Fusidic Acid-Resistant Mutants of *Salmonella enterica* Serovar Typhimurium Have Low Levels of Heme and a Reduced Rate of Respiration and Are Sensitive to Oxidative Stress

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Mutations in the translation elongation factor G (EF-G) make *Salmonella enterica* serovar Typhimurium resistant to the antibiotic fusidic acid. Fus* mutants are hypersensitive to oxidative stress and rapidly lose viability in the presence of hydrogen peroxide. We show that this phenotype is associated with reduced activity of two catalase enzymes, HPI (a bifunctional catalase-hydroperoxidase) and HPII (a monofunctional catalase). These catalases require the iron-binding cofactor heme for their activity. Fus* mutants have a reduced rate of transcription of *hemA*, a gene whose product catalyzes the first committed step in heme biosynthesis. Hypersensitivity of Fus* mutants to hydrogen peroxide is abolished by the presence of δ-aminolevulinic acid, the precursor of heme synthesis, in the growth media and by the addition of glutamate or glutamine, amino acids required for the first step in heme biosynthesis. Fluorescence measurements show that the level of heme in a Fus* mutant is significantly lower than it is in the wild type. Heme is also an essential cofactor of cytochromes in the electron transport chain of respiration. We found that the rate of respiration is reduced significantly in Fus* mutants. Sequestration of divalent iron in the growth media decreases the sensitivity of Fus* mutants to oxidative stress. Taken together, these results suggest that Fus* mutants are hypersensitive to oxidative stress because their low levels of heme reduce both catalase activity and respiration capacity. The sensitivity of Fus* mutants to oxidative stress could be associated with loss of viability due to iron-mediated DNA damage in the presence of hydrogen peroxide. We argue that understanding the specific nature of antibiotic resistance fitness costs in different environments may be a generally useful approach in identifying physiological processes that could serve as novel targets for antimicrobial agents.

Translation elongation factor G (EF-G) is a GTP-binding protein that catalyzes the translocation of peptidyl-tRNA on the ribosome from the A site to the P site (30). After GTP hydrolysis and translocation, EF-G · GDP leaves the ribosome and is regenerated by the spontaneous exchange of GDP for GTP off the ribosome. Fusidic acid is an antibiotic that binds to a complex of EF-G and the ribosome and blocks the release of EF-G · GDP from the ribosome (5, 22). This blockage inhibits further protein synthesis.

Resistance to fusidic acid in *Salmonella enterica* serovar Typhimurium is caused by mutations in *fusA*, the gene encoding EF-G (21). Similar mutations in *fusA* are a common cause of fusidic acid resistance in clinical isolates of fusidic acid-resistant *Staphylococcus aureus* (27). Fus* mutant forms of EF-G, including that encoded by *fusA* studied here, have a reduced rate of nucleotide exchange, and this causes a reduced rate of protein synthesis and cell growth (26). Fus* mutants also have reduced levels of the transcription regulator molecule ppGpp (26). Fus* mutant EF-G apparently interferes with the RelA-dependent synthesis of ppGpp in the ribosomal A site (26). In addition, Fus* mutants have reduced levels of the stress-related sigma factor RpoS (25). The reduced levels of RpoS may also be a consequence of the reduced levels of ppGpp (15, 23) in Fus* mutants. Both ppGpp and RpoS are global transcriptional regulators of gene expression, and consequently, Fus* mutants have phenotypes in addition to translation-rate-related slow growth. These phenotypes include large cell size at division (26) and sensitivity to oxidative stress in vivo (25).

Here we identify reduced production of heme as an important contributing factor to the hypersensitivity of Fus* mutants to oxidative stress. We also show that this has an additional consequence of reducing the rate of respiration in Fus* mutants.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. All strains are derivatives of serovar Typhimurium LT2 (designated as the wild type). This LT2 strain (TT10000) is originally from the lab of John Roth. Transductions were made using P22 HT. Strains were grown at 37°C in Luria-Bertani broth (LB) or, where indicated, in minimal M9 medium or nutrient broth (NB) medium (8). The solid medium was Luria agar (LB supplemented with 1.5% agar; Oxoid, Basingstoke, England).

