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Bacterioopsin-Mediated Regulation of Bacterioruberin Biosynthesis in *Halobacterium salinarum*▽‡

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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 *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 *bop*, the gene that encodes BO. *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 *lye* gene, that catalyzes bacterioruberin biosynthesis. BO-mediated inhibition of bacterioruberin synthesis appears to be specific to the *H. salinarum lye*-encoded enzyme, as expression of a *lye* homolog from *Haloferax 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 *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—*Archaea*, *Bacteria*, and *Eukaryota* (4–6, 15)—raising the possibility that regulatory mechanisms in BR production may be conserved. BR functions as a light-driven proton pump to generate usable cellular energy when low oxygen levels limit aerobic respiration (29). BR is a relatively simple protein-cofactor complex, consisting of a single protein, bacterioopsin (BO), and a single covalently bound retinal cofactor (29) which are maintained in an ~1:1 stoichiometric ratio (40). When production of BR is induced by low oxygen, it accumulates to high levels (30), but BR is not essential under laboratory conditions so mutant strains lacking BR are viable and easily studied (17).

H. salinarum synthesizes retinal *de novo* in a multistep process with distinct opportunities for regulation (Fig. 1). Early steps in the pathway are shared with the biosynthesis of phytanyl, the hydrophobic portion of the polar lipids that make up archaeal cell membranes, with the 20-carbon geranylgeranyl diphosphate (GGPP) being the last shared intermediate (7). In the retinal biosynthetic pathway, two GGPP molecules condense to form the 40-carbon carotenoid phytoene, which undergoes a series of desaturation reactions to form the red carotenoid lycopene (23). For retinal synthesis, lycopene cyclizes to form β-carotene, which is cleaved to form the 20-carbon retinal cofactor (23, 42). Alternatively, lycopene may be used as a precursor for bacterioruberins: tetrahydrobisanhydrobacterioruberin, bisanhydrobacterioruberin, and bacterioruberin (22, 24). These 50-carbon carotenoids function to increase membrane rigidity (25) and provide protection against UV light (36). Therefore, lycopene is a key intermediate that may be used either for retinal or bacterioruberin biosynthesis (Fig. 1).

One mechanism that *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 *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

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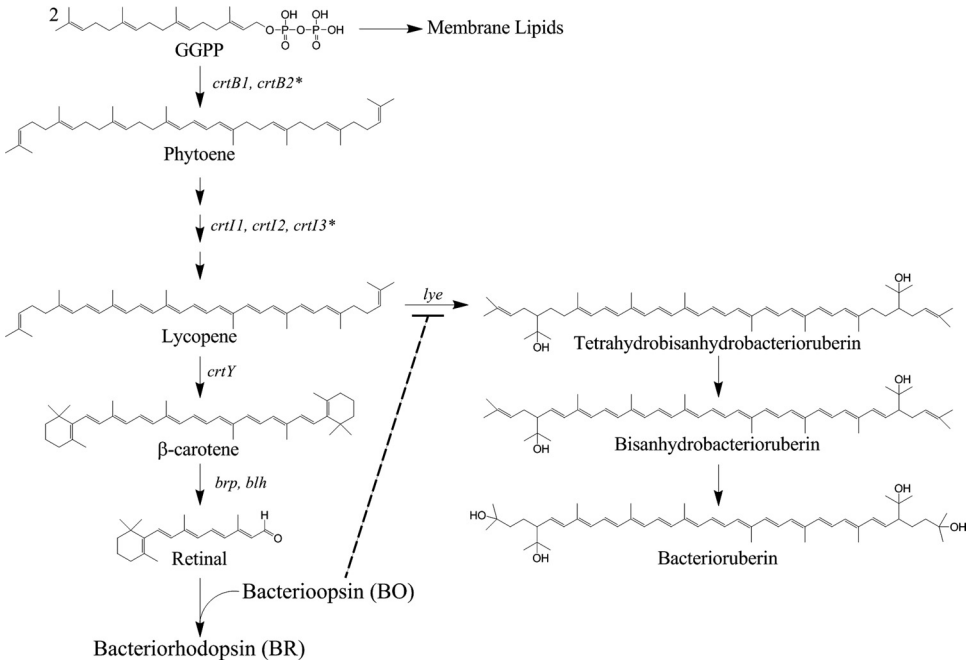

