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Light as an Energy Source in Continuous Cultures of Bacteriorhodopsin-Containing Halobacteria

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The role of light as an energy source for slightly aereated cultures of halobacteria was studied, using continuous cultures with low nutrient concentrations and a low oxygen supply. A series of experiments were carried out with non-illuminated and differently illuminated cultures and with different oxygen transfer rates. Under low oxygen availability, light proved to be a decisively important energy source that allowed the populations to reach higher growth rates and much higher population densities. Oxygen influenced the growth over only a minimal level, below which neither the illuminated nor the dark cultures were affected by the oxygen transfer rate. From these results, it appears that the bacteriorhodopsin-mediated energy supply could have a very important role for the ecology of halobacteria in their microaerophilic habitats. In the illuminated cultures, cells that originated purple colonies on plates appeared. These cells, which could be bacteriorhodopsin-constitutive mutants, are now being studied.

Some of the extremely halophilic species of the genus *Halobacterium* have two alternative ways of producing ATP. When grown aerobically in well-aereated cultures, these bacteria respire, using a respiratory chain (6) and oxygen as an electron acceptor. However, when kept under conditions of very low oxygen supply and illumination, the so-called purple membrane, containing bacteriorhodopsin, is synthesized. This retinal-associated protein is capable of using light energy to create a proton gradient and therefore enables the cell to produce ATP under these conditions (3).

Although this phenomenon has been known for some years, it has not been well established what the actual importance of this photophosphorylation mechanism is for the life and ecology of halobacteria. Brock and Petersen (1) have shown that bacteriorhodopsin can improve the ability of halobacteria to survive under anaerobic, illuminated conditions. However, the halobacteria were considered to be strict aerobes, unable to grow under the conditions needed for the synthesis of the purple membrane. Recently, anaerobic growth of *Halobacterium* species has been described, although in these experiments photophosphorylation allowed growth for only a short time, i.e., while the store of retinal, accumulated during a previous microaerophilic incubation, lasted (5).

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It is generally acknowledged that, in batch cultures of halobacteria, growth stops when the shaking or aereating system is discontinued, even when illumination is provided. However, in batch cultures, only relatively important changes in the growth rate can be detected, and rather unnatural conditions, such as very high nutrient concentrations, are created in such cultures. In continuous cultures, cells can be maintained at very low growth rates and with very low nutrient concentrations. Under these conditions, factors that are negligible in batch cultures, such as maintenance energy, can play a very significant role (10), as probably happens in nature, and can be easily detected. Besides, it has been shown that hypersaline habitats are very poor in oxygen supply (7).

We studied the effect of light on continuous cultures of *H. halobium* aereated very slightly. In this way, we have shown that photophosphorylation plays a very important role in the efficiency of these organisms under these conditions, and, therefore, probably in their natural environment.

MATERIALS AND METHODS

Microorganisms and culture media. The microorganism used for all experiments was *H. halobium* CCM 2090. The culture media contained 25% (wt/vol) salts in the same proportions as those in a previously described artificial seawater (9). For the continuous cultures, this salt solution was supplemented with 0.05% (wt/vol) yeast extract (Difco Laboratories), adjusted to pH 7 with 1 N KOH, and autoclaved. For determination of viable numbers, we used plates with

the same medium but supplemented with 0.5% (wt/vol) yeast extract and solidified with 2% (wt/vol) Bacto-Agar (Difco). All cultures were incubated at 39°C.

Continuous cultures. The culture vessel was a 1,000-ml Erlenmeyer flask stirred by a 4-cm magnetic Teflon-coated rod at ca. 300 rpm. The level of the liquid, and therefore the culture volume and the oxygen transfer rate (OTR), was regulated by a glass tube connected to a ventury vacuum pump through an intermediate flask that acted as a collector of the outlet (Fig. 1). The inflowing medium was pumped in with a four-channel peristaltic pump (Gilson Medical Electronics, Inc.). The lowest aeration conditions were achieved by filling the Erlenmeyer flask up to the neck and introducing a glass finger into the liquid to avoid whirlpools and to diminish the air contact surface (Fig. 1). The medium inlet was always below the liquid level, and the fresh medium in the reservoir was, after autoclaving, covered with a layer of polyesterene scales to minimize oxygen dissolution. In this way, the oxygen supply was regulated by the liquid-air contact surface and kept as constant as possible. Three culture volumes, 500, 850, and 1,130 ml, were used, producing three rates of oxygen transfer.