Antibiotics. Fusidic acid sodium salt was a gift from Leo Pharma, Ballerup, Denmark, and had a final concentration of 800 μg/ml when used in the presence of 1 mM EDTA in the solid medium. Tetracycline (Sigma-Aldrich, Ballerup, Denmark) was used in media at a final concentration of 15 μg/ml.

Hydrogen peroxide sensitivity assays. Bacterial cultures grown in LB to optical densities at 600 nm of 0.2 to 0.4 were treated with 2.5 M hydrogen peroxide (Merck, VWR International, Stockholm, Sweden). To determine viability, aliquots were taken at indicated time points (15, 30, and 45 min), diluted in 0.9% NaCl, and plated onto LB and LB-tetracycline plates. When indicated, cells were
exposed to 2,2'-dipyridil (Sigma-Aldrich) for 15 min prior to treatment with hydrogen peroxide. For δ-aminolevulinic acid (ALA; Sigma Aldrich) supplementation experiments, cells were first grown overnight at 37°C in NB medium with and without 100 μg/ml ALA and diluted 100-fold in fresh medium of the same composition. Cells were then grown in NB medium to OD600 of 0.2 to 0.4 with and without 50 μg of ALA/ml (8) before being exposed to 2.5 mM hydrogen peroxide. Viability was assayed as described above.

**Catalase activity.** Relative catalase activity was quantified by a colorimetric assay based on the use of dicarboxidine ([**γ**]-[**γ**]-diamino-3,3'-biphényl- ylenedioxy) dibutyric acid; Sigma-Aldrich), a sensitive substrate for the detection of catalase activity (29). Dicarboxidine is converted into a colored product in a reaction catalyzed by the activity of lactoperoxidase (EC 1.11.1.7) (170 U/mg of protein; Fluka/Sigma-Aldrich); the amount of color developed is directly proportional to the amount of H2O2 present in the medium. Immediately before each experiment, a solution of 50 μg of lactoperoxidase/ml was mixed with an equal volume of 1 mM dicarboxidine. Relative catalase activity in bacterial cultures was assayed according to a previously established method (36) with minor modifications as described here. Cultures were grown overnight in M9 glucose minimal medium, washed, and then resuspended in prewarmed 37°C M9 medium with 0.2% glycerol as a carbon source (we found that the presence of glucose in the medium interfered with the sensitivity of the reaction). The concentration of cells at the start of the assay was 2 × 10^9 cells/ml. To begin the reaction, H2O2 was added to the bacterial culture to a final concentration of 100 μM. The culture was incubated at 37°C with agitation, and 1-mL samples were taken immediately after the addition of H2O2 and at regular time intervals up to 45 min. Each sample was immediately added to a 200-μl aliquot of the dicarboxidine-lactoperoxidase mixture at room temperature. Within 1 min a stable color developed. The absorbance of the samples was measured at 450 nm. The decrease in the amount of color developed as a function of time is proportional to the amount of H2O2 degraded by catalase and is thus inversely proportional to catalase activity.

