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Review

# Effects of ultraviolet A radiation on survival and growth of Gram negative bacteria

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## ABSTRACT

Ultraviolet A radiation (UVA) exerts a complex action on Gram negative bacteria. Within a variety of cellular components absorbing energy in this wavelength range, flavoproteins and cytochromes of the respiratory chain seem to be the chromophores involved in killing the cell. Damaged components of the respiratory chain would produce hydrogen peroxide and superoxide anion, and these compounds would generate hydroxyl radical, which is likely the main intermediary in the induction of oxidative damage. The identity of the targets remains an open question. For a long time, damage to DNA was considered to be the event leading to cell death, but recently energy depletion due to the inactivation of the respiratory chain has been proposed as an alternative mechanism. At sublethal doses UVA produces, among other effects, a transient inhibition of growth without change in viability. The growth lag is due to the inactivation of some tRNAs, which impairs protein synthesis and triggers the stringent response. A photo-protective function was ascribed to this effect. Global genetic regulators have been implicated in UVA response. The *rpoS* system has a strong influence on bacterial UVA resistance, probably related to the control of the response to oxidative stress, and the quorum sensing system modifies both lethal and sublethal effects of UVA in *Pseudomonas*. The current knowledge of the lethal action of UVA in bacteria could be improved by further analysis of the UVA response in a variety of bacterial species.

**KEYWORDS:** ultraviolet A, oxidative stress, DNA damage, cell membrane damage, *Escherichia coli*

## ABBREVIATIONS

ppGpp, guanosine 5'-diphosphate-3'-diphosphate; $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Glu}$, $tRNA^{Lys}$, transfer ribonucleic acid for phenylalanine, proline, glutamic acid, and lysine, respectively; UVA, ultraviolet A radiation; UVC, ultraviolet C radiation

## INTRODUCTION

Ultraviolet A radiation (320-400 nm) represents the main fraction of the solar ultraviolet radiation reaching the Earth's surface, and is one of the most common stressing agents confronted by bacteria in aquatic environments. Detailed knowledge of the effects of UVA on bacteria is therefore relevant to understanding the fate of bacterial populations in natural waters [1]. This subject was extensively studied in the 1970s, but at present a new interest in the action of solar ultraviolet radiation arises from the use of exposure to sunlight as a technique to improve the microbiological quality of water [2]. The aim of this review is to provide a current compilation of information relevant to this purpose. The effects of UVA on bacteria are complex and strongly dependent on assay conditions. Experimental results

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should be analyzed taking into account the genetic background and physiological state of irradiated bacteria, as well as the characteristics of the incident radiation and the culture conditions before and after irradiation. Therefore, references providing detailed description of experimental procedures are included, even for studies which have been reviewed previously. *Escherichia coli* was widely used as the model organism in the study of the effects produced by UVA in bacteria because detailed information on its physiology and genetics is available and it is related to human health and sanitary problems. Except when indicated in the text the experimental work reviewed here was done using *E. coli*. A section of this review is dedicated to studies concerning the UVA response in *Pseudomonas*.

The effects observed after exposure of bacteria to radiation emitted by germicidal lamps are often considered typical action of ultraviolet radiation on bacteria. Nevertheless, early studies revealed considerable differences between these effects and those produced by UVA irradiation. Hollaender [3] observed that during UVA exposure, the induction of lethal effects requires higher doses, mutagenic effects are absent, sub-lethal effects occur before a significant loss of viability, and survival curves exhibit a shoulder instead of exponential kinetics. Kinetics of cell death suggested that the loss of viability produced by UVA could depend on the accumulation of some toxic substance or the destruction of some essential compound in the cell but, up to a certain limit, this process could be tolerated by bacteria without loss of their ability to divide and develop further. Moreover, the lethal effects of UVA were found to be dependent on temperature, suggesting the occurrence of a secondary process rather than a photochemical reaction. Peak reported that the sensitivity of bacteria to UVA varies widely depending upon the growth phase, and that exponentially growing cells manifest increased sensitivity compared to stationary phase cells [4]. The lethal effect of UVA was found to be dependent on the oxygen concentration in the irradiation medium [5], and survival increased when bacteria were irradiated in presence of glycerol [6], a scavenger for reactive oxygen species. It was also reported that lethality after UVA irradiation depends on fluence rate. While at high fluence rates UVA effects seemed to be independent of it, bacteria became increasingly sensitive as it was decreased [7]. Some authors reported that holding UVA irradiated cells in liquid medium increases survival [4], but the opposite effect was found by others [3, 8]. Since it was observed that the addition of catalase to the media used for post-irradiation culturing increases survival of bacteria exposed to UVA, the formation of reactive oxygen species from hydrogen peroxide was proposed as an explanation for the reduction of viability observed during liquid holding [9]. Early studies clearly indicated a preponderant role of the photodynamic action in the induction of lethal effects by UVA, and therefore a comprehensive description of these effects requires the identification of the chromophores absorbing UVA, the reactive oxygen species generated by UVA irradiation, and the targets altered by this process.

