Expression of OmpC and OmpF porin proteins and survival of *Escherichia coli* under photooxidative stress in Black Sea water

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ABSTRACT: In the present study, the synthesis of OmpC and OmpF porins (outer membrane proteins C and F, respectively) of *Escherichia coli* under photooxidative stress in Black Sea water and the roles of RpoS (the starvation/stationary phase sigma factor, $\sigma^{9}$) and EnvZ (a transmembrane environmental sensor protein) in changing porin protein expression were investigated. The $t_{99}$ (time required for a 2 log reduction) values for wild-type *E. coli* W3110 and MSZ31 (*envZ*) under photooxidative stress were observed to be 14.3 and 16.8 h, respectively, while for porin-deficient mutants MKC505 (*ompC*), MH621 (*ompF*), MKCF36 (*ompF*ompC*), and MSR31 (*ompR*) they were 10.6, 10.2, 9.8 and 9.1 h, respectively. Further, when the porins or *ompR* were deleted, the resistance to photooxidative stress decreased. OmpC and OmpF porin expression increased in all studied transcriptional fusion strains (wild type and mutants) in control microcosms, and almost completely decreased under photooxidative stress in the test microcosms, which consisted of light exposure with methylene blue added as a photosensitizer. Thus, it could be concluded that RpoS and EnvZ regulation of the synthesis of OmpC and OmpF is not photooxidation-dependent. This study determined for the first time that the expression of *ompC* in brackish water is influenced by RpoS.

KEY WORDS: Porin · Photooxidation · *Escherichia coli* · Black Sea · RpoS · EnvZ

INTRODUCTION

In the aquatic environment, various factors are known to affect the survival of enteric bacteria, including temperature (Arana et al. 2010), starvation stress (Muela et al. 2008), osmolarity (Munro et al. 1989), pH (Darcan et al. 2009a), predation by protozoa (Thelaus et al. 2009), and exposure to visible and UV light (İdil et al. 2010, 2011). In this context, oxidative stress may be one of the most serious factors influencing the survival of bacteria, causing damage to cells because of reactive oxygen radicals. Reactive oxygen species (ROS) are produced by light under natural conditions. Light may have either a direct effect or an indirect effect on cells via photosensitizer molecules and generate reactive oxygen radicals (Özkanca et al. 2002, Ziegelhoffer & Donohue 2009). The ROS that cause oxidative stress can be classified as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen ($^1$O$_2$), and hydroxyl (HO) radicals (Storz & Imlay 1999). ROS may also be generated by a number of other biologically relevant processes that do not involve light, e.g. they may be generated by neutrophils to resist bacterial infections.

ROS can cause serious damage to cells in aquatic environments. Bacteria use various protection mechanisms to avoid damage. These mechanisms are either enzymatic or non-enzymatic (Storz & Imlay 1999). The majority of them are controlled by OxyR and SoxRS regulons (Zheng et al. 1999). The protection mechanisms include factors that have direct effects, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione, alpha tocoherol (Storz & Imlay 1999, Zheng et al. 1999), or indirect ones, such as
changes in outer membrane permeability (Özkanca et al. 2002). Gram-negative bacteria adapt their outer membrane permeability by modulating the expression of porins. Porin proteins in the outer membrane of Escherichia coli can occur as both specific and non-specific pore-forming proteins. The OmpC and OmpF porin proteins play an important role in the relationship of the cell with the outer environment and are known to control the permeability of <600 kDa hydrophilic polar solutes across the outer membrane of Gram-negative bacteria (Achouak et al. 2001).

