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O_2-stimulated synthesis of bacteriochlorophyll and carotenoids in marine bacteria

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We examined the effects of a limitation of the O_2-supply on the syntheses of bacteriochlorophyll a_P and carotenoids in isolates of aerobic marine bacteria, OCh 101 and OCh 114, grown heterotrophically. Whereas they formed these pigments fairly well under high aeration in the dark, a limitation of the O_2-supply resulted in the decreased syntheses of bacteriochlorophyll in both strains. Syntheses of carotenoids also were depressed under low aeration but to a lesser extent (especially in OCh 101) than the depression of bacteriochlorophyll synthesis. Aerobic incubation of a culture of OCh 101, that previously had been grown semiaerobically, induced the supplementary synthesis of bacteriochlorophyll. This induction was inhibited almost completely by chloramphenicol. The absorption spectra of suspensions and solvent extracts of cells grown aerobically or semiaerobically are reported.

Key words: Aerobic bacteria — Bacteriochlorophyll a_P — Bacteriochlorophyll synthesis — Carotenoids — Marine bacteria — O_2-stimulation of bacteriochlorophyll synthesis.

Recently Sato and Shimizu reported the presence of minute amounts of Bchl *a* in methanol-utilizing bacteria grown aerobically in the presence of propanediol (*15*, *17*). We also have demonstrated that there are considerable amounts of Bchl *a* in aerobically grown cells of isolates from the marine bacteria, OCh 101 (*7*) and OCh 114 (*18*). Many other strains of aerobic marine bacteria have been isolated which synthesize Bchl (*19*). Since they did not grow under anaerobic conditions, even in the light, they cannot be considered photosynthetic bacteria of known types. On the other hand, O_2 is known to inhibit and repress Bchl synthesis in photosynthetic bacteria. Only trace amounts of Bchl are found in cells of facultative phototrophs growing aerobically. A lowering of the O_2-tension (below threshold levels), in the dark as well as in the light, induces the synthesis of Bchl in cells previously grown aerobically (*2*, *4*, *11*, *14*). Therefore, it is notable that the bacteria isolated by our research group synthesize considerable amounts of Bchl under high aeration. It is important to know, whether the lowering of the O_2-tension stimulates Bchl synthesis in these bacterial strains, as in facultative phototrophs.

Abbreviations: Bchl, bacteriochlorophyll; Bchl a_P, bacteriochlorophyll *a* containing phytol as the esterifying alcohol; Bchl a_{Gg}, bacteriochlorophyll *a* containing, instead of phytol, geranylgeraniol; CAP, chloramphenicol; TLC, thin-layer chromatography.

We here report the results of experiments that examined the effects of the limitation of the O_2-supply on the syntheses of Bchl and carotenoids in these bacteria. Unexpectedly, aerobic conditions were favorable for the Bchl syntheses. Limitation of the O_2-supply appeared to repress (or inhibit) Bchl synthesis. A supplement of O_2 to a culture of OCh 101 previously grown semi-aerobically induced the synthesis of extra Bchl. This induced synthesis of Bchl was inhibited almost completely by CAP. Some depression of carotenoid synthesis also was observed under low aeration.

Materials and methods

Bacterial strains

The bacterial strains, OCh 101 and OCh 114, isolates from the surfaces of thalli of the green seaweed, *Enteromorpha linza* (*7*, *18*), were used. Taxonomical characterization of these strains will be reported elsewhere[3]. They grow well aerobically and synthesize Bchl well, but do not grow anaerobically (e.g., under an atmosphere of N_2) even in the light. Light represses Bchl synthesis in OCh 101 (*7*). A short inspection with a fluorescent lamp has no effect on Bchl synthesis in this bacterium. The effect of light on Bchl synthesis in OCh 114 is not clear. The bacterial strains were stocked on PPES-II agar slants (*21*) at room temperature under room light.

Culture media

The culture medium for OCh 101 (C-G-A medium) was prepared by dissolving 1 g of glucose, 3 g of Casamino Acids (Difco, vitamin-free), 1.55 g of Na-aspartate, 0.05 g of Bacto-yeast extract (Difco), 0.05 g of KH_2PO_4, 0.5 g of tris(hydroxymethyl)methylaminopropanesulfonic acid (Dotite), 10 mg of ferric citrate, 1 mg of thiamine-HCl, 1 mg of Ca-pantothenate, 10 μg of biotin and 10 μg of cyanocobalamin in 1 liter of artificial sea water (*13*), the major components of which were NaCl (20 g/liter), KCl (2 g/liter), $MgSO_4 \cdot 7H_2O$ (4 g/liter) and $CaCl_2 \cdot 2H_2O$ (0.3 g/liter). The pH of the medium was adjusted to 7.6–7.8 before autoclaving the medium at 120°C for 15 min. Vitamins were sterilized separately in a concentrated solution in 0.02 N acetic acid and added before inoculation to the sterilized medium without vitamins. Aspartate was added, because the amount of Bchl synthesized by OCh 101 without added aspartate was smaller by about 25–40% (data not shown).