**β-Galactosidase assays.** Cultures were grown overnight in LB at 37°C and diluted 100-fold in fresh LB medium. Samples were collected from exponentially growing cells (OD600, 0.3) and analyzed as described previously (25). OD600 and OD540 were measured for 16 h at intervals of 5 min in a Bioscreen C machine (Belach, Bioteknik, Stockholm, Sweden). The bioreactor was filled with 10 liters of LB supplemented with 2% glucose and 3 ml of polypropylene glycol (anti-foaming agent; BDH, Poole, United Kingdom). The temperature and pH were adjusted to 37°C and 7, respectively. The bioreactor was inoculated with an appropriate amount of culture to achieve an OD600 of 0.08. The culture used had been grown overnight in a shaking flask of LB supplemented with 0.25% glucose. During the respiration experiment, the OD600 was recorded every 20 min, while dissolved oxygen, carbon dioxide content, and pH were registered continuously. The respiration (oxygen consumption) of the cell population was expressed as the specific respiration rate (d) during one doubling time of the population (the time for the culture’s OD600 to increase from 0.2 to 0.4). d is measured in micromoles of O2 per liter per min per OD600. The overall respiration rate of the population, qX (where X is the cell concentration), is in equilibrium with the oxygen transfer rate (OTR) of the bioreactor, i.e., OTR = qX. The bioreactor OTR was calculated from the formula OTR = kLa(c - c*), where kLa is the volumetric mass transfer coefficient, c* is the saturated oxygen concentration, and c is the measured oxygen concentration at a certain time point. Both kLa and c* were determined prior to the experiment by the sulfite oxidation method (2) and by use of the same value parameters (volume, temperature, aeration, stirrer speed, and pressure) applied to the actual culture. During the experiment, the dissolved oxygen concentration was registered continuously and read when the cell concentration (X) corresponded to an OD600 of 0.4. The specific respiration rate was determined by calculating OTR/X.

## RESULTS

**Fus** mutants reduce HPI and HPII activity. We have previously shown that *Fus* mutants have reduced catalase activity relative to the wild type (25). The relative catalase activity levels associated with the wild type and the strains with the mutations *fusA1-15* and *fusA1* are 1.00, 0.45, and 0.35, respectively. We asked whether the reduced catalase activity in *Fus* mutant JB2110 (*fusA1-15*) is associated with the HPI or HPII catalases. Isofogenic wild-type and *fusA1-15* strains (Table 1) were constructed carrying functional knockouts (*katG::Tn10 and katE::Tn10*) affecting HPI and HPII, respectively. Catalase activity was measured for each strain as the rate of clearance of H2O2 from the growth medium (see Materials and Methods). In strains carrying either one of the catalase genes intact, the presence of the *fusA1-15* mutation reduced the catalase activity to less than half the value found in the equivalent *fusA1* strain (Table 2). This showed that the *fusA1-15* mutation reduced both HPI and HPII catalase activities. We hypothesized that a

### Table 1. Strains used and their genotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>MM14...</td>
<td>Wild-type <em>S. enterica</em> serovar Typhimurium LT2</td>
</tr>
<tr>
<td>MM16...</td>
<td>fusA1 zhb-730::pRR10 (Kan')</td>
</tr>
<tr>
<td>JB2110...</td>
<td>recA1 zhb-730::Tn10 hemA::lacZ (Kan')</td>
</tr>
<tr>
<td>MM122...</td>
<td>hemA::lacZ (Kan')</td>
</tr>
<tr>
<td>MM123...</td>
<td>fusA1 zhb-730::Tn10 hemA::lacZ (Kan')</td>
</tr>
<tr>
<td>MM124...</td>
<td>relA21::Tn10 hemA::lacZ (Kan')</td>
</tr>
<tr>
<td>MM125...</td>
<td>relA21::Tn10 hemA::lacZ (Kan')</td>
</tr>
<tr>
<td>MM126...</td>
<td>hemA::lacZ (Kan')</td>
</tr>
<tr>
<td>MM46...</td>
<td>KatG::pRR10 (Amp')</td>
</tr>
<tr>
<td>MM74...</td>
<td>fusA1 zhb-730::Tn10 KatG::pRR10 (Amp')</td>
</tr>
<tr>
<td>MM106...</td>
<td>katE::Tn10</td>
</tr>
<tr>
<td>MM107...</td>
<td>fusA1 katE::Tn10</td>
</tr>
</tbody>
</table>

All strains are derived from *Salmonella* serovar Typhimurium LT2.
possible cause of the reduced catalase activity might be the molecule heme, which is a cofactor for both HPI and HPII (3).