FIG. 1. The retinal and bacterioruberin biosynthetic pathways in *H. salinarum*. Italics indicate genes that encode catalytic enzymes. Asterisks indicate putative functions based on sequence homology with characterized genes from other organisms. The proposed regulatory mechanism of BO inhibiting bacterioruberin synthesis is indicated by the dashed line.

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 upregulated 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 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 *bop* knockout in the Δ*brp* Δ*blh* 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 pRFP85 for inserting the *H. salinarum lye* gene into the *ura3* locus, the *lye* locus of *Halobacterium* sp. NRC-1 genomic DNA was

TABLE 1. *Halobacterium* strains analyzed in this study

Strain	Reference	Relevant characteristics[a]	Retinal added	Total amt of carotenoid (nmol/liter culture)[b]
MPK407	32	Parental strain (MPK1 Δ*ura3*)	−	116 ± 40
MPK412	32	MPK407 Δ*bop*	−	76 ± 32
MPK414	46	Parental strain (*Halobacterium* sp. NRC-1 Δ*ura3*)	−	553 ± 187
MPK423	33	MPK407 Δ*brp* Δ*blh*	−	42 ± 6
			+	61 ± 7
MPK426	31	MPK407 Δ*crtY*	−	43 ± 14
			+	58 ± 23
RFP29	This study	MPK407 Δ*crtY* Δ*bop*	−	61 ± 31
RFP33	This study	MPK407 Δ*brp* Δ*blh* Δ*bop*	−	71 ± 3
			+	103 ± 26
RFP39	This study	MPK407 Δ*bop* Δ*lye*	−	40 ± 8
			+	43 ± 5
RFP43	This study	MPK407 Δ*lye*	−	106 ± 3
RFP45	This study	MPK407 Δ*crtY* Δ*lye*	−	20 ± 9
RFP49	This study	MPK407 Δ*bop* Δ*lye ura3*::*H. salinarum lye*	−	55 ± 7
RFP53	This study	MPK407 Δ*crtY* Δ*lye ura3*::*H. volcanii lye*	−	57 ± 8
RFP54	This study	MPK407 Δ*lye ura3*::*H. volcanii lye*	−	72 ± 15
RFP55	This study	MPK407 Δ*crtY* Δ*lye ura3*::*H. salinarum lye*	−	38 ± 3
			+	79 ± 10
RFP56	This study	MPK407 Δ*lye ura3*::*H. salinarum lye*	−	77 ± 12
RFP57	This study	MPK407 Δ*bop* Δ*lye ura3*::*H. volcanii lye*	−	83 ± 6
RFP60	This study	MPK407 Δ*brp* Δ*blh* Δ*lye*	−	57 ± 8
			+	45 ± 1
RFP62	This study	MPK414 Δ*bop*	−	845 ± 159

[a] All strains are Δ*ura3* to allow gene replacement by 5-FOA counterselection.
[b] Mean ± 1 standard deviation ($n \geq 3$).

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 *lye* open reading frame, and the 16-bp sequence derived from the *bop* transcription terminator (12). The PCR product was digested with XbaI and BglII and ligated with the XbaI/BglII fragment of the plasmid pMPK424 (33). The plasmid pRFP119, used to insert the *H. volcanii lye* homolog into the *H. salinarum ura3* locus, was constructed similarly. The *lye* locus of *H. volcanii* strain DS70 (45) was amplified using primers RP155 and RP156 from genomic DNA graciously provided by K. Bidle. 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. volcanii lye* homolog open reading frame, and the 16-bp terminator sequence from *bop*.

