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# Growth and bacteriochlorophyll *a* formation in taxonomically diverse aerobic anoxygenic phototrophic bacteria in chemostat culture: Influence of light regimen and starvation.

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Running title: Role of illumination and starvation in aerobic phototrophic bacteria

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## Summary

The influence of light and starvation on bacteriochlorophyll *a* (bchl *a*) and biomass formation of some aerobic anoxygenic photosynthetic bacteria was investigated in chemostat culture. Three species recently isolated from dinoflagellate cultures were compared, *Dinoroseobacter shibae*, which contained relatively high amounts of bchl *a*, and *Stappia* sp. DFL-11 and *Hoeflea phototrophica*, which both have very low amounts of photosynthetic pigments. Simulating day/night alternation *D. shibae* was subjected to 7 light:dark cycles = 8 h:16 h and 3 light:dark cycles = 16 h :8 h. Using a dilution rate of 0.1 $h^{-1}$ and succinate as a substrate (8.5 mmol/l) the concentration of bchl *a* decreased during the light period due to inhibition by light and recovered always to about the previous level during the dark period. Biomass increased during the light period showing that additional energy was generated in the light.

Nutrient deficiency in the absence or presence of light was studied by exposing continuous cultures growing in the dark under the conditions described above to 8 h of illumination, starvation, i.e. interruption of medium supply, or both. The cultures of *D. shibae* exhibited a bchl *a* base level of 2.5 nmol/mg protein, which decreased reversibly in the light and increased significantly during starvation in the dark to reach a level of 4 nmol/mg protein 16 h after medium supply was resumed, indicating a slow regulatory response towards periodic starvation in *D. shibae*. Under simultaneous illumination and starvation conditions, these two effects apparently cancelled one another out, resulting in unchanged levels of bchl *a*. By contrast, the cultures of *Stappia* sp. and *H. phototropica* were characterized by bchl *a* contents of almost zero in the dark, little change under illumination or starvation, but a very marked increase after simultaneous illumina-

tion and starvation, reaching 0.16 and 0.24 nmol bchl*a*/mg protein, respectively, 16 h after medium supply was resumed. These data suggest the presence of a regulatory mechanism integrating light stimuli and starvation related metabolic signals. The different physiological behaviour of the two groups is discussed in terms of the significance of aerobic anoxygenic photosynthesis in their natural environment.

## Introduction

The aerobic anoxygenic photosynthetic bacteria (AAnPs) possess a perfect photosynthetic apparatus including light harvesting systems, reaction center, bacteriochlorophyll *a* (bchl *a*) and carotenoids just as their anaerobic counterparts, the purple nonsulfur bacteria. But whereas the purple bacteria either grow photoheterotrophically under anaerobic conditions or chemoheterotrophically under aerobic conditions, both types of energy generation are active at the same time in the AAnPs. The AAnPs are strict aerobes (with the exception of some strains, e.g. *Roseobacter denitrificans* and *D. shibae,* which are able to use nitrate as an electron acceptor) and, although mechanistically of the anoxygenic type, photosynthesis proceeds under aerobic conditions. Paradoxically the photsynthetic apparatus is synthesized only in the dark and synthesis ceases as soon as light has admission. This seems to restrict the use of light energy, since the photosynthetic complex that is left over from a preceding dark period is gradually diluted by growth. However, in nature with its diurnal light and dark rhythm this does not really constitute a problem as doubling rates per day are small, and thus the photosystem can be restored every night and is available in the morning. There are indications that supply

of organic nutrients has to be limited for photosynthetic pigment formation, suggesting that supplementary energy generation by light would render the AAnPs a selective advantage especially under the oligotrophic conditions of the open ocean (see [1] for more background).

