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ARTICLE

Light stimulates anoxic and oligotrophic growth of glacial *Flavobacterium* strains that produce zeaxanthin

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Abstract

Bacteria that inhabit glaciers usually produce carotenoids. Here, we report that a group of zeaxanthin-producing glacial *Flavobacterium* exhibited light-promoted growth. Of the tested 47 strains, 45 showed increased growths but two died under illumination at 50 μmol photon m^{-2} s^{-1}. Light stimulation occurred mainly in either anoxic or nutrient-poor cultures, while the same levels of light promotion were found for that grown at 14 and 7 °C. Pigment assays identified overrepresentative zeaxanthin but trace retinal in the light promoted 45 strains, while flexirubin was exclusively in the light-lethal two. Genomic analysis revealed the gene cluster for zeaxanthin synthesis in the 45 strains, in which 37 strains also harbored the proteorhodopsin gene *prd*. Transcriptomic analysis found that light-induced expressions of both the zeaxanthin synthesis and proteorhodopsin genes. Whereas, deletion of the *prd* gene in one strain did not diminish light promotion, inhibition of zeaxanthin synthesis did. In comparison, no light promotion was determined in a glacier *Cryobacterium luteum* that produced a non-zeaxanthin-type carotenoid. Therefore, light stimulation on the glacial *Flavobacterium* is mostly likely related to zeaxanthin, which could provide better photoprotection and sustain membrane integrity for the organisms living in cold environments.

Introduction

Glaciers represent extreme cold environments on Earth; however, culture-dependent and -independent investigations indicate that abundant psychrophilic and probably oligotrophic bacteria have inhabited in glaciers [1–7]. Phylogenetically the predominant bacteria in glaciers resembles that of other cold environments, primarily including those affiliated with the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, in which *Arthrobacter* [8], *Hymenobacter* [4], *Deinococcus* [4], *Cryobacterium* [9], *Flavobacterium* [10], *Polaromonas*, and *Sphingomonas* [11, 12] are most represented.

Bacteria that inhabit in cold regions, such as marine surface waters [13], high-altitude soils [14], glaciers [9], and Antarctic [15] are usually pigmented. Shen et al. [16] reported that the yellow-pigmented strains, comprising 47% of the total cultured ones, were dominantly distributed

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throughout a 120 m length of ice core drilled from Yuzhufeng glacier at the Tibetan Plateau. The pigments consisted of multiple types of carotenoids including α- and β-carotenes, diatoxanthin, peridinin, zeaxanthin, lutein, butanoyloxyfucoxanthin, and fucoxanthin, with α-carotene as the most abundant. Klassen and Foght [17] reported the distinctive carotenoid derivatives in the glacier representative bacterium *Hymenobacter*, which include previously unreported presumptive hexosyl, pentosyl, and methyl derivatives of carotenoids. As no convergent phylogenetic pattern was found between the carotenoid derivative distribution and the species 16S rRNA, the carotenoid compositions were assumed to be strain- or eco-variant-specific.

Carotenoids display an array of fundamental functions across diverse organisms [18]. They are well known as an antioxidant, especially in the plants photosynthetic systems they play dual roles as the light-harvesting auxiliary compounds as well as the photoprotective molecules to quench light-generated singlet oxygen species [19]. Therefore, some herbicides are designed to inhibit synthesis of carotenoids [20]. Carotenoids also benefit human health, such as β-carotene, which is referred to as provitamin A, exerting protective effects against diseases via their antioxidant roles [21]. As antioxidants, carotenoids can be particularly important for psychrophiles as more reactive oxygen species (ROS) are generated in cold environments [19, 21, 22]. The photoprotective function of carotenoids, which act as screens against intensive light and UV radiation, highlights their essentiality to the glacial bacteria at high altitudes. Earlier studies also indicated that the carotenoids with polarity, like zeaxanthin and lutein, are usually produced in psychrophiles [23, 24]. This suggests that they could play a role in enhancing the rigidity and homeoviscous adaptation of the psychrophile membranes that are usually in rich of unsaturated fatty acids [24].

Previously, via high-throughput sequencing of community 16S rRNA genes, we found the Family *Flavobacteriaceae* represented the second most abundant bacterial taxa in the supraglacial layers of six Chinese glaciers at the Tibetan Plateau [7]. We also isolated numerous psychrophilic *Flavobacterium* strains from the glaciers [25, 26]. Like other psychrophiles, the glacial *Flavobacterium* also featured non-diffusible yellowish or orange pigments. To detect the pigments' contribution to *Flavobacterium* adaptation to extreme cold and intensive light radiation, we surprisingly found that light promoted the growth of most strains. Genetically, flavobacteria do not encode light-energy harvesting systems like chlorophyll in cyanobacteria and bacterial chlorophyll usually synthesized by photoheterotrophic bacteria. Whereas the phylogenetic relatives of *Flavobacterium*, the non-phototropic marine Bacteroidetes may carry the *prd* gene that encodes proteorhodopsin (PR) [27], a type of bacterial rhodopsin believed as the second light-energy harvesting system after chlorophyll [28]. Two representative *prd*-carrying strains, *Dokdonia* MED134 and *Nonlabens marinus* S1-08, have exhibited light-promoted growth when the bacteria grow in oligotrophic or salinity cultures [29, 30], respectively, that is putatively attributed to the PR proton pump. It is worth noting that PR uses retinal, the cleavage product of *β*-carotene, as the light-harvesting chromophore to convert light energy to a proton motive force [28].

In this study, through integration of pigment identification, genomic and transcriptomic analysis, and genetic and physiological assays on 47 *Flavobacterium* strains isolated from four Chinese glaciers, we found that light illumination promoted the growth of most strains in either of lower oxygen or nutrient deficiency; and the promotion was related to zeaxanthin synthesis. PR did not appear to play an important role in light promotion in the glacial strains, this could be due to the trace retinal synthesis. While, zeaxanthin, a type carotenoid with molecular polarity, appears to be involved in the light stimulation on the glacial *Flavobacterium*, most likely through elevating nutrient metabolism efficiency, yet the mechanisms remain to be elucidated.

Methods

Isolation of glacial bacteria strains

Bacteria were isolated from the supraglacial layers of four Chinese glaciers (Fig. 1). Surface ice at ~0–20 cm depth of the glacier tongues was sampled in September, 2009 as described by Liu et al. [7], and transported in an insulated container filling with dry ice and then stored at −80 °C. Two months before isolation, the ice samples were incubated at 4 °C to revive the bacterial populations, and bacteria were isolated as previously described [26]. Briefly, melted ice samples were diluted into sterile 0.9% (w/v) NaCl and then plated on peptone-yeast extract-glucose (PYG) agar (W/V; bacto peptone (Difco) 0.5%, yeast extract 0.02%, glucose 0.5%, beef extract 0.3%, NaCl 0.05%, $MgSO_4.7H_2O$ 0.15%, and 1.5% agar, pH 7.0). Plates were incubated aerobically at 14 °C in the dark, and single colonies were picked up after 8 weeks of incubation. Purification of the isolates was examined based on homogeneities of the cell morphology and colony shapes. Strains used in this study have been deposited in China General Microorganism Culture Collection Center and listed in Supplementary Table 1.

