Role of *spoT*-dependent ppGpp accumulation in the survival of light-exposed starved bacteria

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In bacteria, cytoplasmic levels of the effector nucleotide ppGpp are regulated in response to changes in growth conditions. This study describes the involvement of SpoT-mediated ppGpp accumulation in the survival of light-exposed bacteria during fatty acid starvation. In contrast to isogenic wild-type strains and *relA* mutants, the '*Vibrio angustum*' S14 *spoT* and *Escherichia coli* *relA spoT* mutants displayed significant losses in viability in response to cerulenin-induced fatty acid starvation under cool-white fluorescent light. However, when starvation experiments were performed in complete darkness, or under light filtered through a UV-resistant perspex sheet, only a minor decline in viability was observed for the wild-type and mutant strains. This finding indicated that the lethal effect was mediated by weak UV emission. In contrast to the *E. coli relA spoT* mutant, which lacks ppGpp, the '*V. angustum*' S14 *spoT* mutant exhibited higher ppGpp levels and lower RNA synthesis rates during fatty acid starvation, features that might be correlated with its lethality. In agreement with this finding, fatty acid starvation lethality also occurred upon induction of ppGpp overaccumulation in *E. coli*. These data suggest that the precise regulation of ppGpp levels in the stressed cell is crucial, and that both the absence and the overaccumulation of ppGpp impair fatty acid starvation survival of light-exposed cells. Moreover, the UV-induced lethal effect during fatty acid starvation was also observed for *E. coli* strains mutated in *rpoS* and *dps*, which, in the wild-type, are regulated directly or indirectly by ppGpp, respectively. The restoration of viability of fatty-acid-starved *spoT* mutant cells through the addition of exogenous catalase suggested that the observed light-dependent lethal effect was, at least in part, caused by UV-imposed oxidative stress. Based on these results, it is proposed that fatty acid starvation adaptation of light-exposed bacterial cells depends on the development of resistance to UV-induced oxidative stress. This stress resistance was found to require appropriate ppGpp levels, ppGpp-induced RpoS expression and, hence, upregulation of RpoS-regulated stress-defending genes, such as *dps*.

Keywords: fatty acid starvation, UV radiation

INTRODUCTION

Bacteria in natural environments are constantly challenged by nutrient limitation and stress conditions. Gram-negative, non-differentiating bacteria, such as *Escherichia coli* and '*Vibrio angustum*' S14 (*Vibrio* sp. CCUG 15956; a model marine bacterium for studying starvation adaptation and stress responses), elicit soph-

istic intracellular reorganization programmes in response to nutrient limitation, especially carbon starvation (Kjelleberg *et al.*, 1993; Matin, 1991). Such programmes are characterized by a series of physiological and genetic changes that facilitate the development of multi-stress resistance, and which ultimately lead to long-term survival (Matin, 2000; Srinivasan & Kjelleberg, 1998). Successful starvation adaptation requires many factors, including ppGpp (Cashel *et al.*, 1996) and the stationary phase sigma factor σ^S (RpoS; Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994).

Abbreviations: ppGppase, ppGpp hydrolase; PSI, ribosome-associated ppGpp synthetase; ROS, reactive oxygen species.

ppGpp is an intracellular signal that accumulates in response to nutritional deficiency in prokaryotes, and its accumulation inhibits the synthesis of rRNA and proteins. The metabolism of ppGpp is mediated by two global regulatory proteins, RelA and SpoT (Cashel *et al.*, 1996). RelA is a ribosome-associated ppGpp synthetase (PSI) that facilitates ppGpp accumulation upon amino acid deprivation. The SpoT protein is a bifunctional enzyme, incorporating a weak ppGpp synthetase (PSII) activity and a ppGpp hydrolase (ppGppase) activity. During carbon starvation, the ppGppase activity of SpoT is inhibited, leading to slow rates of ppGpp accumulation (Cashel, 2000). This *spoT*-dependent ppGpp accumulation occurs within the first few hours of carbon starvation (Flardh *et al.*, 1994), and is essential for subsequent long-term survival (Nystrom, 1994). Furthermore, ppGpp accumulation activates the synthesis of RpoS in *E. coli* cells (Gentry *et al.*, 1993; Lange *et al.*, 1995), which in turn stimulates the expression of many stress-resistance genes (Loewen *et al.*, 1998). For instance, the RpoS-regulated gene *dps* (encoding a DNA-binding protein during starvation) has been reported to be important in oxidative stress resistance (Almiron *et al.*, 1992).

While the downstream events of the carbon starvation adaptive response are relatively well understood, little is known about how carbon starvation is sensed by a bacterium and linked to SpoT-mediated ppGpp accumulation and the consequent upregulation of RpoS (Matin, 2000). Studies of the cellular location of SpoT in *E. coli* have revealed that it is not associated with ribosomes and appears to be a cytosolic protein, which may monitor carbon source availability through a soluble signal molecule (Gentry & Cashel, 1995). Through the use of mutants defective in the fatty acid synthesis pathway and cerulenin, a specific inhibitor of fatty acid synthesis (Omura, 1981), it has been found that fatty acid starvation in the presence of glucose and amino acids stimulates *spoT*-dependent ppGpp accumulation in *E. coli* (Seyfzadeh *et al.*, 1993). The signalling pathway leading to this accumulation, and hence the inhibition of rRNA and protein synthesis, did not involve a significant decrease in the synthesis of either phospholipids or ATP. It may well be part of, or identical to, the still elusive SpoT-controlled carbon starvation response mechanism (Seyfzadeh *et al.*, 1993). The precursor of fatty acid synthesis, acetyl CoA, is mainly produced during glycolysis when glucose is present as the sole carbon source. Therefore, glucose deprivation in bacteria apparently causes inhibition of fatty acid synthesis, leading to fatty acid starvation (DiRusso & Nystrom, 1998). Following this rationale, it is possible that SpoT senses a signal generated during fatty acid metabolism to monitor carbon source availability during growth and carbon starvation (DiRusso & Nystrom, 1998). Interestingly, ppGpp has a negative feedback effect on the fatty acid synthesis pathway, by inhibiting glycerol phosphate acyltransferase (Heath *et al.*, 1994).

