Effects of wavelengths of medium-pressure ultraviolet radiation on photolyase and subsequent photoreactivation
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ABSTRACT
This study aims to investigate the effect of different wavelengths (254, 266, 280 and 365 nm) in polychromatic medium-pressure (MP) UV radiation on the ability of photolyases in repairing dimers and discusses its impact on subsequent photoreactivation. Photolyase was exposed to various doses and irradiances of the UV wavelengths and the dimer repair abilities of the irradiated photolyase were determined via a spectrophotometric assay. At wavelengths below 300 nm, dimer repair rates were not influenced by the UV irradiation between 0.03 and 0.10 mW cm$^{-2}$. For 365 nm, photolyase exhibited enhanced dimer repair at 0.05 mW cm$^{-2}$ and then reduced dimer repair with increasing irradiance. In addition, photolyase was found to have decreasing dimer repair rates when exposed to increasing UV doses at all tested wavelengths. Lower photoreactivation levels after MP UV disinfection as compared to low-pressure (LP) UV disinfection was not attributable to a single wavelength in the polychromatic radiation, but is possibly due to the simultaneous exposure of photolyase to a broad spectrum of radiation, which led to a reduction in the dimer repair ability of photolyase. This study is the first to report the direct effects of UV radiation on photolyase enzyme.

INTRODUCTION
In the natural environment, the repair of UV-induced DNA damage is a common phenomenon which allows for the survival of microorganisms which are exposed to the sunlight. Photoreactivation is a light-mediated enzymatic repair process which specifically targets cyclobutane pyrimidine dimers for removal (Harm 1980). Photolyase, the enzyme responsible for photoreactivation, binds to the DNA at the site of the dimer, absorbs light (300–500 nm) and uses the energy to split the dimers into monomers (Jagger 1967). Although the role of photolyase in photoreactivation has been extensively studied (Heelis et al. 1993; Carell et al. 2001; Byrdin et al. 2003), the exact mechanism is not yet elucidated (Diesenhofer 2000; Weber 2005).

Ultraviolet (UV) disinfection is now a recognized technology for disinfecting drinking water to meet the strict water regulations that are being imposed on water authorities (Mofidi et al. 2002). Typical UV doses in drinking water treatment range from 40 to 60 mJ cm$^{-2}$, and the disinfection technology works by causing damage to the DNA of the microorganisms, the majority of which is the pyrimidine dimers (Harm 1980). The presence of these dimers prevents the proper replication of microorganisms, resulting in cell death. However, photoreactivation of DNA damage in
microorganisms inactivated by UV radiation can cause potential problems, since inactivated waterborne pathogens (e.g. *Vibrio cholerae*, *Escherichia coli* O157:H7, *Salmonella typhimurium*) can regain activity after the DNA damage is removed (Kalisvaart 2004). This leads to a decrease in the efficiency of the disinfection process and recontamination of the treated water. As a result, photoreactivation of various microorganisms, especially *E. coli*, following UV disinfection has been researched to a large extent (Harris et al. 1987; Lindemauer & Darby 1994; Shaban et al. 1997; Sommer et al. 2000; Zimmer et al. 2005; Salcedo et al. 2007). In particular, photoreactivation of *E. coli* following medium-pressure (MP) UV disinfection was found to be lower than that of the traditional low-pressure (LP) UV disinfection (Oguma et al. 2002; Zimmer & Slawson 2002; Hu et al. 2005), suggesting that MP UV disinfection may offer an advantage in photoreactivation suppression. The authors suggested that a possible reason for this was the broad spectrum UV radiation emitted by MP UV lamps which could have caused increased dimer formation, damage to critical replication enzymes and amino acids or damage to photolyase, as opposed to the monochromatic 254-nm LP UV radiation which only causes dimer formation. It is likely that damage to photolyase was the reason since it has been reported that there was no difference between the amount of dimers induced by LP and MP UV radiation (Oguma et al. 2003). In addition, photolyase has absorption maxima at 280 and 384 nm which are emitted by MP and not by LP UV lamps (Sancar & Sancar 1988). However, there has been limited information on how the broad spectrum radiation present in MP UV radiation can affect photolyase. Oguma et al. (2005) previously investigated the effects of several wavelengths in MP UV radiation (230, 254 and 280 nm) on dimer repair in *vivo*, and found that dimer repair was not affected by the individual wavelengths. It was therefore concluded that photoreactivation suppression by MP UV radiation was due to simultaneous exposure to multiple wavelengths, and not attributable to a single wavelength. In particular, photolyase absorbs strongly in the wavelength region below 300 nm, and the combination of wavelengths absorbed by the enzyme could lead to damage to the enzyme or inactivation of the enzyme, although there has not been any concrete evidence to prove this so far. Nevertheless, due to the lack of information in this area, more research needs to be conducted to understand dimer repair following MP UV disinfection. In particular, there is a need to focus on how MP UV disinfection can affect the ability of photolyase to perform dimer repair in more detail. In this study, various wavelengths emitted by MP UV lamps were investigated for their effects on the dimer repair rates of photolyase in *vivo*, quantified using a rapid spectrophotometric assay. The repair rates were also compared to that of photolyase exposed to LP UV radiation and MP UV radiation to understand how the different wavelengths in MP UV radiation can contribute to photoreactivation suppression.

