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Influence of vitamin B_{12} and light on the formation of chlorosomes in green- and brown-colored *Chlorobium* species

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Abstract. The specific Bchl *a* and *c* content of the vitamin B_{12}-dependent *Chlorobium limicola* strain 1230 decreased strongly under vitamin B_{12} limitation. In comparison to a regularly grown culture (20 µg vitamin B_{12}/l) the specific Bchl *c* content of a B_{12}-limited culture was reduced to 20% and the specific Bchl *a* content to 42%. By ultrathin sections it could be clearly demonstrated that B_{12}-deficient cells contained no chlorosomes. After the addition of vitamin B_{12} to a deficient culture, chlorosomes were formed and the Bchl *a* and *c* content increased again to the level of regularly grown cells. The brown-colored *Chlorobium phaeobacteroides* strain 2430 (type strain) and the extremely low-light-adapted strain MN1 were compared with respect to the influence of light on the formation of chlorosomes and the Bchl *e* and carotenoid content. By ultrathin sections it could be demonstrated that strain MN1 produced two-fold larger chlorosomes. Chlorosome dimensions of strain MN1 decreased with increasing light intensities. The number of chlorosomes per cell in both strains did not change with different light intensities. Strain MN1 formed twice as much Bchl *e* as the type strain when grown at 30 or below 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Under comparable light conditions strain MN1 formed 14–57% more carotenoids than the type strain. Low light intensities caused the carotenoid content to increase by 25% in strain 2430 in comparison to high light intensity.

Key words: *Chlorobium limicola* – *Chlorobium phaeobacteroides* – Bacteriochlorophylls – Chlorosomes – Vitamin B_{12} – Light intensities

Green sulfur bacteria are obligately anoxygenic phototrophs which thrive in the anoxic zones of freshwater or marine habitats. In contrast to other phototrophic organisms, green sulfur bacteria are very well adapted to high sulfide concentrations and low light intensities (Trüper and Pfennig 1991; Overmann et al. 1992a). A brown-colored *Chlorobium phaeobacteroides* strain MN1 has recently been isolated from a sample of the chemocline of the Black Sea at a depth of 80 m. This strain is able to grow under severe light limitation (0.25 $\mu mol \cdot m^{-2} \times s^{-1}$). Its adaptation is achieved by an increase of the antenna pigment content and by a very low maintenance energy requirement (Overmann et al. 1992a). Green phototrophic bacteria possess highly effective light-harvesting bodies, the chlorosomes, which contain as antenna pigments either Bchl *c*, *d* or *e* and most of the carotenoids. The antenna Bchls are presumably bound to specific binding polypeptides and are probably arranged in the form of rod-shaped elements (Wagner-Huber et al. 1990; Zuber and Brunisholz 1991). Decreasing light intensities cause an increase in the number of chlorosomes per cell, but their length or width are not affected (Holt et al. 1966). However, thin sections of *Chlorobium limicola* f. *thiosulfatophilum* revealed that the chlorosomes are larger at low light than at high light conditions (Broch-Due et al. 1978).

Besides light intensity, the vitamin B_{12} concentration may influence the formation of chlorosomes. In cultures of vitamin B_{12}-auxotrophic *Chlorobium limicola* strains, the absorption spectra of whole cells varied with the vitamin B_{12} concentration (Pfennig and Lippert 1966). During B_{12}-limited growth the color of the culture changed from dark-green to green-yellow and the Bchl *c* content was extremely reduced. Coenzyme B_{12} is involved in enzymatic transfer of methyl groups and in carbon rearrangement reactions (Taylor 1982; Halpern 1985).

It was the aim of this study to examine the influence of either vitamin B_{12} or light on chlorosome formation and Bchl content in two green-colored *Chlorobium* strains and in a low-light-adapted brown *Chlorobium* strain, respectively.