The culture medium for OCh 114 (enriched PPES-II medium) was PPES-II medium (*21*) modified by doubling the concentrations of polypepton (Daigo Seiyaku), Proteose peptone No. 3 (Difco), Bacto-yeast extract (Difco) and Bacto-soytone (Difco).

Culture

All the cultures were grown at 25°C in the dark. Cells of a stock culture were inoculated into 30 ml of medium in a 300-ml conical flask and cultured aerobically on a rotatory shaker (200 rpm). After 3 days the culture was used for the inoculation.

For aerobic growth, 100 ml of the medium in a 500-ml conical flask was inoculat-

[3] Shiba, T. and U. Simidu, in preparation.

ed with 1 ml of the inoculum, then cultivated on a rotatory shaker (200 rpm). For semi-aerobic growth, 500 ml of medium in a 500-ml conical flask was inoculated with 5 ml of the inoculum, then cultivated on a magnetic stirrer (*3*) (stirring bar 3 cm long, and approximately 400 or 500 rpm for OCh 101 or 400 rpm for OCh 114). When OCh 101 was cultured on a magnetic stirrer at about 500 rpm, the concentration of O_2 dissolved in the culture medium (measured with an oxygen electrode) fell to 0.9 ppm (equivalent to the value in medium saturated with a gas at the partial pressure of O_2: 20 mm Hg) 24 hr after inoculation. Cells grown under the described conditions were designated aerobic and semi-aerobic cells, respectively.

Analysis

After growing for the indicated periods, cultures were analyzed for their Bchl and dry cell weights. Cells in 40 ml of medium were harvested by centrifugation at $10,000 \times g$ for 15 min then washed twice with 10 ml of 3% NaCl. The packed cell mass was used to determine Bchl or the dry weight. To determine the dry weight, we dried the mass of packed cells at 105°C until we found a constant weight. The values for the dry weight were corrected for NaCl (included in the washing solution attached to the tubes for centrifugation) by subtracting 2.4 mg (per 40 ml of culture) from each observed value.

To determine the Bchl *a* we extracted a mass of packed cells 3 times with 1–4 ml (equivalent to 10 times the volume of the mass of the cells) cold methanol. The extracts were separated from the insoluble materials by centrifugation, after which they were combined and stored at −20°C in the dark until the measurement of their light absorbances. The amount of Bchl *a* in an extract was calculated from the absorbance at 770 nm, based on the mM absorption coefficient [Bchl *a*, 42.0 (*20*)]. If necessary, the absorption spectrum was recorded.

Since very little of the carotenoids of OCh 114 were extracted with methanol, a cell mass was extracted several times with cold acetone. These extracts were combined, and the absorption spectrum of the combined solution was recorded.

To obtain the spectra of the cell-suspensions, cells of 3-day cultures were harvested and washed as described above then resuspended in appropriate volumes of 3% NaCl. A 1.6 g portion of sucrose was dissolved in 2 ml of each suspension, and the light absorbances of the solution were recorded against deinonized water.

Visible and near-infrared absorption spectra were recorded with a Hitachi spectrophotometer, Model 124, equipped with a recorder, Model 056. To record the absorption spectra of the cell-suspensions, we attached an integrating sphere, Model 0049, to the photometer.

Isolation of Bchl a

Bchls from cells of OCh 101 and OCh 114 were isolated as reported previously (*7*, *18*). Authentic samples of Bchl a_P and Bchl a_{Gg} were prepared according to Sato and Murata (*16*) from *Rhodopseudomonas capsulata* (NCIB 8254) and *Rhodospirillum rubrum* (NCIB 8255) (*1*, *10*), respectively.

TLC of Bchl a

The TLC of Bchl *a* was carried out as described previously (*7*, *18*). The reversed phase system according to Egger (*5*) was used.

Chemical

CAP was purchased from Sankyo Co. Ltd.

Results

Identity of Bchl a

Previously we identified the Bchl of OCh 101 with the Bchl *a* isolated from *Chromatium vinosum* (*7*) and the Bchl of OCh 114 with Bchl a_P (*18*). Since the reversed phase TLC used to identify the Bchl of OCh 101, according to Egger (*5*), was effective for the separation of Bchl a_P and Bchl a_{Gg} (*18*) and since the Bchl of *C. vinosum* has been identified with Bchl a_P (*6*), the Bchl of OCh 101 must be Bchl a_P. This was confirmed by reexamination by TLC. Fig. 1 shows the results of this experiment. The Bchl of OCh 101 was distinct from Bchl a_{Gg}, but was not separated from Bchl a_P or from the Bchl of OCh 114. Therefore, the Bchl of OCh 101 was unequivocally identified with Bchl a_P.

