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Physiology and tactic response of the phototrophic consortium “Chlorochromatium aggregatum”

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Abstract The phototrophic consortium “Chlorochromatium aggregatum” was enriched from sediment samples of a eutrophic freshwater lake and was maintained at high numbers in anoxic sulfide-reduced medium. Growth of intact consortia was observed only in the light and in the presence of 2-oxoglutarate as an organic carbon source. Consortia of “C. aggregatum” reached maximum growth rates at light intensities ≥ 5 µmol quanta m⁻² s⁻¹. Of ten compounds tested, sulfide, thiosulfate, 2-oxoglutarate, and citrate served as a chemoattractant for “C. aggregatum”. When incubated in the presence of sulfide and in the light, epibions reduced the fluorochrome 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC). Reduction of CTC was not observed in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) or in the dark, indicating that sulfide serves as an electron donor for the phototrophic epibiont. Motile consortia accumulated scotophobically in microcuvettes at a wavelength of 740 nm. Since this wavelength corresponds to the position of the absorption maximum of bacteriochlorophylls c or d, the photosynthetic pigments are most likely the photoreceptors of the scotophobic response. It is concluded that, within the consortia, a rapid interspecies signal transfer occurs between the nonmotile, green-colored epibiont and the motile, colorless central bacterium.

Key words Phototrophic consortia · “Chlorochromatium” · Syntrophy · Phototaxis · Chemo taxis

Abbreviations Behl Bacteriochlorophyll · CCCP Carbonyl cyanide m-chlorophenylhydrazone · CTC 5-Cyano-2,3-di-4-tolyl-tetrazolium chloride

Introduction

Phototrophic consortia are structural associations between a colorless central bacterium and several surrounding cells of pigmented epibions. They have been known since the beginning of this century (Lauterborn 1906; Buder 1914). All attempts to enrich, maintain, and isolate these consortia have been unsuccessful in the past. Based on the color and the presence of chlorosomes in the cells of epibions, it has been concluded that they belong to the group of Green Sulfur Bacteria (Trüper and Pfennig 1971; Caldwell and Tiedje 1974; Pfennig 1980). So far, no additional information on the taxonomic affiliation or metabolism of the epibiont or the central bacterium is available. The isolation of a Green Sulfur Bacterium from “Chlorochromatium aggregatum” has been reported once (Mechsner 1957), but the strain was lost before detailed physiological studies could be made.

The selective advantage of a direct association in consortia may be a syntrophic relationship between both partners. Since Green Sulfur Bacteria can be grown in stable syntrophic cultures with heterotrophic sulfate- or sulfur-reducing bacteria, it has been speculated that a syntrophic sulfur cycle also exists in consortia (Pfennig 1980). Alternative beneficial effects of consortia formation would be chemo tactic and phototactic responses (Hirsch 1984).

Intact consortia exhibit a phobic reaction (“Schreckbewegung”) when suddenly illuminated with light of high intensity (Buder 1914). Although not demonstrated unequivocally, only the central bacterium seems to be motile. If pigments of the epibionts are the photoreceptors of the “Schreckbewegung”, a signal transfer between the nonmotile phototrophic component and the motile chemotrophic central bacterium – and thus between two different unrelated types of bacteria – would be required.

The aim of the present study was to establish and maintain laboratory cultures of phototrophic consortia in defined media. With these newly established cultures, we investigated both the growth and the chemotactic and phototactic behavior of intact consortia in detail.
Materials and methods

Source of organisms

Samples were obtained from the Dagowssee (Neuglobsow, Brandenburg, Germany), a small eutrophic lake north of Berlin. In August 1996 sediment material and overlying water were collected at the deepest site of the lake. Enrichment cultures of phototrophic consortia (see below) were attempted using 2 ml of this sediment suspension per 50 ml of culture medium. The vertical distribution of physical and chemical parameters and of the biomass of phototrophic consortia was determined in a parallel investigation (Oermann et al. 1997).

Culture media and growth conditions

Cultures for the enrichment of phototrophic consortia were set up in air-tight, screw-capped culture tubes (22.5 ml) or in bottles (50 ml). A defined medium (K1) containing the following components (in grams per liter) was prepared under anoxic conditions (Pfennig and Trüper 1989): NaHCO₃, 4.0; KH₂PO₄, 1.0; NH₄Cl, 0.5; MgCl₂·6 H₂O, 0.4; CaCl₂·2H₂O, 0.1; vitamin solution (Pfennig 1978), 1 ml; and trace element solution SL12 (Oermann et al. 1992b), 1 ml. Finally, Na₂S (300 µM) was added as the reducing agent and electron donor for anoxic photosynthesis. The initial pH (before inoculation) of all media was 7.3.

