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*Araştırma Makalesi - Research Article*

# Göl Suyunda *Escherichia coli*'nin OmpC ve OmpF Proteinlerinin Sentezi Üzerine Sıcaklık, Mannitol ve Photooksidatif Stresin Etkisi, EnvZ ve Acp'ın Rolü

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**ÖZ**

Bu çalışma göl suyunda *Escherichia coli*'nin OmpC ve OmpF porinlerinin sentezi üzerine fotooksidatif stres ve mannitolün etkisini araştırmıştır. OmpF sentezi fotooksidatif stresden bağımsız bir şekilde azalırken, OmpC sentezi göl suyunda fotooksidatif stresin bir sonucu olarak azalmıştır. Bu nedenle *E. coli*'de OmpC sentezi fotooksidatif stresden direkt olarak etkilenir. *envZ* ve *pta* genleri mutasyonların göl suyunda fotooksidatif stres altında *E. coli'*de OmpC ve OmpF sentezinin kontrolü üzerine bir etkisi yoktur. Mannitol fotooksidatif stresden korumayı sağlayan bir antioksidant maddedir. Bu çalışmada OmpF sentezinin fotooksidatif stresden bağımsız bir şekilde azaldığını ancak OmpC sentezindeki azalmanın yabani tip *E. coli*'de fotooksidatif stres bağımlı olduğu bulunmuştur. OmpC sentezi bilinmeyen bir mekanizma ile fotooksidatif stres ile düzenlenmektedir. Mannitol porin sentezinin kontrolünde EnvZ ile bir ilişkiye sahiptir.

***Anahtar Kelimeler- Acp, Envz, Göl Suyu, Mannitol, Fotooksidatif***

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# Effect of Photooxidative Stress and Mannitol, Temperature, on Synthesis of OmpC and OmpF Proteins of *Escherichia coli* in Lake Water, the Role of EnvZ and Acp

**ABSTRACT**

This study investigated the effects of photooxidative stress and mannitol on the synthesis of OmpC and OmpF porins of *Escherichia coli* in lake water. The synthesis of OmpF decreased independently of photooxidative stress, whereas the synthesis of OmpC decreased as a consequence of photooxidative stress in lake water. Thus, OmpC synthesis in *E. coli* was directly affected by photooxidative stress. Mutations in the *envZ* and *pta* genes had no effect on the control of OmpC and OmpF synthesis in *E. coli* under photooxidative stress in lake water. Mannitol is an antioxidant substance that provides protection from photooxidative stress. In this study, it was found that OmpF synthesis decreased independently of photooxidative stress in lake water, whereas decreases in OmpC synthesis were dependent on photooxidative stress in wild type *E. coli*. OmpC synthesis was regulated by photooxidative stress via an unknown mechanism.. Mannitol was also found to have a relationship with EnvZ in the control of porin synthesis.

***Keywords- Acp, Envz, Lake Water, Mannitol, Photooxidative***

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## I. INTRODUCTION

The outer membrane in Gram (-) bacterial cells is the first barrier between bacteria and the environment; thus, the outer membrane is a transition region for both harmful and useful molecules. The passage of these molecules through the outer membrane is controlled by porin proteins. Gram (-) bacteria also exhibit adaptive responses that include increased or decreased expression of outer membrane proteins in response to different stresses. In *E. coli*, the OmpC and OmpF porin proteins are most studied proteins in the outer membrane [1]. In addition to nutrient transport, the OmpC and OmpF porins plays important roles in survival during environmental stress [2]. Porin synthesis by *E. coli* is influenced by environmental conditions such as pH, osmolarity, antibiotics, and temperature [1, 3, 4]. There have been many studies of porin proteins [2, 5], however, the molecular mechanisms underpinning OmpC and OmpF porin synthesis under different stress conditions in aquatic environments has not been completely resolved. This is because highly complex transcriptional and post-transcriptional mechanisms control these proteins [5]. The most extensively studied mechanism of porin regulation is the response to osmolarity. EnvZ-OmpR controls transcription during the osmotic regulation of porin genes. EnvZ is an osmosensor, whereas OmpR is a transcriptional response regulator. OmpR activates expression of *ompC* and represses transcription of *ompF* under high osmolarity, whereas OmpR activates expression of the *ompF* gene under low osmolarity. EnvZ is not an absolute necessity, but synthesis of these proteins does not occur in the absence of OmpR, which is a master regulator. Many factors are involved in the phosphorylation of OmpR in the absence of EnvZ. These factors are small molecules, including acetyl phosphate (AcP), phosphoramidate, and carbamyl phosphate [6]. The OmpR is the master regulator of porin genes, but many other regulators are involved such as Lrp, Rob, MarA, SoxS, H-NS, StpA, IHF, and HU [5, 7]. Porins are post-transcriptionally regulated by a variety of sRNAs in addition to MicF. These include such as MicC, IpeX, CyaR, and RybB [8, 9]. Two other phosphorylation systems, i.e., CpxA-CpxR and ArcA-ArcB, control OmpC and OmpF proteins [10, 11]. These factors indicate the high complexity of the mechanism controlling the synthesis of OmpC and OmpF for survival of *E. coli* under different stress conditions. This complex regulation mechanism must be resolved to understand the physiological significance and survival of bacteria under different stress encountered in harsh environments.