The plasmid pRFP65, used to express *H. salinarum lye* in *E. coli*, was made by amplifying the *lye* gene from *Halobacterium* sp. NRC-1 genomic DNA by PCR using primers RP41 and RP42. The resulting PCR product was an 837-bp fragment containing the *lye* gene with a single base change to create an NcoI restriction enzyme site (the base change was a T to G, which resulted in a change in the amino acid sequence from phenylalanine to valine) and 4 bp added to the 3′ end to retain the PmeI restriction site. The PCR product was then cut with NcoI and ligated with the 4-kbp NcoI/PmeI fragment of the plasmid pMPK438 (31). The plasmid pRFP109, used to express *H. volcanii lye* in *E. coli*, was constructed by amplifying the *lye* locus of *H. volcanii* using the primers RP155 and RP156. The PCR product contained three base changes, two of which were upstream of the start site to create an NcoI restriction enzyme site (one base change was an A to G, which resulted in a change in the amino acid sequence from serine to glycine) and 2 bp added to the 3′ end to retain the PmeI restriction site.

Generation of carotenoid-producing *E. coli*. Lycopene-producing *E. coli* cells were generated by transforming *E. coli* TOP10 (Invitrogen, Carlsbad, CA) with pAC-LYC (11), graciously provided by F. X. Cunningham. To test the function of the *H. salinarum* and *H. volcanii lye* genes, plasmid pRFP65, pRFP109, or pMPK454 (identical vector containing no gene for expression) (31) was transformed separately into lycopene-producing *E. coli*.

***H. salinarum* strain construction.** *H. salinarum* strains were constructed using a two-step *ura3*-based gene replacement method as described previously (32). pMPK413 (32), containing a 0.7-kbp internal deletion of *bop* with 4.3 kbp of flanking sequence, was used to make Δ*bop* strains by transforming into MPK426 (31) to make Δ*bop* Δ*crtY* (RFP29) and MPK414 (46) to make *Halobacterium* sp. NRC-1 Δ*bop* (RFP62). The Δ*brp* Δ*blh* Δ*bop* strain (RFP33) was constructed by transforming pRFP33 into MPK423 (33). Δ*lye* strains were constructed by transforming pRFP61 into the following: MPK412 (32) to make Δ*bop* Δ*lye* (RFP39), MPK407 (32) to make Δ*lye* (RFP43), MPK426 (31) to make Δ*crtY* Δ*lye* (RFP45), and MPK423 (33) to make Δ*brp* Δ*blh* Δ*lye* (RFP60). Strains with the *H. salinarum lye* open reading frame integrated at the *ura3* locus were constructed by transforming pRFP85 into the following: RFP39 to make Δ*bop* Δ*lye ura3*::*H. salinarum lye* (RFP49), RFP45 to make Δ*crtY* Δ*lye ura3*::*H. salinarum lye* (RFP55), and RFP43 to make Δ*lye ura3*::*H. salinarum lye* (RFP56). Similarly, strains with the *H. volcanii lye* homolog open reading frame integrated into the *H. salinarum ura3* locus were constructed by transforming pRFP119 into RFP45 to make Δ*crtY* Δ*lye ura3*::*H. volcanii lye* (RFP53), RFP43 to make Δ*lye ura3*::*H. volcanii lye* (RFP54), and RFP39 to make Δ*bop* Δ*lye ura3*::*H. volcanii lye* (RFP57). For all strains, 5-FOA-resistant colonies were screened by PCR to obtain the desired recombinants.

Carotenoid quantification and analysis. Carotenoids were extracted from *H. 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 phenylmethylsulfonyl fluoride, 0.003% NaN_3) 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 acetone, 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. coli* by a procedure similar to that used for *H. 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 *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

(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. *Halobacterium* 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 spectroscopically using an extinction coefficient of $E = 141\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$ (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 bacterioruberins, 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 (*Halobacterium* sp. NRC-1 Δ*ura3*) and MPK412 (MPK407 Δ*bop*) strains and peaks eluting at 38.9 min from *E. coli* expressing *H. salinarum lye* 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 spectroscopically 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 bicinchoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin to generate a standard.