**Fus**<sup>+</sup> mutants have reduced expression of *hemA*. We wanted to determine whether Fus<sup>+</sup> mutants were defective in the production of heme. The first committed step in the biosynthesis of heme is the synthesis of the heme precursor ALA. In *Salmonella*, glutamyl-tRNA reductase, encoded by *hemA*, converts a small fraction of the cells' charged glutamyl-tRNA<sub>Glu</sub> to glutamate-semialdehyde. This in turn is converted by glutamate-1-semialdehyde aminotransferase, encoded by *hemL*, to ALA. We measured expression of *hemA* in the wild type and in two Fus<sup>+</sup> mutants (*fusA1* and *fusA1-15*) by use of a *hemA-lacZ* operon fusion from Tom Elliott (8). Each Fus<sup>+</sup> mutant is associated with a decreased level of transcription of the fusion relative to that of the wild type. In strain MM123, the strain with *fusA1*, the level of *hemA-lacZ* transcription is only 64% of the wild-type level, while in MM124 (*fusA1-15*), it is 85% (Table 3). We conclude that Fus<sup>+</sup> mutants have reduced transcription of *hemA*.

To determine whether the decreased transcription of *hemA* was due to low levels of ppGpp in the Fus<sup>+</sup> mutants (26), we measured the expression of the *hemA-lacZ* fusion in mutants with low ppGpp levels due to mutations in *relA* (31) and *spoT*. The *relA* mutation causes a small reduction in *hemA-lacZ* expression (Table 3). However, while the *fusA1* and *relA21::Tn10* strains have similar basal levels of ppGpp, 5 and 7 pmol/OD<sub>640</sub> unit, respectively (26), they have very different levels of *hemA-lacZ* expression, i.e., 64 and 89% of wild-type levels, respectively. Also, whereas the *fusA1-15* and *relA1 spoT::Tn10* strains have very different basal levels of ppGpp, 20 and <1 pmol/OD<sub>640</sub> unit, respectively (25, 26), they have similar levels of *hemA-lacZ* expression, i.e., 85 and 86% of wild-type levels, respectively. We conclude that the decreased level of transcription of *hemA* in Fus<sup>+</sup> mutants is not fully explained by their decreased levels of ppGpp.

**ALA reverses Fus**<sup>+</sup> sensitivity to oxidative stress. We wanted to determine whether the increased sensitivity of Fus<sup>+</sup> mutants to oxidative stress caused by exposure to hydrogen peroxide (25) could be attributed to a reduced flow through the heme biosynthesis pathway. A recent study has shown that a *Salmonella* *hemA* mutant is very susceptible to hydrogen peroxide (11). We measured the viability of the wild-type strain MM14 and the *fusA1-15* strain JB2110 after exposure to hydrogen peroxide when grown with or without the precursor of heme synthesis, ALA. The wild-type bacteria are relatively insensitive to 30 min of exposure to hydrogen peroxide, and, as expected, the presence of ALA did not significantly affect their survival (Fig. 1). In contrast, the *fusA1-15* strain (JB2110) was sensitive to this level of hydrogen peroxide, with less than 20% surviving 15 min of exposure. However, the *fusA1-15* strain grown with ALA survived as well as the wild type (Fig. 1). The *fusA1* strain MM142 was as sensitive in this assay as the strain carrying *fusA1* (data not shown), and its sensitivity was also reversed by growth in the presence of ALA. The effect of ALA in reversing sensitivity to hydrogen peroxide supports the hypothesis that this sensitivity is due to a low level of heme in the Fus<sup>+</sup> mutant.

