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Inactivation of *Escherichia coli* by Nanoparticulate Zerovalent Iron and Ferrous Ion▽†

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The mechanism of *Escherichia coli* inactivation by nanoparticulate zerovalent iron (nZVI) and Fe(II) was investigated using reactive oxygen species (ROS) quenchers and probes, an oxidative stress assay, and microscopic observations. Disruption of cell membrane integrity and respiratory activity was observed under deaerated conditions [more disruption by nZVI than Fe(II)], and $^{\bullet}$OH or Fe(IV) appears to play a role.

Several recent studies have reported on the antimicrobial activity of nanoparticulate zerovalent iron (nZVI) (1, 2, 8, 11). We previously found that nZVI exhibited a stronger antimicrobial activity than other iron-based nanoparticles and that the inactivation of *Escherichia coli* by nZVI was greater under deaerated conditions than under air-saturated conditions (8). The objective of this study was to gain insight into the mechanism of inactivation of *E. coli* by nZVI and Fe(II) by studying oxidants generated inside and outside cells as well as damage to the bacterial membrane.

The methods for synthesis of nZVI and measurement of Fe(II) concentration were the same as in our previous study (8). The production of intracellular $^{\bullet}$OH was determined by a fluorescence assay with 3′-(*p*-aminophenyl) fluorescein (APF) and 3′-(*p*-hydroxyphenyl) fluorescein (HPF) (Invitrogen) (12, 13). Oxidative stress caused by H_2O_2 and $^{\bullet}O_2^-$ was examined using mutant reporter strains of *E. coli* (3, 6, 10). The *Bac*Light Live/Dead bacterial viability and *Bac*Light RedoxSensor CTC (5-cyano-2,3-ditolyl tetrazolium chloride) vitality kits were used for investigating decreases in cell membrane integrity and respiratory activity, respectively (4, 9, 14). The detailed experimental methods are described in the supplemental material.

Consistent with our previous study (8), the inactivation of all strains of *E. coli* (concentration of *E. coli* at time zero = 2×10^7 CFU/ml) was enhanced under deaerated conditions, and nZVI caused more inactivation than Fe(II) (data not shown; $P = 0.005$). The rapid oxidation of nZVI and Fe(II) in the presence of oxygen may partly explain why no significant inactivation of *E. coli* was observed under air-saturated conditions. The half-lives of nZVI and Fe(II) at pH 8 are reported to be 35 and 5.4 s, respectively, assuming a constant oxygen concentration ($[O_2]_0 = 0.25$ mM) (5, 7). Although the oxidation of nZVI and Fe(II) produces strong oxidants, if these oxidants are produced in the bulk phase, they may not interact directly with *E. coli*. We found that the addition of oxidant scavengers had no significant effect on *E. coli* inactivation, confirming that

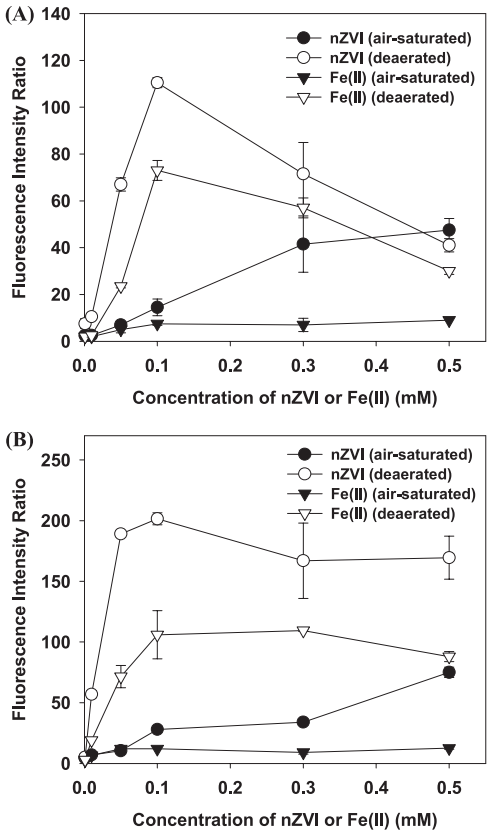

FIG. 1. Detection of intracellular ROS as a function of the concentration of nZVI and Fe(II) using APF (A) and HPF (B).