## Chromophores involved in UVA action

The ultraviolet and visible action spectrum for lethality of *E. coli* shows shoulders or small peaks at 335, 405 and 500 nm in addition to a maximum at 260 nm, suggesting that at wavelengths longer than 313 nm, which are not efficiently absorbed by DNA, the lethal action of the radiation involves other chromophores [10, 11]. Several cellular components could be considered as candidates for these chromophores, including quinones, pteridines, reduced nicotine adenine dinucleotide, 4-thiouridine, pyridoxal phosphate, cytochromes, porphyrins, riboflavin, flavine adenine dinucleotide and flavoproteins. Some of these potential chromophores have been shown to induce strand breaks in isolated DNA irradiated *in vitro* [12]. The unusual nucleotide 4-thiouridine, present in tRNA, was identified as the chromophore and target in the induction of the effect called “growth delay”, which is discussed in another section of this review. A role as a chromophore for the lethal action of UVA was ascribed to 4-thiouridine because strains lacking this nucleotide (*nuv*) exhibit increased resistance to the lethal effect of irradiation at 334 nm, the wavelength corresponding to the absorption maximum of 4-thiouridine [13]. The influence of 4-thiouridine

on survival of *E. coli* is dependent on the presence of oxygen in the irradiation medium and could be related to the induction of single-strand breaks in DNA [14] but, as discussed in the section dedicated to the growth delay effect, is strongly dependent on the irradiation conditions. The identity of chromophores involved in the lethal action of UVA remains an open question, but recent studies support the notion that flavoproteins and cytochromes of the respiratory chain could be responsible for UVA killing of bacteria (see below).

**Involvement of reactive oxygen species**

Dependence of survival on oxygen concentration and photoprotection provided by scavengers for reactive oxygen species indicated that photodynamic action plays an important role in the mechanism of cell death induced by UVA. Results from several lines of research suggested the involvement of hydrogen peroxide in this mechanism. Bacterial sensitivity to UVA was found to increase after addition of hydrogen peroxide to the irradiation medium [15], and hypersensitivity to both UVA and hydrogen peroxide was reported in a mutant lacking exonuclease III (*xthA*) [16]. Moreover, pre-irradiation treatment of bacteria with hydrogen peroxide conferred resistance to UVA, probably due to the induction of anti-oxidative defense systems [17, 18, 19], and a previous treatment with UVA increased the sensitivity of bacteria during subsequent UVA irradiation in the presence of exogenous hydrogen peroxide, as would be expected if radiation were able to produce hydrogen peroxide *in situ* [20]. Additional information was obtained using deletions affecting the structural gene for the OxyR protein (Δ*oxyR*), a positive regulatory element necessary for the expression of genes involved in the response to oxidative stress induced by hydrogen peroxide. UVA hypersensitivity was reported in Δ*oxyR* mutants of *Salmonella enterica* serovar Typhimurium [21] and *E. coli* [22]. Further analysis of the role played by genes whose expression is under OxyR control revealed that mutant strains of *Salmonella enterica* lacking alkyl hydroperoxide reductase (Δ*ahp*) or defective in glutathione synthesis (*ghs*) exhibited increased UVA sensitivity, but the UVA response of a mutant lacking catalase HP-I (Δ*katG*) was similar to that of the wild-type strain [21]. Similar results were obtained with Δ*katG* mutants derived from *E. coli* which exhibit unaltered [23] or slightly increased [19] UVA sensitivity. Interestingly, a constitutive mutant which over-expresses the genes of the *oxyR* system in *Salmonella enterica* (*oxyR1*) was hypersensitive to UVA, but the presence of Δ*ahp* or Δ*katG* mutations in addition to *oxyR1* alleviated this effect [21], and UVA hypersensitivity was also reported in *E. coli* [19] and *Salmonella enterica* [21] strains expressing *katG* from a multicopy clone. It seems that accumulation of UVA-absorbing proteins is responsible for sensitization to killing by radiation in *oxyR1* mutants. On the other hand, negative results were obtained from assays designed to measure hydrogen peroxide accumulation in UVA irradiated cells using chemical methods [20, 21]. The results outlined above led most authors working on this problem to conclude that hydrogen peroxide seems not to be itself the agent responsible for oxidative damage leading to cell death. Nevertheless, the involvement of reactive oxygen species derived from hydrogen peroxide in cell killing by UVA cannot be ruled out.

The role of other components of the anti oxidative defense system of *E. coli* in the UVA response were also analyzed. A mutant strain lacking iron and manganese superoxide dismutase (*sodA sodB*) had significantly greater sensitivity to UVA than the isogenic wild-type strain, suggesting that the superoxide anion radical is generated in response to irradiation and contributes to cell lethality [24]. The generation of hydroxyl radical from hydrogen peroxide was also suggested by the influence exerted by iron metabolism on the UVA response. Increased UVA sensitivity was reported in a mutant that constitutively expresses the iron transport protein enterobactin (Δ*fur*), in a mutant unable to reduce and release iron from enterobactin (*fes*), and in a mutant unable to synthesize enterobactin (*entA*) treated with ferric enterobactin [25]. These results are consistent with the hypothesis that enterobactin is an endogenous chromophore for UVA, which contributes to the lethal effect of the radiation by releasing iron into the cytoplasm and creating favorable conditions for generation of hydroxyl radical from hydrogen peroxide. Nevertheless, additional effects of the

Δ*fur* mutation, including low levels of superoxide dismutase and catalase activities and reduced transcription of the gene *rpoS*, should be taken into account in the interpretation of these results [26, 27].