RESULTS

Deletion of *bop* results in accumulation of bacterioruberins. 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 Δ*ura3*) (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 Δ*bop* strain

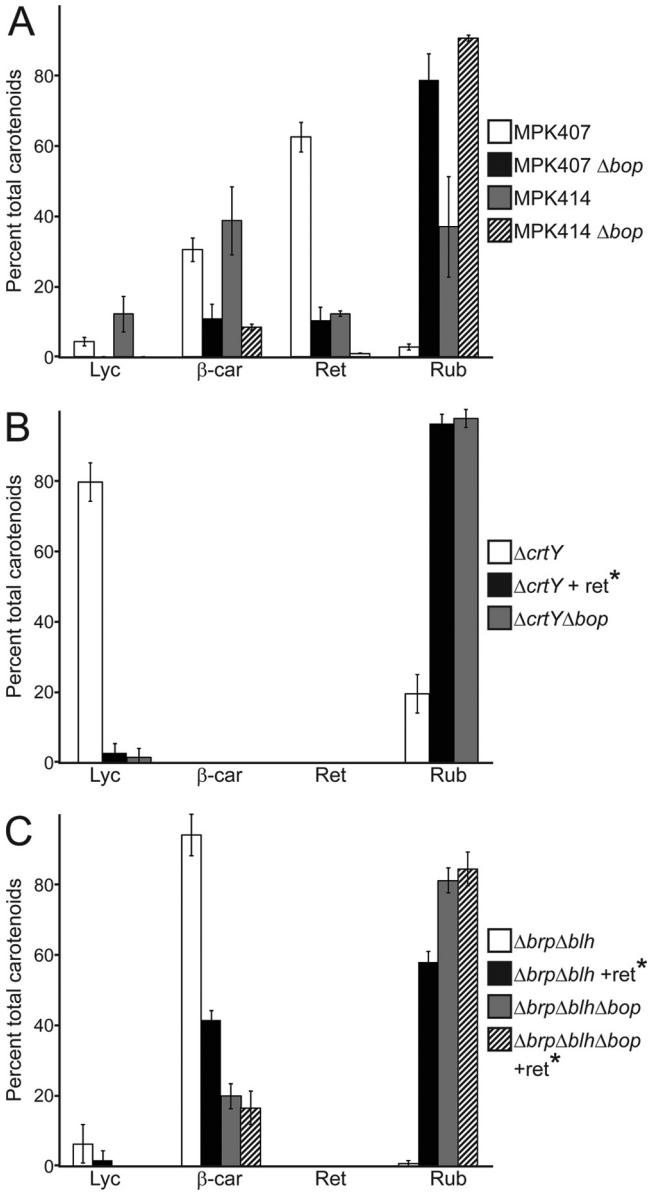

FIG. 2. Deletion of *bop* gene or removal of BO through addition of excess retinal results in accumulation of bacterioruberins. Relative levels of lycopene (Lyc), β-carotene (β-car), retinal (Ret), and bacterioruberins (Rub) as percentages of total carotenoids in strains grown under limiting oxygen conditions to induce BR synthesis. Asterisks indicate that exogenous retinal was added during growth, as described in Materials and Methods, and retinal levels were not quantified in these samples. Error bars indicate 1 standard deviation ($n \geq 3$).

accumulated carotenoids with relative polarity and absorbance spectra suggestive of 50-carbon bacterioruberins (Fig. 3).

