



Complex Effects of Cytochrome P450 Monooxygenase on Purple Membrane and Bacterioruberin Production in an Extremely Halophilic Archaeon: Genetic, Phenotypic, and Transcriptomic Analyses

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OPEN ACCESS

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Specialty section:

This article was submitted to
Extreme Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 12 July 2018

Accepted: 08 October 2018

Published: 26 October 2018

Citation:

Müller WJ, Smit MS,
van Heerden E, Capes MD and
DasSarma S (2018) Complex Effects
of Cytochrome P450 Monooxygenase
on Purple Membrane
and Bacterioruberin Production in an
Extremely Halophilic Archaeon:
Genetic, Phenotypic,
and Transcriptomic Analyses.
Front. Microbiol. 9:2563.
doi: 10.3389/fmicb.2018.02563

Halophilic archaea are known to produce a diverse array of pigments for phototrophy and photoprotection. The aim of this paper was to determine the role of a *Halobacterium* gene encoding the predicted cytochrome P450 monooxygenase (CYP174A1) in pigment synthesis through a combined genetic, phenotypic, and transcriptomic approach. We report on the observed phenotype changes [increased bacterioruberin levels and the loss of purple membrane (PM)] between the *Halobacterium salinarum* R1 and its CYP174A1-deletion mutant. In addition, we report on the whole-genome DNA microarray analysis, which supports the phenotype of PM loss. This work expands our understanding of the *bop*-gene regulon, and its relation to carotenoid biosynthesis, and sheds light on our broader understanding of the role (s) of CYP174A1 in archaeal pigment synthesis. To date, this is the first study in which the physiological role of any cytochrome P450 monooxygenase (CYP450) in extremely halophilic archaea has been reported.

Keywords: purple membrane (PM), bacterioruberin, cytochrome P450, halophilic archaea, bacterioopsin

INTRODUCTION

Members of the *Halobacteriaceae* and *Haloferacaceae* families are extremely halophilic archaea that flourish not only in environments saturated with NaCl but also manage to circumvent harsh environmental factors and survive despite constant exposure to UV- and ionizing radiation and fluctuating levels of desiccation (DasSarma et al., 2006, 2012; Gupta et al., 2015; Rodrigo-Baños et al., 2015). These coping mechanisms, coupled with the fact that these organisms can mostly be cultured with great ease in the laboratory, have sparked much interest due to their potential biotechnological uses (Rodrigo-Baños et al., 2015). The *Halobacterium* and *Haloferax* (Cline et al., 1989; Patenge et al., 2000; Bitan-Banin et al., 2003; Hartman et al., 2010) genera are probably the best studied and most genetically tractable of the *Halobacteriaceae* and *Haloferacaceae* families, respectively – most notable of these is *Halobacterium salinarum* (Sumper and Herrmann, 1976;

Oesterhelt and Krippahl, 1983; Ng et al., 2000; Peck et al., 2000, 2001; Berquist et al., 2006). An outstanding feature of *H. salinarum* and many other members of the *Halobacteriaceae* family is their red pigmentation that can be attributed to bacterioruberin which is the main carotenoid component in these organisms (Rodrigo-Baños et al., 2015). This red pigment protects *H. salinarum* against photo damage due to high light intensities and also aids in photoreactivation and cell membrane reinforcement (Dundas and Larsen, 1963; Shahmohammadi et al., 1998). *H. salinarum* also uses light to its advantage by utilizing bacteriorhodopsin (BR) as a light-driven proton pump to generate cellular energy. BR is a simple protein-cofactor complex comprising bacterioopsin (BO) protein and a covalently bound all-*trans*-retinal co-factor. Under microaerobic conditions, BR formation is induced (Krebs et al., 1991) and accumulates to high levels to form a two-dimensional crystal known as the purple membrane (PM) (Peck et al., 2001; Dummer et al., 2011).

