JB Accepted Manuscript Posted Online 7 August 2017
J. Bacteriol. doi:10.1128/JB.00303-17

Opsin-mediated inhibition of bacterioruberin synthesis in halophilic Archaea

Running Title: opsin-mediated inhibition of bacterioruberin synthesis

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Keywords: cofactor biosynthesis, carotenoid biosynthesis, C50 carotenoid, membrane protein biogenesis, microbial rhodopsin, proteorhodopsin, UbiA prenyltransferase

ABSTRACT

Halophilic Archaea often inhabit environments with limited oxygen, and many produce ion-pumping rhodopsin complexes that allow them to maintain electrochemical gradients when aerobic respiration is inhibited. Rhodopsins require a protein, opsin, and an organic cofactor, retinal. We have previously demonstrated that, in *Halobacterium salinarum*, bacterioopsin (BO), when not bound by retinal, inhibits the production of bacterioruberin, a biochemical pathway that shares intermediates with retinal biosynthesis. In this work, we use heterologous expression in a related halophilic Archaeon, *Haloferax volcanii*, to demonstrate that BO is sufficient to inhibit bacterioruberin synthesis catalyzed by the *H. salinarum* lycopene elongase (Lye) enzyme. This inhibition was observed both in liquid cultures and in a novel colorimetric assay to quantify bacterioruberin abundance based on the colony color. Addition of retinal to convert BO to the bacteriorhodopsin complex resulted in a partial rescue of bacterioruberin production. To explore if this regulatory mechanism occurs in other organisms, we expressed a Lye homolog and an opsin from *Haloarcula vallismortis* in *H. volcanii*. *H. vallismortis* cruxopsin expression inhibited bacterioruberin synthesis catalyzed by *H. vallismortis* Lye, but had no effect when bacterioruberin synthesis was catalyzed by *H. salinarum* or *H. volcanii* Lye. Conversely, *H. salinarum* BO did not inhibit *H. vallismortis* Lye activity. Together, our data suggest that opsin-mediated inhibition of Lye is potentially widespread and represents an elegant regulatory mechanism that allows organisms to efficiently utilize ion-pumping rhodopsins obtained through lateral gene transfer.

IMPORTANCE

Many enzymes are complexes of proteins and non-protein organic molecules called cofactors. To ensure efficient formation of functional complexes, organisms must regulate the production of proteins and cofactors. To study this regulation, we use bacteriorhodopsin from the Archaeon *Halobacterium salinarum*. Bacteriorhodopsin consists of the bacterioopsin protein and a retinal cofactor. In this work, we further characterize a novel regulatory mechanism in which bacterioopsin promotes retinal production by inhibiting a reaction that consumes lycopene, a retinal precursor. By expressing *H. salinarum* genes in a different organism, *Haloferax volcanii*, we demonstrate that bacterioopsin alone is sufficient for this inhibition. We also found that an opsin from *Haloarcula vallismortis* has inhibitory activity, suggesting that this regulatory mechanism might be found in other organisms.

INTRODUCTION

Many integral membrane protein complexes, including those that carry out universal processes such as energy conversion and the response to environmental changes, require organic cofactors. There are compelling reasons for cells to balance the production of proteins and these corresponding cofactors as both proteins and cofactors require energy for synthesis, and an imbalance of proteins and cofactors could potentially harm cells. This balance represents a challenging biological problem since the mechanisms for synthesizing proteins and small molecules are not inherently linked.

To explore this fundamental question, we study the relatively simple microbial rhodopsin complex that consists of a single opsin protein and a 20-carbon retinal cofactor. Microbial rhodopsins undergo a conformational change in response to light and function

to transport ions or mediate phototaxis. After their initial discovery in halophilic Archaea, microbial rhodopsins are now known to be widely distributed (reviewed in (1)), and substantial evidence indicates that this distribution is partially due to frequent lateral gene transfer (2, 3). Although obtaining genes encoding opsins and retinal biosynthetic enzymes allow organisms to better exploit light in their environment, no benefit is gained if insufficient retinal is available to form functional complexes. Retinal biosynthesis may share intermediates with other cellular processes, and it is not known how organisms ensure that resources are devoted to produce retinal needed for rhodopsins. In the work described here, we further characterize a regulatory mechanism in which opsins, when not bound by retinal, inhibit another process to preserve shared intermediates for retinal biosynthesis.

Bacteriorhodopsin (BR), a light-driven proton pump in the halophilic Archaeon *Halobacterium salinarum*, was the first microbial rhodopsin described (4) and has been extensively studied as a model for membrane protein structure and activity (reviewed in (5-7)). Halophilic archaea thrive in high salinity environments such as salt crystallizer ponds and often grow to densities of 10^7-10^8 cells ml^{-1} (8). With this high density, oxygen availability is limited, inhibiting aerobic respiration and necessitating the production of ion-pumping rhodopsins like BR. In conditions of low oxygen, BR production is induced, and the BR apoprotein, bacterioopsin (BO), and retinal are maintained in ~1:1 stoichiometry (9). In *H. salinarum*, retinal is synthesized *de novo* with the final synthesis steps the cyclization of lycopene to form β-carotene (10) and subsequent cleavage of β-carotene to retinal (11). Lycopene is also used to synthesize bacterioruberins (12-14), 50-carbon carotenoids that may function to increase membrane rigidity (15) and provide

protection from ultraviolet light (16). The conversion of lycopene to the first bacterioruberin intermediate is catalyzed by the *lye*-encoded enzyme lycopene elongase (Lye) (14, 17). Thus, lycopene is the last shared intermediate for bacterioruberin and retinal synthesis, and the Lye enzyme is a potential target for regulation of these pathways.