To confirm that the Δ*bop* strain accumulated bacterioruberins, we compared the most abundant carotenoid in Δ*bop* cells to that in cells of MPK414 (*Halobacterium* sp. NRC-1 Δ*ura3*) (46), a strain directly derived from *Halobacterium* sp. NRC-1 which produces characterized bacterioruberins (21, 24). The major carotenoid in the Δ*bop* strain had an HPLC retention time identical to that of the characterized bacterioruberin (Fig. 3A). In addition, this carotenoid exhibited a UV-

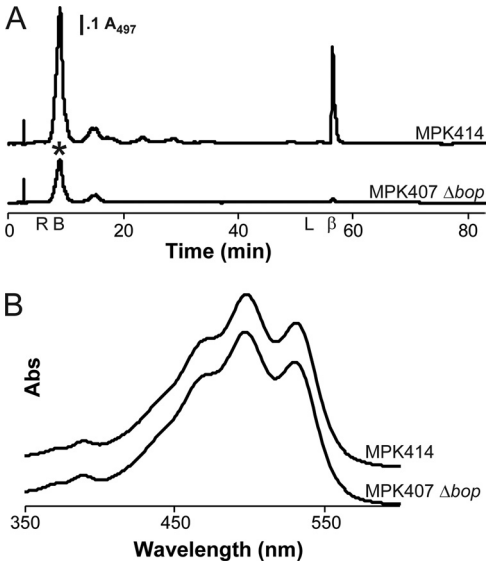

FIG. 3. Bacterioruberin is the major carotenoid in the Δ*bop* strain. (A) RP-HPLC analysis of total carotenoids from MPK414 (*Halobacterium* sp. NRC-1 Δ*ura3*) (top trace) and MPK407 Δ*bop* (bottom trace). Positions of carotenoid standards are noted: retinal (R), bacterioruberin (B), lycopene (L), and β-carotene (β). The asterisk indicates the position of the sample shown in UV-visible absorbance spectra and subjected to MALDI-TOF mass spectrometry analysis. HPLC traces are from carotenoids extracted from similar cell amounts and are offset on the vertical axis for clarity. (B) UV-visible spectra of bacterioruberin from MPK414 (*Halobacterium* sp. NRC-1 Δ*ura3*) (top trace) and MPK407 Δ*bop* (bottom trace). Spectra were obtained with the HPLC diode array detector, normalized to the same peak absorbance to allow comparison, and offset on the vertical axis for clarity.

visible (UV/vis) absorbance spectrum characteristic of a tridecaene chromophore, with a major peak at 497 nm and minor peaks at 533 nm, 475 nm, and 392 nm, and identical to that of bacterioruberin (Fig. 3B). MALDI-TOF mass spectrometry of the major carotenoids yielded mass ion values of 740.4 to 740.5 Da for both carotenoids, which are within the instrumental error of the calculated bacterioruberin mass of 740.6 Da.

Our results demonstrated that parental strain MPK407 produces a small amount of bacterioruberins, but bacterioruberins are the major carotenoids when the *bop* gene is deleted. Since MPK407 is derived from MPK1, a strain previously reported to have reduced bacterioruberin production (17), we examined strains derived from *Halobacterium* sp. NRC-1 to ensure that the deletion of *bop* had the same effect. Although MPK414 (*Halobacterium* sp. NRC-1 Δ*ura3*) produced bacterioruberins with an intact *bop* gene (37% of total carotenoids), the relative abundance increased substantially (91% of total carotenoids) in the corresponding Δ*bop* strain (Fig. 2A). Therefore, the loss of *bop* leads to increased bacterioruberins in strains derived from both MPK1 and *Halobacterium* sp. NRC-1, suggesting a common mechanism in *Halobacterium* for a BO-mediated influence on carotenoid biosynthesis.

Notably, strains derived from *Halobacterium* sp. NRC-1 produce more total carotenoids than MPK1-derived strains (Table 1). To explore if this increased carotenoid production resulted in greater BR production, we analyzed BR production in MPK414 and determined that it had similar levels to MPK407 (see Fig. S1 in the supplemental material). This result suggests that in *Halobacterium* sp. NRC-1-derived strains grown under laboratory conditions, carotenoid production is in excess of that required for biosynthesis of the BR retinal cofactor. This increased production may explain the relatively high levels of bacterioruberins in strains derived from *Halobacterium* sp. NRC-1 compared to MPK1-derived strains (Fig. 2A).

