 0.39	15.95 ± 0.36
	1,000	14.37 ± 0.93	14.20 ± 0.81

[a] These values are not statistically different from the values for the corresponding values ($P = 0.05$).

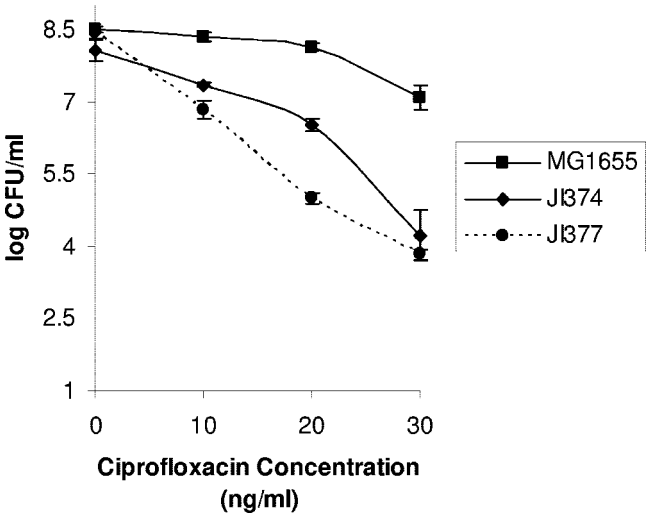

FIG. 3. Roles of *katE*, *katG*, and *ahpCF* in ciprofloxacin sensitivity of strain MG1655 in terms of its viable counts. The numbers of surviving bacteria of different strains at various concentrations of ciprofloxacin were determined as described in Materials and Methods. MG1655 was the control.

Effects of *sod* genes on ciprofloxacin sensitivity of strain MG1655. MG1655 transformants of plasmid pDT1.5, pFeSOD, or pSodC2.3 (having the *sodA*, *sodB*, or *sodC* gene, respectively) showed better survival at 10-ng/ml ciprofloxacin concentration; the increase in survival was ~17% with pDT1.5 and ~24% with pFeSOD or pSodC2.3 over that of MG1655 alone (Table 4). However, at 20- and 30-ng/ml ciprofloxacin concentrations, none of the plasmids decreased the ciprofloxacin susceptibility of MG1655. Superoxide dismutase knockout strains NJ01 (MG1655 *sodA*) and NJ02 (MG1655 *sodB*) did not differ significantly from their parent strain with respect to ciprofloxacin susceptibility (Table 4). However, the ciprofloxacin susceptibility of AS393 (AB1157 *sodC*) was found to be higher by ~12% and 22% compared to AB1157 at 5- and 10-ng/ml ciprofloxacin concentrations, respectively (Table 5). This suggested that O_2^- might have a role in the antibacterial action of ciprofloxacin, particularly at low concentrations.

DISCUSSION

The present study demonstrates the role of reactive oxygen species in the antibacterial action of fluoroquinolones. This statement is supported by the observation that known ROS scavengers, such as glutathione and ascorbic acid, gave protection to *E. coli* MG1655 against ciprofloxacin. Two other independent reports, i.e., stimulation of ROS production by ciprofloxacin in *E. coli*, *Enterococcus faecalis*, and *Staphylococcus aureus* (2) and the presence of increased levels of ROS in ciprofloxacin-sensitive microorganisms (6) substantiate our findings. The inability of other antioxidants, viz., histidine, a $^1O_2$ scavenger (16), mannitol, a known ·OH scavenger (13), and sodium pyruvate, to impart a protective phenotype suggests that only nonspecific scavengers having low redox potential could provide protection. The enhanced protective effect seen with glutathione in comparison to ascorbic acid might be due to the dependence of the protective ability on the redox potential of the given antioxi-

TABLE 4. Effects of knocking out and presence of multicopy *sod* genes on the ciprofloxacin sensitivity of *E. coli* MG1655

