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Fusidic Acid-Resistant Mutants of *Salmonella enterica* Serovar Typhimurium with Low Fitness In Vivo Are Defective in RpoS Induction

Mirjana Macvanin,[1] Johanna Björkman,[2] Sofia Eriksson,[3] Mikael Rhen,[3] Dan I. Andersson,[2] and Diarmaid Hughes[1]*

Department of Cell and Molecular Biology, The Biomedical Center, Uppsala University, S-751 24 Uppsala,[1] Department of Bacteriology, Swedish Institute for Infectious Disease Control, S-171 82 Solna,[2] and Microbiology and Tumour Biology Center, The Karolinska Institute, S-171 77 Stockholm,[3] Sweden

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Mutants of *Salmonella enterica* serovar Typhimurium resistant to fusidic acid (Fus^r) have mutations in *fusA*, the gene encoding translation elongation factor G (EF-G). Most Fus^r mutants have reduced fitness in vitro and in vivo, in part explained by mutant EF-G slowing the rate of protein synthesis and growth. However, some Fus^r mutants with normal rates of protein synthesis still suffer from reduced fitness in vivo. As shown here, Fus^r mutants could be similarly ranked in their relative fitness in mouse infection models, in a macrophage infection model, in their relative hypersensitivity to hydrogen peroxide in vivo and in vitro, and in the amount of RpoS production induced upon entry into the stationary phase. We identify a reduced ability to induce production of RpoS (σ^s) as a defect associated with Fus^r strains. Because RpoS is a regulator of the general stress response, and an important virulence factor in *Salmonella*, an inability to produce RpoS in appropriate amounts can explain the low fitness of Fus^r strains in vivo. The unfit Fus^r mutants also produce reduced levels of the regulatory molecule ppGpp in response to starvation. Because ppGpp is a positive regulator of RpoS production, we suggest that a possible cause of the reduced levels of RpoS is the reduction in ppGpp production associated with mutant EF-G. The low fitness of Fus^r mutants in vivo suggests that drugs that can alter the levels of global regulators of gene expression deserve attention as potential antimicrobial agents.

Fusidic acid is a steroidlike antibiotic that inhibits protein synthesis by binding to a complex of the ribosome and elongation factor G (EF-G) (26). Resistance to fusidic acid in *Salmonella enterica* serovar Typhimurium is caused by mutations in *fusA* encoding EF-G (22). EF-G is a GTP-binding protein that catalyses the translocation of peptidyl-tRNA from the ribosomal A site to the P site (24, 37). After GTP hydrolysis and translocation, EF-G · GDP leaves the ribosome and is regenerated by the spontaneous exchange of GDP for GTP. Fusidic acid blocks the release of EF-G · GDP from the ribosome, thus inhibiting further protein synthesis. Phenotypes of Fus^r mutants of EF-G include a reduced rate of GDP-to-GTP exchange that reduces the rate of protein synthesis and altered levels of the transcriptional regulator molecule ppGpp (guanosine 3′-biphosphate, 5′-biphospate) (29). ppGpp acts as a nutritional stress signal which binds to the β-subunit of RNA polymerase (10, 35) and reduces its affinity for promoters of stable RNA (17, 43) by inhibiting formation of a ternary transcription initiation complex (1, 23). The translational and transcriptional phenotypes of Fus^r mutants can each be expected to have a negative impact on bacterial fitness. Throughout this paper the term fitness is used to describe the relative competitive ability of a mutant versus an isogenic wild type. Depending on the assay, differences in fitness can mean differences in growth rate or differences in survival in a particular environment.

The *rpoS*-encoded σ^s factor (RpoS) is required for expression of a large number of genes in response to various stresses, including nutrient limitation, osmotic challenge, acid shock, heat shock, oxidative damage, and growth into stationary phase (19). RpoS regulates *Salmonella* virulence and is essential during infection (13). *S. enterica* serovar Typhimurium is a facultative intracellular pathogen that, upon infection, resides in macrophages where it is exposed to a wide repertoire of antimicrobial effectors, including the phagocyte NAD(P)H oxidase (Phox). An initial oxidative bactericidal phase, associated with the production of superoxide anion and hydrogen peroxide, is followed by a bacteriostatic phase where nitric oxide is produced (38). The ability of *S. enterica* serovar Typhimurium to survive these stresses is an important determinant of its fitness in vivo (39).

Nutrient deprivation appears to be a critical environmental signal triggering the expression of *Salmonella* virulence genes within the phagosomes of host macrophages (12), and there is evidence that macrophages restrict the growth of phagocytosed organisms by limiting essential nutrients within the phagosome (31). The combination of nutrient restriction and stress conditions in the intracellular environment may be the stimulus for RpoS induction (11). Starvation also elevates the intracellular levels of ppGpp, whereas the synthesis of RpoS is positively regulated by ppGpp (15). In fact, ppGpp-deficient strains fail to synthesize RpoS as cells enter the stationary phase in a rich medium and under starvation (15). The major effects of ppGpp induction are not exerted on *rpo*S mRNA abundance or on protein turnover but instead affect translational efficiency (7). It was proposed that ppGpp indirectly regulates one or more additional factors specifically required for *rpo*S translation. Thus, intracellular *S. enterica* serovar Typhimurium may use

* Corresponding author. Mailing address: Department of Cell and Molecular Biology, Box 596, The Biomedical Center, Uppsala University, S-751 24 Uppsala, Sweden. Phone: 46-18-4714354. Fax: 46-18-530396. E-mail: diarmaid.hughes@icm.uu.se.

ppGpp as a modulator of RpoS expression and thereby activate its adaptation to stress.

