



# Species Widely Distributed in Halophilic *Archaea* Exhibit Opsin-Mediated Inhibition of Bacterioruberin Biosynthesis

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**ABSTRACT** Halophilic *Archaea* are a distinctive pink color due to a carotenoid pigment called bacterioruberin. To sense or utilize light, many halophilic *Archaea* also produce rhodopsins, complexes of opsin proteins with a retinal prosthetic group. Both bacterioruberin and retinal are synthesized from isoprenoid precursors, with lycopene as the last shared intermediate. We previously described a regulatory mechanism by which *Halobacterium salinarum* bacterioopsin and *Haloarcula vallismortis* cruxopsin inhibit bacterioruberin synthesis catalyzed by lycopene elongase. In this work, we found that opsins in all three major *Halobacteria* clades inhibit bacterioruberin synthesis, suggesting that this regulatory mechanism existed in the common *Halobacteria* ancestor. Halophilic *Archaea*, which are generally heterotrophic and aerobic, likely evolved from an autotrophic, anaerobic methanogenic ancestor by acquiring many genes from *Bacteria* via lateral gene transfer. These bacterial “imports” include genes encoding opsins and lycopene elongases. To determine if opsins from *Bacteria* inhibit bacterioruberin synthesis, we tested bacterial opsins and found that an opsin from *Curtobacterium*, in the *Actinobacteria* phylum, inhibits bacterioruberin synthesis catalyzed by its own lycopene elongase, as well as that catalyzed by several archaeal enzymes. We also determined that the lycopene elongase from *Halococcus salifodinae*, a species from a family of *Halobacteria* lacking opsin homologs, retained the capacity to be inhibited by opsins. Together, our results indicate that opsin-mediated inhibition of bacterioruberin biosynthesis is a widely distributed mechanism found in both *Archaea* and *Bacteria*, possibly predating the divergence of the two domains. Further analysis may provide insight into the acquisition and evolution of the genes and their host species.

**IMPORTANCE** All organisms use a variety of mechanisms to allocate limited resources to match their needs in their current environment. Here, we explore how halophilic microbes use a novel mechanism to allow efficient production of rhodopsin, a complex of an opsin protein and a retinal prosthetic group. We previously demonstrated that *Halobacterium salinarum* bacterioopsin directs available resources toward retinal by inhibiting synthesis of bacterioruberin, a molecule that shares precursors with retinal. In this work, we show that this mechanism can be carried out by proteins from halophilic *Archaea* that are not closely related to *H. salinarum* and those in at least one species of *Bacteria*. Therefore, opsin-mediated inhibition of bacterioruberin synthesis may be a highly conserved, ancient regulatory mechanism.

**KEYWORDS** cofactor biosynthesis, carotenoid biosynthesis, C50 carotenoid, membrane protein biogenesis, microbial rhodopsin, proteorhodopsin, UbiA prenyltransferase

Halophilic archaea that make up the class *Halobacteria* grow to such high densities that they provide the brilliant pink, red, and orange colors characteristic of salt lakes and ponds. These colors are primarily produced by 50-carbon carotenoid pigments called bacterioruberins. Although their primary function has not been fully

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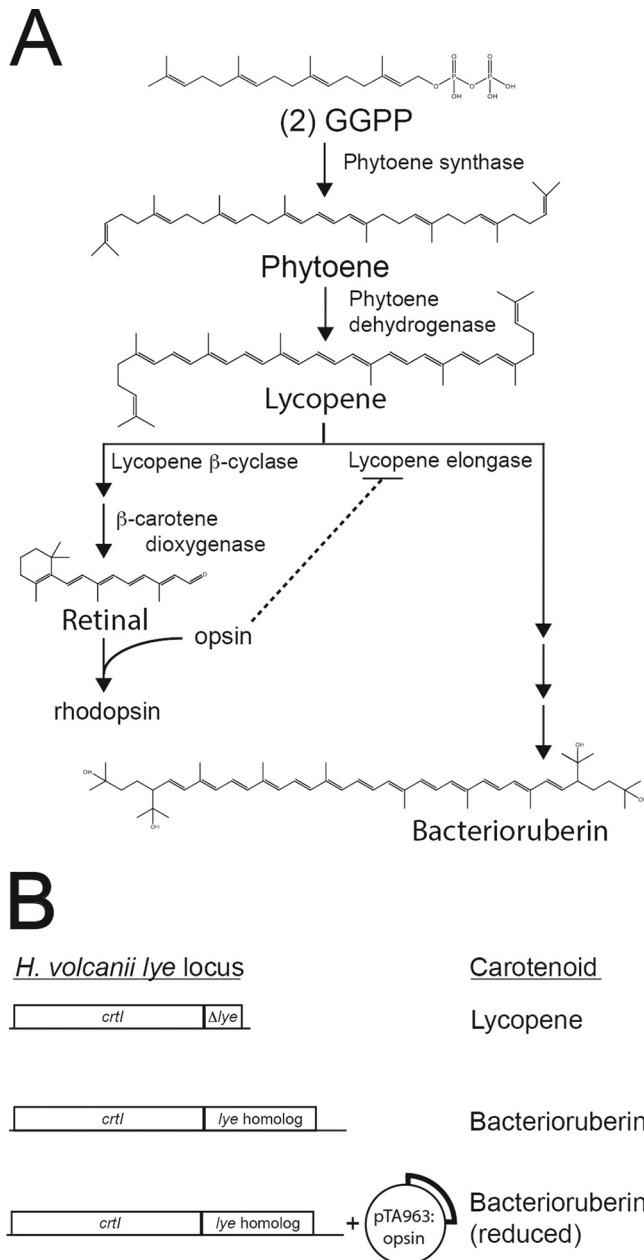
characterized *in vivo*, bacterioruberins are proposed to protect cells from osmotic stress (1) or damage from UV light (2). In *Archaea*, both carotenoids and the hydrophobic tails of membrane lipids are produced from isoprenoid precursors (3). Universally, isoprenoids are derived from the 5-carbon precursor molecules isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which themselves are generated from acetyl coenzyme A (acetyl-CoA) and ATP. Therefore, IPP and DMAPP represent a substantial cellular investment of reduced carbon and energy. IPP and DMAPP are then consumed in consecutive condensation reactions to generate the 20-carbon geranylgeranyl pyrophosphate (GGPP), the last shared intermediate of carotenoid and polar lipid biosynthesis in *Archaea* (3). In the committed step of carotenoid biosynthesis, phytoene synthase catalyzes the condensation of two GGPP molecules to form the 40-carbon phytoene (4). Phytoene is then desaturated by phytoene dehydrogenase (phytoene desaturase) to generate lycopene (5). For bacterioruberin synthesis, lycopene elongase (Lye) catalyzes the addition of two C<sub>5</sub> isoprenoids to generate the 50-carbon bacterioruberin, which may then be modified by further desaturation and hydroxylation (Fig. 1A) (6, 7).