Fatty acid starvation can be mimicked through the

addition of cerulenin, a polypeptide fungal mycotoxin, which binds tightly to two enzymes, 3-oxoacyl-[acyl-carrier-protein] I and II synthases (encoded by *fabB* and *fabF*, respectively). This binding prevents the catalysis of the elongation reaction, thus leading to the inhibition of both saturated and unsaturated fatty acid synthesis and hence growth arrest in bacteria (Moche *et al.*, 1999). Fatty acid starvation halts synthesis of the bacterial lipid membrane, and continued growth of the bacteria results in lethal consequences. Hence, survival of fatty acid starvation depends critically on cessation of cell division and the reduction of growth and net protein synthesis through ppGpp accumulation, together with minimization of existing fatty acid turnover (DiRusso & Nystrom, 1998).

Previous studies in our laboratory have demonstrated that a '*V. angustum*' S14 *spoT* mutant displays a severe loss in viability during carbon starvation (Ostling *et al.*, 1995, 1996), a phenotype similar to that of the *E. coli* *relA spoT* mutant (Nystrom, 1994). Moreover, the carbon starvation lethality of the *spoT* mutant depends on laboratory fluorescent light, which emits extremely weak UV radiation (Ostling *et al.*, 1995). However, ppGpp levels are increased in the carbon-starved '*V. angustum*' S14 *spoT* mutant in contrast to the *E. coli* *relA spoT* mutant, which displays decreased levels of ppGpp (Ostling *et al.*, 1995; Xiao *et al.*, 1991). To further address the above postulated link between carbon starvation and fatty acid starvation, two important factors in carbon starvation survival, *spoT*-dependent ppGpp accumulation and the RpoS regulon, were examined during cerulenin-induced fatty acid starvation. Furthermore, these factors were characterized with regard to their roles in starvation-induced stress resistance. Through the use of various mutant strains of *E. coli* and '*V. angustum*' S14, we report that SpoT-mediated ppGpp accumulation, RpoS and the RpoS-controlled DNA-binding protein (Dps) are required for fatty acid starvation survival in the presence of cool-white fluorescent light. This survival is partly dependent on the resistance to oxidative stress imposed by the weak UV radiation from laboratory fluorescent light. The possible negative effect of ppGpp over-accumulation on fatty acid starvation survival is also discussed.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Marine '*V. angustum*' S14 (CCUG 15956) strains S141, KF21 and J109 have been described previously (Albertson *et al.*, 1990; Flardh *et al.*, 1994; Marden *et al.*, 1987; Ostling *et al.*, 1991, 1995). *E. coli* strains MG1655, CF1652, CF1693, CF5747, CF5748, CF4941 and CF4943 were obtained from Michael Cashel (Laboratory of Molecular Genetics, NICHD, National Institutes of Health, USA). *E. coli* strains ZK126, ZK1000 and ZK1058 were gifts from Roberto Kolter (Department of Microbiology and Molecular Genetics, Harvard Medical School, USA). The plasmid pALS13 (Svitil *et al.*, 1993), carrying a P_{lac} -controlled truncated *relA* gene encoding a 455 aa RelA peptide with PSI activity, was propagated in strain

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Reference
<i>E. coli</i>		
MG1655	λ^- F ⁻	Bachmann & Low (1984)
CF1652	MG1655 $\Delta relA251::kan$	Xiao <i>et al.</i> (1991)
CF1693	MG1655 $\Delta relA251::kan \Delta spoT207::cam$	Xiao <i>et al.</i> (1991)
ZK126	W3110 <i>tna-2 lacU169</i>	Connell <i>et al.</i> (1987)
ZK1000	ZK126 <i>rpoS::kan</i>	Bohannon <i>et al.</i> (1991)
ZK1058	ZK126 <i>dps::kan</i>	Almiron <i>et al.</i> (1992)
CF4941	MG1655 <i>zib563::Tn10, \Delta relA251::kan</i>	Gentry <i>et al.</i> (1993)
CF4943	CF4941 <i>spoT203</i>	Gentry <i>et al.</i> (1993)
CF5747	MG1655 pALS13	Svitil <i>et al.</i> (1993)
CF5748	MG1655 pALS14	Svitil <i>et al.</i> (1993)
<i>'V. angustum' S14</i>		
S141	S14, Sm ^r	Ostling <i>et al.</i> (1991)
KF21	S141, $\Delta relA::kan$	Flardh <i>et al.</i> (1994)
J109	S141, <i>spoT::kan</i>	Ostling <i>et al.</i> (1995)
Plasmids		
pALS13	Ap ^r , P _{lac} :: <i>relA</i> (1–455 aa)	Svitil <i>et al.</i> (1993)
pALS14	Ap ^r , P _{lac} :: <i>relA</i> (1–331 aa)	Svitil <i>et al.</i> (1993)

* Ap^r, Ampicillin resistance; Sm^r, streptomycin resistance; P_{lac}, *E. coli lac* operon promoter.

MG1655, to give strain CF5747. The plasmid pALS14 (Svitil *et al.*, 1993), carrying a P_{lac}-controlled truncated *relA* gene encoding a 331 aa RelA peptide without PSI activity, was propagated in strain MG1655, to give strain CF5748.