The expression levels of Escherichia coli OmpC and OmpF are controlled by the ompB regulon, which is comprised of envZ and ompR genes that form the two-component regulatory system (Hall & Silhavy 1981). The expression of ompC and ompF is upregulated by osmolarity, pH, ionic strength, and temperature (Özkanca & Flint 2002, Darcan et al. 2009b). Additional regulation can also occur through responses elicited by antibiotics, heavy metals, detergents, bile salts, or aromatic compounds, which may decrease the amount of porins in the outer membrane (Pratt et al. 1996, Pagès et al. 2008, Chubiz & Rao 2011). The regulation of porin proteins involves transcriptional and posttranscriptional factors, many of which are only expressed under specific conditions. In addition to EnvZ and OmpR, several other regulators are involved, such as PhoB, Lrp, Rob (Ferrario et al. 1995, Goosen & Van de Putte 1995, Atlung & Ingmer 1997, Painbeni et al. 1997, Deighan et al. 2000, Chubiz & Rao 2011). Porins are also regulated posttranscriptionally by sRNAs, such as MicF and MicC (Delilhas & Forst 2001, Nikaido 1995, De la Cruz & Calva 2010). Porin proteins are controlled by other two-component phosphorelay systems such as CpxR and ArcA (Matsubara et al. 2000, De la Cruz & Calva 2010). Porin-deficient mutants of bacteria were used for survival experiments. In these strains, the porin genes have been knocked out with antibiotic-resistance genes in order to understand the role of porins (Ferrario et al. 1995, Sato et al. 2000). Additionally, transcriptional fusion mutants containing the functional lacZ gene (Liu & Ferenci 2001) were used in order to determine porin expression change under photooxidative stress in Black Sea water (Table 1).

**Survival and β-galactosidase activity assays of Escherichia coli in Black Sea water**

Water samples for each experiment were collected from the Black Sea coast near Samsun, Turkey. Brackish water microcosms were prepared by filtering water samples through Whatman No.1 filter paper and then autoclaved at 121°C in flasks covered with aluminum foil. All Escherichia coli strains were incubated in nutrient broth overnight at 37°C. The culture (10 ml) was harvested by centrifugation at 8000 × g for 5 min. The cells were washed twice with autoclaved brackish sea water (1 ml), and then the pellet was resuspended in autoclaved brackish water (5 ml). The resuspended culture was inoculated into the filtered, autoclaved water microcosm. Beakers

| Table 1. Escherichia coli strains used in this study |
|-----------------|-----------------|-----------------|
| Bacterial strain | Genotype         | Source          |
| E. coli W3110    | Wild type       | Sato et al. 2000|
| MSR31           | W3110 ompR::Tn10| Sato et al. 2000|
| MSZ31           | W3110 envZ::Kan | Sato et al. 2000|
|                 | (16-21) 16-21    |                 |
|                 | (Hyb) ompF−ompC::Kan |               |
| MH621           | MH20 (ompF-lacZ) 16-21 | Ferrario et al. 1995 |
|                 | (Hyb) ompF−ompC::Kan |               |
| MKC505          | MH20 (ompF-lacZ) 16-21 | Sato et al. 2000 |
|                 | (Hyb) ompF−ompC::Kan |               |
| MH225           | MC4100 U(ompC-lacZ+) 10-25 | Liu & Ferenci 2001 |
|                 | (wild type)      |                 |
| MH513           | MC4100 araD+U(ompF−lacZ+) 16-13 (wild type) | Liu & Ferenci 2001 |
| BW3343          | MH513 envZ60::Tn10 | Liu & Ferenci 2001 |
| BW3345          | MH225 envZ60::Tn10 | Liu & Ferenci 2001 |
| BW3301          | MH513 rpoS::Tn10  | Liu & Ferenci 2001 |
| BW3302          | MH225 rpoS::Tn10  | Liu & Ferenci 2001 |
containing filtered, autoclaved water (100 ml) with a final bacterial cell concentration of $5 \times 10^6$ cfu ml$^{-1}$ for survival tests, and $5 \times 10^6$ cfu ml$^{-1}$ for measuring $\beta$-galactosidase activity were used. The tops of the beakers were wrapped with clingfilm to prevent contamination of the microcosm.

