

Organic carbon enhances dark survival of the cyanobacterium *Geitlerinema* sp. isolated from black band disease of corals

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Abstract: Black band disease (BBD) of corals is characterized as a pathogenic, horizontally migrating microbial mat that is dominated by gliding, filamentous cyanobacteria. These cyanobacteria are important in BBD etiology in that they form the dense matrix of the band and are the source of a cyanotoxin that contributes to BBD pathogenesis. The BBD environment is chemically dynamic, fluctuating between oxic and anoxic conditions, and often contains high levels of sulfide. Additionally, it is nutrient and organic carbon rich due to the presence of actively lysing coral tissue and coral mucus. BBD cyanobacteria are physiologically adapted to the BBD environment in both light and darkness. Previous work has shown that BBD cyanobacteria can perform sulfide-insensitive oxygenic photosynthesis during the day. We report here the results of laboratory studies of a culture of the BBD cyanobacterium *Geitlerinema* sp. aimed at investigating metabolism during darkness. This isolate exhibited enhancement of survival supported by exogenous organic carbon sources (both sugars and amino acids) during darkness under both aerobic and anaerobic conditions, with the greatest effect seen under anaerobic conditions. Thus this BBD cyanobacterium is physiologically adapted to the anaerobic, organic rich environment of BBD at night as well as the sulfide-rich, illuminated band environment during the day. Rev. Biol. Trop. 56 (Suppl. 1): 119-126. Epub 2008 May 30.

Key words: Cyanobacteria, fermentation, *Geitlerinema* sp., black band disease, microbial mats.

Black band disease (BBD) of corals affects 64 species of scleractinian and six species of gorgonian corals and is found on coral reefs worldwide (Sutherland *et al.* 2004). It exists as a migrating dark band, several mms to cms wide, that progressively lyses coral tissue, leaving behind exposed coral skeleton (Figure 1). Migration rates are typically an average of 3 mm/day, although some bands have been reported to migrate up to 1 cm/day (Rützler *et al.* 1983). The disease is particularly detrimental to its scleractinian, reef building hosts because most of these species grow on the order of 1 cm in diameter per year (Hudson 1981). As a result of the difference in disease progression vs. coral growth rates BBD often causes coral colony death by massive tissue lysis (Antonius 1981, Rützler and Santavy

1983, Kuta and Richardson 1996, Bruckner and Bruckner 1997).

BBD has many characteristics in common with cyanobacterial dominated microbial mats found in a variety of illuminated, sulfide-rich environments (Carlton and Richardson 1995). Such environments include hot spring outflows and hypersaline ponds and lakes (Stal 1995). Like many microbial mats, BBD exhibits an active sulfur cycle (Jørgensen *et al.* 1979, Carlton and Richardson 1995). Previous studies using oxygen and sulfide sensitive microelectrodes (Carlton and Richardson 1995, Richardson *et al.* 2001) have shown that BBD contains vertical, opposing gradients of sulfide and oxygen that are identical to those found in microbial mats. Additionally BBD microorganisms, most notably the cyanobacteria



Fig. 1. Black band disease on *Diploria strigosa*. a = freshly exposed coral skeleton, b = black band disease, c = apparently healthy coral tissue.

and gliding sulfide-oxidizing bacteria, carry out vertical migrations within the BBD mat (Richardson 1996, Viehman and Richardson 2002), again similar to those found in other microbial mats (Castenholz 1982, Jørgensen 1982, Richardson and Castenholz, 1987a, Castenholz *et al.* 1991, Garcia-Pichel *et al.* 1994). These vertical migrations result in exposure of the migrating organisms to microenvironments of dramatically changing conditions ranging from fully oxic to anoxic and from sulfide-free to sulfide-rich, with accompanying fluctuations in pH and Eh values. Studies have revealed that the physiology of microbial mat and BBD microorganisms are complex and dynamic, and are directly controlled by changes in chemical and light parameters (Jørgensen *et al.* 1979, 1986, Jørgensen 1982, Jørgensen and Revsbech 1983, Castenholz and Utkilen 1984, Richardson and Castenholz 1987a,b, Garcia-Pichel and Castenholz 1990, Castenholz *et al.* 1991, Richardson and Kuta 2003). For example, mat cyanobacteria have been shown to switch between oxygenic and anoxygenic photosynthesis (Jørgensen *et al.* 1986) and between aerobic respiration and fermentation (Richardson and Castenholz 1987b) based on the physical environment at any given time.

