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Thiouridine residues in tRNAs are responsible for a synergistic effect of UVA and UVB light in photoinactivation of *Escherichia coli*

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Summary

Since different wavelengths of light impact different cellular targets, microorganisms exposed to natural sunlight experience a combination of multiple stressors. In order to better understand the effects of sunlight on microorganisms we, therefore, need to understand how different wavelength act alone and in combination. Here, we describe a synergistic effect between UVA and UVB irradiation on viability of *Escherichia coli* bacteria. To investigate the basis of this synergistic effect we analysed mutant strains that were obtained through selection for increased resistance to combined UVA and UVB. By identifying and reconstructing genetic changes in the resistant strains we provide evidence that UVA-absorbing thiouridine residues in tRNAs are the key to the synergistic effect. Our study provides insights into how naturally occurring combinations of stressors can interact, and points to new ways for controlling microbial populations.

Introduction

How combinations of stresses act on organisms is a fundamental question in biology. Our focus, here, is on bacteria, which can be exposed to various stresses including elevated temperature, salinity, light, as well as the presence of chemical and biochemical stressors (Lund, 2013). The impact of one stressor can be modified by the presence of other stressors. Understanding whether and how different stressors interact in their effects on bacteria is an important goal of environmental microbiology, because in natural environments, these stresses often appear in combination.

One important type of stressor that naturally arises in combinations is natural light. Sunlight consists of a continuum of different wavelengths, and different wavelength ranges have different specific effects on organisms, including the incurrence of cellular damage. The main source of damage to organisms comes from the ultraviolet (UV) part, and the nature of the UV damage is dependent on the wavelength. The most energetic part of natural solar UV radiation, the middle ultraviolet (UVB, 290–320 nm) region, can be absorbed by and thereby be damaging to DNA; it is, therefore, often considered the main cause of mortality in cells exposed to sunlight (Cadet *et al.*, 2005; Eischeid and Linden, 2007; Santos *et al.*, 2013; Beck *et al.*, 2014). Most of the UV photons in sunlight are in the near ultraviolet (UVA, 320–400 nm). UVA induces mainly sublethal effects that damage cells without killing them, for example, oxidative stress, protein damage, delayed growth and decreased energy metabolism (Coohill and Sagripanti, 2009; Bosshard *et al.*, 2010; Fisher *et al.*, 2012; Sassoubre *et al.*, 2012; Cadet *et al.*, 2014).

Our goal, here, was to investigate how light of different wavelengths contributes to inactivating bacteria, and how different wavelengths interact in their effects on bacteria. For UVA and UVC irradiation both antagonistic and synergistic interactions have been reported (Webb *et al.*, 1978; Nakahashi *et al.*, 2014). These interactions were associated with the effect UVA has on UVC-induced DNA damage; either through photoreactivation or by impairing the DNA repair machinery (Webb *et al.*, 1978; Nakahashi *et al.*, 2014). For combinations of different wavelengths of UVA antagonistic, additive, or synergistic interactions have been reported, dependent on the combination of wavelength that was used (Tyrrell and Keyse, 1990). Previous reports thus establish that light of different wavelengths can interact in different ways, but the mechanisms underlying these interactions usually remain elusive.

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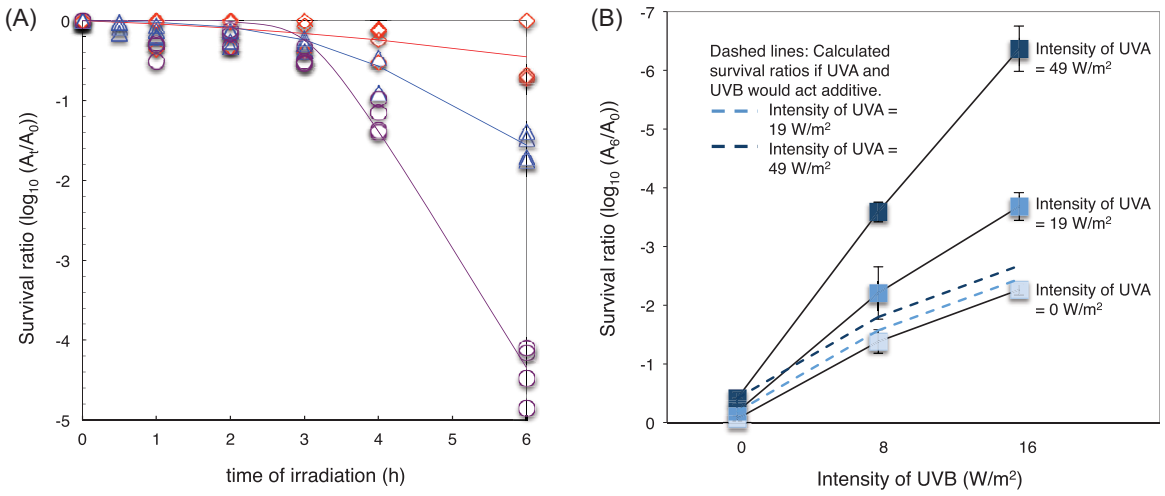