Taken as a whole, the available information suggests that UVA exposure increases the generation of hydroxyl radicals from the relatively harmless reactive oxygen species hydrogen peroxide and superoxide radical, by the Fenton and Haber-Weiss reactions. Catalase inactivation during UVA irradiation [21, 28] probably leaves hydrogen peroxide available for these reactions. If generation of hydroxyl radicals occurs efficiently, the absence of detectable hydrogen peroxide accumulation in irradiated cells [20, 21] is compatible with this process, because intracellular concentration of hydrogen peroxide will depend on the balance between production and consumption. Generation of singlet oxygen from endogenous photo-sensitizers could also occur as a consequence of UVA irradiation in biological systems [29], and a mechanism of photo-damage involving this reactive oxygen species has been proposed in *E. coli* [30, 31]. As discussed below, the occurrence of this process seems unlikely in wild-type cells. In bacteria exposed to natural sunlight, the addition of L-histidine or mannitol to the irradiation medium reproduces for the most part the protective effect provided by oxygen depletion. Since L-histidine is a scavenger for hydroxyl radical and singlet oxygen but mannitol is a hydroxyl radical scavenger which reacts poorly with singlet oxygen, these results indicated that damage depends mainly on hydroxyl radical generation [32].

**Damage to DNA**

Previous knowledge on the mechanisms of action of ultraviolet C and ionizing radiation led most researchers to assume that DNA is also the target for the action of UVA. The effects of UVA on bacterial DNA were studied by comparing the radiation response of strains proficient in DNA repair to that of strains deficient for this function. In these experiments, some strains of *E. coli* deficient in excision repair (*uvrA*) exhibited enhanced UVA sensitivity with respect to the wild-type strain [5, 10, 33]. Similar results were obtained with strains of *Salmonella enterica* [34] and *E. coli* [5, 10] carrying mutations in the structural gene for the RecA protein, which is required for regulation of nucleotide excision repair and for expression of genes involved in the SOS response. Moreover, UVA hypersensitivity was described in *uvrA recA* double mutants and a post-irradiation treatment with acriflavine, an agent which interferes with both excision and recombination repair, strongly enhanced the UVA sensitivity of the wild-type strain, while producing a moderate increase in the sensitivity of the *uvrA* and *recA* mutants, and had limited effect on the response of the *recA uvrA* double mutant [10]. Increased UVA sensitivity was also described in mutants lacking DNA polymerase I (*polA1*) [35], an enzyme which could be involved in repair of radiation-induced single-strand breaks [36]. These results were interpreted as evidence of the involvement of damage to DNA in cell killing by UVA. Nevertheless, other researchers reported similar UVA sensitivity in wild-type strains of *E. coli*, in *uvrA* mutants [37], and in strains unable to perform recombination DNA repair [4, 38, 39]. The results outlined above should be analyzed taking into account that some of the *recA* and *uvrA* strains used carried mutations in the gene *rpoS*, formerly designated *nur* [40, 41], which increase UVA sensitivity. The influence of *rpoS* mutations on bacterial UVA response is discussed below. The effect of *polA1* increasing sensitivity was also found to be dependent on the presence of a wild-type allele of *rpoS* [42]. Slightly increased UVA sensitivity was also reported in other DNA repair deficient strains which carried *recB*, *uvrC*, *lexA* and *polA* mutations [43]. An observation highlighting the importance of DNA damage for the action of UVA is the hypersensitivity to UVA observed in *xthA* mutants of *E. coli* lacking exonuclease III [16]. This enzyme is the main apurinic/apirimidinic site endonuclease in this organism and also exhibits 3' phosphatase and 3'-5' exonuclease activities [44]. It was also reported that in a wild-type background a mutation leading to deficiency of the enzyme formamidopyrimidine DNA *N*-glycosylase (*fpg*::kan$^r$) had little effect on the UVA response of *E. coli*, but the lack of this enzyme increased the sensitivity in a *nuv* mutant lacking 4-thiouridine in its tRNA [33]. The enzyme encoded by *fpg* removes fragmented purine lesions and

8-oxo-7,8-dihydro-2'-deoxyguanosine from DNA [44]. The influence of deficient repair systems on the UVA response of bacteria indicates that, in irradiated cells DNA repair is required in order to overcome the deleterious effects of the radiation. On the basis of these observations it was generally accepted that DNA damage is the key event in determining the loss of bacterial viability during UVA irradiation, as described for ionizing radiation and ultraviolet radiation in the wavelength range efficiently absorbed by nucleotides in DNA. Nevertheless, some alternatives to this interpretation arose from studies concerning other potential targets.

The nature of the lesions induced by UVA in DNA has been discussed for a long time. Photoreactivation was detected in DNA of repair-deficient strains of *E. coli* exposed to UVA [45]. Radiation induced comparable amounts of pyrimidine dimers and single-strand breaks in these strains [46, 47], but the ratio of the yields of dimers generated per unit of dose at 254 and 365 nm was approximately 7 x $10^5$ [46]. Since *uvrA* and *recA* mutations had limited influence on the UVA response in *E. coli* but *uvrA recA* double mutants exhibited hypersensitivity, it was proposed that potentially lethal lesions produced by UVA could be repaired by either of the systems involving those genes [48]. Other clues on the nature of DNA lesions were provided by assays with agents like oxygen and glycerol which modify the induction of single-strand breaks by UVA and lethality in the same way, suggesting that these lesions are relevant for cell killing [49]. The influence of formamidopyrimidine DNA *N*-glycosylase deficiency on the lethal and mutagenic effects of UVA on *E. coli* also suggests oxidative damage to DNA [33, 50]. Using accurate and sensitive chromatographic and enzymatic assays, oxidative lesions to DNA including strand breaks, generation of 8-oxo-7,8-dihydroguanine, and formation of sensitive sites for the action of endonuclease III and formamidopyrimidine DNA *N*-glycosylase have been demonstrated in human cells exposed to UVA [51]. It seems likely that this kind of lesion also occurs in bacteria upon irradiation but, to the best of our knowledge, these techniques have not been applied to analyze the effects of UVA on bacterial DNA to date.

