Bacterioopsin-Mediated Regulation of Bacterioruberin Biosynthesis in \textit{Halobacterium salinarum}^{\dagger, \ddagger}

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Integral membrane protein complexes consisting of proteins and small molecules that act as cofactors have important functions in all organisms. To form functional complexes, cofactor biosynthesis must be coordinated with the production of corresponding apoproteins. To examine this coordination, we study bacteriorhodopsin (BR), a light-induced proton pump in the halophilic archaeon \textit{Halobacterium salinarum}. This complex consists of a retinal cofactor and bacterioopsin (BO), the BR apoprotein. To examine possible novel regulatory mechanisms linking BO and retinal biosynthesis, we deleted \textit{bop}, the gene that encodes BO. \textit{bop} deletion resulted in a dramatic increase of bacterioruberins, carotenoid molecules that share biosynthetic precursors with retinal. Additional studies revealed that bacterioruberins accumulate in the absence of BO regardless of the presence of retinal or BR, suggesting that BO inhibits bacterioruberin biosynthesis to increase the availability of carotenoid precursors for retinal biosynthesis. To further examine this potential regulatory mechanism, we characterized an enzyme, encoded by the \textit{bpe} gene, that catalyzes bacterioruberin biosynthesis. BO-mediated inhibition of bacterioruberin synthesis appears to be specific to the \textit{H. salinarum} \textit{bpe}-encoded enzyme, as expression of a \textit{bpe} homolog from \textit{Haloflexus volcanii}, a related archaeon that synthesizes bacterioruberins but lacks opsins, resulted in bacterioruberin synthesis that was not reduced in the presence of BO. Our results provide evidence for a novel regulatory mechanism in which biosynthesis of a cofactor is promoted by apoprotein-mediated inhibition of an alternate biochemical pathway. Specifically, BO accumulation promotes retinal production by inhibiting bacterioruberin biosynthesis.

Organic cofactors are required for many essential integral membrane protein complexes, including those involved in energy conservation, signal transduction, and light sensing. Despite their importance, relatively little is known about the fundamental problem of how biosyntheses of these cofactors and their corresponding apoproteins are coordinated to ensure the appropriate stoichiometry to form functional complexes. In particular, potential protein-protein and protein-cofactor interactions that mediate coordinated regulation of apoproteins and cofactors remain poorly understood.

To elucidate mechanisms for coordinated protein/cofactor regulation, we study bacteriorhodopsin (BR) in \textit{Halobacterium salinarum} as a model system. BR is the best-characterized member of the microbial rhodopsin family, which includes complexes identified in all three domains of life—\textit{Archaea}, \textit{Bacteria}, and \textit{Eukarya} (4–6, 15)—raising the possibility that \textit{H. salinarum} employs for coordinated regulation of retinal and BO synthesis is the control of gene transcription. Low oxygen tension results in the induction of the \textit{bop} gene, which encodes BO (37, 47). This increased transcription is mediated by the bacterioopsin gene activator (Bat), a transcription factor which also activates transcription of
genes encoding retinal biosynthetic enzymes (2). Recent work suggests that another potential transcription factor, brz, is also required for induction of BO and related enzymes (43). Multiple genome-wide analyses of transcript levels have indicated that genes encoding carotenoid biosynthetic enzymes are up-regulated under low-oxygen conditions (3, 35, 44). Still, these studies revealed that overall differences in transcription are modest compared to the 50-fold increase in BR in only a few hours of growth (30), raising the possibility that regulatory mechanisms that stimulate or inhibit enzyme activities play a key role in the response of H. salinarum to environmental changes. In fact, it has long been known that BR, BO, and/or retinal plays some role in the regulation of biosynthesis of retinal and related precursors (41, 42). These early studies, however, were limited by a lack of knowledge of retinal biosynthetic enzymes and insufficient tools to generate strains that were appropriate to analyze this pathway. Since several retinal biosynthetic enzymes have been characterized and genetic manipulation of H. salinarum is now straightforward, we reexamined the relationship between the BO protein and the biosynthesis of its retinal cofactor.

By utilizing mutant strains that lack the ability to synthesize retinal and therefore BR, we determined that BO itself plays a key role in regulating the synthesis of its retinal cofactor. Our results provide evidence for a novel regulatory mechanism in which the apoprotein, BO, inhibits an alternative biochemical pathway, bacterioruberin synthesis, to increase the availability of shared intermediates for retinal cofactor biosynthesis. We also identified a gene, lye, that likely encodes the lycopene elongase enzyme that catalyzes the committed step in bacterioruberin biosynthesis. BO-mediated inhibition of bacterioruberin biosynthesis is specific to the lye gene of H. salinarum, as expression of the Haloferax volcanii lye homolog resulted in bacterioruberin synthesis that was not inhibited by BO.