Fig. 1. Light exposure experiments where the decrease in the number of viable cells over time was measured.
A. Time course of the inactivation of *E. coli* upon irradiation for 6 h with either UVA irradiation only (red diamonds), UVB irradiation only (blue triangles) or both at the same time (purple circles). For each light condition, one representative experiment with four biological replicates per treatment is shown. Each point represents the number of colony forming units (cfu) relative to the cfu at time zero in one replicate. Solid lines: the fit of a shoulder-log linear model to the inactivation data (Geeraerd *et al.*, 2005).
B. Inactivation after 6 h with different intensities of UVA irradiation and UVB irradiation. While the irradiation with UVB alone (light blue) decrease the survival ratio by one to two orders of magnitude the addition of UVA increase the inactivation up to six orders of magnitude. The error bars represent standard errors of the mean of at least three independent experiments. The synergistic effect between UVA and UVB irradiation manifests as a significant interaction term in an analysis of variance (one way ANOVA, UVA $\times$ UVB interaction, $P < 0.001$, $N = 105$).

How different components interact in their effect on bacteria also has important implications on technical applications like wastewater treatment or drinking water disinfection. For many years, sunlight has been promoted by the World Health Organization (WHO) as an inexpensive alternative to industrial drinking water treatment, especially for developing countries (Fewtrell *et al.*, 2005). A better understanding of the direct and combined effects of different wavelength of solar UV can potentially inform the development of new and improved ways of solar water disinfection.

Our goal was to investigate if and how UVA and UVB irradiation interact in their inhibiting effects on the bacterium *Escherichia coli*, and to elucidate cellular mechanisms underlying a potential interaction. We found that UVA, ineffective on its own, could dramatically increase the effectiveness of UVB. The basis of this synergistic effect was uncovered through the analysis of mutant strains that were obtained through selection for increased resistance to dual wavelength irradiation. The genetic differences between the resistant mutants and the ancestral strain indicated that UVA-absorbing thiouridine residues in tRNAs are the key to the synergistic effect.

Results and discussion

Interaction of UVA and UVB irradiation during inactivation of E. coli

We first analysed the sensitivity of *E. coli* to UVA and UVB irradiation. Specifically, we exposed cultures of *E. coli* MG1655 to UVA (at 365 nm) and UVB irradiation (at 311 nm) and to a combination of both, and determined survival by plating (Fig. 1A). The samples irradiated with UVA showed a small and statistically not significant decrease in viable cell numbers of 21% +/− 36% (mean +/− SE, $P > 0.1$) after 6 h of irradiation (Fig. 1B). UVB irradiation led to an approximately exponential decrease after a lag time of about 3 h (Fig. 1A). After 6 h about 96% +/− 3% (mean +/− SE) of the cells were inactivated (Fig. 1B). If UVA irradiation was applied in addition to the UVB irradiation, the inactivation rate increased dramatically (Fig. 1A). After 6 h of combined UVA and UVB irradiation about 99.94% +/− 0.02% (mean +/− SE) of the cells were inactivated (Fig. 1B). While the applied UVA dose had no clear effect on cell viability alone, it enhanced the inactivation by UVB about 100 times.

One possible explanation for this UVA-induced enhancement of inactivation is that a certain threshold of damage has to be exceeded to inactivate a cell; exposing cells to both UVB and UVA simultaneously increased the number of photons reaching each cell. If this explanation were true, then the inactivation achieved by UVA alone or UVB alone should also demonstrate this threshold effect. To test this, we varied the light intensities of the UVA and UVB individually. A doubling of the UVA light intensity led to a decrease in the number of cultivable cells by around 41% (+/− 22%; SE) (Fig. 1B). This is in accordance with previous findings (Berney *et al.*, 2007). After doubling the UVB light intensity 98.5% +/− 1% (mean +/− SE) of the cells were

inactivated after 6 hours of irradiation (Fig. 1B). For both wavelengths, this is in agreement with the linear relationship between inactivation and light intensity found previously (Bosshard *et al*., 2009). At higher light intensities, UVA and UVB still acted synergistically (Fig. 1B). Therefore, the observed high level of inactivation for cells simultaneously exposed to UVB and UVA was not simply a consequence of increased light intensity. Hence, it appears that UVA and UVB act synergistically.

This raises the question about the cellular basis of this synergistic effect. In order to try to answer this question, it is important to first consider how UVA and UVB irradiation affect the cell individually. UVB damage is mainly attributed to direct light absorption by DNA and subsequent DNA damage. To a lesser extent, UVB is also able to induce protein damage and oxidative damage (Webb *et al*., 1978; Cadet *et al*., 2014). UVA can, at sublethal levels, induce growth delays, a decrease in protein production, slow the cell cycle and induce oxidative stress (Jagger, 1981; Cadet *et al*., 2014). Our results indicate that one or several of these sublethal UVA-induced stress reactions interact synergistically with UVB induced DNA damage. We see two possibilities: Either the additional damage to DNA or proteins induced by UVA adds damage beyond repair, due to amount or nature of the damage. Or the recovery from UVB damage is impaired; either by a specific mechanism or by a general change in cellular state induced by UVA.

