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Extended temperature optimum of photosynthetic reaction centers in Rhodobacterales

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Abstract

Temperature is one of the most important physical factors affecting microbial and biochemical processes. We investigated the performance of photosynthetic apparatus of marine photoheterotrophic bacterium *Dinoroseobacter shibae* under various temperatures. The primary photochemistry and electron transport was measured using variable infra-red fluorometry in the cells grown between 8–35°C. It was found that the photosynthetic electron transport had a broad temperature optimum between 25–50°C. Moreover, the primary charge separation stayed functional even after rising temperature up to 55°C. The same phenomenon was observed also in other phototrophic Rhodobacterales. The psychrotolerant bacterium *Roseisalinus antarcticus* reached its maximum electron transport rate at 48°C, 30°C above its growth temperature. We propose that the extended temperature stability may be crucial to maintain photosynthetic function under situation when photosynthetic membranes heat up above their ambient temperature due to the heat dissipation of the excess light energy.

Additional key words: aerobic anoxygenic phototrophs; *Rhodobaca barguzinensis*; *Roseobacter*; variable fluorescence.

Introduction

Bacterial order Rhodobacterales (Alphaproteobacteria) embraces a large group of species differing significantly in their metabolic properties and ecology. A characteristic feature of Rhodobacterales is the presence of many species performing anoxygenic photosynthesis (Koblížek *et al*. 2013). These species harvest light using type-2 reaction centers (RCs) containing bacteriochlorophyll (BChl) *a* (Selyanin *et al*. 2016). Phototrophic Rhodobacterales are found in many environments including fresh water bodies, soda lakes, marine waters, sediments, and soils. These organisms frequently display a large flexibility in their oxygen or salinity tolerance. Members of the *Rhodobacter* (*Rba*.) and *Rhodovulum* genera represent classical examples of purple nonsulfur bacteria which synthesize pigments and perform photosynthesis under anaerobic or semiaerobic conditions. On the other hand, there exist many marine species belonging to the so called Roseobacter group, which grow and express their photosynthetic complexes in the presence of oxygen (Brinkhoff *et al*. 2008). These aerobic anoxygenic phototrophic (AAP) bacteria are typical photoheterotrophs, they do not fix inorganic carbon and use light energy as a supplement for their mostly heterotrophic metabolism.

It has been reported that AAP abundance positively correlated with chlorophyll concentration, bacterial abundance, and light availability (Koblížek 2015). The seasonal studies conducted in temperate coastal waters of the Baltic Sea (Mašín *et al*. 2006), and the western Mediterranean (Ferrera *et al*. 2014) also documented a positive correlation of AAP abundance and temperature. However, the observed correlations were weak, so the effect may have reflected more complex seasonal trends rather than the temperature alone.

Despite all of their physiological flexibility, phototrophic Rhodobacterales only flourish within a relatively narrow range of temperatures. The majority of cultured Rhodobacterales are mesophilic species with growth optima between 25–35°C. Several species, *Roseovarius tolerans*, *Staleya guttiformis*, and *Roseisalinus* (*Ris.*) *antarcticus*, were isolated from the saline Lake Ekho in East Antarctica, but none of them are true psychrophiles (Labrenz *et al*. 1999, 2000, 2005). A similar situation has been documented for the opposite temperature extreme. Despite numerous attempts, no truly thermophilic species

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Abbreviations: AAP – aerobic anoxygenic phototrophs; BChl – bacteriochlorophyll; F_0 – minimal BChl fluorescence; F_M – maximal BChl fluorescence; F_V – variable BChl fluorescence; F_V/F_M – maximal photochemical yield of RC; HPLC – high performance liquid chromatography; OD_{600} – optical density, turbidity measured at 600 nm; RC – photosynthetic reaction center.
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(growing above 50°C) have been cultured to date (Madigan and Jung 2009, Kalashnikov *et al*. 2014). Most of the research on thermostability of bacterial reaction centers has been conducted with mildly thermophilic species *Thermochromatium tepidum* (Nozawa and Madigan 1991, Kimura *et al*. 2009). However, this purple sulfur bacterium (class Gammaproteobacteria) differs dramatically from Rhodobacterales regarding its ecology, physiology as well as phylogeny.