The benefit of light periods on growth of AAnPs was first shown by Harashima et al. [2] using *Roseobacter denitrificans*. Batch cultures that were grown in the light after a 24 h dark period yielded a cell protein content twice as high as cultures that were grown permanently in the dark or in the light, and the growth rate also increased by a factor of two. Recently Kolber et al. [3] obtained a fivefold higher biomass under a day/night cycle relative to permanently dark conditions with a marine *Erythrobacter* isolate, and a 20 to 40 % higher growth rate. Results that were closer to natural conditions were presented by Yurkov and van Gemerden [4,5] using chemostat cultures of *Erythromicrobium hydrolyticum*. Under a light regimen of 14 h light and 10 h darkness the protein level was 11 % higher at the end of the light period and 4 % at the end of the dark period relative to permanently dark conditions.

Inhibition of bchl a formation by light has been unanimously confirmed by all authors concerned [2,4,6,7,8]. Very low light intensities were sufficient, i.e. half maximum inhibition occurred at 1.2 $W/m^2$ for *Roseobacter denitrificans* [9]. Only from methylotrophic AAnPs there are early reports of a positive action of light on pigment formation [10]. Although these organisms were also not able to form bchl *a* in the light, their bchl *a* content was enhanced if a light period was applied during the first part of the logarithmic growth phase and was then up to three times higher than in a culture grown in complete darkness. Shimada [11] spoke of light as a positive effector for bchl *a* synthesis. Unfor-

tunately this interesting observation has never been verified and further pursued up to now. It could be a starting point for investigations in the area of signal transduction.

The necessity of nutrient deficiency for pigment formation in aerobic anoxygenic phototrophic bacteria is highly plausible, but not well documented in the literature. It was shown that photosynthetic pigments were not formed if cultures were transferred to a rich medium or to a medium with a readily available carbon source [2,3,12]. The chemostat experiments of Yurkov and van Gemerden [4] implied substrate limiting conditions *per se*, but as shown by Suyama et al. [13] for *Roseateles depolymerans* complete absence of nutrients, i.e. periods of starvation, may be required for triggering formation of the photosynthetic complex.

We have isolated a number of marine strains that were shown to harbor the *pufL* and *pufM* genes which code for proteins of the photosynthesis reaction center [14]. One group of these strains, recently described as *Dinoroseobacter shibae* [15], was always distinctly red or pink pigmented, if grown in the dark. Two other phylogenetic groups, i.e. *Hoeflea phototrophica* [16] and *Roseovarius mucosus* [17] contained very small amounts of bchl *a* depending on the growth conditions, another group (*Stappia* sp. DFL-11, Biebl et al. unpublished) contained only traces, and in the fifth group photosynthetic pigments could not be found. Here we studied the influence of light and nutrient deficiency, singly and combined, on cell mass and bchl *a* formation in chemostat cultures in representative strains of the above groups disregarding the one without bchl *a*. Of special interest were the low pigment groups as the bchl *a* amounts hitherto detected are obviously not sufficient to provide an appreciable contribution to energy generation, and it appears possible that these organisms require conditions more relevant to their natural environment to initiate formation of the photosynthetic apparatus. Therefore nutrient

deficiency was not only investigated under substrate limitation typical of chemostat culture, but also by applying periods of total starvation. In addition a series of day/night cycles was investigated for *D. shibae* both under a short day/long night and a long day/short night regimen.

## Methods

The **strains** used in this investigation were isolated from dinoflagellate cultures as described by Allgaier et al. [14]: *Dinoroseobacter shibae* DFL-12 (= DSM 16493 = NCIMB14021) [15], *Stappia* sp. DFL-11 (= DSM 17067 = MCIMB14079) (Biebl et al., unpublished), *Hoeflea phototrophica* DFL-43 (= DSM 17068 = NCIMB 14078) [16], and *Roseovarius mucosus* DFL-24 (= DSM 17068 = NCIMB 14077) [17]. They were maintained and precultured in a medium containing 20 g sea salts (Sigma), 3 g Bacto peptone (Difco), and 0.5 g yeast extract per liter of distilled water.