MATERIALS AND METHODS

Growth conditions. All Halobacterium strains used in this study are listed in Table 1. H. salinarum was grown in culture medium (CM) (10) at 40°C. The medium was supplemented with mevinolin (4 μg/ml) or 5-fluoroorotic acid (5-FOA) (250 μg/ml) when required. Escherichia coli was grown in LB medium at 37°C, except where noted. Ampicillin (50 μg/ml) and chloramphenicol (40 μg/ml) were added when required. All liquid cultures were grown with shaking at 250 rpm.

H. salinarum cultures used for carotenoid and BR analysis were grown as follows: 5-ml cultures were grown to saturation (approximately 4 days), and then 120 ml CM was inoculated with 1.2 ml saturated culture and the culture was grown for 82 to 87 h to induce low-oxygen conditions and in the dark to prevent the possible confounding factor of light. For retinal addition experiments, 30 μl 15 mM retinal in isopropanol was added every 12 h for a total of 7 times during growth. Addition of isopropanol alone was found to have no observable effects on growth or BR or carotenoid production.

Plasmid construction. All plasmids and primers are listed in Table S1 in the supplemental material. The plasmid pRFP33, used for generating a lye knockout in the Δbrp Δblh strain, was constructed by ligating the 9.2-kbp BstXI/EcoRI fragment of pMPK66 (16) and the 1.1-kbp AatII/BstXI fragment of pMPK417 (33). The plasmid pRFP61, used for generating lye-knockout strains, was constructed similarly to plasmids described previously (33). A two-step PCR was conducted with Pfu polymerase (Agilent Technologies, Santa Clara, CA) using Halobacterium sp. NRC-1 (27) genomic DNA as the template. The first PCR was carried out using two sets of primer pairs: primers RP5 and RP7 and primers RP6 and RP8. The resulting PCR products were used as template in the second PCR using primers RP21 and RP22. This PCR yielded a 1,273-bp product with two XbaI restriction enzyme sites at the 5’ end, two HindIII restriction enzyme sites at the 3’ end, and the lye gene with a 508-bp deletion in the center (amino acids 51 to 219) replaced with an insertion that encodes the 6 amino acids EIEIEQ. The PCR product was digested with XbaI and HindIII and ligated with the XbaI/HindIII fragment of the plasmid pMPK428 (31).

To construct the plasmid pRFP65 for inserting the H. salinarum lye gene into the ara3 locus, the lye locus of Halobacterium sp. NRC-1 genomic DNA was

FIG. 1. The retinal and bacterioruberin biosynthetic pathways in H. salinarum. Italics indicate genes that encode catalytic enzymes. Asterisks indicate putative functions based on sequence homology with characterized genes from other organisms. The proposed regulatory mechanism of BO inhibiting bacterioruberin synthesis is indicated by the dashed line.
amplified by PCR using primers RP83 and RP84. The resulting PCR product was an 864-bp fragment containing an XbaI restriction enzyme site at the 5′ end, a BglII restriction enzyme site at the 3′ end, the 
\( \text{lye} \) open reading frame, and the 16-bp terminator sequence at the 3′ end to retain the PmeI restriction enzyme site. The PCR product was digested with XbaI and BglII and ligated with the XbaI/BglII fragment of the plasmid pMPK424 (33). The plasmid pRP56, used to insert the 
\( H. \ \text{volcanii lye} \) homolog into the 
\( H. \ \text{salinarum lye} \) locus, was constructed similarly. The 
\( \text{lye} \) gene from 
\( H. \ \text{volcanii lye} \) was amplified using primers RP155 and RP156 from genomic DNA graciously provided by K. Bidle. To allow gene replacement by 5-FOA counterselection, a two-step procedure was used: the 
\( \text{lye} \) gene was amplified and ligated with the 4-kbp NcoI/PmeI fragment of the plasmid pMPK438 to construct a 
\( \text{lye} \) gene containing the 
\( \text{NcoI} \) restriction enzyme site (the base change was a 
\( T \) to 
\( G \) , which resulted in a change in the amino acid sequence at the 5′ end, a BglII restriction enzyme site at the 3′ end, the 
\( \text{lye} \) open reading frame, and the 16-bp terminator sequence at the 3′ end to retain the PmeI restriction enzyme site.

The plasmid pRP65, used to express 
\( H. \ \text{salinarum lye} \) in 
\( E. \ \text{coli} \), was made by amplifying the 
\( \text{lye} \) gene from 
\( H. \ \text{salinarum lye} \) with a single base change to create an 
\( \text{NcoI} \) restriction enzyme site (the base change was a 
\( T \) to 
\( G \) , which resulted in a change in the amino acid sequence at the 5′ end, a BglII restriction enzyme site at the 3′ end, the 
\( H. \ \text{volcanii lye} \) homolog open reading frame, and the 16-bp terminator sequence from 
\( bop \).