In addition to exhibiting a dynamic oxic/anoxic environment, BBD is nutrient and organic carbon rich due to lysing coral tissue, with the ongoing tissue lysis providing a continual source of organic carbon to the BBD community as it migrates across its host coral. Organic carbon compounds are abundant in coral mucus as well, and include glycoproteins, lipids, and the saccharides fucose, arabinose, galactose, ribose, mannose and glucose (Ducklow and Mitchell 1979, Coffroth 1990). The ability to utilize these organic carbon sources would be physiologically beneficial to BBD microorganisms.

We have been following several lines of research to define the physiological and etiological roles of cyanobacteria in black band disease. To date, physiological studies of BBD cyanobacterial isolates of two genera (*Geitlerinema* and *Leptolyngbya*) have shown that they can conduct oxygenic photosynthesis in the presence of sulfide (Richardson and Kuta 2003; Myers *et al.* 2007), a rare capability (Cohen *et al.* 1986) that would allow survival in the sulfide-rich band environment. Additionally we have shown that several isolates of these two genera from BBD produce the cyanotoxin microcystin (Richardson *et al.*

2007) which contributes to BBD pathogenicity (unpublished). Furthermore, we have previously reported that cultured BBD *Geitlerinema* and freshly collected samples of the entire BBD mat cannot fix dinitrogen, thus would be dependent on fixed nitrogen from the environment, including lysing coral tissue (Richardson and Kuta 2003). We present here new results further defining the physiology of the BBD cyanobacterium *Geitlerinema*, specifically the ability to use organic carbon to enhance survival during darkness.

MATERIALS AND METHODS

Culture isolation and maintenance: The *Geitlerinema* culture used in this study (accession no. AF474001 in GenBank, Ragoonath 2005) was isolated from BBD on a colony of *Montastraea annularis* at Algae Reef in the Florida Keys in 1991. It is maintained photoautotrophically in algal mineral media (ASN III) buffered with EPPS (N-(2-hydroxyethyl) piperazine-N-3-propane sulfonic acid) at 1.2 g/l, with pH adjusted to 8.1. Stock cultures are grown in 125 ml Erlenmeyer flasks at 30° C on a 12:12 light/dark cycle, with low light levels (30 $\mu\text{Ein m}^{-2}\text{s}^{-1}$) of cool white fluorescent light.

Survival experiments: Survival under dark and light aerobic and anaerobic conditions was investigated in the presence of 18 different organic carbon sources. These were arabinose, arginine, asparagine, citrate, fructose, fucose, galactose, glucose, glutamic acid, histidine, lactose, lysine, maltose, mannitol, mannose, ribose, sucrose, and valine. Aerobic experiments were conducted using test tubes with sterile cotton plugs while anaerobic experiments used Hungate tubes in which the media were first heated, boiled, and cooled under a stream of 100 % reagent grade N_2 gas. Tubes for dark incubations were double wrapped in aluminum foil. Light conditions were the same as those for culture maintenance, i.e. 30 $\mu\text{Ein m}^{-2}\text{s}^{-1}$ coolwhite fluorescent light on a 12:12 light/dark cycle. Stock organic carbon source solutions were prepared to a concentration of

1 M in 10 ml dH_2O , filter sterilized, and 100 μl added to 10 ml of ASN III media in each experimental test tube for a final concentration of 10 mM. Carbon sources were selected based on those reported to be present in coral surface mucopolysaccharide (Ducklow and Mitchell 1979, Coffroth 1990). All experiments were carried out in triplicate in a temperature-controlled incubator at 30° C. Dark and light controls consisted of cultures with no exogenous organic carbon source provided. Each experiment was repeated three times and was carried out for a period of 20 days.