Increased UVC sensitivity was found in bacteria exposed to high UVA fluences. This observation, in addition to other reports on the interactions of UVA with UVC and ionizing radiation, suggested that UVA could inactivate DNA repair systems by a mechanism dependent on oxygen, and this mechanism could eventually contribute to the lethal effect induced by UVA itself [52].

An alternative interpretation for the effect of UVA on the clonogenic ability of irradiated bacteria suggests that after UVA exposure, DNA replication is impaired by limited availability of precursors. According to this interpretation the effect of UVA on *E. coli* viability could be related to inactivation of ribonucleoside diphosphate reductase. This complex enzyme converts ribonucleoside diphosphates into the corresponding deoxyribo-nucleoside diphosphates, and some of its components absorb UVA. The involvement of ribonucleoside diphosphate reductase in UVA lethality was proposed because the enzymatic activity disappears upon UVA irradiation *in vivo*. In addition, post-irradiation culturing in the presence of thymine and an exogenous deoxyribosyl source increased UVA survival of a strain which synthesizes deoxythymidilate via the salvage pathway [53].

In *E. coli* UVA is known to be only weakly mutagenic, if at all, and the induction of the SOS response by this kind of radiation has been a subject of controversy. Turner and Eisenstark reported that UVA failed to induce the expression of β-galactosidase when the *recA* promoter of *E. coli* was fused to a *lacZ* structural gene. They proposed that DNA lesions induced during UVA exposure were unable to activate the mechanism of the SOS response [54]. Nevertheless, induction of β-galactosidase expression from an *umuC-lacZ* fusion was detected in wild-type and *uvrA* strains of *E. coli* irradiated with UVA [55]. Upon studying in a mutant unable to synthesize 4-thiouridine (*nuv*) the expression of a fusion to the *sfiA* gene, which is controlled by the SOS system and involved in arrest of cell division, Caldeira de Araujo and Favre concluded that the growth delay effect produced by UVA inhibits the induction of the SOS response [56]. The problem was reexamined in *Salmonella typhimurium* by using an *umuC´-´lacZ* fusion gene and including

post-irradiation incubation in the protocol in order to reduce the influence of the growth delay effect. The induction of the SOS response by UVA was confirmed and shown to require concomitant protein synthesis [57].

**Damage to cell membranes**

Many reports support the notion that UVA produces damage to cell membranes. Morphological changes observed in bacteria exposed to UVA suggested that irradiation could produce changes in the permeability of the cellular envelope [3]. The effects of UVA on the physiological functions of cell membranes were confirmed by several authors, who demonstrated that sub-lethal doses of UVA produce inhibition of the uptake of amino acids [58, 59, 60, 61] and galactosides [58, 62, 63] as a result of alterations in transport proteins, or even of reduced energy availability for the active transport processes [58]. Non-specific increase in the permeability of cell membranes, produced by an unknown mechanism, was also observed in cells exposed to high UVA doses [62]. Additional evidence for the action of UVA on cell membranes was provided by Moss and Smith, who observed that the presence of inorganic salts or detergents in the medium used for post-irradiation culturing reduced survival of bacteria exposed to UVA [64], and by other researchers who detected leakage of material absorbing at 260 nm, labeled thymidine, and $^{86}Rb^{+}$ [65], and decrease in intracellular $K^{+}$ [66] in UVA irradiated bacteria.

In exponentially growing cells of an *E. coli* strain which can neither synthesize nor degrade unsaturated fatty acids, the incorporation of polyunsaturated fatty acids to the cell membrane increased sensitivity to UVA [30, 31]. This change in the UVA response occurred in addition to increased lipid peroxidation and $^{86}Rb^{+}$ leakage, and these effects were enhanced in experiments performed using deuterium oxide [31]. These observations were interpreted assuming that singlet oxygen is generated during irradiation and attacks double bonds of unsaturated fatty acids incorporated in membrane phospholipids. The attack by singlet oxygen would produce peroxidation, and lipid hydroperoxides would induce disorder in the lipid bilayer of the membrane. In this way radiation would modify the permeability of the membrane and this effect could contribute to cell killing [30, 31]. Some aspects of this interpretation require additional considerations. *In vitro* the conjugated acid of superoxide anion, the perhydroxyl radical, can react with double allylic hydrogen atoms of polyunsaturated fatty acids, and the reactivity of fatty acids toward this agent increases with the number of atoms of this kind in the molecule [67]. Thus, changes in UVA sensitivity after incorporation of different fatty acids would be expected if a mechanism mediated by perhydroxyl radical were responsible for the induced damage. Furthermore, since deuterium oxide increases the lifetime of the superoxide anion radical as well as that of singlet oxygen, a mechanism involving this radical seems compatible with the reported results. On the other hand, the action of radiation on membranes modified by the incorporation of polyunsaturated fatty acids could be different from that produced in membranes of wild-type cells which, in the case of *E. coli*, contain mainly saturated and monounsaturated fatty acids [68, 69, 70]. The propagation step of the chain reaction leading to lipid peroxidation in membranes exposed to oxidative stress requires the presence of polyunsaturated fatty acids. Since they are unusual in bacterial membranes, the importance of lipid peroxidation as a mechanism of oxidative damage in bacteria is doubtful [71]. In keeping with the notion that lipid peroxidation is limited in bacteria, colorimetric detection of lipid peroxidation after UVA irradiation of wild-type *E. coli* cells gave negative results [72], and required the incorporation of fatty acids with at least three double bonds and doses higher than those necessary to induce lethal effects in cells unable to synthesize or degrade unsaturated fatty acids [31]. Finally, the incorporation of polyunsaturated fatty acids did not modify the UVA response of *E. coli* cells when they were grown to stationary phase [30, 31]. Since exponential growth is an unusual condition in natural environments, this observation reinforces the notion that a significant contribution of lipid peroxidation to the effect of UVA on bacteria is unlikely during environmental exposure.