Selection of strains with an enhanced survival to the synergistic effect of UVA and UVB

To gain insight to biological basis of the synergistic effects of UVB and UVA, we aimed to obtaining mutants with enhanced survival under combined UVA and UVB irradiation. We reasoned that by identifying bacteria that can evade the synergistic effect of these two wavelengths could help us understand how this effect arises in the first place. We thus performed a selection experiment where we repeatedly subjected populations of *E. coli* to irradiation with combined UVA and UVB irradiation, selecting for strains resistant against this irradiation regime. The selection experiment was done with two ancestor strains derived from *E. coli* MG1655. In one ancestor the *lacZ* gene was replaced with mCherry, a gene encoding a red fluorescence protein (ancestor1); in the other ancestor a gene encoding a green fluorescence protein (GFP) replaced *lacA* (ancestor2). We used two marked strains as ancestors for two reasons. First, they allowed us to perform competition experiments between the ancestors and evolved strains to detect small differences in UV-sensitivity. Second, the use of two differently marked ancestors also served as a control for contamination of the selected populations and cross-contaminations between replicated populations. To initiate the selection experiment, we inoculated six cultures from each of the two ancestors, resulting in 12 parallel selection lines. These selection lines were grown overnight to stationary phase. On day 1, batch cultures were inoculated with the overnight cultures and grown to exponential phase. The cells were harvested and irradiated in buffer with combined UVA and UVB irradiation for 8 h. The survivors were grown over night to stationary phase. On day 2, we diluted the cultures, grew them for one hour to reach the exponential growth phase and exposed them again to combined UVA and UVB irradiation for 8 h. This selection was repeated for eight consecutive days (Fig. 2A). One replicate population of each ancestor was handled the same way but kept in the dark during irradiations. From day 2 on, a fresh clonal isolate of each ancestor was irradiated in parallel to compare to the selection lines. The evolving populations were monitored daily during the selection experiment by measuring the survival by plate count.

We found that, in the course of the eight cycles of the selection experiment, the experimental populations showed substantial adaptation to the selective conditions. At the beginning of the experiment the irradiation led to a decrease in the number of bacteria that were capable of forming a colony by four to five orders of magnitude. Following the seventh cycle, the same light exposure only led to a decrease by one to two orders of magnitude (Supporting Information Fig. S2). This suggested that these populations contained mutants with increased resistance to the light regime we imposed.

Our next goal was thus to isolate such mutants with increased resistance. From each population we obtained between 6 and 30 clonal isolates and analysed their inactivation in combinations of UVA and UVB. From 10 of the 12 evolved populations, we were able to obtain clonal isolates with an enhanced resistance against combined UVA and UVB (strains SP01–SP12; survival under combined UVA and UVB irradiation: Supporting Information Fig. S3). On average, the survival of these isolates was around 100 times higher compared with the ancestor (Fig. 2B). These evolved strains were further analysed by exposing them to UVA and UVB alone. The survival under UVB irradiation alone did not differ between the ancestors and the evolved strains (ancestor: -1.6 $\log_{10}$ reduction (mean) $+/-$ 0.2 (SE); evolved strains: -1.4 $+/-$ 0.2; $P > 0.7$) (Fig. 3B). In the evolved strains, the inactivation after 6 h of irradiation was similar if UVA was applied or not during UVB irradiation (UVA + UVB: -1.3 $\log_{10}$ reduction (mean) $+/-$ 0.2 (SE); UVB only: -1.4 $+/-$ 0.2; $P > 0.5$). Hence, this indicated that in the evolved strains were not sensitive to the synergistic effect of UVA and UVB, and that inactivation was largely due to UVB irradiation alone. The 10 evolved strains tested showed slightly different levels of resistance to the synergistic effect but for all evolved strains, compared with the dark controls, the number of surviving cells