In the present study, we have investigated the fitness costs associated with several fusidic acid-resistant (Fus^r) mutations in vivo. We show that the attenuated in vivo growth of Fus^r mutants is associated with increased sensitivity to H_2O_2. We report that Fus^r mutants have reduced levels of sigma factor RpoS. The relationship between decreased virulence of *Salmonella* with mutant EF-G forms, perturbed levels of ppGpp and reduced levels of RpoS is discussed.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains used are *S. enterica* serovar Typhimurium strains derived from the wild-type strains LT2 (TT10000 from the strain collection of John Roth, University of California, Davis) and ATCC 14028s. LT2-based strains were used in all experiments (in vivo and in vitro), except for competitions in macrophages and C57BL/6 mice, where strains derived from the more-virulent ATTC 14028s were required. LT2 has the advantage of being more defined genetically, whereas with 14028s, it is easier to establish infections in mice and macrophages. We have made comparisons of LT2 and 14028s with respect to growth kinetics in vivo (BALB/c mice), and they behave similarly, i.e., we can extrapolate the 14028s data to LT2. Furthermore, LT2 and 14028s survive stationary-phase and oxidative stresses equally well (41). Fus^r mutations were moved between strains by P22-mediated transduction with a linked marker, *zhb*-736::Tn*10* (21). Within each experiment the strains used were isogenic. We have determined that the *zhb-736*::Tn*10* marker is selectively neutral for growth in our competition experiments in vivo and in vitro, and we have therefore used it to distinguish the wild-type and Fus^r strains in competition experiments. The *katE*::Tn*10* mutation was transduced from TYT3260, ATTC 14028s *katE*::Tn*10* kindly supplied by Stanley Maloy. The *katG* knockout mutation was transduced from the strain TT19901, ATTC 14028s *katG*::pRR10 *karE*::Tn*10* (pRR10 is an RK2-based minireplicon encoding β-lactam resistance), kindly supplied by Kim Bunny and John Roth. The *rpoS-lacZ* fusions used were transduced from the strains TE6253, *putPA1303*::KanR-*rpoS-lacZ* [pr] and TE6127, *putPA1303*::KanR-*rpoS-lacZ* [op], kindly supplied by Tom Elliott (6). Minimal growth medium is M9 salts supplemented with 0.2% glucose, 5 μg of thiamine ml^{-1}, and amino acids at 40 μg ml^{-1} as required. Rich medium is Luria broth (LB). Antibiotics were tetracycline at 15 μg ml^{-1} and fusidic acid (sodium salt) at 800 μg ml^{-1} in the presence of 1 mM EDTA.

Measurement of bacterial viability in the presence of H_2O_2 in vitro. From an overnight culture, 1×10^6 to 2×10^6 cells/ml were inoculated in minimal glucose medium containing 70 μM H_2O_2 and incubated at 37°C without shaking. Samples were taken at each hour over the course of 23 h, diluted, and spread onto LB plates. After overnight incubation at 37°C, CFU were counted. The remainder of each culture was further incubated for several additional days to determine whether any living cells remained after the H_2O_2 treatment. H_2O_2 was diluted in water from a 30% stock (Merck).

Competition assays in vivo. BALB/c mice, C57BL/6 wild-type and isogenic *Cybb* knockout mice (34), and stock 002365 (Jackson Laboratory, Bar Harbor, Maine) were housed at the Microbiology and Tumor Biology Center, Karolinska Institute (Stockholm, Sweden) in accordance with both institutional and national guidelines. Animal experiments were performed as described previously (2, 3) by using an intraperitoneal challenge. Competitions were run for one cycle of 3 to 4 days corresponding to about 10 generations of bacterial growth (3).

Competition assays in cell culture: J774-A.1 macrophages. J774-A.1 cells (ATCC TIB 67) were cultivated in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), L-glutamine (10 mM final concentration; Gibco), and HEPES (10 mM final concentration; Gibco). Batches of RPMI and fetal bovine serum were screened before use to ensure they did not contain endotoxin. Cells were infected with *S. enterica* serovar Typhimurium at a multiplicity of infection of 1. Briefly, bacteria were suspended in phosphate-buffered saline, opsonized for 30 min in vitro with 10% mouse serum, diluted in HEPES-buffered RPMI, and subsequently seeded onto J774-A.1 cells. Plates were centrifuged for 5 min at 1,000 × *g*. After 1 h of infection, extracellular bacteria were killed by treatment for 45 min with 50 μg of gentamicin/ml. For continued incubations, killing medium was replaced by maintenance medium containing gentamicin (10 μg/ml). The amount of intracellular bacteria was determined, at the indicated time intervals, by hypotonic lysis to release intracellular bacteria, after which viable cells were counted on agar plates. For the second growth cycle (16 to 32 h), intracellular bacteria were grown first in one set of cells, then released from host cells by hypotonic lysis, enriched, recoated with complement, and fed to fresh cells.