Strain identification based on 16S rRNA gene sequencing

Genomic DNAs were extracted from the glacial strains using Genomic DNA Rapid Isolation Kit for bacterial cells

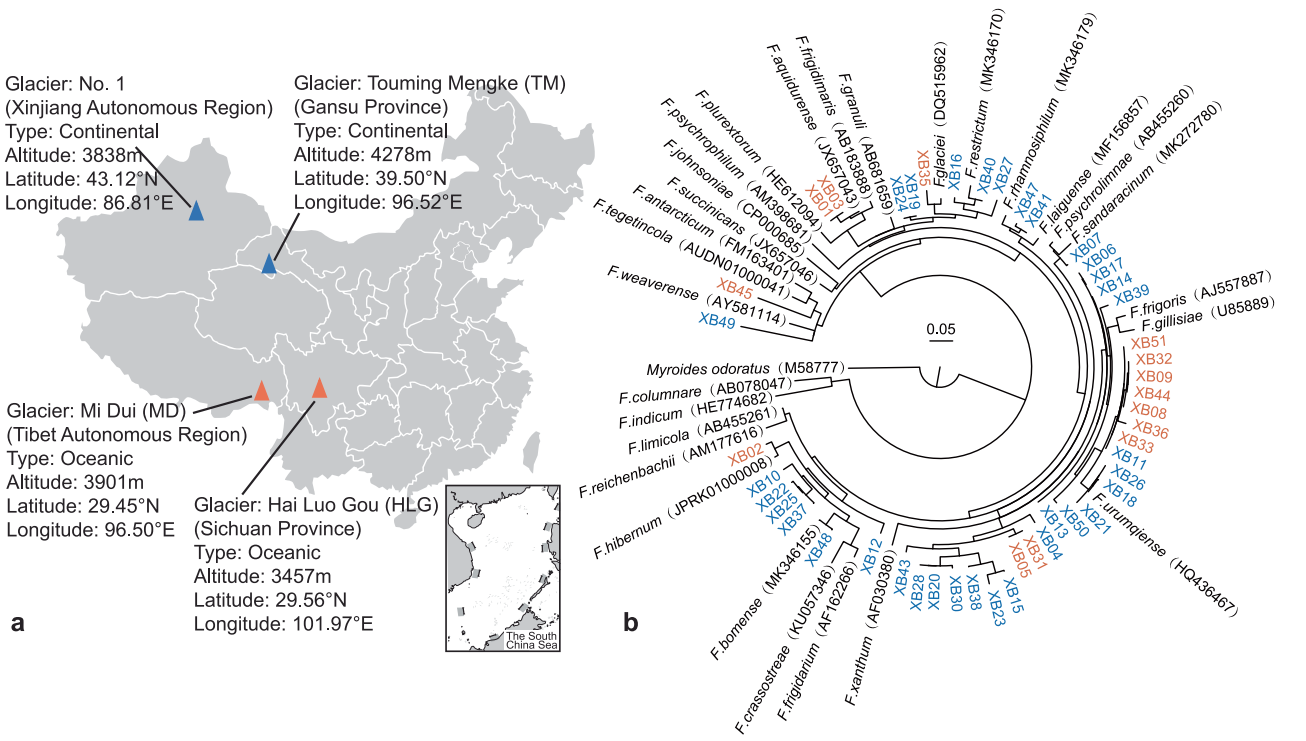


Fig. 1 Locations from where the glacial *Flavobacterium* strains were isolated and a phylogenetic analysis of their genus-level relationships. a A map showing the four glaciers where the psychrophilic *Flavobacterium* strains were isolated from, with additional geographical information for each glacier. **b** A neighbor-joining phylogenetic tree of 16S rRNA genes constructed using PHYLIP and with node support evaluated by 1000 bootstrap replicates. Branch lines and strain numbers colored in orange and blue indicate the strains isolated from the southern and northern glaciers, respectively, while those in black are reference taxa. Inside the parenthesis are the 16S rRNA gene accession numbers of the reference strains, while those of the glacial strains are provided in Supplementary Table 1.

(BioDev-Tech, China) by following the manufacturer's instructions. The universal bacterial PCR primers 27f and 1492r were used to amplify the 16S rRNA genes. Sequencing of the 16S rRNA gene amplicons was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on an automated DNA analyzer system (PRISM 3730XL DNA analyzer, Applied Biosystems) at SinoGenoMax Corporation (Beijing, China). The 16S rRNA gene sequences were deposited in the GenBank database and compared against those of the recognized type strains using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/) [31]. The accession numbers for the 16S rRNA genes are listed in Supplementary Table 1.

Light and dark growth experiments

The glacial strains were routinely cultivated statically in 5 ml of PYG broth within 12 × 140 mm culture tubes and incubated at 14 °C in a Saifu incubator (Ningbo Saifu Experimental Instrument Co., Ltd, Zhejiang, China) outfitted with LUMILUX daylight lamps (OSRAM, L36W/865). Oligotrophic growth was cultured in a mineral solution (0.2% $(NH_4)_2SO_4$, 0.05% $NaH_2PO_4{\cdot}H_2O$, 0.05% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, and 0.01% $CaCl_2{\cdot}2H_2O$) containing 0.4 % (w/v) of glucose. Aerobic and anoxic cultures were built in 100-ml flask filled with 10- and 100-ml medium, respectively, and the dissolved oxygen levels were measured with an OXY Meter (Unisense, Denmark). Illuminated cultures were constantly exposed to light fluxes of 10 and 50 µmol photons m^{-2} s^{-1}, respectively, which are determined with Quantum Light Meters (3415FXSE, Spectrum Technologies, Inc, USA). Dark cultures were wrapped by aluminum foil. Culture optical densities at wavelength of 600 nm (OD_{600}) were recorded in a 2-day interval to monitor growth rate. All experiments were carried out in triplicate. To probe the carotenoid-based light stimulation, 100 µM (final concentration) 2-(4-methylphenoxy) triethylamine hydrochloride (MPTA) [32, 33], the lycopene cyclization inhibitor, were added to inhibit zeaxanthin synthesis in the light-dark experiments.

Pigment identification

Cells in the late exponential phase were collected by centrifugation at 12,000 × *g*. An aliquot of cell pellet was first exposed to 20% KOH for identification of flexirubin [34] and the other aliquot was extracted with acetone. Pigments in the acetone extract were subjected to whole wavelength spectral

scanning using a Lambda 35 UV/VIS spectrometer (PerkinElmer, Inc, USA) for preliminary identification of the pigments. The light absorbance spectra of a variety of carotenoids and flexirubin were used as references [35]. Pigments were further identified by HPLC (P1201, Dalian Elite Analytical Instruments Co., Ltd., China) equipped with a YMC-Pack ODS-A column (150 × 4.6 mm) using an elution of acetonitrile:dichloromethane (2:1) at a flow rate of 1 mL/min, followed by detection of UV absorbance at wavelength of 450 nm, except of retinal determination at 380 nm, as described by Sabehi et al. [36]. Pure chemicals of zeaxanthin, lycopene, and retinal (Aladdin®, China) were used as references.

Phylogenetic analyses

A total of 29 16S rRNA sequences from 28 *Flavobacterium* species and *Myroides odoratus* were retrieved from GenBank as references for phylogenetic analysis. Overall, 47 newly sequenced 16S rRNA genes of the glacial strains and the 29 sequences were used to generate a dataset. Sequence alignment and editing were performed using the CLC Main Workbench (CLC Bio, Aarhus, Denmark). A phylogenetic tree, using the 16S rRNA sequence of *Myroides odoratus* as the outgroup, was reconstructed using neighbor-joining methods and the support for nodes were evaluated with 1000 bootstrap replicates using the PHYLIP version 3.6 program.

A phylogenetic tree for rhodopsin genes was also constructed using the same methods as described. The dataset included 37 rhodopsin sequences from the glacial *Flavobacterium* strains in this study and 12 other rhodopsin sequences that were retrieved from GenBank.