To start the cultures, an inoculum of 5 ml was grown with 0.5% (wt/vol) yeast extract in a screw-capped tube horizontally positioned in an orbital incubator. This inoculum was transferred to the culture vessel with 1,000 ml of the medium used for continuous culture and incubated with aeration provided by a soft humidified air flow and by magnetic stirring until an optical density (OD) of ca. 1 was achieved. The culture was then deprived of oxygen by stopping aeration and stirring and illuminated for 72 h. Afterwards, the medium was divided in two equal parts and placed into two identical flasks, one of which was completely covered with aluminum foil to impede the entrance of light. Experiments were always run in parallel, with one illuminated and one non-illuminated culture. The culture vessels were then filled with fresh medium to the required volume, and afterwards the inflowing medium was introduced at the flow rate required to produce the different dilution rates (D values) used. Cultures were run until the OD remained constant for 3 to 4 days. This was considered to be the steady state for the conditions used. OD readings were done at 520 nm in a Bausch & Lomb Spectronic 20 spectrophotometer. Viable counts on plates were also done for each steady state. All the experiments done under the same illumination conditions and the lowest oxygen transfer rate were carried out in the same culture. When a steady state, as determined by the above criteria, was reached, the D value was changed, and then we waited for the new steady state. For all other experiments, we started at the inoculation stage.

Illumination was provided by one or two 60-W filament lamps located at 5 cm from the widest perimeter of the culture vessel. Light passed through the transparent wall and a few centimeters of the bath water. The illumination intensity reached with one of the lamps, measured with a photometer at the culture location, was 5,000 lx.

OTR determinations. The OTRs produced at the different air-liquid contact surfaces were determined by the sulfite oxidation method (2), using a salt solution as in the culture medium. This salt solution was saturated with $NaSO_3$, filtered, and then placed in the culture vessel; $CuSO_4$ was added to a final concentration of 0.001 M. After starting the stirring, and keeping all the conditions the same as those in the cultures, samples of 5 ml were withdrawn every 6 h and the sulfite concentration was determined by an iodimetric method.

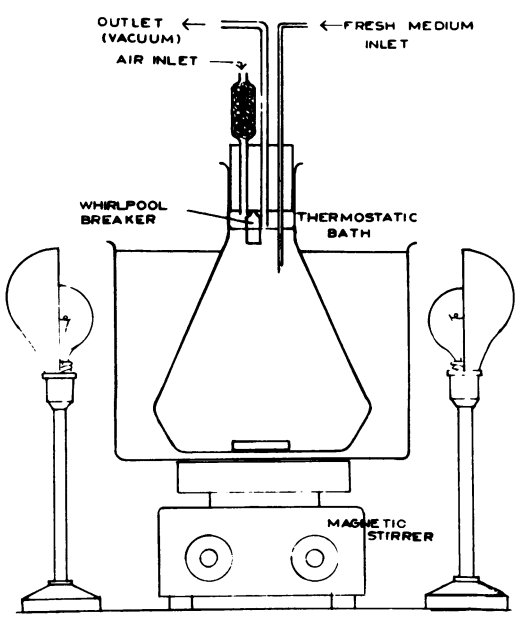

FIG. 1. Culture vessel used for the continuous culture of halobacteria at the lower aeration level.

RESULTS

In Fig. 2 are shown the values of the population density in the steady state, represented by the OD, at different D values and in three differently illuminated cultures. All these experiments were done with the lowest OTR (0.044 mM O_2 per liter/h). Both illuminated populations developed much higher population densities and could also withstand higher D values without being washed out. Furthermore, the population density approximately doubled when the light intensity was doubled. The first steady state was achieved after a long incubation time (10 to 15 days), and then, by changing the D value, a new steady state was created much sooner (4 to 5 days). Once the steady state was reached at D = 8.10^{-3} h^{-1}, the illuminated culture was covered with aluminum foil, and then the OD dropped to a value similar to that of the culture kept in darkness. This procedure ensured that there had been no selection in the illuminated flask of a mutant with a more efficient dark metabolism. At the end of this group of experiments, two kinds of colonies appeared on the plates for viable cell enumeration. At the steady state

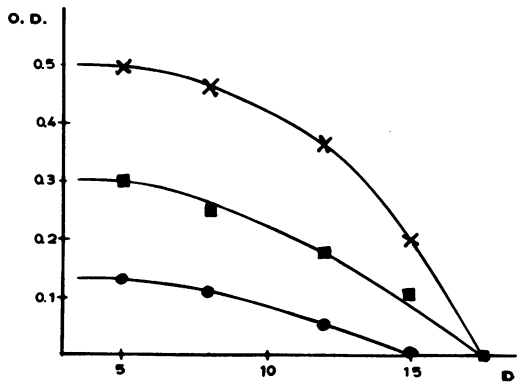

FIG. 2. Population densities at the steady state of continuous cultures of halobacteria very slightly aereated (0.044 nM O_2 per liter/h), at different D values (10^{-3}/h), and at three levels of illumination. ●, Culture kept in darkness; ■, culture illuminated at 5,000 lx; ×, culture illuminated at 10,000 lx. OD was measured at 520 nm.

reached when D = 15.10^{-3} h^{-1}, 60% of the colonies were the usual type for this strain, but 40% had a deep purple pigmentation. Representatives of these colonies were isolated, and the pigmentation proved to be stable after several transfers. On the plates from the dark cultures, all the colonies were normal, red-pigmented ones.