The **continuous cultures** were run in a 1 l glass fermenter with a water coat for temperature control using a working volume of 0.5 l. The culture was stirred at a rate of 120 rounds/min and aerated with a flow of 40 ml/min corresponding to 0.08 vvm. Temperature was maintained at 30 °C by a cryostat to lead the heat produced by the lamps away. For illumination three 60 W krypton incandescent lamps (Osram) were mounted at a distance of 8 cm from the fermentor wall and dimmed electronically to 60 % intensity. The resulting illuminance measured at the fermenter wall was 1400 Lux (400 – 700 nm), corresponding to about 30 µE $m^2$ $sec^{-1}$.

143 The culture medium consisted of 20 g Sigma sea salts, 0.3 g $(NH_4)_2SO_4$, 0.2 g $KH_2PO_4$
144 1.37 g di-Na-succinate, 0.1 g yeast extract, 1 ml of trace element solution SL12 [18] and
145 1 ml 1M $H_2SO_4$ per litre. For strain DFL-43, which required complex nutrients and lower
146 salinity, 10 g sea salts, 2 g Bacto peptone, 0.5 g yeast extract, 1 ml SL 12 and 0.15 ml
147 1M NaOH were used. The pH stably adjusted itself to 8.0 to 8.2 and was not regulated.
148 The medium was supplied and the culture fluid was removed by peristaltic pumps ad-
149 justed to obtain a dilution rate of 0.1 (± 0.02) $h^{-1}$. The bioreactor was autoclaved and
150 inoculated with 10 % of a dark grown batch culture. Steady state was reached after
151 appr. 4 residence times.
152 **Analytical methods.** Cell density was measured as optical density at 650 nm. Cell pro-
153 tein was determined using the bicinchoninic acid (BCA) method [19] as supplied by the
154 Pierce company (Rockford, Illinois, USA). In the variation applied („micro-BCA“) cell
155 desintregration by chemical lysis and analysis were performed in one step. 0.1 ml of a
156 culture having an $OD_{650}$ of 0.5 to 1.0 was washed in PBS buffer. The pellet was sus-
157 pended in 1 ml of distilled water, and 0.5 ml of this suspension was mixed with the alka-
158 line BCA reagent (0.1 M NaOH) in a 1.5 ml Eppendorf tube. The small amount of cells
159 present was completely dissolved by the NaOH. The mixture was kept at 60 °C for 1 h
160 and then cooled to room temperature. Its absorption was measured at an OD of 562 nm
161 within 10 min. For calibration, serum albumin V (Roth, Karlsruhe, Germany) was used in
162 a concentration range from 6 to 24 mg/l. In one experiment (day/night regimen), protein
163 concentration was determined with coomassie blue [20] using the protocol of Biorad
164 (München, Germany). The cells were microscopically examined at each sampling date.
165 Bacteriochlorophyll *a* content was determined by extraction with acetone-methanol (7:2).
166 10 to 30 ml culture were centrifuged at 9000 g for 10 min. The pellet was suspended in

the drop of medium that remained after decanting, and 1 ml of the solvent was added. The volume of the pellet, which slightly dilutes the added solvent, was estimated from its weight (about 0.1 ml). Absorption was measured at 772 nm after 1 h of incubation at room temperature in absolute darkness. If very weak absorption was expected, a complete spectrum was recorded between 340 and 900 nm using an Ultrospec Plus spectrophotometer (Pharmacia, Uppsala, Sweden) to account for baseline deviations. For calculation of bchl *a* concentration an extinction coefficient of 75 mmol/(l cm) [21] was used.