Genome sequencing, assembly, and annotation

Genomic DNA was extracted and purified using the Genomic DNA Rapid Isolation Kit for bacterial cells (BioDev-Tech, China), as described above. All genomes were sequenced using a whole-genome shotgun strategy on the Illumina Genome Analyzer IIx platform (Illumina, USA). The DNA libraries were constructed using the Illumina paired-end (PE) sequencing method after genomic DNA was fragmented by sonication using a Bioruptor (Bioruptor NGS; Diagenode, Liège, Belgium). DNA libraries (15–30 ng/μl) were then prepared by ligating specific Illumina adapters designed for PE sequencing, selecting the ligated DNA fragments of ~300–500 bp with Agencount AMPure XP Beads (Beckman Coulter, Beverly, USA), and enriching the ligated DNA by PCR amplification. The quantity and quality of the DNA libraries were determined by real-time PCR (ABI 7500) and an Agilent 2100 Bioanalyzer (Agilent Technologies). The PE module was then used to sequence the quality-filtered libraries on the GA IIx platform. To generate PE reads, distinct Illumina adapters were ligated to each end of the fragments with PCR primers that allowed the assignment of each end as separate runs. Reads for each of the indexed samples were then separated using a custom Perl script. Imaging analysis and base calling were conducted using the Illumina GA Pipeline software package.

Low-quality reads were filtered from the sequence dataset using the DynamicTrim and LengthSort Perl scripts within the SolexaQA program. The remaining short reads were then assembled using SOAPdenovo (http://soap.genomics.org.cn), which is a genome assembler specifically developed for assembling next-generation short-read sequences. Genomic gaps were then filled using the SOAP GapCloser after assembly. The complete genomes of strains XB07 and XB36 were sequenced using the Mgiseq2000 PE150 and Nanopore-PromethION platform, and assembled using the SPAdes program [37]. Protein-coding genes were predicted using Glimmer 3.02, while tRNAscan-SE and RNAmmer were used to identify tRNA and rRNA genes, respectively. The genome sequences were uploaded to the Rapid Annotation using Subsystem Technology (RAST) platform to evaluate annotated sequences.

RNA sequencing, read mapping, and quantification

Each three *prd*-containing strains (PR+), XB07, XB26, and XB30 and *prd*-lacking strains (PR−), XB05, XB36, and XB43, were chosen for transcriptomic sequencing. Mid-exponential cells were harvested from 5 mL of light and dark cultures in triplicate by centrifugation at 12,000 × *g* for 5 min at 4 °C. Total RNA was then extracted using the Qiagen RNeasy Mini Kit (Qiagen, Melbourne, VIC, Australia) according to the manufacturer's instructions. RNA quality and quantity were determined on 1% agarose gel, and one unit of RNase-free DNase I and 50 units of RNase Inhibitor (Invitrogen) were then added to 2 μg RNA preparation to digest the residual chromosomal DNA.

The RNA preparation (1.5 μg) was used as input for rRNA removal using the Ribominus Transcriptome Isolation Kit for bacteria (Invitrogen Life Technologies, Carlsbad, CA, USA). The KAPA Stranded RNA-Seq Library Preparation Kit for the Illumina Platform (KAPA, USA) was used to generate the sequencing libraries following the manufacturer's recommendations. Index adapters were then added to multiplex sequence the samples. Agencount AMPure XP Beads (Beckman Coulter, Beverly, USA) were used to purify cDNA fragments and the libraries. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform, generating PE reads.

The RNA-seq reads were first aligned to the rRNA sequences from each strain with Bowtie v2.2.4 [38], using the assembled genomes as references. The remaining unaligned reads at the last step were mapped to the whole

genome sequences. Alignment results were then subjected to gene quantification using Cufflinks v2.0.2 [39] using the default parameter settings. Fragments per kilobase of transcript per Million fragments mapped values were calculated based on the length of the fragments and the read counts mapped to fragments.

Gene expression profiles were compared for each strain that was grown under light vs. dark. Genes were annotated against the NR database and only homologous genes among all six strains were considered for further analysis. Genes that were differentially expressed by >1.4-fold change in at least two strains in the PR+ or PR− groups were chosen and further filtered to only include those genes with average fold change values >1.6 among the six strains. A gene expression heatmap was then constructed using the R pheatmap package.

PCR and quantitative RT-PCR

Total RNA was used as template for reverse transcription to generate cDNA using the GoldScript cDNA synthesis kit (Invitrogen). Quantitative PCR (qPCR) experiments were carried out in a Mastercycler ep realplex2 machine (Eppendorf, Hamburg, Germany) using Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) as the reporter dye. Tenfold serial dilutions of pGM-T Vector containing the target gene were quantified using the NanoDrop ND-1000 spectrophotometer. The fragments were also tenfold serially diluted (10^{-1} to 10^{-8}) in triplicate to generate a standard curve for quantification. qPCR primers (Supplementary Table 2) were designed using OligoArchitect (www.oligoarchitect.com). The 16S rRNA gene copies of the corresponding strain were used as a copy number reference that the gene transcripts were normalized against. The qPCR cycling program was as follows: 1 cycle of 95 °C for 30 s and then 40 cycles of 95 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s.

Construction of the *prd* gene deletion mutant

The *prd* gene deletion mutant was constructed in strain XB07 by conjugation as described by Li et al. [40]. The resultant mutant was sensitive to tetracycline. Strain *E. coli* S17-1 λpir and the plasmid pMS75 were kindly provided by Prof. Pin Nie at the Institute of Hydrobiology at Chinese Academy of Sciences. The genomic DNA of strain XB07 was used as template and a 2.1 kbp fragment comprising 56 bp of the 5′ proximal end of the *prd* gene (XB07GL000621) and the 2073 bp upstream sequence were amplified with the primers PRup-SalF (embedding the SalI site) and PRup-SphR (embedding the SphI site) using *Pfu* polymerase (TIANGEN, Beijing, China). A 2.2 kbp fragment comprising 60 bp 3′ proximal end of *prd* and 2115 bp of downstream sequence were also amplified using the primers PRdn-KpnF (introducing a KpnI site) and PRdn-SalR (introducing a SalI site) listed in Supplementary Table 2. The purified PCR product, PRup, was first cloned into the pMS75 plasmid, which carried the tetracycline resistance gene TetQ, to ultimately generate the pMS75-PRup plasmid. The PCR product PRdn was cloned into the pMS75-PRup plasmid to generate the pMS75-PRup-RPdown plasmid that was then introduced into *E. coli* S17-1 λpir. *E. coli* S17-1 λpir-pMS75-PRup-RPdown was cultured overnight in 20 ml of LB broth containing 100 mg/ml ampicillin at 37 °C, while strain XB07 was cultured in 20 ml PYG at 14 °C for 24 h. *E. coli* S17-1 λpir and XB07 cells were collected by centrifugation, washed twice with PYG, and then mixed with 100 µl of PYG liquid. The cell suspension mixture was then spotted on nitrocellulose membranes overlying PYG agar and incubated for 5 days at 14 °C to allow plasmid transfer by conjugation. The conjugation product was then spread on R2A plates containing 15 µg/ml of tetracycline and incubated at 14 °C for another 5 days. The tetracycline-resistant clones were then inoculated into 4 ml of antibiotic-free PYG liquid and shaken at 14 °C for 24 h. Cultures (100 µl) were then spread on R2A agar supplemented with 10% sucrose. Ten colonies were selected and inoculated onto R2A agar with or without tetracycline, and those that only grew on R2A agar without tetracycline were selected for further verification. The mutants were confirmed by PCR amplification using the PR_delF and PR_delR primers (Supplementary Table 2), yielding a 1.2 kbp product from wild-type cells and a 0.6 kbp product from the deletion mutant cells. The mutant was also verified via the *prd* gene fragment sequencing and the absence of *prd* transcript through quantitative RT-PCR.