Cytochrome P450 monooxygenases (CYP450s) are of special interest due to their versatile biocatalytic repertoire: they can perform an array of reactions including hydroxylation, epoxidation, dealkylation and dehalogenation. (McLean et al., 2005; Bernhardt, 2006). Archaeal CYP450s have received less scrutiny than Bacterial and Eukaryal enzymes and the only two well studied archaeal CYP450s to date are from the hyperthermo-acidophiles *Sulfolobus acidocaldarius* and *Sulfolobus tokodaii* (Koo et al., 2000; Yano et al., 2000; Oku et al., 2004) and the thermo-acidophile *Picrophilus torridus* (Futterer et al., 2004; Ho et al., 2008). Due to the genetic tractability and ease of culturing, we investigated the role of CYP450s in extremely halophilic archaea using the model archaeon *H. salinarum*. A simple DELTA-BLAST, using the CYP174A1 amino acid sequence from *H. salinarum* R1 as query against the non-redundant database, produced positive hits of more than 460 putative CYP450s spanning 34 genera in total from both the *Halobacteriaceae* and *Haloferacaceae* families. Surprisingly there is no literature available on this specific CYP450 in *H. salinarum* or any other CYP450s from extremely halophilic archaea for that matter.

Currently the physiological function of *CYP174A1* in *H. salinarum* and CYP450s in other extremely halophilic archaea is unknown. Our results provide a possible first clue: the *CYP174A1* from *H. salinarum* appears to play a role in pigment metabolism. The role of CYP450s in pigment metabolism is not a novel one and has been well documented in the cheese ripening bacterium *Brevibacterium lines* (Dufossé and de Echanove, 2005), the green algae *Haematococcus pluvialis* (Schoefs et al., 2001), the thermophilic, yellow-pigmented bacterium *Thermus thermophilus* HB27 and the heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous*. In the two aforementioned organisms, their CYP450 encodes for a β -carotene hydroxylase (Blasco et al., 2004; Mandai et al., 2009) and astaxanthin synthase (Ojima et al., 2006; Barredo et al., 2017) respectively.

In this paper we present the very first data that could provide a clue in elucidating the role of CYP450 in the *Halobacterium* genus and specifically *H. salinarum*. The sole and

putative CYP450 gene from *H. salinarum* named *CYP174A1*, was deleted from the chromosome of *H. salinarum* and the effect of this deletion was evaluated with *inter alia* DNA-microarray analyses.

EXPERIMENTAL PROCEDURES

Culturing Conditions

Propagation of pGEM-T® Easy in TOP10 *Escherichia coli* (Invitrogen) was performed in Luria-Bertani (LB) broth (Sambrook et al., 1989) at 37°C with agitation at 160 rpm. Selective pressure was maintained by supplementing the LB broth with ampicillin (final concentration 100 μ g/mL). Solid media cultivations were performed by supplementing the growth media with 15 g/L bacteriological agar and selective pressure was maintained with 60 μ g/mL ampicillin (final concentration).

Halobacterium salinarum R1 (Stoeckenius and Kunau, 1968; Strahl and Greie, 2008; DasSarma et al., 2018) was cultured in complete medium described by Oesterhelt and Krippahl (1983) that contained (per 1 L): 20 g MgSO₄ 7H₂O, 3 g tri-sodium citrate, 250 g NaCl, 2 g KCl and 10 g peptone. Strains were cultured at 40°C at a shaking speed of 200 rpm. Solid media cultivations were performed by supplementing the broth with 15 g/L bacteriological agar and selective pressure was maintained with a final concentration of 10 μ g/mL mevinnolin (lovastatin) dissolved in dimethyl sulfoxide (DMSO) (final DMSO concentration in medium was 0.1% v/v). For blue/red selection experiments, plates were spread with 40 μ L of 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; 40 mg/mL dissolved in dimethyl formamide and diluted with water).

Deletion Construction

Halobacterium salinarum R1 (Table 1) was cultured in liquid medium until late stationary phase (OD₆₀₀ = 1.2) and genomic DNA extraction was performed as described by Labuschagne and Albertyn (2007). Sequence specific oligonucleotide primer sets (US_Hind_F and Prom_R) and (Term_F and DS_Bam_R) (Supplementary Table S1) and Expand Long Template Polymerase (Roche Molecular Biochemicals) were used to amplify ca. 1kb directly upstream (US) and directly downstream