Stock solutions of organic substrates were prepared and added aseptically to the growth media. A set of enrichment cultures was supplemented with different carbon substrates (final concentration, 5 mM). In all experiments, growth was initiated with 5% (v/v) inoculum. All culture vessels were incubated vertically without shaking at 22°C and at 20 µmol quanta m⁻² s⁻¹ of a daylight fluorescent tube (Osram daylight 5000 de lux, 18 W). Growth was monitored microscopically by counting in a Thoma counting chamber. Light intensities were measured with an LI-189 quantum meter plus LI-200 SA pyranometer sensor (LI Cor, Lincoln, Neb., USA).

Chemotaxis assays

Chemotactic responses were analyzed by a modified capillary assay (Adler 1973). Ten holes of 3-mm diameter were drilled in one side wall of a 100-ml flat Meplats glass bottle (Fig. 1). Bottles were filled with 20 ml of sediment slurry (surface sediment diluted four times with overlying water) or enrichment cultures in the late exponential phase of growth (containing approximately 10⁶ consortia ml⁻¹). During filling, the head space was gassed with N₂/CO₂ (80:20%) to maintain anoxic conditions. Stock solutions of various carbon and sulfur compounds were diluted to a final concentration of 1 mM using centrifuged and filtered (0.2-µm-pore-size membrane filters; Nalgene, New York, USA) supernatant of the same culture. Capillaries (length, 50 mm; inside diameter, 0.1 x 1.0 mm; capacity, 5 µl; Vitro Dynamics, Rockaway, N.J., USA) were filled with these solutions by capillary action and sealed at one end with plasticine (Idena, Berlin, Germany). Two parallels were used per substrate. Finally, each capillary was inserted into a hole so that its end extended into the bacterial suspension, and then it was fixed with plasticine. The complete chemotaxis chamber was incubated for 1 h in dim light (2 µmol quanta m⁻² s⁻¹) at 22°C. Afterwards, the capillaries were removed, their open ends were sealed with plasticine, and the number of consortia was counted by light microscopy (dark field, x 200 magnification). Because of their large size and conspicuous morphology, consortia were easily distinguishable from single bacterial cells or detritus particles.

Scotophotic response and bacteriospectrogram

Small microscopic chambers of 100-µm depth were prepared using pieces of cover slips as spacers between the microscope slide and the cover slip. After addition of aliquots from an enrichment culture containing motile consortia, the chambers were sealed with a paper/moisture barrier capillary.

Scotophotic accumulation was stimulated by closing the field stop of a Leitz DMR microscope (Wetzlar, Germany) such that only a small portion of the visible field was illuminated at a magnification of x 100. After 30 min of incubation, the field stop was opened and a photograph was taken immediately with a camera.

The wavelength dependence of the scotophotic response of intact consortia was examined in a similar way, except that an interference filter with a spectral range of 570–1100 nm (Oriel, Langenberg, Germany) was inserted in the light path. The spectrum generated by the filter was focused in the optical plane of the cover slip of the microscope chamber. Photomicrographs were recorded at a magnification of x 100 in dark field at the beginning of the experiment and after 1 h of incubation using a video camera (CF 8/1 RCC; Kappa, Gleichen, Germany). The camera was connected to a computer-based image analysis system (Image P2; H & K Melbysteme, Berlin, Germany). The bacteriospectrogram was then derived by subtracting the initial image from the image at the end of the exposure. For quantification, the numbers of consortia accumulated in the illuminated field were counted at intervals of 10 nm.

For comparative purposes, an absorption spectrum of the same enrichment culture was recorded using a Lambda 2S UV/VIS spectrometer (Perkin Elmer, Überlingen, Germany).

Reduction of CTC

Experiments with the fluorescent electron acceptor 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC) were employed to determine and distinguish the metabolic activities of the phototrophic and heterotrophic components of the consortia. The tetrazolium salt is reduced by the respiratory chain and precipitates as a fluorescent formazan derivative inside the cells; thus, it can be used as an indicator of respiratory activity (Rodriguez et al. 1992).