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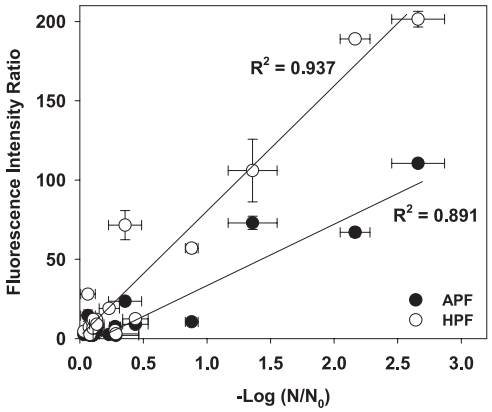

FIG. 2. Correlation between inactivation of *E. coli* and intracellular ROS detected with APF and HPF [concentrations of nZVI and Fe(II) greater than 0.1 mM are excluded from this figure due to concerns about reagent limitations].

the generation of oxidants in the bulk phase did not contribute to inactivation (data not shown).

Figure 1 shows the fluorescent intensity ratio, as measured by APF and HPF, resulting from reactive oxygen species (ROS) produced inside or on the surface of *E. coli*. Higher signal intensities were measured under deaerated conditions than under air-saturated conditions ($P = 0.000026$), indicating that ROS were produced via reactions of nZVI or Fe(II) with H_2O_2 or other species in the cells. Although nZVI does not produce more oxidants than Fe(II) at neutral pH values in the bulk solution (5), nZVI generated more intracellular ROS than Fe(II) ($P = 0.0001$). One explanation for the higher generation of intracellular oxidants by nZVI than by Fe(II) is that nZVI has an affinity with the *E. coli* cells, facilitating the delivery of Fe(II) to the cells. The decreased signal intensity at concentrations above 0.1 mM nZVI and Fe(II) under deaerated conditions occurred likely because the amount of the probe compounds inside the cells was limited, and further oxidation of the probe compounds by nZVI and Fe(II) may have produced secondary products with no significant fluorescence activity. This explanation was supported by the observation that the intensity of the fluorescence signal generated by HOCl was higher when more APF was employed (see Text S1 in the supplemental material). The results of ROS measurements using APF and HPF correlated well with *E. coli* inactivation by nZVI and Fe(II), as shown in Fig. 2, suggesting that oxidant generation was either directly involved with or closely related to processes that cause inactivation of *E. coli* by nZVI and Fe(II).

nZVI and Fe(II) can generate oxidants such as $^{\bullet}OH$, Fe(IV), $^{\bullet}O_2^-$, and H_2O_2 if oxygen is present. However, it is not clear which reactive oxidants are mainly responsible for *E. coli* inactivation, especially under deaerated conditions. The effects of $^{\bullet}O_2^-$, and H_2O_2, which may be produced inside the cells and are less reactive oxidants than $^{\bullet}OH$ and Fe(IV), were examined by an oxidative-stress assay with *E. coli* reporter mutants. As shown in Fig. S2 in the supplemental material, no response from either strain was observed under either condition, regardless of air saturation, suggesting that the bactericidal activities of nZVI and Fe(II) are not related to the direct effects of intracellular $^{\bullet}O_2^-$ and H_2O_2.

In Fig. 3A, microscope images are shown for *Bac*Light Live/Dead-stained *E. coli* bacteria that were treated with 0.05 mM nZVI and Fe(II) under air-saturated and deaerated conditions. Figure 3B shows the dead-cell ratios obtained by setting the value for total cells to 100%. A larger decrease in cell membrane integrity induced by nZVI and Fe(II) was observed