TABLE 1 | Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source (reference)
TOP 10 <i>Escherichia coli</i>	Plasmid propagation	Invitrogen
<i>Halobacterium salinarum</i> R1	Laboratory strain Gas vesicle deficient	Stoeckenius and Kunau, 1968; Strahl and Greie, 2008
pGEM-T® Easy	Blue/White selection, TA-cloning, Amp ^R	Promega
pMKK100	Blue/Red selection, shuttle and suicide vector, bgaH, Amp ^R , Mev ^R	Koch and Oesterhelt, 2005; del Rosario et al., 2007

(DS) of the open reading frame of the *CYP174A1* gene. Sub-cloning of the individual US and DS regions and ultimately the US/DS fusion (deletion cassette) were performed in pGEM-T[®] Easy (Promega). All sub-cloned plasmids were extracted as described by Sambrook et al. (1989). The final US/DS deletion cassette was liberated from pGEM-T[®] Easy with *Hind*III and *Bam*HI endonucleases and directionally cloned into the suicide vector pMCK100 (Koch and Oesterhelt, 2005). The resulting pMCK100 construct was transformed into competent *H. salinarum* cells to perform blue/red clone selection (**Supplementary Figure S1**).

Competent *H. salinarum* R1 cells were prepared as described by Cline and Doolittle (1987) with some minor modifications as described by Koch and Oesterhelt (2005). After transformation cultures were streaked on plates containing mevinolin and X-gal and incubated at 40°C for 5–7 days or until colonies appeared. Successfully transformed cells *i.e.*, cells harboring the integrated pMCK100 plasmid containing the *bgalH* gene (halophilic β -galactosidase) formed bright blue colonies on X-gal containing plates (Patenge et al., 2000).

When the single colonies became large enough, four blue colonies were picked and transferred to four test tubes containing 5 mL complete medium devoid of mevinolin. Cultures were incubated at 40°C at 100 rpm and after the cultures reached an OD₆₀₀ = 0.3–0.4, they were diluted 200-fold in 35 mL complete medium in 100 mL Erlenmeyer flasks without any mevinolin. Cells were cultured 3 consecutive times to a cell density of OD₆₀₀ = 0.3–0.4 and then finally cultured to a density of OD₆₀₀ = 0.5–0.8 at a shaking speed of 100 rpm. After the final round of culturing, the cell suspensions were diluted 10⁻⁵ and 10⁻⁶ fold with complete medium to a final volume of 100 μ L. The entire 100 μ L was plated out onto complete medium containing X-gal and no mevinolin. Plates were then incubated at 40°C for 5–7 days or until colonies became visible. Plates typically yielded 40–50 colonies and at least 10 red colonies from each plate were picked for the deletion screening experiment and all blue colonies were excluded.

Δ CYP174A1 Screening

Red colonies were inoculated in 5 mL complete medium without selective pressure at 40°C until OD₆₀₀ = 0.4. Genomic DNA was extracted as described above. To assess if (i) successful *CYP174A1* deletion and (ii) deletion occurred at the correct locus, two separate PCR reactions were performed. Oligonucleotides (421-F and 424-R) based on gene sequences adjacent to *CYP174A1* were used for the first round of PCR using *Taq* DNA Polymerase (New England Biolabs). Clones that displayed the correct deletion genotype were then subjected to a second round of PCR using locus specific oligonucleotide primers (Int-F and DS-Bam-R) (**Supplementary Table S1**) and Expand Long Template polymerase (Roche Molecular Biochemicals). Clones that displayed the correct amplicon size were then finally designated as Δ CYP174A1.

Pigment Extraction

Halobacterium salinarum R1 and Δ CYP174A1 strains were cultured in 35 mL complete medium as described previously. Five milliliter samples were taken during late stationary phase at 86 h (OD₆₀₀ = 1.4) and 96 h (OD₆₀₀ = 1.5) and centrifuged at 17 000 \times g for 10 min at ambient temperature. Supernatants were removed and the wet weights of the pellets were normalized. Pellets were extracted with ice cold acetone for 1 h at 4°C with agitation. After each extraction, the pellet was collected by centrifugation at 20 000 \times g and the red supernatant was collected. The extraction was repeated until the pellets appeared white. One milliliter of the supernatant was dried under N₂-gas and resuspended in 0.3 mL fresh acetone. The concentrated extracted pigments were subjected to a wavelength scan (200–750 nm, 2 nm intervals) in 96-well UV microtitre plates using a SpectraMax M2 (Molecular Devices).