**Media dependence of Fus**<sup>+</sup> sensitivity to hydrogen peroxide. In the ALA assay described above, cells were grown in a rich LB medium. We noted that in LB, the extreme sensitivity to H<sub>2</sub>O<sub>2</sub> of strains carrying *fusA1*, previously noted in M9 medium (25), is partly ameliorated. We asked which constituent of LB medium was responsible for this reversal of sensitivity. We made these assays as pairwise competitions between the wild type and Fus<sup>+</sup> mutants because this is a very sensitive way to measure relative improvements in survival or growth rate. Note that no ALA was added in these assays. We found that the addition of Casamino Acids to M9 glucose minimal medium ameliorated the *fusA1* mutant’s sensitivity to H<sub>2</sub>O<sub>2</sub> (Table 4). The pathway of heme biosynthesis begins with the conversion of glutamyl-charged tRNA<sub>Glu</sub> by the product of the

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**TABLE 2. Impact of fusA1-15 genotype on catalase activity in Serovar Typhimurium strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Active catalase(s)</th>
<th>Catalase activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM14</td>
<td>Wild type</td>
<td>HPI + HPII</td>
<td>100</td>
</tr>
<tr>
<td>JB2110</td>
<td>fusA1-15</td>
<td>HPI + HPII</td>
<td>45</td>
</tr>
<tr>
<td>MM106</td>
<td>katE::Tn10</td>
<td>HPI</td>
<td>43</td>
</tr>
<tr>
<td>MM107</td>
<td>katE::Tn10 fusA1-15</td>
<td>HPI</td>
<td>12</td>
</tr>
<tr>
<td>MM46</td>
<td>katG::pRR10</td>
<td>HPII</td>
<td>73</td>
</tr>
<tr>
<td>MM74</td>
<td>katG::pRR fusA1-15</td>
<td>HPII</td>
<td>31</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are an average of at least 10 independent assays with a standard error of 5%.

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**TABLE 3. Gene expression from a hemA-lacZ operon fusion in wild-type, Fus<sup>+</sup>, and relA/spoT mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>β-Galactosidase activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM14</td>
<td>Wild type</td>
<td>98.0</td>
<td>1.1</td>
</tr>
<tr>
<td>MM124</td>
<td>fusA1-15</td>
<td>83.6</td>
<td>1.1</td>
</tr>
<tr>
<td>MM123</td>
<td>fusA1</td>
<td>62.5</td>
<td>2.6</td>
</tr>
<tr>
<td>MM125</td>
<td>relA21::Tn10</td>
<td>87.4</td>
<td>0.8</td>
</tr>
<tr>
<td>MM126</td>
<td>relA1 spoT::Tn10</td>
<td>83.9</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relevant genotype: full genotypes are listed in Table 1.

<sup>b</sup> β-Galactosidase activities are given in Miller units (10<sup>3</sup>) and are the average of results of at least four independent assays.

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FIG. 1. Effect of H<sub>2</sub>O<sub>2</sub> on bacterial viability of the wild type and a Fus<sup>+</sup> mutant as a function of the presence or absence of ALA, a precursor in the heme biosynthesis pathway. The standard error of these measurements is ±10% for the wild type and ±4% for the *fusA1-15* mutant.
with H2O2 and measured the relative synthesis pathway. Accordingly, we added several individual fusA1 strain to H2O2 (Table 4). The magnitude of the increase tophane, had a large effect in reducing the sensitivity of glutamine or glutamate, but not proline, histidine, or tryp-

tophan, had a large effect in reducing the sensitivity of the standard anaerobe (28). It grows faster aerobically because a protoporphyrin molecule which leaves a protoporphrin in strain MM14 (wild type) and the Fusr mutant MM142 (fusA1) strains each and incubated in M9 minimal medium with 0.2% glucose (and the indicated additions) for 18 h at 37°C with shaking. The final cell number was approximately 10⁷/ml, which corresponds to about 10 generations of growth.