Materials and methods

Organisms, culture conditions and harvest of cells

Chlorobium limicola strain 1230 is strictly dependent on vitamin B_{12} for growth. It was grown photolithoautotrophically at 28 °C

Abbreviation: Bchl, bacteriochlorophyll

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and 260 μmol · m^{-2} · s^{-1}. The culture medium (prepared and modified after Pfennig and Trüper 1989) contained in 1 l: 0.25 g KH_2PO_4; 0.4 g NH_4Cl; 0.34 g KCl; 0.5 g $MgSO_4 \times 6\,H_2O$; 0.25 g $CaCl_2 \times 2\,H_2O$; 20 μg vitamin B_{12}, 1 ml of a H_3BO_3-free trace element solution SL 12 (Overmann et al. 1992b); 0.06 mg H_3BO_3; 1.5 g $NaHCO_3$, and 0.36 g $Na_2S \times 9\,H_2O$. Sterile stock solutions of vitamin B_{12} (0.002%), SL 12, H_3BO_3 (0.03%), $NaHCO_3$ (7.5%), and sodium sulfide (24%) were prepared separately and were added to the autoclaved basal growth medium in the concentration required. The pH of the freshly prepared medium was adjusted to pH 6.9 with sterile HCl or Na_2CO_3 (2 mol/l each).

Vitamin B_{12}-limited cell cultures were obtained by transfer of 0.5–2 ml of a regularly grown culture to a 100 ml screw cap bottle containing vitamin B_{12}-free medium. In order to achieve high population density and vitamin B_{12} limitation, repeated additions of neutralized sodium sulfide solution (Siefert and Pfennig 1984) were necessary.

Chlorobium vibrioforme strain NCIB 8327 (DSM No. 263) does not require vitamin B_{12} for growth. It was grown under the same conditions as mentioned above in the medium described by Steinmetz and Fischer (1982). Enhancement of cell yield was obtained by feeding the cultures several times with a mixture of 0.6 g sodium sulfide and 0.1 g bicarbonate, dissolved in 40 ml dist. water and partially neutralized with 1.5–2 ml sterile 2 M H_2SO_4.

Chlorobium phaeobacteroides strain 2430 (type strain) and strain MN 1 (low-light-adapted strain from the Black Sea) were grown photolithoautotrophically in 100 ml screw cap bottles at 20 °C and 0.25, 0.5 and 30 μmol · m^{-2} · s^{-1} in the media described by Overmann (1991). Cells of all *Chlorobium* strains were harvested with a Beckman centrifuge J2–21 (Beckmann Instruments, München, Germany) at 4 °C and at 15,000 × *g* for 20 min. The pellet was washed once in 0.1 M Tris-HCl buffer, pH 7.5 and, if not used directly, stored at −18 °C.

Spectrophotometric determination

Absorption spectra were recorded in the range of 300–850 nm and were determined in a Kontron Uvikon 860 double beam spectrophotometer (Kontron Instruments, Neufahrn, Germany) using 1 cm quartz cells. Spectra of whole cells were obtained by adding 50% sucrose to cultures suspended in 50 mM potassium phosphate buffer, pH 7.0.

Determination of Bchls and protein

Cell material was harvested by centrifugation in an Eppendorf 5415C centrifuge at 15,000 × *g* for 20 min. Bchls were obtained by extracting the pellet with methanol (96%) in the dark for 1 h at room temperature before the suspension was centrifuged again (see above). Bchl concentrations were determined from the supernatant at 658 nm (Bchl *d*), 670 nm (Bchl *c*), 659 nm (Bchl *e*), and 770 nm (Bchl *a*) using extinction coefficients ($g^{-1} \cdot l \cdot cm$) of 82.3 (Bchl *d* and *e*), 86.0 (Bchl *c*), and 84.1 (Bchl *a*) (Stal et al. 1984). The pellet of the methanol extraction was heated at 95 °C for 5 min in 1 N NaOH, allowed to dissolve over night and was then used for protein determination according to Bradford (1976).

Electron microscopy

For ultrathin sections the cells were fixed in 6% glutardialdehyde for 90 min at 4 °C, postfixed in 2% OsO_4 for 2 h, dehydrated and embedded in the low viscosity embedding medium of Spurr (1969). Electron micrographs were taken with a Zeiss EM 109 electron microscope (Zeiss, Oberkochen, Germany).

Calculation of chlorosome size and number

Ultrathin longitudinal and cross sections were used to determine cell size, chlorosome area, dimensions and chlorosome numbers per cell. The calculated values represent an average of 8–17 evaluations of micrographs.