DNA Microarrays

Strains of *H. salinarum* R1 and Δ CYP174A1 strains were cultured with agitation at 100 rpm in complete medium in triplicate, at 40°C. Samples for total RNA extraction were taken at the logarithmic- and stationary phases of growth which corresponds to OD₆₀₀ of *ca.* 0.3–1.3, respectively (see **Supplementary Figure S3**). Total RNA extraction, cDNA synthesis, Cy3-dCTP (used for parental strain) and Cy5-dCTP labeling (used for deletion strain) of the cDNA and computational analyses of results was performed as previously described (Coker et al., 2007; DasSarma et al., 2012). Agilent microarray data were analyzed using GeneSpring software, version 11.5.1 and Agilent Feature Extraction Software, version 10.5.1.1, was used for background subtraction and LOWESS normalization. The extracted RNA from the three parallel cultures for each strain was standardized to 6 μ g and pooled to minimize biological noise. Fluorescently labeled cDNA targets from each strain (representing each phase of growth) were combined in a 1:1 ratio and hybridized in duplicate at 65°C for 15 h on a single Agilent slide containing replicated genes from *Halobacterium* sp. NRC-1 (Müller and DasSarma, 2005).

Membrane Analysis

Halobacterium salinarum R1 parental and Δ CYP174A1 strains were cultured in 1 L complete medium at 40°C at 200 rpm until an OD₆₀₀ = 1.2 was reached (late stationary phase). Cells were harvested at 6 000 \times g for 10 min at 4°C and the resulting pellets were used for purple and red membrane isolation using sucrose cushion gradients essentially as described by DasSarma et al. (2012). Pellets were resuspended in basal salts solution and the resulting cell lysate was transferred into dialysis tubing and dialyzed against 5 L of water at 4°C with three changes. The cell paste was treated with 50 μ L DNase (10 μ g/ μ L) and incubated at 37°C for 1 h while shaking at 180 rpm. The DNaseI digested cell lysates were gently layered onto the sucrose gradients and placed in a balanced SW32 Ti rotor and spun at 132 000 \times g

for 17 h at 18°C in a Beckman Coulter Optima™ L-100 Ultracentrifuge.

Microarray Data Accession Number

Array data were deposited in the GEO database¹ under series accession number GSE104012.

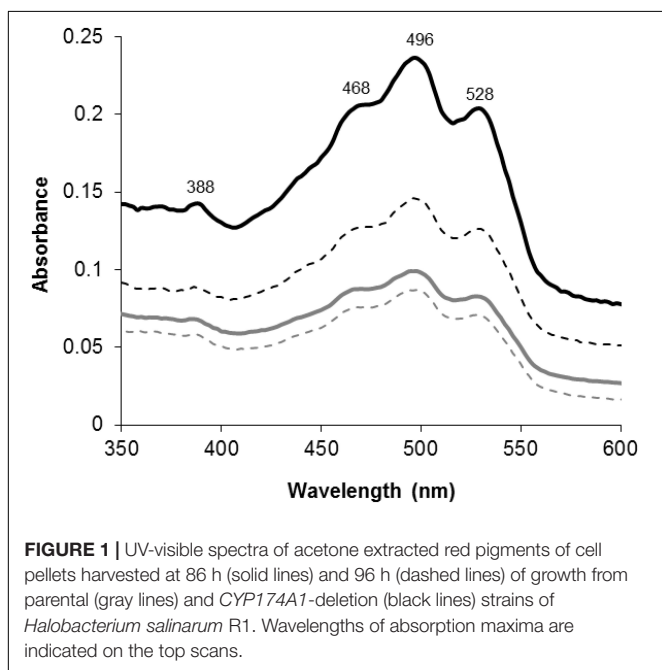
RESULTS

Deletion of *CYP174A1* Appears to Influence Bacterioruberin Synthesis

When *H. salinarum* R1 parental and the P450 deletion strains were cultured on solid medium and in liquid medium a marked difference in pigmentation was observed. In the liquid cultures the deletion strain first appeared darker red-orange in comparison to the parent R1 strain after about 40 h of growth (early stationary phase). Normalized wet-weight samples of each strain was collected at 86 h and 96 h (late stationary phase) and the red pigments were extracted with acetone and subjected to an UV-visible wavelength scan. **Figure 1** illustrates the UV-visible spectra of the extracted pigments from both parental- and deletion strains.