In general, temperature has a profound effect on microbial growth and activity since it directly affects the rate of biochemical reactions. Temperature may also affect the metabolic patterns, the nutritional requirements, and the composition of microbial cells. Low temperatures are known to limit respiration and restrict bacterial growth. On the other hand, a high temperature may disrupt cellular metabolism and regulation, damage enzymes and affect the overall cellular apparatus.

To learn more about the performance of bacterial photosynthetic RCs at various temperatures, we investigated the temperature preference of pigment synthesis, primary photochemistry, and electron transport in marine AAP bacterium *Dinoroseobacter* (*Drb.*) *shibae* (Biebl *et al*. 2005). In addition, we also used other photoheterotrophic Rhodobacterales differing in their temperature preference. The psychrotolerant bacterium *Ris*. *antarcticus* EL-88^T was isolated from Antarctic lake Ekho, the haloalkaliphile *Rhodobaca* (*Rca.*) *barguzinensis* alga-05 was isolated from a soda lake in Siberia (Boldareva *et al*. 2008), while the marine bacterium *Roseobacter* sp. COL2P was isolated from Mediterranean coastal waters (Koblížek *et al*. 2010). The function of their photosynthetic RCs at various temperatures was investigated using kinetic BChl fluorometry.

Materials and methods

Microbial cultures: *Drb. shibae* DFL-12^T, *Ris*. *antarcticus* EL-88^T, and *Roseobacter* sp. COL2P were grown in complex organics seawater media described before (Koblížek *et al*. 2010). *Rca. barguzinensis* alga-05 was grown in organic medium designed for haloalkalophic species (Boldareva *et al*. 2008). The aerobic cultures were incubated in 250-mL Erlenmayer flasks with cotton plugs on an orbital shaker at specified temperatures. Illumination was provided by a bank of *Dulux L 55W/865* luminescent tubes (*Osram*, Germany, spectral temperature of 6500 K) delivering approx. 100 μmol(photon) m^{-2} s^{-1}. If not stated otherwise, the cultures were grown under 12-h dark/12-h light regime. During the experiment investigating the growth of *Drb. shibae* cultures at different temperatures, the late exponential phase inoculum grown in the dark at 20°C was diluted to OD_{600} = 0.01 with fresh growth medium and distributed into Erlenmayer flasks. The obtained subcultures were transferred into incubators with temperatures set at 8, 15, 20, 30, 35, 40, and 45°C. The growth was followed two times per day by turbidity measurements at 600 nm.

Fluorescence measurement: Infra-red fluorescence measurements were performed using a kinetic fluorometer *FL-3000* (*Photon Systems Instruments Ltd.*, Brno, Czech Republic) equipped with the 'Superhead' optical unit populated with an array of blue-green 505 nm *Luxeon Rebel* diodes. The infra-red (BChl) fluorescence signal (λ > 850 nm) was detected using a silicon photodiode registering with a 10 MHz resolution. The cells harvested at late exponential phase were diluted 10 times into fresh medium and dark adapted for half an hour at room temperature prior to probing their photosynthetic activity. Cell suspension (2.3 mL) was first cooled down to 10°C and then in the dark linearly heated to the selected temperature over a period of 10 min. Heating period was chosen as a minimal time required for inducing a stable half inhibition of the yield of primary photochemistry of the reaction centers at 70°C (Fig. S1, supplement). After reaching the chosen temperature, the BChl fluorescence was elicited by a 140 μs-long square-wave pulse of light with an intensity of ~ 0.1 mol(photon) m^{-2} s^{-1}. The yield of primary photochemistry was calculated as $F_V/F_M = (F_M - F_0)/F_M$. Here, F_0 is the minimal BChl fluorescence yield at time 1 μs emitted by the open reaction centers capable of charge separation. The F_M is the maximal BChl fluorescence yield obtained after 100 μs of illumination that is emitted by the closed reaction centers that have undergone charge separation and stabilization of the electron at the quinone primary acceptor Q_A. The fluorescence relaxation following the maximal BChl fluorescence level F_M was detected by 124 logarithmically spaced probing flashlets placed after the saturating pulse. The normalized BChl fluorescence decays were fitted with three exponential-decay curves by least square numerical fitting. The rate of fluorescence relaxation was expressed as a sum of three components $f(t) = a_1 exp(-k_1 t) + a_2 exp(-k_2 t) + a_3 exp(-k_3 t)$, where $f(t)$ is the fluorescence response at time t; k_1, k_2, and k_3 represent the rate constants of reoxidation of the primary quinone electron acceptor Q_A^-, and a_1, a_2, and a_3 their corresponding amplitude. The individual measurements were conducted over a range from 5 to 90°C with 2.5°C increments with a fresh sample for each individual measurement (*i.e.*, in total of 35 measurements).