Results

Influence of vitamin B_{12} on the pigment content of green Chlorobium cultures

Chlorobium limicola strain 1230 and *C. vibrioforme* strain 8327 were grown regularly with vitamin B_{12} (20 μg/l) and under vitamin B_{12}-limiting conditions. In comparison to a regularly grown culture of *C. limicola* strain 1230, the specific Bchl *c* content of the cells from a culture lacking vitamin B_{12} was reduced to 20%, while the Bchl *a* content was reduced to 42% only. As already observed by Pfennig and Lippert (1966), the color changed from dark-green

Table 1. Comparison of the specific Bchl *a*, *c* and *d* content per mg protein of *Chlorobium limicola* strain 1230 and *Chlorobium vibrioforme* strain 8327 under different culture conditions

Specific Bchl content (mg/mg protein)	Strain and growth time (days)					
	1230			8327		
	5[a]	21[b]	10[c]	3[a]	3[b]	31[b]
Bchl *a*	0.028	0.012	0.015	0.018	0.014	0.015
Bchl *c*	0.216	0.042	0.2	–	–	–
Bchl *d*	–	–	–	0.31	0.164	0.18

[a] Cultures with 20 μg/l vitamin B_{12}
[b] Vitamin B_{12}-deficient cultures
[c] Addition of 20 μg/l vitamin B_{12} to the culture lacking vitamin B_{12}

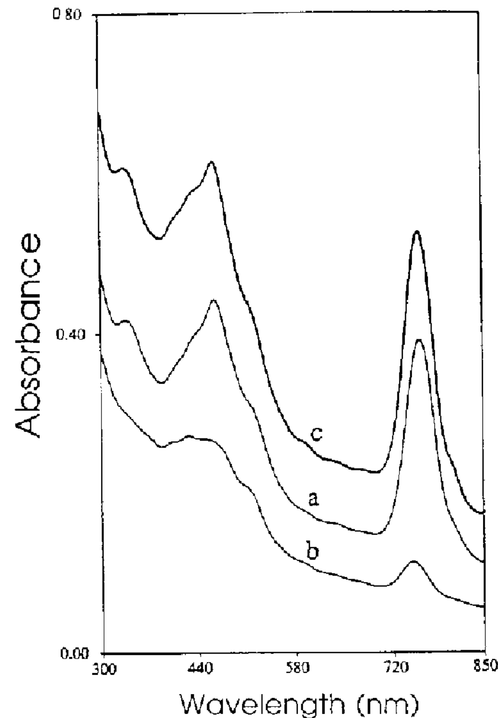

Fig. 1. Absorption spectra of whole cells of *Chlorobium limicola* strain 1230; *a* culture with 20 μg/l vitamin B_{12} (growth time: 3 days; specific Bchl *c* content: 0.216 mg/mg protein); *b* vitamin B_{12}-deficient culture (growth time: 21 days; specific Bchl *c* content: 0.042 mg/mg protein); *c* addition of 20 μg/l vitamin B_{12} to the culture lacking vitamin B_{12} (growth time: 10 days; specific Bchl *c* content: 0.2 mg/mg protein)

to green-yellow. Addition of B_{12} to the deficient culture caused an increase of Bchl *c* content up to the level of a regularly grown culture, but the Bchl *a* content reached only 54% of the initial concentration (Table 1). Decrease and increase of Bchl *c* (≈ 755 nm) and carotenoid content (absorption maxima between 400 and 530 nm) can clearly be seen in the absorption spectra of whole cells, shown in Fig. 1.

Although *Chlorobium vibrioforme* strain 8327 does not require vitamin B_{12} for growth, the Bchl content of the cells was somewhat affected by B_{12}. Growth without vitamin B_{12} did not cause a change in the color of the culture, and absorption spectra of intact cells grown with or without vitamin B_{12} were similar (data not shown). However, the Bchl *d* content of cells grown in the absence of vitamin B_{12} decreased by about 40–50% and the Bchl *a* content was only reduced to 79–83% of vitamin B_{12}-grown cells regardless of growth time (Table 1).