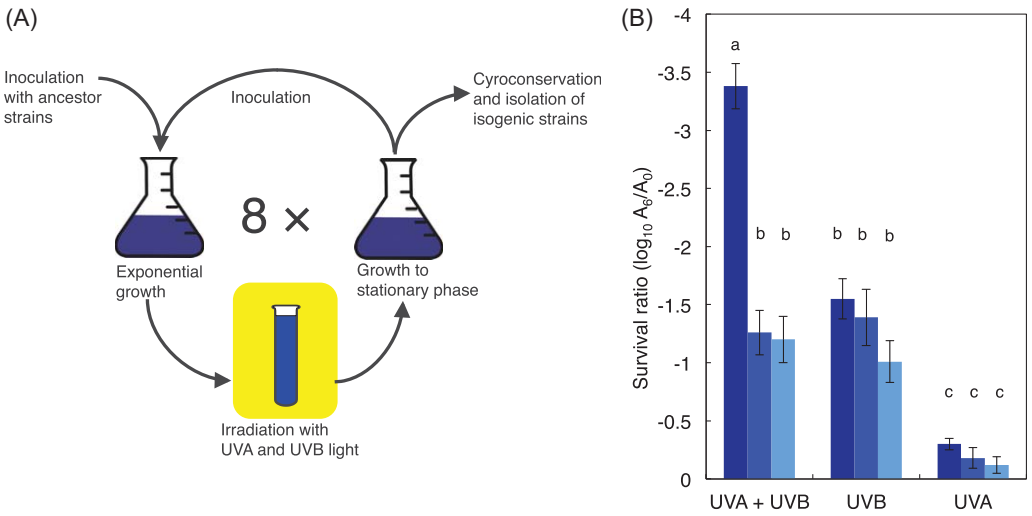

Fig. 2. A. Schematic representation of the selection experiment designed to enrich for mutants that are more resistant to combined UVA and UVB irradiation. 12 parallel selection lines were started with wild type *E. coli* and irradiated with combined UVA and UVB irradiation. The survivors were grown to stationary phase. This stationary phase cells were used as the inoculum for the next round of UV treatment. This was repeated eight times. After the eighth round we obtained clonal isolates from each of the 12 selection lines. B. Inactivation data after 6 h of irradiation of the ancestor strains (dark blue), the evolved strains (blue) and the ancestor strains with a mutated *thiI*-allele (light blue) for each of the three light conditions. A Tukey HSD post hoc test in an analysis of variance revealed three subsets where the means are not significantly different (a, b: $P = 0.37$, c: $P = 0.98$). These homogeneous subsets are marked with the letters a, b and c. The bars represent the mean of at least three experiments with a minimum of four biologically independent samples each. Error bars represent standard errors of the mean. The strain and the light treatment had a significant influence on the photoinactivation, and the effect of the light treatment differed between strains (two way ANOVA, light, $P < 0.001$; strain, $P < 0.001$; light × strain interaction, $P < 0.001$, $N = 176$).

was at least 70 times higher after 6 h of simultaneous exposure to UVA and UVB irradiation (Supporting Information Fig. S3).

While the survival under the conditions of the selection experiment (irradiation with a mix of UVA and UVB) was enhanced, no significant change in susceptibility to UVB could be detected (Fig. 2B). Since the effect of UVB is mainly attributed to direct DNA damage, we hypothesize that the selection experiment did not change the amount of UVB-induced DNA damage. Hence, the increased survival is probably a consequence of increased resistance against UVA, which on its own did not significantly affect cellular viability. To study the basis of this effect we aimed to find genetic differences between the ancestor strains and the evolved strains.

Sequencing of strains with an enhanced survival to UV

We used whole-genome sequencing to identify the genomic modifications responsible for the phenotypic changes in the evolved strains. We sequenced the genome of the two ancestors, the corresponding dark controls and of the 10 evolved strains with enhanced resistance to UV from our selection experiment. We identified 1–15 genomic changes (excluding synonymous mutations) in each evolved strain compared with the ancestor, all single point mutations or short deletions of up to four bases (Supporting Information Table S2). No genetic difference between the ancestors and the dark controls could be observed. Deletions accounted for 28% of all genetic changes, insertions for 4% and point mutations for 67%. The point mutations had an over representation of C->T::G->A (62%). This implicated a mutagenic effect of the applied light (Brash, 2015). One gene stood out because it was mutated in all evolved strains: *thiI*. No other gene was mutated in more than one evolved strain. The mutant *thiI* alleles were characterized by six different point mutations and in one case a change in two adjacent bases, hence, we identified seven mutant alleles of *thiI* (Table 1). The fact that a mutation in *thiI* was found in every evolved strain we sequenced pointed toward an important role of the gene product ThiI in coping with the applied light stress.

ThiI: The critical enzyme for the interaction of UVA and UVB irradiation

To test whether the mutations in *thiI* were necessary and sufficient for the increased resistance to the combination of UVA and UVB irradiation, we engineered strains that only differed from the ancestor by one of the seven different mutations in *thiI* found in the evolved strains. We then tested whether these mutations alone could explain the phenotypic change we observed by measuring the survival of these strains under our different light conditions. The evolved strains all showed a decreased sensitivity towards