Proteins of the respiratory chain have been proposed as potential targets for the action of UVA [3]. UVA irradiation produced partial growth inhibition in cultures of *E. coli* when glucose was used as

the only carbon and energy source, but under similar conditions growth inhibition was complete when bacteria grew with other carbon sources which allow energy to be obtained solely by oxidative phosphorylation. Moreover, growth of the strongly aerobic bacterium *Pseudomonas aeruginosa*, was severely inhibited by UVA even when glucose was used as the carbon source. These results and the analysis of the products generated by glucose degradation in irradiated and non-irradiated cultures led Kashket and Brodie to propose that UVA destroys photosensitive components which are essential for obtaining energy from oxidative metabolism [73]. Inhibition of the activity of several components of the respiratory chain after UVA irradiation was further demonstrated using fractionated bacterial systems [74, 75]. Pre-irradiation growth conditions seem to modify the UVA response of *E. coli* according to their influence on the activity of the respiratory chain. It was reported that growth under anaerobic conditions enhances UVA resistance whilst in bacteria grown under aerobic conditions, resistance depends on the carbon source used, increasing with the use of acetate, succinate, glucose or lactose [37]. The action of UVA on the respiratory chain was also demonstrated by diminished oxygen uptake in irradiated bacteria [37, 58]. Reduction of oxygen uptake was found to be dependent on the substrate used by irradiated bacteria and interestingly, it does not seem to be dependent on generalized damage to cell membranes [58]. Biochemical studies revealed that the activity of NADH dehydrogenase and the ATP concentrations per cell [76] as well as the electrochemical proton gradient [77] diminish after UVA exposure in *E. coli*. The involvement of porphyrin components of the respiratory chain in inactivating events produced by UVA was suggested by experiments carried out with an *E. coli* strain carrying the mutation *hemA8*, which blocks the synthesis of δ-aminolevulinic acid, one of the first steps in the synthesis of porphyrin, and ultimately in the synthesis of cytochromes, catalases and peroxidases. This strain was resistant to UVA, but its sensitivity was restored when it was supplemented with δ-aminolevulinic acid [78]. The effect of supplementation was shown to be photodynamic and not accompanied by an increased number of single-strand breaks in DNA. Nevertheless, increased $^{86}Rb^{+}$ leakage was observed after this treatment and it was suggested that damage to the proton-$K^{+}$ pump or lipid peroxidation could be responsible for UVA sensitivity [79]. Further evidence for a role of components of the *E. coli* respiratory system as endogenous photosensitizers was provided by UVA hypersensitivity observed in a strain of *E. coli* overproducing cytochrome $b_{558}$ from a cloned *cydB* gene, and in another strain containing the cloned *cydA* and *cydB* genes which overproduced cytochromes $b_{558}$, $b_{595}$, and *d* [80]. In recent years oxidative damage to proteins has been demonstrated in *E. coli* exposed to UVA and the relevance of this kind of damage in the sequence of events leading to cell killing has been reexamined [26, 81]. The temporal inactivation pattern of different cellular functions during UVA exposure was analyzed using flow cytometry and viability stains. It was found that a breakdown of respiration precedes a depletion of cellular ATP pool, a loss of ATPase activity, and the loss of the ability to grow. Membrane depolarization and increased permeability were observed only later in the inactivation process. Additional experiments showed that within the enzymes involved in energy metabolism, those associated to membranes are the most susceptible to the effects of radiation [28, 82]. The observed effects of UVA on energy production led Egli and co-workers to propose that a breakdown of energy metabolism is probably the cause of cell death in *E. coli* exposed to UVA, and the sequence of events leading to the loss of viability could start with inactivation of $F_1F_0$ATPase and proteins of the respiratory chain [28]. In early studies concerning the effects of radiation on bacteria, the respiratory chain was proposed as the target for the lethal effect exerted by visible light on the obligate aerobe *Micrococcus luteus* (*Sarcina lutea*) [83]. In this bacterium, which is totally dependent on aerobic metabolism, the loss of viability was ascribed to inactivation of the oxidative phosphorylation and the consequent fall in the ATP/ADP ratio [84]. UVA-induced damage to the respiratory chain has been clearly demonstrated in *E. coli*, but a significant contribution of such damage to the loss of viability was generally thought to be unlikely because it is a facultative anaerobe bacterium.