We previously described a novel regulatory mechanism in *H. salinarum* in which BO, when not bound by retinal, inhibits Lye activity so bacterioruberin production is reduced and lycopene is available for synthesis of the retinal cofactor (Fig. 1). To determine the specificity of this inhibition, we then compared the activity of *H. salinarum* Lye to the Lye homolog from *Haloferax volcanii*, a related halophilic Archaeon that does not express an opsin but does produce bacterioruberins. When expressed in *H. salinarum, H. volcanii* Lye also converted lycopene to the first bacterioruberin intermediate but was not inhibited by BO (14). Here, we build on this previous work by expressing opsin and Lye homologs in *H. volcanii*. We demonstrate that BO is sufficient to inhibit *H. salinarum* Lye in the absence of any other factors from *H. salinarum*. We also provide insight into potential features of Lye and BO that are required for BO-mediated inhibition of bacterioruberin synthesis. Lastly, we demonstrate that *Haloarcula vallismortis* cruxopsin inhibits bacterioruberin synthesis catalyzed by the *H. vallismortis lye* homolog. This finding suggests that interactions between opsins and other proteins may be widespread and raises the possibility that this regulatory mechanism confers a selective advantage to organisms that express ion-pumping rhodopsins.

RESULTS

The *H. volcanii lye* homolog is required for bacterioruberin synthesis. We previously determined that the *H. volcanii lye* homolog (HVO_RS16895, formerly HVO_2527) rescued the Δ*lye* phenotype in *H. salinarum*, and expression of *H. volcanii lye* in lycopene-producing *Escherichia coli* resulted in a bacterioruberin precursor (14). To test if the *lye* gene was required for bacterioruberin synthesis in *H. volcanii*, we constructed an in-frame deletion of residues 45-226 from the 301 amino acid (aa) predicted protein. The *H. volcanii* Δ*lye* strain lacked detectable bacterioruberin production and resulted in an accumulation of lycopene (Fig. 2). Bacterioruberin synthesis was restored with the introduction of either *H. volcanii* or *H. salinarum lye* on an expression vector (Fig. 2), demonstrating that Lye is required for the conversion of lycopene to bacterioruberins.

BO expression in *H. volcanii* is sufficient to inhibit *H. salinarum* Lye activity. *H. volcanii* lacks genes for any opsins (18) and is highly unlikely to have any native factors that interact with opsins. *H. volcanii* therefore represents an ideal system to test the possibility that BO expression does not require other proteins from *H. salinarum* to inhibit lycopene elongase activity. We first used site-directed recombination (19) to place the *H. salinarum lye* gene at the *H. volcanii lye* locus. The *H. salinarum* Lye enzyme was functional as bacterioruberin synthesis was restored in this strain (Fig. 3A). To test if the presence of BO was sufficient to inhibit the *H. salinarum* Lye enzyme, we transformed the strain expressing *H. salinarum lye* with an expression vector harboring *bop*, the gene that encodes BO. In *H. volcanii* with its native *lye* gene, bacterioruberin synthesis was unaffected by BO expression (Fig. 3A). In contrast, when *H. salinarum lye* replaced *H.*

volcanii lye, BO expression reduced bacterioruberin to about 16% of the total carotenoids (Fig. 3A) with the remaining 84% as lycopene. To determine if this inhibition was a specific effect of the opsin, we added retinal during growth of the culture to convert the BO to BR. Retinal addition resulted in a partial rescue of bacterioruberin levels to 63% of the total carotenoid (bacterioruberin and lycopene) in the cells (Fig. 3A). These results indicated that BO is sufficient to inhibit the *H. salinarum* Lye enzyme.

Developing a colony color analysis to assess bacterioruberin production. To more rapidly and efficiently quantify bacterioruberin production in *H. volcanii* strains, we developed an assay based on colony color. Bacterioruberin imparts a pink color, and this color is more intense in colonies with a greater concentration of bacterioruberin (Fig. 3B). Large colonies were grown on solid media, supplemented with retinal where noted, and pictures taken with a digital camera mounted to a dissection microscope under controlled lighting. Using image analysis software, a variety of color measures were recorded. These measures were modeled using beta regression for their ability to predict bacterioruberin as a percentage of total carotenoid in cultures as measured by RP-UHPLC for multiple strains (Supplemental Materials). Using the parameters of the model, we calculated a metric for bacterioruberin proportion. This Ruberin Metric facilitated high-throughput screening and comparisons of strains in the absence or presence of retinal (Fig. 3C). Although colony color analysis exhibited greater variability compared to RP-UHPLC, our mean values were strikingly similar to those determined by analysis of carotenoids from liquid culture (compare Fig. 3A and 3C).