For the many halophilic *Archaea* that produce rhodopsin complexes for energy conversion, lycopene is the final shared intermediate for two end products, namely, bacterioruberin and retinal, the prosthetic group (sometimes called “cofactor”) of rhodopsin. To generate retinal, lycopene is cyclized to form  $\beta$ -carotene, which then undergoes oxidative cleavage to form retinal (8–10). Given the energy input required for carotenoid biosynthesis and the requirement for retinal to form functional rhodopsins, these organisms must have regulatory processes to optimally allocate lycopene for bacterioruberin or retinal synthesis. We previously described a mechanism of regulation in which *Halobacterium salinarum* bacterioopsin (BO), when not bound by retinal, inhibits bacterioruberin synthesis catalyzed by Lye (6). We subsequently identified a similar inhibition by the opsin of *Haloarcula vallismortis* (11). This inhibition likely ensures that lycopene is conserved for retinal biosynthesis when opsins are available. In the work described here, we examined opsins and Lyes from multiple halophilic *Archaea*. Our findings demonstrate that opsin-mediated inhibition of bacterioruberin synthesis is distributed throughout the *Halobacteria* and indicate that this mechanism may have existed in the last common *Halobacteria* ancestor.

We next explored the possibility that opsin-mediated inhibition of bacterioruberin synthesis existed at the origin of halophilic *Archaea*. Substantial evidence indicates that halophilic *Archaea* arose from a methanogenic species (12, 13). The transition from a presumably anaerobic, autotrophic metabolism to the generally aerobic, heterotrophic metabolism of halophilic *Archaea* has been proposed to be driven by acquisition of bacterial genes (14, 15). A recent study examining this proposed acquisition predicted Lye and bacterioopsin of *Halobacteria* to be among these laterally transferred genes from *Bacteria* (15). To gain insight into the evolution of this mechanism, we examined the possibility that opsins from *Bacteria* inhibit Lye homologs. Of two bacterial opsins analyzed, one, an opsin homolog from a member of the *Actinobacteria*, *Curtobacterium* sp. strain ER1/6, inhibited its own Lye. In addition, the *Curtobacterium* sp. ER1/6 opsin also inhibited the Lye from several *Archaea* species, suggesting some level of functional and structural conservation even across domains. To analyze if Lyes from a species lacking opsins could be subject to opsin-mediated inhibition, we used the Lye from *Halococcus salifodinae*, a species isolated from Permian halite deposits (16, 17) that lacks opsin homologs (18). We found that the *H. salinarum* bacterioopsin could inhibit bacterioruberin synthesis catalyzed by *H. salifodinae* Lye, suggesting that this enzyme retains structural features allowing inhibition in this organism devoid of opsin homologs.

## RESULTS AND DISCUSSION

**Bacterioruberin and retinal synthesis is common and widely distributed in *Halobacteria* species.** To assess if the need to coordinate bacterioruberin and retinal production is common in halophilic *Archaea*, we analyzed *Halobacteria* genome se-



**FIG 1** (A) Carotenoid biosynthesis in halophilic *Archaea*. Enzyme names are indicated next to reactions catalyzed. A broken line indicates the regulatory mechanism of an opsin inhibiting the committed step in bacterioruberin synthesis. GGPP, geranylgeranyl pyrophosphate. (B) Experimental approach for testing opsin-mediated inhibition of Lye in *H. volcanii*. The *H. volcanii* *lye* locus is shown approximately to scale on the left and the carotenoid produced is indicated on the right. The *lye* gene encoding lycopene elongase is directly downstream and in a putative operon with *crtI*, the gene that encodes phytoene dehydrogenase. No bacterioruberin is produced in the *H. volcanii*  $\Delta$ *lye* strain (Fig. 2). *lye* homologs are incorporated into the *lye* locus, maintaining the integrity of *crtI*, and bacterioruberin synthesis is restored (Fig. 2). Opsins are then expressed by introducing pTA963 (34), harboring the gene encoding the opsin, and bacterioruberin production is quantified to determine if the opsin inhibits Lye activity.

quences to determine the prevalence of organisms with the necessary genes for synthesis of these molecules (Table 1; homolog accession numbers listed in Table S1 in the supplemental material). We conducted a preliminary examination of all 348 *Halobacteria* genome sequences available on GenBank. To allow consistent analysis, we selected one representative genome for each species and excluded genomes with too many contigs and those missing highly conserved, essential genes. Among the 220

**TABLE 1** Number of *Halobacteria* genomes<sup>a</sup> harboring genes allowing synthesis of lycopene, bacterioruberin, retinal, and/or opsins

Product(s) (enzyme[s] encoded)	Complete genomes (no.)	Incomplete genomes <sup>b</sup> (no.)	Total (no.)
Lycopene (phytoene synthase, phytoene dehydrogenase)	43	175	218
Bacterioruberin (lycopene elongase)	43	172	215
Retinal (lycopene cyclase, $\beta$ -carotene dioxygenase)	25	114	139
Opsin	29	123	152
Retinal and opsin	24	112	136
Retinal, opsin, and bacterioruberin	24	111	135

<sup>a</sup>*Halobacteria* genome sequences were obtained from the National Center for Biotechnology Information (NCBI; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) in August 2018, and homologs were identified as described in Materials and Methods. All homologs identified are listed in Table S1 in the supplemental material. In total, 220 genomes were analyzed, 43 complete and 177 incomplete.

<sup>b</sup>Genome assembly levels "contig" and "scaffold," as labeled in the NCBI database, were considered to be incomplete.

genomes that remained after this selection, we found that carotenoid synthesis is nearly universally conserved, as 218 genomes harbor the necessary genes, encoding phytoene synthase and phytoene dehydrogenase, needed to catalyze the conversion of GGPP to lycopene. Of these genomes that have genes for lycopene production, only 3 lack a gene encoding Lye, needed for bacterioruberin synthesis. All 43 completely assembled genomes contain all genes necessary for bacterioruberin production, suggesting that incomplete sequences may account for genomes that lack these genes (Table 1). Overall, our analysis indicates that the ability to generate bacterioruberin from isoprenoid precursors is a nearly universal characteristic of halophilic *Archaea*.

