Molecular assessment of UVC radiation-induced DNA damage repair in the stromatolitic halophilic archaeon, *Halococcus hamelinensis*

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**ABSTRACT**

The halophilic archaeon *Halococcus hamelinensis* was isolated from living stromatolites in Shark Bay, Western Australia, that are known to be exposed to extreme conditions of salinity, desiccation, and UV radiation. Modern stromatolites are considered analogues of very early life on Earth and thus inhabitants of modern stromatolites, and *Hcc. hamelinensis* in particular, are excellent candidates to examine responses to high UV radiation. This organism was exposed to high dosages (up to 500 J/m²) of standard germicidal UVC (254 nm) radiation and overall responses such as survival, thymine–thymine cyclobutane pyrimidine dimer formation, and DNA repair have been assessed. Results show that *Hcc. hamelinensis* is able to survive high UV radiation dosages and that intact cells give an increased level of DNA protection over purified DNA. The organism was screened for the bacterial-like nucleotide excision repair (NER) genes uvrA, uvrB, uvrC, as well as for the photolyase phr2 gene. All four genes were discovered and changes in the expression levels of those genes during repair in either light or dark were investigated by means of quantitative Real-Time (qRT) PCR. The data obtained and presented in this study show that the uvrA, uvrB, and uvrC genes were up-regulated during both repair conditions. The photolyase phr2 was not induced during dark repair, yet showed a 20-fold increase during repair in light conditions. The data presented is the first molecular study of different repair mechanisms in the genus *Halococcus* following exposure to high UVC radiation levels.

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1. Introduction

It is critical for organisms to maintain their genome, a process that requires the protection and repair, if necessary, of their DNA. Ultraviolet radiation (UVR), the most prevalent environmental mutagen, has been an important environmental control during the evolution of life on Earth, and still has a profound impact on modern day life [1]. However, in addition to its harmful effects, UVR plays an important role in the evolution of life, as it is a potent naturally occurring mutagen generating genetic polymorphisms, the basis of evolution [1,2].

For life to thrive in the Archaean (3.8–2.5 Ga ago), organisms had to cope with a UV radiation climate (>220 nm) exhibiting a biological effectiveness 1000 times higher than present today (>290 nm), due to the lack of an stratospheric ozone layer, which after its build up about 2 Ga ago, acts as an effective UV screen [3,4].

Halophilic archaea may have already existed in the Archaean and are therefore ideal candidates to study the effects of high UVC radiation on microbes. Nowadays, halophilic archaea flourish in evaporitic hypersaline environments under intense solar radiation [5]. Representatives of the archaeal family *Halobacteriaceae* have been studied extensively for their response to elevated UVR (e.g., *Halobacterium* sp. [6–9] and *Haloferax volcanii* [10]). Members of this family have optimized their genome and proteome structures to reduce damage induced by UVR [11]. For example, *Halobacterium salinarum* NRC-1 not only survives high dosages of UVC, but also possesses effective mechanisms to detect and repair DNA damage following UVR exposure [9,12]. Similar studies of other members of the *Halobacteriaceae*, the genus *Halococcus*, are rare. Yet members of *Halococcus* are excellent organisms in which to study resistance to environmental extremes as their unique cell wall composition makes them extremely resistant to environmental stress factors [13,14]. In general, archaea lack murein, a peptidoglycan with numerous chemical variations that forms rigid cell walls in almost all taxa of bacteria with only a few exceptions [14]. The cell wall of *Halococcus morrhuae* consists of a mixture

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of neutral amino sugars, uronic acids and a particular aminouronic acid, gulosaminouronic acid [14]. They are known for their ability to survive in unfavorable conditions such as low NaCl concentration [15], in subterranean salt deposits [16] and a simulated Martian atmosphere [17,18]. Furthermore, the species of interest in the present study, Hcc. halimelanus, was isolated from modern stromatolites that thrive in a high UVR environment [19].