Results

The *Flavobacterium* strains isolated from glaciers are psychrophiles

To better understand the contributions of the pigments produced by glacial bacteria to their adaptations to extreme cold and high UV radiation, 47 glacial *Flavobacterium* strains (Supplementary Table 1) were selected for investigation. These strains were isolated from the upper 20-cm ice layers of four Chinese glaciers, with 14 and 33 strains from southern and northern glaciers of China, respectively (Fig. 1a). Most of the strains (except two) grew between 0 and 20 °C and optimally at 14 °C, in PYG broth (Supplementary Table 3) having growth rates of 0.01–0.28 µ/day. Therefore, these glacial *Flavobacterium* strains were assigned as psychrophiles according to the psychrophiles definition of optimal growth below 15 °C [41].

Phylogenetic analysis based on the 16S rRNA gene homology indicated that the glacial *Flavobacterium* strains

and those from polar regions were primarily clustered, but distantly related to the mesophilic species like *F. johnsoniae* and *F. indicum* as well as the fish pathogens like *F. columnare* and *F. psychrophilum* (Fig. 1b). This implies that the glacial strains could contain distinct biological and ecological features.

Most glacial *Flavobacterium* strains exhibit better growth with light exposure

To investigate the effects of light radiation on the glacial *Flavobacterium* growth, a light-dark growth experiment was performed for the 47 glacial strains. Both the light-exposed and aluminum foil-wrapped culture (5 ml) in test tubes were incubated statically at 14 °C in an incubator equipped with a color 865 LUMILUX light, which provides cold white light source covering the entire light spectrum but overrepresented by green light at 520–620 nm. Under the illumination of 50 µmol photon $m^2 s^{-1}$, growth was monitored by measuring OD600 until 12 days, and growth rates of both illuminated and dark cultures were calculated for each strain. Surprisingly, of the tested strains, 45 strains displayed better growth in light-exposed cultures (Fig. 2), exhibiting 1.2–4-fold higher growth rates and 1.1–2.1-fold higher final biomass in light cultures than the corresponding dark cultures. Whereas, light appeared to be lethal to strains XB41 and XB47, the two could only grow in the dark, and a parallel tested *Cryobacterium luteum*, which was isolated from the same glacier, did not show light-stimulated growth (Supplementary Table 3). To preclude a possibility that light-induced growth may be derived from photolysis of the culture nutrients, two strains that exhibited light-stimulated growth were cultured in preilluminated PYG broth for light and dark experiments. However, higher growth was still observed for light-exposure cultures (Supplementary Table 3). This verifies the light-promoted growth but not due to nutrient photolysis related stimulation.

Furthermore, we grew strain XB07 under different photon fluxes, and found a higher light-promoted growth (2.2-fold) under 50 µmol photon $m^2 s^{-1}$ than that under 10 µmol photon $m^2 s^{-1}$ (1.6-fold) (Fig. 3a). This determined that the enhanced growth is related to the illumination intensity, so confirms the light promotion on the growth of the glacial *Flavobacterium* strains.

Light-promoted glacial *Flavobacterium* growth occurs under lower levels of oxygen or nutrients

To find the environmental parameters that are possibly related to the light-stimulation, we first tested the aerobic levels as *Flavobacterium* spp. are obligate aerobes. By growing strain XB07 in 10- and 100-ml medium within a 100-ml flask that contain dissolved oxygen of 296.03 ± 2.59 µmol and 240.10 ± 3.61 µmol, respectively, lower light promotion fold (1.14-fold) was found in 10-ml culture (more dissolved oxygen) than that of the 100-ml culture (less dissolved oxygen, 1.5-fold), though much higher growth rates were achieved in 10-ml culture (Fig. 3b). This suggests that light could only affect the growth of the glacial *Flavobacterium* at lower oxygen availability.

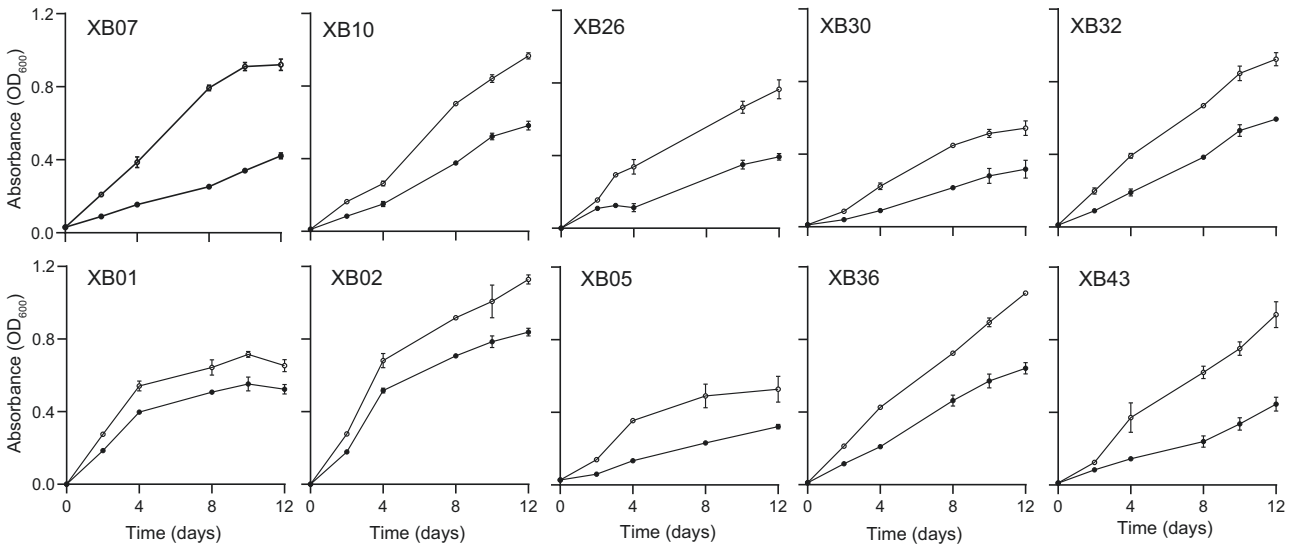


Fig. 2 Light stimulated growths of the glacial *Flavobacterium* strains. Strains were statically cultured in 5 ml of peptone-yeast extract-glucose broth within test tubes at 14 °C in an incubator installed with a color 865 LUMILUX light that generates cold white light, with overrepresentation of 520–620 nm wavelengths. Triplicate cultures of each strain were illuminated and cultured in dark wrapped by aluminum foil, respectively. OD values at 600 nm were measured in 2-day intervals, and growth curves of representative 10 strains are shown by the empty and solid symbols showing growth under light and dark conditions, respectively. Growth rates and final cell mass of all the strains are shown in Supplementary Table 3. The averages of three replicates and standard deviations are shown.

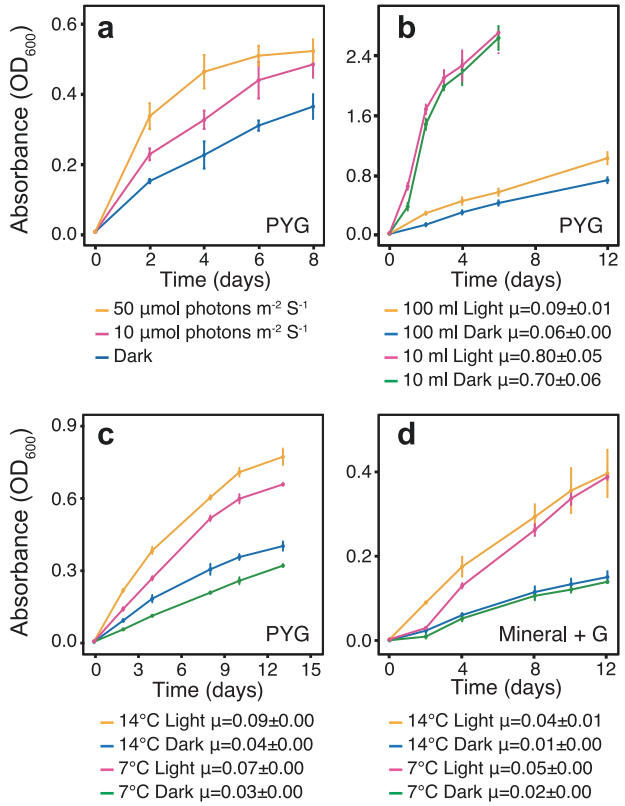


Fig. 3 Environmental parameters that affect the light stimulation on strain XB07. Light/dark experiments are conducted as in Fig. 2 unless indicated otherwise. **a** The light culture was respectively cultivated in 5-ml medium in test tubes at light fluxes of 10 and 50 µmol photon m^2 s^{-1}. **b** Strain XB07 was statically grown in 10- and 100-ml medium in a 100-ml flask, respectively, which contain dissolved oxygen of 296.03 ± 2.59 µmol and 240.10 ± 3.61 µmol as determined using an OXY Meter (Unisense, Denmark), respectively. **c** Strain XB07 was statically cultured in PYG at 7 °C and 14 °C, respectively. **d** The strain was grown in 5 ml mineral medium containing 0.4% glucose (Mineral +G). Each growth experiment was performed in triplicate, and the averages of triplicate and standard deviations were shown.