In contrast, the capacity to produce rhodopsin complexes is widespread but not universal in *Halobacteria* species. We found that 152 genomes out of 220 harbor a gene for at least one opsin, and 136 of these also have genes encoding enzymes necessary for generating retinal from lycopene, lycopene  $\beta$ -cyclase (9) and  $\beta$ -carotene dioxygenase (8, 10). Comparing these values, 16 species, including 5 completely assembled genomes, have genes encoding opsins but apparently do not have the ability to generate retinal. These proteins may perform functions that do not require retinal binding, and a role in responding to stimuli other than light has been previously proposed for these putatively "blind" opsins (19). Interestingly, three genomes that have genes for retinal synthesis, including the completely assembled *Halopenitus persicus* genome, lack identifiable opsin homologs, suggesting that retinal functions in other roles aside from serving as an opsin prosthetic group. In total, 135 genomes out of the 220 analyzed contained genes for bacterioruberin, retinal, and rhodopsin synthesis (Table 1), indicating that approximately half of the known *Halobacteria* species require a mechanism to direct available lycopene toward retinal or bacterioruberin.

Although the phylogeny of the *Halobacteria* is not fully characterized, a combination of 16S rRNA and RNA polymerase subunit B' sequence comparisons defined two clades, with other organisms placed in a third group (20). With many more genome sequences available, a subsequent study using 40 concatenated conserved marker genes broadly supported the earlier phylogeny and clearly defined three clades within *Halobacteria* (18). We had previously found that opsins from two organisms in clade 3, *H. salinarum* and *H. vallismortis*, inhibit their respective Lyes. To examine if this mechanism occurs throughout *Halobacteria*, we selected nine organisms distributed throughout the class and included at least two members from each of the three clades (Table 2). Since our previous work indicated that opsins from proton pumps mediated this activity, we chose opsin homologs that had residues equivalent to aspartates at positions 85 and 96, demonstrated to be required for proton translocation in *H. salinarum* bacteriorhodopsin (21). Very few halophilic *Archaea* have been developed to be genetically tractable, so our approach to study potential interaction between these proteins was heterologous expression and assessment of bacterioruberin production in *Haloferax volcanii* (Fig. 1B).

**Lycopene elongases are functionally expressed in *H. volcanii*.** As a first step, we obtained codon-optimized synthetic genes encoding Lye from the nine *Halobacteria* species. These genes were integrated into the chromosome of *H. volcanii*, exactly replacing the open reading frame of the native *lye* gene (Fig. 1B) to allow similar

**TABLE 2** Source species of Lye and opsin homologs used in this study

Species or strain	Domain	Archaeal clade <sup>b</sup>	UniProtKB accession no. for:		Genome reference or source
			Lye homolog	Opsin homolog(s)	
<i>Halobacterium salinarum</i>	Archaea	3	Q9HPD9	P02945	38
<i>Haloarcula marismortui</i> ATCC 43049	Archaea	3	Q5V532	Q5UXY6, Q5V0R5	39
<i>Halorhabdus utahensis</i> DSM 12940	Archaea	3	C7NVL7	C7NQP3	40
<i>Natronomonas moolapensis</i> DSM 18674	Archaea	3	M1X573	M1Y5I7	41
<i>Halomicrobium mukohataei</i> DSM 12286	Archaea	3	C7P310	C7P2T2	42
<i>Haloarcula vallismortis</i> ATCC 29715	Archaea	3	A0A1H2XMF1	P94854	Unpublished
<i>Haloquadratum walsbyi</i> DSM 16854	Archaea	2	G0LJV2	G0LFX8, G0LFY1	43
<i>Halorubrum distributum</i> JCM 10118	Archaea	2	M0F851	M0F390 <sup>a</sup>	18
<i>Halorubrum</i> sp. A07HR67	Archaea	2	V4XXE3	V4ZQH6	44
<i>Natrinema pellirubrum</i> DSM 15624	Archaea	1	L0JK84	L0JG40	18
<i>Haloterigena mahlil</i> H13	Archaea	1	A0A179EP62	A0A179EFG5	45
<i>Haloferax volcanii</i> DS2	Archaea	1	D4GTV9	None	46
<i>Halococcus salifodinae</i> DSM 8989	Archaea	NA	M0N6J9	None	18
<i>Rubrobacter xylanophilus</i> DSM 9941	Bacteria	NA	Q1ATY0	Q1AUE6	Unpublished
<i>Curtobacterium</i> sp. ER1/6	Bacteria	NA	A0A1E5MG13	A0A1E5MMK0	26

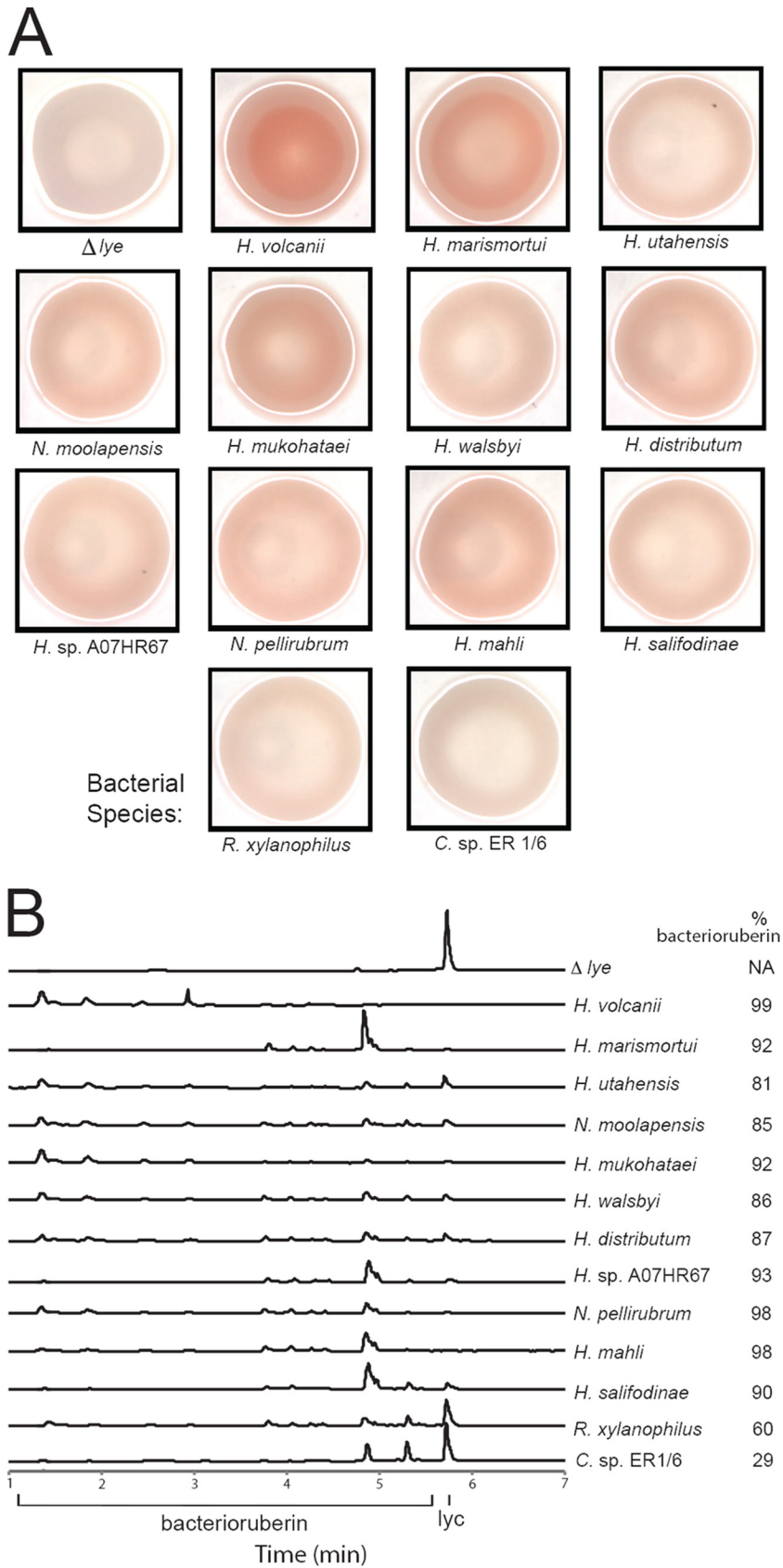
<sup>a</sup>*H. distributum* opsin was modified to match the longer protein predicted in reference 19.