Media and growth conditions. *E. coli* strains were grown at 30 °C on Luria–Bertani (LB) agar (Sambrook *et al.*, 1989). '*V. angustum*' S14 strains were grown at 26 °C on VNSS agar (Marden *et al.*, 1985). For liquid cultures of *E. coli* strains, culture side-arm flasks were inoculated with freshly grown colonies and grown at 30 °C on a rotary shaker (160 r.p.m.) in MOPS medium supplemented with 0.2% glucose, 10 µg thiamin ml⁻¹, 40 µg uridine ml⁻¹ and 0.1% Casamino acids (Neidhardt *et al.*, 1974). For liquid cultures of '*V. angustum*' S14 strains, culture side-arm flasks were inoculated with freshly grown colonies and grown at 26 °C on a rotary shaker (160 r.p.m.) in marine minimal medium (MMM) supplemented with 0.2% glucose, 10 µg thiamin ml⁻¹, 40 µg uridine ml⁻¹ and 0.1% Casamino acids (Ostling *et al.*, 1991). Growth was monitored as a function of optical density measured at 600 nm (OD₆₀₀) with a Pharmacia LKB Novaspec II spectrophotometer. Where appropriate, antibiotics were used at the following concentrations: 100 µg ampicillin ml⁻¹; 12.5 µg tetracycline ml⁻¹; 50 µg kanamycin ml⁻¹ for *E. coli* strains and 85 µg ml⁻¹ for '*V. angustum*' S14 strains; 50 µg chloramphenicol ml⁻¹; and 200 µg streptomycin ml⁻¹.

Starvation conditions and light source. Fatty acid starvation was achieved by adding cerulenin (Sigma) to a final concentration of 200 µg ml⁻¹ to cultures at OD₆₀₀ 0.2. Cultures subjected to starvation regimens were stored statically in glass flasks positioned on the laboratory bench top, either directly under the light of a cool-white fluorescent lamp (F15T8CW, Philips Lighting) or under the light covered by a sheet of UV-cutoff perspex (GE Lexan XL-10, Cadillac). The distance between the lamp and the flasks was 50 cm. According to the information provided by the manufacturer, the UV emission spectrum of the light source was mainly identical in the UVA

Fig. 1. Spectrum of the cool-white fluorescent lamp used in this study (a) and UV-visible light transmission of the perspex (□) and the glass flask (△) used in this study (b).

(320–400 nm) region with a peak at 366 nm (Fig. 1a). The intensities of UVB (290–320 nm) and UVC (< 290 nm) light were much lower than that of the UVA in the cool-white fluorescent light (Cebula *et al.*, 1995). The perspex sheet and the glass flask used in this study were placed into the beam of a visible–UV spectrophotometer (DU640, Beckman), and their

UV-visible light transmittance spectra in the 250–500 nm range were obtained (Fig. 1b). The perspex sheet was essentially opaque at all wavelengths below 400 nm (UVA, UVB and UVC). The glass flask was opaque to UV wavelengths of 290 nm or less (UVC). Measured by a wavelength UV-meter (UV products) outside the glass flasks, the mean fluence rate used for the cool-white fluorescent light exposure in this study was 0.2 W m^{-2} . In all experiments, non-illuminated flasks covered with aluminium foil were used as controls. Restoration experiments were performed with illuminated starvation-stressed cells, to which 26 U catalase ml^{-1} (Sigma) was added at 0 h of starvation. Viability was assessed at appropriate time intervals in terms of c.f.u. ml^{-1} on appropriate agar plates, by the drop-plate method (Hoben & Somasegaran, 1982).

Measurement of the rates of total RNA synthesis. The rate of total RNA synthesis was determined as the rate of pulse-labelled radioactive uridine incorporation into trichloroacetic acid (TCA)-insoluble material (Flardh *et al.*, 1992). Aliquots of an experimental culture were labelled with 0.13 mM [5,6- ^3H]uridine [$37.8 \text{ Ci mmol}^{-1}$ ($1.4 \text{ TBq mmol}^{-1}$), Amersham]. At appropriate intervals, 50 μl of the culture was removed and added to 800 μl ice-cold 10% TCA; the resulting preparations were kept on ice for 1 h prior to collection of the precipitated material on polycarbonate membrane filters (pore size 0.2 μm , Millipore). The precipitates were washed three times with ice-cold 5% TCA and then transferred to scintillation vials (Crown Scientific). Opti-Phase 'Hi-Safe' 3 (Fisher Chemicals) was used as the scintillation cocktail, and the radioactivity was determined with a Packard series 2000 liquid scintillation counter. The rates of uridine incorporation were determined from the slopes of the linear incorporation graphs (Nystrom *et al.*, 1986).

Measurement of ppGpp. The method used to assay ppGpp was performed as described by Cashel (1994). Briefly, cells were grown in the appropriate minimal medium with a low phosphate concentration (0.4 mM). When OD_{600} 0.05 was reached, [^{32}P]orthophosphate (Amersham) was added to a culture aliquot at a concentration of 100 $\mu\text{Ci ml}^{-1}$, and incubation was continued for about 120 min. Cell densities were monitored in an unlabelled culture aliquot. Sampling for ppGpp began at OD_{600} 0.2–0.5. The samples were then mixed with 13 M formic acid and three freeze-thaw cycles were performed. Afterwards, the samples were centrifuged and 5 μl samples of supernatant were subjected to one-dimensional TLC on polyethyleneimine sheets (Merck) and developed with 1.5 M potassium phosphate (pH 3.4). Nucleotide abundance was quantified by a Phosphor-Imager Analysis System (Bio-Rad). Amounts of ppGpp were expressed as the total activity (c.p.m.) of blank-corrected ppGpp per OD_{600} unit of culture.