However, similar light-stimulation levels were found for strain XB07 grown at 7 and 14 °C in PYG (Fig. 3c), suggesting that temperature is not a parameter inducing the light utilization. Comparing with that of the PYG culture, the oligotrophic culture of strain XB07 exhibited both reduced growth rate and final OD600, however, it had higher light-promoted growth (fivefold in 7 °C and threefold in 14 °C cultures) than in the rich medium (Fig. 3d). This indicates that the light can also be used as a supplemented energy in nutrient-poor environments.

Most light-stimulated strains produce zeaxanthin but trace retinal

Next, the pigments that are produced by the glacier strains were identified. The tested 47 strains all produced non-water-diffusible orange or pink pigments (Fig. 4a upper panel), conforming to the characteristics of the genus *Flavobacterium*. Three types of pigments, zeaxanthin, lycopene, and flexirubin, were identified (Fig. 4a lower panel) using a combination of 20% KOH treatment on the late exponential cells (Supplementary Fig. 1), optical absorption spectral scanning (Fig. 4b), and HPLC assays (Fig. 4c) of the acetone extracts from the late exponential cells. Strains XB41 and XB47 exclusively produced flexirubin, strain XB49 produced mainly lycopene and less zeaxanthin, and the remaining 44 strains primarily produced zeaxanthin. Of the 44 strains, five synthesized both flexirubin and zeaxanthin. However, retinal contents were only detected in marginal concentrations relative to the detection thresholds even in light-exposed strain XB07 either in earlier or later exponential growth (Fig. 4c lower panel), making the PR proton pump in mediating light stimulation on the glacier *Flavobacterium* unlikely. The parallel tested *Cryobacterium luteum* produced a non-zeaxanthin type carotenoid (Supplementary Fig. 2) that could be C.p.450 as indicated by Vila et al. [15]. Illumination elevated zeaxanthin yields in some glacial *Flavobacterium* strains (Supplementary Fig. 3), implying a role of zeaxanthin in light promotion of the glacial bacteria.

Zeaxanthin synthesis pathway and the proteorhodopsin gene are present in most glacial strain genomes

To better understand the genetic basis of the light stimulation on the glacial psychrophilic *Flavobacterium*, the genomes of all 47 glacial strains were sequenced on the Illumina GAII platform. The high-quality draft genome assemblies resulted in the total sizes of ~3.6 ± 0.7 Mbp per genome, except for strains XB01 and XB03 that have genomes of around 6.5 Mbp and which contain more copies of some genes than the other isolates (Supplementary Table 4). The 47 genomes have 98.23–99.65% completeness and no contamination.

As expected, genes that have been conventionally believed to involve in cold adaptation, like cold shock protein CspA and DEAD RNA helicase, are all present in the glacial strain genomes (Fig. 5a). Overrepresented gene groups included those encoding the enzymes for the synthesis of carotenoids (especially zeaxanthin and rhodopsin), transmembrane transporters like TonB-dependent receptors, and proteins involved in anti-oxidative stress, including an ortholog of the *E. coli* transcriptional regulator OxyR, thioredoxin-disulfide reductase TrxB, and two-component signal transfer systems (Supplementary Table 4). Except for strains XB41 and XB47, 45 genomes carry the *crtIBZ* operon for the zeaxanthin synthesis pathway: *crtB* encoding phytoene synthase, *crtI* encoding phytoene dehydrogenase, *crtY*