Other analyses and statistics: The rate of respiration was measured with Clark-type concentration electrode in a thermostated, magnetically stirred cuvette (*Hansatech Instruments Ltd*., UK). Electrical current at a polarization potential of 700 mV was monitored by *Oxycorder* (*Photon Systems Instruments Ltd.*, Czech Republic) with a sampling frequency of 30 Hz. Cell suspensions of 2 mL were homogenously illuminated by *KL2500 LED* cold light source (*Schott*, Germany) providing white light of 200 μmol(photon) m^{-2} s^{-1}. The respiration was measured at 10, 20, 30, 40, 48, and 55°C. Calibration of the measured signals was carried out against a sterile growth medium equilibrated with the ambient air and then depleted of oxygen by bubbling the chamber with nitrogen. Tabulated values of oxygen concentration in saline water were used to convert measured signals into oxygen concentration [mmol(O_2) L^{-1}]. The rate of respiration in mmol(O_2) g(BChl *a*)$^{-1}$ h^{-1} was determined as a slope of linear regression

of a plot of O_2 concentration *vs*. time normalized to BChl *a* concentration. All experiments were carried out in quadruplicates.

BChl *a* concentration was determined using the high performance liquid chromatography (HPLC) as described before (Selyanin *et al*. 2016). Protein content was determined spectrophotometrically using a Lowry assay kit (*Merck*, Germany).

All statistical analyses were performed using *SigmaPlot v. 13.0*. Data are presented as mean ± standard deviation. A value of $P<0.05$ was considered statistically significant.

Results and discussion

Temperature preference of *Drb. shibae*: First we investigated the temperature preference of *Drb. shibae* grown between 15 and 45°C. To remove the inhibitory effect of light on pigment synthesis observed previously (Piwosz *et al*. 2018), we incubated all the cultures in the dark. The best growth was observed in cultures grown between 30 and 40°C, with the maximum growth rate (~ 3.4 d^{-1}) and biomass obtained at 35°C (Fig. 1*A*). The cells incubated at 45°C showed only a transient increase in turbidity during the first 22 h. On the course of the following cultivation, the turbidity showed a slow but gradual decline.

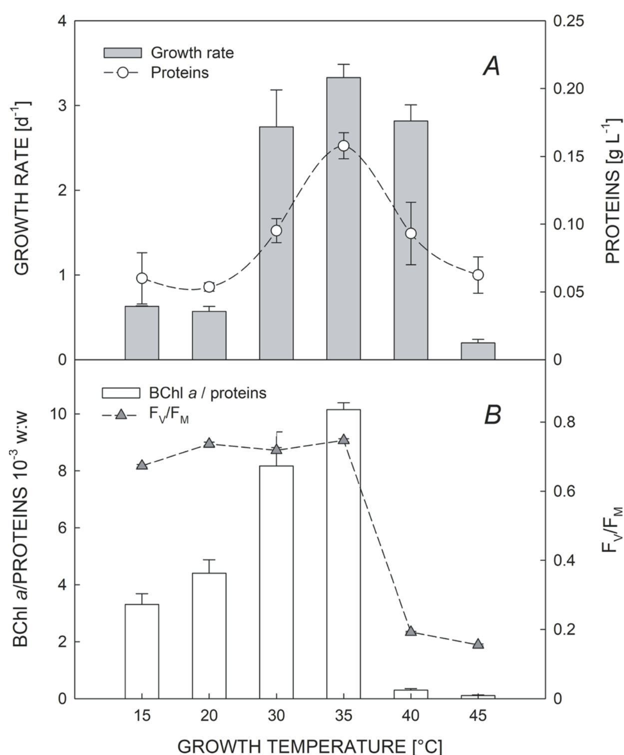

Fig. 1. Influence of cultivation temperature on *Dinoroseobacter shibae* grown in the dark. The initial growth rates (*bars*) and protein concentration reached at steady state (*circles*) (*A*). BChl *a* per protein ratio (*bars*) and yield of photochemistry F_V/F_M (*triangles*) (*B*). The error bars report the standard deviations calculated from three parallel cultures.