The plasmid pRP65 was constructed by replacing the 
\( \text{lye} \) gene with 
\( H. \ \text{volcanii lye} \) homolog open reading frame and the 16-bp terminator sequence from 
\( bop \). The resulting PCR product was a 955-bp fragment containing an XbaI restriction enzyme site at the 5′ end, a BglII restriction enzyme site at the 3′ end, the 
\( H. \ \text{volcanii lye} \) homolog open reading frame, and the 16-bp terminator sequence from 
\( bop \).

Carotenoid quantification and analysis. Carotenoids were extracted from 
\( H. \ \text{salinarum} \) strains essentially as described previously (13). Cultures were centrifuged for 15 min at 12,500 × g. The pellet was washed in 100 ml medium salts and centrifuged for 15 min. The pellet was centrifuged again for 2 min, and the remaining liquid was removed by aspiration. Cell pellets were occasionally stored at −20°C prior to lysing, which had no observable effect on carotenoid content. The pellet was lysed in 2 ml lysis solution (0.004 mg/ml DNAse, 0.5 mM phe- nylmethylsulfonyl fluoride, 0.003% Na2S2O4) for 1 h by shaking at 250 rpm at room temperature in the dark in tubes purged with nitrogen. Lysate (0.3 ml) was removed and set aside for analysis of BR if necessary. Remaining lysate was then mixed with 13 ml acetonitrile, and the mixture was stirred vigorously for 20 min. After the mixture was stirred, a mixture of 7 ml hexane–1.5 ml water was added, and that mixture was stirred for two additional minutes. The upper layer was transferred to a new tube and then evaporated to dryness under nitrogen. Extraction with hexane and water was repeated if additional color was visible in the lower layer. The pigments were resuspended in 100 μl ethyl acetate for analysis.

Carotenoids were extracted from 
\( E. \ \text{coli} \) by a procedure similar to that used for 
\( H. \ \text{salinarum} \). Five-milliliter cultures were grown to saturation and used to inoculate 50 ml LB medium. These cultures were grown at 28°C with shaking for approximately 4 h. Arabinose was added to a final concentration of 1% (wt/vol) to induce expression of the 
\( \text{lye} \) gene. Cultures were incubated overnight (14 to 16 h) at 28°C. Cultures were then centrifuged for 15 min at 12,500 × g. The supernatant was removed, and the pellet was resuspended in 1.5 ml 10 mM Tris

**Table 1.** 

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Relevant characteristics</th>
<th>Retinal added</th>
<th>Total amt of carotenoid (nmol/liter culture)</th>
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<tbody>
<tr>
<td>MPK407</td>
<td>32</td>
<td>Parental strain (MPK1 Δura3)</td>
<td>–</td>
<td>116 ± 40</td>
</tr>
<tr>
<td>MPK412</td>
<td>32</td>
<td>MPK407 Δbop</td>
<td>–</td>
<td>76 ± 32</td>
</tr>
<tr>
<td>MPK414</td>
<td>46</td>
<td>Parental strain (Halobacterium sp. NRC-1 Δura3)</td>
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<td>553 ± 187</td>
</tr>
<tr>
<td>MPK423</td>
<td>33</td>
<td>MPK407 Δbop Δblh</td>
<td>–</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>MPK426</td>
<td>31</td>
<td>MPK407 ΔcrtY</td>
<td>+</td>
<td>61 ± 7</td>
</tr>
<tr>
<td>RFP29</td>
<td>This study</td>
<td>MPK407 ΔcrtY Δbop</td>
<td>–</td>
<td>43 ± 14</td>
</tr>
<tr>
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<td>This study</td>
<td>MPK407 Δbop Δblh Δbop</td>
<td>+</td>
<td>58 ± 23</td>
</tr>
<tr>
<td>RFP39</td>
<td>This study</td>
<td>MPK407 Δbop Δlye</td>
<td>+</td>
<td>103 ± 26</td>
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<td>This study</td>
<td>MPK407 Δlye</td>
<td>+</td>
<td>40 ± 8</td>
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<tr>
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<td>+</td>
<td>43 ± 5</td>
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<td>RFP49</td>
<td>This study</td>
<td>MPK407 Δbop Δlye Δura3::H. salinarum lye</td>
<td>–</td>
<td>106 ± 3</td>
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<tr>
<td>RFP53</td>
<td>This study</td>
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<td>20 ± 9</td>
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<td>This study</td>
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<td>–</td>
<td>55 ± 7</td>
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<tr>
<td>RFP55</td>
<td>This study</td>
<td>MPK407 ΔcrtY Δbop Δura3::H. salinarum lye</td>
<td>–</td>
<td>57 ± 8</td>
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<td>MPK407 Δbop Δlye Δura3::H. volcanii lye</td>
<td>–</td>
<td>72 ± 15</td>
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<td>38 ± 3</td>
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<td>79 ± 10</td>
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<td>77 ± 12</td>
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<td>MPK407 Δbop Δlye Δura3::H. salinarum lye</td>
<td>–</td>
<td>83 ± 6</td>
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* All strains are Δura3 to allow gene replacement by 5-FOA counterselection.