Influence of vitamin B_{12} on the ultrastructure of green Chlorobium strains

A comparison of the cellular ultrastructure of regularly grown cells with those grown under vitamin B_{12} limitation clearly demonstrated that no chlorosomes occurred in the latter ones (Fig. 2b). Obviously vitamin B_{12} deficiency caused an immediate cessation of the formation of Bchl and chlorosomes, while growth still proceeded for some time. The decrease of chlorosomes was accompanied by a formation of granular structures, probably an accumulation of proteins or polysaccharides (Fig. 2b). If vitamin B_{12} was added to a deficient culture, Bchl and chlorosomes were formed again and the granular structures diminished (Fig. 2c). Contrary to *C. limicola* strain 1230 ultrathin sections of *C. vibrioforme* strain 8327 showed no change of the ultrastructure during growth without vitamin B_{12} (micrographs not shown).

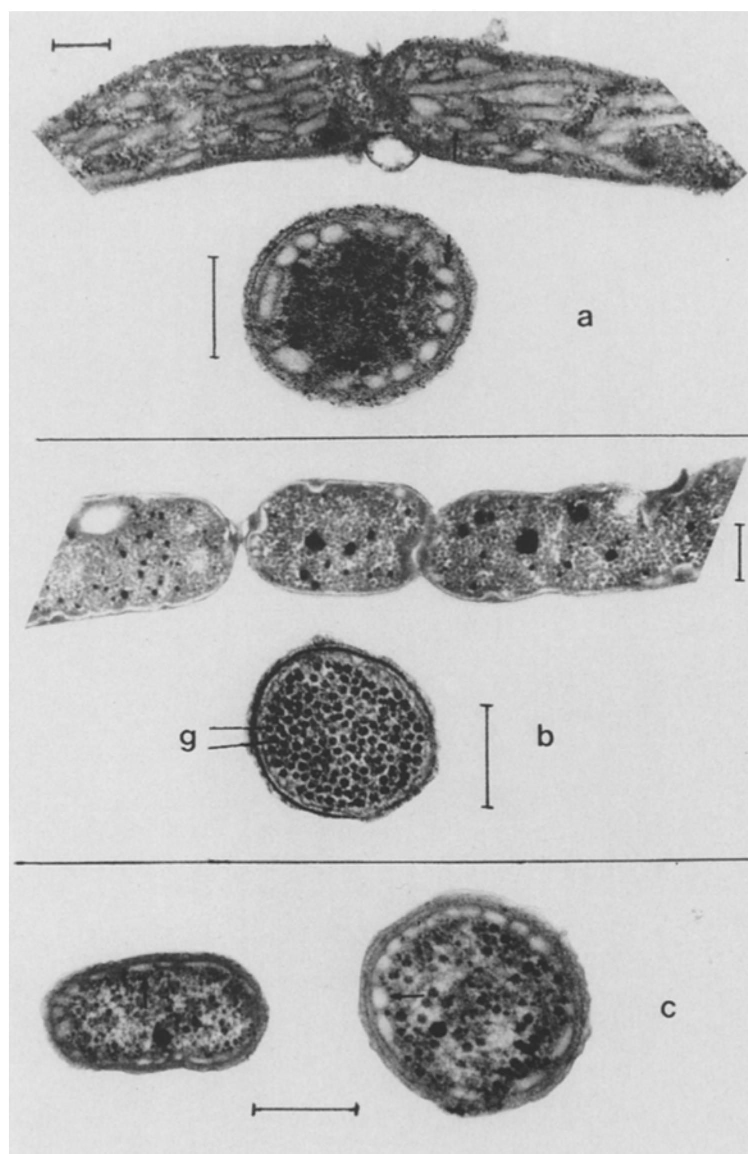

Fig. 2a–c. Ultrathin sections of *Chlorobium limicola* strain 1230 grown under different culture conditions; **a** cells grown with 20 μg vitamin B_{12}/l (growth time: 3 days); **b** vitamin B_{12}-deficient cells (growth time: 21 days); **c** addition of 20 μg/l vitamin B_{12} to the culture lacking vitamin B_{12} (growth time: 10 days). Each *bar* represents 0.2 μm; *arrows* point to chlorosomes; g, granular structures