Another set of experiments was done at only one D (5.10^{-3} h^{-1}), keeping one culture in the dark and the other illuminated at 10;000 lx, but varying the OTR. Although different culture volumes were used, the D value was kept constant by adjusting the flow rate. Three OTRs, 0.044, 0.48, and 0.8 mM O_2 per liter/h, were obtained. At an OTR of 0.044 (Fig. 2), the OD reached at the steady state was 0.50 for the illuminated culture and 0.13 for the culture kept in darkness. When the OTR was increased to 0.48, very little change occurred; the corresponding ODs were 0.51 and 0.15. However, the next OTR increase produced a drastic drop in the OD of the illuminated culture, which decreased to 0.21, whereas the dark culture increased its steady-state OD to 0.22. Therefore, under these conditions, the effect of light was null or slightly negative.

One last experiment was done, in which, by keeping the OTR at 0.044 and D = 5.10^{-3} h^{-1}, the nutrient concentration in the reservoir was decreased to 0.005% yeast extract. Under these conditions, wash-out occurred in both the illuminated and the non-illuminated cultures.

DISCUSSION

It seems clear, on the basis of our results, that *H. halobium* cells are able to grow under conditions of very low oxygen supply. Although the growth rates which developed were very low, they may be meaningful in the natural environment. The conditions we created in the continuous cultures are probably very similar to those occurring in nature, where the microorganisms grow slowly in low-nutrient solutions and with a scarcity of oxygen. Therefore, the bacteriorhodopsin-mediated energy supply could be of remarkable importance for these microorganisms.

The effect produced by illumination was similar to that obtained when the concentration of the limiting nutrient was increased (10). Although the limiting nutrient is unknown in this case owing to the complex composition of the yeast extract, it is very probably the energy source. We therefore concluded that light was acting as an important energy source for the cells. The population density of the culture receiving 10,000 lx was about fourfold higher than that of the non-illuminated culture (Fig. 2). This difference could represent a significant advantage for competition in the natural environment. In fact, the light intensity in nature is much higher and could therefore act as a more effective energy source. However, the highest growth rate developed in the 10,000-lx illuminated culture was still very low, corresponding to a doubling time of 46 h, whereas in well-aereated cultures this organism can grow with a doubling time of 6 to 7 h (4).

Although respiration could have been taking place in both the illuminated and the non-illuminated populations, the oxygen supply did not seem to be limiting. In any case, changing from a low to an intermediate aereation level, while increasing the OTR by more than 10-fold, did not increase the population density. However, the next increase in the oxygen supply, to a level twice as high as the previous one, produced a drastic change in the situation. The enhancement of growth by light no longer existed, and the population densities were similar in both cultures, increasing significantly in the non-illuminated one and decreasing to less than half of the earlier level in the other one. All this may be explained if we consider the oxygen availability as a regulatory signal as well as a nutrient. It is well known that the purple membrane is synthesized only under microaerophilic conditions; therefore, it can be expected that over a certain limit, light would not be useable as an energy source. However, it remains to be explained why, in the dark cultures, the population density did not increase when the OTR increased from 0.044 to 0.48. It is possible that under these aereation conditions respiration occurs at a very low level or is even completely absent, and the cells derive their energy from some fermentative pathway (5). Most of the physiological work

done with halobacteria refers to cells grown in intensively aereated cultures. Therefore, very little is known about their capabilities and behavior under microaerophilic conditions. It seems possible, on the basis of results described in this work, that the role of respiration in the ecology of halobacteria is not as important as has been thought previously. We have determined the oxygen concentrations in the ponds of a marine saltern and, in the more concentrated ones where halobacteria predominate (9), we have found extremely low values. However, since the halobacteria possess gas vacuoles that allow them to float, they could place themselves on the surface and permit respiration to occur, especially at night when the strong solar radiation that would make the surface too exposed is not present and photophosphorylation cannot occur.

The examination of the colonies that appeared on plates from lengthily maintained illuminated and non-illuminated cultures showed two unexpected results. First, purple colonies appeared in the illuminated cultures. A study of these colonies is now under way in our laboratory. Possibly, their color could correspond to bacteriorhodopsin-constitutive mutants that would produce the purple membrane even under highly aerobic conditions and without light. Second, no nonpigmented colonies appeared on plates from the dark cultures, although nonpigmented variants appear spontaneously at a high rate in these microorganisms (8). Theoretically, these nonpigmented cells could be expected to have an advantage in these cultures and therefore to displace the pigmented ones. It could be that carotenoids have, in this case, another role in addition to a strictly photoprotective one.

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