One of the most important factors affecting bacteria in natural aquatic environments is the exposure to sunlight [12]. Sunlight damages cells directly or indirectly via reactive oxygen species that interact with photosensitizer molecules. Singlet oxygen ($O^-$), hydroxil radicals ($HO^-$) and hydrogen peroxides ($H_2O_2$) are produced when oxygen ($O_2$) and organic matter are exposed to sunlight [12]. Bacteria possess various mechanisms that prevent such damage. The majority of these mechanisms are controlled by the OxyR and SoxRS regulons. These mechanisms include directly affected factors such as catalase, SOD, glutathione, α-tocopherol, and indirect factors such as changes in outer membrane permeability. Also the OmpF protein is regulated by MicF via SoxRS [13]. However, studies of the underlying molecular mechanisms and importance of porins under photooxidative stress in aquatic environments remain inconclusive. Therefore, the purpose of this study was to investigate changes in OmpC-OmpF synthesis of *E. coli* and to elucidate the role of EnvZ, AcP, and the effects of mannitol under photooxidative stress in lake water.

## II. MATERIALS AND METHODS

Lake water was obtained from a lake in the Dumlupinar University campus area, and filtered through a Whatman No. 1 filter paper followed by autoclaving at 121°C. The bacterial strains used in this study are shown in Table 1. All strains were routinely grown in nutrient broth (Merck) overnight at 37°C. Cultures (10 mL) were harvested by centrifugation at 8000×*g* for 5 min. The cells were washed twice by sterilized lake water, and the pellet re-suspended in autoclaved lake water (10 mL). The re-suspended culture was then inoculated into the autoclaved lake microcosm (at a final concentration of $5 \times 10^8$ CFU $mL^{-1}$). The tops of the beakers were covered with cling film to prevent contamination of the microcosm. Methylene blue (MB) dye (Merck; final concentration, 1.5 µM) was added to all beakers (except for the control) as a photosensitizer under illuminated conditions. As a positive control, a series of samples was incubated in the dark with and without MB. Mannitol was added to illuminated conditions with MB. The microcosms were incubated at different temperatures (10, 24 and 37 °C). The bacterial samples in lake water were exposed to four visible light sources (fluorescent lamp, white wavelength 400–700 nm). The light intensity was measured using a radiometer, and determined as 2680 ± 49 lux [14].

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β-galactosidase activity was measured according to the method of Miller (1992) [16]. Each result was expressed as the mean ± standard deviation of three independent experiments. Mannitol was used as an antioxidant and added to the lake water at a 10 mM final concentration MB+L . All primary data are presented in mean of standard deviations. Differences between treatments and the time-dependent change were analyzed using the Student's t-test (results considered significant at $p < 0.05$).

**Table 1** *Esc10herichia coli* strains used in this study

| Bacteria | Genotype | Source |
|---|---|---|
| MH225 | MC4100 U(*ompC-lacZ*$^{+}$) 10-25 (wild type) | [15] |
| MH513 | MC4100 *araD*+U(*ompF*$^{'}$*-lacZ*$^{+}$) 16-13 (wild type) | [15] |
| BW3343 | MH513 *envZ60*::Tn*10* | [15] |
| BW3345 | MH225 *envZ60*::Tn*10* | [15] |
| BW3601 | MH513 *pta*: :*kan* | [15] |
| BW3602 | MH225 *pta*: :*kan* | [15] |

## III. RESULTS AND DISCUSSION

The expression of *ompF* and *ompC* genes of *E. coli* under photooxidative stres in Lake Water: The survival of *E. coli* under different stress requires activation of specific genes at sufficient levels. This synthesis is controlled by multiple regulatory mechanisms. An understanding of the interplay between these multiple regulatory mechanisms would provide insights into the survival of *E. coli* in natural environments. This study investigated whether the expression of *E. coli ompF* and *ompC* genes changed under photooxidative stress in lake water. The measurement of β-galactosidase enzyme activity was performed using *ompC::lacZ* and *ompF::lacZ* transcriptional fusions. The role of EnvZ and AcP in the synthesis of porins under photooxidative stress was also investigated. The effects of temperature on effect of the photooxidative stress were examined.