<sup>b</sup>Archaeal clades are from reference 18. NA, not applicable.

expression to the native Lye protein. All resulted in a shift in colony color to pink, compared to the off-white of the *lye* deletion strain (Fig. 2A), indicating a rescue of bacterioruberin synthesis. To confirm bacterioruberin production, carotenoids were extracted from liquid cultures and analyzed by high-performance liquid chromatography (HPLC). In wild-type *H. volcanii* cells, bacterioruberin constitutes ~99% of the total lycopene-derived carotenoid. While exhibiting slightly reduced activity compared to that of native Lye, all Lyes from *Halobacteria* restored at least 86% of bacterioruberin synthesis in *H. volcanii* (Fig. 2B). Thus, heterologous expression in *H. volcanii* is a suitable technique to assess Lye activity.

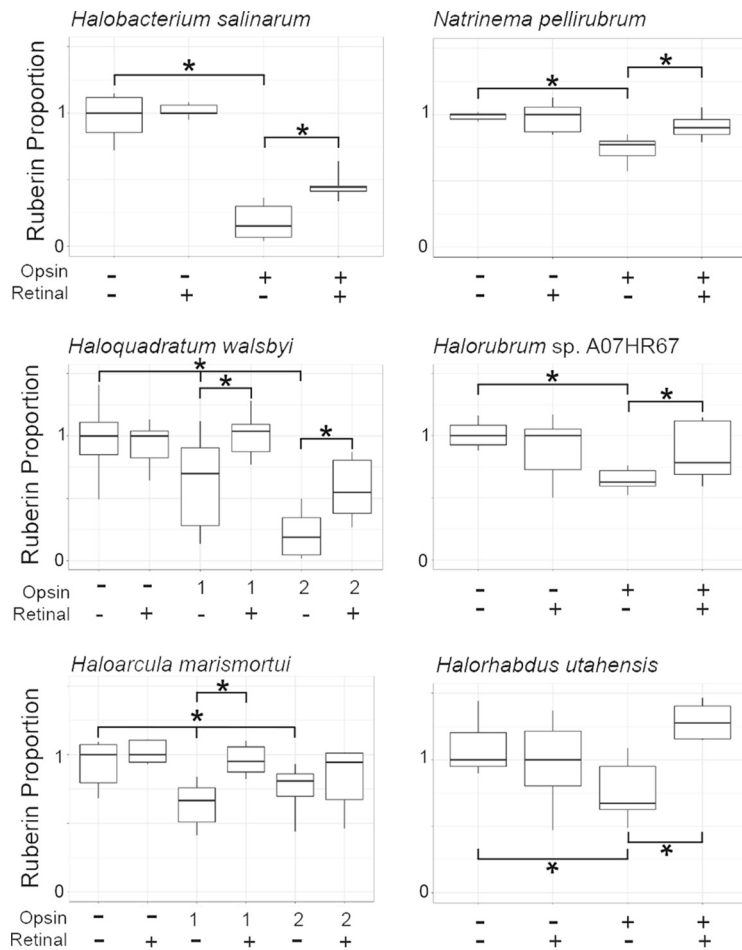
**Expression of opsins often inhibits the Lyes of their respective species.** We had previously determined that expression of *H. salinarum* BO in *H. volcanii* cells allowed effective inhibition of bacterioruberin synthesis catalyzed by *H. salinarum* Lye (11). To assess potential opsin-mediated inhibition of bacterioruberin synthesis across the *Halobacteria* class, we expressed opsins in the *H. volcanii* strains harboring the *lye* genes from their respective organisms. Lyes from five organisms out of the nine tested exhibited a reduction in bacterioruberin production upon expression of an opsin from their genome (Fig. 3). The most active opsin tested was Bop2 from *Haloquadratum walsbyi*, a clade 2 species, which resulted in an 82% reduction in bacterioruberin synthesis. *H. walsbyi* Bop1 also reduced bacterioruberin, but only by 30%. A similar difference in activity was observed in the two opsins tested for *Haloarcula marismortui*, a clade 3 species, as Bop1 inhibited bacterioruberin production by 33% (Fig. 3), compared to only 19% inhibition by Bop2. Expression of opsins from the clade 1 species *Natrinema pellirubrum*, clade 2 species *Halorubrum* sp. strain A07HR67, and clade 3 species *Halorhabdus utahensis* also significantly inhibited bacterioruberin production from their respective Lye enzymes. When the *H. volcanii* strains expressing these opsins were grown in the presence of retinal to convert the available opsin to the rhodopsin complex, bacterioruberin synthesis was partially, but significantly, restored in nearly all strains, with only the *H. marismortui* Bop2-expressing strain lacking a statistically significant increase in bacterioruberin (Fig. 3). Since *H. volcanii* lack any known proteins that interact with retinal, this increase in bacterioruberin is strong evidence that inhibition of Lye is caused by opsin. Moreover, this reduction in inhibition with retinal indicates that the opsins folded into structures that allowed at least a partial formation of the rhodopsin complex.