Methylene blue (MB) dye (Merck; final concentration 1.5 µM) was added as a photosensitizer to all test microcosms exposed to light (L+MB) and to the positive control that was incubated in the dark (D+MB). As positive controls, a series of samples without the photosensitizer were also incubated in the dark (D) and light (L). All microcosms were incubated at 24°C. L+MB and L microcosms were then exposed to 6 visible light sources (white wavelength 400 to 700 nm). The light intensity was measured in the beakers using a radiometer and determined to be 4800 ± 76 lx, mean ± SD.

Survival tests were performed using the surface spread plate technique, and total bacteria counts were performed by acridine orange direct count for transcriptional fusion mutants (Hobbie et al. 1977). Bacterial samples (5 ml) for total bacterial counts were filtered through black nuclepore track-etched membrane (Whatman) and treated with acridine orange dye (0.01% final concentration). Filters were then dried and examined using a UV microscope (Nikon Eclipse E600). $\beta$-galactosidase ($\beta$-gal) activity was measured using the method of Miller (1992).

RESULTS

Survival of wild-type Escherichia coli and its porin mutants

The present study was performed to investigate how different porin proteins influenced the survival of $E. coli$ in Black Sea water under photooxidative stress. The numbers of wild-type and mutant $E. coli$ cells gradually decreased during a 10 h incubation period when exposed to the test treatment (L+MB; Table 2). Survival experiments showed that the culturable count of $E. coli$ strains declined faster in the L+MB treatment than in the other treatments (Table 2). No significant decrease was observed in the dark or light control samples for the period of experimentation (L, D, D+MB; Table 2). The envZ mutant displayed a longer survival time compared to the wild type in the L+MB treatment ($p < 0.05$; Table 3). Further, the $ompCompF$ and $ompR$ mutants displayed shorter survival times when compared to wild-type $E. coli$ and $ompC$ and $ompF$ mutants ($p < 0.05$; Table 3).

Effect of photooxidative stress on porin protein synthesis

A change was observed in the synthesis level of OmpF and OmpC of Escherichia coli under photooxidative stress in brackish water. As shown in Fig. 1, both OmpF and OmpC synthesis decreased after exposure to photooxidative stress. While porin protein synthesis increased in control samples (D, D+MB, L), the expression in L+MB samples decreased very quickly. This reduction was not a result of bacteria death as the numbers of all bacteria in the enzyme experiments did not decline by more than 1 log (total bacteria with acridine orange were also counted; total bacteria counts and culturable counts in all bacteria were approximately the same, data not shown). The expression of $ompC$ genes (192 ± 11 MU) of $E. coli$, which were transferred after 18 h incubations in nutrient broth, was greater than those of $ompF$ (110 ± 13 MU) during the stationary phase. While OmpF synthesis decreased from 110 ± 13 to 42 ± 5 MU after 8 h under photooxidative stress in brackish water ($p < 0.05$), OmpC synthesis decreased from 192 ± 11 to 57 ± 10 MU ($p < 0.05$). On the other hand, OmpC synthesis increased by approx. 1.5 times in all control samples (L, D, D+MB; $p < 0.05$), and OmpF synthesis also increased approx. 2.7 times in the light control samples (L) and 3 times for all dark samples in brackish water (D, D+MB; $p < 0.05$).

Data analysis and statistics

All count data were expressed as mean (± SD) values of log counts from 3 replicated experiments. The survival time was expressed as $t_{99}$ values (the time for a 2 log decrease in the culturable count from the initial inoculum size). Differences between treatments and the time-dependent change were analyzed using the Student’s $t$-test (results considered significant at $p < 0.05$).
Effect of RpoS and EnvZ on ompC and ompF expression under photooxidative stress