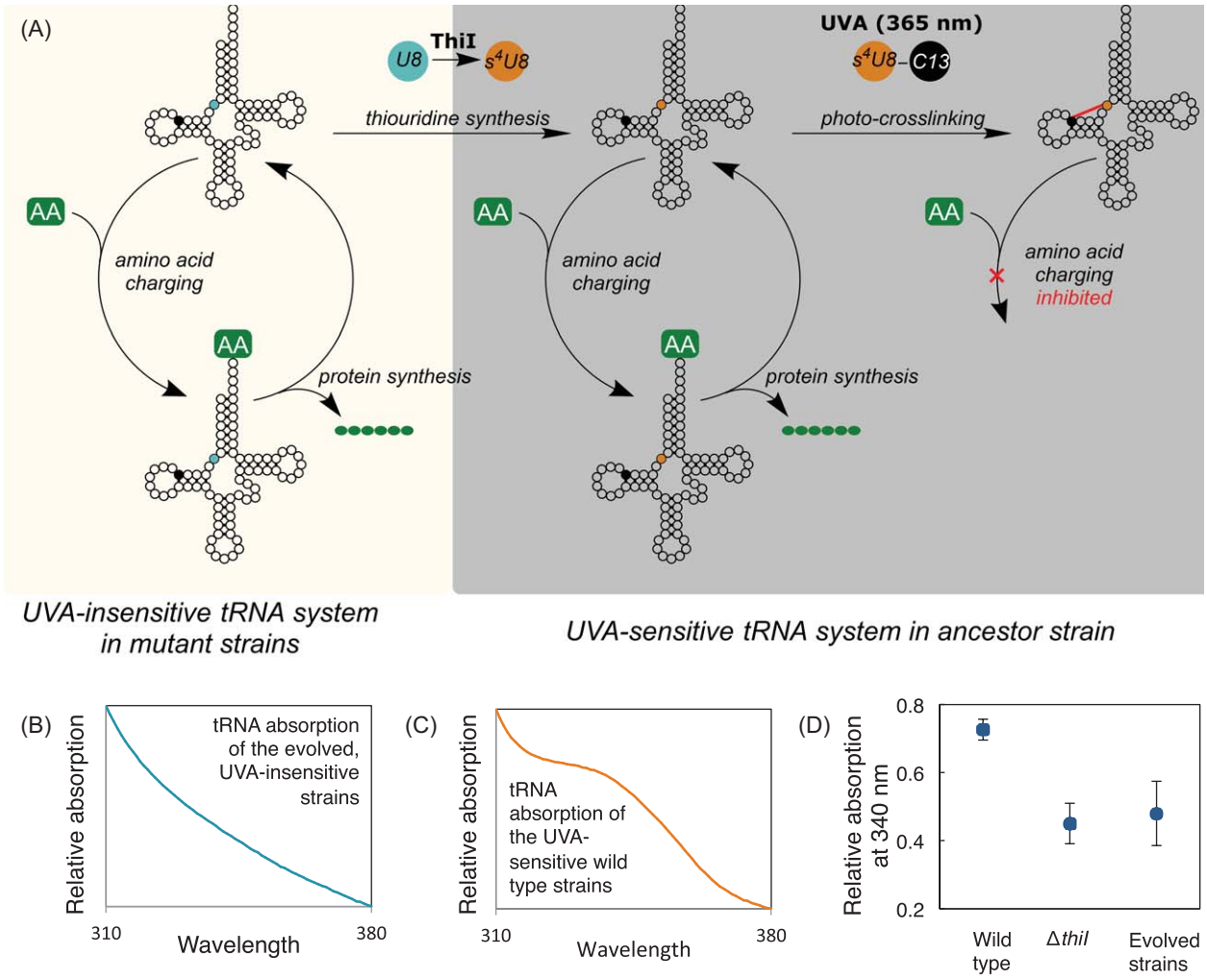

Fig. 3. A. Schematic representation of the proposed mechanism for tRNA-inactivation by UVA. Right panel (grey): situation with the wild type *thil*-allele. s^4U_8 is photo-crosslinking under UVA irradiation, thereby inhibiting amino acid charging; Left panel (white): In the evolved UVA-insensitive strains U_8 is not modified. Amino acid charging is not inhibited upon UVA irradiation. B. Relative absorption spectra between 310 and 380 nm of tRNA extracted from the evolved strains. C. Relative absorption spectra between 310 and 380 nm of tRNA extracted from the ancestor strains. D. Relative absorbance at 340 nm of extracted tRNA. For the ancestor strains, two tRNA extracts of each ancestor were measured. For SP40 (ancestor1 Δ*thil*) we measured four tRNA extracts and for each evolved strain two tRNA extracts (20 in total). Blue dots represent the mean; the error bars the standard deviation.

Table 1. *thil*-alleles isolated from the populations selected for increased resistance to the combined effect of UVA and UVB.

Position	Mutation	Strain	Annotation
440800	GA→AG	SP12	E7R (GAA→AGA)
440813	A→T	SP01	K14I (AAA→ATA)
441326	C→T	SP03 and SP04	S185F (TCC→TTC)
441350	C→T	SP07	S193F (TCC→TTC)
441587	C→A	SP02	A272E (GCA→GAA)
441634	G→A	SP08 and SP09	E288K (GAA→AAA)
441796	G→A	SP10 and SP11	E342K (GAA→AAA)

Position in the genome of *E. coli* wt (column 1), differences in the nucleotide sequence compared with the wild type (column 2), strains of the selection experiment in which this mutation was found (column 3), change in the amino acid sequence (column 4).

the combination of UVA and UVB irradiation (Fig. 2B). The inactivation of the ancestor strains with any of the mutant *thil*-allele that we tested was not significantly different from the evolved strains with the same allele in *thil* (Supporting Information Fig. 3). This indicates that the mutations found in *thil* of the evolved strains indeed were responsible for the disappearance of the synergistic effect of UVA and UVB irradiation.

