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The Effect of Bacterioruberin Deletion on Production of Bacteriorhodopsin in *Halobacterium salinarum* R1

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ABSTRACT

Bacteriorhodopsin is a retinal protein located in purple membrane of *Halobacterium salinarum* which acts as light-dependent proton pump. Bacterioruberin is a by-product in bacteriorhodopsin biosynthesis pathway in *Halobacterium salinarum*. In order to study the effects of bacterioruberin deletion on quantity of active cellular bacteriorhodopsin production, random mutation by UV radiation on *Halobacterium salinarum* R1 has been carried out. Afterwards, mutated strains which lacked bacterioruberin were selected and production of cellular active bacteriorhodopsin in both mutated and normal (with bacterioruberin) strains were evaluated. The results of this study indicated that the bacterioruberin deletion had insignificant effects on bacteriorhodopsin production. Hence, the biosynthesis pathway of bacteriorhodopsin basically has to be considered independently from the bacterioruberin synthesis.

Keywords: Bacterioruberin, Bacteriorhodopsin, *Halobacterium salinarum* R1, UV tolerance, Absorption spectrum

INTRODUCTION

Halobacterium salinarum is an extreme halophile microorganism of archaebacteria domain. *Halobacterium salinarum* consists of 4 retinal proteins which are photosynthetic pigments and playing role in converting photo energy and signal transduction[1]. Rhodopsin sensor type I and II are also retinal proteins in halorhodopsin. Bacteriorhodopsin is a retinal protein existent in the purple membrane of *Halobacterium salinarum*. In the presence of light, this protein pumps protons out of cell and provides them with the energy for survival[2]. Bacteriorhodopsin is a complex containing equal ratio of membranous apo-protein (Bacterio-opsin) and an all-trans retinal chromophore[3]. Bacterio-opsin protein is coded by bop gene; in contrast, retinal is produced intracellularly of geranyl diphosphate (Figure 1)[3].

As illustrated in Figure 1, the bacterioruberin is constructed as a by-product by lycopene elongase enzyme (lye) from lycopene[4] in bacteriorhodopsin biosynthesis pathway. Most of produced carotenoid pigments in *Halobacterium salinarum* are categorized as bacterioruberins and minority of them are other pigments[5].

The aim of this study is to examine whether bacterioruberin deletion from its biosynthesis pathway results in system energy charge increment and consequently leads to improvement of bacteriorhodopsin production or the production of bacteriorhodopsin is

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carried out by a constant level of energy which is independent from the production rate of its by-product (bacterioruberin). In order to obtain strains with favorable characteristic (lack of bacterioruberin), two methods are available, random and site-directed mutagenesis. Due to feasibility and accessibility, the former method with implementation of UV radiation (random mutagenesis) was used in current study. The selection of desired strain which lacked bacterioruberin carotenoid pigment has been performed by phenotypic selection of such strain among wild-types which were being exposed to UV radiation.

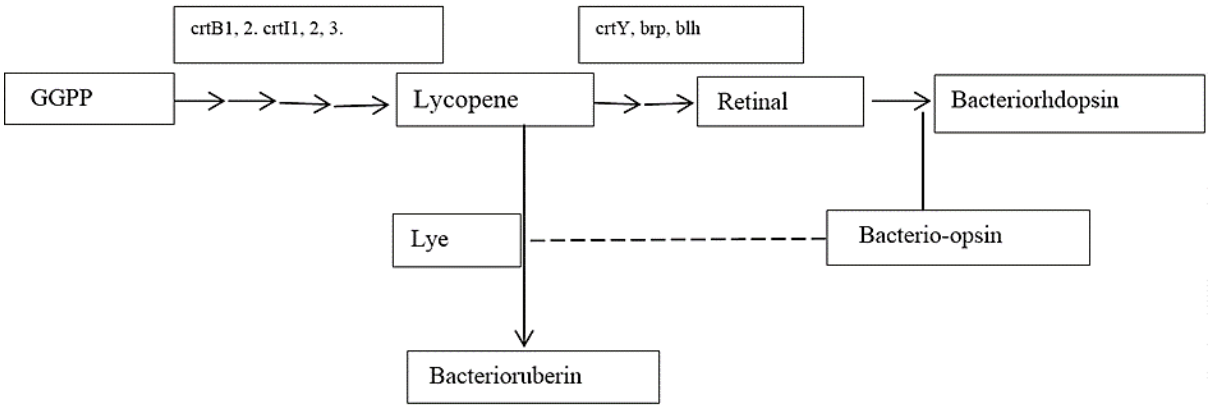

Figure 1. Biosynthesis of bacteriorhodopsin mechanism in *Halobacterium salinarum* R1[3]

EXPERIMENT

Chemicals and instrumentation

Bacteria and Cell Culture requirements: In current study, *Halobacterium salinarum* R1 purchased from DSMZ company and 4 mutant strains (lack or fractional amount of bacterioruberin) achieved from random mutation method were cultured in a culture medium enriched with 250 g/L sodium chloride, 20 g/L magnesium sulphate, 2 g/L potassium chloride, 3 g/L trisodium citrate, 5 g/L yeast extract, 3 g/L bacterial peptone and pH of which was adjusted with implementation of KOH (pH=7-7.2). 20 g of agar was added to the mentioned mix to culture bacteria in a solid phase medium.