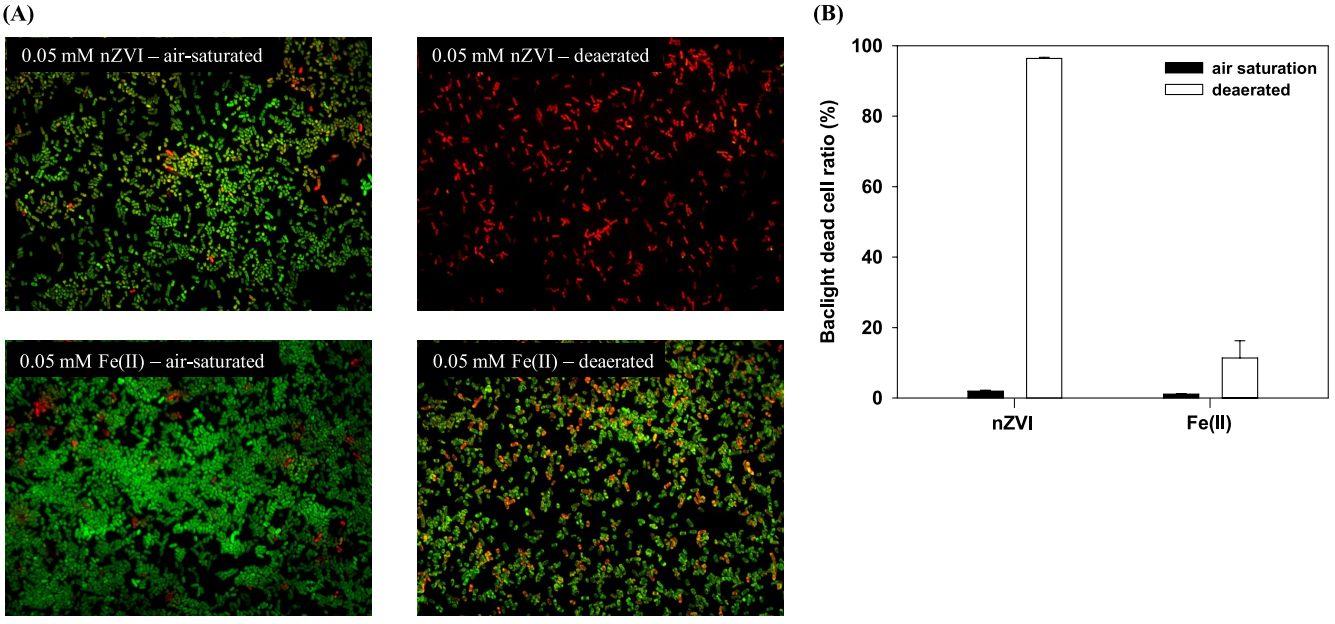

FIG. 3. Microscopic images of *Bac*Light Live/Dead staining (A) and *Bac*Light-measured dead-cell ratios (B) for *E. coli* inactivated by nZVI and Fe(II) {inactivation efficiencies, 0.1 log [0.05 mM nZVI—air saturated], 2.2 log [0.05 mM nZVI—deaerated], 0.1 log [0.05 Mm Fe(II)—air saturated], and 0.4 log [0.05 mM Fe(II)—deaerated]}.

under deaerated conditions. The enhanced production of intracellular oxidants under deaerated conditions appears to be involved in more-serious damage to the cell membrane integrity by oxidizing the cellular components associated with the cell membrane. Under air-saturated conditions, *E. coli* treated with nZVI and Fe(II) was mostly observed as green, indicating nonpermeable cell membranes. *E. coli* was also stained with CTC–DAPI (4′,6-diamidino-2-phenylindole) to investigate the effect of nZVI and Fe(II) on respiratory activity (see Fig. S3 in the supplemental material). Nonrespiring-cell ratios were much higher in samples treated under deaerated conditions than in those treated under air-saturated conditions. nZVI resulted in slightly more damage to the respiratory activity of *E. coli* than Fe(II).

From these results, we conclude that the bactericidal activities of nZVI and Fe(II) involve the generation of intracellular oxidants, $^{\bullet}$OH or Fe(IV), produced by the reaction with hydrogen peroxide or other species. Serious damage to cell membrane integrity and respiratory activity was observed. The effective inactivation of *E. coli* under deaerated conditions indicates that nZVI and Fe(II) have potential for use as antimicrobials. nZVI has several advantages, such as low cost, easy preparation, and high reactivity, compared to other metal nanoparticles. However, its low-level activity under air-saturated conditions could limit its widespread use in environmental applications.

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