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# METABOLISM OF CYANOBACTERIA IN ANAEROBIC MARINE SEDIMENTS

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ABSTRACT - Two cyanobacteria isolated from marine microbial mats metabolized endogenous carbon reserves anaerobically in the dark. *Microcoleus chthonoplastes* reduced elemental sulfur to sulfide and *Oscillatoria* sp. additionally produced lactate in the absence of elemental sulfur. Under nitrogen fixing conditions, however, no sulfur reduction occurs. Lactate fermentation appeared to be the mechanism of anaerobic carbon degradation under these conditions. With a nitrogenase-reducible substrate, e.g. acetylene, added, lactate fermentation as well as sulfur reduction stops in cultures of *Oscillatoria* containing nitrogenase. In this case only ethylene production was observed. These cyanobacteria seem to possess the capability to carry out anaerobic dark metabolism constitutively. No lag-period was observed in utilization of carbon reserve polymer or in product formation after transferring the cultures from the light to the dark under anaerobic conditions.

*Key words :* cyanobacteria, anaerobic, lactate fermentation, sulfur reduction, nitrogen fixation

RÉSUMÉ - Deux cyanobactéries isolées de tapis bactériens marins métabolisent des réserves carbonées endogènes en anaérobiose et à l'obscurité. *Microcoleus chthonoplastes* réduit le soufre élémentaire en sulfure et *Oscillatoria sp.* produit en plus du lactate en l'absence de soufre élémentaire. Toutefois, sous conditions de fixation de l'azote, la réduction du soufre ne se produit pas. La fermentation du lactate semble être, dans ce cas, le mécanisme de dégradation anaérobie du carbone. Par addition d'un substrat nitrogénase réductible tel que l'acétylène, la fermentation du lactate tout comme la réduction du soufre s'arrêtent dans les cultures d'*Oscillatoria* contenant une nitrogénase. Dans ce cas, seule la production d'éthylène est observée. Ces cyanobactéries semblent pouvoir effectuer leur métabolisme de constitution en anaérobie et à l'obscurité. Aucun délai n'est observé dans l'utilisation des polymères carbonés de réserve ou dans la formation de produits, après transfert des cultures de la lumière à l'obscurité sous conditions anaérobies.

*Mots clés :* cyanobactéries, anaérobie, fermentation du lactate, réduction du soufre, fixation d'azote.

## INTRODUCTION

In recent years, microbial mat systems have received increasing attention (Cohen *et al*, 1984 ; Jorgensen *et al*, 1983; Stal *et al*, 1984b). The majority of microbial mats usually are vertically stratified communities of distinct populations of microorganisms. They are small scale microbial ecosystems. Although not exclusively, they are particulary abundant in marine intertidal environments (Walter, 1976).

Cyanobacteria (blue-green algae) play a key role in the development of the microbial mat and are the main mat building organisms (Jorgensen *et al*, 1983; Stal *et al*, 1984b). Cyanobacteria are predominantly oxygenic phototrophic organisms. They use water as electron donor, producing oxygen. Photosynthesis by cyanobacteria presents the main input of organic material in intertidal sandy sediments. As organic material accumulates, heterotrophic bacteria will decompose it, hereby producing anaerobic conditions beneath the cyanobacterial mat. Anaerobic conditions allow sulfate reducing bacteria to develop.

This group of bacteria produces sulfide by oxidizing simple organic compounds, using sulfate as electron acceptor. The sulfide produced precipitates as iron-sulfide, which forms the black layer, typical for anoxic marine sediments. Sulfide may act as electron donor for anoxygenic photosynthesis by purple sulfur bacteria. Conditions given these bacteria form a red layer between the cyanobacteria and the sulfate reducing bacteria.

Very marked gradients of oxygen and sulfide exist in microbial mats. In the light, cyanobacteria produce oxygen and purple sulfur bacteria consume sulfide. The interface of oxygen and sulfide can found at the lower border of the cyanobacteria mat. A coexistence of low concentrations of oxygen and sulfide has been found in several mat systems (Jorgensen *et al*, 1979; 1983; Krumbein *et al*, 1979; Revsbech *et al*, 1983). In the dark, oxygen production by oxygenic photosynthesis of cyanobacteria as well as sulfide oxidation by the purple sulfur bacteria stops. The oxygen wich eventually accumulated during the day is rapidly consumed by heterotrophic organisms. Sulfide production by the sulfate reducing bacteria presumably continues. Therefore also a chemical reduction of oxygen by sulfide is expected. After some period of darkness the oxygen/sulfide interface moves up and, finally, can be found at or above the mat surface.