Cell death was used to document (absence of) survival. Cell death was determined by observation of the release of the red water-soluble pigment phycoerythrin to the surrounding medium as a result of filament lysis. Additionally, dead cells appeared green due to the maintenance of membrane-bound chlorophyll *a* along with the loss of phycoerythrin. Cell survival was confirmed by microscopic observation of intact, motile filaments. Growth during the survival period was not quantifiable because heterotrophic bacteria living in close association with the polysaccharide sheath could not be removed from the culture.

Statistical analysis: All experiments were run in triplicate. The Shapiro-Wilk test was used to test for normality. A one-way ANOVA was performed on pooled carbon sources as well as Tukey-HSD Post-Hoc test for comparison of means.

RESULTS

Survival with exogenous organic carbon: The addition of organic carbon enhanced the period of survival of BBD *Geitlerinema* under both dark aerobic and dark anaerobic conditions. Under light aerobic (control) conditions there was survival in the presence of all organic carbon sources for the entire length of the experiment (20 days) with the exception of arabinose, in which cultures survived an average of 19.9 (± 0.1) days (Figure 2a). Cultures incubated in dark aerobic conditions did not survive as long

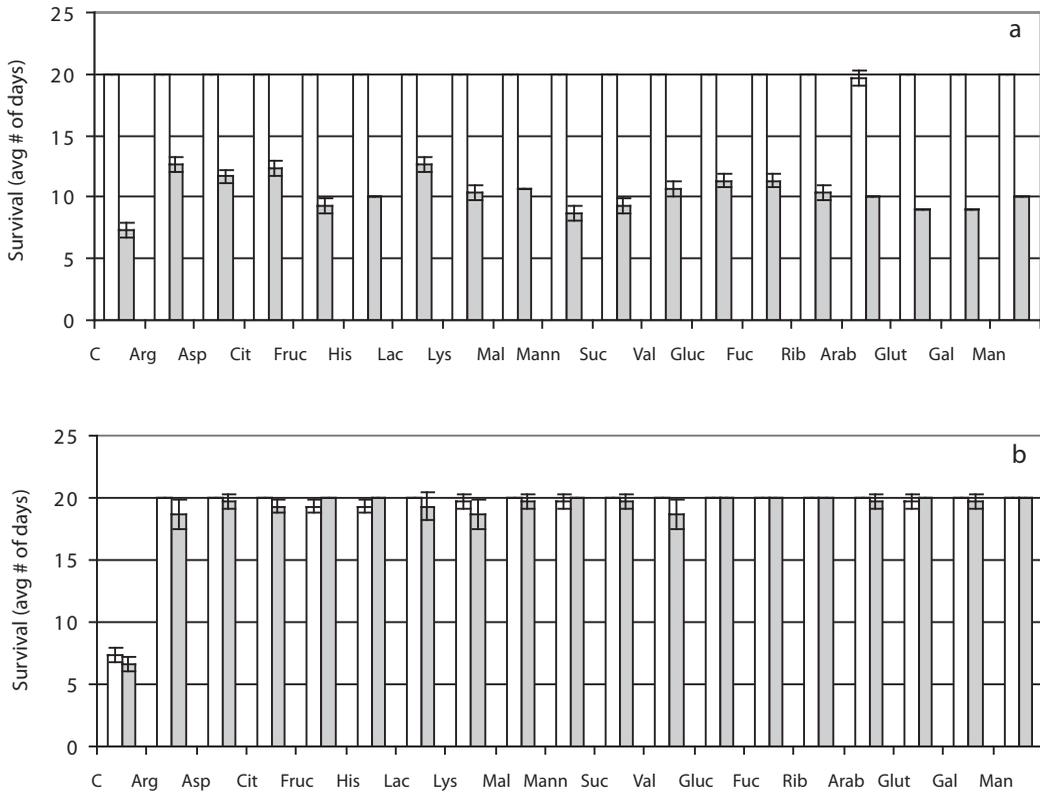


Fig. 2. Survival in light and dark aerobic (a) and anaerobic (b) conditions. Survival was monitored over a 20-day period. White bars represent survival in light conditions, shaded bars in dark conditions. C = control (no organic carbon); Arg = arginine; Asp = asparagine; Cit = citric acid; Fruc = fructose; His = histidine; Lac = lactose; Lys = lysine; Mal = maltose; Mann = mannitol; Suc = sucrose; Val = valine; Gluc = glucose; Fuc = fucose; Rib = ribose; Arab = arabinose; Glut = glutamic acid; Gal = galactose; Man = mannose. Error bars indicate standard deviation.

as the controls. The greatest survival in dark aerobic conditions was supported by arginine and lactose, with average survival values of 12.7 days (± 0.6) and 12.6 days (± 0.5), respectively (Figure 2a). When exogenous organic carbon sources were excluded the cultures in dark aerobic conditions survived on endogenous carbon for 7.3 days (± 0.5).