Ciprofloxacin concn (ng/ml)	% Survival of *E. coli* (mean ± SD)					
	MG1655	NJ01	NJ02	MG1655/pDT1.5	MG1655/pFeSOD	MG1655/pSodC2.3
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
10	67.3 ± 6.4	70.2 ± 7.8	61.5 ± 1.7	84.0 ± 2.6[a]	90.9 ± 1.9[a]	91.2 ± 10.0[a]
20	41.4 ± 1.3	36.3 ± 5.7	32.1 ± 10.1	35.8 ± 5.2	49.9 ± 7.9	39.8 ± 11.5
30	3.6 ± 1.6	7.3 ± 1.9	2.1 ± 1.0	4.9 ± 0.9	1.00 ± 0.1	2.7 ± 0.7

[a] Numerical values that are significantly different from the values for the corresponding controls ($P = 0.05$).

dant for one electron reduction pathway (22). Such selectivity of the antioxidants for e protection against oxidative stress has recently been reported (22). The inability of glutathione to protect cells against the antibacterial action of nonfluoroquinolone antibiotics demonstrates that the glutathione-mediated protection is not a general phenomenon but is specific to fluoroquinolones.

Our finding that glutathione gave protection against other fluoroquinolones as well implies that reactive oxygen species may have a similar role in the antibacterial action of all these fluoroquinolones. These observations are in contrast to those of Alba et al. (1) who, on the basis of unaltered in vitro bactericidal effect of norfloxacin by the presence of β-carotene, suggested that ROS do not have a role to play in the bactericidal effect of fluoroquinolones. However, it is important to note here that *E. coli* is a noncarotenogenic microorganism (4) without any reported transport protein for β-carotene. Hence, the observations of Alba et al. could be attributed to the inefficient transport of β-carotene across the *E. coli* cell membrane due to its lipophilic nature and the absence of specific transporters for it. On the other hand, compounds such as glutathione and ascorbic acid can readily cross the cell membrane because of their hydrophilic nature, low molecular weight, and presence of specific transporters for these antioxidants on the cell membrane (31, 39), which enables them to manifest their antioxidant action in the cytosol.

Our observation that intact *katG* or *ahpCF* is required by dividing *E. coli* cells for protection against the antibacterial action of ciprofloxacin confirms the involvement of oxidative stress in this phenotype, and the presence of wild-type *katE* alone is not sufficient to protect the dividing cells against the H_2O_2-mediated antibacterial action of ciprofloxacin. It is important to note here that both *ahpCF* and *katG* lie under the control of the *oxyR* regulon that plays an important role in overcoming the oxidative stress caused by H_2O_2 (11, 36). JI374 cells that have severely compromised H_2O_2 scavenging function (35) show increased ciprofloxacin sensitivity. Further increased ciprofloxacin sensitivity of JI377 demonstrates that the complete elimination of the H_2O_2 scavenging function in *E. coli* makes cells hypersensitive to ciprofloxacin. Mutations of *kat* genes in combination with *ahpCF* could alter the ciprofloxacin sensitivity of the cells, implying that *ahpCF* has an equally important role in scavenging of endogenous H_2O_2, which is in agreement with the findings of Seaver and Imlay (35).

The presence of the multiple H_2O_2 scavenging activities ensures that the remaining functional H_2O_2 metabolizing activities protect the cells from ciprofloxacin when *ahpCF* alone is knocked out or when one or both of the catalases are mutated in *E. coli*. An unaltered ciprofloxacin sensitivity of a strain carrying mutations in *katE* and *ahpCF* is in line with the findings of Seaver and Imlay (35) that mutations in *katE* and *ahpCF* together do not hamper the H_2O_2 detoxification ability of the cells.

Superoxide dismutases present in the cell are sufficient to take care of the significant increase of superoxide anions inside the cell (15, 36) that are generated as the by-products of normal metabolic processes. The modulation of ciprofloxacin sensitivity either by the multiple copies of *sod* genes or by *sod* mutants shows that superoxide anions also have a role in the antibacterial action of this antibiotic (Table 2 and 3). All the *sod* transformants showed better survival in comparison to strain MG1655 (Table 2) at low ciprofloxacin concentration (10 ng/ml). The unaltered ciprofloxacin sensitivity of *sodA* and *sodB* mutant derivatives (NJ01 and NJ02, respectively) may be due to the redundancy of cytosolic superoxide dismutase activities where the absence of one activity can be compensated by the presence of the other one. On the other hand, the slightly increased ciprofloxacin sensitivity of a *sodC* mutant derivative (AS393) at a low antibiotic concentration shows the distinctive importance of *sodC*, as it encodes the sole superoxide dismutase activity present in the periplasm of *E. coli*. However, the concentration dependence of this effect needs further characterization.