RESULTS

Light-exposed '*V. angustum*' S14 *spoT* and *E. coli relA spoT* mutants display severe losses in viability during fatty acid starvation

It has previously been found that fatty acid starvation, induced by the addition of cerulenin in the presence of glucose and amino acids, stimulates *spoT*-dependent ppGpp accumulation in *E. coli* (Seyfzadeh *et al.*, 1993). To further investigate the consequences of this ppGpp accumulation in response to fatty acid starvation in bacteria, the survival abilities of *E. coli* and '*V. angustum*' S14 *relA* mutants and *spoT* mutants were

compared to those of their respective isogenic wild-type strains during fatty acid starvation (Fig. 2). After 55 h fatty acid starvation under cool-white fluorescent light, both the '*V. angustum*' S14 *spoT* mutant and the *E. coli relA spoT* mutant displayed a pronounced loss in viability (6.6 and 4.1 log, respectively) in comparison to the wild-type strains and the *relA* mutants (Fig. 2). Interestingly, these data also demonstrated that '*V. angustum*' S14 strains are more sensitive to fatty acid starvation than *E. coli* strains (Fig. 2). When the same starvation experiments were performed in complete darkness, much smaller losses in viability were observed, for all strains tested, in comparison to those exposed to light (Fig. 2). When the same starvation experiments were performed under the fluorescent light filtered by a UV-resistant perspex sheet, only minor losses in viability were observed for all strains (data not shown), which were similar to those observed in the dark (Fig. 2). These results demonstrate that the *spoT*, but not the *relA* mutations, in '*V. angustum*' S14 and *E. coli* exacerbate the decline in viability during fatty acid starvation in the light. This *spoT*-dependent increased rate of loss in viability is dependent on the UV emission from the normal cool-white fluorescent lamp. In this experiment, no significant loss in viability was observed in all strains tested under the control conditions (without fatty acid starvation in the light and dark) within 55 h (data not shown). These results specifically relate the lethal effect of light on the *spoT* mutants to fatty acid starvation, as opposed to any implication of lethality due solely to light exposure. After 5 d treatment in the light or dark, nearly all of the fatty-acid-starved cells of the tested strains were dead, whereas the non-starved cells maintained viability (data not shown), confirming the final lethal consequences of fatty acid starvation.

The '*V. angustum*' S14 *spoT* mutant displays high ppGpp levels during fatty acid starvation

While both the '*V. angustum*' S14 *spoT* mutant and the *E. coli relA spoT* mutant exhibited light-dependent fatty acid starvation lethality, the alterations in their respective ppGpp levels were distinct during fatty acid starvation (Fig. 3). Wild-type *E. coli* and '*V. angustum*' S14 displayed an initial increase in ppGpp levels upon fatty acid starvation in the presence of glucose and amino acids, followed by a gradual decrease in the ppGpp levels after 1 h starvation (Fig. 3). The '*V. angustum*' S14 *spoT* strain J109 displayed a threefold higher level of ppGpp in comparison to that of the isogenic wild-type strain S141 after 1 h fatty acid starvation, whereas the *E. coli* ppGpp⁰ strain CF1693 did not display ppGpp accumulation during fatty acid starvation (Fig. 3). In contrast, the *relA* mutants displayed the same alterations in ppGpp levels as those of the wild-type strains (Fig. 3). The higher ppGpp level observed in the '*V. angustum*' S14 *spoT* mutant during fatty acid starvation is similar to that observed during carbon starvation (Ostling *et al.*, 1995). Furthermore, an inverse correlation was observed between ppGpp levels and the synthesis rates of stable RNA during the fatty

Fig. 2. Survival of '*V. angustum*' S14 strains and *E. coli* strains under cool white fluorescent light (□) or in complete darkness (■) during fatty acid starvation. (a) Wild-type '*V. angustum*' S141, (b) '*V. angustum*' S14 *relA* mutant KF21, (c) '*V. angustum*' S14 *spoT* mutant J109, (d) *E. coli* wild-type MG1655, (e) *E. coli relA* mutant CF1652, and (f) *E. coli relA spoT* mutant CF1693. Incubation was continued for 55 h subsequent to growth arrest when the c.f.u. counts were approximately 10^8 cells ml⁻¹ (see Methods for details). Representative data from one of three replicate experiments are shown.

Fig. 3. Alterations in the ppGpp levels during fatty acid starvation under light in strains of '*V. angustum*' S14 and *E. coli*. (a) □, '*V. angustum*' S141 wild-type; Δ, '*V. angustum*' KF21 (*ΔrelA::kan*); ○, '*V. angustum*' J109 (*spoT::kan*). (b) □, *E. coli* MG1655 wild-type; Δ, *E. coli* CF1652 (*ΔrelA::kan*); ○, *E. coli* CF1693 (*ΔrelA::kan ΔspoT::cam*). Cellular ppGpp was labelled with [³²P]orthophosphate, extracted and quantified as c.p.m. per OD₆₀₀ unit by TLC. See Methods for details. Representative data from one of three replicate experiments are shown.

acid starvation conditions described above (data not shown), corroborating previous reports that have demonstrated RNA synthesis rate measurements to reflect variation in ppGpp levels (Cashel *et al.*, 1996).