* Mean ± 1 standard deviation (\( n \geq 3 \)).
(pH 8) and then mixed with 11 ml acetone. After the mixture was vigorously stirred for 20 min, a mixture of 6 ml hexane–1 ml water was added and stirring was continued for two additional minutes. The rest of the extraction was carried out as described for *H. salinarum*.

Carotenoid extracts were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). Samples were fractionated on a Varian ProStar HPLC system using a Microsorb MV reverse-phase C_{18} column (4.6-mm inner diameter, 250-mm length, 5-μm particle size). The mobile phase was a gradient of solvent A (90% acetonitrile, 9.9% water, 0.1% triethylamine) and solvent B (ethyl acetate) eluting at 1 ml/min. The solvent change over an 83-min sample run was programmed as follows: elution with 90% solvent A–10% solvent B for 2 min, gradient to 30% solvent B for 45 min, gradient to 45% solvent B for 2 min, gradient to 75% solvent B for 15 min, isocratic elution with 75% solvent B for 3 min, gradient to 10% solvent B for 6 min, and reequilibration with 10% solvent B for 10 min.

Standard curves for lycopene, β-carotene, and retinal were generated with commercial carotenoids. Bacterioruberin standard curves were generated with carotenoid isolated from cell extracts. *Halobaculum* sp. NRC-1 carotenoids were extracted and fractionated by HPLC. The peak eluting at 8.9 min was collected and evaporated to dryness under nitrogen. This bacterioruberin was then resuspended in acetone, and the concentration was calculated spectrophotometrically using an extinction coefficient of ε = 141 M^{-1} cm^{-1} (24). These samples were then analyzed by HPLC to produce bacterioruberin standards. All peaks eluting at between 4 min and 45 min which displayed similar absorbance spectra to characterized bacterioruberins (24) were included as bacterioruberins. “Total carotenoids” refers to the sum of lycopene and the lycopene-derived carotenoids β-carotene, retinal, and bacteriorubers, unless noted. Although total carotenoid production exhibited substantial variability (Table 1), we found that the relative percentages of carotenoids were consistent among cultures within the same strain. Other carotenoids that may be present in minor amounts and lycopene precursors were not included in our analysis.

Peaks eluting at 8.9 min from carotenoids extracted from the MPK414 (*Halobaculum* sp. NRC-1 Δura3) and MPK412 (MPK407 Δbop) strains and peaks eluting at 38.9 min from *E. coli* expressing *H. salinarum* βop or containing an empty expression vector were isolated by HPLC and analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a Bruker MALDI/TOF-MS Reflex IV mass spectrometer operating in positive reflection mode. The matrix was made by dissolving 2,5-dihydroxybenzoic acid (DHB; 10 mg/ml) in 80% acetonitrile. The carotenoids were dried completely under nitrogen and resuspended in 4 μl matrix, and 2 μl was spotted onto a well of a 96-well stainless steel MALDI-TOF MS target plate for analysis. A nitrogen laser emitting at 337 nm was used to generate ions. A source voltage of 25 kV and an extraction voltage of 20.7 kV were used. The mass spectrometer was calibrated with DHB [M + H]^+ (m/z = 155) and Bruker Peptide Calibration Standards II (Bruker Daltonics, Billerica, MA).

**BR quantification.** BR was quantified spectrophotometrically from whole-cell lysates by light/dark analysis as described previously (17). Briefly, cell lysates were dark adapted for 12 to 16 h at room temperature and the absorbance spectrum from 300 nm to 800 nm was obtained. Samples were then light adapted by illumination for 4 min with a 150-W lamp with a 530-nm-long-pass-cutoff filter, and then the absorbance spectrum was obtained again. The BR concentration (in mg/ml) was calculated using the formula 2.4 × change in A_{587} (ΔA_{587}). Total cellular protein was determined by bichinchoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin to generate a standard.

**RESULTS**

**Deletion of bop results in accumulation of bacteriorubers.** To begin examining the role of BO in regulating retinal synthesis, we analyzed how knockout of *bop*, the gene that encodes BO, affected the relative abundance of carotenoids derived from lycopene, the common precursor to retinal and bacterioruberins (Fig. 1). Upon growth under limiting oxygen conditions known to induce synthesis of BO, β-carotene, and retinal (42), the parental strain, MPK407 (MPK1 Δura3) (32), primarily accumulated retinal and β-carotene, as quantified by reverse-phase HPLC (Fig. 2A). In contrast, when the *bop* gene was ablated by an internal deletion of 721 bp from the 789-base-pair open reading frame, β-carotene and retinal levels were substantially reduced (Fig. 2A). Instead, the Δbop strain accumulated carotenoids with relative polarity and absorbance spectra suggestive of 50-carbon bacteriorubers (Fig. 3).