The obtained spectra all displayed the characteristic so-called “three-finger” shape that is typical of the C₅₀ bacterioruberin-like carotenoids (D’Souza et al., 1997; Fang et al., 2010). Based on the spectral data, all deletion strains always produced more red pigment when compared to the parental strains. Deletion of *CYP174A1*

¹<http://www.ncbi.nlm.nih.gov/geo>



in *H. salinarum* R1 appears to have influenced carotenoid-metabolism and *inter alia* caused the accumulation of bacterioruberins.

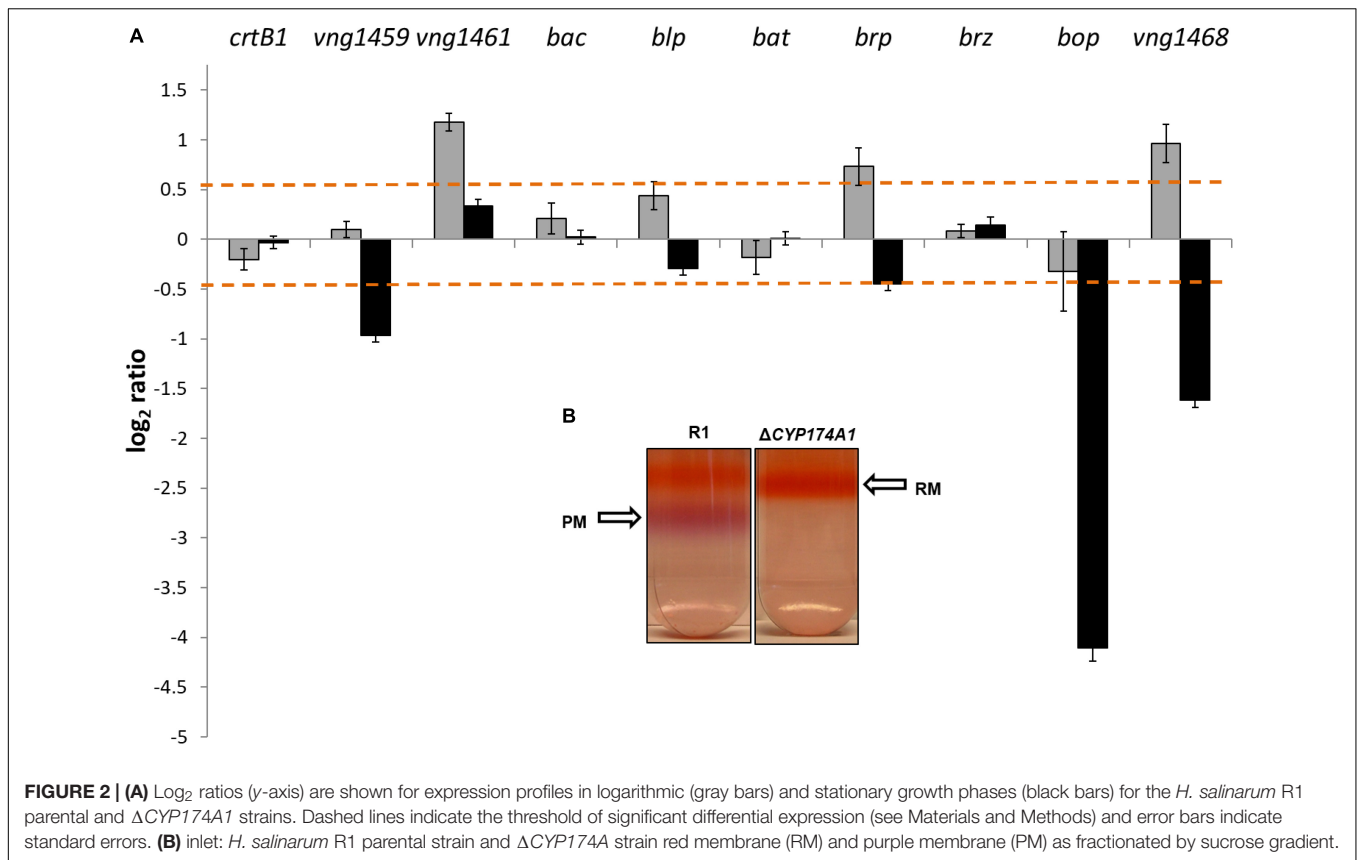
DNA Microarray and Purple Membrane Analyses