Overaccumulation of ppGpp in *E. coli* causes fatty acid starvation lethality for light-exposed cells

The fatty acid starvation lethality of the *E. coli* ppGpp⁰ strain CF1693 demonstrated that ppGpp is required for survival under light. On the other hand, the higher ppGpp levels and the severe loss in viability of the '*V. angustum*' S14 *spoT* mutant during fatty acid starvation implied that overaccumulation of ppGpp also impairs successful resistance. To further investigate this hypothesis, *E. coli* RelA overexpression strains CF5747 and CF5748 (Svitil *et al.*, 1993) were assayed for fatty acid starvation survival during light exposure. IPTG-induced RelA overexpression in strain CF5747 elicited ppGpp overaccumulation: a 20-fold increase in the ppGpp levels of the IPTG-treated cells in comparison to a threefold increase in the untreated cells was observed after 1 h fatty acid starvation (Fig. 4a). This overaccumulation of ppGpp reduced the synthesis rates of total RNA (data not shown) and impaired the ability of the cells to survive fatty acid starvation in the light. A 3.2 log drop in the c.f.u. count of the IPTG-treated cells in comparison to a 0.2 log drop in the c.f.u. count of the untreated cells after 40 h fatty acid starvation was observed (Fig. 4b). In contrast, the overexpression of an inactive 331 aa RelA peptide in strain CF5748 (Svitil *et al.*, 1993) had no such effect (data not shown). The 455 aa truncated RelA peptide expressed in strain CF5747 is

Fig. 4. (a, b) Effect of RelA overexpression on the ppGpp accumulation (a) and the survival ability (b) of *E. coli* strain CF5747 during fatty acid starvation under light. RelA was induced by adding 1 mM IPTG to *E. coli* strain CF5747 carrying *P_{lac}::relA* on plasmid pALS13 (Svitil *et al.*, 1993). □, ■ denote the data for cells without and with IPTG, respectively. (c) Survival of *E. coli* strains CF4941 (Δ*relA*::*kan*) (◆) and CF4943 (Δ*relA*::*kan* *spoT203*) (●) during fatty acid starvation under light. Further details are described in Results and Discussion. Representative data from one of two replicate experiments are shown.

metabolically unstable, which avoids the toxic effect of protein overexpression (Svitil *et al.*, 1993). Taking strain CF5748 as a negative control, the data suggest that the light-dependent fatty acid starvation lethality of strain CF5747 may be due to ppGpp overaccumulation, rather than to the toxic effects of protein overexpression, plasmid maintenance, or IPTG itself. Moreover, the artificial overproduction of ppGpp resulted in reduced lethality in strain CF5747 in the absence of starvation, as well as during starvation in the dark (data not shown). In *E. coli*, the *spoT203* allele has been found to reduce ppGpp degradative activity, resulting in an eightfold elevation of ppGpp levels (Sarubbi *et al.*, 1988). During fatty acid starvation under light exposure, *E. coli* CF4943, containing the *spoT203* mutation, displayed a significant decline in viability in comparison to its isogenic parent strain, CF4941 (Fig. 4c). These results support our hypothesis that overaccumulation of ppGpp impairs the survival ability of the illuminated fatty-acid-starved cells.

Light-exposed *E. coli* strains with mutations in *rpoS* and *dps* display severe losses in viability during fatty acid starvation

It has been found that ppGpp upregulates the synthesis of the stationary-phase sigma factor RpoS in *E. coli* (Gentry *et al.*, 1993; Lange *et al.*, 1995). RpoS and RpoS-regulated proteins (e.g. Dps) are crucial for starvation-induced stress resistance and starvation survival (Almiron *et al.*, 1992; Eisenstark *et al.*, 1996; Hengge-Aronis, 1996; McCann *et al.*, 1991). To investigate the role of the RpoS regulon in fatty acid starvation, *rpoS* and *dps* mutant *E. coli* strains were compared in survival ability to the isogenic wild-type strain during fatty acid starvation (Fig. 5). After 55 h fatty acid starvation under cool-white fluorescent light, both mutants displayed significantly greater losses in viability in comparison to the wild-type strain. The *rpoS* mutant exhibited a 2.9 log

decrease in viability, whereas the corresponding values for the *dps* mutant and the wild-type strain were 5.6 log and 0.5 log, respectively (Fig. 5). When these starvation experiments were performed in complete darkness, much smaller losses in viability were observed for all strains tested in comparison to those kept in the light (Fig. 5). When these starvation experiments were performed under the fluorescent light filtered through a UV-resistant perspex sheet, the minor losses in viability, for all strains, were identical to those observed in the dark (data not shown). These data indicated that *rpoS* and the RpoS-regulated stress-resistance gene *dps* are involved in fatty acid starvation survival during light exposure. The main challenge to the cells under these conditions appears to be the UV radiation from the cool-white fluorescent lamp used. Moreover, the difference between the losses in viability of the *rpoS* and *dps* mutants during fatty acid starvation suggests a potential direct role for DNA-binding protein in starvation (Dps) in fatty acid starvation survival under light.

Addition of catalase to the illuminated culture largely restores viability of '*V. angustum*' S14 and *E. coli* *spoT* mutants during fatty acid starvation

The main fraction of the UV emission from the cool-white fluorescent light is UVA radiation (Cebula *et al.*, 1995), which causes oxidative damage to DNA and proteins in bacteria through photo-oxidation (Eisenstark, 1989). Therefore, the light-dependent lethality of the *spoT* mutants during fatty acid starvation is likely to be related to the UV-induced oxidative stress. To address the role of oxidative stress resistance in the fatty acid starvation response, we investigated the effect of catalase, an H₂O₂-degrading enzyme, on the survival of the fatty-acid-starved *E. coli* and '*V. angustum*' S14 cells. Addition of catalase resulted in an increase in viability for illuminated *spoT* mutant cultures of '*V. angustum*' S14 and *E. coli* that were fatty acid starved for 35 h, in