Survival under light vs. dark anaerobic (100% N₂ gas phase) conditions was not significantly different (Figure 2b). All cultures survived for an average of 19.3 (± 0.1) days and 19.0 (± 0.3) days, respectively. In both light and dark anaerobic conditions when organic carbon sources were excluded, cultures survived for no more than one week.

In the presence of exogenous organic carbon dark, anaerobic survival was significantly different (greater) than survival under dark, aerobic conditions (Tukey HSD Post-Hoc test, $r^2 = 0.99$).

DISCUSSION

Dark metabolism of cyanobacteria: As discussed in a review by Stal and Moezelaar (1997) very little work has been carried out on cyanobacterial metabolism under anaerobic conditions and darkness. This is because all cyanobacteria generate oxygen during their preferred mode of metabolism, oxygenic photoautotrophy, and thus are often found in

aerobic environments. Under dark aerobic conditions it is well known that all cyanobacteria can carry out aerobic respiration of glycogen (produced during photosynthesis), which is degraded via the oxidative pentose phosphate pathway (Matthijs & Lubberding 1988). However, as pointed out in Stal and Moezelaar (1997), there are a variety of natural environments in which cyanobacteria experience prolonged periods of anoxia and darkness. These include anoxic hypolimnia of lakes and ponds, microbial mats, sediments of lakes and estuaries, surface water blooms, and in symbiotic associations with higher plants. It has been demonstrated repeatedly in laboratory experiments that cyanobacteria isolated from these environments are capable of fermentation of stored glycogen when exposed to darkness and anoxia (reviewed in Stal and Moezelaar 1997). Despite this widespread physiologic capability studies have found that only a very few cyanobacteria can make use of exogenous (as opposed to endogenous) organic carbon to support the ability to ferment. These include members of the genera *Nostoc*, *Oscillatoria* (with the relevant species reclassified as belonging to *Geitlerinema*), and *Microcystis*, as well as symbiotic species (Stal and Moezelaar 1997).

Enhanced survival of BBD *Geitlerinema* during darkness: Black band disease represents another example of a natural environment in which cyanobacteria experience prolonged periods of anoxia and darkness. Microelectrode studies of intact black band incubated in darkness revealed that the entire band becomes anoxic and sulfide rich, and that at times the oxygen/sulfide interface extends above the band into the water column (Richardson *et al.* 2001). Thus BBD cyanobacteria are exposed to an anaerobic (and reducing), organic-rich environment every night.

Our results reported here show that the BBD cyanobacterium *Geitlerinema* can use a variety of amino acids, sugars, and other organic carbon compounds to enhance survival in the dark under anaerobic (as well as aerobic) conditions. Under dark anaerobic conditions

this cyanobacterium survived an average of 19 days with exposure to exogenous organic carbon, as opposed to a maximum of 12 days under aerobic conditions and seven days with no added carbon source.

Ecological significance of BBD cyanobacterial physiology in darkness: The observation that exogenous organic carbon enhances survival under both aerobic and anaerobic dark conditions, and that the most pronounced enhancement occurred in dark anaerobic conditions, is important for the ecology of BBD cyanobacteria since these conditions reflect the natural microenvironments of BBD at night. Microelectrode studies (Richardson *et al.* 2001) have shown that oxygen is present near the surface of the band and extends to a depth of several hundreds of microns into the band during the period following dusk, resulting in a period every day in which parts of the band are aerobic and dark. During these times, BBD cyanobacteria would carry out the usual metabolic mode of aerobic respiration. After prolonged darkness, however, as sulfide accumulates and aerobic respiration consumes oxygen, the oxygen/sulfide interface moves to the band surface. As a result, for most of the night the entire band is anoxic (Richardson *et al.* 2001). During this period BBD cyanobacteria switch to an anaerobic metabolic mode. Thus, the metabolism of this cyanobacterium is flexible and adaptive to the dynamic environment of the band.