***H. salinarum* Lye residues 69-120 are necessary for BO-mediated inhibition**. To identify the region of *H. salinarum* Lye required for BO-mediated inhibition, we

constructed 'hybrid' genes that contained portions of both *H. salinarum* and *H. volcanii lye* (Fig. 4A). These genes were integrated into the *H. volcanii lye* locus and inhibition was tested by expression of BO. BO-mediated inhibition of Lye was not observed when the enzyme only included the N-terminal 68 amino acid residues from *H. salinarum* (Fig. 4B). However, hybrid Lye enzymes that included larger sections of *H. salinarum* Lye were inhibited by BO expression (Fig. 4B). Together, our data demonstrate that the *H. salinarum* Lye residues 69-120 are necessary to allow inhibition by BO. Unfortunately, hybrid genes that encoded an N terminus from *H. volcanii* did not confer substantial bacterioruberin production (data not shown) so we could not test if this region was sufficient to allow BO-mediated inhibition.

To assess potential structural differences in the region required for inhibition, we generated template-based secondary structure predictions (22) of *H. salinarum* and *H. volcanii* Lye proteins (Fig. 4C). Both homologs were predicted to be integral membrane proteins with nine transmembrane domains. For both predictions, the highest template match was to a structure of 4-hydroxyboenzoate octaprenyltransferase, a UbiA superfamily member from the thermophilic Archaeon *Aeropyrum pernix* (25) with at least 90% confidence score over more than 85% of the length of the Lye proteins. Similar to the predicted activity of Lye, this enzyme uses isopentenyl pyrophosphate as a substrate to attach prenyl groups to secondary metabolites. Based on this model, the 52 aa region necessary for inhibition has a similar overall structure in both *H. salinarum* and *H. volcanii* enzymes and consists of a portion of a cytoplasmic loop, a transmembrane helix, a very short extracellular loop, and another transmembrane helix (Fig. 4C).

Ablation of BO-mediated inhibition of bacterioruberin biosynthesis requires

covalent binding of retinal. After observing that retinal addition to convert BO to BR largely reversed the inhibition of bacterioruberin biosynthesis (Fig. 3), we sought to determine if this reversal required covalent binding of retinal to BO. We expressed BO with the lysine residue at position 216 in the mature protein changed to alanine. This lysine (K216) is required for the Schiff-base linkage to retinal (26, 27). The K216A BO inhibited *H. salinarum* Lye activity to a similar degree as wild-type BO (Fig. 5A). Retinal addition resulted in a slight, but not statistically significant (Permutation test, Bonferroni adjusted p=0.11), increase in bacterioruberin, but to a substantially lesser degree than wild-type BO (Fig. 5A).

***H. salinarum* sensory opsin II does not inhibit Lye.** In addition to BR, *H. salinarum* produces three other rhodopsins: the chloride ion pump halorhodopsin and two sensory rhodopsins. Since these opsins also require retinal, we tested if they inhibited Lye activity. We constructed *H. volcanii* strains expressing each *H. salinarum* opsin, but immunoblotting of C-terminal his-tagged versions of these proteins revealed that only sensory opsin II (SOII), the protein component of sensory rhodopsin II (SRII), was expressed at levels reduced but comparable to BO (Fig. 5B). Like BO, SOII was detected in the membrane fraction, indicating that it was appropriately targeted to the plasma membrane of *H. volcanii* (Fig. S3). Expression of SOII did not affect bacterioruberin synthesis, and the addition of retinal to this strain also did not change bacterioruberin production (Fig. 5A). This result indicates that BO has specific structural features that allow inhibition of Lye activity, and these features are not present in SOII.

Evidence of an opsin and Lye interaction in another organism. As a first step to determine if opsins in other organisms also inhibit the activity of lycopene elongase

enzymes, we analyzed potential inhibition caused by cruxopsin-3 (CO), an opsin identified in *H. vallismortis* (28). Like BO, CO covalently binds retinal to form a rhodopsin that functions as a proton pump (29). An expression vector encoding CO was constructed and introduced into *H. volcanii* strains with *H. volcanii* or *H. salinarum lye*. CO expression was confirmed by immunoblotting of his-tagged CO, although at approximately half the abundance of BO (Fig. 5B), and the protein localized to the *H. volcanii* membrane (Fig S3). In *H. volcanii*, CO had no observable effect on bacterioruberin production catalyzed by *H. volcanii* or *H. salinarum* Lye enzymes (Fig. 6).