*E. coli* produces two outer membrane proteins, OmpC and OmpF, and these porins play very important roles for survival in aquatic environments [4,17]. Many studies have investigated the effects of light or photosensitizer/light on bacterial survival in aquatic environments [18-20]. However, insufficient data is available regarding the effects of photooxidative stress on porin synthesis in aquatic environments.

The counts of all tested mutants have been checked both in dark and illuminated microcosms. We did not observe reduction more than 1 log at survival of strains according to initial counts (6.5 CFU $mL^{-1}$) after 9 h incubation (data not shown). Therefore, decrease in the expression of *ompC-ompF* was not due to the death of mutants. The expression of *ompF* in wild-type *E. coli* decreased independently of photooxidative stress in the lake water, may be due to lack of nutrition [21] (Fig. 1A). OmpF synthesis decreased equally in the dark controls and illuminated samples in the lake water at all temperatures tested. The expression of *ompF* decreased from 25±1 to 4±2 Miller units at 37°C in the illuminated microcosms containing MB, and to a similar extent at other temperatures. In the dark, the expression of *ompF* decreased from 25±1 to 5±2 Miller units at 37°C. The expression of *ompF* decreased more than 2 times at 10°C and 37°C than that of at 24°C in control samples ($P < 0.05$).

OmpC synthesis by wild-type and mutant *E. coli* decreased in the dark controls at all temperatures tested; however, synthesis of OmpC decreased significantly more in the illuminated samples (Light + MB) than that of in the dark controls (Fig. 2A-C) at all temperatures ($P < 0.05$). Figure 2A shows that synthesis of OmpC in wild-type *E. coli* decreased from 308±7 to 19±1 Miller units in the illuminated+MB microcosm at all temperatures (approximately similar). However, synthesis of OmpC decreased from 308±7 to 122±6 Miller units in the dark control, 132±4 in the light control, and 118±5 in the dark+MB samples at 37°C. The synthesis of OmpC was more decreased 3 times compared to 10°C, 2 times according to 37°C at 24°C of control samples ($P < 0.05$). So, expression of *ompC* in lake water was decreased by direct photooxidative stress, whereas *ompF* expression was not affected.

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Gram (-) bacteria protect themselves from stress by reducing or increasing the synthesis of OmpF-OmpC porin proteins [10]. The physiological importance of OmpC and OmpF in the complex cellular responses to photooxidative stress is unknown. MB should be able to pass through the porins and cross the bacterial membrane because of its low molecular weight and positive charge. Decreased porin expression may prevent the uptake of reactive oxygen species and MB into cells. Synthesis of OmpC and OmpF may be decreased to reduce the entry of radicals and MB dye. Darcan (2012) showed that both OmpC and OmpF porin levels are reduced in black sea water under photooxidative stress [21]. In our present study, OmpC porin synthesis was only reduced by photooxidative stres in lake water environment.