Repression and induction of Bchl synthesis in OCh 101 due to a decrease and increase in O_2-supply, respectively

Fig. 2 shows the time courses of growth and Bchl synthesis in OCh 101 under aerobic and semi-aerobic conditions. Although both growth and Bchl synthesis were depressed by limiting the O_2-supply, the decrease in the rate of Bchl synthesis was greater than the decrease in the growth rate. Whereas, at the early logarithmic growth phase (27 hr after inoculation), the cell-yields of the semi-aerobic culture (0.33 mg as dry weight/ml) was about 2/3 that of the aerobic culture (0.49 mg as dry weight/ml), Bchl content in the former (0.27 nmole/ml) was less than 1/3 of that in the latter (0.88 nmole/ml). Consequently, the specific Bchl content (per mg dry

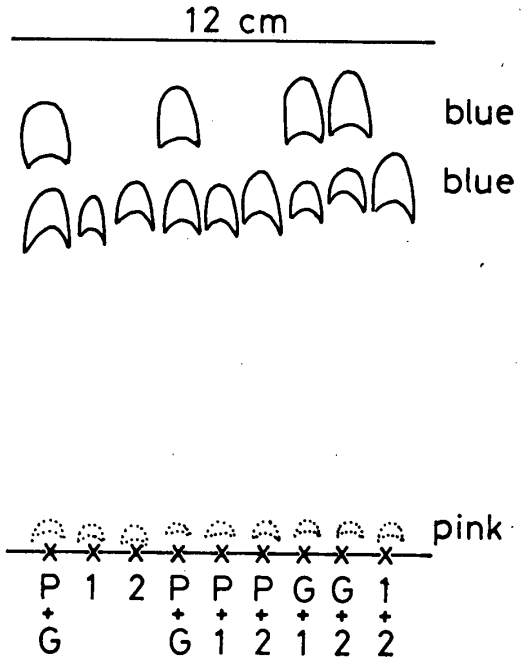

Fig. 1. *TLC of the Bchl of OCh 101 and OCh 114.* Hyflosuper Cel-triolein/methanol-acetone-water (80 : 16 : 9). The solvent mixture was saturated with triolein. 1: Bchl of OCh 101; 2: Bchl of OCh 114; P: Bchl a_P; G: Bchl a_{Gg}.

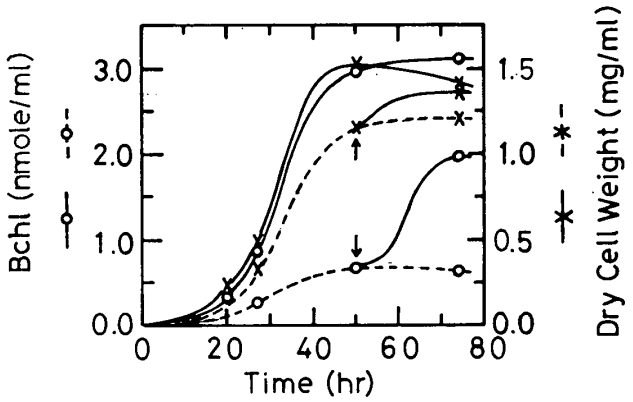

Fig. 2. *Time courses of growth and Bchl synthesis in OCh 101 under aerobic and semi-aerobic conditions.* ——: aerobic growth, ----: semi-aerobic growth. After 50-hr of cultivation (arrows), part (90 ml) of the semi-aerobic culture, which had been aerated by stirring at about 500 rpm, was withdrawn and cultivated for another 24 hr on a rotatory shaker in a 500-ml conical flask (——).

weight) in the semiaerobic cells (27-hr culture, 0.82 nmole; 50-hr culture, 0.57 nmole) was considerably lower than that in the aerobic cells (27-hr culture, 1.78 nmoles; 50-hr culture, 1.90 nmoles) throughout the growth cycle.