Influence of light on the pigment content in brown Chlorobium strains

Chlorobium phaeobacteroides strains 2430 and MN1 were cultured at high (30 μmol · m^{-2} · s^{-1}) and low (0.25 to 0.5 μmol · m^{-2} · s^{-1}) light intensities. The absorption spectrum of the low-light-adapted *C. phaeobacteroides* strain MN1 was very similar to the absorption spectrum obtained with the type strain 2430 at higher light intensities (Overmann et al. 1992a). Growth under low light conditions caused a remarkable increase of the Bchl *e* content in both strains in comparison to cultures grown under high light conditions. At 30 or below 1 μmol × m^{-2} · s^{-1} the low-light-adapted strain MN1 produced nearly twice as much Bchl *e* as the type strain (Table 2). Under comparable light intensities *C. phaeobacteroides* strain MN1 produced 14–57% more carotenoids than the type strain. In comparison to high light conditions, the carotenoid content in the type strain increased by 25% at low light intensities (Table 2).

Influence of light on the ultrastructure of brown Chlorobium strains

Under comparable low light conditions *Chlorobium phaeobacteroides* strain MN1 formed 2-fold larger chlorosomes than the type strain as demonstrated by ultrathin sections (Fig. 3a, b). Chlorosome size of strain MN1 decreased with increasing light intensity, but was still considerably larger in comparison to the type strain 2430. Calculations of the chlorosome area in relation to the whole cell area confirmed the above-mentioned results

Table 2. Comparison of chlorosome area, dimensions, number per cell, Bchl, and carotenoid content of *Chlorobium phaeobacteroides* strain MN1 and strain 2430 under various light conditions (average values)[a]

Strain and light conditions (μmol · m^{-2} · s^{-1})	MN 1 30	2430 30	MN1 0.25	2430 0.5
Dimensions of chlorosomes (nm)[b] (length × width)	59 × 34	97 × 23	128 × 36	100 × 26
Chlorosome area per cell (%)	13.8	7.2	23.3	11.4
Number of chlorosomes per cell (cs/ls)	9/21	9/15	9/16	9/16
Bchl *e* (mg/mg protein)	0.20	0.088	0.54	0.25
Car/Bchl *e* (E_{476}/E_{660})	4.4	2.8	4.0	3.5

[a] Calculations were established by means of micrographs of ultrathin sections

[b] Values were determined with longitudinal ultrathin sections. cs. cross section; ls: longitudinal section; car: carotenoids