Preparation of cells for radiation and selection of favorable strain was carried out by monoclonal cultivation from *Halobacterium salinarum* R1 in 30 mL liquid medium and incubated at 39 °C, rotated in 180 rpm for 3-4 days until cells reached mid-logarithmic phase. After reaching mid-logarithmic phase (OD = 0.8 and CFU = 1×10^9 cells/mL), the cells were exposed to radiation. The suspension of mentioned mix (15 mL) was centrifuged for 10 minutes at 10,000 rpm rate. The precipitation was rinsed with yeast extract and culture medium lacking peptone.

UV radiation of bacterial cells: In dark ambient conditions, cells (15 mL with concentration of CFU 1×10^9 cells/mL) in an uncapped sterile plate was exposed to UV under UV-C lamp (260 nm) with a distance of 30 cm and radiation intensity of 0.4 J/s/m^2 by UV cross linker (Link BLX-Bio) apparatus. After radiation, at certain doses, 250 µL was added to 10 mL enriched liquid culture medium and then incubated for 7 days at 39 °C at 180 rpm. After preparation of monoclonal culture, mutated strains were selected regarding to phenotypic characteristics.

Screening of strains for measurement of carotenoids: All examined strains (with or without mutation) were grown and maintained in 30 mL *Halobacterium salinarum* - specialized medium culture at 200 rpm for 7 days and at 39 °C.

Extraction and analysis of carotenoids: When wild and mutated carotenoid strains reached the stationary phase, 2 mL of cellular suspension was precipitated by 10,000 rpm centrifugation for 15 minutes. Sterile water was added to lyse the cells and then acetone and hexane solvents were implemented to extract carotenoids [6]. Analysis of extracted carotenoids was carried out in 400-600 absorption range by spectrophotometer apparatus (Unicame UV 300). The total carotenoid content was assessed by measuring the absorbance at 490 nm[7].

Bacteriorhodopsin quantification: In order to measure wild-type and mutated bacteriorhodopsin in stationary phase, five replicates were prepared. 2 mL of each replica was added to micro-tube. Then they were centrifuged for 5 minutes at 12000 rpm. Afterwards, 1 mLof double-distilled water containing 0.1 microgram DNAse 1(sigma) was added to the precipitate and homogenized by gently mixing. Regarding to the fact that bacterioruberin interferes in bacteriorhodopsin quantification[3], in order to eliminate the effect of bacterioruberin, lysate, 4 M NaOH and 4 M NH_2OH was added and mixed in a ratio of 9:0.5:0.5, respectively[8]. Final mix was scanned by spectrophotometer and absorption was calculated at initial time and after being exposed to light for 24 hours. The amount of bacteriorhodopsin was calculated in mg/L according to formula 1[9].

(Formula 1): $BR\ (mg/L) = (X \times \frac{A^0_{568} - A^{24}_{568}}{\varepsilon}) \times 1000$

X in Formula 1 indicates molecular weight of bacteriorhodopsin which is 26000 KDa [10] and ε indicates extinction coefficient of purple membrane (ε568 =63000 M^{-1} cm^{-1})[11].

RESULT AND DISCUSSION

Estimation of *Halobacterium salinarum* R1 sensitivity to UV radiation was conducted by counting colonies exposed to different doses of UV. The survival rate has been calculated as Figure 2. *Halobacterium salinarum* R1 is a microorganism resistant to ultraviolet radiation. As described in Figure 2, amounts lower than 90 J/m^2 has no significant effect on the number of colonies and D37 indicator (37% rate of survival) occurs in 265 J/m^2. Figure 2 showed that there is no linear correlation between the intensity of UV dose and survival rate, which is in accordance with reports by others researchers[12,13]. Such linear correlation could be interpreted as requirement of more than a hit to a cell for UV-driven damages. By examining the phenotypes of all colonies exposed to UV radiation, 4 strains which lacked bacterioruberin pigment were selected for bacteriorhodopsin quantification.

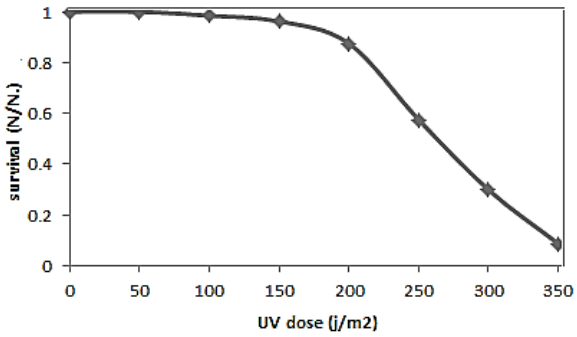

Figure 2. Survival rate graph of *Halobacterium salinarum* R1 exposed to different doses of ultraviolet radiation; N=the number of observed cells exposed to UV; N0= the number of cells observed in control sample.