Measurements of ppGpp. (i) Basal ppGpp levels. Bacterial cultures were grown in M9 minimal medium for at least 15 generations of exponential growth to an optical density at 460 nm (OD_{460}) of 0.3 to 0.4. Cells (60 ml) were fixed with 6 ml of 1.9% formaldehyde, and nucleotides were extracted according to a published method (27). High-performance liquid chromatography analysis and quantification of ppGpp levels were performed as described previously (29).

(ii) Starvation-induced ppGpp levels. Bacteria were grown in buffered morpholinepropanesulfonic acid (MOPS) minimal medium (5) with 0.2% glucose and 100 μCi of $^{32}P_i$ (Amersham) ml^{-1} in a BioscreenC reader (Labsystems). Starvation was induced during exponential growth at an OD_{600} of 0.2 to 0.3 by the addition of α-methyl glucoside to a final concentration of 2.6% (18). Aliquots (20 μl) were removed every 15 s to microcentrifuge tubes containing 20 μl of cold 20% formic acid. Zero time points were taken immediately before the addition of α-methyl glucoside. Acid extracts were incubated on ice for 30 min and then centrifuged in a Microfuge. Samples (5 μl) of supernatant were applied to polyethyleneimine-cellulose plates (Macherey-Nagel) and chromatographed in 1.5 M KH_2PO_4, pH 3.0. Chromatograms were analyzed and quantified with a PhosphorImager with Molecular Dynamics software.

β-Galactosidase assays. For measurements of *rpoS-lacZ* fusion induction upon entry into stationary phase, cultures were initially grown overnight at 37°C in LB medium and then diluted 100-fold in fresh LB medium. Samples from exponentially growing (E) and stationary-phase (S and S + 2) cultures were collected and assayed for β-galactosidase activity (30). Exponentially growing cells were collected at an OD_{600} of 0.3. Stationary-phase samples were taken from the cultures that were left to grow for an additional 1 h (S) or 3 h (S + 2) after reaching an OD_{600} of 0.5 (20). Appropriate dilutions of S and S + 2 samples were made in order to be approximately equal to the OD_{600} of the exponentially growing cells. The OD_{420} and OD_{540} were measured at intervals of 5 min in a BioscreenC machine. Miller units of β-galactosidase activity were calculated from the linear part of the curve $OD_{420} = f$(time [in minutes]), at approximately the same OD_{420} for all of the samples analyzed, with the formula $OD_{420} - 1.75 \times OD_{550}/OD_{660} \times$ time (in minutes) × volume of the sample (in milliliters) × 1,000. For measurements of *lacZ-rpoS* fusion induction upon glucose starvation, cultures were grown overnight in minimal M9 medium with 0.2% glucose. Cultures were diluted 50-fold in fresh media and grown to an OD_{600} of 0.2 to 0.3, at which time α-methyl glucoside was added to a final concentration of 2.6% (18). The cultures were left to incubate with shaking at 37°C for a further 5 and 30 min, at which times samples were taken and subjected to a standard β-galactosidase assay as described above.

RESULTS

Fitness of Fus^r mutants in vitro does not correlate with their fitness in vivo. EF-G Fus^r mutants with reduced translation and growth rates in vitro (29) show, as expected, reduced fitness in vivo (3). To determine whether factors other than translation rate are relevant for fitness in vivo, we studied a collection of Fus^r mutants for which the rate of protein synthesis was similar. Thus, we selected, from a strain carrying the unfit mutation *fusA1*, a set of strains carrying secondary mutations within EF-G that restore fitness in vitro, measured as exponential growth rate in glucose minimal media (21). These growth-rate-compensated (GRC) mutants retained, in most cases, resistance to fusidic acid, and the original *fusA1* mutation and the alleles are referred to as *fusA1-1* and *fusA1-2*, etc. (Table 1). The fitness of strains carrying these mutations in vivo was measured in competition against a fusidic acid-sensitive (Fus^s) wild-type strain in a BALB/c mouse infection model (see Materials and Methods). The degree of fitness restoration in vitro versus in vivo for these GRC strains showed a very poor correlation (Table 1). Thus, while GRC mutants in vitro are restored to within a few percent of the wild-type growth rate, in vivo these same strains, although improved relative to the parental strain, have in many cases very slow growth rates. We concluded that Fus^r mutations in EF-G can reduce fitness

TABLE 1. Relative fitness of the wild type and Fus^r mutants in vitro and in vivo[d]

fusA allele	EF-G mutation(s)	Growth rate, in vitro[a]	Generation time, in vivo[b]	MIC (μg/ml)[c]
Wild type	Wild type	1.00	1.00	100–200
fusA1-1	P413L, G13C	0.98	0.94	200
fusA1-2	P413Q	1.02	0.85	400
fusA1-7	P413V	0.98	0.68	800
fusA1-8	P413L, A66V	0.98	0.66	2,400
fusA1-11	P413L, F444L	0.98	0.59	2,400
fusA1-14	P413L, V291E	0.98	0.36	800
fusA1-15	P413L, T423I	0.98	0.33	>3,200
fusA1	P413L	0.52	0.00	2,400

[a] The in vitro growth rate is the relative growth rate in M9 glucose minimal medium, with that of the wild type set at 1.00.
[b] The in vivo generation time is the relative generation time in BALB/c mice, with that of the wild type set at 1.00. Values are calculated from growth competition assays in a mouse intraperitoneal infection model as described previously (3) and are taken from this reference.
[c] The MIC is the minimal inhibitory concentration of fusidic acid (in micrograms/milliliter) required to inhibit bacterial growth in a microtiter well assay (21).
[d] All values are the arithmetic means of the results from at least four independent measurements.

in vivo by a mechanism that does not correlate with the effects on the growth rate measured in vitro.