The experimental evidence outlined in the preceding sections indicates that both energy supply and DNA repair are required for survival of bacteria exposed to UVA, but it does not establish whether the loss of viability is determined by DNA damage or by inactivation of the respiratory chain in cells able to repair DNA and obtain energy by fermentation. Deficiencies in DNA repair systems could exacerbate the effect of lesions which wild-type cells usually overcome. On the other hand, it seems difficult to understand an influence of DNA repair on a killing process dependent on protein damage. Alterations induced by radiation in DNA and in membrane proteins could contribute independently to the loss of viability, and a preponderant role for some kind of lesion could be dependent on the characteristics of the exposed cells and the irradiation conditions. Alternatively, the effects of UVA on DNA and the respiratory chain could be related if lesions to DNA are produced by reactive oxygen species generated by damaged components of the respiratory chain. Many types of mutations were induced in logarithmic cells of *E. coli* exposed to UVA, whereas mutations induced in stationary or synchronized cells were specific. These observations, and the dependence of bacterial survival on oxygen availability during pre-irradiation growth observed in the same experimental conditions, led Webb and Tai to propose that DNA damage occurs in a part of the genome which is in contact with the cytoplasmic membrane, and components of the respiratory chain are the chromophores involved in this effect [37]. If this were the case, any factor affecting the activity of the respiratory chain would be expected to modify UVA sensitivity, even when the event leading to the loss of viability was the subsequent DNA damage.

### Influence of *rpoS* mutations

In a study of the influence of DNA repair on the UVA response of *E. coli*, Tuveson and Jonas described a mutation in the *rpoS* gene, formerly designated *nur* or *katF*, which increased UVA sensitivity without any apparent effect on the response of the bacteria to the radiation emitted by germicidal lamps [40]. High sensitivity to sunlight was found in *E. coli* strains carrying this mutation [48, 85], and the same characteristic was reported in *rpoS* mutants derived from *Pseudomonas syringae* [86] and *Salmonella enterica* [87, 88]. Mutations in the *rpoS* gene were also related to the occurrence of an increased number of DNA single-strand breaks upon UVA irradiation in *E coli* [39]. The RpoS protein, also known as $\sigma^{38}$ or $\sigma^{s}$, is an alternative sigma transcription factor that controls the expression of a number of genes involved in responses to environmental stress in bacteria [89]. The *rpoS* system plays a key role in the defense against oxidative damage in *E. coli*, and the sensitivity of *rpoS* mutants to UVA is likely related with a deficient ability to overcome the effects of oxidative damage. In fact, the expression of some genes whose mutation alters the UVA response in *E. coli* is under *rpoS* control (*xthA*, *katE*), or is regulated by the *oxyR* system or the *rpoS* system according to the physiological conditions (*katG*, *ahpCF*) [90]. An increase in RpoS concentration was observed in *E. coli* during the transition from the exponential phase to the stationary phase [91]. Such an increase in RpoS concentration could be a reason for the change in UVA resistance described by several authors during this transition [34, 40]. In keeping with this notion, dependence of UVA sensitivity on specific growth rate was found to be correlated with the intracellular level of RpoS [85]. Additional data on the influence of *rpoS* on bacterial UVA response were obtained employing microarray technology to analyze global gene expression. Induction of the *rpoS* gene was detected when bacterial growth was stopped by UVA exposure in continuous cultures of *E. coli*. However, when bacteria resumed growth under irradiation, *rpoS* transcription was repressed rather than induced, suggesting that RpoS might be important for UVA resistance under transient exposure, but not in the adaptation of *E. coli* to grow under this stress [92].

### Growth delay effect

When cells of *E. coli* or related bacterial species are exposed to a low dose of UVA they undergo a transient inhibition of growth without any change in viability. This phenomenon, designated “growth delay effect”, was observed by Hollaender during early studies on the effects of UVA [3]. The action spectrum for the growth delay effect

exhibits a maximum at 334 nm [93]. When tRNA isolated from *E. coli* was exposed to radiation of this wavelength, a photoreaction was observed in molecules containing the unusual nucleotide 4-thiouridine in the 8th position and cytosine in the 13th position leading to a cross-link between these residues [94]. The same photoproduct, 5-(4'-pyrimidin 2'-one) cytosine, was detected after *in vivo* UVA irradiations [95, 96]. Whilst biological functions of most of the tRNA molecules modified by UVA were found to be preserved, a significant decrease of the acceptor activities of $tRNA^{Phe}$ and $tRNA^{Pro}$ was observed after UVA exposure *in vitro* [97] and *in vivo* [98]. Comparing the action spectrum for growth delay induction to the absorption spectrum of 4-thiouridine, and the dose dependence of the cross-linking photoreaction *in vivo* with that of the growth delay induction, Thomas and Favre proposed that this photo-reaction is the first event leading to the growth delay effect [95]. The same notion was proposed independently by Ramabhadran who observed that UVA irradiation produces cessation of RNA synthesis and the action spectrum for this effect is similar to the absorption spectrum of 4-thiouridine [99]. Another important observation was that UVA irradiation of growing *E. coli* cultures stimulates the synthesis of ppGpp in addition to inhibiting RNA synthesis and cell growth, resembling the effects produced by amino acid starvation, and reduced effects of radiation on growth and tRNA synthesis take place when mutants deficient in the synthesis of ppGpp in response to amino acid starvation (*relA*) were exposed to UVA [100]. A model was proposed assuming that 4-thiouridine is the chromophore and the target for the growth delay effect. This effect would be the result of decreased protein synthesis, due to the lack of active $tRNA^{Phe}$ and $tRNA^{Pro}$, and the triggering of the stringent response, due to the presence of uncharged tRNAs and the consequent synthesis of ppGpp. The role of 4-thiouridine was confirmed by studying the UVA response in mutants lacking this rare nucleotide (*nuv*), in which the growth delay effect was found to be almost absent [13, 101]. The involvement of the stringent response in the induction of growth delay was found to be limited to cells in exponential phase, and dependent on the activity of the SpoT protein, which hydrolyses ppGpp when its synthesis has been triggered by amino acid starvation. The length of the lag induced by UVA was found to be determined by the burst in ppGpp concentration during irradiation, and the stringent response was assumed to be an amplifier of the effect of radiation [102]. A repair process removing 5-(4'-pyrimidin 2'-one) cytosine from cross-linked tRNA was proposed [103]. The mechanism of growth delay described in *E. coli* was also found in *Salmonella enterica*, and a detailed analysis of its response revealed that adenosine 5',5'''-triphosphoguanosine-3''' -diphosphate was synthesized in addition to ppGpp upon irradiation, and proteins required for UVA resistance but not involved in the stringent response were induced during growth delay [104].