This raises the question about the mechanistic link between ThiI and UV sensitivity. ThiI, the protein relevant for the synergistic effect of UVA and UVB damage, is a sulfur transferase common to the biosynthetic pathway to both thiamine and 4-thiouridine (s^4U) in tRNAs (Palenchar, 2000). Unlike the four standard nucleosides, this thiolated

uridine has an absorbance that extends into the UVA range. s^4U is common in bacterial tRNA. In *E. coli*, about 70% of the tRNA molecules contain this thionucleoside (Favre, 1974). The absorption of UVA triggers a photoreaction that results in the formation of an intra-molecular cross-linking (Thomas and Favre, 1980). Cross-linked tRNAs are restricted in the amino acid charging capacity resulting in an accumulation of uncharged tRNAs (Carre *et al.*, 1974) (Fig. 3A). If the concentration of uncharged tRNAs reaches a critical level in bacterial cells they induce growth arrests and shut down protein production by triggering the stringent response (Ramabhadran and Jagger, 1976). The decrease in protein production could lead in turn to a decreased DNA damage repair ability. Indeed, it has been shown that UVA slowed down the recovery of thymine dimers in *E. coli*, a type of DNA damage also caused by UVB irradiation (Nakahashi *et al.*, 2014). Therefore, it is plausible that reduced activity of ThiI leads to reduced DNA repair capacity and therefore to UV sensitivity.

To test this notion, we analysed whether the mutations in *thiI* indeed decrease the cellular level of s^4U, and could thereby reduce sensitivity towards UVA irradiation. Thiouridine-containing tRNAs absorb in the UVA range and have a peak between 320 and 380 nm with a maximum at 334 nm in the absorption spectrum (Favre, 1974). UVA irradiation at 365 nm lies to the red of the absorption maximum of s^4U, but lies within the absorption band. It has previously been shown that 365 nm light is able to induce crosslinking reactions involving thiouridines (Hajnsdorf *et al.*, 1986; Woisard *et al.*, 1994). We measured the absorption spectrum of total tRNA extracts from the different strains with the wild type *thiI* allele and the different mutant *thiI* alleles. A peak between 320 and 380 nm could only be observed in tRNA extracted from ancestor stains and the dark controls (Fig. 3C) but not in the evolved strains (Fig. 3B). The absorption spectrum of the extracted tRNA of the evolved strains (SP01–SP12) was comparable to the absorption spectrum of the ancestor strain with a clean deletion of the *thiI* locus (SP40) (Fig. 3D). Therefore, we concluded that the mutations in *thiI* lowered the ability of ThiI to modify uridine in tRNA, potentially by decreasing or eliminating the activity of the enzyme. This observation is consistent with the interpretation that the UVA chromophore s^4U is responsible for sensitivity towards UVA in *E. coli*, and that a reduction in this chromophore in the evolved strains explains their increased resistance.

As *thiI* is also involved in the biosynthesis of thiamine, it is reasonable to ask whether thiamine is involved in the UVA-sensitivity of *E. coli*. While we do not have a definitive answer, we currently disfavour a role of thiamine in the UVA-induced photosensitivity for several reasons. First, if thiamine would be responsible for the observed synergistic effect, it would be likely that mutations in other genes coding for proteins in the thiamine biosynthesis pathway would be detected as well, and this was not the case. Second, *thiI* and *iscS* are the only genes common to the biosynthetic pathway of thiamine and s^4U. Both were first characterized in screenings for minimalized UV-sensitivity (as nuvA and nuvC) (Ramabhadran and Jagger, 1976; Ryals *et al.*, 1982; Taylor *et al.*, 1998); to our knowledge, no other enzyme of the biosynthetic pathway of thiamine has been reported to influence the UV sensitivity of *E. coli*. Finally, thiamine is not a UVA chromophore and is therefore not a likely locus of UVA photosensitivity.

From our data, we propose the following mechanism for the synergistic effect of UVA and UVB for inactivation of *E. coli*. Most tRNAs of *E. coli* contain thiouridines, which are UVA-sensitive chromophores. Light absorption of thiouridine causes tRNA crosslinking, which blocks the charging of the tRNA with amino acids and reduces protein synthesis. A reduction in protein synthesis then leads to a decrease in DNA repair capability, rendering direct DNA damage by UVB irradiation more effective and explaining the synergistic effect.

In conclusion, we have found that UVA and UVB irradiation can act synergistically in the photoinactivation of *E. coli*, and that the 4-thiouridine normally present in the tRNA of *E. coli* is most likely responsible for its UVA-sensitivity. This system represents an exemplary case of naturally occurring multiple stressors, and of synergistic interaction between multiple stressors in their effects on organismal viability. The results are also potentially relevant from an applied perspective, for example, in the context of solar water disinfection. The findings emphasize the importance of different wavelengths and their interaction during solar water disinfection. Shielding of light from certain wavelength, either by the container used or by the water column, can be a critical factor to the success of solar water disinfection.