Fus^r mutants have reduced fitness in macrophages. The capacity to survive within macrophages is an absolute requirement for *Salmonella* virulence and, therefore, for fitness in vivo (14). We tested the relative fitness of the wild type and four Fus^r strains during competition in a macrophage infection model (see Materials and Methods). Three Fus^r mutants (*fusA1*, *fusA1-14*, and *fusA1-15*) previously found to be unfit in vivo (3) were also unfit in competition against the wild type in the macrophage assay (Fig. 1). In contrast, the Fus^r mutant carrying *fusA1-7*, although unfit in vivo (3), competed effectively with the wild type in the macrophage assay. The lower fitness of the mutant with *fusA1-7* in the mouse competition assays (Table 2) suggests that, in the more complex in vivo environment, it is subjected to stresses it does not meet in the macrophage assay. The order in which these four Fus^r mutants were ranked in fitness under macrophage growth conditions was the same as that observed in the BALB/c in vivo model (Table 1).

Fus^r mutants lose viability in the presence of H_2O_2 in vitro. Resistance to oxidative stress may be an important characteristic in the ability of *Salmonella* to withstand killing in phagocytic cells (31). One of the main determinants for the killing of *Salmonella* by macrophages is H_2O_2 (40). We tested whether Fus^r mutants were sensitive to hydrogen peroxide in vitro by measuring survival in glucose minimal medium supplemented with 70 μM hydrogen peroxide. This concentration of hydrogen peroxide was used because it approximates the concentration generated during the respiratory burst (16, 25, 40) and because it distinguishes clearly between the different Fus^r mutants. The experiment showed that bacterial growth was initially inhibited for several hours, after which a decrease in the viable count (CFU) was observed (Fig. 2). For the LT2 wild type and the fittest Fus^r strain (*fusA1-1*), the CFU decreased from the initial $\sim 2 \times 10^6$ cells/ml to 1×10^4 (wild type) or 1.7×10^5 (*fusA1-1*) cells/ml. Thus, the Fus^r mutant carrying *fusA1-1* is more resistant than the wild type to exposure to

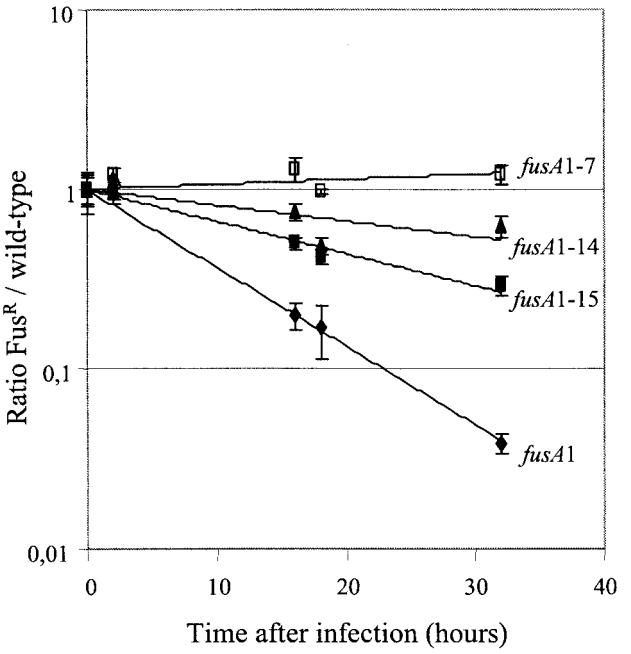

FIG. 1. Relative competitive ability of the wild type versus four different Fus^r mutants in a macrophage infection. Conditions are described in Materials and Methods. With the exception of the time zero points (four independent measurements per assay), each point is the mean of the results from 7 to 11 independent measurements. Standard error bars (standard deviations of the means) are shown for each point.

H_2O_2, although it is slightly less fit in growth competition both in vitro and in vivo (Table 1). In contrast, the CFU of the Fus^r mutant carrying *fusA1-15* decreased from $\sim 2 \times 10^6$ cells/ml to only 50 cells/ml after 22 h of exposure to H_2O_2, before growth resumed. Although this number of cells is very small, multiple experiments confirmed that beginning with 10^6 cells results typically in about 5 logs of killing, with the survivors resuming growth. The Fus^r mutant with the least fit allele, *fusA1*, was so sensitive in this assay that no cells survived. Multiple experiments confirm that this strain is so sensitive to H_2O_2 that reproducibly no cells survive in assays where $\sim 10^6$ to 10^7 cells/ml are initially inoculated. With the exception of the strain carrying *fusA1*, each of the strains eventually resumed growth and, by 36 h, had reached a density of at least 10^9 CFU/ml (Fig. 2). We concluded that the oxidative stress caused by H_2O_2 reduced the viability of the unfit Fus^r mutants relative

TABLE 2. Competition between the wild type and different Fus^r mutant *Salmonella* strains in two strains of mice, wild-type C57BL/6 and *Cybb* mice

Fus^r mutant	Competition index (SE) in mouse strain[a]:		Fold improvement
	C57BL/6	*Cybb*	
fusA1-7	5.1×10^{-1} (1.6×10^{-1})	2.2 (5.0×10^{-1})	4
fusA1-14	2.2×10^{-3} (4.4×10^{-3})	9.2×10^{-2} (2.2×10^{-2})	42
fusA1-15	1.2×10^{-3} (7.8×10^{-5})	4.4×10^{-2} (1.5×10^{-2})	37
fusA1	$<10^{-6}$	4.6×10^{-5} (2.4×10^{-5})	>46

[a] Each result is expressed as a competition index, which is the ratio of mutant to wild type at the end of one growth cycle/the ratio of mutant to wild type at time zero. Each data point is the arithmetic mean of the bacterial ratios from the livers of six mice.