After cultivation for 50 hr (shown by arrows) a part (90 ml) of the semi-aerobic culture (of a late logarithmic growth phase) was withdrawn and further cultivated for 24 hr on a rotatory shaker in a 500-ml conical flask. Thus, the culture condition was shifted (from semi-aerobic) to aerobic, after which stimulation of growth and Bchl synthesis took place. Although, the increase in dry cell weight was small (0.21 mg/ml), a considerable amount of Bchl (1.32 nmoles/ml) was synthesized. The specific Bchl content in the cells rose (from 0.57) to 1.44 nmoles per mg of dry

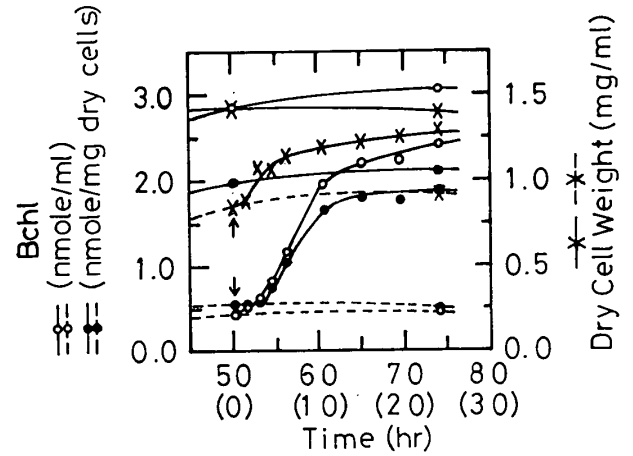

Fig. 3. *Time course of O_2-stimulated synthesis of Bchl in OCh 101 previously grown semi-aerobically.* ——: aerobic growth, ----: semi-aerobic growth. After 50-hr of semi-aerobic cultivation by magnetic stirring at about 500 rpm (as described in **Materials and methods** and as indicated by the arrows) 100 ml portions of the culture were cultured further aerobically (i.e. aerated by rotatory shaking at 200 rpm in 500-ml conical flasks) for the indicated periods (figures in parentheses) then analyzed for Bchl and dry cell weight. Dry cell weights and Bchl contents in cultures grown only aerobically or semiaerobically are also plotted.

Table 1 *Inhibition by CAP of the O_2-slimulated synthesis of Bchl in cells of OCh 101 previously grown semi-aerobically*

Condition of aeration	CAP[a]	Bchl increased (nmole/ml)	Dry cell weight increased (mg/ml)
High[b]	−	1.32	0.21
	+	0.02	0.11
Low[c]	−	−0.03	0.05
	+	−0.03	−0.01

An OCh 101 culture, which had been grown for 50 hr semi-aerobically (i.e. aerated by magnetic stirring at about 500 rpm as described in **Materials and methods**) and which contained 1.16 mg of dry cells and 0.67 nmole of Bchl per ml, was aerated for another 24 hr under the indicated conditions.
[a] CAP (10 μg/ml of the culture) was added at the start of aeration.
[b] A 90-ml portion of the culture was aerated by rotatory shaking (200 rpm) in a 500-ml conical flask.
[c] A 300-ml portion of the culture was aerated by magnetic stirring at about 400 rpm in a 300-ml conical flask.

weight. This increase in Bchl is not explainable by the meager growth during aerobic cultivation (following the previous semi-aerobic culture for 50 hr).

Fig. 3 shows results of a separate experiment tracing the time course of Bchl synthesis induced by a supplement of O_2 to a culture of OCh 101 previously grown semi-aerobically. The increase in the specific content of Bchl (per mg dry cells) began after a time lag (3 hr). This time lag varied from experiment to experiment, but was always within 8 hr. The stimulation of Bchl synthesis by a supplement of O_2 was inhibited almost completely by CAP (Table 1). Therefore, O_2-stimulated synthesis of Bchl in cells previously grown semi-aerobically probably requires protein synthesis and is adaptive, as in the response of aerobic cells of facultative phototrophs to low aeration (*8, 11*).

The above results are evidence that Bchl synthesis in OCh 101 was repressed by limiting the O_2 supply and was induced by restoring it.

Alteration of the absorption spectrum in a suspension of OCh 101 cells due to a limitation of the O_2 supply

Fig. 4 shows visible and near-infrared absorption spectra of suspensions of cells of OCh 101 grown aerobically (curve I) and semi-aerobically (curve II). A difference spectrum (I minus II, curve III) also is given[4]. In addition to a broad and intense band of carotenoids (λ_{max}: 465 nm), a sharp band (λ_{max}: 865 nm) and a minor band (λ_{max}: 802 nm) were observed in the near-infrared region of the spectrum of aerobic cells. From the differences between the light absorbances at λ_{max} and those at 780 nm, we judged that the intensities of the near-infrared bands in the spectrum of semi-aerobic cells were only about 1/7 those of aerobic cells. Since the

[4] O_2 was introduced into the semi-aerobic culture with stirring at about 400 rpm (slower than in the experiment of Fig. 2). Therefore, the cell-yield and the specific Bchl content in the semi-aerobic cells were lower than those described in the preceding section.