Data from the two color microarrays for samples from the logarithmic and stationary phases of growth were plotted on a scatter plot (**Supplementary Figure S2**). Differentially expressed genes that were statistically significant were defined as genes that displayed a *P*-value < 0.05 and a fold change cutoff threshold of ≥ 1.5 (\log_2 ratio of ≥ 0.5). Based on the aforementioned criteria, 41 and 101 genes were differentially expressed during the logarithmic and stationary phases of growth, respectively. The *brp* gene (encoding the bacterio-opsin-related protein) and two other genes with unknown functions, *vng1461* and *vng1468*, were significantly expressed during the logarithmic growth stage. *Brb*, in conjunction with *crtB1* are involved in the first and last committed steps of the retinal chromophore biosynthetic pathway, respectively (Baliga et al., 2001; Peck et al., 2001; DasSarma et al., 2012). Most strikingly of the gene expression profile, was the very low expression levels (linear fold change of -19.10 or \log_2 ratio of -4.10) of the *bop* gene (encoding for the bacterio-opsin protein) during stationary phase in the $\Delta CYP174A1$ strain. The *bop* gene forms part of a cluster of genes, called the *bop*-gene regulon (Peck et al., 2001; Tarasov et al., 2008, 2011; DasSarma et al., 2012) that is involved in the biosynthesis and regulation of BR in PM. **Figure 2A** illustrates the gene expression profiles of *bop* and other genes associated with the *bop*-regulon during both phases of growth as mentioned above. The very low levels of *bop*-expression imply the possible abolishment of PM synthesis and this was confirmed with a subsequent sucrose gradient: PM was present in the parental R1 strain but absent in the $\Delta CYP174A1$ strain (**Figure 2B**).

DISCUSSION

Bacterioruberin Accumulation

Deletion of the *CYP174A1* gene in *H. salinarum* R1 appears to have influenced bacterioruberin and PM biosynthesis. Red colored bacterioruberins accumulated (**Figure 1**) and PM became absent in the $\Delta CYP174A1$ strain when compared to the parental strain (**Figure 2B**). An increase in bacterioruberin levels in *H. salinarum* was also observed by Dummer et al. (2011) but this was due a deliberate *bop* gene deletion. The authors discovered that the *lye* (lycopene elongase) gene catalyzed the committed step in bacterioruberin biosynthesis and that the *bop* gene product, bacterio-opsin, inhibited lycopene elongase and consequently the production of bacterioruberins. In the current study, the very low expression of *bop* in the $\Delta CYP174A1$ strain has likely rendered the lycopene elongase enzyme completely uninhibited and caused the increase in bacterioruberin biosynthesis. It has been previously proposed that free retinal (when not bound to



bacterio-opsin) could potentially regulate bacterioruberin biosynthesis; however, Dummer et al. (2011) found that free retinal had no significant effect on bacterioruberin biosynthesis.