To assess if inhibition was correlated to opsin abundance, we constructed opsin genes with C-terminal hexahistidine (His<sub>6</sub>) tags and analyzed expression via immunoblotting. We had previously used a His<sub>6</sub>-tagged *H. salinarum* BO to assess expression (11), and this BO also inhibited bacterioruberin synthesis catalyzed by *H. salinarum* Lye



**FIG 2** Expression of Lye homologs in *H. volcanii* restores bacterioruberin production. (A) Photographs of representative colonies of *H. volcanii* strains with the native *lye* deleted ( $\Delta$ lye) or replaced with *lye* (Continued on next page)



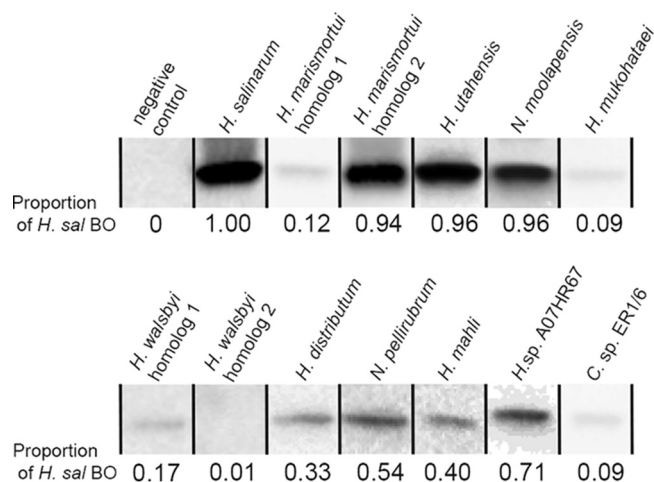


**FIG 3** Expression of opsins inhibits Lye-catalyzed bacterioruberin production in *Halobacteria* species. Box plot (Tukey's [37]) indicating proportionate bacterioruberin levels compared to those of the empty vector control in *H. volcanii* strains expressing Lye homologs from the indicated species. Bacterioruberin levels were determined by colony pigment analysis, as described previously (11). Heavy horizontal bars indicate the median value, and boxes demarcate the upper and lower quartiles. Whiskers extend to the smaller value of 1.5 times the interquartile range or to the most extreme value. Asterisks indicate a Bonferroni adjusted  $P$  value of  $<0.05$  ( $n \geq 6$ ).

(data not shown). Among the opsins that exhibited inhibitory activity, BO homologs from *N. pellirubrum*, *Halorubrum* sp. A07HR67, and *H. utahensis* all had abundances at 50% or greater relative to that of *H. salinarum* BO (Fig. 4). Intriguingly, for organisms with two BO homologs, abundance was inversely correlated to inhibitory activity; that is, *H. marismortui* Bop1 was much less abundant than *H. marismortui* Bop2. For *H. walsbyi*, the less active Bop1 was measured at 17% of *H. salinarum* BO abundance, but the *H. walsbyi* Bop2, highly active in inhibiting bacterioruberin, was not detected in our immunoblots (Fig. 4). Although data relating to the native physiological abundance of

#### FIG 2 Legend (Continued)

homologs from the indicated species. Colonies were prepared from cultures, grown for 4 days, and photographed as described in Materials and Methods. (B) Reverse-phase ultra-high-performance liquid chromatography (RP-UHPLC) traces of carotenoid extracts from *H. volcanii*  $\Delta$ lye, *H. volcanii* H1209 (parental strain), and strains expressing Lye homologs from the indicated species. Positions of bacterioruberin and lycopene (lyc) standards are noted. Traces were normalized for total carotenoid concentration, corrected for slight differences in retention time using an internal standard, and offset along the vertical axis for clarity. Traces are representative of at least 2 replicates for each strain, except for *Halorubrum* sp. A07HR67, where only 1 trace was available. Values indicate bacterioruberin as a molar percentage of total lycopene and bacterioruberin.



**FIG 4** Expression of opsins in *H. volcanii*. *H. volcanii* strains harboring expression plasmids for indicated opsins with C-terminal His<sub>6</sub> tags were grown in cultures using the same procedures as that for carotenoid analysis. Cell lysates were normalized by total protein concentration and separated by polyacrylamide gel electrophoresis. Blots were probed with anti-His<sub>6</sub> primary antibody. For standardization across multiple blots, cell lysates from *H. volcanii* expressing *H. salinarum* BO were included on all blots. Subsequently, each immunoblot image was adjusted (as an entire image) so that the BO bands from all blots had identical net (band subtracting background) mean gray values. The proportion indicated on the image was determined by dividing the net mean gray value of each opsin band into the net mean gray value of *H. salinarum* BO. Bands shown are from different blots, as indicated by black lines, and each is representative of at least 3 biological replicates. All full immunoblots are shown in Fig. S1 in the supplemental materials.

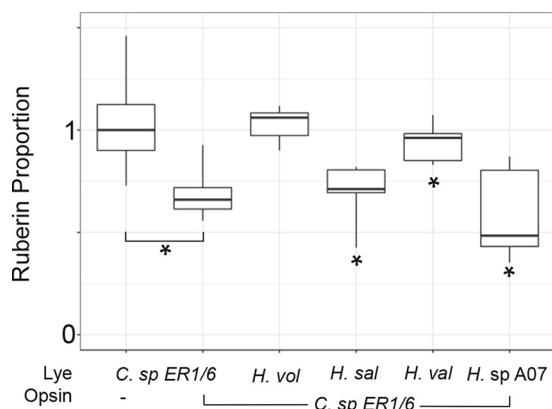
opsins and Lye in these species are not available, our results suggest that even a small amount of opsin can inhibit bacterioruberin production.

The expression of opsins from four species, *Haloterrigena mahlii*, *Halorubrum distributum*, *Halomicrobium mukohataei*, and *Natronomonas moolapensis*, did not result in a significant decrease in bacterioruberin synthesis (data not shown), but expression of all proteins was detected at various levels. Given that the expression of these opsins was generally reduced compared to that of opsins that exhibited inhibition of bacterioruberin synthesis, it may be that these proteins have structural differences when expressed in *H. volcanii* that prevent normal inhibition of Lye activity. Alternatively, this regulatory mechanism may not occur in these species.

Taken together, our results indicate that opsin-mediated inhibition of bacterioruberin production is not confined to clade 3 species and is likely common in *Halobacteria*. Overall, we have evidence that this mechanism occurs in 7 out of 11 total species tested. Opsin-mediated inhibition is nearly as likely to occur in clade 1 species, (1 of 2 tested) and clade 2 species (2 of 3 tested) as it is in clade 3 species (2 of 4 tested in this report and 4 out of 6 when including the previously reported species *H. salinarum* and *H. vallismortis*). Additionally, due to differences in environmental conditions, some of the opsin-Lye pairs tested by heterologous expression in *H. volcanii* are likely false negatives. From our results, we think the simplest explanation is that this regulatory mechanism existed in the last common ancestor of the *Halobacteria* and may have been present when the halophilic *Archaea* arose from an influx of bacterial genes into a methanogen.

