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# Lipid profile: a useful chemotaxonomic marker for classification of a new cyanobacterium in *Spirulina* genus

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**Abstract**

The morphological, physiological and genetic characteristics of an isolate cyanobacterium from hard sand of the lake Venere in the Pantelleria island (Italy) were described. The isolate with a small-size coiled helix shape, growing optimally at pH 9.2–9.5 at 30°C under continuous illumination and aeration, possessed a 61.5 mol% of Guanine + Cytosine content of DNA. The lipid profile showed the presence of mono-, di-glycosyl, sulphoquinovolosyl and phosphatidyl (MGDG, DGDG, SQDG and PG). The fatty acid profile was also studied, characterized by the absence of γ-linolenic acid and the presence of saturated and monounsaturated C16 and C18. The latter was also present as a dienoic component. The fatty acid composition was affected by growth temperature by increasing the degree of desaturation at a lower temperature and the biosynthesis of shorter acyl chains. The effects of growth conditions other than temperature, physical, nutritional and chemical on lipid composition were also studied. The overall features of the cyanobacterium isolated from Pantelleria clustered it into *Spirulina* genus. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Cyanobacteria; *Spirulina*; *Arthrospira*; Chemotaxonomy; Lipid profile; Fatty acids

## 1. Introduction

Cyanobacteria, the oldest oxygenic photosynthetic organisms known so far, are classified into five subgroups and constitute a major group of prokaryotes (Holt et al., 1994). They are a diverse group of prokaryotes, whose common feature is their oxygenic photosynthesis, similar to that in algae and higher plants. They continue to make a large contribution to the equilibrium of the Earth's atmosphere by producing oxygen and removing carbon dioxide

In cyanobacteria, a large variety of species with diverse morphological and physiological properties are comprised; their genetic diversity is retraived by Guanine + Cytosine (G + C) contents of DNA, which cover the entire range of values found for prokaryotes (Tandeau de Marsac and Houmard, 1993).

As previously noted (Holt et al., 1994), classification of cyanobacteria is in transition and may be subjected to great modification. Criteria for classification include morphology, physiology, chemistry, genetics and, more recently, molecular biology (Viti et al., 1997).

There is much confusion in cyanobacterium taxonomy for the *Arthrospira* and *Spirulina* genera. Until now, many papers have described properties of *S. platensis* and *S. maxima*, while both species are moved in *Arthrospira* genus; *S. major* was already described as the type strain of *Spirulina* genus in 1993 (Rippka and Herdman, 1993). The G + C content of DNA, lipid analyses and some physiological features help in the assignment of a new species to genus *Arthrospira* or *Spirulina* (Rippka and Herdman, 1993; Viti et al., 1997).

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Nowadays, there is a great interest for biotechnological applications of cyanobacteria in general and for "Spirulina", in particular (Mahajan and Kamat, 1995; Mathew et al., 1995; Tadros et al., 1995), in health chemoprevention and as food and cosmetic additives, because it is rich in proteins and in unsaturated fatty acids. Cyanobacterium "Spirulina" is the commercial name used in the products, which generally contains *Arthrospira* strain(s) (Gambacorta, personal communication).

Cyanobacteria, named also blue–green algae, were found in almost all the ecosystems examined so far (Tandeau de Marsac et al., 1993; Holt et al., 1994). Recently, interest in microorganism strains obtained from extreme environments has grown considerably, because they represent an innovative approach to obtain new underexplored resources.

In the screening program to obtain new prokaryotes from extreme environments, we have collected samples from hard sand of the lake Venere in the Pantelleria island (Sicily, Italy), that showed high salinity and alkalinity.

Alkaline-halophilic environments are rich in photosynthetic primary producers, mainly the result of the dense population of cyanobacteria, that presumably support the rest of the microbial community. These blooms of cyanobacteria are usually dominated by Oscillatoriales members (Jones et al., 1998). From the haloalkaliphilic environment of Pantelleria, we have isolated new strains of Oscillatoriales, belonging to *Oscillatoria* and *Spirulina* genera as well as an aerobic bacterium classified as *Halomonas pantelleriense* (Romano et al., 1996).

It is well known that lipids are useful chemotaxonomic markers for classification of prokaryotes (Ratledge and Wilkinson, 1988). In particular, for cyanobacteria, it was proposed that the fatty acid profiles permit to divide these microorganisms in five groups (Cohen et al., 1995).

To survive in extreme or variable environments, cyanobacteria have developed specific regulatory systems, in addition to those of other prokaryotes or photosynthetic eukaryotes (Tandeau de Marsac et al., 1993).