Surviving high levels of UVR depends upon three factors: avoidance, protection and repair [20,21]. Organisms use sand, evaporites and other forms of shielding as protection from solar radiation [22]. For example, organisms can survive high dosages of UVC when covered by only 1 mm of rock [23,24]. Protection is the second essential factor where organisms, such as some cyanobacteria, produce mycosporine-like amino acids (MAA) and the pigment sycytomin to shield them from damaging UVR from 310–370 nm [25,26].

Repair of UV-induced damage, the third essential factor, allows an organism to restore its genetic information. UVR-induced DNA damage includes strand breakage, photoproducts either produced by the direct effects of UVB or UVC radiation, or indirect damage through the photochemical production of reactive oxygen species, with mechanism of repair dependent on the type of damage. In nature, the two major photoproducts induced by UVB are cyclobutane pyrimidine dimers (CPD), the most common of which are thymine dimers, and (6–4) photoproducts [9].

There are several pathways that allow cells to detect and repair DNA damage. Nucleotide excision repair (NER) is universal in the bacterial and the eukaryotic domains and has been extensively studied [27,28]. Briefly, in bacteria the NER comprises of four key proteins: UvrA and UvrB (for recognition of the damage), Uvvc (which act as exonucleases to make incisions either side of the lesions) and UvrD (a DNA helicase II) [9]. It has been shown previously that the proteins UvrA, UvrB, and Uvvc are essential for UVR-induced damage repair in Hbt. sp. NRC-1 [29].

An alternative to the complex multi-step pathway of nucleotide excision repair is photoreactivation, which is energetically cheaper, more direct, and thus a less error-prone mechanism of reversing pyrimidine dimers to their monomeric form [30,31]. Photolases are taxonomically widespread including such vertebrates as fish, reptiles and marsupials [31].

Furthermore, other known repair pathways are the base excision repair (BER) and the alternative DNA excision repair (AER), both of which have been described in detail elsewhere [28,32].

With the variety of repair pathways known, we used Hcc. halimelanus as a model to study how an ancient phototroph may have repaired UVC-induced damage. To distinguish between photoreactivation and light-independent repair mechanisms, we compared repaired UVC-induced damage in Hbt. sp. NRC-1 [29].

2.2. Exposure to UVC radiation

Ten milliliters of washed samples were exposed in a petri dish (90 mm diameter) to UVC radiation using a GE Germicidal lamp. All experiments were carried out in biological triplicates. The intensity of the beam was 42 μW/cm² at 254 nm, measured with a high-resolution spectrometer (HR4000, Ocean Optics). The dosage was calculated according to the formula 1 mW/m²/s = 1 mJ/m². Samples were exposed (for up to 30 min to reach higher fluences) in white-light room illumination to the following UV fluences: 0, 25, 50, 75, 100, 150, 200, 300, 400, and 500 J/m², respectively, at room temperature.

Following exposure, samples were immediately placed on ice, covered with aluminum foil to prevent photo-enzymatic repair, and were processed immediately to minimize repair reactions. To determine survival following exposure, several dilutions of the samples were spotted onto DSM 97 modified agar plates and incubated at 37 °C for two weeks. Survival was evaluated after two weeks by counting colony-forming units (cfu).

2.3. DNA isolation

DNA was isolated using the XS-buffer method as described previously [33]. In brief, cell pellets were re-suspended in 500 μL XS-buffer (1% potassium ethyl xanthogenate, 100 mM Tris–HCl pH 7.4, 20 mM EDTA pH 8, 1% sodium dodecylsulfate and 800 mM ammonium acetate). Samples were vortexed vigorously and incubated for 120 min at 65 °C followed by a phenol:chloroform:isoamyl alcohol (25:24:1) extraction and isopropanol purification [34]. The purified DNA was re-suspended in 100 μL dH₂O and stored at −30 °C until further use.