To confirm that the Δbop strain accumulated bacteriorubers, we compared the most abundant carotenoid in Δbop cells to that in cells of MPK414 (*Halobacterium* sp. NRC-1 Δura3) (46), a strain directly derived from *Halobacterium* sp. NRC-1 which produces characterized bacteriorubers (21, 24). The major carotenoid in the Δbop strain had an HPLC retention time identical to that of the characterized bacterioruberin (Fig. 3A). In addition, this carotenoid exhibited a UV-
Bacterioruberin accumulation is not due to the presence of free retinal. One possible explanation for the accumulation of bacterioruberins upon bop deletion is that free retinal, lacking available BO for binding, influences the carotenoid biosynthetic pathway (Fig. 1). To test this possibility, we examined the effects of bop deletion in strains lacking the ability to synthesize retinal. We have previously demonstrated that deletion of both bop and blh or the single deletion of crtY abolishes retinal synthesis but does not substantially affect production of the BO protein, resulting in a large pool of BO unbound by cofactor (31, 33). Consistent with these previous findings, the ΔcrtY strain primarily accumulated lycopene (Fig. 2B), and the Δbop Δblh strain primarily accumulated β-carotene (Fig. 2C). When BO was eliminated by deleting bop in either strain, bacterioruberins were the most abundant carotenoids: ~99% of the total carotenoid in the ΔcrtY Δbop strain (Fig. 2B) and ~84% in the Δbop Δblh Δbop strain (Fig. 2C). These results demonstrate that the loss of BO directly results in increased levels of bacterioruberins, even when retinal is eliminated.

Bacterioruberin accumulation results from elimination of unbound BO by retinal addition. As an alternative method to remove BO without disrupting the bop gene, H. salinarum retinal-deficient strains were grown with repeated addition of excess retinal to convert BO to BR. We had previously determined that retinal addition to the Δbop Δblh strain during growth resulted in an ~35% greater BR accumulation than the native level of the parental MPK407 strain (33), indicating that the pool of unbound BO is dramatically reduced or abolished by the addition of exogenous retinal. In the Δbop Δblh strain, growth in the presence of excess retinal resulted in a sharp increase in the accumulation of bacterioruberins: ~57% of total carotenoids compared to less than 1% in growth without retinal (Fig. 2C). When retinal was added to the ΔcrtY strain to deplete BO, lycopene constituted only ~3% of total carotenoids, and the remaining ~97% were bacterioruberins (Fig. 2C). To confirm that the observed effect of exogenous retinal was mediated by converting BO to BR, the Δbop Δblh Δbop strain, lacking BO, was grown with retinal. As predicted, retinal addition had no significant effect on the relative abundance of any carotenoids compared to growth without retinal (Fig. 2C). Therefore, BO depletion due to the addition of excess retinal produces the same effect as deletion of the bop gene: the loss of BO leads to increased bacterioruberins.

Identification of a Halobacterium bacterioruberin biosynthetic enzyme. A simple mechanism for the observed accumulation of bacterioruberins is that BO, when not bound to its retinal cofactor, inhibits bacterioruberin synthesis. As a first step to examine this possibility, we sought to identify the enzyme that catalyzes the conversion of lycopene to the first bacterioruberin intermediate. On the basis of biochemical studies (21, 22), this biosynthesis is proposed to proceed via a condensation reaction that adds two 5-carbon isoprene units to
each end of the 40-carbon lycopene to generate the 50-carbon tetrahydrobisanhydrobacterioruberin. This reaction is similar to that for the formation of flavoxanthin in Corynebacterium glutamicum catalyzed by the lycopene elongase enzyme (19, 20). A search for similar genes in the H. salinarum genome (34) using BLAST (1) revealed a homologous gene, OE3380R (Vng1682C in the Halobacterium sp. NRC-1 genome sequence [27]), which we designated “lye.” The H. salinarum lye gene encodes a putative protein of 275 amino acid residues with 31% identity and 48% similarity over 211 amino acids to the C. glutamicum lycopene elongase (see Fig. S2 in the supplemental material). The lye gene product is a member of the ubiC protein superfamily, which includes enzymes that transfer 5-carbon prenyl groups to various substrates (8). A membrane topology analysis by the TMHMM tool (18) predicted that lye encodes an integral membrane protein with 7 transmembrane domains (see Fig. S2 in the supplemental material), consistent with a proposed catalytic function using hydrophobic carotenoid substrates that likely reside in the cell membrane. The H. salinarum lye gene is located within a carotenoid biosynthetic gene cluster, as it is directly downstream of a putative phytoene desaturase enzyme, crtI1, and directly upstream of a hypothetical carotenoid biosynthetic enzyme, OE377R, and a putative phytoene synthase enzyme, crtB2. Homologs of lye are found in other halophilic archaea that have been demonstrated to produce bacterioruberins as well as bacterial species that produce other 50-carbon carotenoids (see Fig. S2 in the supplemental material).