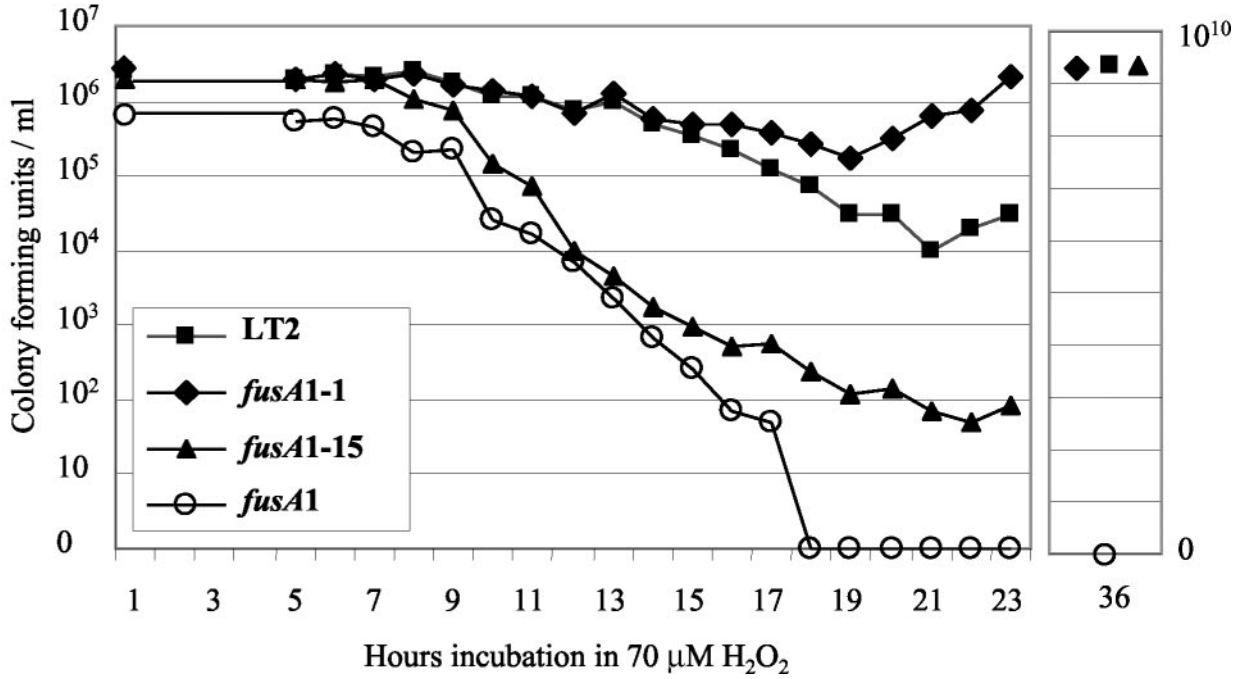

FIG. 2. Growth inhibition and loss of viability of the wild type and Fus^r mutants in the presence of H_2O_2. Approximately 10^6 CFU of each culture was inoculated into M9 glucose with 70 μM H_2O_2 and incubated at 37°C without shaking. Samples were taken at the indicated intervals, diluted, and plated onto LB plates to determine the number of CFU for each strain. The 36-h sample shows that growth had resumed for three of the four strains after the initial killing period. No growth occurred in the culture with the mutant carrying *fusA1* even after several days of incubation. This experiment was repeated two to five times for each strain, and results from a representative experiment are shown.

to the wild type, inhibiting growth and causing cell death. Furthermore, the relative sensitivity of different Fus^r mutants to H_2O_2 correlated with their relative in vivo fitness measured in the BALB/c mouse model (Fig. 2; additional data for the other Fus^r mutants are not shown).

In vivo sensitivity to hydrogen peroxide. To test whether sensitivity to H_2O_2 is an important in vivo determinant of the fitness of Fus^r mutants, we measured competitive ability in vivo in two different mouse strains: a wild-type strain, C57BL/6, and an isogenic strain carrying a targeted mutation in NADPH cytochrome b oxidase (*Cybb*). *Cybb* mice are unable to undergo a phagocyte oxidative burst. We observed that the fitness of three unfit Fus^r mutants was improved in the *Cybb* mice by about 40-fold (Table 2). The strain carrying the *fusA1-7* mutation was restored to wild-type fitness. We conclude that sensitivity to oxidative stress is a significant fitness parameter of the Fus^r mutants. However, the fitness of the three least-fit Fus^r mutants was not fully restored in the *Cybb* mice. The incomplete restoration of fitness may be because the *Cybb* mice still produce some H_2O_2 and almost twice as much nitric oxide as the wild-type mice (40). However, there may be additional factors that contribute to the low fitness of the Fus^r mutants in vivo.