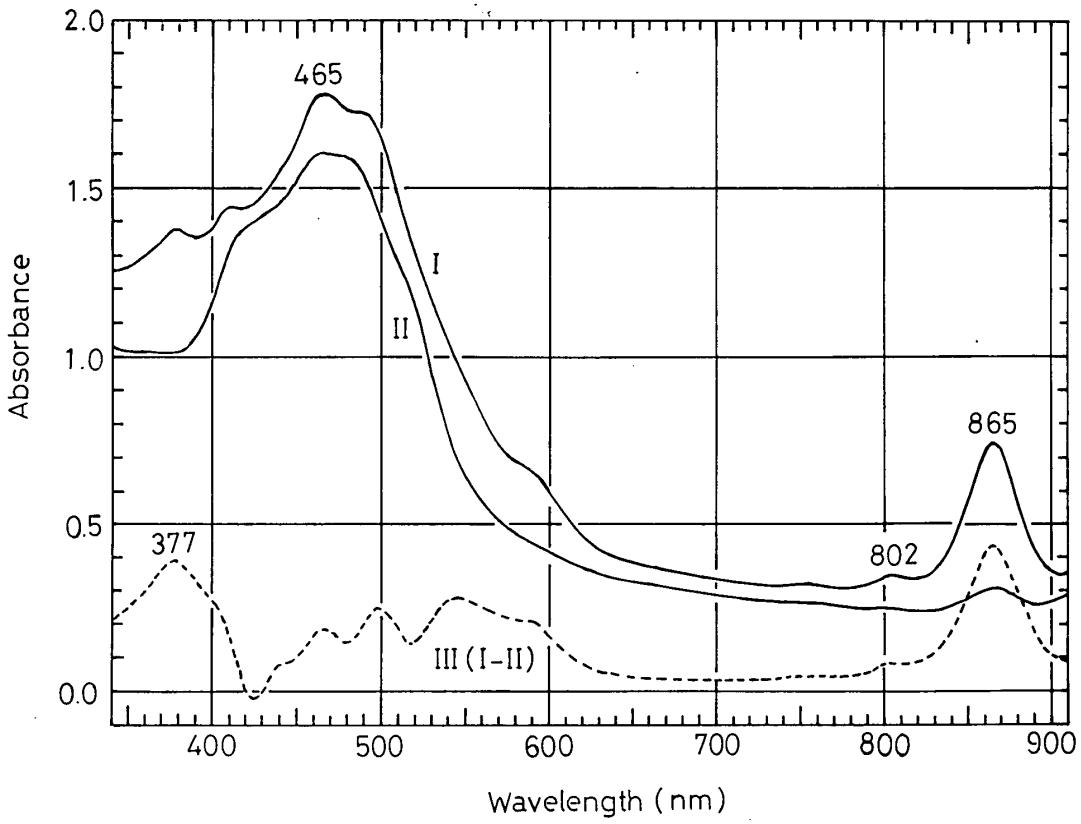

Fig. 4. *Visible and near-infrared absorption spectra of suspensions of aerobic and semi-aerobic cells of OCh 101.* I: aerobic cells (2.25 nmoles Bchl/mg dry weight), II: semi-aerobic cells (0.38 nmole Bchl/mg dry weight), III: absorption spectrum of the suspension of aerobic cells recorded against the suspension of the semi-aerobic cells (difference spectrum, I minus II). Cell suspensions[a] (4.0 mg dry cells/ml for aerobic cells and 3.9 mg dry cells/ml for semi-aerobic cells) in 3% NaCl were prepared and their light absorbances were recorded as described in **Materials and methods.**

[a] Cell portions were harvested from each of the 3-day aerobic and semi-aerobic cultures of OCh 101. O_2 was supplied to the semiaerobic culture by stirring the culture at about 400 rpm.

specific Bchl content in the semiaerobic cells was about 1/6 that in the aerobic cells, (calculated from the values in the legend of Fig. 4) the intensities of the two near-infrared bands were roughly proportional to the Bchl contents. Evidently, the 802- and 865-nm bands belonged to Bchl *a*, which was probably bound to a protein (or proteins), as reported for typical purple bacteria (*22*). Very weak bands also were found near 750 nm in the spectra of both aerobic and semiaerobic cells. In the difference spectrum (curve III, I—II) a Solet band (λ_{max}: 377 nm) of Bchl *a* was observed.