The proposed mechanism of growth delay explains most of the experimental results obtained, though some of its aspects require further analysis. Mutant strains lacking 4-thiouridine exhibit some alteration in their growth after UVA exposure [13, 101], and the occurrence of additional phenomena in the induction of growth delay was suggested by reexamination of the action spectra for this effect and for 4-thiouridine-cytosine cross-linking [105]. The alteration of $tRNA^{Glu}$ and $tRNA^{Lys}$ by photoreaction of the unusual nucleotide 5-methylaminomethyl-2-thiouracil was proposed as an explanation for the effect of UVA on growth of *nuv* mutants, but this photoreaction requires radiation of relatively short wavelengths [106]. The effect of UVA on glucose and succinate transport was found to parallel the effect on growth, suggesting that cell membrane could be an additional target for the growth delay effect [107]. This notion was also supported by the reports describing modifications of the extent of growth delay produced by environmental conditions known to affect the cell membrane in *E. coli*. Thus, the lag time after UVA irradiation was found to be shorter in bacteria grown in the presence of glycerol or sucrose, which retain their succinate dehydrogenase activity and alanine uptake ability in spite of the exposure to UVA [108]. The same effects were observed when bacteria were grown at low temperature or in the presence of ethanol [72]. In addition, the extent of the lag period, the inactivation of succinate and lactate dehydrogenases, and the emission of

ultra-weak chemiluminescence decreased after irradiations performed under a nitrogen atmosphere, suggesting the involvement of oxidative damage in the sub-lethal effects of UVA [72]. Given the fatty acid composition of *E. coli* cell membranes, the involvement of lipid peroxidation in these effects seems unlikely (see above). The influence of oxidative stress on post-irradiation growth could be related to damage to proteins. As it was described in *E. coli* mutants, UVA induced a growth lag independent of 4-thiouridine in a *nuv* strain derived from *Salmonella enterica*. In experiments performed using growing cells of this strain, the addition of branched chain amino acids to the medium delayed the entry into the growth lag [104]. This could be explained by taking into account that UVA inactivates the enzyme dihydroxyacid dehydratase involved in the synthesis of valine, leucine and isoleucine, by a mechanism dependent on oxidative damage [109]. The concomitant inactivation of dihydroxyacid dehydratase and the transport systems for valine, leucine and isoleucine [59] could make these amino acids transiently unavailable for protein synthesis, impairing culture growth even in a complex medium. The influence of oxidative damage on the growth delay effect was undetectable in *Enterobacter cloacae*, which exhibits increased resistance to the lethal and sub-lethal effects of UVA and undergoes a short growth delay triggered by tRNA modification [110].

The ability of UVA to protect irradiated bacteria against the effects of a subsequent exposure to 254 nm radiation was associated with the induction of the growth delay effect, and this effect was therefore assumed to be a photoprotective mechanism [93]. Nevertheless, it was reported that the absence of 4-thiouridine increased survival of bacteria exposed to 340 nm radiation [13, 14]. In order to evaluate the photoprotective function of 4-thiouridine, a *nuv* mutant of *Salmonella enterica* and an isogenic strain proficient in tRNA thiolation were exposed to UVA at a fluence rate resembling those expected during environmental irradiations. The response of both strains was the same during the first four hours of treatment, but when the survival fractions were below 1% survival curves exhibited a tail effect and increased resistance was apparent in the wild-type strain [104]. The inhibition of the SOS response by the growth delay effect was also proposed as a protective mechanism reducing the induction of mutations in *E. coli* exposed to sunlight [56]. Transient depletion of 4-thiouridine leading to suppression of the growth delay effect was reported in *Enterobacter cloacae* after exposure to a sub-lethal dose of UVA [111], and the tRNA sulfur transferase activity was reduced in *E. coli* after the same treatment [112]. This characteristic of the 4-thiouridine metabolism seems strange considering its potential role as a photo-protector. The synthesis of 4-thiouridine requires the IscS protein, which contains pyridoxal phosphate as a cofactor and absorbs radiation at 363 nm [113]. This protein seems to be a potential target for the action of UVA on tRNA thiolation, but IscS inactivation should introduce requirements for thiamine and nicotinic acids and supplementation with these compounds had no effect on growth of irradiated cells [112]. When exponentially growing cells of *E. coli* were exposed to a conditioned medium, they became resistant to the lethal effects of UVA and the extent of the growth delay induced by a fixed UVA dose increased. Both effects seem related to the presence of hydrogen peroxide in the conditioned medium [114], and this observation is in keeping with the notion of a protective role exerted by growth delay.

### Effects of UVA on *Pseudomonas*

Most of our knowledge on the effects of UVA on bacteria has been obtained from studies performed with *E. coli* and *Salmonella enterica*. However, some information on the UVA response in organisms belonging to the genus *Pseudomonas* is also available.