Reduced catalase activity associated with Fus^r mutants. The sensitivity of Fus^r mutants to H_2O_2 in vitro and in vivo suggested to us that they might have reduced levels of catalase activity. We measured the rate of clearance of H_2O_2 from the growth medium (33, 42) and found that Fus^r mutants, relative to the wild type, are slow at clearing H_2O_2 (data not shown). As a control, we showed that strains carrying insertion mutations in *katE* or *katG* had catalase activities reduced to 33 and 77% of the wild-type level, respectively. These experiments showed that Fus^r mutants also had reduced catalase activity, down to 35% of wild-type activity in the case of *fusA1*. However, others have reported that catalase activity per se is not an important virulence factor (8). To assess directly the significance of catalase activity to in vivo fitness, we performed competition experiments with BALB/c mice. The wild type was competed against isogenic strains carrying either of two unfit Fus^r mutations (*fusA1* or *fusA1-15*) or carrying insertions inactivating *katE* or *katG*. The competition results (Table 3) showed that both Fus^r strains were very unfit, as expected, but that the catalase mutations had little or no effect on the in vivo competition index. Our conclusion is that while Fus^r mutants have reduced catalase activity, this phenotype does not explain their reduced fitness in vivo. This is in agreement with previous results showing that an *S. enterica* serovar Typhimurium double mutant (*katE* and *katG*) unable to produce either HPI or

TABLE 3. Relative fitness of Fus^r and catalase mutants competing against wild-type 14028s in BALB/c mice

Relevant mutation	Competition index[a]	SE
katE::Tn*10*	1.4	0.2
katG::pRR10	0.4	0.08
fusA1-15	0.002	0.001
fusA1	<0.0008	

[a] The competition index is the ratio of mutant to wild type at the end of one growth cycle/the ratio of mutant to wild type at time zero. Each result is the arithmetic mean of the bacterial ratios from at least four mice.

TABLE 4. ppGpp level in Fus[r] strains

fusA allele	Relative ppGpp level[c]	
	Basal[a]	Induced[b]
Wild type	1.0	1.0
fusA1-1	0.9	1.3
fusA1-2	1.4	ND
fusA1-7	1.7	1.1
fusA1-8	1.0	ND
fusA1-11	1.2	ND
fusA1-14	1.0	0.7
fusA1-15	1.3	0.6
fusA1	0.3	0.3

[a] The basal level of the wild type is 15 pmol of ppGpp/OD_{460} of exponentially growing cells.
[b] Induced levels are measured as the percent GTP converted into ppGpp 90 s after induction by the addition of α-methylglucoside. The value for the wild type is 29%. ND, not determined.
[c] All results are the arithmetic means of the results from at least three independent experiments. Our detection level in measurements of basal levels of ppGpp was 1 pmol/OD_{460}, and the variation between experiments is approximately ±2 pmol/OD_{460}.

HPII catalase activity retains full virulence in macrophage and mouse assays (8).

Basal and starvation-induced levels of ppGpp in Fus[r] mutants. The *fusA1* mutation, associated with low fitness both in vitro and in vivo, has reduced basal and starvation-induced levels of ppGpp (29). We assayed ppGpp levels in several GRC Fus[r] mutants to determine whether the ppGpp levels had been restored to wild-type levels. Basal levels of ppGpp were measured in exponentially growing cells by high-performance liquid chromatography analysis (see Materials and Methods). Wild-type LT2 had 15 pmol/OD_{460} while in *fusA1* it was 5 pmol/OD_{460}. In the GRC mutants, basal levels were restored (but not always exactly to the wild-type level) and ranged from 13 to 26 pmol/OD_{460}, with no obvious correlation with their fitness in vivo (Table 4). Under glucose starvation conditions, the *fusA1* strain converted only 10% of GTP into ppGpp compared with about 30% conversion for the wild-type strain. Conversion of GTP into ppGpp was restored to the wild-type level in the most-fit GRC Fus[r] mutants but not in the less fit mutants, *fusA1-14* and *fusA1-15* (Table 4). Thus, altered ppGpp-mediated gene regulation might be one factor in determining the relative fitness of these strains under stress conditions.

Expression *rpoS-lacZ* fusions in Fus[r] mutants. Synthesis of RpoS is positively regulated by ppGpp (15). The *rpo*S-encoded σ^S factor regulates *Salmonella* virulence and is essential during infection (13). We measured the expression of *rpo*S in various Fus[r] mutants with perturbed starvation levels of ppGpp by using translational [pr] and transcriptional [op] *rpoS-lacZ* fusions (6). Expression of *rpoS* was measured on samples taken at three different points during growth. Samples from exponentially growing cultures (E) were taken at an OD_{600} of 0.3. Samples from cultures entering stationary phase (S) were taken 1 h after the time at which the OD_{600} reached 0.5 (20). This definition of S compensated for the slower growth rate of *fusA1*. A second stationary-phase sample (S + 2) was taken 3 h after the OD_{600} reached 0.5 (20). The β-galactosidase activity of the translational fusion, *rpoS-lacZ* [pr], in the wild-type strain was low during exponential growth but increased dramatically after entrance into stationary phase. In the wild type, the induction ratio (S + 2)/E was ~30-fold (Fig. 3A), in agreement with published data (11, 20). The level of induction at S + 2 was close to maximal, and only a small further increase was associated with overnight incubation (data not shown). Relative to the wild type, each of the Fus[r] mutants tested induced *rpoS-lacZ* expression to a lesser extent upon entry into stationary-phase growth. Thus, at S + 2, the inductions associated with the various *fusA* mutations were 76, 54, and 23% of the wild-type level for *fusA1-1*, *fusA1-15*, and *fusA1*, respectively (Fig. 3A).