Fig. 6. Effect of catalase on the survival of illuminated fatty acid starved cells of '*V. angustum*' S14 and *E. coli*. (a) '*V. angustum*' S141 wild-type (squares) and '*V. angustum*' J109 (*spoT::kan*) (circles). (b) *E. coli* MG1655 wild-type (squares) and *E. coli* CF1693 ($\Delta relA::kan \Delta spoT::cam$) (circles). Experiments were performed with illuminated fatty-acid-starved cells in non-supplemented medium (solid symbols) or medium supplemented with catalase (open symbols). Representative data from one of three replicate experiments are shown.

comparison to non-catalase-supplemented cells (Fig. 6). Specifically, the '*V. angustum*' S14 *spoT* mutant displayed an increase in viability of three orders of magnitude (Fig. 6a), whereas the *E. coli* *relA spoT*

Fig. 5. Survival of *E. coli* strains during fatty acid starvation under cool-white fluorescent light (□) or in complete darkness (■). (a) *E. coli* ZK126 wild-type; (b) *E. coli* *rpoS* mutant ZK1000; (c) *E. coli* *dps* mutant ZK1058. Incubation was continued for 55 h subsequent to growth arrest when the c.f.u. counts were approximately 10^8 cells ml^{-1} (see Methods for details). Representative data from one of three replicate experiments are shown.

mutant displayed an increase of two orders of magnitude (Fig. 6b) upon the addition of catalase. The c.f.u. counts of the '*V. angustum*' S14 and *E. coli* wild-type strains were also increased slightly by catalase addition after 35 h of starvation (Fig. 6), indicating that catalase has a general effect on the fatty acid starvation survival of the illuminated cells. These results suggest that the UV-imposed oxidative stress accounts for the severe decline in viability of the fatty-acid-starved *spoT* mutant cells.

DISCUSSION

It has been reported that fatty acid starvation in the presence of glucose and amino acids stimulates a *spoT*-dependent ppGpp accumulation in *E. coli* (Seyfzadeh *et al.*, 1993). The present report demonstrates that a '*V. angustum*' S14 *spoT* mutant and an *E. coli* *relA spoT* mutant display severe losses in viability in response to cerulenin-induced fatty acid starvation under cool-white fluorescent light in the presence of glucose and amino acids (Table 2). In contrast, the respective *relA* mutants and wild-type strains exhibited little or no loss in viability under the same conditions. On the other hand, the mutant cells starved in the dark or under the light filtered by a UV-cutoff perspex sheet did not display this pronounced loss in viability. Therefore, the *spoT*-dependent increased rate of decline in cell viability during fatty acid starvation is provoked by fluorescent light illumination, in particular the weak UV emission from the light used. This light-dependent lethal effect during fatty acid starvation also occurred in the *E. coli* strains with mutations in *rpoS* and *dps* (Table 2).

Role of SpoT during carbon and fatty acid starvation

We propose that SpoT senses a signal generated in fatty acid metabolism to monitor carbon source availability during growth, and during carbon starvation. The SpoT-mediated ppGpp accumulation (Seyfzadeh *et al.*, 1993) and the *spoT*-dependent survival during fatty acid starvation are similar to the characteristics of the carbon-starved cells (Flardh *et al.*, 1994; Nystrom, 1994; Ostling *et al.*, 1995; Xiao *et al.*, 1991). These data suggest that the signalling pathway leading to ppGpp accumulation upon fatty acid starvation may be part of, or identical to, the as yet elusive SpoT-controlled carbon starvation response mechanism (Seyfzadeh *et al.*, 1993). Consistent with this idea, it was found that carbon-starved *E. coli* cells with a mutation in *accD* (a gene encoding acetyl-CoA carboxylase, a key enzyme in fatty acid biosynthesis) display an aberrant pattern of ppGpp accumulation, excessive cell division, and progressive failure to respond rapidly to a nutritional up-shift (Seyfzadeh, 1994). It is well known that the precursor of fatty acid synthesis, acetyl-CoA, is mainly produced during glycolysis when glucose acts as the sole carbon source. Therefore, glucose deprivation in bacteria apparently causes inhibition of fatty acid synthesis, leading to fatty acid starvation (DiRusso & Nystrom, 1998). Following this rationale, it is possible that SpoT normally responds to a signal produced in fatty acid metabolism to monitor carbon source abundance, and optimizes growth economy of the cell accordingly (DiRusso & Nystrom, 1998; Gentry & Cashel, 1995). This putative signal may be synthesized from fatty acid derivatives, such as fatty acyl-CoA, and inhibit the SpoT

Table 2. Relative viability of the strains in fatty acid starvation

When catalase was present it was added at 26 U ml⁻¹. All strains exhibited no differences during growth in light vs growth in dark.

Strain	Fatty acid starvation*	
	Light	Dark
' <i>V. angustum</i> ' S14 wild-type	++	+++
' <i>V. angustum</i> ' S14 wild-type + catalase	+++	+++
' <i>V. angustum</i> ' S14 <i>relA</i> mutant	+	++
' <i>V. angustum</i> ' S14 <i>spoT</i> mutant	---	+
' <i>V. angustum</i> ' S14 <i>spoT</i> mutant + catalase	+	++
<i>E. coli</i> wild-type	+++	+++
<i>E. coli</i> wild-type + catalase	+++	+++
<i>E. coli</i> <i>relA</i> mutant	++	++
<i>E. coli</i> <i>relA spoT</i> mutant	--	++
<i>E. coli</i> <i>relA spoT</i> mutant + catalase	++	+++
<i>E. coli</i> <i>rpoS</i> mutant	-	++
<i>E. coli</i> <i>dps</i> mutant	---	+
<i>E. coli</i> wild-type P _{tac} :: <i>relA</i>	+++	+++
<i>E. coli</i> wild-type P _{tac} :: <i>relA</i> + 1 mM IPTG	--	+