A sample of a late exponential enrichment culture was harvested by centrifugation (10,000 x g, 10 min), was washed once in sulfide-free medium K1, and finally was resuspended in the same medium. One-milliliter samples were then transferred to rubber-stoppered tubes (10 ml) and gassed with Na₂CO₃ (80-20%) for 10 min. After the addition of substrates, the reaction was started by adding CTC at a final concentration of 2 mM. After 2 h of incubation in darkness or at a light intensity of 10 µmol quanta m⁻² s⁻¹, samples were concentrated tenfold by centrifugation and were transferred to an agar-coated slide (Pfennig and Wagener 1986). During the subsequent swelling of the agar layer, the consortia were disintegrated, thereby exposing the central rod surrounded by the epibionts. The accumulation of fluorescent formazan crystals was then monitored separately for the centrals rods and the epibionts using a Leitz DMR epifluorescence microscope (rhodamine filter set N2.1). For each sample, a total of 100 consortia was counted.

Fig. 1 Glass bottle modified for chemotaxis experiments with phototrophic consortia
Results

Enrichment and growth characteristics

During earlier studies, phototrophic consortia could be enriched only transiently either in sulfide-reduced synthetic media or in natural water samples (Hirsch 1984; Cancenella 1996; Overmann et al. 1997; J. Overmann, unpublished results). Therefore, we initially performed a series of chemotaxis experiments to identify suitable carbon sources for the cultivation of consortia. In these experiments, surface sediment from the Dagowsee, which contained high numbers of the phototrophic consortia, was used.

Of the four substrates tested, only 2-oxoglutarate caused a chemotactic accumulation of consortia (see Fig. 5A). Although “Pelochromatium roseum” was the dominating consortium in the sediment sample, it was mainly “Chlorochromatium aggregatum” (= 80%) and “Chlorochromatium glebulum” (= 10%) that exhibited chemotaxis to 2-oxoglutarate. Based on these results, 2-oxoglutarate was used as a substrate during the subsequent enrichment experiments.

After 2 weeks of incubation, we observed growth of the green-colored “C. aggregatum” in the mineral medium K1 supplemented with 2-oxoglutarate (Fig. 2). Growth of “C. aggregatum” was dependent on both light and the presence of 2-oxoglutarate (Fig. 3). No growth was observed either in the absence of 2-oxoglutarate or in the dark after an incubation of even 2 months (data not shown in Fig. 3). Obviously, entire consortia are not able to grow photoheterotrophically or chemotrophically. All attempts to obtain enrichment cultures with formate, acetate, propionate, butyrate, methanol, ethanol, propanol, butanol, pyruvate, lactate, malate, fumarate, succinate, isocitrate, and citrate were unsuccessful and did not result in any significant growth of consortia. For a successful enrichment, an illumination of cultures in a cycle of 12 h light and 12 h darkness was not necessary (Fig. 3).

![Fig. 2](image)

**Fig. 2** Phase-contrast photomicrograph of “Chlorochromatium aggregatum” enriched on 2-oxoglutarate during the exponential growth phase (bar, 10 μm)

![Fig. 3](image)

**Fig. 3** Photobacteriogenic growth of “Chlorochromatium aggregatum” in enrichment cultures. Cultures were incubated in the presence of 2-oxoglutarate ■ for a 12-h light/12-h dark cycle, (▲) in continuous light, or ◆ in darkness. Parallel cultures were incubated in the absence of 2-oxoglutarate (5 mM) □ for a 12-h light/12-h dark cycle, △ in continuous light, or ○ in darkness

Cultures of “C. aggregatum” have now been maintained for more than 20 transfers using 2-oxoglutarate as the carbon source in the mineral medium K1.

Compared to the total number of phototrophic consortia in the Dagowsee (3.4 × 10^4 ml^-1; Overmann et al. 1997) and considering the small fraction of “C. aggregatum” among the consortia (< 10%), the number of “C. aggregatum” in our enrichments (3 × 10^6 ml^-1; Fig. 3) is more than three orders of magnitude higher than in the natural habitat.
Furthermore, we investigated the dependence of the growth rate of the enrichment cultures on the light intensity (Fig. 4). Light limitation of growth was observed only at light intensities ≤ 3 µmol quanta m⁻² s⁻¹, while maximum growth rates and doubling times of 1 day were observed at between 5 and 20 µmol quanta m⁻² s⁻¹.