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 *brp* 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 Δ*crtY* strain primarily accumulated lycopene (Fig. 2B), and the Δ*brp* Δ*blh* 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 Δ*crtY* Δ*bop* strain (Fig. 2B) and ~84% in the Δ*brp* Δ*blh* Δ*bop* 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 Δ*brp* Δ*blh* 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 Δ*brp* Δ*blh* 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 Δ*crtY* 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 Δ*brp* Δ*blh* Δ*bop* 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 flavuxanthin 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 *ubiA* 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, *OE3377R*, 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 Δ*bop* 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, Δ*lye ura3*::*Halobacterium lye* and Δ*bop* Δ*lye 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 bacterioruberins was restored to ~77% of total carotenoids in the Δ*bop* Δ*lye* background and ~3% of total carotenoids in the Δ*lye* background (Fig. 4A and B), very similar to the levels observed in MPK407 and MPK407 Δ*bop* 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

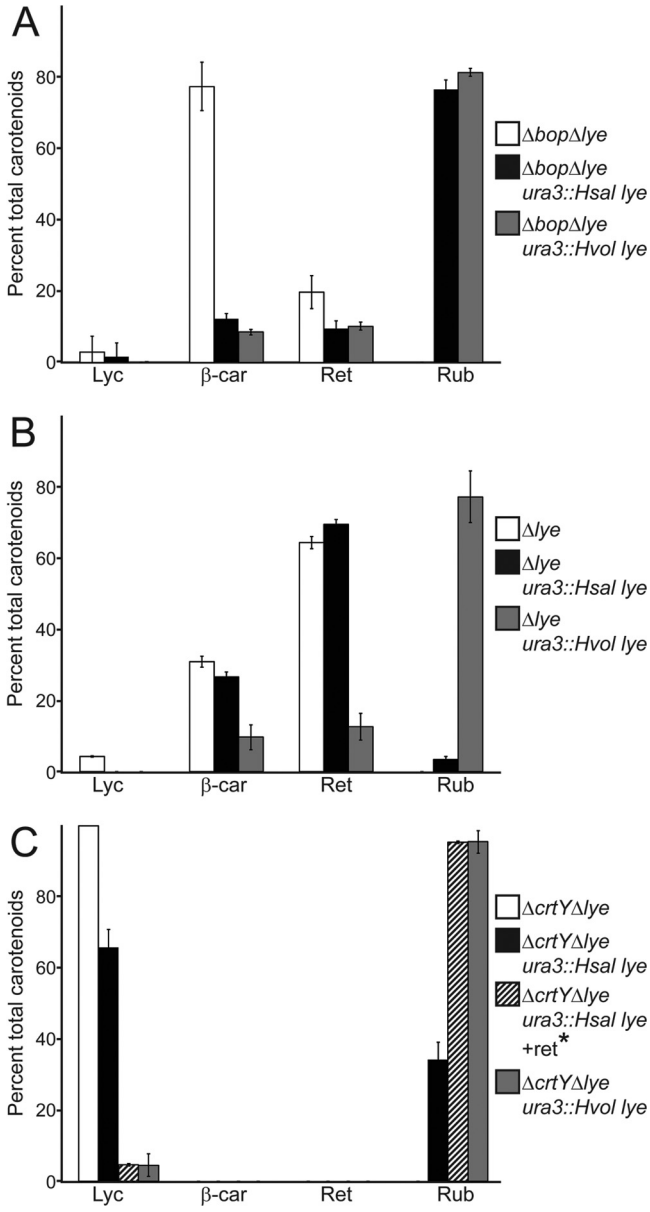

FIG. 4. The *lye* gene is required for synthesis of bacterioruberins, and bacterioruberin synthesis is rescued with *H. salinarum lye* or the *H. volcanii lye* homolog. Relative levels of lycopene (Lyc), β-carotene (β-car), retinal (Ret), and bacterioruberins (Rub) as percentages of total carotenoids in strains grown under limiting oxygen conditions to induce BR synthesis. *Hsal lye*, *lye* from *H. salinarum*; *Hvol lye*, *lye* homolog from *H. volcanii*. The asterisk indicates that exogenous retinal was added during growth, as described in Materials and Methods, and retinal levels were not quantified in this sample.