Hence, the cyanobacteria in these systems will be faced by anaerobic conditions in the dark. Thus far, dark metabolism of cyanobacteria generally is believed to be aerobic respiration (Smith, 1982). Only in a few occasions anaerobic dark metabolism of a cyanobacterium was reported. *Oscillatoria limnetica* has shown to be able to ferment its carbon reserve polymer polyglucose to lactate. In the presence of elemental sulfur this organism carries out a sulfur respiration (Oren and Shilo, 1979). Oren and Shilo (1979) observed sulfide production in *Aphanothece halophytica* under dark anaerobic conditions. An other type of anaerobic respiration in cyanobacteria was observed in *Synechococcus lividus*. This organism respires endogenous carbon reserves using sulfate or thiosulfate as terminal electron acceptors. Sulfide and in the case of thiosulfate as electron acceptor, also sulfite are the products (Sheridan and Castenholz, 1968; Sheridan, 1973).

The Shallows of the southern North Sea (Wadden Sea) are characterized by large intertidal flats. In the upper littoral zone of these intertidal flats microbial mats develop. Because of their green-red-black lamination Schulz (1936) and Schulz and Meyer (1939) called this biotope "Farbstreifen-Sandwatt" (colour-striped-sand).

The North Sea mats we investigated are dominated by two species of cyanobacteria. *Oscillatoria* sp. is particulary important in freshly colonized sediments (Stal *et al*, 1984b). *Microcoleus chthonoplastes* is the main mat builder and is the dominant organism in well-developed cyanobacterial mats (Stal *et al*, 1984c). There is a good deal of evidence that nitrogen fixation is the critical process responsible for initial sediment colonization (Stal *et al*, 1984b). Previously, we showed that *Oscillatoria* sp. strain 23, a filamentous, non-heterocystous cyanobacterium, isolated from this environment, can fix nitrogen even under aerobic conditions (Stal and Krumbein, 1981). We also found that this organism can fix nitrogen in the dark under anaerobic conditions. Here we report anaerobic dark energy metabolism in cultures of *Oscillatoria* sp. strain 23 and *Microcoleus chthonoplastes*.

## MATERIALS AND METHODS

### *Organisms and culture conditions*

*Oscillatoria* sp. strain 23 and *Microcoleus chthonoplastes* strain 11 were isolated from the

cyanobacterial mat on the island of Mellum, southern North Sea (Stal and Krumbein, 1981; Stal *et al*, 1984c). Both strains are typical marine cyanobacteria: no growth was observed in freshwater medium. *Oscillatoria* sp. strain 23, a filamentous, non-heterocystous cyanobacterium, fixes molecular nitrogen even under complete aerobic conditions (Stal and Krumbein, 1981). This organism shows good growth in media depleted of a source of fixed nitrogen. Both organisms were grown in artificial seawater medium $ASN_{III}$ (Rippka *et al*, 1979). *Oscillatoria* was also grown on the combined nitrogen free variant of this medium ($ASN_{III}^{o}$) for some experiments, to induce nitrogenase. Organisms were grown in a Gallenkamp (London) illuminated shaking incubator at 20°C with 1300 and 2500 lux light intensity for *Oscillatoria* and *Microcoleus* respectively.

### *Anaerobic dark incubation*

Cultures were centrifuged at 10,000 g and washed twice in a buffered medium containing 25 $g.l^{-1}$ NaCl and 10mM TES (N-Tris (hydroxymethyl)-methyl-2-aminoethane-sulfonic acid), pH 7.9. The filaments were then suspended in this medium. Ten ml of suspension was filled in a 12 ml tube and 1 ml of a suspension of elemental sulfur or 1 ml $H_2O$ was added. The tubes were wrapped in alumina foil and bubbled with oxygen free helium for 5 minutes. The tubes were closed with a serum stopper and incubated at room temperature. After 6, 12, 24 and 54 hours of incubation tubes were centrifuged and pellet and supernatant analyzed for chlorophyll *a*, glycogen, $H_2S$ and lactate. The tubes contained 60 $\mu$g, 200 $\mu$g and 50 $\mu$g chlorophyll *a* for *M. chthonoplastes, Oscillatoria* grown on $ASN_{III}$ and *Oscillatoria* grown on $ASN_{III}^{o}$, respectively. For acetylene reduction measurements, 10 ml of *Oscillatoria* were transferred into a 40 ml serum bottle. The bottles were wrapped in alumina foil and bubbled with helium. After closing the bottle, 15% of acetylene was injected, using a gastight syringe.