Chemotaxis

Following the initial chemotaxis experiments with sediment suspensions from the Dagowsee (Fig. 5A), enrichment cultures of “C. aggregatum” were used to test additional carbon and sulfur compounds as chemoattractants. “C. aggregatum” exhibited chemotaxis toward sulfide, thiosulfate, 2-oxoglutarate, and citrate (Fig. 5B). In comparison with the sediment samples (Fig. 5A), the chemotactic response of consortia in enrichment cultures showed a higher degree of variation (compare standard deviations for 2-oxoglutarate in Fig. 5A and B). This fact can be explained by residual 2-oxoglutarate that was still present in the enrichment cultures during the exponential phase and interfered with the chemotaxis assay. Consortia never showed any chemotactic response to acetate. The consortia that accumulated in capillaries containing sulfide were mostly nonmotile at the end of the experiment, while those observed in capillaries with thiosulfate still exhibited motility.

Reduction of CTC

Because of the pronounced chemotactic response towards sulfide, we investigated whether the green epibionts use sulfide as an electron donor (Fig. 6). Reduction of the artificial electron acceptor CTC in epibionts and in central bacteria was monitored separately by epifluorescence.

In the absence of sulfide, a considerable fraction of the central bacteria showed fluorescence (19 and 43%, Fig. 6A). Since residual substrates in the culture fluid had been removed in a washing step prior to the experiments, this in-

![Diagram A](image)

![Diagram B](image)

**Fig. 5A, B** Chemotactic accumulation of “*Chlorochromatium aggregatum*” in capillaries filled with various substrates. Substrate concentrations were 1 mM each or, in the case of peptone, 0.01%. Three or four parallel experiments were run. Vertical bars indicate standard deviations. A sediment sample from the Dagowsee A or an enrichment culture of “*C. aggregatum*” (B) was incubated in the chemotaxis chamber.

**Fig. 6** Fraction of A central rods and B epibionts exhibiting reduction of the fluorochrome CTC in the presence of different combinations of sulfide, formaldehyde (HCHO), and the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Acetone served as a solvent for CCCP; its effect was therefore tested separately. For each substrate combination, consortia were incubated in the dark and in dim light.
dicates the presence of endogenous electron donors. Addition of 2-oxoglutarate did not increase the fraction of red fluorescent cells (data not shown). However, the percentage of fluorescent central bacteria increased from 24 to 66% during incubation in the presence of sulfide (Fig. 6A). With one exception, incubation in dim light increased the CTC reduction by the central bacteria significantly (Fig. 6A). Controls containing formaldehyde (final concentration of 3.7%) or the uncoupler CCCP (100 µM) demonstrated that reduction of CTC was biologically mediated.

Epibionts of “C. aggregatum” showed light-dependent electron transfer to CTC only in the presence of sulfide and in the light (Fig. 6B). Similar to the central bacterium, formaldehyde and CCCP completely inhibited the electron transfer from sulfide to CTC.

Phototaxis
A scotophobic response of “C. aggregatum” was observed in white light. Consortia accumulated in the illuminated part of the microscope sample after an initial incubation period of 10 min (Fig. 7A,B). This was observed only at very low light intensities (0.35–0.7 µmol quanta m⁻² s⁻¹). Consortia exposed to light intensities higher than 1.5 µmol quanta m⁻² s⁻¹ did not show this scotophobic accumulation. “C. aggregatum” exhibited a wavelength-dependent accumulation in the spectral range between 700 and 770 nm (Fig. 8). No consortia were found at positions that had been illuminated with light of longer wavelengths up to 1,100 nm (this wavelength range is not shown in Fig. 8).
The shapes of the absorption spectrum and of the scotophobic accumulation curve were very similar, but the wavelength of the absorption maximum and the maximum of cell accumulation differed by approximately 10 nm (747 nm and 730–742 nm, respectively). The apparent discrepancy of 10 nm can be explained by a slight deviation from linearity of the interference filter employed.

Discussion

Former studies of phototrophic consortia have relied on natural sample material and have focused on morphological features [see, e.g., Caldwell and Tiedje (1974) and Croome and Tyler (1984)] because it was not possible to maintain intact consortia in the laboratory. Here, we present a technique to enrich and maintain “C. aggregatum” in a defined culture medium with a composition based on the results of chemotaxis experiments. This is a first step toward the elucidation of symbiotic interactions between both partners of this outstanding association. Similar experiments had been initialized by one of us (J. Overman) previously, but no definite results were obtained using the conventional capillary method (Canganella 1996). As a consequence, no stable enrichment cultures could be established. Our newly designed chemotaxis chamber permits (1) simultaneous tests of several chemotactants in one and the same bacterial culture or natural sample, and (2) a direct quantification of the consortia accumulated inside the capillary.