**MATERIALS AND METHODS**

**Preparation and purification of photolyase**

Photolyase was prepared using *E. coli* DH5α (Invitrogen, California, USA) inserted with the plasmid pMS969S containing the *phr* gene. Six liters of cells were grown in Luria broth containing 50 μg ml⁻¹ ampicillin until optical density at 600 nm reached 0.6, and photolyase production was induced with isopropylthio-β-D-galactoside (IPTG) for 3–5 hours. The cells were harvested by centrifugation (4,000 × g, 10 min, 4 °C). Purification of photolyase was performed using the rapid purification method as described by Sancar & Sancar (1988). Briefly, the cells were lysed by sonicating on ice, and the protein was precipitated with ammonium sulfate (65% saturation). The solution was then loaded onto a blue sepharose fast flow column (CL-6B, Amersham Biosciences AB, Uppsala, Sweden). Photolyase eluted at approximately 1 M concentration and was dialyzed and stored at −80 °C. The UV-VIS absorption spectrum of the purified photolyase is presented in Figure 1.

**UV irradiation**

The purified photolyase was diluted with assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 100 μg/ml DNase-free bovine serum albumin) to achieve a final concentration of between 1 × 10⁻⁷ and 5 × 10⁻⁷ M. The actual concentration was determined by measuring the absorbance of the mixture at 280 nm and applying the
Beer-Lambert law with an extinction coefficient of $1.28 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm (MacFarlane & Stanley 2001). One hundred microliters of the diluted photolyase was pipetted onto the cap of a micro-centrifuge tube and placed under a collimated beam apparatus (Calgon Carbon Corporation, Pittsburgh, USA) equipped with a 1 kW MP UV lamp for irradiation in vitro. In order to investigate the various wavelengths in the MP UV spectrum, optical band-pass filters (Melles Griot, USA; Bandpass interference filters, UV; Part nos. F10-253.7-4-25.0M; F10-266.0-4-25.0M; F10-280.0-4-25.0M; F10-365.0-4-25.0M) were attached to the base of the collimating tube to select for the specific wavelength of interest (254, 266, 280 or 365 nm) and allow it to pass through the collimating tube to the photolyase. The spectra of the unfiltered and filtered MP UV radiation are shown in Figures 1 and 2, respectively.

Spectrophotometric assay for determination of photolyase activity in vitro

Photolyase activity was measured using a spectrophotometric assay modified from that developed by Jorns (1985). Briefly, photolyase was mixed with dimer-containing oligo-dT$_{18}$ (prepared by irradiating with LP UV lamp for 1 hour), incubated in the dark for 3–5 min for the attachment of photolyase to the dimers, and then exposed to photoreactivating light (9 W, 365 nm) at a distance of 5 cm away from the light source. Continuous repair of dimers by photolyase was monitored by observing the increase in absorbance of the photolyase-substrate mixture at 260 nm every 2 min up to 20 min of exposure to photoreactivating light. The increase in absorbance at 260 nm was then used to calculate the dimer repair rate achieved per mole of photolyase used.
experiments were also conducted three to five times for each condition to ensure reproducibility of the results.

RESULTS

Figure 3 shows the dimer repair rates of photolyase exposed to different wavelengths filtered from MP UV radiation, at different irradiances. For the filtered wavelength of 254 nm, there was no significant difference in dimer repair rates of photolyase exposed to the two irradiances tested (analyzed by t-test, \( P = 0.64 \)). There was also no difference in dimer repair rates for the different irradiances tested for 266 nm (analyzed by t-test, \( P = 0.24 \)) and 280 nm (analyzed by t-test, \( P = 0.82 \)). However, for these wavelengths tested (Figures 3(a)–3(c)), there was a slight decrease in dimer repair rates of between 8 and 25% as compared to the unexposed photolyase, i.e., at UV dose of 0 mJ cm\(^{-2}\). This suggests that the exposure of photolyase to these wavelengths in vitro caused an adverse effect in the ability of photolyase to repair dimers. For photolyase exposed to 365 nm radiation filtered from MP UV radiation, three irradiances were tested and the results (Figure 3(d)) show that there was a difference in the dimer repair rates at different irradiances and different UV doses applied (\( P = 0.18 \), tested by analysis of variance). At the lowest irradiance of
0.05 mW cm\(^{-2}\), exposure to radiation at 365 nm increased the dimer repair rates of photolyase with increasing UV dose. As the irradiance was increased to 0.1 mW cm\(^{-2}\), the dimer repair rates increased at 5 mJ cm\(^{-2}\), and then decreased slightly when higher UV doses were applied. For the highest irradiance of 0.2 mW cm\(^{-2}\), the dimer repair rates showed only a decrease with increasing UV doses.