The spectrum of the semi-aerobic cells did not differ distinctly in the carotenoid region (400–550 nm) from that of the aerobic cells. However, the difference spectrum (III, I—II) shows, that the aerobic cells were more bathochromic than the semiaerobic cells. This difference was observed more clearly between the spectra of the methanolic extracts of the semi-aerobic and the aerobic cells (Fig. 5). Since the carotenoids in this bacterium can be extracted from the cells almost completely with methanol, the carotenoid composition in the semi-aerobic cells must differ

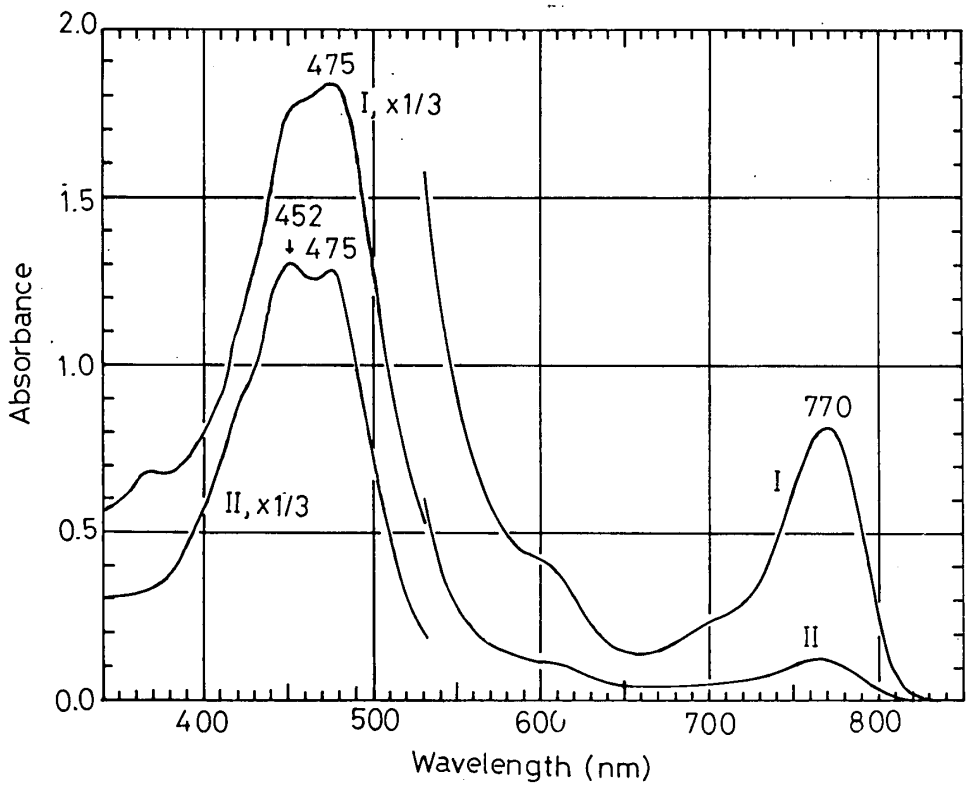

Fig. 5. *Absorption spectra of methanolic extracts of aerobic and semi-aerobic cells of OCh 101.* I: aerobic cells (2.25 nmoles Bchl/mg dry weight), II: semi-aerobic cells: (0.38 nmole Bchl/mg dry weight). Aerobic or semi-aerobic cells[a] equivalent to 87.8 mg of dry weight were extracted with methanol as described in **Materials and methods.** Each of the combined extracts was brought up to 10 ml and its light absorbances were recorded. Spectra in the carotenoid region were recorded after a 3-fold dilution.

[a] For the cultures from which the cells were harvested, see the legend to Fig. 4.

from that in the aerobic cells. Fig. 5 also shows, that the total amount of carotenoids in the semi-aerobic cells was somewhat lower than that in the aerobic cells. However, the extent of the decrease in the carotenoid content caused by the decline in the O_2-supply was limited. Because of the difference in the absorption spectra, i.e. the difference in carotenoid composition, the color of the semiaerobic culture (orange-yellow) was differed from that of the aerobic culture (orange-red).

Biosynthesis of Bchl and carotenoids in OCh 114

As with OCh 101, the syntheses of Bchl and carotenoids were depressed when O_2 was limited in OCh 114, although the depression was not extensive. Fig. 6 shows the absorption spectra of methanolic extracts of aerobic (solid line, I) and semi-aerobic cells (solid line, II). Although the aerobic cells contained (per mg dry weight) 8.15 nmoles of Bchl, the semiaerobic cells contained only 2.61 nmoles of Bchl (slightly lower than 1/3 the specific content in aerobic cells). The absorption spectra of acetone extracts of the aerobic (broken line, I) and the semi-aerobic cells (broken line, II) are also shown in this figure. From the light absorbances at λ_{max} (480 nm), we judged that the total amount of carotenoids in the semi-aerobic cells