These results, together with the results of our previous studies of sulfide-resistant oxygenic photosynthesis by BBD *Geitlerinema* (Richardson and Kuta 2003, Myers *et al.* 2007), demonstrate that BBD *Geitlerinema* is physiologically capable of taking advantage of the BBD environment in both light and darkness and is therefore well adapted to this unique and highly fluctuating ecological niche. Both metabolic abilities are important in the microbial ecology of the band. For example, most other (oxygenic) photosynthetic microorganisms, including most cyanobacteria, cannot tolerate sulfide and would thus be excluded from the

nutrients available in the band, yielding a competitive advantage to *Geitlerinema*. The ability to use exogenous organic carbon when the band is anaerobic allows BBD *Geitlerinema* to physiologically take advantage of the organic-rich BBD environment during darkness, and may allow them to avoid death. Stal and Moezelaar (1997) report that cyanobacterial species that could not carry out fermentation of carbon (endogenous or exogenous) died and lysed within 2-3 hours after exposure to dark anoxic conditions. They propose that the capability of fermentation is "essential" for those cyanobacteria that are naturally exposed to conditions of anoxia and darkness, as is the case with BBD cyanobacteria. Although we did measure fermentation byproducts in our experimental cultures, the source of these byproducts could be the sheath-associated bacteria present in the cultures and those results are not presented here. Therefore, we can only suggest that the BBD *Geitlerinema* sp. is capable of fermenting. However, the prolonged nature of the enhanced survival of BBD *Geitlerinema* (19 days) suggests that this isolate is capable of growing heterotrophically on the exogenous organic carbon provided. Again, although we did measure positive growth rates via a protein assay during dark incubations, sheath-associated heterotrophic bacteria in the *Geitlerinema* culture may have contributed to these results and those data are not presented here. It has been calculated that 5-30 mM of exogenous carbon would be required to support anaerobic chemoorganotrophic cyanobacterial growth (Stal and Moezelaar 1997). Since the horizontal migration of BBD over coral provides a continual source of lysing coral tissue as well as continued access to coral mucus, the requirement for a constant supply of organic carbon substrate appears to be fulfilled. The observed enhanced survival of BBD *Geitlerinema* may be due to an environmentally relevant case of cyanobacterial heterotrophy.

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RESUMEN

La enfermedad de la banda negra que afecta a los corales (BBD, por sus siglas en inglés) está compuesta de una "alfombra" de microbios patógenos dominado por cianobacterias filamentosas que se deslizan horizontalmente. Estas cianobacterias son fundamentales en la etiología de la BBD porque forman la matriz densa de la banda y son el origen de una cianotoxina que contribuye a la patogénesis de la BBD. El ambiente de la BBD es químicamente dinámico, con cambios entre condiciones oxigenadas y anóxicas, y con altas concentraciones de sulfuro. Además, la BBD es rica en nutrientes y en carbono orgánico debido a la presencia de tejidos de corales que liberan activamente el contenido de las células. Las cianobacterias de la BBD están adaptadas al ambiente de la BBD, en luz y oscuridad. Trabajos anteriores han demostrado que durante el día, las cianobacterias de la BBD pueden hacer fotosíntesis oxigénica sin verse afectadas por la presencia del sulfuro. Comunicamos los resultados de un estudio de laboratorio sobre el metabolismo en la oscuridad de un cultivo de la cianobacteria de la BBD *Geitlerinema* sp. La supervivencia de esta estirpe, tanto en condiciones oxigenadas como en condiciones anóxicas, fue sustentada por fuentes exógenas de carbono orgánico (azúcares y aminoácidos). El mayor efecto se produjo en condiciones anóxicas. De noche esta cianobacteria está fisiológicamente adaptada al ambiente de la BBD, rico en sustancias orgánicas, mientras que de día está adaptada al ambiente iluminado rico en sulfuro.

Palabras clave: cianobacteria, fermentación, *Geitlerinema* sp., enfermedad de banda negra, tapetes microbianos.

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