We then tested for CO-mediated inhibition of the *H. vallismortis* Lye enzyme. A similarity search of the *H. vallismortis* genome sequence (available from the Joint Genome Institute [http://genome.jgi.doe.gov]) revealed a gene, BLV10_RS12820, predicted to encode a 294 aa protein homologous to *H. salinarum* Lye (60% identity over 276 amino acids) and *H. volcanii* Lye (60% identity over 273 amino acids). The *H. vallismortis lye* homolog rescued bacterioruberin synthesis when expressed from a plasmid (Fig. 2) or integrated into the *H. volcanii lye* locus (Fig. 6), confirming the functional identity of the gene. Expression of CO sharply reduced bacterioruberin production when catalyzed by *H. vallismortis* Lye, and this inhibition was partially relieved when retinal was added (Fig. 6). BO had no significant effect on bacterioruberin synthesis in the strain with *H. vallismortis* Lye (Fig. 6), indicating that the opsin-Lye interaction is specific. This result shows that opsin-mediated inhibition of Lye is not unique to *H. salinarum* and may be a common mechanism to regulate retinal production.

DISCUSSION

BO expression is sufficient to inhibit *H. salinarum* Lye. By expression of *H. salinarum* genes in *H. volcanii*, we determined that BO is sufficient to inhibit the bacterioruberin biosynthesis catalyzed by *H. salinarum* Lye, and this inhibition is released by addition of retinal to convert BO to BR (Fig. 3). The most plausible explanation for these findings is that BO, in the absence of its retinal cofactor, directly binds Lye. When sufficient retinal is available to convert BO to BR, Lye is released and catalyzes the conversion of lycopene to bacterioruberins.

We also demonstrated that BO with the K216A point mutation, which does not covalently bind retinal, inhibits Lye activity even in the presence of retinal (Fig. 5A). Therefore, BO must undergo a structural change upon retinal binding that eliminates its inhibiting function. We are currently working towards identifying mutations in BO that allow simultaneous covalent binding of retinal and inhibition of Lye activity. These modified BR complexes may, as for wild-type BR, form the two-dimensional crystal lattice that allows relatively straightforward purification and structural determination of BR. These structural analyses would provide insight into the structure of the BO apoprotein and changes that occur upon retinal binding.

Inhibition of activity is likely mediated by a specific region of Lye

By expression of hybrid genes that contained regions of both *H. salinarum* and *H. volcanii lye*, we determined that *H. salinarum* Lye residues 69-120 were necessary for inhibition (Fig. 4A, 4B). This region does not encompass either of the two conserved aspartate-rich motifs that likely mediate substrate binding or catalysis in UbiA prenyltransferases (25, 30), indicating that variability in this region could allow regulation

while maintaining the catalytic core of the enzyme. For both Lye proteins, the transmembrane helices in the region required for inhibition are predicted to be accessible to possible interactions with other membrane proteins (Fig. 4C), suggesting that a potential BO-Lye interaction could occur between transmembrane regions. Interestingly, the signaling by sensory rhodopsins to their cognate transducers are known to be mediated through contacts within the membrane (31), raising the possibility of similar interactions between BO and Lye. Although the *H. salinarum* and *H. volcanii* Lye proteins are predicted to have high structural similarity in the portion of the protein necessary for regulation (Fig. 4C), the largest section of predicted disordered structure lies in the cytoplasmic loop within this region. This finding suggests that conformational changes may occur in this area that regulate enzyme activity.

Based on sequence similarity, Lye is a member of the UbiA superfamily, integral membrane proteins found throughout all domains of life that catalyze the addition of a prenyl group to a variety of substrates (reviewed in (32)). Intriguingly, UbiA enzymes, including octaprenyltransferases encoded by CoQ, UbiA, and MenA genes, catalyze key steps in quinone biosynthesis and use polyprenyl pyrophosphate molecules that could also be used as precursors for retinal synthesis. Ion-pumping rhodopsins are often upregulated in low-oxygen conditions when the need for quinones for respiration and oxygen scavenging is reduced. Therefore, our findings raise the possibility that opsins could inhibit these UbiA enzymes in a similar mechanism as BO and CO inhibit Lye. The UbiA superfamily is highly relevant to human health as dysfunctional UbiA enzymes have been implicated in a variety of disease including Schnyder Crystalline Corneal Dystrophy (33), infantile multisystem disease (34), and urologic cancer (35). In addition,

selective inhibitors of these prenyltransferases have been shown to be effective against *Mycobacterium tuberculosis* (36). Our characterization of opsin-mediated Lye inhibition may allow insight into the regulation and activity of other members of the UbiA superfamily.

Opsin-mediated inhibition of Lye and similar enzymes represents an ideal regulatory mechanism for genes obtained through lateral transfer

In this work, we determined that *H. vallismortis* CO specifically inhibited the *H. vallismortis* Lye (Fig. 6) suggesting that *H. vallismortis*, like *H. salinarum*, inhibits bacterioruberin synthesis to conserve lycopene for retinal production. This raises the possibility that the described mechanism, in which an opsin inhibits a pathway that consumes retinal precursors, may be commonly found in organisms with ion-pumping rhodopsins. As further evidence that ion-pumping rhodopsins have specific features that mediate inhibition, we determined that expression of *H. salinarum* SOII had no effect on bacterioruberin accumulation (Fig. 5A). Although we cannot exclude the possibility that SOII folding is different when expressed in *H. volcanii*, our results are consistent with previous observations in *H. salinarum* that conversion of lycopene to bacterioruberin was nearly quantitative in the absence of BO (14). BR and SRII are structurally very similar (37, 38), but they have stark differences in expression patterns in *H. salinarum*; BR production is induced to high levels (~10% of total cell protein) under microaerobic conditions, and SRII is produced in much lower amounts (0.01-0.1% of total cell protein) under both aerobic and microaerobic conditions (39). Given these differences in expression, BO-mediated inhibition of Lye activity may have evolved to ensure that all available lycopene is directed towards retinal synthesis when cells required BR activity

to survive microaerobic conditions.