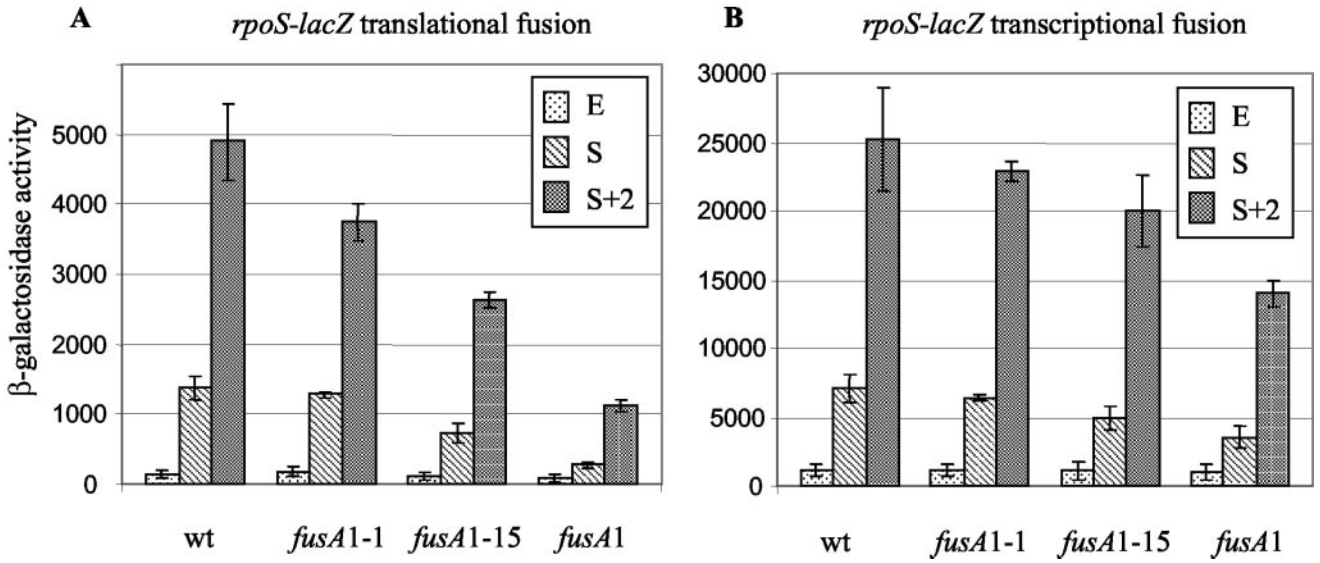

FIG. 3. (A) Expression of *rpoS-lacZ* translational fusion in the wild type (wt) and Fus[r] mutants as a function of growth stage. E is exponential growth, S is 1 h after the OD_{600} reached 0.5, and S + 2 is 3 h after the OD_{600} reached 0.5. Values shown are the means of the results from three independent measurements. Standard error bars (standard deviations of the means) are shown for each point. The data in panel B are the same as described for panel A, except that the transcription activity from the *rpoS* promoter is being measured.

Similar assays were made with an *rpoS-lacZ* transcriptional fusion [op] in wild type and Fus^r mutants (Fig. 3B). These showed that in the wild type, *rpoS* expression increased upon entry into the stationary phase (Fig. 3B). For the wild type, the transcription induction ratio (S + 2)/E was 22. This induction ratio is similar to published data (20). Of the three Fus^r mutants, only the strain with the *fusA1* mutation had significantly slower induction kinetics than the wild type, having 50 to 55% of wild-type levels at S and S + 2 (Fig. 3B). Taken together, the measurements of *rpoS-lacZ* fusions suggested that Fus^r mutants with reduced in vivo fitness were defective in inducing *rpoS* upon entry into the stationary phase and that the defect is more pronounced at the posttranscriptional level.

The β-galactosidase assays on cells entering the stationary phase were made in LB medium to facilitate a direct comparison with published results (20) on *rpoS* induction upon entry into the stationary phase. We also made β-galactosidase assays on the *rpoS-lacZ* fusions in cells growing exponentially in minimal M9 glucose medium, where carbon starvation was induced by the addition of α-methyl-glucoside (see Materials and Methods). In the wild type, the *rpoS-lacZ* induction ratio after 30 min of starvation was ~5-fold, as expected from the literature (19), while in the strains with *fusA1* or *fusA1-15*, virtually no induction was detected (<2-fold). We conclude that Fus^r mutants are defective in RpoS induction both under conditions of entry into the stationary phase and starvation stress, in rich and minimal medium.