## Results

*Influence of a day / night regimen in a strongly pigmented species*

To simulate natural conditions in marine environments, a chemostat culture of *Dinoroseobacter shibae* DFL-12 was exposed to a 24 h day / night regimen, running 7 cycles at 8 h light and 16 h darkness (winter conditions) and 3 cycles at 16 h light and 8 h darkness (summer conditions). The dilution rate was 0.1 $h^{-1}$ corresponding to a cell doubling time of 6.9 h. The succinate concentration in the inflow medium was 8.5 mmol/l. Fig. 1 shows that cell densities and bchl *a* concentrations showed the same course for each of the ten cycles. The OD increased somewhat during the light periods and decreased during the dark periods, reaching a constant average level after about two cycles. It was slightly below that found in the initial dark period, probably due to cell aggregates that were observed at that time. Thus, a long term increase of cell density as

a result of light /dark alternation could not be ascertained. The protein data recorded during the long day cycles corresponded to the OD values. As expected, bchl *a* concentrations decreased during illumination and increased in the dark. At the end of the longer light periods during the last three cycles, bchl *a* levels were correspondingly lower. The bchl a levels reached at the end of the dark periods were always in the same range as the level in permanent darkness or only slightly higher, indicating that in both type of cycles bchl *a* is produced up to a certain level which cannot be surpassed under steady state conditions. Accordingly, the bchl *a* level of the final dark period after 10 day/night cycles was almost the same as that of the initial dark period. The bchl *a* content at the end of each light period was somewhat higher than calculated for the case that bchl *a* formation ceased completely in the light and thus the bchl *a* present was diluted during growth. For instance, for the end of the first light period the bchl *a* content was calculated to be 0.35 nmol/ml based on complete inhibition of *de novo* synthesis and a mean residence time in the chemostat of 6.9 h, but actually a value of 0.45 nmol/l was measured. This corresponds to a bchl *a* formation rate of 0.013 nmol/h which is about 15 % of the rate of bchl *a* synthesis in *D. shibae* in permanent darkness (0.079 nmol/h). Thus, small amounts of bchl *a* were still formed during illumination, indicating that the light intensity used was not sufficient to entirely suppress pigment formation.

*Influence of light and starvation in a strongly pigmented species*

Although chemostat cultures are substrate limited *per definitionem,* they do not simulate the starvation conditions encountered in oligotrophic aquatic environments. Therefore, the effect of the complete absence of a carbon source was investigated in a continuous

culture with and without light by stopping the medium supply for 7 h (D = 0 $h^{-1}$); the culture was then under stationary conditions. All other parameters were as described above.

Fig. 2 shows the results obtained with *D. shibae*. In the first perturbation period the effect of illumination under continued nutrient supply investigated above (see Fig. 1) was reproduced. As found previously, protein concentration was higher and bchl *a* concentration was lower at the end of the perturbation period. The OD also showed a temporary decrease. After 16 h of darkness the culture had returned to the initial dark steady-state levels.

Starvation under dark conditions ($2^{nd}$ perturbation period) paradoxically resulted in an increase in protein concentration and a decrease in the OD. Probably storage material accumulated previously in the cells was used up and transformed into protein, since protein content increased. After nutrient supply was resumed, both the OD and protein concentrations returned to their original values. Interestingly, bchl *a* concentrations first declined, but after 24 h reached a higher value than before. The basic level of bchl *a* in *D. shibae* under constant darkness was 2.5 nmol/l. 24 h after the starvation period, 4 nmol/l were found in the cells. Thus, starvation must have triggered a regulatory response resulting in synthesis of bchl *a* independent of light. The repetition of this chemostat experiment showed again a large increase in bchl *a* concentration following dark starvation (data not shown).

Combined illumination and starvation ($3^{rd}$ perturbation period) showed similar effects on OD and protein concentration as starvation alone, while the bchl *a* content remained unchanged. Obviously, the stimulation of bchl *a* synthesis by starvation was compen-

sated by its light inhibition. All three parameters gradually returned to their initial values in the restored continuous culture.