EnvZ is an osmosensor of a two-component phosphorelay system and is responsible for the phosphorylation and dephosphorylation of OmpR. The synthesis of OmpC and OmpF in envZ-mutant Escherichia coli under photooxidative stress decreased, similar to that of the wild type. While ompC expression of envZ-mutant E. coli under photooxidative stress decreased from 55 ± 3 to 6 ± 1 MU, it also decreased to 44 ± 3 MU, 34.8 ± 4.6 MU, and 40 ± 7 MU in the D, L and D+MB control microcosms, respectively (p < 0.05; Fig. 2). In contrast, while the synthesis of OmpF also decreased from 57 ± 8 to 7 ± 2 MU in the L+MB microcosm, this porin increased in all 3 control microcosms, to 76 ± 0.58, 927 ± 99, and 863 ± 39 MU in the L, D and D+MB microcosms, respectively (p < 0.05; Fig. 2). The envZ-deficient E. coli could not synthesize the OmpC porin protein in the control microcosms like the wild type; however, there was a remarkable proportion of envZ-independent OmpF synthesis in Black Sea water.

The synthesis of OmpF and OmpC in rpoS-mutant Escherichia coli was found to decrease remarkably under photooxidative stress in the water microcosm, similar to the wild type. While OmpF synthesis in rpoS-mutant E. coli decreased from 578 ± 57 to 63 ± 17 MU, OmpC synthesis decreased from 185 ± 10 to 32 ± 13 MU under photooxidative stress in brackish water (Figs. 3 & 4). On the other hand, OmpF synthesis in the control microcosms increased from 586 ± 83 to 927 ± 99 MU in the light control, 983 ± 54 MU in the D+MB control, and 863 ± 39 MU in the dark control (Fig. 3A). The expression of ompC in rpoS-deficient E. coli was found to be the same as the wild type at 0 h (190 ± 23 MU for wild type, Fig. 4B; 186 ± 11 MU for the rpoS mutant, Fig. 3B), but the
expression of *ompC* in wild-type and *rpoS*-mutant *E. coli* was approximately $320 \pm 20$ (Fig. 1B) and $248 \pm 20$ MU (Fig. 3B), respectively, after 8 h of incubation in all control Black Sea water microcosms ($p < 0.05$).

**DISCUSSION AND CONCLUSIONS**

Aquatic environments contain very variable stress factors, such as sunlight, that affect the survival of bacteria. Bacteria must use regulatory mechanisms for survival when exposed to stress conditions in environments such as brackish water. One such mechanism in Gram-negative bacteria is the regulation of outer membrane permeability provided by porins (Gauthier et al. 1992).

In the present study, the survival time of *Escherichia coli* was found to decrease under photooxidative stress when compared with control microcosms. There are several existing studies on the effects of photooxidative stress on the survival of bacteria (Muela et al. 2002, Sinton et al. 2007, Ziegelhoffer & Donohue 2009, Darcan & Aydin 2012). Highly reactive oxygen species attack cell compartments, resulting in cell death or viable but nonculturable cell forms (Idil et al. 2011).

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**Fig. 1.** *Escherichia coli*. Synthesis of 2 porin proteins, (A) OmpF (MH513) and (B) OmpC (MH225), by wild-type *E. coli* (strain in parentheses) under photooxidative stress as measured by mean (± SD) $\beta$-galactosidase activity from 3 replicated independent experiments. Methylene blue (MB) was added as a photosensitizer to the light-exposed test microcosm (L+MB) and the dark control microcosm (D+MB). Light control (L) and dark control (D) microcosms without photosensitizer were also used. MU: Miller Units

**Fig. 2.** *Escherichia coli*. Synthesis of 2 porin proteins, (A) OmpF (BW3343) and (B) OmpC (BW3345), by *envZ*-mutant *E. coli* (strain in parentheses) under photooxidative stress as measured by mean (± SD) $\beta$-galactosidase activity from 3 replicated independent experiments. See Fig. 1 for treatment descriptions and abbreviations.
A few studies determined that the deletion of 6 different genes had a great effect on the survival of *E. coli* in sea water or brackish water; 2 of these genes are *ompC* and *ompF* porin genes (Rozen & Belkin 2001, Darcan et al. 2003). In the present study, porin proteins (OmpC and OmpF) were shown to be important in providing *E. coli* with resistance against photooxidative stress in Black Sea water. The culturability of *E. coli* was more affected by *ompC-ompF* and *ompR* mutations compared to the other mutations and the wild type. Another study indicated that the decrease in synthesis of OmpA porin under photooxidation was caused by visible irradiation (Muela et al. 2008).