DISCUSSION

Translation factor EF-G drives ribosomal movement through its interaction with the ribosomal A site. The A site on the ribosome is also where the transcription regulator molecule, ppGpp, is produced by the RelA protein. Fusidic acid is an antibiotic that targets EF-G in the ribosomal A site. Fusidic acid-resistant mutants (Fus^r) of *Salmonella* have alterations in EF-G that decrease their sensitivity to the antibiotic (21, 22). It was previously shown that many of these Fus^r mutants reduce growth and translation rate as could be expected for mutants of EF-G (29). More intriguingly, it was noted that Fus^r mutants were also frequently disturbed in their production of ppGpp on the ribosome (29), suggesting that mutant EF-G can perturb not only translation, but also transcription regulation. Fus^r mutants have also been shown to be unfit in vivo (3). Because of the perturbation of ppGpp levels in Fus^r strains, we asked whether the loss of fitness associated with a Fus^r phenotype in vivo could be associated with altered expression of one or more important genes, rather than simply being the result of a reduced growth rate. To determine this, we have made use of Fus^r mutants with growth rates similar to those of the wild type (21). We measured the relative fitness of these Fus^r mutants and found that many still have severe fitness defects in vivo (Table 1).

Why are Fus^r mutants with a normal growth rate unfit in vivo? Upon infection, *Salmonella* evokes a host immune response and is targeted and engulfed by macrophages (36). Here we showed that Fus^r mutants could be similarly ranked in fitness in mice (Table 1) and in macrophages (Fig. 1). The relative fitness of Fus^r mutants is improved in *Cybb* mutant mice that are incapable of mounting a normal phagocyte oxidative response (Table 2). This identifies sensitivity to oxidative attack as one factor determining the relative fitness of Fus^r mutants in vivo. This link between in vivo fitness and sensitivity to the oxidative response is supported by the fact that Fus^r strains are growth inhibited, and lose viability, in the presence of micromolar concentrations of hydrogen peroxide in vitro (Fig. 2). Sensitivity to hydrogen peroxide suggested to us that Fus^r mutants might have a decreased catalase activity. We measured catalase activity in Fus^r mutants and found that it was reduced in strains with low fitness in vivo. However, reduced catalase levels by themselves do not reduce *Salmonella* fitness in vivo (Table 3), as has also been observed by others (8). This showed that while Fus^r mutants are sensitive to oxidative stress in vivo (Table 2), the cause of this sensitivity is not their reduced catalase activity per se.

One critical factor for *Salmonella* virulence is the stationary-phase sigma factor, RpoS (13). The Fus^r strains are defective in ppGpp production (Table 4), a molecule that is proposed to be a positive regulator of RpoS levels (15). Thus, the Fus^r mutants might have reduced levels of RpoS in the stationary phase or other stress conditions, and that may be the cause of their low fitness in vivo. In accordance with this idea, we found that *fusA* mutations were associated with reduced induction levels of *rpoS*. The effect was mainly at the level of *rpoS* translation, and the magnitude of the effect correlated with the in vivo fitness associated with a particular *fusA* mutation (Fig. 3). From these experiments we conclude that the reduced in vivo fitness of the Fus^r mutants resulted from their failure to respond appropriately to stress conditions with a rapid induction of expression of RpoS sigma factor. The low level of induction may, in turn, be due to the reduced levels of ppGpp produced in Fus^r mutants in response to stress signals (Table 4).

The RpoS sigma factor is induced in response to a variety of different stress conditions (19, 28), including nutrient starvation, growth phase shift, and oxidative damage. Cellular levels of ppGpp increase in response to each of these stress conditions (9). Thus, immunoblots revealed a 25- to 50-fold increase in RpoS when ppGpp was artificially induced, without starvation, and that a complete ppGpp^0 deficiency blocked RpoS induction during starvation. The major effect of ppGpp induction on RpoS levels is exerted on the translational efficiency of the RpoS mRNA rather than on the rate of transcription or protein turnover (7). Expression of an *rpoS-lacZ* translational fusion increased rapidly in *S. enterica* serovar Typhimurium after phagocytosis, with over 70% of maximal induction occurring during the first 2 h (11). This suggests that the regulatory system mediated by RpoS is activated by the intracellular environment of eukaryotic cells (11). Our results suggest that some Fus^r mutants reduce ppGpp induction levels under stress conditions and that one result of this is a reduced RpoS induction. A consequence for *Salmonella* is a reduction in the in vivo fitness of Fus^r mutants.

Exploiting knowledge of in vivo fitness costs. There have been several reports associating fitness costs in vivo with antibiotic resistance mutations (2–4, 32). In none of these cases has the specific nature of the in vivo fitness cost been identified. In terms of the Fus^r mutants described here, we have found that there are at least two significant fitness costs associated with the resistance mutations. One cost, a reduced rate of protein synthesis, is relevant both in vivo and in vitro. The second cost identified here is reduced virulence associated with the failure of Fus^r strains to

properly induce RpoS expression in response to stress signals and is primarily relevant in vivo. Indeed, as shown here, Fus[r] mutants with a very small reduction in growth rate in vitro, are often significantly impaired in growth or survival in vivo. Determining the nature of the specific fitness costs associated with antibiotic resistance in vivo provides potential tools for improving how we deal with antibiotic-resistant strains. Such information could inform the choice of targets to be explored in screening programs for novel antibiotic drugs. Specifically, drugs that can alter the levels of ppGpp and/or RpoS, or indeed any other global regulator of gene expression, deserve attention as potential antimicrobial agents. In addition, we have noted that Fus[r] mutants disturb two central processes, translation and transcription, and it may be that this double hit makes it difficult for bacteria to genetically compensate for the resulting fitness loss. Thus, a second class of targets to be considered in drug screening programs would be those that occupy functional intersections between different important cellular processes.

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