In this paper, we report the isolation, characterization and classification of a new cyanobacterium as *Spirulina* strain "pantelleria", able to produce exopolysaccharide (Nicolaus et al., 1999) together with the effect of growth conditions on its lipid pattern.

## 2. Results and discussion

The cyanobacterial strain "pantelleria" was isolated from saline–alkaline hard sand of costal lake Venere in Pantelleria island (Sicily, Italy). The culture, selected mainly on alkaline medium at pH 10.0, was finally purified by picking, under microscope, with needle a single filament and by inoculating in a vial with 2 ml of medium. The procedure was carried out twofold.

The purified trichome appeared to be composed of small-size cell and a tightly coiled helix; because of the trichome shape, the strain could be assigned to *Spirulina* genus. The above morphological features did not change significantly with growth conditions. Species of *Spirulina* have a known large distribution in fresh, marine and brackish waters (Staley et al., 1989), but species are also seen in inland saline lakes, such as the case of our strain.

The temperature growth range was from 20 to 35°C with an optimum growth at 30°C. At pH 9.2–9.5, the strain exhibited optimal growth and can grow at pH ranging from 8.0 to 10.0. The enrichment medium named AO was the medium normally used for growth and maintenance of the strain, although it also grew in media with a lower salt concentration (A and MN) as well as in media in which the ionic strength was higher than that of optimal medium (ASN III). The BG11 and $BG11_0$ media did not sustain the growth. These growth features are different from those reported for the type strain *S. major* and also for *S. subsalsa* (Cohen et al., 1987; Staley et al., 1989; Rippka and Herdman, 1993). The optimal growth conditions of pH, temperature and salinity reflected the environmental parameters of the site from which the cyanobacterium was isolated.

The cyanobacterium from Pantelleria was not able to fix nitrogen and it is an obligate photoautotroph, as was reported before for related strains (Staley et al., 1989; Kenyon et al., 1972). In fact, the growth in the presence of 1% glucose plus DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl-urea) in light as well as in 1% glucose under darkness scored negative results. The optimal growth conditions were under continuous illumination and aeration at 30°C, pH 9.2–9.5.

The mol% G + C content of DNA was determined to be 61.5%, by HPLC. With the same procedure, the G + C content of *Spirulina* PCC 6313 was determined to be 53.0%, value similar to that reported in literature (Staley et al., 1989; Rippka and Herdman, 1993).

The pattern of polar lipids was characterized by the presence of glycolipids, as major components; ninhydrine-positive compounds were absent, contrary to previous reports on related strains by Kenyon et al. (1972) but as reported from Rippka and Herdman (1993), and the phospholipids were present in a lesser extent. The glycolipids and the phospholipid present in the "pantelleria" strain were mono-, di-glycosyl, sulphoquinovolosyl (MGDG, DGDG, SQDG) and phosphatidyl (PG) derivatives of 1,2-diacylglycerol; the PG was present in low amount. The identification was obtained by staining tests and Rf comparison on TLC with appropriate standards.

The profile of fatty acids under optimal growth conditions, 30°C with continuous illumination and aeration (Table 1), showed more abundant acyl chains *n*C16:0 (44.4%), and C16:1 with both isomers (19.4%). Other components were *n*C18:2 (10.4%); C18:1 (15.2 %), present with both isomers, and *n*C18:0 (10.6%). The profile of the fatty acids of *S. major* PCC 6313 is quite similar to that published by Kenyon et al. (1972), but was very different from the profile observed in our strain. In fact, short acyl chains, as C14 (C14:1, 15%; and C14:0, 26%) and C16 (C16:1, 34%; and C16:0, 24%) were the main components, while C18 compounds, both saturated and mono-unsaturated, occurred only in trace amount, less than 2% of the total acyl chains. Moreover, C18:2 was totally absent and the ratio of saturated/unsaturated acyl chains is ca. 1 in PCC 6313 instead of ca. 1.2 for the ''pantelleria'' strain.

The ''pantelleria'' strain was grown under different conditions and the polar lipids and fatty acid profile were examined. Under all tested conditions, the biomass yield as well as lipid content did not change significantly; a similar result was also reported for other species (Tomaselli et al., 1997).

By modulating the growth conditions, as reported in Table 1, the main effect of such variations on polar lipids was the small decrease of PG in all tested culture conditions. Some new spots, positive for sugar staining, at Rf between mono- and di-glycolipids for the culture starved for nitrogen, and phosphorus and at lower temperature, were observed.

When the sole variation to standard growth conditions was the absence of aeration (4) and the culture was in a static state, the degree of saturation is higher than under optimal growth parameters, with the major effect on C18:0 that increased at the expense of C18:2. Surprisingly, in this condition, the *trans* isomer of C16:1 was no longer detected, while the ratio of both isomers of C18:1 remained quite constant.