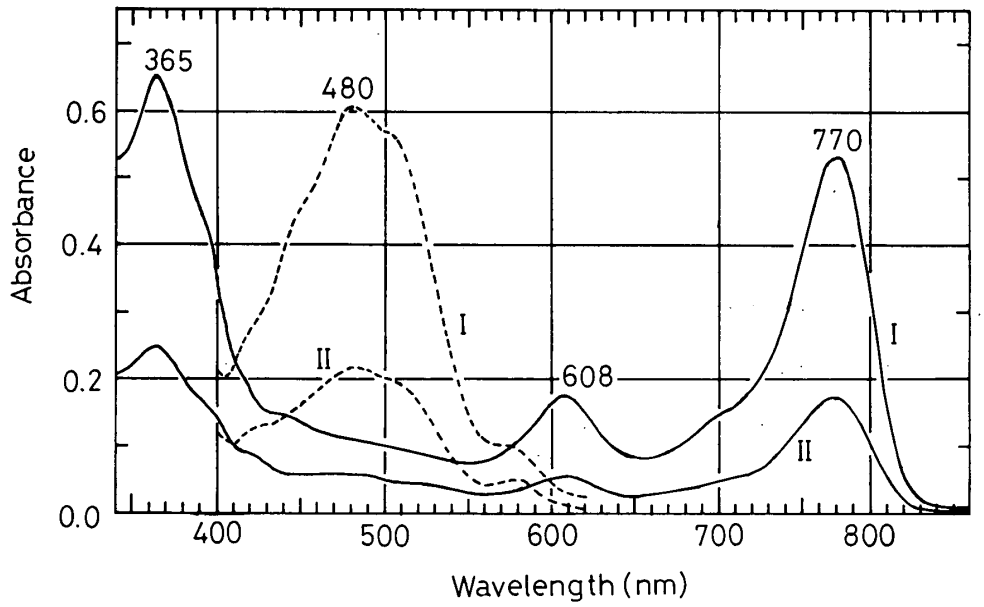

Fig. 6. *Absorption spectra of solvent extracts of aerobic and semi-aerobic cells of OCh 114.* I: aerobic cells (8.15 nmoles Bchl/mg dry weight), II: semi-aerobic cells (2.61 nmoles Bchl/mg dry weight). Aerobic or semi-aerobic cells[a] equivalent to 30.5 mg of dry weight were extracted with methanol (——) or acetone (----) as described in **Materials and methods**. Each of the combined extracts was brought up to 20 ml and its light absorbances were recorded.

[a] Cell portions were harvested from each of the 3-day aerobic and semi-aerobic cultures of OCh 114. O_2 was supplied to the semi-aerobic culture by stirring the culture at about 400 rpm.

was slightly higher than 1/3 that in the aerobic cells. Thus, the depression of Bchl synthesis due to a limtation of the O_2 supply was accompanied by the depression of carotenoid synthesis. However, the depression of carotenoid synthesis was slightly less than that of Bchl synthesis.

Whether any difference in carotenoid composition existed between the aerobic and semi-aerobic cells is not clear since no remarkable difference was observed between the shapes of the spectra of the acetone extracts.

Absorption spectra of suspensions of the aerobic (curve I) and the semi-aerobic cells (curve II) are shown in Fig. 7. A broad band (λ_{max}: 510 nm) in the carotenoid region and two sharp bands (λ_{max}: 806 and 873 nm) in the near-infrared region were seen in the spectrum of the aerobic cells. Intensities of all the bands in the spectrum of the semi-aerobic cells were considerably lower than those of the aerobic cells. As with OCh 101 and purple bacteria (*22*), the two near-infrared bands were attributable to Bchl *a*, which was probably bound to proteins. Relative intensities of the two bands were altered depending on the culture conditions. Conditions that depressed Bchl synthesis (e.g. low aeration) lowered the intensity of the 806-nm band relative to that of the 873 nm-band. In one case, the 806-nm band shifted to 810 nm (data not shown). A very weak band was observed near 750 nm in the spectrum of aerobic cells as well as in the spectrum of semi-aerobic cells.

Discussion

The rate of Bchl synthesis in facultative phototrophs is reversely related to O_2-