To test whether lye is required for bacterioruberin biosynthesis, we constructed strains containing in-frame deletions of lye. In the Δbop and the parental strains, lye deletion eliminated production of bacterioruberins (Fig. 4A and B). To ensure that this effect was not due to spurious mutations at the DNA level, we constructed complementation strains, Δlye ura3::Halobacterium lye and Δbop Δlye ura3::Halobacterium lye, in which the lye open reading frame, lacking any upstream or downstream sequence, was incorporated into the ura3 locus. In these strains, the production of bacterioruberin was restored to ~77% of total carotenoids in the Δbop Δlye background and ~3% of total carotenoids in the Δlye background (Fig. 4A and B), very similar to the levels observed in MPK407 and MPK407 Δbop strains, respectively (Fig. 2A). These results confirmed that lye is required for bacterioruberin synthesis in H. salinarum.

To examine the potential catalytic function of the lye gene product, we expressed the lye gene in Escherichia coli containing pAC-LYC (11), an expression vector encoding enzymes to allow lycopene production. Expression of H. salinarum lye in these E. coli strains resulted in the accumulation of several new carotenoids in addition to lycopene (Fig. 5A). One new carotenoid product (Fig. 5A, asterisk) was significantly more polar than lycopene, exhibiting a retention time of 38.9 min, compared to a retention time of 55.6 min for lycopene, in our reverse-phase HPLC system. This relative polarity is consistent with that expected for the proposed bacterioruberin precursor tetrahydrobisanhydrobacterioruberin, a 50-carbon molecule containing hydroxyl groups (Fig. 1) near both ends of the molecule. In addition, the carotenoid exhibited a UV/vis spectrum characteristic of an aliphatic undecaene chromophore with a major peak at 475 nm and minor peaks at 506 nm, 450 nm, and 367 nm (Fig. 5B), nearly identical to a published UV/vis spectrum for tetrahydrobisanhydrobacterioruberin (28). To further characterize this carotenoid, the product with a retention time of 38.9 min was collected and analyzed by MALDI-TOF mass spectrometry. The most abundant mass ion in the sample was 708.6 ± 0.08 Da (standard error), identical to the calculated mass for tetrahydrobisanhydrobacterioruberin. A control sample was generated by extracting carotenoids from a lycopene-producing E. coli strain containing an
empty expression vector and collecting a fraction at 38.9 min in the HPLC separation (Fig. 5A, asterisk). This negative-control sample yielded no mass ions above background level. Thus, the _H. salinarum_ _lye_ gene encodes a functional enzyme that catalyzes the conversion of lycopene to a likely bacterioruberin precursor.

**BO-mediated inhibition of bacterioruberin biosynthesis is specific to the _H. salinarum_ _lye_ gene product.** Our results provided evidence that the _lye_-encoded enzyme catalyzes the committed step in bacterioruberin synthesis and represents a potential target for regulation of this pathway by BO (Fig. 1). To examine the specificity of BO-mediated inhibition of bacterioruberin synthesis, we compared the _H. salinarum_ _lye_ gene to a homolog from another halophilic archaeon, _H. volcanii_. Like _H. salinarum_, _H. volcanii_ produces bacterioruberins (26) but does not produce any known rhodopsins. A BLAST search of the _H. volcanii_ genome sequence (14) revealed an open reading frame, _HVO_._2527_, predicted to encode a 301-amino-acid protein with little production of the retinal cofactor.

Expression of the _H. volcanii_ _lye_ homolog rescued the _Δlye_ phenotype, as the _Δbop Δlye ura3::H. volcanii_ _lye_ strain produced levels of bacterioruberins very similar to those produced by the _Δbop Δlye ura3::H. salinarum_ _lye_ strain (Fig. 4A). To examine if the _H. volcanii_ _lye_ product is inhibited by BO, we constructed strains expressing _H. volcanii_ _lye_ and BO, _Δlye ura3::H. volcanii_ _lye_ and _ΔcrtY Δlye ura3::H. volcanii_ _lye_. In the _Δlye ura3::H. volcanii_ _lye_ strain, bacterioruberins constituted ~78% of the carotenoids, much greater than the ~3% of total carotenoids observed in the corresponding strain with _H. salinarum_ _lye_, _Δlye ura3::H. salinarum_ _lye_ (Fig. 4B). In the _ΔcrtY Δlye ura3::H. volcanii_ _lye_ strain, bacterioruberins accumulated ~96% of total carotenoids (Fig. 4C). Again, this bacterioruberin production was much higher than the ~34% of total carotenoids observed in the _ΔcrtY Δlye ura3::H. salinarum_ _lye_ strain (Fig. 4C). These results demonstrated that bacterioruberin synthesis catalyzed by the _H. volcanii_ _lye_ homolog was not inhibited by BO. Therefore, BO-mediated inhibition of bacterioruberin synthesis is specific to the _H. salinarum_ _lye_ gene product, suggesting that this inhibition may be a regulatory mechanism to direct the available lycopene to be used for retinal synthesis.