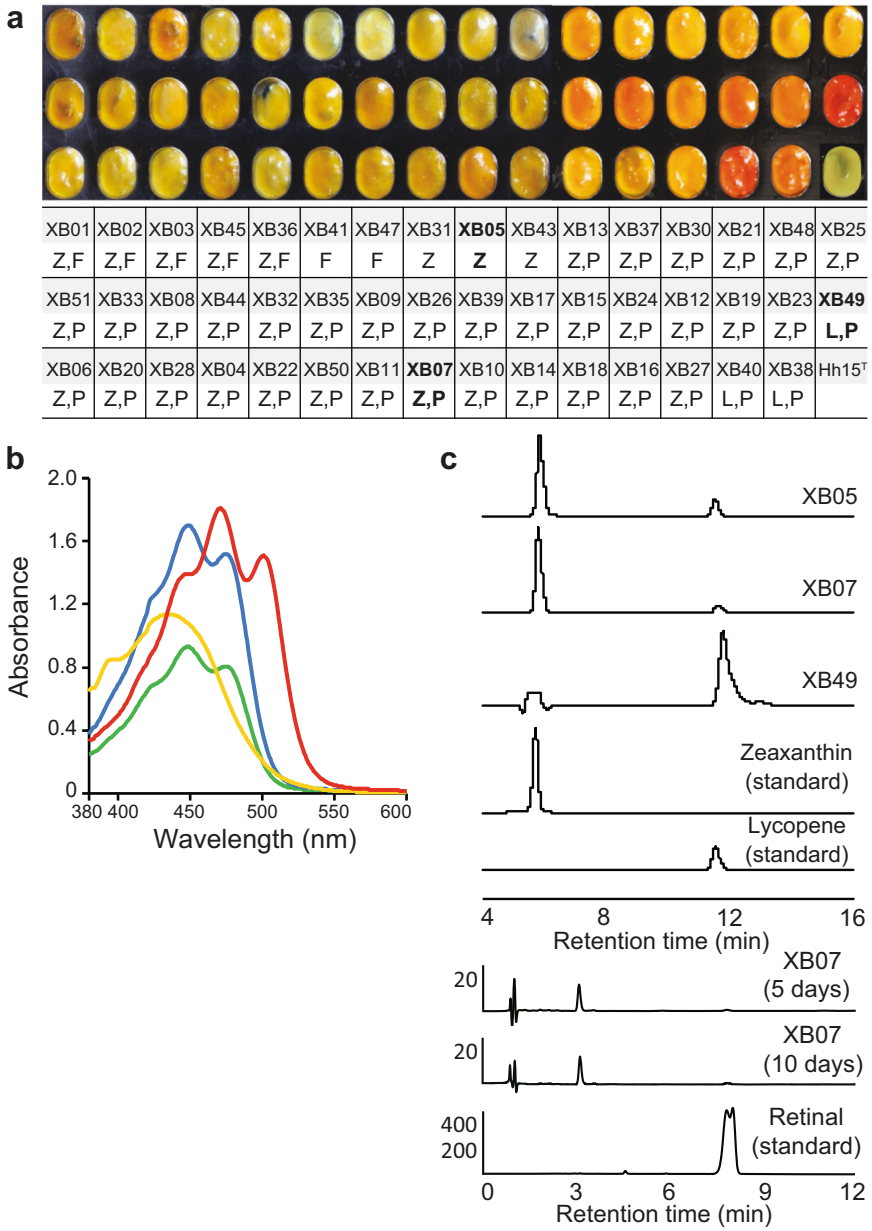
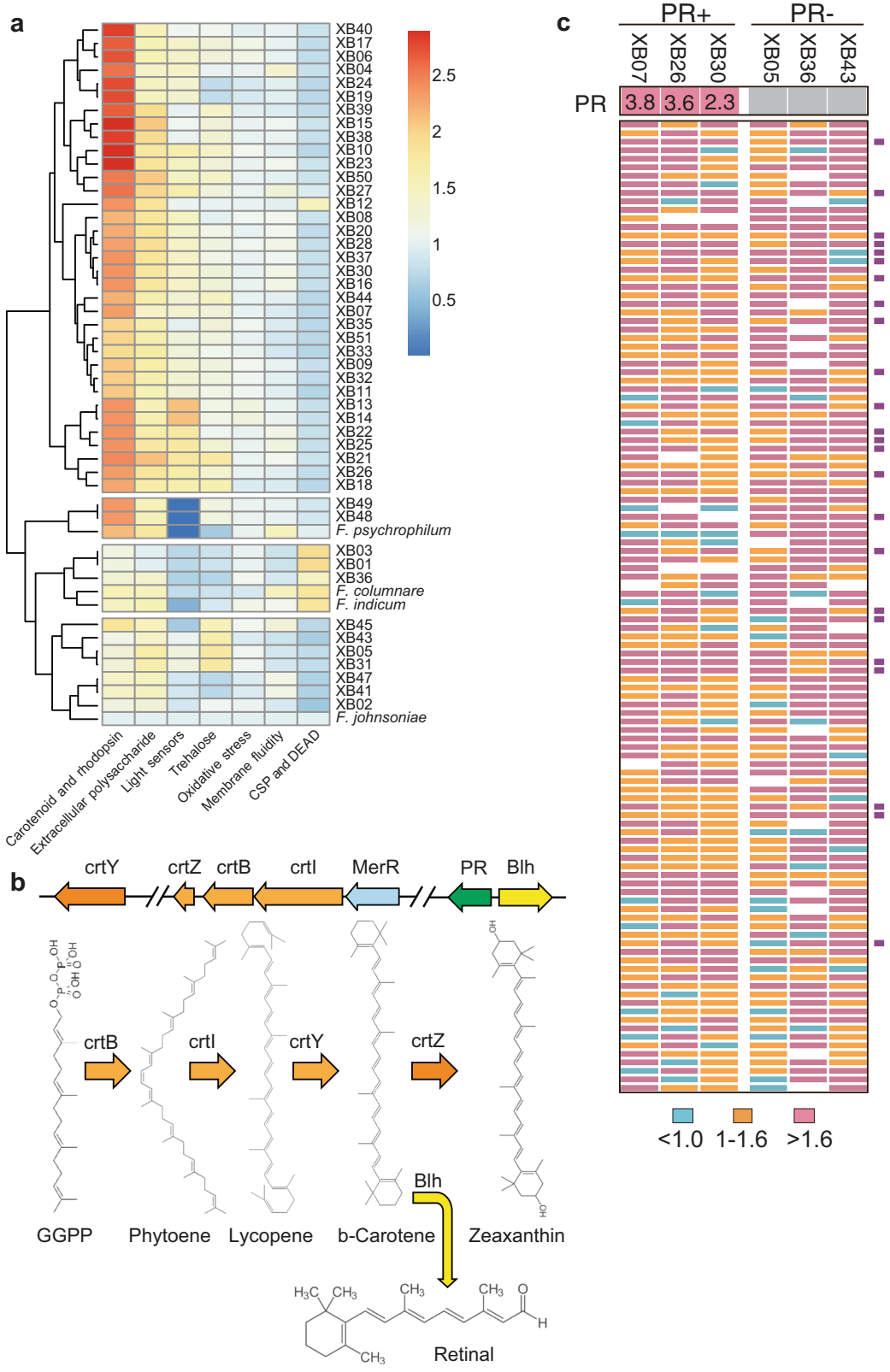


Fig. 4 Identification of the carotenoid species produced by the glacial *Flavobacterium* strains. a The colored culture lawns were collected from the late exponential cultures of the 47 glacial *Flavobacterium* strains (upper panel) and *Cryobacterium luteum* Hh15^T, which was isolated from the same glacier. Strain numbers are listed in the corresponding rows and columns (lower panel). The uppercase letters beneath the strain numbers indicate the pigment identifications: Z zeaxanthin, F flexirubin, P proteorhodopsin, L lycopene. **b** Representative spectra of the pigment extracts from strains XB49 (red), XB07 (blue), XB05 (green), and XB47 (yellow). **c** HPLC identification of the carotenoids produced by strains XB49 (mainly lycopene), XB07, and XB05 (mainly zeaxanthin) at a wavelength of 450 nm (upper panel), and retinal contents within XB07 in different growing days at 380 nm (lower panel).

encoding lycopene β-cyclase and *crtZ* for β-carotene hydroxylase (Fig. 5b). The *crtIBZ* operon situated adjacent to a MerR family transcriptional regulator that is believed to respond to environmental stimuli including oxidative stress [35]. Among the 45 strains, 37 genomes encode the rhodopsin (Supplementary Table 4) gene *prd* that is co-localized with *blh* that encodes *β*-carotene dioxygenase, an enzyme that converts *β*-carotene to retinal. Phylogenetic analysis indicated that the rhodopsin proteins from the glacial strains are PRs (Supplementary Fig. 4), as evidenced by the phylogenetic relatedness to that of *Dokdonia* sp. MED134, a marine flavobacterial strain exhibiting light-stimulated growth. To preclude a possibility of undetected *prd* gene in some genomes due to incompleteness, we obtained the complete genomes of each representative *prd*-present and -absent strains, XB07 and XB36, which confirmed the absence of *prd* in the draft genomes. PCR amplification of *rpd* was further performed and confirmed the absence in the ten strains (Supplementary Fig. 5).

The *dar* gene cluster that encodes proteins for synthesizing flexirubin was observed in the genomes of XB41 and XB47 and five other strains (Supplementary Table 4), while neither zeaxanthin/retinal synthesis pathways nor *prd* gene was observed in the XB41 and XB47 genomes. Thus, flexirubin alone may not sustain *Flavobacterium* survival to illumination.


a
XB40
XB17
XB06
XB04
XB24
XB19
XB39
XB15
XB38
XB10
XB23
XB50
XB27
XB12
XB08
XB20
XB28
XB37
XB30
XB16
XB44
XB07
XB35
XB51
XB33
XB09
XB32
XB11
XB13
XB14
XB22
XB25
XB21
XB26
XB18
XB49
XB48
F. psychrophilum
XB03
XB01
XB36
F. columnare
F. indicum
XB45
XB43
XB05
XB31
XB47
XB41
XB02
F. johnsoniae
2.5
2
1.5
1
0.5
Carotenoid and rhodopsin
Extracellular polysaccharide
Light sensors
Trehalose
Oxidative stress
Membrane fluidity
CSP and DEAD
b
crtY
crtZ
crtB
crtI
MerR
PR
Blh
crtB
crtI
crtY
crtZ
Blh
GGPP
Phytoene
Lycopene
b-Carotene
Zeaxanthin
Retinal
c
PR+
PR-
XB07
XB26
XB30
XB05
XB36
XB43
PR
3.8
3.6
2.3
<1.0
1-1.6
>1.6

◀ **Fig. 5 Overrepresented gene categories in the glacial *Flavobacterium* genomes and the light-responding genes. a** Hierarchically clustered dendrogram with corresponding heatmap showing the relative abundances of the overrepresented gene categories among the 47 glacier strains. Color intensity represents the abundance fold difference of the corresponding genes in the genomes of the glacial *Flavobacterium* strains over that of *F. johnsoniae*, the type species of the genus. Blue and red coloring represent the minima and maxima abundance fold differences, respectively. Gene categories that may be related to cold adaptation are shown beneath each column. **b** A schematic shows gene organization of the *crtIBZY* operon that encodes enzymes for synthesizing zeaxanthin, proteorhodopsin (PR), and β-carotene dioxygenase (Blh) in the upper panel. The lower panel shows the predicted pathways of zeaxanthin and retinal synthesis in the glacial strains. **c** Heatmap showing the consensus upregulated genes in each of the three glacial *prd*-containing (PR+) and *prd*-lacking (PR−) *Flavobacterium* strains in light vs. dark cultures. Colored blocks show the fold changes of transcripts, as indicated beneath the heatmap. Numbers at the top of the heatmap indicate the fold changes of the *prd* gene (PR) expression in light vs. in dark cultures, as measured with quantitative RT-PCR. The purple blocks at the right specify the genes that primarily responded to light illumination, that are shadowed in Supplementary Dataset 2.