Fluorescence assay of heme levels. The experiments described above suggest that Fus' mutants have defects in the synthesis of heme. We decided to measure, using a fluorescence assay (see Materials and Methods), the relative amount of heme in strain MM14 (wild type) and the Fusr mutant MM142 (fusA1). For each strain, we assayed 10 independent cultures, using 1 OD₆₀₀ unit of cells for each measurement. The assay is based on stripping the iron from the heme moiety, which leaves a protoporphyrin molecule which fluoresces following excitation at 400 nm (see Materials and Methods). We normalized fluorescence to biomass, measured as either optical density of the starting culture or dry weight of the cells; the two methods gave the same result. We found that the Fus'(fusA1) strain has significantly less heme than the wild-type strain (mean ± standard error of the mean) of 0.69 ± 0.02 [wild type] and 1.00 ± 0.05, respectively). This provides direct evidence that the Fus' phenotype can cause a heme deficiency.

Respiration rate. Salmonella serovar Typhimurium is a facultative anaerobe (28). It grows faster aerobically because a proton motive force is generated across the bacterial membrane with oxygen as a terminal electron acceptor (19, 28). This process drives the efficient synthesis of ATP (13). Heme-containing cytochromes are an essential part of this respiratory process. We considered the possibility that Fus' mutants, being limited in the amount of heme they produce, might also be defective in aerobic respiration. We measured the rate of oxygen consumption for the wild type and for two Fus' mutants, strains MM142 (fusA1) and JB2110 (fusA1-15), during exponential growth in LB medium. We found that each of the mutants had a significantly lower respiration rate than the wild type (Fig. 2). The respiration rate for the fusA1 mutant is only 41% of that measured for the wild type, and for the fusA1-15 mutant, the respiration rate is 74%. We conclude that these Fus' mutants have a significantly reduced rate of respiration.

Iron-mediated DNA damage. It was proposed that one consequence of a respiratory deficiency could be the accumulation of NADH, which ultimately provides free iron to the electrons that drive the Fenton reaction (11). Hydroxyl radicals are generated through the Fenton reaction, in which ferrous iron transfers an electron to hydrogen peroxide. These radicals then directly oxidize DNA, producing strand breaks and changes in DNA topology (16). Thus, inhibition of respiration results in an increased amount of cytosolic reductants available to reduce free ferric iron. In the presence of hydrogen peroxide, the ferrous iron thus formed will donate an electron and generate a hydroxyl radical that can damage DNA. This argument is supported by findings that inhibition of any step in the respiratory chain dramatically accelerates the rate at which hydrogen peroxide kills Escherichia coli (16, 33). To test this hypothesis with respect to Fus' strains defective in respiration, we incubated cultures of the wild type and a Fus' mutant with 2,2'-dipyridyl (an iron chelator that penetrates cells and chelates intracellular ferrous iron as well as iron outside the cells). Cultures were incubated together with 2,2'-dipyridyl for 30 min prior to exposure to 2.5 mM H₂O₂ for 45 min. In cultures treated directly with 2.5 mM H₂O₂, the percentages of cells viable after 45 min were 41% (wild type) and 20% (Fus' mutant). In contrast, over 70% of the cells in each of the cultures pretreated with 2,2'-dipyridyl survived 45 min of exposure to 2.5 mM H₂O₂. These results suggest that the decreased survival of the Fus' mutant in the presence of H₂O₂ is, at least in part, associated with iron-mediated DNA damage, since a decrease in the ferrous iron concentration improves survival.

Previous studies have shown that killing by micromolar concentrations of H₂O₂ (mode I bacterial killing) is mediated by iron-dependent DNA damage, while killing by millimolar H₂O₂ concentrations (mode II killing) is caused by oxidative

![FIG. 2. Respiration (rate of oxygen consumption) in the wild type and two different Fus' mutants. The standard error of these measurements is less than ±2 U for each strain.](http://aac.asm.org/)

### Table 4. Medium composition reverses the sensitivity of the Fus' mutation fusA1 to hydrogen peroxide

<table>
<thead>
<tr>
<th>Growth competition medium</th>
<th>CI (fusA1 mutant/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9</td>
<td>3.2 × 10⁻²</td>
</tr>
<tr>
<td>M9 + 70 μM H₂O₂</td>
<td>&lt;1 × 10⁻⁶</td>
</tr>
<tr>
<td>M9 + 70 μM H₂O₂ + 1% Casamino Acids</td>
<td>1.4 × 10⁻³</td>
</tr>
<tr>
<td>M9 + 70 μM H₂O₂ + 0.2% glutamine</td>
<td>1.4 × 10⁻³</td>
</tr>
<tr>
<td>M9 + 70 μM H₂O₂ + 0.2% glutamic acid</td>
<td>3.5 × 10⁻⁴</td>
</tr>
</tbody>
</table>