### *Nitrogenase activity*

Nitrogenase activity was measured as acetylene reduction (Stewart *et al*, 1967). Acetylene and ethylene were determined gas chromatographically as described previously (Stal and Krumbein, 1981; Stal *et al*, 1984b).

### *Preparation of fine dispersed elemental sulfur*

A suspension of finely dispersed elemental sulfur was prepared according to Roy and Trudinger (1970). This suspension contained approximatively 1.3 mg $S.ml^{-1}$ as was determined by the method of Stal *et al.* (1984a).

### *Determination of sulfide*

Sulfide was determined colorimetrically after Pachmayr (Trüper and Schlegel, 1964).

### *Determination of L-lactate*

L-lactate was determined enzymatically using the Boehringer Test-Combination (Boehringer, W-Germany) (Noll, 1974).

### *Determination of glycogen*

Glycogen was determined as glucose after hydrolyzing the cells in 10 ml 2 N HCl for 6 hours at 100°C. The hydrolysate was neutralized with 10 ml 2 N NaOH and 16 ml 200 mM phosphate buffer, pH 7.0 was added. The volume was adjusted to 50 ml with distilled water. Glucose was determined by the GOD-Perid-Method, Boehringer Test-Com-

*M. chthonoplastes* does not show lactate fermentation when incubated under dark anaerobic conditions. In the presence of elemental sulfur, however, this organism produces sulfide (Fig. 4).

In Table 2 we listed the amounts of product formed and glycogen utilized in *Oscillatoria* and *M. chthonoplastes*, incubated anaerobically in the dark. It is striking that always 1 mol glucose utilized yielded 0.8 mol product, be it ethylene, sulfide or lactate.

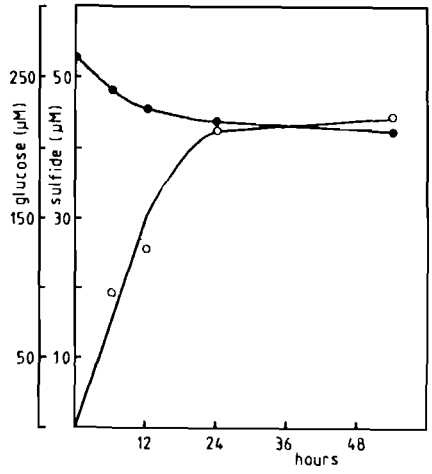


Figure 4 : Sulfide production and glycogen degradation in cultures of *M. chthonoplastes*, strain 11, incubated anaerobically in the dark in the presence of elemental sulfur. Glucose and sulfide are expressed as μMol/mg chl *a*. Closed symbols: glucose; open symbols: sulfide.

| Organism and additions | Total sugar | Glycogen used | $C_2H_4$ formed | $S^{2-}$ formed | Lactate formed |
|---|---|---|---|---|---|
| *Oscillatoria* sp $N_2$-grown culture | | | | | |
| $+C_2H_2$ | 91.2 | 61.7 | 52.8 | n.d. | 0 |
| $C_2+H_2+S^\circ$ | 91.2 | 61.7 | 52.9 | 0 | 0 |
| *Oscillatoria* sp. $NO^-_3$-grown culture | | | | | |
| no additions | 25.0 | 13.3 | n.d. | n.d. | 10.7 |
| $+S^\circ$ | 25.0 | 12.8 | n.d. | 9.9 | 0 |
| *M. chthonoplastes* $NO^-_3$-grown culture | | | | | |
| no additions | 266 | 0 | n.d. | n.d. | 0 |
| $+S^\circ$ | 266 | 51 | n.d. | 44.9 | 0 |

Table 2 : The amounts of product formed and glycogen degraded by *Oscillatoria* sp. strain 23 and *M. chthonoplastes* strain 11 incubated for 54 hours anaerobically in the dark. In the experiments with acetylene added, cultures were grown with $N_2$, other cultures were grown on nitrate. 15 % acetylene was added in the helium gas phase. Elemental sulfur was added in excess. All amounts are expressed as μMol/mg chl *a*.