2.4. Thymine–thymine cyclobutane pyrimidine dimers (TT-CPD) Identification

Thymine dimers were detected according to the protocol of Sinha et al. [35] with some modifications. Purified DNA was quantified (λ = 260, nm) using a SpectraMax Plus 384 ( Molecular Devices) spectrophotometer (OD260 nm of 1.0 equals 50 ng of DNA). Samples were denatured by adding 1/10 volume 1 M NaOH and incubated at 80 °C for 30 min. Equal amounts (200 ng) were blotted onto a wetted Amersham HybondTM-LFP membrane and the membrane was baked for 30 min at 80 °C. Non-specific binding sites were blocked by incubating the membrane in PBS-T buffer + 5% ECL advancedTM blocking reagent (GE Healthcare) for 1 h at room temperature. The membrane was then incubated at 37 °C for 2 h with the primary antibody (mouse anti-thymine dimer, Kamiya Biomedical; 1:3000 dilution in PBS-T). The membrane was washed three times with PBS-T and incubated for 1 h with the secondary antibody (sheep anti-mouse IgG with Cy3, Kamiya Biomedical; 1:1000 diluted in PBS-T + 5% ECL advancedTM blocking reagent). The membrane was washed again and thymine dimers were visualized on a Typhoon Trio Scanner (Amersham Bioscience).

2.5. Repair mechanisms

In order to ascertain if the bacteria-like NER pathway and the photolyase gene phr2 are present in this organism, degenerate primers were designed to screen for the bacteria-like excision repair genes uvrA, uvrB, and uvvc, respectively, as well as for the photolyase phr2. Several known protein sequences were obtained from the NCBI database and aligned using CLUSTALW to identify highly conserved regions. Those alignments were submitted to the web-based primer design program CODEHOP, which generates a set of degenerate primers according to conserved regions [36] (Table 1). For amplification of the different genes the following gra...
dient PCR conditions were used: initial denaturation step at 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, gradient annealing temperatures between 65 °C and 55 °C for 30 s, and 72 °C for 1.5 min followed by a final extension at 72 °C for 10 min. Products were visualized on a 1% agarose gel stained with GelStar® Nucleic Acid Gel Stain. Subsequent to PCR amplification, the products were cloned into TOPO TA cloning Kit for Sequencing (Invitrogen) according to the manufacturer’s manual. Positive clones were picked and analyzed by sequencing with the primers T7 and T3 provided by the manufacturer. To confirm sequence identity, sequences were checked against the NCBI database [37]. Sequences with high similarity to those of uvrA, uvrB, uvrC and phr2 were subsequenly employed to design quantitative Real-Time PCR (qRT-PCR) primers (Table 1).

To elucidate differences between repair in the presence or absence of light, replicate 10 ml of sample were exposed to an overall dosage of 75 J/m² under the germicidal lamp as described above. Sensc of light, replicate 10 ml of sample were exposed to an overall dosage of 75 J/m² under the germicidal lamp as described above. The samples were centrifuged for 5 min at 10,000 g and the remaining thymine dimers quantified using the dot-blot Acid Gel Stain. Subsequent to PCR amplification, the products were visualized on a 1% agarose gel stained with GelStar.

2.6. RNA extraction and qRT-PCR

To further investigate the involvement of the uvrA, uvrB, uvrC and phr2 genes in UV-induced DNA damage repair, samples (10 ml) were exposed to 75 J/m² UVC radiation. Following exposure, triplicate samples were centrifuged (5000g for 5 min), transferred into 20 ml pre-warmed DSM97 modified media and incubated at 37 °C with either fluorescent light or in darkness. Samples (1.5 ml) were taken after 0, 10, 12, 16, 20, 24, 36, and 48 h, respectively. DNA was extracted as described above and the remaining thymine dimers quantified using the dot-blot system as described above.