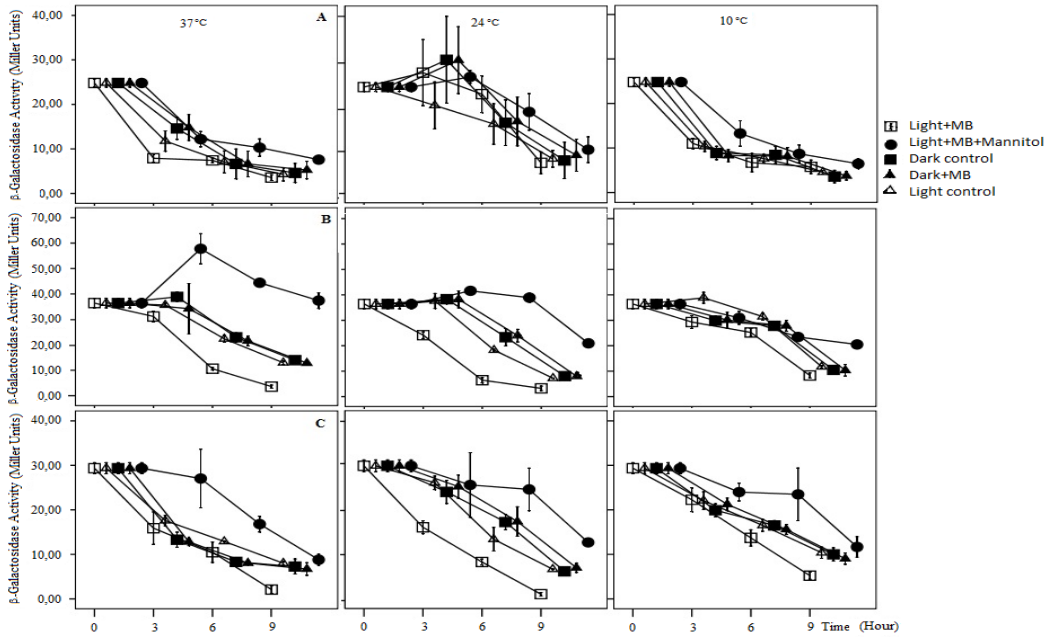


**Fig. 1.** Expression of *ompF* in wild type *E. coli, EnvZ and pta mutants* under photooxidative stress at different temperatures in lake water. **A)** MH513 (Wild-type *E. coli*) **B)** BW3343 (*envZ*⁻ *E. coli*) **C)** BW3601 (*pta*⁻ *E. coli*)

While OmpC and OmpF synthesis in sea water without photooxidation was increased, the production of two porins decreased in lake water without photooxidative stress. As a result, photooxidation caused decrease in the OmpC and OmpF synthesis in both the lake water and seawater. Özkanca *et al.* (2002) showed that the synthesis of both OmpC and OmpF decreased in *Salmonella typhimurium* by approximately 50% after 5 h incubation under photooxidative stress induced by light/toluidine blue O in seawater [22]. Another study showed that the synthesis of OmpA was decreased by visible irradiation [20]. A study of oxidative stress showed that the amount of OmpW protein was increased 1.7 fold when *Salmonella typhimurium* was treated with the superoxide-generating agent menadione [23]. Chou *et al.* (1993) also showed that *micF* transcription was strongly inducible, but wholly-dependent, on the *soxRS* locus in response to treatment with the superoxide-generating agent Paraquat [24]. The OmpF porin level decreased because this transcript prevented *ompF* mRNA translation, whereas OmpC expression had no effect [24]. In contrast, the current study showed that the reduction in *ompF* was not associated with photooxidative stress in lake water, whereas *ompC* was decreased by photooxidative stress. The outer membrane is the structure that is first affected by photooxidation because it is an easy target for free radicals. Thus, our study is the first to demonstrate that expression of *ompC* in *E. coli* is greatly affected by photooxidation stress in lake water.

OmpF synthesis in wild type increased temporally after 3 hours at 24ºC. 24 ºC is a more ideal temperature than 37 ºC in oligotrophic environments such as lake water. Lundrigan and Earhart (1984) demonstrated that OmpF synthesis in *E. coli* was higher at 27 ºC and 20 ºC than that 37 ºC [25]. Also Özkanca and Flint (2002) shown that expression of *ompF* in *E. coli* was higher at 25 ºC than that 10 ºC in lake water [26]. Similar results

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were obtained in our study too. Natural environments can be obtained to different results from those obtained in studies conducted in known mediums because many of different stress factors affects at same time in natural environments.

OmpF synthesis in the *envZ* mutant *E. coli* (Fig. 1B) and the *pta* mutant *E. coli* (Fig. 1C) was also decreased under the same conditions; however, synthesis in these two mutants decreased slightly more under light+MB microcosms compared with that in the dark control. Synthesis of OmpC in the absence of *envZ* or AcP was similarly decreased at all temperatures (not at 10ºC) in lake water. Synthesis of OmpC in the *pta* mutant decreased from 550±27 to 2±2 Miller units in the light + MB, to 319±8 in the light control, to 313±25 in the dark control, and to 323±43 in the dark+MB ($P < 0.05$). EnvZ regulates porin gene expression simply by controlling the levels of OmpR-P and that it does so by varying the ratio of its kinase and phosphatase activities [27]. When *envZ* is deleted, both *ompF* and *ompC* expression in all conditions such as our study are significantly reduced [20, 28]. However, without *envZ*, both *ompC* and *ompF* can be expressed due to other factors that phosphorylate OmpR [29]. In our study, the synthesis decreased without EnvZ, but at 37 ºC and 24 ºC compared to 10 ºC, there was a further decrease than that of dark controls. Acetyl-P is also known as a global regulator by phosphorylate various response regulators, including OmpR [30]. AcP is synthesized from acetyl coenzyme A and Pi with the release of free coenzyme A by phosphotransacetylase [31]. In our study, it was determined that the absence of Acp was resulted in a considerable decrease compared to the initial rate. This decreased was entirely due to the photooxidative stress. In previous studies, this decrease in *pta* mutants has not been shown when exposed to osmolarity, pH and starvation stress [14, 21, 32]. In addition, many factors control the synthesis of porins by providing to control OmpR [33].