**A bacterial opsin is capable of inhibiting bacterioruberin production.** To determine if opsin-mediated inhibition of Lye could occur in *Bacteria*, we conducted similarity (BLAST) searches of the UniProt microbial proteomes database to identify genomes in *Bacteria* with homologs to *H. salinarum* bacterioopsin and Lye. To focus our search on organisms that likely produced the retinal cofactor of opsin from a lycopene precursor, searches of the same database were conducted using enzymes that catalyze the conversion of lycopene to  $\beta$ -carotene, namely, *H. salinarum* lycopene cyclase (9) (UniProtKB accession number [Q9HNE5](#)), *Solanum lycopersicum* chloroplastic lycopene cyclase (22) (UniProtKB accession number [Q43503](#)), and *Synechococcus* sp. lycopene cyclase (23) (UniProtKB accession number [A5A545](#)). Similarity searches were then





**FIG 5** A bacterial opsin inhibits its own Lye and several archaeal Lyes. Box plot indicating proportionate bacterioruberin levels of *H. volcanii* strains expressing the indicated Lye and, if noted, the *Curtobacterium* opsin homolog. Relative bacterioruberin levels were determined by colony color analysis, and data were plotted as described for Fig. 3. Asterisks indicate a Bonferroni adjusted *P* value of  $<0.05$  compared to empty vector controls ( $n \geq 6$ ).

conducted with  $\beta$ -carotene dioxygenases from *H. salinarum* (10) (UniProtKB accession number [Q9HNE6](#)) and *Salinibacter ruber* (24) (UniProtKB [Q2S6A1](#)), which both catalyze the oxidative cleavage of  $\beta$ -carotene to retinal but are not homologous. From this analysis, 13 bacterial genomes were identified that encode homologs of bacterioopsin, enzymes that allow retinal synthesis from lycopene, and homologs of Lye. The opsin sequences were analyzed to identify those with the required residues to allow light-driven proton translocation. The region of the genomes containing the *lye* homologs was explored to identify genes potentially involved in carotenoid synthesis. Lastly, we conducted a literature search to determine if the identified species or close relatives produced a red or pink pigment that could indicate a modification of lycopene.

The best matches for potential opsin-mediated inhibition of Lye activity from this screen were genes from two species (Table 1), both in the *Actinobacteria* phylum, namely, *Rubrobacter xylanophilus*, a thermophilic freshwater organism (25), and *Curtobacterium* sp. ER1/6, a citrus tree endophyte (26). The *R. xylanophilus* opsin has demonstrated proton-pumping activity (27). The *Curtobacterium* sp. ER1/6 protein is not annotated as an opsin but includes a lysine residue at position 241 for likely retinal binding and acidic residues at positions 101 and 112 involved in proton translocation. Both *R. xylanophilus* and *Curtobacterium* sp. ER1/6 *lye* homolog open reading frames (ORFs) are upstream and overlap genes homologous to *Haloarcula japonica crtD*, a gene demonstrated to encode a carotenoid 3,4-desaturase, which catalyzes the processing of the first bacterioruberin intermediate to mature bacterioruberin (7). Interestingly, this potential operon mirrors that observed in *Halobacteria* genomes, except that the orientation is reversed, with the *crtD* homolog upstream of *lye* homologs in *Halobacteria*.

To examine potential opsin-mediated inhibition, we obtained genes encoding Lye and opsin homologs codon optimized for expression in halophilic *Archaea*. Incorporation of the *R. xylanophilus* or *Curtobacterium* sp. ER1/6 *lye* homolog into the *H. volcanii* *lye* locus resulted in partial recovery of bacterioruberin production (Fig. 2) despite the dramatically increased salinity compared to the environments in the native organisms. Expression of the *R. xylanophilus* opsin in the *H. volcanii* strain with the *R. xylanophilus* *lye* homolog resulted in no reduction in bacterioruberin production (data not shown). In contrast, expression of the *Curtobacterium* sp. ER1/6 opsin homolog resulted in a small, but significant, reduction in bacterioruberin synthesis catalyzed by the *Curtobacterium* sp. ER1/6 Lye homolog (Fig. 5). To examine potential cross-domain interactions, we tested the ability of the *Curtobacterium* sp. ER1/6 opsin to inhibit Lye from halophilic *Archaea*. While expression of *Curtobacterium* sp. ER1/6 opsin had no significant effect on *H. volcanii* Lye, it significantly reduced bacterioruberin production catalyzed by *H. salinarum*, *H. vallismortis*, and *Halorubrum* sp. A07HR67 Lye (Fig. 5). This result raises the

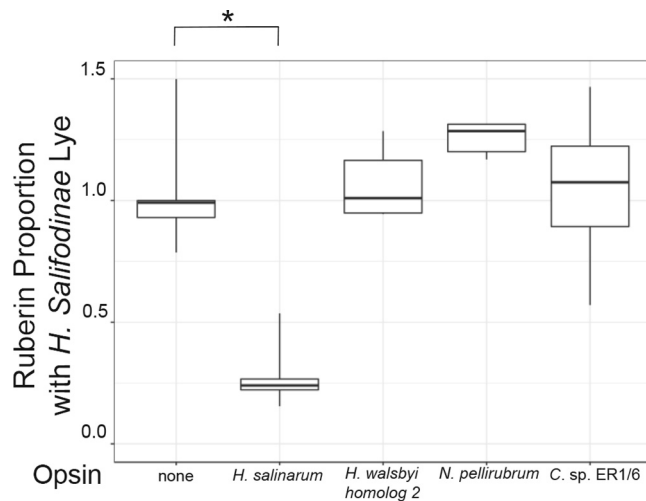
possibility that the opsin-Lye interaction existed in the ancestor that gave rise to both bacterial and archaeal proteins.