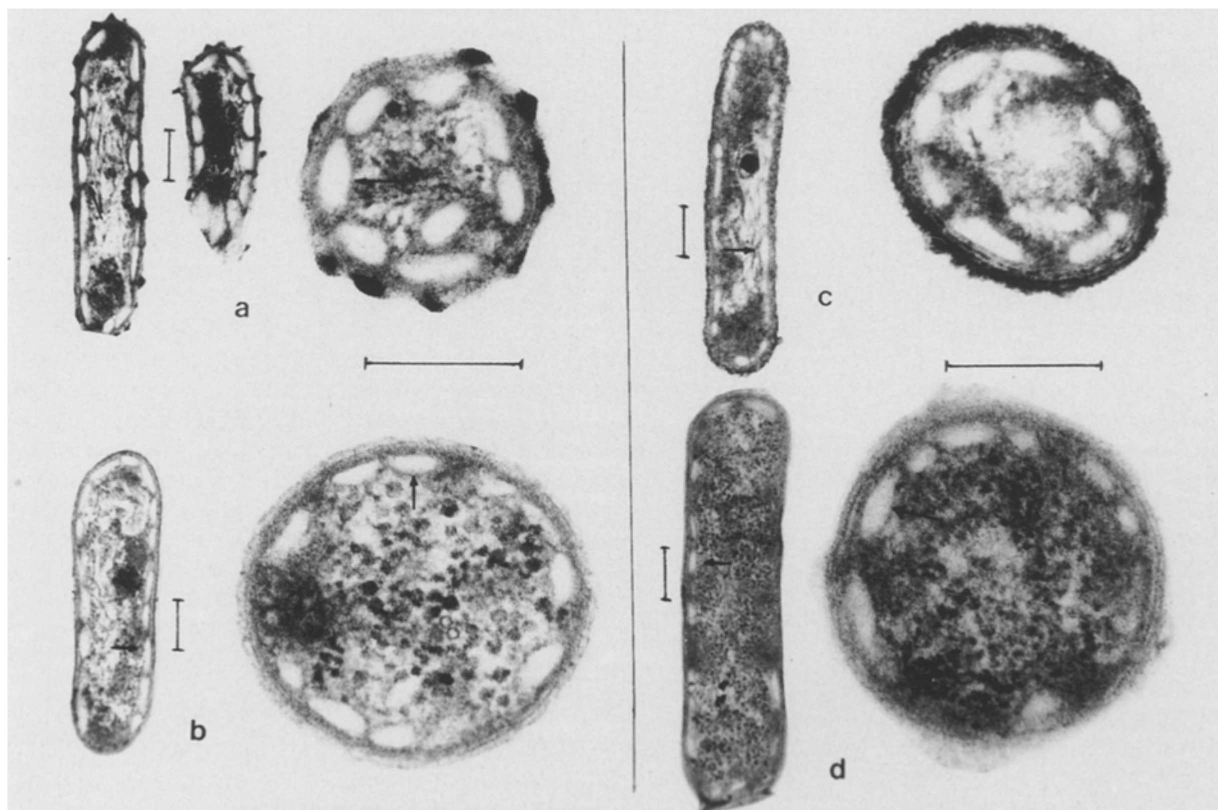

Fig. 3. Ultrathin sections of *Chlorobium phaeobacteroides* strain MN1 and strain 2430 grown at low and high light intensities; **a** strain MN1 grown at 0.25 μmol · m^{-2} · s^{-1}; **b** strain 2430 grown at 0.5 μmol · m^{-2} · s^{-1}, **c** strain MN1 and **d** strain 2430 grown at 30 μmol · m^{-2} · s^{-1}. Each *bar* represents 0.2 μm; *arrows* point to chlorosomes

(Table 2). A statistical examination (*t*-test and Welch-test, see Lorenz 1984) of the obtained average chlorosome area values in relation to the whole cell area indicated significant differences. These differences were proved with both strains under comparable light conditions and within one strain grown at different light intensities. In both strains different light intensities did not change the number of chlorosomes per cell (Table 2). Certainly, cells of *C. phaeobacteroides* strain MN1 grown under low light conditions showed a distinct increase in the length of their chlorosomes, while the width of the chlorosomes was not changed. Strain MN1 formed clearly larger chlorosomes than the type strain regardless of light intensities (Table 2, Fig. 3).