Experimental procedures

Bacterial strains

All strains used in this study were derived from the *E. coli* K12 strain MG1655 and are listed in Supporting Information Table S1. For the light exposure experiments MG1655 was used. For the selection experiment and consecutive experiments, two strains carrying a fluorescent marker were constructed as described previously (Datsenko and Wanner, 2000). In the first strain, which we called ancestor1, *lacZ* was replaced with the gene encoding the red fluorescent protein mCherry. For the second strain, which we called ancestor2, *lacA* was replaced with the gene encoding the green fluorescent protein GFP.

Light conditions

As the UVA light source, we used a 200 W medium-pressure Hg-vapour lamp (Newport, APEX1) with a band pass filter

(340–380 nm; Thorlabs; FB360-10) that only passed the 365 nm line. To reduce light intensity, a neutral density filter (Thorlabs; NE201B) was used when appropriate. As the UVB light source, we used a 40 W narrow band 311 nm UVB lamp (Housing: Chongqing Derma Optic and Electronic Technique Co., Ltd; bulbs: Philips; PL01). Two UVB lamps (2 × 40 W) were used simultaneously to increase light intensity, as indicated. The light spectra of the used lamps were measured with a fiber optic spectrometer (Jaz Spectrometer, Ocean Optics) (Supporting Information Fig. 1). The lamps were placed in front of a turntable to allow equal irradiation of max. 16 test tubes simultaneously. To determine the light intensity, we used a two-component chemical actinometer solution of p-nitroanisole (PNA) and pyridine (pyr) (Dulin and Mill, 1982). The decrease of the PNA concentration was determined by HPLC (Dionex, UltiMate 3000 LC) with an Ascentis RP-Amide C18, 4.6 × 250 mm, 5 μm particle size column, with a mobile phase composition of 65:35 ACN:H_2O and a flow rate of 1 ml/min. The absorbance was monitored with a PDA detector at 314 nm.

Light exposure experiments and plate count assay

For each experiment, 4 cultures were prepared by adding a single bacterial colony to 5 ml sterile M9 minimal medium [M9 minimal salts (Sigma-Aldrich), 10 mM glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$] and incubated at 37°C overnight. For batch cultivation, 25 ml of M9 minimal medium were inoculated in an Erlenmeyer flask with 3 ml of the overnight culture and incubated with vigorous shaking until an OD_{600} of 0.6–0.7 was reached. The cells from the batch culture were harvested by centrifugation (4000 g for 10 min) and washed two times with sterile M9. The cells were then diluted with M9 buffer (1 × M9 minimal salts, Sigma-Aldrich) to an OD_{600} of 0.02. Aliquots of 4 ml were exposed to light in 6 ml borosilicate test tubes (diameter 13 mm). The tubes were placed in a turntable, which enabled the equal irradiation of all test tubes.

To directly compare the photoinactivation of different strains, competition experiments were performed. The probes were prepared as described above but for irradiation, two strains were mixed at equal ratios to a total OD_{600} of 0.02. In each case, one strain was ancestor1 or its descendant (no functional *lacZ*), the other a wt, ancestor2 or its descendant (functional *lacZ*) to enable blue-white screening.

Bacterial inactivation was quantified using a colony-forming assay. The irradiated samples and the dark controls were sampled every hour. The bacterial suspensions were diluted in M9 buffer, plated on M9 agar plates [M9 media with 1.5% agar (Sigma-Aldrich)] for light exposure experiments or on LB plates containing X-Gal (40 μg/ml, Promega) and IPTG (0.5mM, Sigma-Aldrich) for competition experiments and incubated at 37°C for 18 h. Following the incubation, the number of colonies was counted. The survival ratio was calculated using the following equation:

$$\text{survival ratio} = \log_{10}(A_t/A_0)$$

where A_0 is the colony count at the begin and A_t the colony count at a certain time point t.

To assess the significance of the effect of the light used, P values of interaction tests were determined in an analysis of variance of the type of light (UVA, UVB, UVA × UVB as factors). To compare different strains, the P values of interaction tests were determined in an analysis of variance including genotype and type of light (strain, light, strain × light) as factors. All statistical analysis was done with SPSS.