On the basis of our results, we conclude that the antibacterial action of fluoroquinolones involves reactive oxygen species, such as superoxide anions and hydrogen peroxide. However, the exact mechanism of this phenomenon is yet to be worked out. We have shown that the presence of antioxidants rescues bacteria against the antibacterial action of fluoroquinolones. These observations are of significance, as fluoroquinolones are important antibiotics with immense therapeutic value, and further investigations surrounding the intake of antioxidants on the effects of fluoroquinolones for the treatment of infections caused by *E. coli* are warranted in the future.

TABLE 5. Effect of knocking out *sodC* on the ciprofloxacin sensitivity of *E. coli* AB1157

Ciprofloxacin concn (ng/ml)	% Survival of *E. coli* (mean ± SD)	
	AB1157	AS393
0	100 ± 0	100 ± 0
5	83.3 ± 1.2	71.7 ± 2.7[a]
10	51.1 ± 8.1	28.7 ± 2.3[a]
15	8.6 ± 1.40	4.0 ± 1.2
20	0.05 ± 0.05	ND[b]

[a] Numerical values that are significantly different from the values for the corresponding controls ($P = 0.05$).
[b] ND, not detected.

ACKNOWLEDGMENTS

We are grateful to James Imlay for providing the strains and plasmids related to this study. We also thank A. V. S. S. N. Rao, D. Rath, and S. Uppal for valuable discussion and critical reading of the manuscript.