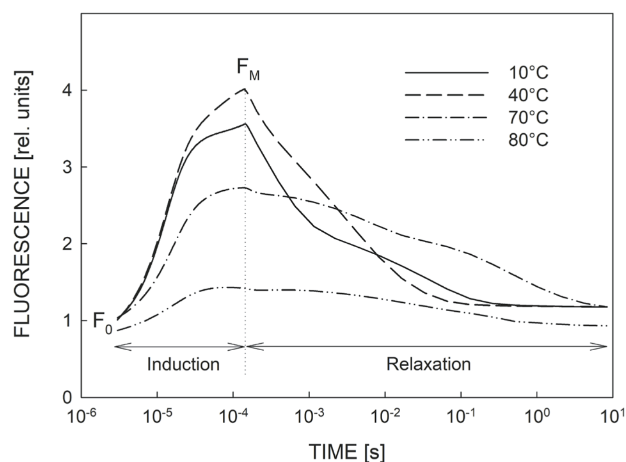

Fig. 2. *In vivo* bacteriochlorophyll fluorescence induction-relaxation kinetics of *Dinoroseobacter shibae* grown at 35°C and briefly exposed to elevated temperatures of 10 to 80°C. The induction of bacteriochlorophyll fluorescence from F_0 to F_M elicited by short pulses of actinic light corresponds to a gradual closure of the photosynthetic reaction centers. The subsequent relaxation from the F_M reflects the reopening of the reaction centers. The kinetics were analysed to obtain electron transfer rate constants k_1, k_2, and k_3.

A somewhat different picture was obtained when we looked at synthesis of photosynthetic pigments and photosynthetic RCs. Here the cultures grown at 40°C had significantly reduced pigmentation when compared to cultures grown at 35°C (3 10^{-4} *vs*. 1 10^{-2} BChl *a* per protein, w:w) (Fig. 1*B*). To further investigate this phenomenon, we tested the function of their RCs using variable BChl fluorometry. While the cells grown at or below 35°C exhibited normal high photochemical yields, F_V/F_M ~ 0.7, the cells grown at 40 or 45°C had F_V/F_M < 0.2 (Fig. 1*B*). Similar phenomenon has been reported in marine AAP bacterium *Erythrobacter* sp. NAP1 or the slightly thermophilic bacterium *Rubritepida flocculans*, which may grow up to 35°C or at 48°C, respectively, but they only express BChl *a* at much lower temperatures (Alarico *et al*. 2002, Koblížek *et al*. 2003).

Performance of photosynthetic reaction centers at varying temperatures: The fact that *Drb. shibae* cells can still grow well at 40°C, but their photosynthetic apparatus is largely reduced and not functional may indicate that the photosynthetic apparatus is either unstable at higher temperatures or is not assembled at super-optimal temperatures. We therefore used the *Drb. shibae* culture grown at 35°C and tested the functionality of the RCs *in vivo* at various temperatures. In the set of measurements, cells were exposed to temperatures of 5–80°C and their photosynthetic performance was tested using variable BChl fluorescence (Fig. 2). The observed influence of elevated temperature was approximately biphasic. The recorded F_V/F_M ratios remained constant up to approximately 55°C (Fig. 3). At higher temperatures, it gradually declined, signalizing an impaired function of the primary photochemistry. To test whether the observed