The addition of 2-oxoglutarate was pivotal for the maintenance of the structural integrity of consortia during growth. Our results so far do not indicate whether 2-oxoglutarate itself or any metabolic degradation product is utilized for growth. Furthermore, it is unclear whether the carbon substrate is utilized by the epibiont or by the central rod. Photoorganoheterotrophic growth was reported for one isolate of Green Sulfur Bacteria (“Chlorobium chlororochromatii”) that originated from “C. aggregatum” samples (Mechsner 1957) and that was assumed to represent the epibiont. However, 2-oxoglutarate did not stimulate CTC reduction of epibionts in our experiments. Acetate, propionate, or pyruvate are the only organic compounds that can be assimilated by the presently available pure cultures of Chlorobiaceae species (Pfennig and Trüper 1989). However, these carbon compounds did not elicit chemotaxis and also did not support growth of consortia. Therefore it appears unlikely that the epibiont cells directly perceive and assimilate carbon substrates such as 2-oxoglutarate and citrate.

The pronounced chemotactic response of whole consortia toward sulfide raised the question of whether the epibionts or the central rod perceive and oxidize sulfide. All species of Green Sulfur Bacteria presently existing in pure culture are obligately photolithotrophic and use sulfide as the electron donor for anoxygenic photosynthesis (Pfennig and Trüper 1989). The epibionts of “C. aggregatum” reduced CTC with sulfide exclusively in the light. Furthermore, some of the consortia of the “C. aggregatum” type in water samples from the Dagowsee contained one-to-three highly refractile, yellowish globules between the cells. These globules are very similar to the extracellular droplets of sulfur that transiently accumulate during sulfide oxidation in cultures of Green Sulfur Bacteria. Taken together, these results indicate that the epibionts of “C. aggregatum” oxidize sulfide photosynthetically as do free-living Green Sulfur Bacteria.

The mineral medium used for successful enrichment of consortia contained much less sulfide (300 µM) than did the conventional medium for Green Sulfur Bacteria (2.5 mM). At higher sulfide concentrations, free Chlorobium cells grew profusely in the cultures. Still, free cells of Green Sulfur Bacteria were observed at high numbers in our enrichment cultures, especially at the end of the exponential growth phase of “C. aggregatum”. Obviously, a strong limitation of external sulfide is of competitive advantage for intact consortia.

The growth of Green Sulfur Bacteria in pure culture is light-limited below 10 µmol quanta m⁻² s⁻¹ (Overmann et al. 1991, 1992a, 1997). In dialysis cultures, consortia sampled in the Dagowsee stayed intact at low light intensities of 5–10 µmol quanta m⁻² s⁻¹ (Overmann et al.,

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**Fig. 8** A Bacteriospectrogram of “Chlorochromatium aggregatum”. Computerized photomicrograph of the section of the phototaxis chamber illuminated with light of wavelengths between 570 and 790 nm. Each dot represents one consortium. B Integrated number of accumulated consortia per 12-nm interval (diagonal hatching) as compared to the absorption spectrum of whole cells of the same enrichment culture (line)
The growth of “C. aggregatum” in our enrichment cultures was light-limited only below 5 μmol quanta m⁻² s⁻¹. Moreover, scotophobic response experiments revealed that consortia accumulate only at light intensities of the microscope lamp below 1.5 μmol quanta m⁻² s⁻¹. It can be concluded that phototrophic consortia are adapted to even lower light intensities than are free-living Green Sulfur Bacteria. This corresponds well to the conditions in their natural habitat, the Dagowsee, where a maximal underwater irradiance of 7 μmol quanta m⁻² s⁻¹ has been measured (Overmann et al. 1997).

Phototrophic consortia are motile by means of polar flagella that could be visualized with crystal violet (Overmann et al. 1997). The type of movement of partly disintegrated consortia with only one or two epibionts indicates that the central bacterium is the motile component (J. Overmann, F. Canganella, and P. Egland, unpublished results). The maximum of the scotophobic accumulation of “C. aggregatum” occurred at wavelengths that correspond to the absorption maximum of bacteriochlorophylls e or d. Our data for the first time provide strong evidence for a signal transduction between the epibionts and the central rod in the consortia.

In a parallel investigation (Overmann et al. 1997), the physical and chemical parameters of the natural habitat of “C. aggregatum” were determined in detail. Based on the high buoyant density of consortia and their sedimentation rates, it was postulated that consortia must exhibit some sort of phototaxis under natural conditions. At least for “C. aggregatum”, this property could be demonstrated in the present work. It remains to be elucidated whether chemotaxis toward reduced sulfur species or organic carbon compounds is of similar ecological relevance for the successful colonization of lakes by phototrophic consortia.

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