Comparative transcriptomics detects the genes that directly or indirectly respond to light illumination

To identify the light-responding genes in order to predict those involved in light-promoted growth, a light-dark culture differential transcriptomic assay was conducted on six strains that exhibited better growth under light, with each three carrying the *prd* gene (PR+) or not (PR−). The exponential cells of the light- and dark cultures were collected for total RNA extraction, and RNA-seq was performed on the Illumina Hiseq platform. Comparative transcriptomic analysis detected 86–93% of the ORFs within the six genomes (Supplementary Dataset 1). For each strain, genes were selected for those displayed >1.4-fold differentially expressed in at least two strains of each of the PR (+) and PR (−) groups, and then further screened by those with averages >1.6-fold changes among the six strains. Only orthologs that were expressed in all six strains were further analyzed, and all differentially expressed genes were annotated using the NR database.

A total of 114 genes exhibited altered transcription in response to light among the six strains (Fig. 5c and Supplementary Dataset 2). Genes with increased expression generally belonged to the categories of (1) those within the operon encoding zeaxanthin synthesis, including *crtI* for phytoene dehydrogenase (1.3–5.6-fold change), *crtB* for phytoene synthesis (1.1–3.2-fold), and *crtY* for β-carotene hydroxylase (1.2–3.1-fold). This is consistent with increased contents of zeaxanthin-like pigment in the cultures grown under light (Supplementary Fig. 3); (2) diguanylate cyclase/phosphodiesterase (GGDEF and EAL domains) with PAS/PAC sensor(s) (1.4–2.6-fold) that may constitute bacteriophytochromes; (3) those involving in energy conservation and acquiring nutrients, including membrane-associated TonB receptor (1.3–2.4-fold), a DMT transporter (1.4–2.8-fold), neopulluanase (0.9–2.7-fold), and β-glucanase (1.3–3-fold); (5) regulatory proteins including an ECF-type sigma factor (1.1–3.5-fold), cAMP-binding proteins (1.8–4.7-fold), and the two-component system sensor histidine kinase (1.3–4.1-fold); and (6) oxidative resistance protein genes like those encoding the thiol: disulfide interchange protein (1.1–4.6-fold), MsrAB (1.4–3.7-fold) and catalase (0.7–5.8-fold) in addition to the DNA damage repair protein AlkB (0.9–6-fold). As expected, light also consistently induced *prd* gene expression (2.3–3.8-fold) among the three *prd*-carrying strains, However, the *blh* gene that encodes β-carotene dioxygenase in converting β-carotene to retinal was very poorly expressed in the strains, hinting that the glacial *Flavobacterium* PR proton pump might not be active under light stimulation.

Overall, the comparative transcriptomic analysis on light-/dark-grown cultures suggests that light-stimulated growth could be related to the enhanced synthesis of zeaxanthin; illumination could increase energy metabolism, and imposed oxidative stress on the cells. Thus, it is reasonable to predict that zeaxanthin plays a similar role in protecting glacial *Flavobacterium* strains from ROS generated by an intensive illumination.

Zeaxanthin is related to light promotion on the glacial *Flavobacterium* strains

To investigate the contributions of PR and zeaxanthin to the light-promoted growth of the glacial strains, the *prd* gene (XB07GL000621) was first deleted in strain XB07 using the conjugation approach developed by Li et al. [40]. Successful deletion was confirmed via sequencing of the DNA fragment embedded *prd* coding region (Supplementary Fig. 6) and failure of determining the *prd* transcript via qPCR (Ct = 25 of the wild-type strain vs. Ct = 36 of the mutant). However, a comparable light-promoted growth was observed for the *prd* deletion mutant and the wild-type strain, as both exhibited similar increased growth rates in the light- and dark-grown cultures (Fig. 6a).

The correlation of zeaxanthin production with light promotion was then experimentally investigated. The lycopene cyclase inhibitor, MPTA, was used to block zeaxanthin synthesis, as the larger gene cluster for zeaxanthin synthesis makes genetic manipulation difficult. Upon addition of a final concentration of 100-μmol MPTA, generally reduced growth was observed in both the light and dark cultures, but greater reductions of growth were observed in light-incubated cultures (Fig. 6a). It is worth noting that MPTA suppressed light-stimulated growth of strain XB07 was only observed in the first 2 days. In contrast, MPTA continuously suppressed the light-stimulated

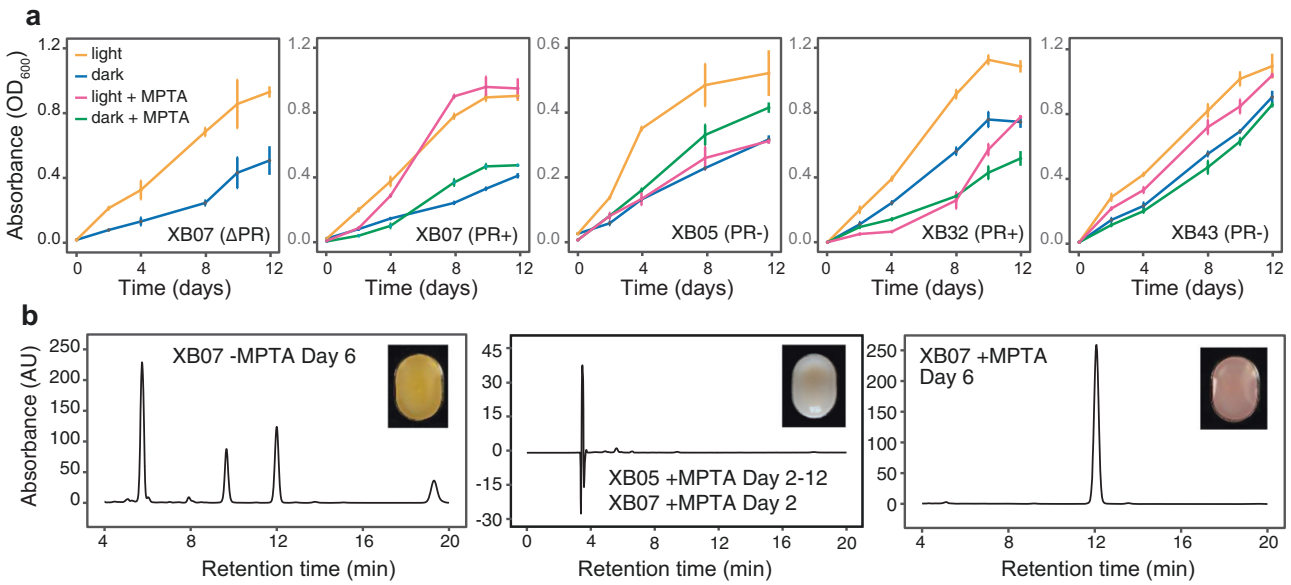


Fig. 6 Zeaxanthin-associated light stimulation of representative glacial *Flavobacterium* strains. a Light/dark growth assays of *prd*-carrying (PR+) or *prd*-lacking (PR−) strains, and the *prd* gene deletion mutant of XB07 (ΔPR) were statically cultured in PYG at 14 °C with incubation of triplicate cultures for each strain under green light (520–620 nm) or wrapped with aluminum foil. MPTA was added to a final concentration of 100 µmol to inhibit zeaxanthin synthesis in experimental cultures. OD values at 600 nm were measured in 2-day intervals, and the averages of triplicate cultures are shown along with standard deviations. **b** HPLC identification of the accumulated pigments in cultures with (+) or without (−) MPTA added by referencing the retention times in Fig. 4c. Inserts are the corresponding culture lawns.