Genes allowing rhodopsin production are commonly spread by lateral gene transfer, and many organisms may produce substantial amounts of ion-pumping rhodopsins to survive in conditions of limited resources (1). After genes are obtained, retinal production may be in competition with other processes in these organisms for shared intermediates like lycopene. Opsins that inhibit these processes to promote retinal synthesis may confer a selective advantage, especially when functional ion-pumping rhodopsins are needed for energy conversion in the absence of respiration. Therefore, this regulatory mechanism first described for *H. salinarum* could be found among organisms with ion-pumping rhodopsins in all three domains of life. More broadly, apoproteins, like BO, lacking their cofactors may have activities that have not been characterized.

MATERIALS AND METHODS

Growth conditions. *H. volcanii* was grown in complete (Hv-YPC) medium (40) supplemented with 40 µM thymidine. Liquid cultures were grown at 40 °C shaking at 250 rpm. Plates were incubated at 42 °C. For *H. volcanii* cultures used for carotenoid quantification or opsin expression analysis, 10 mL cultures were started from individual colonies and grown for 24 h, diluted to 0.05 A_{660} in new medium, and grown for an additional 48 h to an A_{660} value of 1.7-2.0. There were no obvious growth differences in any *H. volcanii* strains. 4 mL of these saturated cultures were used to inoculate 96 mL Hv-YPC in 250 mL flasks, and these 100 mL cultures were grown for 18 h. Where noted, 20 µL of 20 mM retinal (Sigma-Aldrich, St. Louis, MO) in isopropanol (or isopropanol alone for cultures without retinal) were added to the 100 mL cultures to a final concentration of 4 µM retinal.

***H. volcanii* strain construction.** *H. volcanii* strains are listed in Table 1. Plasmids and primers used to construct strains are listed in Tables S6 and S7, respectively. Plasmids were propagated in *E. coli* DH5α (New England Biolabs, Ipswich, MA) grown in LB medium supplemented with ampicillin (100 µg/mL). *H. volcanii* transformation was conducted as previously described (41). Gene knockout and replacement mutations were made in *H. volcanii* strain H1209 (42) and derivative strains. For genomic recombinants, selection and counterselection of *pyrE2* was conducted as described using plasmids derived from pTA131 (40). Plasmids for gene expression in *H. volcanii* were constructed using pTA963 (42). All plasmids introduced into *H. volcanii* were confirmed by sequencing the added or modified DNA regions. If appropriate, proper integration into the genome was confirmed by PCRs both upstream and downstream of

the integrated region that included one primer not present on the integrative plasmid. Opsin- or Lye-encoding open reading frames introduced into *H. volcanii* were amplified by PCR and resequenced to confirm strain fidelity. T. Allers generously provided H1209, pTA131, and pTA963. *H. volcanii* DS70 (43) was graciously sent by K. Bidle.

Carotenoid quantification and analysis from cultures. Carotenoids were extracted from *H. volcanii* similarly to the previously described method for *H. salinarum* (14). Cultures were centrifuged for 15 min at 11,000 × g. The pellet was washed in 100 mL media salt solution, centrifuged for 15 min and supernatant removed. To facilitate removal of as much supernatant as possible, the pellet was centrifuged for an additional 5 min, and remaining liquid was removed by aspiration. Pellets were resuspended in 1 mL media salt solution, transferred to screw-cap microcentrifuge tubes, and centrifuged for 5 min at 16,100 × g. The supernatant was removed, and tubes purged with N_2. Cell pellets were stored at -80 °C until carotenoid extraction.

For extraction, the pellet was lysed in 1 mL lysis solution (0.016 mg/mL DNase (Sigma-Aldrich); 0.05% NaN_3) for 1 h by shaking at 1,500 rpm in the dark. The cell lysate, ~1.5 mL, was then vigorously mixed in an amber vial with 9 mL of acetone for 20 min. Hexane (5 mL) and water (1 mL) were added and briefly vigorously mixed. The organic (upper) layer was collected, and the remaining sample was extracted again with 3 mL of hexane. The organic layers were combined and evaporated to dryness under an N_2 stream. The pigments were resuspended in 250 µL ethyl acetate and filtered through a 0.45 µm polytetrafluoroethylene filter before analysis with reverse-phase ultra high performance liquid chromatography (RP-UHPLC).