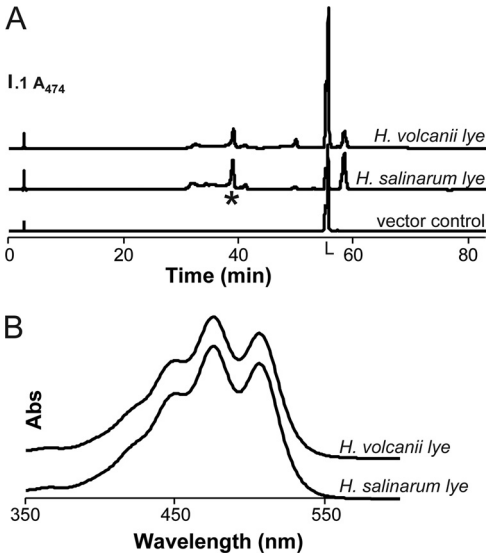

FIG. 5. Expression of *H. salinarum lye* and *H. volcanii lye* homolog in lycopene-producing *E. coli* results in production of a possible bacterioruberin precursor. (A) RP-HPLC analysis of carotenoids from lycopene-producing *E. coli* containing an empty expression vector (bottom trace), a vector with *H. salinarum lye* (middle trace), or a vector with *H. volcanii lye* (top trace). The position of lycopene is indicated (L). The asterisk indicates the position of samples shown in UV-visible absorbance spectra and subjected to MALDI-TOF mass spectrometry analysis. Traces from the *E. coli* strains containing vectors with *H. salinarum lye* or *H. volcanii lye* show extracts from normalized cell amounts. The vector control trace shows an extract from 4-fold fewer cells. (B) UV-visible spectra of the potential bacterioruberin precursor produced by expression of *H. salinarum lye* or the *H. volcanii lye* homolog. Spectra were obtained with the HPLC diode array detector, normalized to the same peak absorbance to allow comparison, and offset on the vertical axis for clarity.

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 65% identity over 272 amino acids to the protein encoded by *H. salinarum lye* (see Fig. S2 in the supplemental material). Expression of this *H. volcanii lye* homolog in lycopene-producing *E. coli* resulted in the production of new carotenoids with identical HPLC retention times and spectral absorbance properties seen with expression of *H. salinarum lye* (Fig. 5), suggesting that the enzymes have similar catalytic properties using lycopene as a substrate.

To compare the activities of these enzymes in *H. salinarum*, we generated strains with either *H. salinarum lye* or the *H. volcanii lye* homolog integrated into the *ura3* locus. As noted previously, expression of *H. salinarum lye* from this locus restored production of bacterioruberins in the Δ*bop* Δ*lye* background (Fig. 4A). To determine if bacterioruberin production was still inhibited by BO when the *lye* open reading frame was expressed at the *ura3* locus, we analyzed the Δ*crtY* Δ*lye ura3*::*H. salinarum lye* strain, which produces BO but lacks retinal synthesis. In this strain, ~34% of total carotenoids were bacterioruberins, with the remainder being lycopene (Fig. 4C). However, when the Δ*lye* Δ*crtY ura3*::*H. salinarum lye* strain was grown in the presence of excess retinal to deplete BO, the lycopene was nearly quantitatively converted to bacterioruberins (Fig. 4C), indicating that the *H. salinarum lye* gene product was subject to BO-mediated inhibition even when it was expressed at a different locus.