The dimer repair rates of photolyase exposed to the various wavelengths filtered from MP UV radiation at the irradiance of 0.05 mW cm\(^{-2}\) are shown in Figure 4. Based on the trends observed, the dimer repair rates decreased with increasing UV doses when exposed to wavelengths less than 300 nm; the decrease in repair rates was as high as 23% of the repair rate of un-irradiated photolyase at the highest UV dose tested (20 mJ cm\(^{-2}\)). On the other hand, photolyase exposed to radiation at 365 nm exhibited an increased dimer repair rate of about 7% beyond a UV dose of 10 mJ cm\(^{-2}\) for the irradiance of 0.05 mW cm\(^{-2}\). A slight increase in dimer repair rate was also observed for the irradiance of 0.1 mW cm\(^{-2}\) between UV doses of 0–5 mJ cm\(^{-2}\). In addition, Figure 4 shows that the shortest wavelength of 254 nm resulted in the lowest rate of dimer repair by photolyase (dimer repair rate was only 76.8% of that of un-irradiated photolyase, as compared to 91.6 and 87.8% for wavelengths of 266 and 280 nm, respectively).

In Figure 5, the dimer repair rates of photolyase irradiated with MP UV-filtered 254 nm radiation were compared to those irradiated with LP UV radiation (254 nm) and MP UV radiation (200–400 nm). The irradiiances of the LP UV radiation and the 254 nm radiation filtered from the MP UV lamp were 0.045 and 0.05 mW cm\(^{-2}\), respectively. Although the irradiiances were similar, the response of the irradiated photolyase was markedly different between the two types of radiation. For LP UV irradiation, the photolyase repaired dimers at the same rate up to a UV dose of 5 mJ cm\(^{-2}\), after which the repair rate decreased to only about 84% of the un-irradiated photolyase at a UV dose of 20 mJ cm\(^{-2}\). When exposed to MP UV-filtered 254 nm radiation, the dimer repair rate of photolyase decreased continuously with increases in UV dose, similar to the trend of MP UV irradiated photolyase. It can also be seen from Figure 5 that photolyase exposed to MP UV radiation exhibited the lowest rates of dimer repair at each UV dose.

**DISCUSSION**

Photoreactivation of bacteria following UV disinfection has been of great interest to researchers, especially in
recent decades as the technology is rapidly being adopted for drinking water treatment. Although treated water is typically delivered via enclosed distribution systems with minimal light exposure, disinfected water may sometimes be stored in tanks before use, or collected and stored in containers before consumption as in the case of rural communities, which may lead to possible light exposure after disinfection. More importantly, disinfected water that is drained into surface waters is exposed to light and photoreactivation is most likely to take place. Therefore, the effects of photoreactivation should be properly understood. Other than the reduction in the overall efficiency of the process, the possible reactivation of pathogenic bacteria is also a cause for concern. While MP UV radiation has been reported to be able to prevent photoreactivation (Oguma et al. 2002; Zimmer & Slawson 2002; Hu et al. 2005), there is limited information on the reason behind it. Recently, Hu & Quek (2008) observed that dimer repair rates by photolyase were reduced more significantly following exposure to MP UV radiation than exposure to LP UV radiation, and provided some evidence to show that the cause of the reduction in dimer repair rates was due to oxidation of the flavin adenine dinucleotide in photolyase. The results from that study suggest that the additional wavelengths present in MP UV disinfection could have caused the additional decrease in dimer repair rates by irradiated photolyase. Therefore, in this study, various wavelengths were selected and filtered from MP UV radiation in an attempt to determine which wavelengths were responsible for photoreactivation prevention in bacteria. These wavelengths were selected based on the peaks of the emission spectrum of the MP UV lamp and the absorption spectrum of photolyase. This is because in order to achieve maximum damage to the photolyase, not only must the particular wavelength be emitted by MP UV lamps at a relatively high irradiance, photolyase must also be able to absorb the radiation at that particular wavelength. In Figure 1, the overlapping spectra from the MP UV lamp emission and the absorption of photolyase show that the maximum absorption of photolyase is at 280 nm where an emission peak is observed for the MP UV lamp, while high emission peaks were found at 266 and 365 nm. There was also significant absorption at these wavelengths by photolyase, so they were chosen for this study. Although wavelengths above 400 nm are emitted by MP UV lamps, they were not taken into consideration since the absorption by photolyase at these wavelengths was negligible. In addition to the wavelengths mentioned above, 254 nm was also selected as a wavelength to be filtered from MP UV radiation, because this would allow for comparisons with the 254 nm radiation emitted by LP UV lamps.