**A Lye from a deeply rooted branch of *Halobacteria* that natively lack opsins is capable of being inhibited by an opsin.** *Halococcus* is the only genus within the *Halococcaceae* family, and a multimarker phylogeny places the halococci as a deep-rooted branch outside the three major *Halobacteria* clades (18). There are seven *Halococcus* genome sequences available, and all of them encode enzymes that allow bacterioruberin production from GGPP (see Table S1 in the supplemental material). None of the available genomes encode detectable homologs to opsins or retinal biosynthetic enzymes. The *Halococcus* common ancestor may have lost genes for rhodopsins and retinal biosynthesis before radiation. An alternative explanation is that opsin and retinal biosynthetic genes were acquired by other *Halobacteria* species after the *Halococcus* lineage diverged. We chose to study the Lye of *Halococcus salifodinae*, an organism cultured from halite deposited in the Permian period (16), suggesting that the species has been isolated for approximately 250 million years. A gene encoding *H. salifodinae* Lye was integrated into the *H. volcanii* lye locus, and bacterioruberin synthesis was restored to 90% of wild-type levels (Fig. 2B). Expression of BO from *H. salinarum*, a clade 3 species, sharply reduced bacterioruberin synthesis by 74%. To determine if *H. salifodinae* Lye could be inhibited by other opsins, we tested active opsins from *Halobacteria* clade 1, *N. pellirubrum* bacterioopsin, and from clade 2, *H. walsbyi* bacterioopsin 2. Neither opsin significantly inhibited *H. salifodinae* Lye. In addition, we tested the bacterial opsin from *Curtobacterium* sp. ER1/6, and no inhibition was observed. Therefore, a halobacterial Lye that has not been exposed to selective pressure from opsin expression for a long period may retain features that allow specific opsin-mediated inhibition.

**Conclusion.** We previously demonstrated that opsin-mediated inhibition of bacterioruberin biosynthesis occurs in two relatively closely related species, *H. salinarum* and *H. vallismortis*, from clade 3 of the *Halobacteria*. In this work, we demonstrate that opsins throughout the *Halobacteria* have the ability to inhibit the activity of their Lye enzymes. We also demonstrate that a bacterial opsin inhibits its Lye and those of at least three *Archaea* species. In addition, a Lye homolog from an early diverged species outside the 3 major clades, *H. salifodinae*, can be inhibited by a modern opsin. Together, our results demonstrate that opsin-mediated inhibition of Lye is widely conserved in *Halobacteria* species and may occur between similar proteins in *Bacteria* species.

Halophilic *Archaea* likely arose from a methanogenic species (12, 13), and this emergence is correlated to lateral acquisition of many bacterial genes (14, 15). One explanation for the widespread distribution of opsin-mediated inhibition of Lye in *Halobacteria* is that this regulatory mechanism existed when both opsin and Lye were acquired together in the common ancestor of *Halobacteria*. A recent paper explored the genomes of two members of *Methanonatronarchaea*, newly isolated halophilic methanogens that are proposed to form a class most closely related to *Halobacteria* (28). This study suggests that both opsins and Lye were acquired by the common ancestor of *Halobacteria*. In this scenario, some lineages of *Halobacteria* lost genes encoding opsins, while, as we found in our analysis, *lye* and other genes allowing bacterioruberin synthesis were nearly universally maintained. This interpretation is supported by the finding that the Lye from *H. salifodinae*, a species from a genus that lacks any known opsins, retains the ability to be inhibited by opsin (Fig. 6). We also have evidence that this mechanism is found in *Bacteria*, as we observed that expression of the *Curtobacterium* sp. ER1/6 opsin inhibits its Lye activity. However, we have determined that not all Lyes are inhibited by all or any opsins, a phenomenon that indicates that other selective pressures on the genes may have resulted in the loss of this inhibitory interaction.

An alternative hypothesis is that this inhibition arose after radiation of the *Halobacteria* and spread by lateral gene transfer. To examine the possibility that genes encoding opsin or Lye exist on horizontally transferred regions, we examined predicted genomic islands (29) for the species listed in Table 1. Although many genomic islands were predicted, none of the archaeal Lye or opsin homologs used in our study were in



**FIG 6** Lye from a *Halobacteria* species that lacks opsins is inhibited by *H. salinarum* BO. Box plot indicating proportionate bacterioruberin levels of *H. volcanii* strains expressing *H. salifodinae* Lye and the indicated opsin homologs. Relative bacterioruberin levels were determined by colony color analysis, and data were plotted as described for Fig. 3. The asterisk indicates a Bonferroni adjusted *P* value of  $<0.05$  compared to empty vector controls ( $n \geq 6$ ).

these regions. In the bacterial genes used in our study, only the *R. xylanophilus* opsin, which notably did not show inhibition activity, was predicted to be in the region of the genome inherited by lateral transfer. We also think that inheritance by lateral transfer of smaller genome regions is unlikely, as the *lye* and *bop* homologs are not closely associated in any genome we examined. Our data also do not exclude the possibility that this mechanism is so favorable that it arose independently in multiple lineages. We plan to continue exploring required structural motifs in both opsin and Lye proteins to provide additional insight into the evolution of this regulatory interaction.

## MATERIALS AND METHODS

**Genomic analysis.** As of August 2018, 348 genomes were classified as *Halobacteria* in the GenBank database (30). For analysis, we eliminated genomes assembled to more than 200 contigs or scaffolds and chose one genome from each available species. If genomes from multiple strains in a species were available, we chose the type strain genome if available but otherwise chose the genome sequence with the most complete assembly. BLAST (31) databases for each genome were constructed from FASTA amino acid (.faa) files for proteins and FASTA nucleotide files (.fna) for nucleotides, using makeblastdb version 2.6.0 with default options. BLASTP (version 2.6.0) searches to determine the presence of proteins in each genome were conducted using default options and an E value cutoff of  $1 \times 10^{-10}$ . To check for missed annotations, genomes were subjected to TBLASTN (version 2.6.0) searches with default options of nucleotide sequences with an E value cutoff of  $1 \times 10^{-10}$ . Since our initial analyses revealed that many incompletely assembled genomes were likely missing significant sections of DNA sequence, we assessed genome completeness by performing BLAST and TBLASTN searches for essential, highly conserved genes, as reported in reference 32. Out of the 93 genes identified as highly conserved in both *Archaea* and *Eukarya* (see Tables S2 and S3 in reference 32), we chose a subset of 10 genes that represented a variety of cellular processes. The *H. salinarum* homolog of the following genes were used for these searches (protein name [NCBI gi number, UniProtKB accession number for *H. salinarum* homolog]): aspartate-tRNA ligase (6323011, [O07683](#)), RIO-type serine/threonine protein kinase Rio1 (6324693, [B0R7D7](#)), RNase L inhibitor (6320296, [Q9HMC1](#)), RNA polymerase subunit A' (6324690, [P0CX02](#)), ribosomal protein S3 (6324151, [P15009](#)), rRNA small subunit methyltransferase A (6324989, [Q9HQH1](#)), guanine nucleotide exchange factor SDO1 (6323050, [Q9HQ87](#)), protein translocase subunit SecY (6323411, [Q9HPB1](#)), signal recognition particle 54-kDa protein (6325345, [Q9HMN5](#)), and TATA box-binding protein E (6320996, [Q9HN56](#)). Notably, all 43 genomes labeled with an assembly level of "complete" harbored homologs for all 10 of these genes, indicating that genomes with assembly levels of "contig" or "scaffold" may be missing substantial portions of the actual genome sequence.