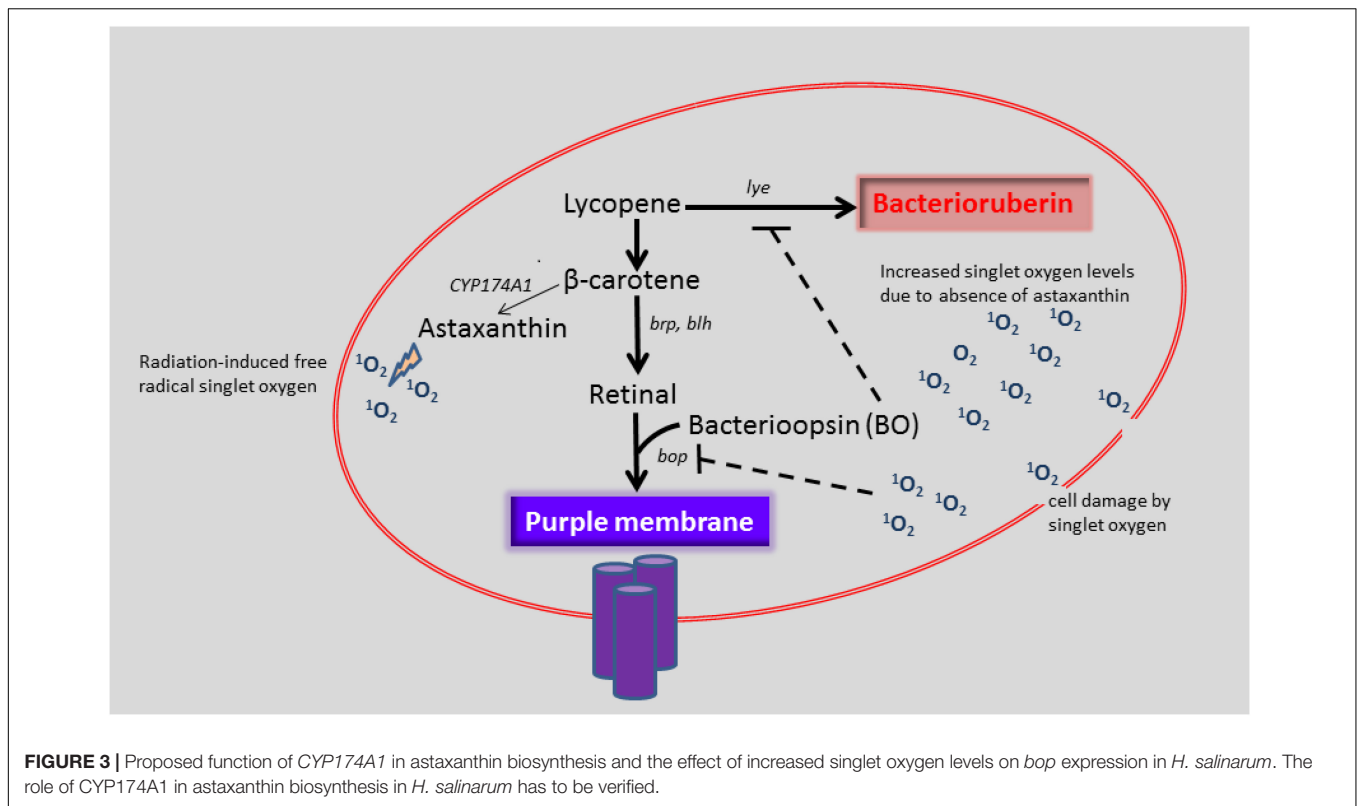
Decreased *Bop* Gene Transcription Levels

The *bop* gene forms part of a tightly regulated cluster of genes referred to as the *bop*-regulon (Tarasov et al., 2008, 2011; DasSarma et al., 2012). *Bop* is regulated by a sensor regulator gene called *bat* and potentially also by a small zinc-finger containing protein called *brz*. The *bat* gene encodes for a *trans*-acting factor that induces *bop* transcription at low oxygen tension, which naturally occurs in the stationary phase. DasSarma et al. (2012) illustrated that a *bat* deletion caused a marked drop in transcription levels of several genes in the *bop*-regulon including *bop*. The subsequent drop in *bop* transcription directly caused the loss of PM. **Figure 2A** illustrates that neither *bat* nor *brz* were significantly expressed in stationary phase and that instead of *bop* being induced, the transcript levels of *bop* dropped dramatically, which in turn caused the loss of PM (**Figure 2B**). In addition, there was no significant gene expression and considerable decrease in transcription levels of e.g., *crtB1* or *blp* as observed when *bat* is deleted (DasSarma et al., 2012). Although a similar phenotype for loss of PM was observed for this study, the crucial difference is that no *bat* deletion was ever introduced as was the case with DasSarma et al. (2012).

In rare cases, the abolishment of *bop* can be attributed to spontaneous insertions either in *bop* itself or in the *brp* gene. Since the early 1980s, several insertion sequences (IS) have been identified in *bop* from various PM-deficient *Halobacterium* strains (Simsek et al., 1982; DasSarma et al., 1983; Ovchinnikov et al., 1984; Pfeiffer et al., 1984; Pfeiffer and Betlach, 1985; Ebert et al., 1987). Typically, the IS called ISH 1 (1 118 bp in size) integrates into a single site in the *bop* gene and ISH 2 (520 bp in size) at several sites of *bop*. In the current study, the *bop*, *brp* and *bat* genes from both the parental and Δ CYP174A1 strain, were PCR amplified with gene specific oligonucleotide primers (**Supplementary Table S1**) to assess their ORF size. All the aforementioned genes displayed the correct amplicon size (see **Supplementary Figure S4**). For the current study, we concluded that IS was most probably not responsible for the decreased *bop* transcription levels and loss of PM in the Δ CYP174A1 strain.