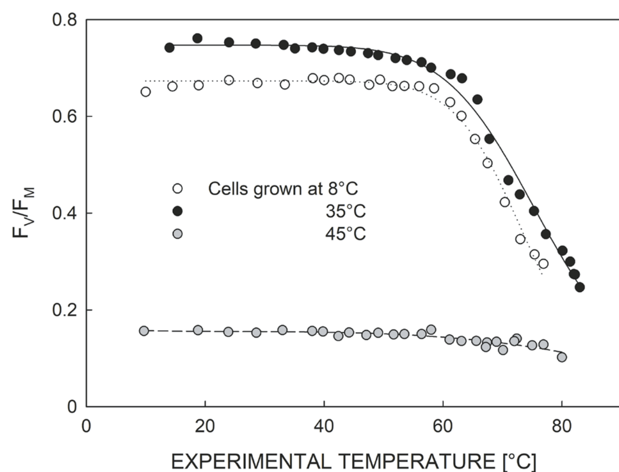

Fig. 3. Temperature dependence of the yield of primary photochemistry F_V/F_M recorded in *Dinoroseobacter shibae* cells grown at suboptimal, optimal, and super-optimal temperatures briefly exposed to temperatures varying between 5 to 81°C.

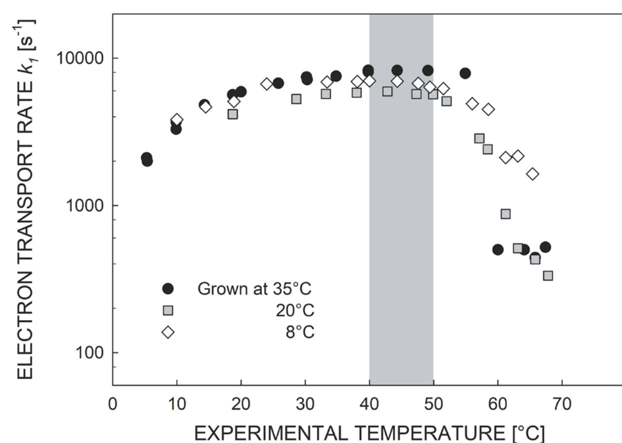

Fig. 4. Temperature dependence of electron transport rate k_1 recorded in *Dinoroseobacter shibae* cells grown at 8, 20, and 35°C and briefly exposed to experimental temperatures varying between 5 to 68°C. The *grey box* marks the temperature optimum.

thermal stability of the photochemical reactions depends on growth temperature, we performed a control measurement using *Drb. shibae* cells grown at suboptimal temperatures. The cells grown at 8°C retained constant F_V/F_M ratios (~ 0.66) up to 58°C and declined only at experimental temperatures above 60°C (Fig. 3).

Photosynthetic reactions catalysed by the reaction centers that follow the primary charge separation and stabilization involve the forward transport of the separated charges from the primary electron acceptor Q_A to the secondary acceptor Q_B. We used the relaxation part of the variable fluorescence measurements (Fig. 2) to deconvolute the Q_A^- reoxidation rate constants for *Drb. shibae* cells grown at 35°C. Interestingly, the determined electron transport rate constant k_1 had a broad temperature optimum between 25 to 50°C, reaching its maximum rate (8,263 s^{-1}) at 44°C (Fig. 4). At temperatures above 55°C, the electron transport rapidly declined.

Since we wanted to learn whether the extended electron transport optimum is an internal characteristic of the reaction centers or it is affected by the fluidity of the photosynthetic membranes, we conducted the measurements of electron transport rates in cultures grown at 8 and 25°C, which is significantly lower than optimum temperature. We saw the same temperature dependencies of electron transport rates as in the culture grown at 35°C (Fig. 4). There was only a small shift of the k_1 maximum which was found at 40 and 43°C for cultures grown at 8 and 20°C, respectively. These measurements indicate that the observed high temperature optima originate from the inherent characteristics of the RCs and they are not significantly modulated by the thermal adaptation of the individual cultures.