After eliminating genomes lacking one or more of these essential genes, we assessed the ability to produce carotenoids and opsins by conducting BLAST searches using the following genes: to assess the ability to generate lycopene from GGPP, *H. salinarum* phytoene synthase 2 (UniProtKB accession number [Q9HPE1](#)) and *H. salinarum* phytoene dehydrogenase 1 ([Q9HPD8](#)); to identify species with the capability to synthesize bacterioruberin, *H. salinarum* Lye ([Q9HPD9](#)); and to determine the ability to synthesize retinal, *H. salinarum* lycopene  $\beta$ -cyclase ([Q9HNE5](#)) and  $\beta$ -carotene dioxygenase ([Q9HNE6](#)). Genes encod-

ing opsins were identified using *H. salinarum* bacterioopsin (P02945), haloopsin (P0DMH7), sensory opsin-1 (P0DMH8), and sensory opsin-2 (P71411).

***H. volcanii* strain construction.** *H. volcanii* strains are listed in Table S2 in the supplemental material. Synthetic gene sequences and details of plasmid construction are given in Tables S3 and S4, respectively, in the supplemental material. Synthetic genes encoding Lyes and opsins were designed using codon optimization for *H. marismortui*, using the IDT DNA Codon Optimization Tool (<https://www.idtdna.com/CodonOpt>), and ordered from GenScript (Piscataway, NJ). Plasmids all harbored an ampicillin resistance marker and were propagated in *Escherichia coli* DH5 $\alpha$  cells (New England Biolabs, Ipswich, MA) in LB media with 100  $\mu$ g/ml ampicillin (Gold Biotechnology, St. Louis, MO). For integration into the *H. volcanii* *lye* locus, synthetic genes encoding Lye were amplified with appropriate primers (Table S5 in the supplemental material) and ligated into pRFP128 (11), an integrative plasmid derived from pTA131 (33), using NdeI and BglII sites.

For expression of opsins, codon-optimized synthetic genes (Table S3) were inserted into pTA963 (34), using NdeI and EcoRI sites, to allow expression from the *p.tnaA* promoter. As in our previous studies (11), we relied on the moderate level of expression of this promoter when *H. volcanii* strains with pTNA963-based plasmids are grown in *H. volcanii*-yeast extract-peptone-Casamino Acids (Hv-YPC) medium (35). Genes encoding opsins with C-terminal His<sub>6</sub> tags were amplified with primer RP268 (anneals to pTA963-based plasmids) and appropriate primers (Table S5) to encode His<sub>6</sub>, using the previously constructed opsin expression plasmids as the template. These PCR products were inserted into pTA963 using NdeI and EcoRI sites. All plasmid modifications were confirmed by sequencing. Genetic modification of *H. volcanii* was carried out in derivatives of strain H1209, as previously described (34). Proper integration of *lye* homologs into the *lye* locus was confirmed by PCRs that included one primer from the *lye* homolog and one primer, RP558 (Table S5), with a binding site on the chromosome but not in the integrative plasmid. The Lye-encoding region, and plasmid inserts in all *H. volcanii* strains, were sequenced to confirm accurate genotypes.

**Carotenoid analysis.** For carotenoid analysis from liquid cultures, *H. volcanii* cultures were grown in Hv-YPC medium (33) supplemented with 40  $\mu$ g/ml thymidine. The carotenoids were extracted and analyzed by reverse-phase ultra-high-performance liquid chromatography, as previously described (11).

For calculation of bacterioruberin as a proportion of total bacterioruberin and lycopene, colonies were grown on Hv-YPC solid medium supplemented with thymidine (40  $\mu$ g/ml) and supplemented with retinal (50  $\mu$ M) where noted. Images were obtained, and a Ruberin metric was calculated as described previously (11). Briefly, colonies were grown from cultures normalized by turbidity for 4 days and photographed with a Moticam 5.0-megapixel (MP) camera (Motic, Hong Kong, China) mounted on a dissection microscope (VistaVision; VWR, Radnor, PA). Color quantification was obtained by sampling approximately 15% of the total colony area with the Adobe Photoshop CS6 (Adobe, San Jose, CA) eyedropper tool to determine values for hue and saturation. These values were normalized to those of *H. volcanii* H1209 colonies grown under identical conditions on the same plates. The ruberin metric was calculated using the inverse-logit function previously determined to accurately predict bacterioruberin content, as follows:

$$R' = 8.0061 - (5.8748 \times \text{normalized hue}) + (2.3428 \times \text{normalized saturation})$$

$$\text{ruberin metric} = e^{R'} / (1 + e^{R'})$$

Since Lye enzymes from different organisms had various levels of activity when expressed in *H. volcanii*, ruberin proportions were calculated for each *H. volcanii* strain by dividing the ruberin metric for strains expressing an opsin by the ruberin metric from the corresponding control strain with the empty vector. For statistical comparisons between strains, we used the permutation test as implemented by the R package perm (36).

**Quantification of opsin abundance in *H. volcanii*.** Cultures of *H. volcanii* strains harboring expression plasmids for opsins with C-terminal His<sub>6</sub> tags were grown, cell lysates prepared, and total protein quantified as described previously (11). Cell lysates were diluted to 2 mg of protein per ml using Laemmli sample buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol (355 mM) as a reducing agent. Protein samples (30  $\mu$ l) were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Opsin levels were quantified by immunoblotting with THE His tag mouse antibody (GenScript) diluted to a concentration of 0.2  $\mu$ g/ml. The blot was subsequently probed using the One-Hour Western advanced kit (GenScript) according to the manufacturer's protocol. Chemiluminescence for visualization was developed using LumiSensor horseradish peroxidase substrate (GenScript) following the manufacturer's instructions, and images were obtained with an Azure c600 imaging system (Azure Biosystems, Dublin, CA). Bands were quantified by densitometry using the "mean gray value" determination in Photoshop CS6 (Adobe).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00576-18>.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.05 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.04 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.04 MB.

**SUPPLEMENTAL FILE 4**, XLSX file, 0.03 MB.

**SUPPLEMENTAL FILE 5**, XLSX file, 0.03 MB.

**SUPPLEMENTAL FILE 6**, PDF file, 0.9 MB.

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R.F.P. designed the research, directed the biological interpretation of the data, and wrote the majority of the manuscript. S.M.G. assisted in the experimental design, performed much of the strain construction, and directed data collection and analysis. A.M.G. performed strain construction and data collection and assisted in biological interpretation. All authors read and approved of the paper.

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