3. Results

3.1. Survival and DNA damage following UV exposure

The survival of Hcc. hamelinensis following exposure to UVC radiation as high as 500 J/m² was evaluated, and the formation of thymine dimers was also compared in intact cells and naked DNA (Fig. 1). Data indicate a steady decline in survival with the increase of UV dosage until 300 J/m². At dosages higher than 300 J/m², the number of CFU remained constant with no significant variation up to a dosage of 500 J/m². A concomitant steady increase in thymine dimer formation is observed, with the naked DNA suffering more damage than the DNA within the intact cells. This suggests that the DNA in the intact cell appears to be protected against UVC radiation when compared to naked DNA. The higher induction rate of photoproducts in naked DNA compared to DNA in cells may...
also be the consequence of starting DNA repair processes under the latter conditions.

3.2. Repair mechanisms

The repair efficiency of UVC-induced cyclobutane pyrimidine dimers during light and dark recovery conditions is illustrated in Fig. 2 which shows a decrease of dimers over time. The results indicate that Hcc. hamelinensis is able to repair UVC-induced damage (75 J/m²) within 16 h when exposed to visible light, and within 24 h without light. After 36 h of recovery, no thymine dimers could be detected (data not shown). The data suggest the presence of a photoreactivation system within the organism as well as a system that does not rely on the presence of light, but takes longer to remove the thymine dimers.

3.3. Screening for uvrA, uvrB, uvrC, and phr2 genes

Degenerate primers were designed for the bacteria-like NER genes uvrA, uvrB, uvrC, and the photolyase gene phr2 (Table 1) employing protein alignments from known sequences obtained from the NCBI database. With this method, partial sequences of all four genes could be identified in Hcc. hamelinensis. All sequences have been deposited in the NCBI database with the following accession numbers: uvrA HM063473, uvrB HM063474, uvrC HM063475 and phr2 HM063472.

3.4. Gene expression during light and dark repair

Expression levels of putative repair genes were measured following 75 J/m² UV-C exposure and repair for up to 60 min. Expression levels of uvrA, uvrB, uvrC, and phr2 were measured (in triplicate) following 0, 10, 30 and 60 min exposure employing qRT-PCR. Values obtained following 0 min incubation were set as 1 (starting amount of molecules within the samples), and values were compared to this as either to be up-regulated (>1) or down-regulated (<1).

During dark repair, the genes uvrA, uvrB and uvrC were moderately up-regulated during the first 10 min of repair with approximately the same ratio observed (Fig. 3). This trend increased following a 30 min incubation, however, the ratio between these three genes changed. uvrA and uvrB were up-regulated 1.78-fold and 2.41-fold, respectively, while the uvrC gene was found to be up-regulated 3.7-fold. No significant further up-regulation of these genes was found following incubation for 60 min. The photolyase gene phr2 was marginally up-regulated following 10 min incubation, however, the expression returned to the starting ratio following 30 min of incubation.

During light repair, all four investigated genes were up-regulated following 60 min incubation with the phr2 gene (19.66-fold) showing the highest up-regulation (Fig. 4). Comparable to the results obtained during dark repair, the uvrA (5.44-fold) and uvrB (4.98-fold) genes showed similar levels of up-regulation, while the uvrC gene was up-regulated by 7.22-fold. Both the phr2 and the uvrC gene showed the highest increase in expression between 30 and 60 min.

4. Discussion

This study is the first to explore responses to UV damage in a stromatolitic microorganism, and thus has important implications for understanding the potential evolution and adaptation of early life. To the best of our knowledge, this is the first molecular study of any member of the genus Halococcus investigating different repair mechanisms following exposure to high UVC radiation. The data presented clearly show that Hcc. hamelinensis is able to survive high dosages of UVC radiation (up to 500 J/m²), possesses the uvrA, uvrB, and uvrC genes of the bacteria-like nucleotide excision repair (NER) system, as well as the photolyase gene phr2. Results also show that these genes are all up-regulated during DNA damage repair.