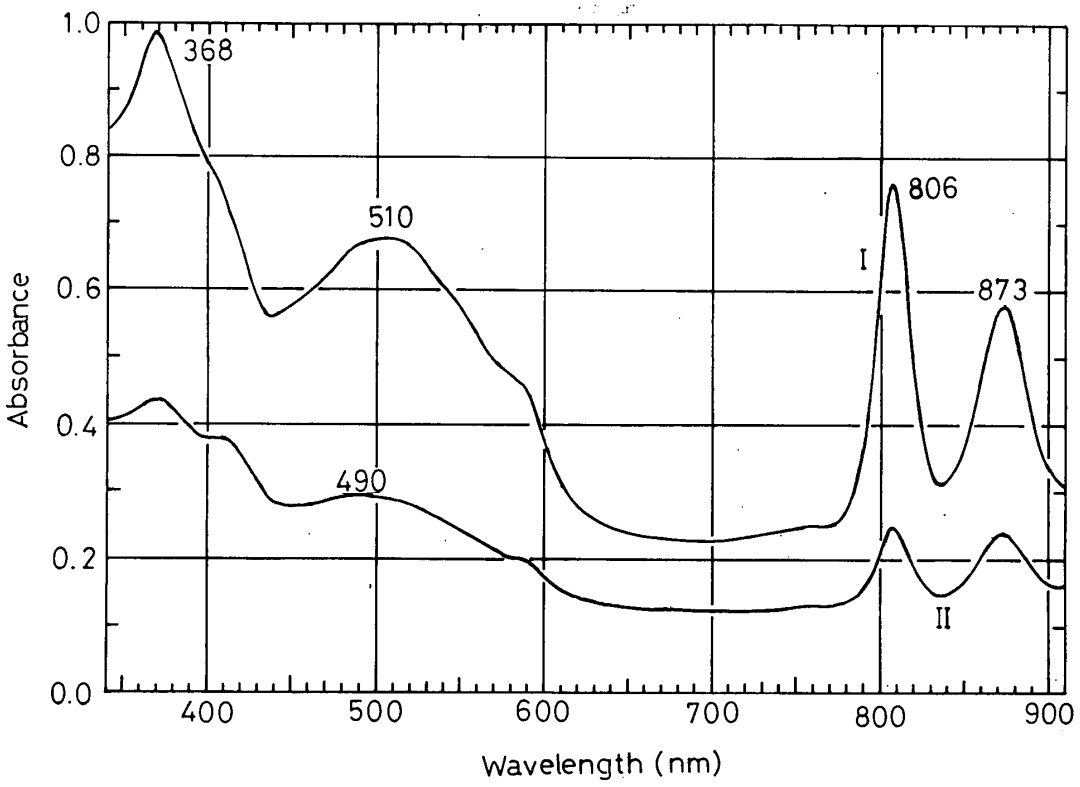

Fig. 7. *Visible and near-infrared absorption spectra of aerobic and semi-aerobic cells of OCh 114.* I: aerobic cells (8.15 nmoles Bchl/mg dry weight), II: semi-aerobic cells (2.61 nmoles Bchl/mg dry weight). Cell suspensions[a] (1.45 mg dry cells/ml for both aerobic and semi-aerobic cells) were prepared and their light absorbances were recorded as described in **Materials and methods.**

[a] For cultures from which cells were harvested, see the legend to Fig. 6.

tension. Even mutants of *Rhodopseudomonas spheroides*, which are capable of synthesizing Bchl under aerobic conditions, synthesize Bchl in larger amounts semi-aerobically than aerobically (*12*). Therefore, our observations, that Bchl syntheses in OCh 101 and OCh 114 were repressed (or inhibited) by a decline in the O_2 supply and were induced by aerobiosis, rule out the possibility that these bacterial strains might be mutant derivatives of known facultative phototrophs, in which the threshold levels of the O_2-tension that allows Bchl synthesis are unusually high. In spite of some similarlities of OCh 101 and OCh 114 to non-sulphur purple bacteria[5], they must be taxonomically different from the photosynthetic bacteria of hitherto known types. Whereas facultative phototrophs adapt to anaerobiosis by developing intracytoplasmic membranes carrying Bchl and carotenoids and by utilizing light energy instead of respiration (*4*, *14*), OCh 101 and OCh 114 do not adapt to anaerobiosis in a similar manner. Conversely, high aeration induces these bacteria to produce Bchl. Therefore, the possibility is very low that they might be able to grow anaerobically in the light under any unknown conditions.

Nevertheless, whether they can utilize light energy is not clear. Bchl may function under aerobic conditions. The contents of Bchl in aerobic cells of OCh 101

[5] See the footnote 3 (p. 1284)

(2 nmoles/mg dry weight) and OCh 114 (8 nmoles/mg dry weight) are sufficiently high for us to expect this. Moreover, in vivo absorption spectra of Bchl *a* in cells of OCh 101 and OCh 114 were characteristic of the protein-bound forms functioning in typical purple bacteria (*22*). However, the roles of Bchl in these bacteria must be determined by further investigation.

The mechanism that regulates Bchl synthesis in these bacteria by the O_2 supply is unknown. However, inhibition of the O_2-stimulation of Bchl synthesis in cells previously grown semiaerobically, by CAP, suggests that Bchl synthesis is inducible by high aeration and O_2 regulates Bchl synthesis through protein synthesis, probably either through the synthesis of enzymes involved in Bchl synthesis or through the synthesis of the proteins that will bind to Bchl. The possibility that O_2 is a direct reactant for Bchl synthesis [for example an electron acceptor for dehydrogenation processes in the pathway for Bchl synthesis (*9*)] cannot be ruled out. However, the presence of a lag time for the initiation of Bchl synthesis in semi-aerobic cells of OCh 101 induced by high aeration cannot be explained by the direct participation of O_2 as a reactant in the pathway for Bchl synthesis.

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