High levels of resistance to ultra-violet in *Halobacterium salinarum* is maintained by many factors such as genetic mutation repairing mechanism, genome ploidy level [14], presence of carotenoids pigments like bacteriorhoberin [15,16] high concentration of intracellular KCl ion[17] and some other factors.

Comparison of absorption spectrum of pigments in studied strains. After extracting pigments from *Halobacterium salinarum* R1 and mutated strains, mentioned suspension was analyzed for estimation of absorption spectrum by spectrophotometer in a 400-600 nm range. The results are depicted in Figure 3. As illustrated in Figure 3, bacterioruberin carotenoid pigment (with maximum absorption in 468, 479 and 530 nm) is the dominant pigment in *Halobacterium salinarum* R1[18]. However, the amounts of this carotenoid were reduced significantly in mutated strains (Table 1). As shown in Table 1, the *Halobacterium salinarum* R1 strain has the most capability for carotenoid pigment's production; whereas such capability is severely reduced in mutated strains to such an extent that the pigment production of *Halobacterium salinarum* R1 is 57 times more than that of *Halobacterium salinarum* R1M3.

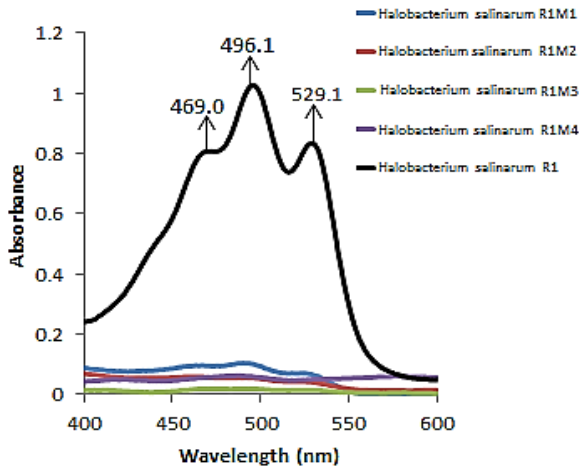

Figure 3. Absorption spectrum of carotenoid pigments of mutated and wild-type *Halobacterium salinarum* R1.

Absorption spectrum analysis indicated three absorption peaks related to bacterioruberin in 469, 496 and 529 nm of the light spectrum of *Halobacterium salinarum* R1; however, such peaks were absent in the graph of mutated strains.

Table 1. The pigment production in *Halobacterium salinarum* R1 and mutant strains

Strain	**Degree of pigment** *OD_{490}/**OD_{600}[14])
R1	0.573
R1M1	0.057
R1M2	0.030
R1M3	0.010
R1M4	0.033

*OD_{490}=is OD_{490} of pigments in hexane,** OD_{600}=is OD_{600} of culture

Bacteriorhodopsin quantification of wild-type and mutants strains of *Halobacterium*. The averages of five replicas amount of bacteriorhodopsin for wild-type and mutant strains of *Halobacterium salinarum* R1 are shown in Table 2. As depicted in Table 2, the deletion of bacterioruberin in *Halobacterium salinarum* R1M1 and R1M2 has no impact on the process of bacteriorhodopsin production in relation to wild-type strain; however, unexpectedly, bacteriorhodopsin biosynthesis is noticeably reduced in *Halobacterium salinarum* R1M3 and R1M4 strains.

Table 2. The bacteriorhodopsin production in *Halobacterium salinarum* R1 and mutant strains (strain type BR (mg/L))

Strain type	BR(mg/L)
H. salinarum R1M1	35.26
H. salinarum R1M2	34.38
H. salinarum R1M3	30.37
H. salinarum R1M4	28.37
H. salinarum R1	37.62

Degree of pigmentation (OD490/OD600) differs considerably between wild and mutated strains. Pigment production level in *Halobacterium salinarum* R1 was 10, 19, 57 and 17 times higher than mutated strains *Halobacterium salinarum* R1M1, *Halobacterium salinarum* R1M2, *Halobacterium salinarum* R1M3 and *Halobacterium salinarum* R1M4, respectively. Due to the fact that there are similar chemical biosynthesis pathways for bacteriorhoberin and bacteriorhodopsin, to estimate cellular active bacteriorhodopsin, mutated strains not capable of producing bacteriorhoberin are more suitable choices.

CONCLUSION

There is no significant difference between bacteriorhodopsin production in mutated strains and *Halobacterium salinarum* R1.Therefore it can be concluded that despite common biosynthesis pathways for bacteriorhodopsin and bacteriorhoberin, there are some peculiar cellular regulatory systems which control levels of active cellular bacteriorhodopsin. In shortage of bacteriorhodopsin production, such mechanisms alter the energy flow in such a way that energy will not trigger an increase in bacteriorhodopsin production and certain amounts of bacteriorhodopsin will be produced in presence of light. Researching protein coding genes for these pathways will be accomplished by present research group. The results of this study shown that deletion of bacterioruberin production pathway has no significant effect on the amount of active bacteriorhodopsin produced in cells.

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