In order to investigate the effects of UV radiation on photolyase, the spectrophotometric assay was employed to assess the level of photolyase activity in vitro, by means of its dimer repair rate. The method measures the increase in absorbance of the oligonucleotide as an indication of dimer removal (Jorns 1985; Sancar & Sancar 1988), and calculations for dimer repair rates are based on this absorbance increase. The spectrophotometric assay was chosen because it allows for rapid detection of photolyase activity and is simple in its application. Due to the fact that the ability of photolyase to repair dimers is highly dependent on the oxidation state of the photolyase (Payne & Sancar 1990), this rapid and simple detection prevents external factors such as the addition of other oxidizing/reducing chemicals during the detection process from interfering with the results. This allows the assay to be sensitive to small changes in the dimer repair ability of photolyase. However, we also note that large deviations were obtained during the experiments even though more duplicate experiments were conducted to try to ensure that the data were representative. This was due to the sensitivity of the method which made it difficult to obtain small variations between experiments.

Although the effect of various wavelengths of UV radiation on the inactivation of microorganisms has been reported (Linden et al. 2001), there has been limited research on their effects on photolyase (Oguma et al. 2005). Here, the dimer repair rates of photolyase were found to be reduced when exposed to wavelengths below 300 nm (254, 266 and 280 nm), indicating that photoreactivation of bacteria can be suppressed when inactivated with these wavelengths. A possible reason for this may be that the shorter wavelengths have higher energy photons which when absorbed by photolyase, can result in greater damage to the enzyme, thereby reducing its ability to perform dimer repair. This is consistent with the conclusions of Oguma et al. (2005) who...
investigated wavelengths at 230, 254 and 300 nm. In addition, the data show that reduced dimer rates can also occur with exposure to 365 nm radiation, but only at a UV irradiance of 0.1 mW cm$^{-2}$ or higher.

Photolyase exposed to 365 nm radiation was found to exhibit both enhanced and reduced dimer repair abilities depending on the UV irradiation used, and indicates that the effect of 365 nm on photolyase was highly dependent on the UV irradiance, where the higher UV irradiances and higher UV doses reduced the ability of photolyase to perform dimer repair. The enhanced repair rates observed for low 365-nm irradiation may be because photolyase uses light energy between 300 and 500 nm to perform photoreactivation. In particular, photolyase has an absorption maximum at 384 nm, which is close to the wavelength of 365 nm (see Figure 1, dashed line). Therefore, exposure to light energy at 365 nm is likely to aid in the photoreactivation process.

In this study, a distinct difference was observed between the effects of 254 nm radiation from LP UV lamps and that of 254 nm radiation filtered from MP UV lamps on the dimer repair ability of photolyase. One reason for this may be because the filtered radiation contains a few peaks at 250, 260 and 266 nm, which together may have accounted for the additional decrease in dimer repair rates as compared to that of the LP UV irradiated photolyase.

With an increase in UV dose, the dimer repair rate of photolyase was also reduced. This is also consistent with other studies where the photoreactivation enzyme (or photolyase) was found to be destroyed by 365 nm radiation in E. coli at high UV doses of above 2 $\times$ 10$^2$ mJ cm$^{-1}$ (Tyrrell et al. 1973; Brown 1978; Linden et al. 2001). Overall, the dimer repair rate of photolyase was the lowest with exposure to MP UV radiation, instead of the filtered individual wavelengths investigated in the study. This suggests that the simultaneous exposure of photolyase to radiation of different wavelengths could contribute to a greater decrease in dimer repair ability of photolyase than exposure to a single wavelength. It also indicates that photoreactivation can be suppressed with exposure to a broad spectrum of radiation with the largest contribution coming from the shortest wavelength. The photoreactivation suppression by MP UV radiation previously reported (Oguma et al. 2002; Zimmer & Slawson 2002; Hu et al. 2005) was therefore not attributable to any single wavelength emitted by the MP UV lamps.

In summary, the results of this study have shown that photolyase activity can be affected by exposure to different wavelengths in MP UV radiation by decreasing the dimer repair rates achieved. In particular, the shorter wavelengths have a greater impact on photolyase activity. The simultaneous exposure of photolyase to many different wavelengths in MP UV disinfection could likely have resulted in a reduced ability of photolyase to perform dimer repair, thereby leading to lower photoreactivation levels in E. coli. Therefore, this provides further evidence that MP UV disinfection has an advantage over LP UV disinfection in terms of photoreactivation suppression.

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**REFERENCES**


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