Results of cell survival and the formation of thymine dimers in this species are shown in Fig. 1. The data indicate that the survival rate of Hcc. hamelinensis stays relatively stable following exposure of 300, 400 and 500 J/m², however this effect is more likely due to the experimental setup, with cells on the upper layer shading the cells underneath, thereby protecting them against UVC radiation. The results in the present study also indicate a difference between exposure of naked DNA and DNA within the cell with re-
spect to the formation of thymine dimers (Fig. 1). It can be clearly seen that DNA within a cell is significantly less damaged than naked DNA. This effect may be due to the protection of the DNA in the cell by carotenoids, namely bacterioruberin. The absorption maxima ($\lambda_{\text{max}}$) of bacterioruberin are at 388, 468, 495 and 530 nm (in acetone) [41], therefore it is not likely that bacterioruberin (or carotenoids) directly protect the DNA in terms of damage prevention, however those pigments might aid in the recovery from UV damage by supplying energy indirectly to photoreactivating enzymes for the reversal of thymine dimers [41,42].

Data obtained during the course of this study revealed that *Hcc. hamelinensis* is able to completely repair damage inflicted by 75 J/m² UVC radiation within 16 h when exposed to fluorescent light and within 24 h when incubated in darkness (Fig. 2). Subsequent molecular screening for a *phr2* photolyase gene as well as the *uvrA*, *uvrB*, and *uvrC* genes revealed the presence of all four of these genes. Expression studies employing qRT-PCR for these genes during dark/light repair are presented in Figs. 3 and 4, respectively. During dark repair, the *uvrA*, *uvrB*, and *uvrC* genes were moderately up-regulated with *uvrC* showing the highest expression after 30 min. The *phr2* gene was not significantly up-regulated during dark repair. Previous studies reported similar findings during UV damage repair in *Hbt. NRC-1*, in that only a fraction of repair genes were up-regulated during repair [12]. This is due to the fact that lesions from oxidative damage by reactive oxygen species, stalled replication forks, and double-stranded DNA breaks also occur naturally as by-products during general metabolism, and thus the constitutive presence of DNA repair proteins may be necessary to repair these damages [12,43].

The results indicate that *uvrC* is higher expressed compared to *uvrA* and *uvrB*, which could be attributed to the possibility that *uvrA* and *uvrB* may have other roles in addition to DNA repair. This has previously been shown to be the case in *Escherichia coli*, where UvrA and UvrB, but not UvrC, are essential for an alternative replication system in which UvrA, UvrB and UvrD – together with other unidentified proteins such as polymerases and exonucleases, can take over the function of the Poll enzyme in DNA replication [27,44]. It is not clear at this stage if this scenario is true for halo-philic archaea as well, and further studies are necessary to elucidate additional functions, if any, of the uvrABC system in these microorganisms. Another reason for these results may be the presence of another repair mechanism not examined here (e.g., a eukaryotic-like NER) within the cell.

During light repair a similar pattern of the uvrABC system was observed compared to dark repair with the difference that gene expression levels were higher (Fig. 4). Not surprisingly, the *phr2* gene was strongly up-regulated following 60 min incubation and
is most likely the reason for the more efficient repair observed during light conditions (Fig. 2).

This study provides an initial insight into molecular responses of *Hcc. hamelinensis* to high UVC radiation and provides the basis for further investigations, such as the possible presence of eukaryotic-like nucleotide excision repair and metabolic changes observed during different repair conditions. Furthermore, by using an organism that was isolated from stromatolites [19], a niche that is a modern analogue to early ecosystems [45,46], we further our understanding on what may have been required to survive and thrive in early earth’s environments. Combined with previous studies on the robustness and longevity of other members of *Halococcus* spp., [17] it draws an intriguing picture of the possibility that *Halococcus* spp. may be modern day representatives of early life on earth.

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