* ++, <0.2 log loss; +, 0.5–1 log loss; -, 1–2 log loss; --, 2–3 log loss; ---, 3–4 log loss; ---, >4 log loss.

function responsible for ppGpp accumulation and further starvation survival, perhaps by directly binding to the SpoT protein or by binding to another positive factor required for SpoT function (Seyfzadeh *et al.*, 1993). Hence, fatty acid starvation leads to the loss of this signal and upregulation of SpoT-mediated ppGpp accumulation. Alternatively, carbon starvation and fatty acid starvation may use independent signal transduction mechanisms to trigger *spoT*-dependent ppGpp accumulation and further starvation adaptive responses. Further studies of the signalling pathway(s) leading to ppGpp accumulation during fatty acid starvation are currently being undertaken.

UV-induced oxidative stress and fatty acid starvation

It is interesting that the fatty acid starvation lethality of the cells with mutations in *spoT*, *rpoS* and *dps* is provoked by standard laboratory cool-white fluorescent light. Furthermore, this lethal effect was found to be mostly due to the weak UV emission of the light. According to the information provided by the manufacturer, the main fraction of the UV emission from the cool-white fluorescent lamp is UVA radiation (Fig. 1a). The lamp also emits very low levels of UVB and UVC that may cause direct damage to DNA (Cebula *et al.*, 1995). However, the effect of UVC can be ruled out in this study since the glass flasks we used block UVC radiation (Fig. 1b). UVA and UVB produce many reactive oxygen species (ROS) through photo-oxidation and hence cause oxidative damage to DNA, proteins and membrane lipids in bacteria (Eisenstark, 1989). ROS are produced during normal cellular metabolism, but can be augmented after cellular exposure to UV, especially in the presence of various photo-sensitizers (Jagger, 1981). The light-dependent lethal effects reported here are suggested to be mainly due to UV-induced oxidative stress rather than to direct DNA damage. This hypothesis was also supported by the following findings: (i) the *spoT* mutants were found to be more sensitive to UV radiation as well as to H₂O₂ exposure in comparison to their isogenic parents during fatty acid starvation (unpublished data); (ii) addition of exogenous catalase to the growth medium during fatty acid starvation in this study reversed most of the loss in cell viability of the *spoT* mutants provoked by fluorescent light illumination (Fig. 6). We note that since only UVA and UVB of solar UV irradiation reach marine surface waters, the natural habitats of marine *Vibrio* species, our findings have clear ecological implications (Joux *et al.*, 1999). Moreover, our observed lethal effects occur in starved cells exposed to extremely weak UV radiation from laboratory cool-white fluorescent light. Its intensity (0.2 W m⁻²) is much lower than that of full sunlight (33 W m⁻²), and is typical of the intensities found in marine surface waters, where the UV radiation is quenched (Jeffrey *et al.*, 1996).

One of the factors that appear to play an important role in the response of bacterial cells to UV irradiation is the mechanism known as stringent response. It is well known that UV exposure affects tRNA containing a 4-

thiouridine modified base, which cross-links with cytidine, losing its charge capacity and triggering the stringent response with RelA-mediated ppGpp accumulation and the consequent arrest of rRNA and protein biosynthesis. This mechanism induces a growth delay, including impairment of replication and membrane integrity in *E. coli* (Jagger, 1981; Thiam & Favre, 1984), *Salmonella typhimurium* (Kramer *et al.*, 1988) and *Pseudomonas aeruginosa* (Fernandez & Pizarro, 1999). It may be questioned whether the UV emission employed in this study, rather than fatty acid starvation, provoke the ppGpp accumulation observed in this study. However, our experimental conditions are distinct from the UV radiation used in the studies reported above. Firstly, the fluence rate of UV emission used here is 0.2 W m⁻², which is much lower than the levels reported before: 216 W m⁻² for *E. coli* (Jagger, 1981; Thiam & Favre, 1984), and 35 W m⁻² for *S. typhimurium* (Kramer *et al.*, 1988) and *P. aeruginosa* (Fernandez & Pizarro, 1999). Secondly, in contrast to the findings reported in the papers cited above, we did not observe growth delay or significant ppGpp accumulation in the growing cells illuminated by the cool-white fluorescent light (data not shown). Thirdly, the patterns of ppGpp accumulation we observed for the fatty-acid-starved cells in the light and dark are similar (data not shown), suggesting that starvation, instead of light, is needed as a means of inducing ppGpp. It is therefore suggested that the extremely weak UV emission used in this study do not serve as a factor to trigger RelA-mediated ppGpp accumulation in the starvation adaptive response, but act as a stress challenge to the starved cells.