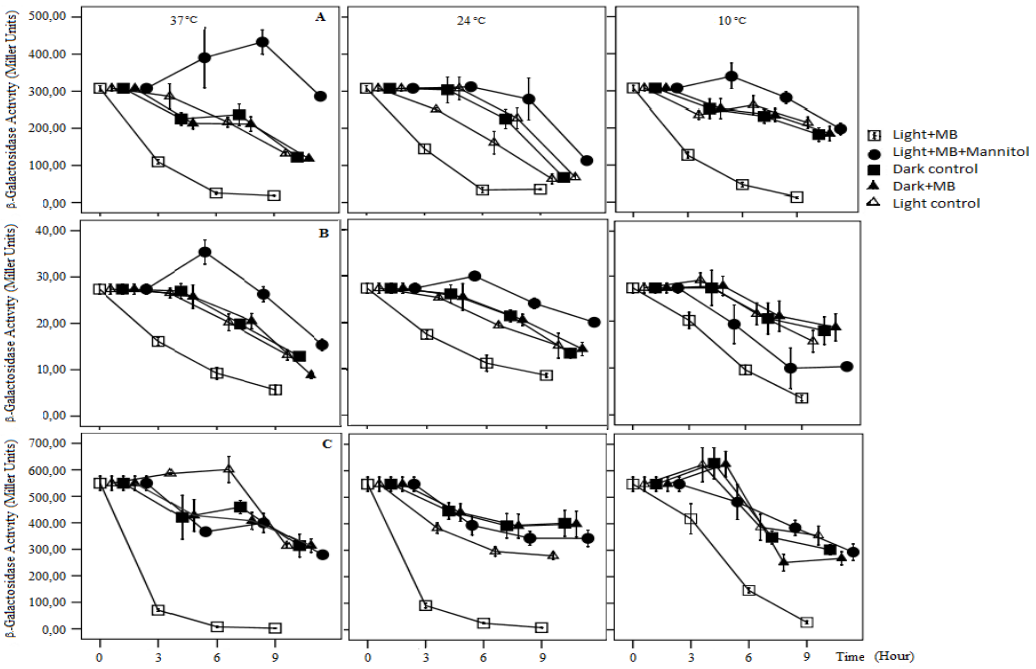


**Fig. 2.** Expression of *ompC* in wild type *E. coli EnvZ and pta mutants* under photooxidative stress at different temperatures in lake water. **A)** MH225 (wild-type *E. coli*) **B)** BW3345 (*envZ*⁻ *E. coli*) **C)** BW3602 (*pta*⁻ *E. coli*)

The effect of Mannitol on expression of porins under photooxidative stres in lake water: We compared the wild type and mutants with and without mannitol under photooxidative stres in lake water. The results showed that mannitol prevented a reduction in the expression of porin proteins during photooxidative stress. Mannitol had a greater effect *ompC* expression than on *ompF* expression (Fig. 1 and 2). The expression of *ompC* was more affected by photooxidative stress in lake water (Fig. 1 and 2). *ompF* expression in the wild type in the

light+MB microcosm decreased from 25±0.9 to 3.6±0.6 at 37°C. However, when mannitol was added to the light+MB microcosm, expression of *ompF* in the wild type was not significantly different from that without mannitol (from 25±0.9 to 7.6±0.7, $P < 0.05$). Disruption of *envZ* had a slight effect on the expression of the *ompF*. The expression of *ompF* in *envZ*-deficient *E. coli* (from 36±1.9 to 37.4±3.1 at 37°C) was five times higher than that in the wild-type (at 37°C) after 9 h incubation with mannitol, and the results were similar at other temperatures (Fig. 1, $P < 0.05$). There was a significant level of EnvZ-independent *ompF* transcription under photooxidative stress in lake water containing mannitol. Mannitol was used as an antioxidant to protect against oxidative inactivation by radicals in the light microcosms. When mannitol was added to the lake water containing the wild-type, expression of *ompF* was not a serious improvement. Thus, *E. coli ompF* expression was independent of photooxidative stress in lake water, indicating that *envZ* and AcP do not play important roles in this process. OmpF synthesis in the *envZ* mutant was also higher than that in wild-type *E. coli*. Mannitol slowed the rate of decrease of OmpF porin synthesis in *envZ* and *pta* mutant under photooxidative stress.