growth of strain XB05, although light was not lethal (Fig. 6b). The simultaneous pigment assays showed zeaxanthin disappearance in MPTA-amended cultures of XB07 after the first 2 days, and lycopene accumulation in the sixth day, contrasting with the persistent existence of zeaxanthin in the cultures not amended with MPTA. A lasting absence of zeaxanthin was observed in the MPTA-amended XB05 cultures. These results suggest that zeaxanthin could contribute to the light promotion due to unknown mechanisms.

However, we failed to detect the proton pump trait of strain XB07 (Supplementary Fig. 7a), instead more glucose was consumed by the light exposed than the dark culture (Supplementary Fig. 7b) as determined using 3,5-dinitrosalicylic acid reagent [42]. This suggests that unlike PR and other membrane pumps, zeaxanthin via an unknown pathway transfers the captured light energy to bioenergy.

Discussion

Bacteria inhabiting in glaciers must cope with, in addition to low temperatures, multifaceted stresses including oxidative stress derived from increased oxygen solubility in cold, and oligotrophic stress by decreased solubility of solutes and nutrients, and extreme light exposure at high altitudes. Previous studies have found that the glacial bacteria are featured with production of various carotenoids [16], and this study reports that a group of glacial *Flavobacterium* strains that produces predominantly zeaxanthin (Fig. 4) are not only the representatives of intrinsic psychrophiles by growing optimally at 14 °C, but also exhibit light-promoted growth in deficiency of oxygen or nutrient (Figs. 2 and 3), resembling PR-facilitated light energy usage by some marine flavobacteria in oligotrophic growth [29] or salinity stress [43]. However, the mechanisms of zeaxanthin related light promotion on the glacial *Flavobacterium* remain to be elucidated.

In addition of producing zeaxanthin, 37 of the 45 tested strains that display light promotion also carry the *prd* gene encoding PR (Supplementary Table 4). PR appears to be an inherited membrane protein of some gram-negative bacteria including flavobacteria, which has been revealed by metagenomic analyses to be widely distributed among numerous marine bacteria and archaea [27, 44]. The PR proteins, through binding retinal, generate proton motive force pumping through retinal captured light energy, and convert it to ATP production. Such as *prd* overexpressed *E. coli* exhibits light-powered flagellar motor activity and swimming when respiration is inhibited [45]. Up to date, the reported PR-mediated light-energy conservation appears to be restricted to marine bacterial species [28–30, 43]. While, the poor expression of the β-carotene dioxygenase gene *blh* (Supplementary Dataset 1) and trace retinal yields (Fig. 4c) in the glacial *Flavobacterium* comparing with that in the sea-ice psychrophile *Psychroflexus torquis* [43], in addition to that deletion of the *prd* gene did not change the light

stimulation (Fig. 6a), the PR proton pump appears not to work as a light-driven system in the glacial strains. Given that the *prd* gene is transcribed at similar levels as that of *crtIYZ* as well as is induced by light illumination, PR should perform unidentified functions in the glacial *Flavobacterium*.

Although association between zeaxanthin and light stimulation is determined in the glacial *Flavobacterium*, the underpinning mechanisms are largely not known. Carotenoids, especially the hydroxylated carotenoid zeaxanthin, could play roles in integrating the psychrophilic cell membranes that usually have increased fluidity due to the unsaturated membrane lipids [46]. Bacteria in the genus *Flavobacterium* have been identified primarily as psychrophiles growing between 4 and 30 °C, and distributed in Antarctica, glaciers, permafrost, deep-sea environments, or other cold aquatic ecosystems [47], thus carotenoids with polarity like zeaxanthin and lutein would be more important in maintaining the cell integrity in the cold. Increased oxygen solubility usually occurs in lower temperatures, and thus elevates ROS stress to the psychrophiles; while the bacteria in high-altitude glaciers are suffered another oxidative stress from the singlet oxygen species generated by the intensive light radiation. Carotenoids rendered antioxidation could quench ROS and thus provide a photoprotection to the glacial bacteria. The hydroxylated carotenoid zeaxanthin has been shown to effectively induce dissipative states of the PSI and PSII photosystems of plants, in addition to quenching a portion of the excitation energy through binding of the PSI components to form carotenoid radical cations, thereby playing a key role in photoprotection to plants [48]. Although no proton pump activity was detected in the glacier *Flavobacterium* (Supplementary Fig. 7a), Kupisz et al. reported that zeaxanthin likely functions as a proton pump to transport protons across an artificial membrane [33]. Based on the Fourier transform infrared spectroscopy analysis of β-carotene and zeaxanthin, Kupisz et al. also suggested that the polyene chains of carotenoids form weak hydrogen bonds with water molecules [49].

Nevertheless, differential transcriptomic analysis detected that light induces expression of the genes encoding a diguanylate cyclase/phosphodiesterase (GGDEF and EAL domains) with PAS/PAC sensor(s), which may constitute bacteriophytochromes to capture and use light energy. In addition, light also induced electron transfer chain components (e.g., cytochrome C oxidase) and polysaccharide hydrolysis proteins (Supplementary Dataset 2), and the light illuminated culture did consume more glucose than the dark counterpart (Supplementary Fig. 7b). Illumination appears also elevating the transcription of other transmembrane transporters, including ABC transporters and TonB-dependent receptors, the other type of proton pump that specifically transports sugars, siderophores, and vitamin B [50]. Therefore, several membrane transporters may facilitate sugar uptake and metabolism so promote growth of the glacial *Flavobacterium* strains when exposed to light. However, no other known light-responsive membrane protein domains, like the *Xanthomonas* LOV and MED134 BLUF were found in the glacier *Flavobacterium* strains.

Zeaxanthin, a polar carotenoid, could be an over-representative pigment in psychrophiles and bacteria inhabiting in cold areas. The genera of *Flavobacterium*, *Chryseobacterium* and *Zobellia* that are affiliated with Bacteroidetes all possess pigment profiles composed of zeaxanthin, β-cryptoxanthin, and β-carotene [15]. Zeaxanthin is also found to be the major carotenoids produced by some marine flavobacteria [51], including *Dokdonia* MED134, which exhibits light-stimulated growth, also carries the zeaxanthin synthetic gene cluster in the genome. A recently described marine genus and species, *Mesoflavibacter zeaxanthinifacien*, is named after zeaxanthin [52]. In addition, zeaxanthin along with β-cryptoxanthin and β-carotene was found to be the predominant carotenoids in an Antarctic psychrotolerant bacterium, *Sphingobacterium antarcticus* [24]. Therefore, the multifaceted roles of zeaxanthin that benefit to psychrophiles may drive the light-promoted growth of the glacial *Flavobacterium*.

Data availability

All the genomic and RNA-seq data have been deposited under the NCBI BioProject accession number PRJNA382862, in which the complete genomic data of strains XB07 and XB36 are under the accession numbers of CP049329 and CP062159, respectively.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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