A study of oxidative stress indicated that the elimination of *ompF* did not increase the resistance of *E. coli* to the superoxide-generating agent menadione (Greenberg 1989). Porins might affect several biological functions, thus allowing a rapid adaptation and survival of bacterial cells. However, the physiological importance and requirement of OmpC and OmpF for the survival of *E. coli* under photooxidative stress remains unclear. In the present study, the *envZ*-deficient *E. coli* had longer survival times than wild-type *E. coli* under photooxidative stress in brackish water, which agrees with other studies on survival of *envZ*-mutated *E. coli* under stress conditions (Darcan et al. 2003, 2009a).

Fig. 3. *Escherichia coli*. Synthesis of 2 porin proteins, (A) OmpF (BW3301) and (B) OmpC (BW3302), by rpoS-mutant *E. coli* (strain in parentheses) under photooxidative stress as measured by mean (± SD) β-galactosidase activity from 3 replicated independent experiments. See Fig. 1 for treatment descriptions.

Fig. 4. *Escherichia coli*. Synthesis of 2 porin proteins, (A) OmpF (BW3301) and (B) OmpC, by 6 different strains of *E. coli*, including 2 wild-type (MH513 and MH225) and 4 mutant (*envZ*-mutant BW3343 and BW3345 and *rpoS*-deficient BW3301 and BW3302) strains under photooxidative stress as measured by mean (± SD) β-galactosidase activity from 3 replicated independent experiments. Only data from the test microcosm (light-exposed with methylene blue photosensitizer, L+MB) are shown.
Gram-positive bacteria are sensitive to photosensitization by various dyes, while Gram-negative bacteria are more resistant. This difference can be explained by the structural differences in the cell walls and outer membrane (Dahl et al. 1989). The control of the outer membrane plays a role in Gram-negative bacteria protection against stress conditions. In the present study, expression of \textit{ompC} and \textit{ompF} in \textit{Escherichia coli} was reduced under photooxidative stress in Black Sea water. However, the porin expression of \textit{E. coli} increased in the control microcosms. The expression of \textit{ompC} and \textit{ompF} decreased because of photooxidative stress despite the high osmolality and alkaline pH (pH 8.2) in brackish water. Özkanca et al. (2002) determined that the synthesis of both OmpC and OmpF in \textit{Salmonella typhimurium} was decreased by 50% after 5 h incubation under photooxidative stress in seawater.

Nevertheless, the molecular mechanisms of expression of porin proteins under the effect of photooxidative stress in natural aquatic environments remain unclear. Chou et al. (1993) showed that \textit{micF} transcription was strongly inducible but wholly dependent on the \textit{soxRS} locus in response to treatment with the superoxide-generating agent paraquat. The OmpF porin level decreased as this transcript prevented \textit{ompF} mRNA translation, whereas OmpC expression had no effect (Chou et al. 1993). In the present study, the expression of \textit{ompF} and \textit{ompC} was observed to decrease because of the effect of photooxidative stress in Black Sea water.