*Influence of light and nutrient deficiency in weakly pigmented species*

The two strains treated in this section, *Stappia* sp. DFL-11 and *Hoeflea phototrophica* DFL-43 [16] appeared colorless in flask cultures, but the latter turned pink if grown at sea salt concentration below 1 %, particularly on agar plates. In extracted cells of liquid dark batch cultures up to 0.3 and 0.9 nmol bchl/mg protein were measured, respectively, compared to 6 nmol in *Dinoroseobacter shibae* DFL-12. Under continuous cultivation in the chemostat in the dark the bchl *a* content was close to zero and only slightly affected by illumination or starvation periods (Fig. 3 and 4). But when both factors were applied together, a sharp increase of bchl *a* concentration was obseved for both organisms 16 h after medium supply and dark conditions had been restored, resulting in 0.16 and 0.24 nmol/mg protein/ml, respectively. A minor bchl *a* peak was found for DFL-43 under starvation conditions in the dark. Cell mass parameters (OD and protein concentration) drastically decreased during starvation in DFL-11, while they increased somewhat in DFL-43. In DFL-11, cell decay most probably occurred. In the culture of DFL-43, residual nutrients may have been assimilated during starvation, since a complex medium had to be used for that strain which possibly did not establish conditions of complete organic substrate limitation.

A third strain, *Roseovarius mucosus* DFL-24, in which traces of bchl *a* had frequently been found [17], did not form any bchl *a* under the continuous culture conditions used,

and bchl *a* production could not be induced, neither by light or starvation periods nor by both.

## Discussion

In the past years it has become obvious that the photosynthetic system in strictly aerobic bacteria has an adaptive advantage only under conditions of the natural diurnal cycle of light and dark [1,3]. During the light period the bacteria have the chance to generate additional metabolic energy from light and thus save organic substrate, and during the dark period they replenish their photosystem which is necessary because of their inability to synthesize the photosynthetic apparatus in the presence of light. This is most adequately simulated in a chemostat culture under a light / dark regimen, as demonstrated by Yurkov and van Gemerden [4] for *Erythromicrobium hydrophilum*. In our experiments using *Dinoroseobacter shibae* the inhibiting effect of light periods on bchl *a* formation and the recovery of bchl *a* concentration during the dark periods was confirmed. The correspondence between the two studies is noteworthy inasmuch as the organisms studied are taxonomically not closely related. While *E. hydrophilum* is placed within the radiation of the *Sphingomonas* species (α-4 subgroup of the *Proteobacteria*), *D. shibae* is a typical member of the *Roseobacter* clade (α-3 subgroup). In addition, it was shown that bchl *a* was not only not decomposed in the light but that its formation was even continued at a low rate. In spite of the relatively high irradiation the light intensity was not sufficient to suppress bchl a formation completely, probably due to self-shadowing of the

cells in the densely grown fermenter culture. Such conditions might also occur in the natural environment, e.g. within biofilms or in very dense algal blooms.

Of special interest for the formation of the photosynthetic complex was the role of nutrient deficiency and starvation in the presence and in the absence of light as it had not yet been investigated in continuous culture and therefore at environmentally relevant conditions. We have to discriminate between changes that occurred within the starvation and/or the illumination period itself and those that occured after restoration of nutrient supply and darkness. The latter effects might be caused by restarting the general metabolism but might also be a consequence of signals induced during the starvation/illumination period. For both effects, major differences between strongly and weakly pigmented AAnPs have been found.

For the strongly pigmented species, *D. shibae*, it was demonstrated for the first time under defined conditions that the absence of organic nutrients actually enhances the bchl *a* level of the cells. As expected, simultaneous illumination attenuated the effect of starvation. The continuous cultures of the weakly pigmented strains, *Stappia* sp. DFL-11 and *Hoeflea phototrophica*, formed only traces of bchl *a* under continuous cultivation in the dark, and light or starvation had either no effect at the end of the perturbation period and thereafter or it was relatively small. However, when both factors acted at the same time, a considerable increase in bchl *a* content was observed 16 h after the culture was returned to darkness and feeding. The level was still low compared to that obtained with *D. shibae* (by a factor of 10), but the experiments showed that conditions exist that induce formation of the photosynthetic apparatus even in cultures that usually do not express their photosynthesis genes under laboratory conditions. And it might well be that for strains which have been shown to posess reaction centre protein genes and traces