For RP-UHPLC, samples were fractionated on a Shimadzu (Kyoto, Japan) UHPLC system using a C_{18} column (2.1 x 50 mm, 1.9 µm particle size), and absorbance at 490 nm was monitored with a photo diode array detector. The mobile phase was a gradient of solvent A (0.05% ammonium acetate) and solvent B (acetonitrile:methanol:chloroform 75:15:10) eluting at 0.4 mL min^{-1}. The solvent change over an 11.5-min sample run was programmed as follows: elution with 70% B for 1 min, gradient to 100% B for 2.5 min, isocratic elution with 100% B for 4.5 min, gradient to 70% B for 0.1 min, and reequilibration with 70% B for 3.4 min. The standard curve for lycopene was generated with commercial lycopene (Sigma-Aldrich). The bacterioruberin standard curve was generated from pooled *H. volcanii* (H1209) cell extracts. Bacterioruberin concentration was calculated spectroscopically using an extinction coefficient in acetone of E = 141 mM^{-1} cm^{-1} at 490 nm (44) The lycopene present in the samples used for the bacterioruberin standards was calculated to contribute less than 0.5% to the absorbance at 490 nm. All peaks eluting between 1.2 and 5.2 min that displayed spectra characteristic of bacterioruberins were included in the bacterioruberin concentration. Lycopene precursors and other carotenoids potentially present in minor concentrations were not included in the analysis.

Colony color analysis to assess bacterioruberin production. Cultures were grown from colonies for 48 h and the A_{660} value adjusted to 1.5 prior to plating. 5 µL droplets of culture were placed on Hv-YPC plates supplemented with 100 µL isopropanol (control) or 100 µL 20 mM retinal (dissolved in isopropanol) for a final concentration of approximately 50 µM retinal. The droplets were allowed to dry and the plates incubated at 42 °C. Each colony was photographed at approximately 24-hour increments with a

373 Moticam 5.0 MP camera (Motic, Hong Kong, China) mounted on a VistaVision
374 dissection microscope (VWR, Radnor, PA). Consistent lighting was achieved with a ring
375 light connected to a light source with a 150-watt halogen bulb with a 380-780 nm
376 emission spectrum. Images were obtained with Motic Image Plus, version 2.0 software.
377 The field was "white balanced" prior to each session to ensure photographic
378 consistency. Wild-type (H1209) *H. volcanii* was included on every plate to allow
379 normalization of the data.

380 For color quantification of colonies, each image was sampled using Photoshop CS6
381 software (Adobe, San Jose, CA). The eyedropper sampling tool was set to the
382 maximum area (101 x 101 pixels), and the sampled area was chosen for color
383 consistency, avoiding any visible shadows or highlighting artifacts. After sampling, the
384 color window was used to display the measurements of that color: hue, saturation,
385 brightness, red, green, blue, cyan, magenta, yellow and black. Values were normalized
386 by comparison to wild-type (H1209) colonies on the same plate. These measurements
387 were recorded for each genotype and used in beta regression modeling (45) to predict
388 bacterioruberin levels as determined by RP-UHPLC analysis. The regression analysis
389 indicated that a combination of hue and saturation after 4 days of colony growth was the
390 best predictor of bacterioruberin content. This model was used to estimate parameters
391 for a function incorporating hue and saturation for the calculation of a "Ruberin Metric,"
392 which predicts the proportion of bacterioruberin for genotypes grown in the absence or
393 presence of retinal. Comparisons of colonies based on their Ruberin Metric were made
394 using a permutation test as implemented by the R package perm (46). For comparisons
395 involving group sample sizes of 9 or less, the permutation test was made with exact

computation. Asymptotic approximation was used for larger sample sizes. Bonferroni adjustment was applied when multiple comparisons were conducted using the same data. Details of model development and calculation of the Ruberin Metric are included in Supplemental Materials.

Quantification of opsin expression in *H. volcanii*. *H. volcanii* strains harboring plasmids for expression of opsins with C-terminal His_6 tags were constructed. Cultures were grown and cell pellets were obtained as described for carotenoid analysis. Pellets were lysed in 1.5 mL of lysis solution (1.3 mM Benzonase Nuclease (Sigma-Aldrich), 1X protease inhibitor cocktail (Sigma-Aldrich P2714), 10 mM Tris-HCl, pH 8.5), and incubated with rotation at 37 °C for approximately 1.5 h to allow degradation of DNA. Total protein was quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). Cell lysates were diluted to protein concentrations of 12 mg/mL using Laemmli Sample Buffer (Biorad, Hercules, CA), with 2-mercaptoethanol (355 mM) as a reducing agent. Protein samples (30 µL) were separated by polyacrylamide gel electrophoresis, and opsin levels determined by immunoblotting with α-his antibody (Genscript, Piscataway, NJ). The blots were subsequently probed with α-mouse IgG secondary antibody conjugated to horseradish peroxidase (Genscript) and developed with Picosignal Chemiluminescence kit (Pierce) following the manufacturer's instructions. Densitometry quantification was performed using Quantity One software (Biorad).