Discussion

The present study has revealed that vitamin B_{12} deficiency obviously causes a decrease not only in the bacteriochlorophyll content but also in the number of chlorosomes or even a total cessation of their formation in vitamin B_{12}-dependent green colored *Chlorobium* strains. Studies by Pfennig and Lippert (1966) with vitamin B_{12}-dependent purple and green phototrophic bacteria have shown already that the pigment content was correlated to the vitamin B_{12} concentration offered. As shown in Table 1 and Fig. 1, vitamin B_{12}-limited growth of *Chlorobium* is mainly caused by a strong decrease in the specific content of Bchl *c* and carotenoids. Although *C. vibrioforme* strain 8327 requires no vitamin B_{12} for growth, small effects of B_{12} on the Bchl *a* and *c* content were observed (Table 1). This is in good agreement with the observations of Broch-Due et al. (1978) and Ormerod et al. (1990) obtained with *Chlorobium* cultures grown under various light conditions or in the presence of anesthetic gases. These results contradict a common regulation of antenna Bchl and Bchl *a* synthesis. In addition to the above-mentioned findings we could show by ultrathin sections that no chlorosomes occur in cells of *C. limicola* strain 1230 lacking vitamin B_{12} and that they are reformed after the addition of the vitamin (Fig. 2). Ormerod et al. (1990) reported similar effects on antenna Bchl content and chlorosome formation in *C. vibrioforme* caused by anesthetic gases such as N_2O, ethylene or acetylene. These authors observed that the Bchl *d* biosynthesis was inhibited, but not that of Bchl *a*, and that the number of chlorosomes was reduced when the cultures were treated with the above-mentioned substances. Until now nothing is known about the extent to which vitamin B_{12} is directly involved in the formation of intact chlorosomes. Recently, Stupperich and Schurr (1991) enriched the coenzyme B_{12} form of a corrinoid with the membrane fraction of chlorosomes from *Chloroflexus aurantiacus*. This corrinoid could be a component of a methylmalonyl-CoA mutase involved in the autotrophic CO_2 fixation pathway (Holo 1989; Stupperich et al. 1990). Regarding CO_2 fixation similar observations were made in *Chlorobium limicola*. If the culture medium was supplemented with propionate besides CO_2 as main carbon source, propionate was assimilated by the organism via methylmalonyl-CoA into succinyl-CoA (Fuchs et al. 1980). Therefore it would be of interest, whether vitamin B_{12} will accumulate in *Chlorobium* also in the chlorosome fraction as mentioned above for *Chloroflexus*. In contrast to *C. vibrioforme* strain 8327, the formation of whole chlorosomes is extremely affected in *C. limicola* strain 1230 under vitamin B_{12}-limited conditions. Granular structures, possibly consisting of proteins or polysaccharides, are present in vitamin B_{12}-deficient cells of *C. limicola* strain 1230, as shown by ultrathin sections (Fig. 2b). Similar accumulations of polysaccharide granules were found in vitamin B_{12}-deficient cells of *Chromatium okenii* (Pfennig and Lippert 1966).

Upon low light conditions green phototrophic bacteria react by increasing the antenna pigments and the size or number of chlorosomes (Holt et al. 1966; Broch-Due et al. 1978; Schmidt et al. 1980; Holo et al. 1985). *C. phaeobacteroides* strain MN1 is very well adapted to low light intensities and photooxidizes sulfide under these conditions much faster than the type strain 2430. At light saturation it is just the opposite way (Overmann et al. 1992a). At 30 and below 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensities, strain MN1 forms about twice as much Bchl *e* as the type strain (Table 2 and Overmann et al. 1992a). This is in good agreement with the findings of Holt et al. (1966), who observed a 5-fold increase of the antenna Bchl content in "*Chloropseudomonas ethylicum*" under low light conditions. Similar results were obtained with cultures of *Chloroflexus* (Pierson and Castenholz 1974; Schmidt et al. 1980), and with *C. limicola* f. *thiosulfatophilum* (Broch-Due et al. 1978; Holo et al. 1985). Different light intensities do not cause a change in the Bchl *a* content in the latter organism. This is in contrast to the findings observed in *Chloroflexus* (Pierson and Castenholz 1974; Schmidt et al. 1980) and *C. phaeobacteroides* strain MN1 (Overmann et al. 1992a), where low light intensity caused an increase of Bchl *a* content. An increase in the carotenoid/Bchl *e* ratio was found in high light grown cells of *C. phaeobacteroides* strain MN1, while the type strain 2430 showed an opposite reaction. The Black Sea *Chlorobium* MN1 had a higher carotenoid content, which, however, did not change significantly with the light intensity. The higher carotenoid content of this strain may indicate the importance of these pigments for the adaptation to low light conditions. Changes in the pigment content in green bacteria are attributed also to variations of chlorosome number per cell (Holt et al. 1966; Broch-Due et al. 1978). By ultrathin sections (Fig. 3) of *C. phaeobacteroides* strain 2430 and strain MN1 we could show that chlorosome sizes increased with decreasing light intensities. In both strains different light intensities do not change the number of chlorosomes per cell (Table 2).

The results obtained with *C. phaeobacteroides* strain MN1 have shown that Bchl *e* content and chlorosome dimensions are dependent on light conditions and thus confirm earlier findings with *Chlorobium* containing Bchls other than Bchl *e* (Holt et al. 1966; Broch-Due et al. 1978). Apparently the variations in chlorosome size can be considered to be an adaptic response of *C. phaeobacteroides* strain MN1 to severe light limitation.

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