To directly explore the balance between bacterioruberin and BR synthesis, we determined how expression of the _H. volcanii_ _lye_ homolog affected BR levels in comparison to the native _H. salinarum_ _lye_. In _Δlye_ and _Δlye ura3::H. salinarum_ _lye_ strains, BR levels were nearly identical, and in both strains, BR levels were slightly less than the level in the parental strain, MPK407 (Fig. 6). In contrast, expression of the _H. volcanii_ _lye_ homolog resulted in an ~8-fold decrease in BR (Fig. 6), suggesting that the _H. volcanii_ _lye_ enzyme consumed the limiting resource of lycopene for bacterioruberin synthesis and allowed relatively little production of the retinal cofactor.

**Free retinal or BR does not inhibit β-carotene synthesis.** It has been proposed that free retinal, when not bound to BO, could potentially regulate β-carotene synthesis or bacterioruberin synthesis (42), and identification of the _lye_ gene allowed us to examine this hypothesis in strains that lacked the ability to use lycopene for bacterioruberin synthesis. In the _Δbop Δlye_ strain, which lacks the ability to synthesize BO or bacterioruberins, β-carotene and retinal constitute ~96% of
the total carotenoids (Fig. 7). When retinal was added to the Δbop Δlye strain, β-carotene accumulation decreased modestly to ~87% of total carotenoids (Fig. 7), indicating that retinal addition did not substantially affect β-carotene synthesis. To examine if free retinal stimulated bacterioruberin synthesis, retinal was added to Δbrp Δbh Δbop cells during growth. Retinal addition had no significant effect, as bacterioruberins accumulated to ~84% of total carotenoids (Fig. 7), nearly the same as the ~80% observed without retinal addition (Fig. 2C).

To determine if BR inhibits the conversion of lycopene to β-carotene, we examined production of carotenoids in a Δbrp Δbh Δlye strain, which lacks the ability to make bacterioruberins and retinal. When this strain was grown with repeated addition of retinal, BR accumulated to 0.5 nmol/mg, about 45% greater than the level observed in the parental strain. This high level of BR did not appear to substantially inhibit β-carotene synthesis, as ~88% of the total carotenoid was β-carotene (Fig. 7), nearly the same as the ~92% observed (Fig. 7) when no retinal was added during growth, and therefore no BR was present. Altogether, our results indicate that neither free retinal nor BR has a substantial effect on the retinal or bacterioruberin biosynthetic pathway.

**DISCUSSION**

**Role of BO in the regulation of bacterioruberin and retinal synthesis.** The role of BO in retinal biosynthesis has been the subject of previous studies (13, 40, 42). Importantly, these studies utilized strains reported to be bacterioruberin negative, so the proposed regulatory mechanism of BO inhibiting bacterioruberin synthesis would not have been observed. Deshpande and Sonar concluded that BO acts to stimulate the conversion of lycopene to β-carotene (13). In contrast, our results demonstrate that BO is not required to activate the conversion of lycopene to β-carotene in vivo. When deletion of lye ablated synthesis of bacterioruberins, nearly all carotenoids were retinal or β-carotene, even when BO was absent (Fig. 4A and 7). We cannot rule out the possibility that BO may stimulate β-carotene production, but our data show that this reaction occurs efficiently in vivo in the absence of BO.

An alternative suggested mechanism for the regulation of retinal biosynthesis is feedback inhibition by retinal or BO on the committed step in retinal synthesis, the cyclization of lycopene to form β-carotene (40). We found that a BO-mediated regulatory mechanism exists in the absence of retinal, as the deletion of bop resulted in increased bacterioruberins in strains lacking the ability to synthesize retinal (Fig. 2B and C). In addition, neither free retinal nor BR has a significant effect on the production of β-carotene. Addition of exogenous retinal did not inhibit β-carotene synthesis in vivo (Fig. 7), nor did it affect the relative levels of β-carotene or bacterioruberin (Fig. 2C), indicating that retinal does not indirectly prevent β-carotene synthesis by stimulating increased bacterioruberin synthesis. It may be possible that biosynthetic retinal could have effects that cannot be restored by exogenous retinal, but we consider that to be unlikely, as exogenous retinal is sufficient to convert BO to BR in vivo. Finally, BR does not appear to inhibit β-carotene synthesis, as lycopene was efficiently converted to β-carotene even when abundant BR was generated by the addition of exogenous retinal (Fig. 7).