ACKNOWLEDGMENTS

R.F.P. designed the research, directed the biological interpretation of the data, and wrote the manuscript. A.M.P. assisted in the experimental design and data analysis and performed much of the strain construction. S.M.G. developed the colony colorimetric method, conducted data analysis, and constructed some of the strains used in the study. D.R.A. generated the statistical model for colony colorimetry and conducted data analysis. E.L.S. conducted experiments to quantify carotenoids and proteins. All authors read and approved of the paper.

We would like to thank Drs. Kang Mo Ku and Moo Jung Kim for conducting the RP-UHPLC analysis. Sara Lagerholm, Vanesa Silvestri, and Marie Estock provided valuable technical assistance. Many undergraduate students contributed their time to this project including Adam Lavertu, Antoinette Dummer, Kyle Hughes, Ray Nakada, Katherine Metayer, Erika Smith, Gracey McGrory, Christine Wamsley, Abby Gregory, Sarah Lane-Reticker, Emily Hoylman, Mary Schulz, and Nick Waldner. We are indebted to Frank Fekete, David Hall, and James Scott for insightful scientific advice during our discussions. Research reported in this project was supported by an Institutional Development Award from the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health under grant number P20GM103423. Additional funding was provided by the Colby College Natural Science Division and award 5GM094725-02 from NIGMS to R.F.P.

FIGURE LEGENDS

FIG. 1 Lycopene is the last shared intermediate in retinal and bacterioruberin biosynthesis. CrtY, the lycopene cyclase that catalyzes the conversion of lycopene to β-carotene that is subsequently cleaved to form retinal. Lye, lycopene elongase that catalyzes the prenylation of lycopene to the first bacterioruberin intermediate. The regulatory mechanism of BO inhibition of Lye activity is indicated by the dashed line.

FIG. 2 The *H. volcanii lye* gene is required for bacterioruberin synthesis. RP-UHPLC traces of carotenoid extracts from *H. volcanii* Δ*lye*, *H. volcanii* H1209 (parental strain), and strains harboring plasmids expressing *lye* from *H. volcanii*, *H. salinarum*, and *H. vallismortis* as labeled. Positions of bacterioruberin and lycopene (*lyc*) standards are noted. Traces were normalized for total carotenoid concentration, corrected for slight differences in retention time using an internal standard, and offset along the vertical axis for clarity. Traces are representative of at least 3 replicates for each strain.

FIG. 3 Expression of BO in *H. volcanii* specifically inhibits *H. salinarum* Lye. A. Box plot (Tukey's (20, 21)) indicating bacterioruberin levels as determined by RP-UHPLC analysis of *H. volcanii* cultures with *H. volcanii* or *H. salinarum lye*. Strains harbored a BO-expression vector or empty vector (control) as indicated. Where noted, retinal was added during the growth of the culture. Carotenoid levels were quantified by RP-UHPLC as described in Methods. Heavy horizontal bar indicates the median value and boxes demarcate the upper and lower quartiles. Whiskers extend to the smaller value of 1.5 times the interquartile range or the most extreme value, n≥3. **B.** Photographs of representative colonies of *H. volcanii* strains with native *lye* replaced with *H. salinarum lye*. Empty vector control (left), strain with plasmid allowing BO

expression (middle), strain with plasmid allowing BO expression with retinal added to the plate to convert BO to BR (right). **C.** Box plot indicating bacterioruberin levels (Ruberin Metric) as determined by analysis of colony color. *H. volcanii* cultures were plated on Hv-YPC media supplemented with retinal as noted. Four-day old colonies were photographed and digital images analyzed as described in Methods. Heavy horizontal bar indicates the median value and boxes demarcate the upper and lower quartiles. Whiskers extend to the smaller value of 1.5 times the interquartile range or the most extreme value, n≥9. * indicates Bonferroni adjusted $p<0.05$. *NS*, not significantly different

FIG. 4 A specific region within *H. salinarum* Lye is necessary for BO-mediated regulation. A. Protein alignment map of *H. salinarum* and *H. volcanii* Lye homologs and hybrid. The x-axis represents the consensus alignment amino acid position, and the vertical lines within the protein sequences represent gaps in the alignment. Dashed lines delineate the *H. salinarum* Lye region required for inhibition by BO. **B.** Box plot indicating bacterioruberin levels of strains expressing different versions of *lye* in the absence or presence of BO. Images were obtained, analyzed and data plotted as described in Fig. 3. n≥9. * indicates Bonferroni adjusted $p<0.05$. *NS*, not significantly different. **C.** Template-based modeling of *H. salinarum* and *H. volcanii* Lye proteins. Structure predictions of *H. salinarum* (left) and *H. volcanii* (right) Lye. Structures are oriented so that the cytoplasm (*in)* is at the bottom and the extracellular environment (*out*) is at the top of the image. The regions highlighted in blue (*H. salinarum*) and cyan (*H. volcanii*) represent the identified 52-aa region required for inhibition by BO. The structural models for the two Lye homologs were generated using PHYRE2 (22) and

superimposed using SuperPose (23). The structure files were highlighted and exported to image files using Geneious version 7 (24).