Thermal stability of phototrophic Rhodobacterales: To test whether the observed extended temperature stability is a more universal phenomenon, we decided to perform several similar experiments on closely related phototrophic species. Two Rhodobacterales species (*Rca. barguzinensis* and *Roseobacter* sp. COL2P) were mesophilic, Antarctic bacterium *Ris. antarcticus* was psychrotolerant (Table 1). In spite of the differing temperature preferences, the recorded F_V/F_M ratios remained high well above 50°C for all studied Rhodobacterales strains (not shown). The same trend was observed for the electron transfer. The examined species maintained high electron transfer rates above 40°C. The electron transfer then typically declined above 50°C (Fig. 5). Moreover, the psychrophilic bacterium *Ris. antarcticus* showed its electron transfer maximum at 48°C (k_1 = 5,482 s^{-1}; Fig. 5), which was 30°C above its growth temperature. All these results demonstrate that the photosynthetic reaction centers in all investigated Rhodobacterales share the same temperature optima, which are not related to the growth optimum of the particular strain. Similar trends were observed also in Alphaproteobacterium *Erythrobacter* sp. NAP1 (Fig. S2, supplement), which documents that the extended temperature optimum is not restricted only to phototrophic Rhodobacterales.

Analogy of mitochondrial membranes: It is very interesting to note that the observed temperature maxima of the rapid electron transfer between 40 and 50°C corresponds well with the optimum temperature reported recently for key enzymes involved in oxidative phosphorylation in mitochondria (Chrétien *et al*. 2018). It was found that mitochondrial cytochrome *c* oxidases had temperature optima around 50°C, while ATPase optimum was around 46°C (Chrétien *et al*. 2018). The higher temperature optimum in mitochondria was put in context with the fact that highly metabolically active mitochondria elevate their internal temperature by up to 10°C above the ambient.

A similar situation may actually also occur in photosynthetic membranes, which may internally overheat due to the fact that a large part of the absorbed light energy

Table 1. Optimum growth temperatures, the cultivation temperature, and the temperature of the maximal electron transport rate k_1 (T_{MAX}) of the investigated organisms. Optimum growth temperatures were taken from the literature (Labrenz *et al*. 2005, Boldareva *et al*. 2008, Koblížek *et al*. 2010).

Species	Optimum growth temperature [°C]	Cultivation temperature [°C]	T_{MAX} [°C]
Dinoroseobacter shibae DFL-12^T	25–37	8	40
		20	43
		35	44
Rhodobaca barguzinensis alga-05	20–35	25	45
Roseisalinus antarcticus EL-88^T	16–26	18	48
Roseobacter sp. COL2P	20–33	23	47

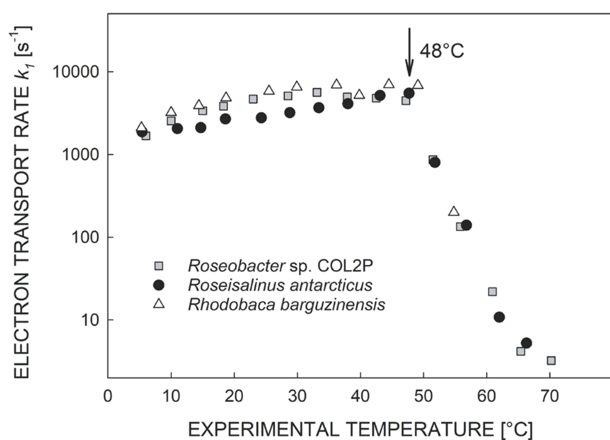

Fig. 5. Temperature dependence of electron transfer rate k_1 recorded in marine bacterium *Roseobacter* sp. COL2P grown at 23°C, psychrotolerant bacterium *Roseisalinus antarcticus* grown at 18°C, and haloalkaliphilic bacterium *Rhodobaca barguzinensis* grown at 25°C.

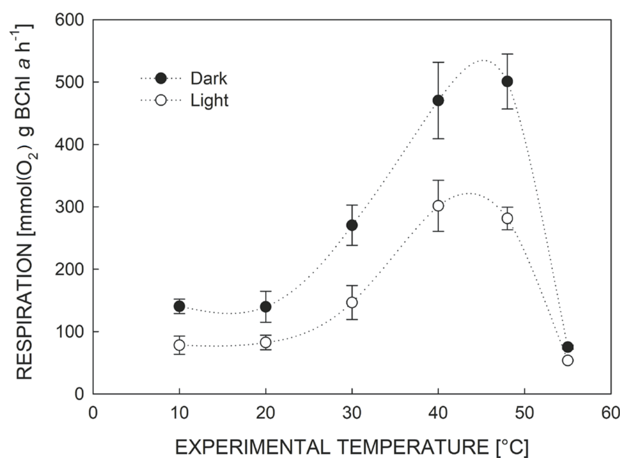

Fig. 6. Temperature dependence of respiration of *Dinoroseobacter shibae* grown at 35°C. The rate of O_2 respiration was measured in the dark and under illumination of 200 μmol(photon) m^{-2} s^{-1}.