**Possible mechanisms for BO-mediated inhibition of bacterioruberin synthesis.** In all strains capable of bacterioruberin synthesis, the removal of BO through either bop deletion or addition of excess retinal resulted in a dramatic increase in the abundance of bacterioruberins relative to other carotenoid precursors (Fig. 2). A simple explanation for the observed accumulation of bacterioruberins is that, when BO levels are high, bacterioruberin synthesis is inhibited, which increases the pool of lycopene available for retinal synthesis. On the basis of biochemical studies, the committed step in bacterioruberin synthesis is the addition of two 5-carbon dimethylallyl pyrophosphate (DMAPP) or isopentyl pyrophosphate (IPP) molecules to the 40-carbon lycopene to make the 50-carbon tetrahydrobisanhydrobacterioruberin (22, 24). Although a possible mech-
anism for our observed results is that BO inhibits this reaction by binding one of the reactants, we do not favor this explanation. It is highly improbable that BO would sequester DMAPP or IPP, since these are essential building blocks for membrane polar lipids as well as carotenoids. BO binding to lycopene is plausible but unlikely due to the high levels of specificity required for this interaction; lycopene is a 40-carbon carotenoid differing only in the number of C=C double bonds compared to its immediate precursors phytoene, phytofluene, and neurosporene, and our current and previous studies (31, 33) show no evidence for BO inhibiting lycopene production.

We favor a model in which BO inhibits the activity of bacterioruberin biosynthetic enzymes by direct interaction or through other protein intermediates. As a first step to characterize the BO-mediated inhibition of bacterioruberin synthesis, we identified the *H. salinarum* lye enzyme, which likely catalyzes the conversion of lycopene to the first bacterioruberin intermediate (Fig. 1). *H. salinarum* lye-knockout strains produced no detectable bacterioruberins, and this phenotype was rescued by integrating *lye* at the *ura3* locus (Fig. 4). Expression of *H. salinarum* lye in lycopene-producing *E. coli* resulted in the formation of new carotenoids, including the major product whose polarity, UV/vis absorbance, and mass were consistent with those of the bacterioruberin precursor tetrahydrosibianhydrobacterioruberin (Fig. 5). To our knowledge, the *H. salinarum* protein encoded by *lye* is the first enzyme shown to catalyze the synthesis of bacterioruberins.

It is unlikely that BO inhibits transcription or translation of *lye*, as depletion of BO by addition of exogenous retinal to strains in which the *H. salinarum* *lye* open reading frame is expressed from the *ura3* locus resulted in a dramatic increase of bacterioruberin production (Fig. 4C). These results indicate that inhibition of bacterioruberin synthesis by BO occurs even when *H. salinarum* *lye* lacks any potential upstream or downstream regulatory sequences. Additional support for a specific protein-protein regulatory action by BO is provided by evidence that BO inhibits bacterioruberin synthesis only when it is catalyzed by the *H. salinarum* *lye*-encoded enzyme and not when it is catalyzed by the *H. volcanii* *lye* homolog (Fig. 4). Correspondingly, expression of the *H. volcanii* *lye* homolog decreased production of BR substantially (Fig. 6). This specificity suggests that there are differences between these proteins that allow the *H. salinarum* enzyme itself to be inhibited by mechanisms involving BO.

The described regulatory role of BO is interesting in the context of the broad distribution of proteins homologous to BO in all three domains of life. Some of these homologs lack the lysine residue required for retinal binding and are unlikely to have photoactivity (38). Although the functions of these opsins-related proteins are not well understood, several members of this family, notably, *Saccharomyces cerevisiae* Hsp30, are thought to be chaperones (9). In addition, BR, with the introduction of only three point mutations, has been shown to be capable of activating HtrII, an integral membrane transducer protein (39). Therefore, it is plausible that evolutionary precursors to BO and its homologs interacted with other proteins in the membrane, in addition to their photoactive roles.

This potential regulatory mechanism represents an elegant solution to maintaining the stoichiometry of BO and its retinal cofactor. In its native environment of salt ponds, *H. salinarum* grows to high densities, quickly depleting available oxygen and halting aerobic respiration. Under these conditions, BR is necessary to maintain the electrochemical gradient that represents an important portion of the energy storage in a cell. Producing large amounts of BR is likely of more immediate importance than maintaining high levels of bacterioruberins to provide protection. When all the unbound BO protein has received a retinal cofactor to become a functional BR complex, this inhibition will be released and lycopene will be made available for synthesis of bacterioruberins. This mechanism allows a more immediate response to low oxygen without the need to precisely regulate the levels of carotenoid biosynthetic enzymes. Additionally, when there is no longer a need for retinal, synthesis is halted as lycopene becomes consumed in bacterioruberin synthesis.

In total, our findings demonstrate that BO inhibits bacterioruberin biosynthesis and suggest that this mechanism allows regulation of retinal biosynthesis. Further studies are needed to characterize this regulation at the molecular level. To our knowledge, this mechanism, in which an apoprotein inhibits an alternative biochemical pathway to direct intermediates to cofactor biosynthesis, has not been described for any protein cofactor complex, and similar mechanisms may regulate cofactor biosynthesis in other systems.

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