Selection experiment and isolation of mutants with increased resistance to UV

As ancestors for the selection experiment, seven clonal cultures from ancestor1 and seven clonal cultures from ancestor2 were grown over night to stationary phase (OD_{600} around 1) in M9 media. On the first day of the selection experiment, the cultures were diluted to an OD_{600} of 0.02 in 4 ml fresh M9 medium and grown again for 1 h to reach exponential growth. Afterwards, the cells were washed 2 times with M9 buffer and resuspended in 4 ml M9 buffer. Six cell suspensions of each ancestor were then exposed to both UVA (49 W/m^2) and UVB (8 W/m^2) light for 8 h in the turntable. One cell suspension of each ancestor was covered and placed in the turntable as dark control. One tube with a PNA/pyr actinometer was irradiated simultaneously. Bacterial inactivation was determined as described above. After the irradiation the bacterial suspensions were incubated with M9 medium over night as well as a culture of the ancestor strain that had not been exposed to light. The next morning, the overnight cultures were used to inoculate a new batch culture, grown to exponential phase and irradiated as described above. 300 μl of the overnight culture was mixed with glycerol to a final concentration of 10% and stored at −80°C. The whole procedure was repeated for eight consecutive days.

From plate streaks of the 12 populations clonal isolates were obtained and tested for their sensitivity to combined UVA and UVB. Clonal isolates from populations derived from ancestor1 (populations 1, 3, 5, 7, 9, 11) were competed against ancestor2; clonal isolates derived from ancestor2 (populations 2, 4, 6, 8, 10, 12) were competed against ancestor1. Six clonal isolates from each population were tested for their sensitivity to combined UVA and UVB and strains with at least 70 times higher survival than the ancestor were considered as resistant. If no strain showed the resistant phenotype, up to 30 clonal isolates were tested. From 10 of the 12 evolved populations, we were able to obtain clonal isolates with an enhanced resistance against combined UVA and UVB (strains SP01–SP12; survival under combined UVA and UVB irradiation: Supporting Information Fig. 3). On average, the survival of these isolates was around 100 times higher compared with the ancestor (Fig. 2B). These evolved strains were further analysed by exposing them to UVA and UVB alone.

Sequencing and bioinformatics

Genomic DNA was extracted using DNeasy kits (Qiagen) and the DNA concentrations were measured with a Qubit (1.0; Life Technologies, Carlsbad, California). The library synthesis was conducted at the Functional Genomics Center Zurich (http://www.fgcz.ch/) using a Nextra XT DNA Library Prep Kit (Illumina). Paired-end (2 × 150nt) sequencing was performed on

a MiSeq (Illumina, San Diego, California, USA) at the Genetic Diversity Centre (GDC) in Zurich.

Quality control was performed with FastQC Version 0.10.1 (Andrews, 2010). Quality filtering was performed with Prinseq 0.20.3 (Schmieder and Edwards, 2011) (Parameters: min quality mean 25; min length 50nt; max 1 ambiguous nucleotide; de-replicated; trim ambiguous nucleotide from the end; trim end quality 28). Trimmed reads were mapped to the reference genome (*E. coli* K-12 MG1655 m56 reference genome, RefSeq Accession Number NC_000913.2). Mutations were identified with gdtools as part of breseq r34 (Deatherage and Barrick, 2014) by pairwise genome comparison of the evolved strain with the corresponding ancestor.

Allelic replacement

To induce scar-free point mutations found in the evolved strains into the ancestral strain, we used homologous recombination as previously described (Lindsey *et al*., 2013). Primers were designed using Geneious 7.0.5. All primers are listed in Supporting Information Table S3. After the second round of homologous recombination, the strains were tested by temperature switch PCR (TSP) (Tabone *et al*., 2009) and *thiI* was sequenced to verify the desired mutant strains.

Extraction and spectral analysis of tRNA

We extracted the total tRNA of the two ancestors (ancestor1 and ancestor2), the 10 evolved strains and the SP40 (ancestor1 Δ*thiI*) by chloroform/isoamyl alcohol extraction (Rajakovich *et al*., 2012). The extractions were done in duplicate. The absorbance at 260 nm (A_{260}) was measured and an aliquot of the sample was diluted to 0.5 ml with a solution of 0.3 M sodium acetate 10 mM EDTA (pH 4.5) to an equivalent *A*260 of 40 and then filled to a volume of 1.5 ml with cold ethanol (100%). The sample was stored at −20°C until further use. A UV-vis absorbance spectrum from 200 to 800 nm was recorded for each probe (Cary 100 spectrophotometer, Varian). The spectra were normalized to 1 at 315 nm and to 0 at 380 nm. The analysis was done with the absorbance at 340 nm.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Relative light intensity spectra of the UVA lamp (a) and the UVB lamp (b). The light intensity was normalized to 1 at 365 nm (UVA lamp) and 311 nm (UVB lamp) respectively.
Fig. S2. The shown survival rates represent the sensitivity towards UV irradiation after eight hours of irradiation. The 12 populations were irradiated the eighth consecutive day while ancestor1 was not irradiated previously.
Fig. S3. Comparison of the survival ratio for strains with different alleles in *thiI*. The strains between SP01 and SP12 are evolved strains from the selection experiment. The strains between SP21 and SP32 are ancestor strains harboring a mutant version of *thiI*. Error bars represent the standard errors of the means.
Table S1. Strains used in this study.
Table S2. List of all mutations identified in the 10 evolved strains compared to the corresponding ancestor.
Table S3. Primer list of all primers used for the construction of strains SP21-SP32.