*a* Media (10 ml) were inoculated with (per milliliter) 10⁶ cells of the wild-type (MM14) and fusA1 (MM142) strains each and incubated in M9 minimal medium with 0.2% glucose (and the indicated additions) for 18 h at 37°C with shaking. The final cell number was approximately 10⁷/ml, which corresponds to about 10 generations of growth.

*b* The competition index (CI) is the ratio of the number of viable fusA1 mutant CFU to the number of wild-type (WT) CFU after 18 h of competition in each medium. A value of <1 × 10⁻⁶ means that no viable cells were recovered. Results are the means of results of three independent assays.
damage to multiple targets (17). To compare the relative sensitivities of serovar Typhimurium Fus' mutants to mode I and mode II killing, low (750 μM) and high (4 mM) H₂O₂ concentrations were used according to a published method (35). Exposure to 750 μM H₂O₂, 50% of the wild-type cells (MM14) and 3% of cells of the fusA1 mutant (MM142) and the fusA1-15 mutant (JB2110) survived after 3 h of incubation (Fig. 3A). In contrast, exposure to 4 mM H₂O₂ for 2 h reduced the viability of the wild type to 1%, of JB2110 to 0.2%, and of MM142 to a level below the detection limit (<0.001%). Loss of viability after exposure to 4 mM H₂O₂ is believed to involve oxidative modification of lipids and proteins (17). These results suggest that MM142 (the fusA1 mutant) is hypersensitive to the oxidative damage to DNA, to proteins, and to lipids caused by mode II killing, whereas in the case of JB2110 (the fusA1-15 mutant), the DNA damage caused by Mode I killing might be the more important factor for survival.

**DISCUSSION**

Fus' strains of *Salmonella* serovar Typhimurium with mutations in EF-G have reduced catalase activity and are sensitive to oxidative stress caused by H₂O₂ (25). Detoxification of H₂O₂ in *E. coli* and *Salmonella* is due mainly to two distinct species of catalase. HPI, a bifunctional catalase-hydroperoxidase, is encoded by the katG gene and is active as a tetramer of 81-kDa subunits (9). HPI is transcriptionally induced by OxyR as part of a genetic response to H₂O₂ (34). HPI activity is observed in the periplasm and in cytoplasmic membrane fractions (14). HPII, a monofunctional catalase, is encoded by the katE gene (24), is active as a tetramer of 78-kDa subunits (10), and is under the control of rpoS (32). HPII is localized in the cytoplasm (14). Here we have shown that the reduced catalase activity in Fus' mutants is associated with a reduction in activity for both HPI and HPII. This association suggested to us that it might reflect a deficiency in something common to both enzymes, such as the iron-binding cofactor heme. We found that expression of hema, as measured from a hemaA-lacZ fusion, is reduced in Fus' mutants, which supports this hypothesis. *Salmonella* Serovar Typhimurium uses the enzyme glutamyl-tRNA reductase (encoded by hema) to convert a small fraction of the cells' glutamyl-tRNAᵣ₃₃ to glutamate semialdehyde. This in turn is converted by the enzyme glutamate-1-semialdehyde aminotransferase (encoded by hemL) to ALA (1, 12). We found that providing cells with ALA, the precursor in the biosynthesis of heme, improved the viability of Fus' strains exposed to H₂O₂ to the wild-type level. It has been reported for *E. coli* that induction of the stringent response (7) results in the RelA-dependent excretion of glutamate from cells (6). Because the levels of ppGpp are disturbed in Fus' mutants (26), we wondered whether the ALA deficiency might be due in part to excretion of glutamate, which possibly leads to insufficient charging of tRNAᵣ₃₃. We hypothesized that the external addition of either glutamate or glutamine (which can be converted into glutamate inside cells) might decrease the sensitivity of Fus' mutants to H₂O₂. In support of this, we found that the addition of Casamino Acids, glutamate, or glutamine dramatically improved the survival and growth of the least fit Fus' mutant, relative to the wild type, in the presence of H₂O₂. Other amino acids tested had no positive or negative effect on the Fus' mutant. Because glutamyl-tRNAᵣ₃₃ is the substrate of the hemaA product, a reasonable interpretation of our results is that the insufficient availability of glutamate is, together with reduced transcription of hemaA, a factor limiting heme synthesis in Fus' mutants. Finally, we measured relative heme levels by a fluorescence assay and found that a Fus' mutant (with a fusA1 genotype) has significantly less heme than the wild type (69% of that of the wild type). Thus, heme deficiency is a phenotype of Fus' mutants.