When mannitol was added to the light+MB lake water, the OmpC synthesis decreased from 550±27 to 280±1.5 Miller units at 37°C, to 344±31 at 24°C, and to 293±31 at 10°C ($P < 0.05$). Thus, OmpC synthesis was affected by photooxidative stress to a greater extent than OmpF synthesis in lake water. Mannitol inhibited the reduction in OmpC synthesis under photooxidative stress. Mannitol also increased the synthesis of OmpC by all strains at 37°C compared with the controls. OmpC synthesis in the wild-type and *pta* mutants were higher than that in the absence of mannitol (Fig. 2). OmpC synthesis in the wild type increased at 3 h and 6 h in lake water containing mannitol. However, this increase was not observed in *envZ*$^-$ *E. coli*. This suggests a relationship between mannitol and the expression of porin (especially EnvZ), because the production of OmpF and OmpC was higher than that in the dark controls, especially at 37°C. *ompF* expression increased in *envZ*-deficient mutants when mannitol was added, whereas *ompC* expression did not.

EnvZ possesses kinase and phosphatase activity and it controls the concentration of OmpR-P in the cell. EnvZ plays an important role under osmotic stress and entry of viable but non culturable (VBNC) [4, 34]. The current study found no direct relationship between EnvZ and porin control under photooxidative stress in lake water. However, there was a relationship between mannitol and EnvZ. The reason for this effect of mannitol is not known. However, *E. coli* may have a relationship between outer membrane permeability and mannitol transport system in cytoplasmic membrane [35], and may play a role of molecules such as EnvZ, AcP which control OmpR at this regulation. AcP can function as a phosphate donor for OmpR, and it regulates porin synthesis under certain conditions [6]. However, the role of AcP under photooxidative stress remains unknown. The current study showed that AcP plays no role in the photooxidative stress response in lake water. In a rich medium, OmpC synthesis was very high when the *pta* gene was mutated under certain conditions and nutrient broth [4, 14].The initial level in lake water was also quite high. OmpC and OmpF porins are believed to work in an opposing fashion; however, the levels of both these porins can increase or decrease at the same time under certain conditions [22], such as those found in the situations tested in this study.

Mannitol is a hydroxyl scavenger that protects from oxidative stress [36]. Sabbahi *et al.* (2008) found that the survival of *S. aureus* under MB/light photooxidation conditions increased by about 27% in the presence of mannitol [37]. Chen *et al*. (2015) shown that wild type M5 strain of *E. coli* and a mutant L5 (*ahpCF katEG* mutant) strain cause DNA damage by chloro-benzoquinones in the presence of ferric ions [38]. In the same study, it was shown that this damage is much reduced in the presence of mannitol and ascorbic acid, thus protecting mannitol from ROS.

The photodynamic activity of MB is known to act by the type 1 mechanism (hydroxyl radicals) and also by the type 2 mechanism (singlet oxygen) [39]. The current study showed that porin synthesis (especially that of OmpC) was not greatly affected by photooxidation in the presence of mannitol. Thus, mannitol provided protection from photooxidative stress in lake water. However, there was also a relationship between porin expression and mannitol. The expression of *ompF* in wild-type *E. coli* was unchanged when mannitol was added. The synthesis of *ompF* in the *envZ* mutant was approximately five times higher than that in the wild-type in the presence of mannitol. The synthesis of *ompF* was suppressed in the wild-type by the EnvZ osmosensor. Synthesis of OmpC increased in the wild-type and *pta* mutant in the presence of mannitol, especially at 37°C. It is possible that the kinase activity of EnvZ is increased by mannitol, which also increases OmpR-P.

## IV. CONCLUSION

The results showed that OmpF synthesis decreased independently of photooxidative stress in lake water, whereas decreases in OmpC synthesis were dependent on photooxidative stress in wild type *E. coli*. OmpC synthesis was regulated by photooxidative stress via an unknown mechanism. There was also a relationship between EnvZ and mannitol. The results of this study indicate that there is an unknown relationship between OmpC expression and photooxidative stress, and that there is also a relationship between the EnvZ osmosensor and mannitol.

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