SpoT-mediated ppGpp accumulation has been shown to be important for carbon starvation survival (Nystrom, 1994; Ostling *et al.*, 1995). The stationary-phase sigma factor RpoS is required for resistance to nutrient starvation, UVA radiation and oxidative stress (Eisenstark, 1998; Eisenstark *et al.*, 1996; Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994). The RpoS-regulated protein Dps (DNA-binding protein in starvation) is also known to be important in oxidative stress resistance, via protection of DNA against ROS attack (Almiron *et al.*, 1992). Hence, the light-dependent lethality of the *spoT*, *rpoS* and *dps* mutants during fatty acid starvation may not be surprising. However, it is worthwhile to note the differences among the losses in viability during fatty acid starvation for the *relA spoT* mutant, the *rpoS* mutant and the *dps* mutant. The greater lethality for the *relA spoT* mutant in comparison to that for the *rpoS* mutant can be explained by the recent finding that ppGpp is required for the expression of RpoS-controlled genes in a RpoS-independent manner (Kvint *et al.*, 2000). Furthermore, McGlynn & Lloyd (2000) demonstrated that ppGpp plays a direct role in promoting the ability of *E. coli* cells to survive UV-induced DNA damage. This increased survival may be explained by the destabilization of RNA polymerase promoter open complexes, or by stalled elongation complexes (Bartlett *et al.*, 1998; Zhou & Jin, 1998), thereby minimizing stalled RNA polymerase blocks to

replication fork progression and excision repair. The greater lethality of the *dps* mutant in comparison to that of the *rpoS* mutant indicates that Dps may play a more direct role in protecting cells against stress attack in starvation. This assumption is also supported by the multi-factorial regulation of Dps expression, i.e. it is activated by OxyR during exponential phase and by IHF and RpoS in stationary phase (Altuvia *et al.*, 1994). Together, these results suggest that the fatty acid starvation adaptation of the illuminated bacterial cells requires successful defence against UV-imposed oxidative stress, and that the starvation-induced stress resistance is dependent on *spoT*-dependent ppGpp accumulation, as well as the upregulation of *rpoS* and RpoS-controlled stress resistance genes such as *dps*.

Accumulation of ppGpp and fatty acid starvation survival

The light-exposed *E. coli relA spoT* mutant CF1693 loses cell viability and fails to accumulate ppGpp following fatty acid starvation. However, the light-exposed *E. coli relA* mutant maintains cell viability and accumulates ppGpp as the wild-type strain does during fatty acid starvation. This suggests a correlation between the loss in viability and the lack of *spoT*-dependent ppGpp accumulation. A similar correlation has been found in carbon starvation adaptation, in which ppGpp accumulation during the first few hours of starvation has a long-term effect on the survival ability for many days (Matin, 2000; Nystrom, 1994). The fatty acid starvation lethality of the *E. coli relA spoT* deletion mutant is not surprising, since this ppGpp⁰ strain is more sensitive to many other stresses, including carbon starvation and amino acid starvation (Nystrom, 1994; Xiao *et al.*, 1991). The '*V. angustum*' S14 *spoT* mutant J109 displayed threefold higher ppGpp levels and a severe loss in viability in comparison to the wild-type strain S14 during fatty acid starvation. J109 contains a *Tn10* insertion in the middle of the *spoT* gene and exhibits higher ppGpp accumulation as well as light-dependent loss in viability during carbon starvation (Ostling *et al.*, 1995). Therefore, the transposon insertion *spoT* mutant of '*V. angustum*' S14 is different from the *relA spoT* deletion mutant of *E. coli*. Although the mechanism of the ppGpp overaccumulation in J109 is unknown, the similar transposon disruption mutants in *E. coli* (Sarubbi *et al.*, 1989) suggest that it may be due to the reduced ppGppase activity of SpoT. Thus, the fatty acid starvation lethality of light-exposed '*V. angustum*' S14 *spoT* mutant cells appears to depend on a greater and prolonged elevation of ppGpp levels, caused by the truncated SpoT protein.

Artificial overproduction of ppGpp by overexpression of a recombinant *relA* in *E. coli* during fatty acid starvation under light led to a marked loss in cell viability, which was analogous to the results observed for the '*V. angustum*' S14 *spoT* mutant. Also, the *E. coli spoT203* allele, found to reduce ppGpp degradative activity resulting in an overelevation of ppGpp levels

(Sarubbi *et al.*, 1988), significantly exacerbates the decline in viability during fatty acid starvation under light. These results support our hypothesis that overaccumulation of ppGpp impairs the survival ability of the illuminated fatty-acid-starved cells. Apirakaramwong *et al.* (1999) reported a similar decline in cell viability in stationary phase due to artificial ppGpp overaccumulation. After overexpression of a recombinant constitutive *relA* in *E. coli*, a rapid increase in the levels of ppGpp was observed, accompanied by a severe loss in viability and a decrease in the RpoS level (Apirakaramwong *et al.*, 1999). This finding is unexpected because it has been reported that RpoS synthesis is upregulated by ppGpp (Gentry *et al.*, 1993; Lange *et al.*, 1995). However, it suggests that the prolonged ppGpp accumulation may become toxic for cells in late-phase starvation through the reduction of RpoS levels. Since accumulation of ppGpp has the dual consequences of stimulating and inhibiting gene expression, it is likely that the proper balance of these effects must be set for an appropriate physiological response. In other words, overaccumulation of ppGpp may inhibit rRNA synthesis and down-regulate the protein synthetic capacity to a level which is below that required for sufficient translation of stress resistance proteins. Although the lethal effect of ppGpp overaccumulation with fatty acid starvation was more significant than that without starvation, the sole killing effect of ppGpp overproduction might not be ruled out, which was supported by recent studies (Aizenman *et al.*, 1996; Sat *et al.*, 2001) showing that MazEF-mediated programmed cell death is induced by the artificial overproduction of ppGpp, leading to higher concentrations than those of physiological conditions.

Based on the results presented in this study, we propose that successful fatty acid starvation survival of the light-exposed bacterial cells depends on an adaptive response, which requires appropriate *spoT*-dependent ppGpp accumulation, the upregulation of RpoS and hence the induction of RpoS-controlled stress resistance proteins such as Dps. Lack, or overaccumulation, of ppGpp impairs the fatty acid starvation survival of light-exposed cells. These early-phase starvation-induced proteins are able to exert prevention and/or repair of the UV-induced oxidative damage, leading to effective protection against naturally occurring weak UV radiation.

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