*Pseudomonas aeruginosa* is a versatile micro-organism, present in aquatic or soil environments, and is an important opportunist human pathogen. One of the most intriguing aspects of its response to UVA is its high sensitivity when compared with *E. coli* [73, 115]. *P. aeruginosa* possesses several antioxidative strategies for defense against reactive oxygen species generated by its strongly aerobic metabolism. Nevertheless, cell death by UVA exposure, which is dependent on oxidative damage, is observed in *P. aeruginosa* at doses at which *E. coli* cell viability is not affected.

The action of UVA on the proteins of the respiratory chain could be more deleterious for a bacterium that depends on respiration to obtain energy than for a facultative one [73]. In *P. aeruginosa*, UVA indirect damage mediated by oxygen was found to be responsible for cell killing, inhibition of respiration and inhibition of transport systems, while inactivation of succinate and lactate dehydrogenases was produced by the direct action of the radiation and not involved in the lethal effect [115]. In contrast to the effects described in *E. coli* [64], survival of *P. aeruginosa* after UVA irradiation was found to increase by the addition of salts to the plating media, suggesting that radiation induces membrane damage expressed as increased osmotic sensitivity [116]. Protection against the lethal action of UVA was observed in cells submitted to nutritional stress and in cells irradiated in presence of pyocyanine, a phenazine pigment synthesized by *P. aeruginosa* and related to unfavourable growth conditions and virulence. The effect of nutritional stress depends on protein synthesis, while the effect of pyocyanine was attributed to UVA absorption by the pigment [116].

Some genetic mechanisms involved in the UVA response were studied in *Pseudomonas*. The RecA protein is an important factor in this response, perhaps by the induction of the SOS system [117]. Moreover, in *P. aeruginosa* the concentration of the RecA protein was found to increase after exposure to UVA, suggesting that radiation induces the expression of the *recA* gene. In *Pseudomonas syringae* the alternative sigma factor RpoS is involved in the defense against the lethal action of sunlight [86]. At present, the genes involved in *rpoS* mediated defense in *Pseudomonas* are unknown, and it has been proposed that *rpoS* is more important for general stress survival in *E. coli* than in *Pseudomonas*, where RpoS protein has more specific roles related to virulence and colonization [118]. High sensitivity of *P. aeruginosa* could be related to the functional role of *rpoS*.

A prominent feature of the genus *Pseudomonas* is its ability to form robust biofilms in nature and as a pathogen. Biofilms are very resilient structures and are difficult to destroy. They cause persistent infections and contamination of medical devices, as well as fouling in industrial pipelines. UVA disinfection of both planktonic and biofilm cells of *P. aeruginosa* was studied using photocatalytic and non-photocatalytic techniques. As expected, biofilms were more resistant to UVA than planktonic cells [119]. DNA damage induced by UVA in biofilms of *P. aeruginosa* was monitored by employing a strain carrying a plasmid-borne fusion of the *recA* gene promoter to a promoterless *lux* operon. Neither induction of the *lux* operon nor significant cell death was observed upon exposure to UVA of biofilms formed by this strain, except when the irradiation was applied in presence of the photosensitizer psoralen. Resistance could be due to the absorption of UVA by the alginate biofilm matrix [120].

Quorum sensing is a signalling mechanism employed by bacteria to regulate gene transcription in response to population size. The influence of the quorum sensing systems *las* and *rhl* of *P. aeruginosa* on the effects induced by UVA was studied using mutant strains deficient in the production of the corresponding signals, 3OC12-HSL and C4-HSL. It was demonstrated that both systems are essential in the response to lethal UVA doses, providing protection against oxidative damage [121]. A strong correlation was observed between the levels of catalase, whose expression depends on quorum sensing system [122], and UVA sensitivity, supporting the hypothesis of an important role of this enzyme in the UVA response of this microorganism. A *P. aeruginosa katA* strain, deficient in the production of its main catalase, exhibited marked UVA sensitivity, showing a survival level similar to a mutant impaired for the synthesis of both quorum sensing signals [121]. The influence of catalase on UVA resistance is an interesting feature which distinguishes the UVA response of *Pseudomonas* from those of *E. coli* and *Salmonella enterica*. A growth delay induced by sub-lethal doses of UVA and dependent on the quorum sensing system *rhl* was observed in stationary cells of *P. aeruginosa*. It was also shown that, besides promoting growth delay, low doses of UVA are able to induce an increment in the level of the C4-HSL signal independent of cell density. It was suggested that the induction of the quorum sensing system by UVA could act as an adaptive mechanism against the toxic effects of

radiation by inducing genes involved in protective functions [121].

## FINAL REMARKS

The quantitative description of bacterial survival during UVA exposure, and ultimately the prediction of the UVA response of a bacterium, requires the formulation of a comprehensive model for the action of the radiation. This model should assess the contributions of different damage mechanisms to cell killing and the influence of environmental conditions on the balance between these contributions. Considerable differences in sensitivity to UVA and sunlight were reported among *E. coli* and the related bacterial species *Enterobacter cloacae* [110] and *Salmonella enterica* [123], while the kinetics of cell death described in *P. aeruginosa* [115] and *Vibrio colera* [123] are notably different from that corresponding to *E. coli* under the same irradiation conditions. These observations and the characteristics of the UVA response found in *P. aeruginosa* [115, 116, 121] highlight the need for model organisms used to test the performance of a disinfection procedure to be carefully selected, and further studies concerning the action of UVA on other bacterial species would be useful for this purpose.

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