Possible Physiological Role of CYP174A1 in *H. salinarum*

Calo et al. (1995) reported that some species of *Haloarcula hispanica* and *H. salinarum* contain *trans*-astaxanthin. In *H. salinarum* about 11% of the pigment (per weight basis) was *trans*-astaxanthin while 24% was 3-hydroxy-echinenone (Calo et al., 1995). Astaxanthin biosynthesis can occur *via* a 3-hydroxy-echinenone intermediate by the addition of two keto and two hydroxyl moieties at the 4,4' and 3,3'-positions of the β -ionone rings of β -carotene, respectively (Martín et al., 2008). Given the fact



that 3-hydroxy-echinenone and astaxanthin have been identified in *H. salinarum* (Calo et al., 1995), we speculate that, as in the case of the CYP450 from *X. dendrorhous*, CYP174A1 from *H. salinarum* acts as both a ketolase and a hydroxylase to produce astaxanthin.

Astaxanthin is a potent anti-oxidant capable of quenching the highly reactive oxygen species (ROS) called singlet oxygen (Makino et al., 2008; Glaeser et al., 2011). Singlet oxygen is the product of photo-oxidative stress due to cells being exposed to high light intensities and can cause severe cell damage by rapidly reacting with *inter alia* proteins, lipids and DNA (Glaeser et al., 2011). Interestingly, microarray analyses by Facciotti et al. (2010) revealed that when *Halobacterium* sp. strain NRC-1 was grown in rich medium, the *CYP174A1* transcript levels significantly increased in the transition from the exponential to stationary phase of growth. Stationary phase is also the period when *bop* expression is increased to biosynthesize PM for the purpose of phototrophic growth (Baliga et al., 2001; DasSarma et al., 2012).

If we assume that CYP174A1 catalyzes the biosynthesis of astaxanthin in *H. salinarum*, it could be argued that increasing levels of singlet oxygen induces the formation of astaxanthin. Interestingly, Schroeder and Johnson (1995) observed that carotenoid biosynthesis was induced by singlet oxygen and other peroxy radicals in *X. dendrorhous*. The deletion of *CYP174A1* and presumably the consequent decrease in astaxanthin will cause a potential detrimental increase in singlet oxygen levels for *H. salinarum* (Figure 3).

We speculate that when *CYP174A1* is deleted, that increasing singlet oxygen levels and possibly other ROS act as a chemical signal that necessitates *H. salinarum* to utilize an auxiliary mechanism: by inhibiting the *bop* gene product and thereby lifting the inhibition on the *lye* gene. This in turn will result in increased biosynthesis of bacterioruberin, which also has anti-oxidant properties (Rodrigo-Baños et al., 2015). The higher levels of bacterioruberin could further potentially aid in the protection of *H. salinarum* against oxidative damage from singlet oxygen and other ROS.

The role(s) of CYP450s in extremely halophilic archaea is still unknown. This study paves the way for future work that could shed more light on the complex physiological role of CYP450s in not only *H. salinarum* but possibly other extremely halophilic archaea. In particular, astaxanthin levels need to be measured in both the $\Delta CYP174A1$ and parental strains and the possible role of CYP174A1 in the synthesis of astaxanthin needs to be verified. The complex regulation of pigment synthesis is not only of interest from a molecular genetic perspective but is also of increasing interest for astrobiology (Schwieterman et al., 2018; Walker et al., 2018).

AUTHOR CONTRIBUTIONS

WM performed the research and wrote the paper. MC assisted with the microarray experiments and analysis. MS, EvH, and SD coordinated the study. MS and EvH provided funding for the study.

ACKNOWLEDGMENTS

The authors wish to thank the DasSarma laboratory for their useful inputs and the National Research Foundation (South Africa) for funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.02563/full#supplementary-material>

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