REFERENCES

1. **Alba, M. A., R. R. Sanchez, G. P. Cervantes, F. B. Moreno, R. F. Paz, and E. G. Jimenez.** 2000. Antimutagenesis of β-carotene to mutations induced by quinolone on *Salmonella typhimurium*. Arch. Med. Res. **31:**156–161.
2. **Albesa, I., M. C. Becerra, P. C. Battan, and P. L. Paez.** 2004. Oxidative stress involved in the antibacterial action of different antibiotics. Biochem. Biophys. Res. Commun. **317:**605–609.
3. **Appelbaum, P. C., and P. A. Hunter.** 2000. The fluoroquinolone antibacterials: past, present and future perspectives. Int. J. Antimicrob. Agents **16:**5–15.
4. **Armstrong, G. A.** 1997. Genetics of eubacterial carotenoid biosynthesis: a colourful tale. Annu. Rev. Microbiol. **51:**629–659.
5. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standard single disk method. Am. J. Clin. Pathol. **45:**493–496.
6. **Becerra, M. C., and I. Albesa.** 2002. Oxidative stress induced by ciprofloxacin in *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **297:**1003–1007.
7. **Benov, L. T., and I. Fridovich.** 1994. *Escherichia coli* expresses a copper and zinc containing superoxide dismutase. J. Biol. Chem. **269:**25310–25313.
8. **Berlett, B. S., and E. R. Stadtman.** 1997. Protein oxidation in aging, disease and oxidative stress. J. Biol. Chem. **272:**20313–20316.
9. **Brummett, R., and K. Fox.** 1989. Aminoglycoside-induced hearing loss in humans. Antimicrob. Agents Chemother. **33:**797–800.
10. **Butler, J., and B. M. Hoey.** 1993. Redox cycling drugs and DNA damage, p. 243–273. *In* B. Halliwell and O. I. Aruoma (ed.), DNA and free radicals. Ellis Horwood Ltd., West Sussex, England.
11. **Cabiscol, E., J. Tamarit, and J. Ros.** 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. **3:**3–8.
12. **Chen, C. R., M. Malik, M. Snyder, and K. Drlica.** 1996. DNA gyrase and topoisomerase IV on the bacterial chromosome: quinolone induced DNA cleavage. J. Mol. Biol. **258:**627–637.
13. **Desesso, J. M., A. R. Scialli, and G. C. Goeringer.** 1994. D-mannitol, a specific hydroxyl free radical scavenger, reduces the developmental toxicity of hydroxyurea in rabbits. Teratology **49:**248–259.
14. **Drlica, K., and X. Zhao.** 1997. DNA gyrase, topoisomerase IV and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61:**377–392.
15. **Gort, A. S., and J. A. Imlay.** 1998. Balance between endogenous superoxide stress and antioxidant defenses. J. Bacteriol. **180:**1402–1410.
16. **Hartman, P. E., Z. Hartman, and K. T. Ault.** 1990. Scavenging of singlet molecular oxygen by imidazole compounds: high and sustained activities of carboxy terminal histidine dipeptides and exceptional activity of imidazole-4-acetic acid. Photochem. Photobiol. **51:**59–66.
17. **Hawkey, P. M.** 2003. Mechanism of quinolone action and microbial response. J. Antimicrob. Chemother. **51**(Suppl. S1)**:**29–35.
18. **Imlay, J. A.** 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. **57:**395–418.
19. **Imlay, J. A., and S. Linn.** 1987. Mutagenesis and stress response induced in *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **169:**2967–2976.
20. **Imlay, K. R. C., and J. A. Imlay.** 1996. Cloning and analysis of *sodC*, encoding the copper-zinc superoxide dismutase of *Escherichia coli*. J. Bacteriol. **178:**2564–2571.
21. **Keele, B. B., Jr., J. M. McCord, and I. Fridovich.** 1970. Superoxide dismutase from *Escherichia coli* B: a new manganese-containing enzyme. J. Biol. Chem. **245:**6176–6181.
22. **Koziol, S., M. Zagulski, T. Bilinski, and G. Bartosz.** 2005. Antioxidants protect the yeast *Saccharomyces cerevisiae* against hypertonic stress. Free Radic. Res. **39:**365–371.
23. **Loewen, P. C., J. Switala, and L. Triggs-Raine.** 1985. Catalase HPI and HPII in *Escherichia coli* are induced independently. Arch. Biochem. Biophys. **243:**144–149.
24. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244:**6049–6055.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mingeot-Leclercq, M. P., and P. M. Tulkens.** 1999. Aminoglycosides: nephrotoxicity. Antimicrob. Agents Chemother. **43:**1003–1012.
27. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. Approved standard M7–A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
28. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial susceptibility testing. Eighth informational supplement M100S12. National Committee for Clinical Laboratory Standards, Villanova, Pa.
29. **Oliphant, C. M., and G. M. Green.** 2002. Quinolones: a comprehensive review. Am. Family Physician **65:**455–464.
30. **Park, H. R., T. H. Kim, and K. M. Bark.** 2002. Physicochemical properties of quinolone antibiotics in various environments. Eur. J. Med. Chem. **37:**443–460.
31. **Parry, J., and D. P. Clark.** 2002. Identification of a *cysB* regulated gene involved in glutathione transport in *Escherichia coli*. FEMS Microbiol. Lett. **209:**81–85.
32. **Pomposiello, P. J., and B. Demple.** 2000. Oxidative stress, p. 526–532. *In* J. Lederberg et al. (ed.), Encyclopedia of microbiology, 2nd ed., vol. 3. Academic Press, San Diego, Calif.
33. **Sakamoto, H., and D. Touati.** 1984. Cloning of iron superoxide dismutase gene (*sodB*) in *Escherichia coli* K-12. J. Bacteriol. **159:**418–420.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Seaver, L. C., and J. A. Imlay.** 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. J. Bacteriol. **183:**7173–7181.
36. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188–194.
37. **Umezawa, N., K. Arakane, A. Ryu, S. Mashiko, M. Hirobe, and T. Nagano.** 1997. Participation of reactive oxygen species in phototoxicity induced by quinolone antibacterial agents. Arch. Biochem. Biophys. **342:**275–281.
38. **Yost, F. J., and I. Fridovich.** 1973. An iron containing superoxide dismutase from *Escherichia coli*. J. Biol. Chem. **248:**4905–4908.
39. **Zhang, Z., M. Aboulwafa, M. H. Smith, and M. H. Saier, Jr.** 2003. The ascorbate transporter of *Escherichia coli*. J. Bacteriol. **185:**2243–2250.