is dissipated as heat. Therefore it is important that the RC centers can operate even at higher than ambient temperatures. Also, it is important to note that mitochondria evolved from Alphaproteobacteria which entered the eukaryotic host (Gray 2012). So, mitochondria and Alphaproteobacteria share many common characteristics, such as membrane organization and composition. We may speculate that they also inherited the same temperature preference.

To test this hypothesis, we investigated the thermal stability of enzymes involved in the oxidative phosphorylation by measuring the rate of O_2 respiration in the dark and also under illumination of 200 μmol(photon) m^{-2} s^{-1}. Fig. 6 shows the respiration activity in *Drb. shibae* rose from 20–40°C with an optimum between 40–48°C that was followed by a steep decline. The photophosphorylation activity reflected in the difference between dark and light respiration (Koblížek *et al*. 2010) has also the maximum extent at 48°C. This confirms that also enzymes involved in respiration exhibit extended temperature stability in the same range as photosynthetic electron transport. These findings also positively support the temperature optima documented for ATPase and cytochrome *c* oxidases by Chrétien *et al*. (2018).

Heat production model: We are not aware of any study which would determine the internal temperature of the active photosynthetic membranes and this topic certainly deserves closer attention. Therefore, we attempted to estimate the heat produced by illuminated photosynthetic membranes inside of a thermally isolated cell of *Drb. shibae*. The single cell contains *ca*. 3,000 reaction centers (Piwosz *et al*. 2018) that represent moderately pigmented cells when compared with the values of ~ 150–30,000 RC per cell found in a representative sample of purple nonsulfur and AAP bacteria by Selyanin *et al*. (2016). The investigated species living in the aquatic environments where they are exposed mostly to the blue-green light spectrum (infra-red part of the spectrum is rapidly removed in the water column). Approximately half of the energy of a blue photon (λ = 440 nm), which has been absorbed by a reaction center (~ 870 nm), is dissipated as heat (E = 1.4 eV RC^{-1} = 2.25 10^{-19} J RC^{-1}). A moderate light intensity of 400 μmol(photon) m^{-2} s^{-1} of blue light will cause an effective flux of approx. 100 hits per reaction center per second. Each RC will dissipate heat of 22.5 aJ per second. Collectively, all reaction centers in the cell produce a heat of 67.5 fJ s^{-1}. With a volume of the cell being estimated to 0.5 μm^3 it contains an approximated 0.5 fL of water

weighting 0.5 pg. Assuming that the heat capacity of the cell is mostly dictated by water (C_{H2O} = 4.2 J K^{-1} g^{-1}), we estimate the photosynthetic membranes illuminated with blue light will receive thermal energy corresponding to 0.032 K s^{-1} or 1.92 K min^{-1}. This is somewhat lower than the estimated heat production of 4.58 K min^{-1} in mitochondria by Chrétien *et al*. (2018), but in more pigmented cells or under more intense irradiance the produced heat may increase several fold. The assumption of the thermally isolated cell is, obviously, a theoretical construct. In the real world, the heat is (after the temperature gradient builds up) transferred to the surrounding water. Still, our calculation provides an information of the extent of the heat production in the photosynthetic membranes.

Conclusions: The main observation of this study is that the electron transport optimum of the photosynthetic reaction centers in Rhodobacterales is extended to significantly higher temperatures than that of their growth optima. This phenomenon was observed in all the studied species irrespective of the cultivation temperature. This suggests that it is an intrinsic characteristic of photosynthetic RCs. Similarly, an extended temperature optimum was also found in enzymatic system responsible for oxidative phosphorylation. We speculate that the extended temperature optimum of the photosynthetic reactions may have evolved to cope with the risk of internal overheating due to heat released from light absorbed during photosynthesis.

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