Expression of the *H. volcanii lye* homolog rescued the Δ*lye* phenotype, as the Δ*bop* Δ*lye ura3*::*H. volcanii lye* strain produced levels of bacterioruberins very similar to those produced by the Δ*bop* Δ*lye 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, Δ*lye ura3*::*H. volcanii lye* and Δ*crtY*Δ*lye ura3*::*H. volcanii lye*. In the Δ*lye 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*, Δ*lye ura3*::*H. salinarum lye* (Fig. 4B). In the Δ*crtY* Δ*lye ura3*::*H. volcanii lye* strain, bacterioruberins accumulated to ~96% of total carotenoids (Fig. 4C). Again, this bacterioruberin production was much higher than the ~34% of total carotenoids observed in the Δ*crtY* Δ*lye 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 Δ*lye* and Δ*lye 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* 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 Δ*bop* Δ*lye* strain, which lacks the ability to synthesize BO or bacterioruberins, β-carotene and retinal constitute ~96% of

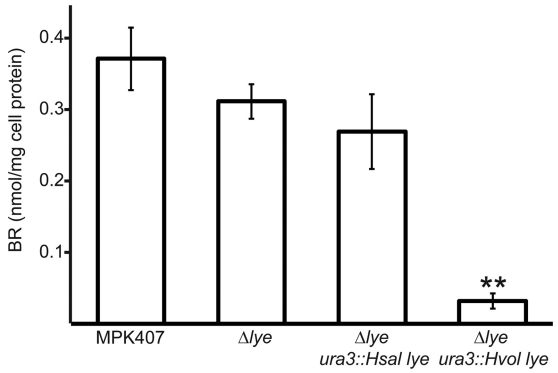

FIG. 6. Expression of the *H. volcanii lye* homolog reduces BR production. BR levels in the parental MPK407 strain, the Δ*lye* strain, and strains in which the Δ*lye* mutation was complemented by integration of *H. salinarum* (*Hsal*) *lye* or the *H. volcanii* (*Hvol*) *lye* homolog into the *ura3* locus. Cultures were grown under limiting oxygen conditions to induce BR synthesis, and BR levels were determined by the difference in absorbance at 587 nm between light-adapted and dark-adapted cell lysates as described in Materials and Methods. Double asterisks indicate a significant difference in BR levels compared to all other strains ($P \leq 0.01$, Student's *t* test). Error bars indicate standard errors ($n \geq 4$).

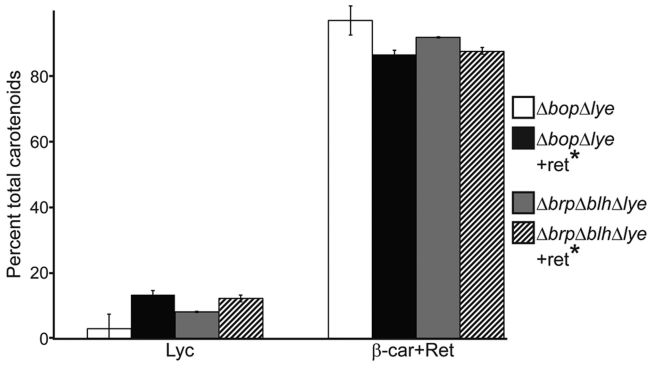

FIG. 7. β-Carotene synthesis is not substantially inhibited by retinal or BR. Relative levels of lycopene (Lyc) and combined β-carotene (β-car) and retinal (Ret) as percentages of total carotenoids in strains grown under limiting oxygen conditions to induce BR synthesis. Bacterioruberins were not observed in either strain. Retinal percentage was added to β-carotene percentage for the Δ*bop* Δ*lye* strain; no retinal was observed in the Δ*brp* Δ*blh* Δ*lye* strain. Asterisks indicate that exogenous retinal was added during growth, as described in Material and Methods, and retinal levels were not quantified in these samples. Error bars indicate 1 standard deviation ($n \geq 3$).

the total carotenoids (Fig. 7). When retinal was added to the Δ*bop* Δ*lye* 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 Δ*brp* Δ*blh* Δ*bop* 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 Δ*brp* Δ*blh* Δ*lye* 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 BR 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 dimethylall 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 tetrahydrobisanhydrobacterioruberin (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 opsin-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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