## Discussion

Mat-building cyanobacteria in intertidal marine sediments are temporarily faced with anaerobic conditions (Jorgensen *et al*, 1983; Revsbech *et al*, 1983). Several cyanobacteria have developed mechanisms in order to survive and grow in the absence of oxygen. Some cyanobacteria are known to carry out anoxygenic photosynthesis under anaerobic condi-

tions in the light, using sulfide as electron donor (Cohen *et al*, 1975a; Cohen *et al*, 1975b; Garlick *et al*, 1977).
In the dark, cyanobacteria usually generate energy for maintenance and other purposes by aerobic respiration (Smith, 1982). They oxidize their reserve polymer glycogen via the oxidative pentose phosphate pathway (Smith, 1982). The NADPH formed is oxidized via the respiratory chain with oxygen as terminal electron acceptor.

Cyanobacteria in microbial mats have to cope with anaerobic conditions in the dark. The metabolism of cyanobacteria under such conditions was subject of this study. We have shown that two cyanobacteria isolated from a marine microbial mat possess mechanisms of anaerobic dark metabolism. *Oscillatoria* strain 23 can ferment endogenous carbon reserves to lactate or, alternatively, reduces elemental sulfur to sulfide. *M. chthonoplastes*, on the other hand, only reduces sulfur.

There are several other reports on anaerobic dark metabolism in phototrophic organisms. Lactic acid fermentation is known in some green algae (Gibbs, 1962). Dark sulfide production is known in purple sulfur bacteria (Trüper and Pfennig, 1966). Van Gemerden (1968) reported the oxidation of storage polyglucose to poly-$\beta$-hydroxybutyric acid using endogenous sulfur as electron acceptor. The cyanobacterium *Synechococcus lividus* reduces sulfate to sulfide or thiosulfate to sulfide and sulfite in the dark anaerobically (Sheridan and Castenholz, 1968; Sheridan, 1973). Oren and Shilo (1979) reported sulfur respiration and lactate fermentation in the cyanobacterium *Oscillatoria limnetica* and sulfur respiration in *Aphanocapsa halophytica*.
*Oscillaroria* sp. strain 23 is an interesting organism to study because of its capabality to synthesize nitrogenase. Nitrogenase activity is also observed under dark anaerobic conditions. The observation, that, in the presence of a nitrogenase reducable substrate (e.g. acetylene), neither lactate nor sulfide is produced, but exclusively ethylene, raises several questions. The production of lactate serves probably not only as a sink for electrons, but the excretion of lactate into the medium eventually is an energy generating process (Otto, 1981). The absence of lactate production when acetylene is present, however, makes it likely that lactate serves predominantly as a sink for electrons. The same argument applies for sulfur reduction. On the other hand, in the absence of a nitrogenase-reducable substrate, but in cells containing nitrogenase enzyme, lactate is the product even in the presence of elemental sulfur. This is different in cells grown on nitrate and thus not containing nitrogenase. In this case we find sulfide when elemental sulfur is added and lactate in the absence of sulfur. We do not have a satisfactory explanation for this observation. One possibility is that nitrogenase is coupled to an electron transport system which also transfers electrons to sulfide. Nitrogenase may block electron transfer to sulfur in that case.

Many organisms are able to reduce elemental sulfur to sulfide (Pfennig and Biebl, 1981). However, in many organisms it is not clear whether this is a true dissimilatory sulfur reduction. In 1976 the first true dissimilatory sulfur reducing bacteria were isolated. Pfennig and Biebl (1976) isolated *Desulfuromonas acetoxidans*. All strains oxidize acetate to $CO_2$, using elemental sulfur as electron acceptor. In our organism we cannot decide whether the sulfur reduction is dissimilatory or just a sink for electrons, without the additional generation of energy. Also in *Oscillatoria limnetica* there is no evidence for a true dissimilatory sulfur reduction.
*Oscillatoria limnetica* produced twice as much lactate per glucose metabolized than *Oscillatoria* sp. strain 23. The amount of lactate produced per glucose metabolized in our strain was about 0.8 moles. The same applies for the production of sulfide. For *Oscillatoria* as well as for *M. chthonoplastes* we found approximately 0.8 moles of sulfide formed

per glucose metabolized which is almost ten times as much as in *O. limnetica*. Moreover, *O. limnetica* produced concomitantly sulfide and lactate, which we did not observe in our strain.

As in *O. limnetica, Oscillatoria sp.* strain 23 and *M. chthonoplastes*, seem to possess the enzymes for anaerobic dark metabolism constitutively. Product formation was linear for about 12 hours and started without a lag period, immediately after the cultures were transferred into dark anaerobic conditions.
We conclude that sulfur reduction and lactate fermentation may occur in many, if not all, cyanobacteria that are frequently exposed to anaerobic conditions in the dark. The importance of this phenomenon in natural environments as well as the elucidation of the physiology of this process are currently under investigation.

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