Because heme is also a component of the cytochromes involved in respiration (3), we suspected that heme-deficient Fus' mutants might also be defective in respiration. We measured respiration as the rate of oxygen consumption and found that the Fus' mutants had a significantly reduced rate compared to the wild type. Similarly, it was previously shown that a heme-deficient mutant (hemA::Tn10) of *Salmonella* serovar Typhimurium was both hypersensitive to hydrogen peroxide and respired at half the rate of wild-type cells (11). Mutants with defects in respiration have been shown to suffer from an increased rate of iron-mediated DNA damage (11, 16). Inhibition of any step in the respiratory chain, either by cyanide or by genetic defects in respiratory enzymes, dramatically accelerates the rate at which hydrogen peroxide kills *E. coli* (18, 33, 38). It has also been established that respiratory blocks accelerate the rate of DNA damage (38). The respiratory blocks do
not substantially affect the levels of intracellular free iron or H$_2$O$_2$, indicating that they accelerate damage because they increase the availability of the electron donor. Indeed, a respiratory deficiency causes the accumulation of NADH, which provides to free iron the electrons that drive the Fenton reaction. It was proposed that respiratory inhibitors, by blocking the oxidation of NADH, increase the amount of cytotoxic reactants available to reduce free ferric iron. In the presence of hydrogen peroxide, the ferrous iron thus formed will donate an electron and generate a hydroxyl radical that can damage DNA. We found that the presence of 2,2'-dipyridyl, a chelator that penetrates cells and sequesters intracellular ferrous iron as well as iron outside the cells, prior to exposure to H$_2$O$_2$, increases the viability of a Fus$^+$. mutant.

Thus, amino acid alterations giving an antibiotic resistance phenotype are, at least in the case of some fusidic acid-resistant mutants, associated with pleiotropic phenotypes which reduce bacterial fitness in a variety of environments. These phenotypes, including the defect in heme biosynthesis and the reduced heme levels identified in this study, are interesting in that they provided insight into the Achilles’ heel of resistant strains. Importantly, this Achilles’ heel is not limited to Fus$^+$ mutation associated with a severe growth defect in vitro, such as fusA1. We have shown here that a Fus$^+$ mutation, fusA1-15, with an in vitro growth rate equivalent to that of the wild type, is also sensitive to oxidative stress. Sensitivity to oxidative stress is of interest because (i) aerobic growth is associated with the generation of reactive oxygen species and (ii) intra-cellular stress is of interest because (i) aerobic growth is associated with an in vitro growth rate equivalent to that of the wild type, reviewed previously (20). It has been suggested that a mutation in hemA associated with antibiotic resistance may result in exploitable DNA. We found that the presence of 2,2'-dipyridyl, a chelator that penetrates cells and sequesters intracellular ferrous iron as well as iron outside the cells, prior to exposure to H$_2$O$_2$, increases the viability of a Fus$^+$ mutant.

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