of bacteriochlorophyll adequate conditions for supplementary energy generation by light have not yet been found. Our culture of *Roseovarius mucosus* that failed to form bchl *a* under the conditions used for the other three strains may be taken as an example for that. Since the bchl *a* increase was observed during the dark period that followed the light and starvation stress it can be assumed that a signal transduction system such as bacteriophytochrome may be involved, which was recently discovered in *Rhodopseudomonas palustris*, *Bradyrhizobium sp*. and *Deinococcus radiodurans* to regulate the synthesis of the photosynthetic complex or of the light protecting carotenoids [22, 23]. Whether the observed induction of bchl *a* formation can be compared to the increased bchl *a* formation after light exposure during the initial growth phase previously described in *Methylobacterium* batch cultures is doubtful, as these cultures encountered optimal nutritional supply during the illumination period [10].

The great differences in the reaction to nutrient deficiency and light in species with high and low bacteriochlorophyll content possibly reflect different ecological adaptations. Species like *Dinoroseobacter shibae*, *Roseobacter denitrificans* and *Erythromicrobium hydrolyticum* maintain a high level of bchl *a*, which is more or less independent of nutrient supply and only controlled by light. Their preferred habitats are probably the costal areas with fast turnover of organic matter. Species like *Stappia sp.* DFL-11 and *Hoeflea phototrophica* on the other hand, which contain only traces of bchl *a*, use their photosynthesis genes only under conditions of extreme shortage of organic nutrients and are thus better adapted to oligotrophic environments such as the pelagic zones of the open ocean. As discussed by Giovannoni et al. for the role of proteorhodopsin in SAR11 [24], the slightest selective advantage might be significant under permanent starvation condi-

tions. In *Roseateles depolymerans* [25] the use of the photosystem is restricted to periods of absolute nutrient deficiency during which it assists to maintain basic metabolic functions.

**Conclusion.** This work deals with the role of light and nutrient deficiency in photosynthetic pigment formation in AAnP. It is shown for the first time under close to natural conditions (chemostat) that nutrient deficiency is a positive inducer of bchl *a* synthesis which may compensate for the pigment synthesis inhibiting action of light. Continuous cultures of strains which normally produce only traces of bchl *a* do not immediately react to light and starvation periods, but form pigments after resumption of the original conditions if both pulses are simultaneously given, indicating a signal transduction effect. These experiments are a starting point for a deeper understanding of the ecological role of aerobic photosynthesis using traces of bchla, a process which is of large importance in the world oceans.

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## Legends to the figures

**Fig. 1.** Optical density, protein and bacteriochlorophyll *a* concentrations in a chemostat culture of *Dinoroseobacter shibae* under conditions of a dark/light regimen, using 7 light/dark cycles of 8 h:16 h and 3 light/dark cycles of 16 h: 8 h. The dilution rate was 0.1 $h^{-1}$, the light intensity 1400 Lux at the external reactor wall, and the organic substrate was 1.37 g/l di-Na-succinate.

**Fig. 2.** Biomass and bacteriochlorophyll *a* level in a chemostat culture of *Dinoroseobacter shibae* under dark conditions interrupted by periods of 8 h illumination, starvation (medium inflow set to zero) or both. Culture conditions as in Fig. 1.

**Fig. 3.** Biomass and bacteriochlorophyll *a* level in chemostat cultures of *Stappia* sp. DFL-11 under dark conditions interrupted by 8 h illumination, starvation or both. The periods shown are from 2 fermenter runs, periods a and c are from the same, period b from a separate one. Culture conditions as in Fig. 1.

**Fig. 4.** Biomass and bacteriochlorophyll *a* level in chemostat cultures of *Hoeflea phototrophica* DFL-43 under dark conditions interrupted by 8 h illumination, starvation or both. Culture conditions as in Fig. 1, with the exception of organic substrate being 2 g/l Bacto peptone and 0.5 g/l yeast extract.