Porin proteins are regulated by the EnvZ-OmpR 2-phosphorelay system (Forst et al. 1989). If \textit{ompR}, which is a master regulator, is deleted, porin proteins cannot be synthesized (Forst et al. 1989, Darcan et al. 2009b). However, if \textit{envZ}, which is an osmosensor, is deleted, both OmpC and OmpF can be synthesized (Liu & Ferenci 2001, Darcan et al. 2009b). The present study showed that EnvZ had no role in photooxidation-dependent porin expression. However, \textit{EnvZ} was required for the expression of \textit{ompC}, which has a very important role in \textit{E. coli} survival. While the expression of \textit{ompC} in wild-type \textit{E. coli} increased in control microcosms, the expression of \textit{ompC} in \textit{envZ}-deficient \textit{E. coli} decreased. The expression of \textit{ompC} is dependent on osmolality in brackish water. EnvZ is an osmosensor (Slauch et al. 1988), and therefore, the expression of \textit{ompC} was regulated by EnvZ. The expression of \textit{ompF} is expected to decrease in brackish water (Lan & Igo 1998) but the expression of \textit{ompF} was found to increase in brackish water in the present study, which is probably related to the alkaline pH (8.2) and osmolality in Black Sea water. Darcan et al. (2005, 2009b) determined that the expression of \textit{ompF} and \textit{ompC} was approximately the same amount in alkaline pH (8.3) and high osmolality in minimal medium or brackish water. As a result, the expression of \textit{ompF} was EnvZ-dependent, and the expression of \textit{ompF} played a role in a lot of factors such as H-NS, IHF (Fig. 5) other than EnvZ in Black Sea water.

The RpoS plays a very important role in the survival of \textit{Escherichia coli} under stress conditions (Liu & Ferenci 2001, Rozen & Belkin 2001). The expression of \textit{ompF} is controlled via MicF RNA, which is negatively affected by RpoS, and the expression of \textit{ompF} is decreased by RpoS (Pratt et al. 1996, Liu & Ferenci 2001). In contrast, the relationship between RpoS and \textit{ompC} is not mechanistic (Liu & Ferenci 2001). The present study found that the expression of \textit{ompC} was not regulated by RpoS under photooxidative stress in Black Sea water. However, although a previous study showed that the expression of \textit{ompC} was not con-
trolled by RpoS (Liu & Ferenci 2001), in the present study a knock-out in rpoS influenced ompC expression in the control microcosms. According to my results, RpoS has an unknown role in the expression of ompC in brackish water; however, rpoS has an indirect effect on the expression of ompC. The expression of ompF in rpoS-deficient E. coli was higher than that of wild-type E. coli under different conditions in several studies (Liu & Ferenci 2001, Darcan 2005, Darcan et al. 2009b), including the present study (rpoS-deficient E. coli was 63 ± 17 MU, wild-type E. coli was 42 ± 5 MU after 8 h incubation). RpoS has a strong negative effect on the expression of ompF. When rpoS-mutant E. coli was produced in rich medium, the rate of OmpF synthesis was considerably higher than that of the wild type, and about the same amount of OmpC was produced as in the wild type in nutrient broth medium after 18 h incubation (Darcan & Özkanca 2008).

The survival time of Escherichia coli was observed to decrease when porin genes (ompC and ompF) were mutated under photooxidative stress in Black Sea water (Tables 2 & 3). However the expression of ompC and ompF of E. coli decreased under photooxidative stress (Figs. 1–4). Therefore, when comparing the synthesis of porin and the culturability results with each other, a conflict between the decrease in porin synthesis and the need for porin proteins for culturability under photooxidative stress in brackish sea water arises, one that is rather difficult to explain. Aquatic environments include various factors (such as pH, osmolarity, and starvation) that affect the survival of bacteria. The reduction in synthesis of porins is not a consequence of oxidative damage under photooxidative stress. Reduction of ompC and ompF is likely under transcriptional or translational control. As shown in Fig. 5, porin synthesis is very complex and includes many other factors. In addition, the detailed porin control mechanism under photooxidative stress is unknown.

The present study determined that the expression of ompC and ompF decreased under photooxidative stress in Black Sea water. The expression of porins was not regulated by RpoS and EnvZ under photooxidative stress. For the first time, this study determined that the expression of ompC was positively and indirectly controlled by RpoS in brackish water.

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