FIG. 5 BO lacking covalent retinal binding inhibits Lye; *H. salinarum* sensory opsin II does not inhibit Lye. A. Box plot indicating bacterioruberin levels of *H. volcanii* strains with *H. salinarum lye* expressing BO, BO K216A, or SOII in the absence or presence of retinal. Images were obtained, analyzed and data plotted as described in Fig. 3. n≥9. * indicates Bonferroni adjusted $p<0.05$. *NS*, not significantly different. **B.** Quantification of *H. salinarum* BO, SOII, and *H. vallismortis* CO expression. *H. volcanii* strains harboring expression plasmids for indicated opsins with C-terminal His_6 tags were grown in cultures with the same procedures as used for carotenoid analysis. Cell lysates were normalized using total protein concentration and separated by polyacrylamide gel electrophoresis. Blots were probed with α-his antibody and bands quantified by densitometry. Median values as compared to BO median set to 1. Error bars indicate 1 standard deviation, n=3. *Inset*, representative immunoblot of his-tagged BO, SOII, and CO.

FIG. 6 *H. vallismortis* Lye is specifically inhibited by *H. vallismortis* CO. Box plot indicating bacterioruberin levels of *H. volcanii* strains with *H. volcanii*, *H. salinarum*, or *H. vallismortis lye* and the indicated opsin in the absence or presence of retinal. Images were obtained, analyzed and data plotted as described in Fig. 3. n≥9. * indicates Bonferroni adjusted $p<0.05$. *NS*, not significantly different.

503 **TABLE**

504 **TABLE 1. *H. volcanii* strains used in this study**

Strain	Genotype	Construction or Reference
Parental strain, Δ*lye*, and *lye* complementation strains		
H1209	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ Δ*mrr*	(42)
RFP58	H1209 Δ*lye*	H1209 - pRFP125 genomic recombinant
RFP191	RFP58 p{*H. volcanii lye*}	RFP58 transformed with pRFP244
RFP208	RFP58 p{*H. salinarum lye*}	RFP58 transformed with pRFP256
RFP212	RFP58 p{*H. vallismortis lye*}	RFP58 transformed with pRFP258
Strains to test BO-mediated inhibition of *H. salinarum* Lye activity		
RFP79	H1209 p{*H. salinarum bop*}	H1209 transformed with pRFP126
RFP80	H1209 p{empty vector}	H1209 transformed with pTA963
RFP152	RFP58 *lye*::*H. salinarum lye*	RFP58 - pRFP224 genomic recombinant
RFP158	RFP152 p{empty vector}	RFP152 transformed with pTA963
RFP159	RFP152 p{*H. salinarum bop*}	RFP152 transformed with pRFP126
Strains with hybrid *H. salinarum* - *H. volcanii lye* genes		
RFP181	RFP58 *lye*::*H. salinarum lye* aa 1-120; *H. volcanii lye* aa 147-301	RFP58 - pRFP237 genomic recombinant
RFP182	RFP58 *lye*::*H. salinarum lye* aa 1-192; *H. volcanii lye* aa 225-301	RFP58 - pRFP238 genomic recombinant
RFP188	RFP58 *lye*::*H. salinarum lye* aa 1-68; *H. volcanii lye* aa 95-301	RFP58 - pRFP241 genomic recombinant
RFP192	RFP181 p{empty vector}	RFP181 transformed with pTA963
RFP193	RFP181 p{*H. salinarum bop*}	RFP181 transformed with pRFP126
RFP194	RFP182 p{empty vector}	RFP182 transformed with pTA963
RFP195	RFP182 p{*H. salinarum bop*}	RFP182 transformed with pRFP126
RFP200	RFP188 p{empty vector}	RFP188 transformed with pTA963
RFP201	RFP188 p{*H. salinarum bop*}	RFP188 transformed with pRFP126
Strains expressing *H. salinarum sopII* or *H. salinarum bop*^{K216A}		
RFP162	RFP152 p{*H. salinarum sopII*}	RFP152 transformed with pRFP222
RFP174	RFP152 p{*H. salinarum bop*^{K216A}}	RFP152 transformed with pRFP152
Strains expressing opsins with C-terminal His$_6$ tags		
RFP166	RFP152 p{*H. salinarum bop*-His$_6$}	RFP152 transformed with pRFP227
RFP168	RFP152 p{*H. salinarum sopII*-His$_6$}	RFP152 transformed with pRFP230
RFP169	RFP152 p{*H. vallismortis cop3*-His$_6$}	RFP152 transformed with pRFP231
Strains expressing *H. vallismortis lye* and/or *H. vallismortis cop3*		
RFP131	RFP58 *lye*::*H. vallismortis lye*	RFP58 - pRFP207 genomic recombinant
RFP139	RFP131 p{empty vector}	RFP131 transformed with pTA963
RFP140	RFP131 p{*H. salinarum bop*}	RFP131 transformed with pRFP126
RFP141	RFP131 p{*H. vallismortis cop3*}	RFP131 transformed with pRFP201
RFP144	H1209 p{*H. vallismortis cop3*}	H1209 transformed with pRFP201
RFP163	RFP152 p{*H. vallismortis cop3*}	RFP152 transformed with pRFP201

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