Under growth condition where nitrogen supply was lowered to one fifth (2), the major effect was an increase in the degree of desaturation, mainly at the level of C16:1 that reached ca. 31% of the total fatty acids, such an increase was at the expense of C16:0, and in a lesser extent of C18:0 and C18:2. A quite similar behavior was observed when the culture was deprived totally of phosphorus (3); in this case, a small increase in C18:1 abundance was also observed. Under nitrogen starvation and lack of aeration (5), the total absence of the *trans* isomer of C16:1 occurred, as it was observed for the experiment in which only aeration was eliminated (4). Moreover, there was a large increase of C18:0 paralleled by a decrease of C18:2 with a resulting increase in the degree of saturation. In stressed conditions without air, phosphorus and nitrogen starvation (6), the pattern of fatty acids was quite similar to that described in the previous experiment, with the sole exceptions of a little increase of the di-unsaturated component and of the presence of *trans* isomer of C16:1. Under the last two stressed growth conditions, a strong increase of C18 acyl chains with respect to C16 was observed; in fact, the C16:0/C18:0 ratio decreased ca. threefold in comparison with the optimal growth conditions (Table 1).

By decreasing the temperature to 26°C (7), a doubling of C16:1 was found with respect to 30°C at the

Table 1
Fatty acid composition (% total fatty acids) and relative ratio of *Spirulina* strain ''pantelleria'' cultivated under different growth conditions

| Experiments[a] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| [b]C16:1 trans | 8.2 | 9.6 | 9.6 | 0.0 | 0.0 | 4.8 | 10.8 | 7.7 |
| [b]C16:1 cis | 11.2 | 21.8 | 20.3 | 18.6 | 13.9 | 12.2 | 25.7 | 20.0 |
| C16:0 | 44.4 | 37.6 | 35.4 | 42.4 | 38.8 | 34.9 | 40.7 | 38.4 |
| [b]C18:2 | 10.4 | 6.8 | 8.8 | 6.6 | 2.0 | 5.5 | 1.4 | 16.3 |
| [b]C18:1 trans | 11.7 | 13.5 | 15.0 | 11.9 | 15.6 | 15.9 | 10.9 | 7.6 |
| [b]C18:1 cis | 3.5 | 2.8 | 3.2 | 2.8 | 3.9 | 3.7 | 3.3 | 2.5 |
| C18:0 | 10.6 | 7.9 | 7.8 | 17.8 | 25.7 | 22.9 | 7.2 | 7.5 |
| % saturated | 55.04 | 45.47 | 43.17 | 60.17 | 64.44 | 57.86 | 47.9 | 45.95 |
| % monoenoic | 34.59 | 47.74 | 48.06 | 33.25 | 33.56 | 36.63 | 50.7 | 37.77 |
| % dienoic | 10.37 | 6.79 | 8.77 | 6.59 | 2.00 | 5.5 | 1.39 | 16.28 |
| C16:0/C18:0 | 4.19 | 4.74 | 4.57 | 2.39 | 1.51 | 1.52 | 5.66 | 5.11 |
| C16:1/C18:1 | 1.28 | 1.92 | 1.64 | 1.27 | 0.71 | 0.86 | 2.56 | 2.77 |
| saturated/unsaturated | 1.22 | 0.83 | 0.76 | 1.51 | 1.81 | 1.37 | 0.92 | 0.85 |
| C18:2/C(n):1 | 0.30 | 0.14 | 0.18 | 0.2 | 0.06 | 0.15 | 0.03 | 0.43 |

[a] *Experiments*: (1) optimal growth conditions, OGC (AO medium, 30°C, continuous aeration and illumination); (2) OGC with 1/5 of $NaNO_3$; (3) OGC with 1/5 of $NaNO_3$ and without $K_2HPO_4$; (4) OGC without aeration; (5) OGC with 1/5 of $NaNO_3$ and without aeration; (6) OGC with 1/5 of $NaNO_3$ and without aeration and $K_2HPO_4$; (7) 26°C with light and aeration; (8) 26°C with dark/light cycle and without aeration.

[b] Double bond positions not determined.

expense of C18:2, that was lowered ca. sevenfold, now withstanding this the ratio saturated/unsaturated acyl chains decreased, as well as the C18/C16 ratio (Table 1). At this incubation temperature, shorter chains and unsaturated ones were preferred. When at the culture incubated at this lower temperaure a light/dark (12 h/12 h) cycle was applied and aeration was eliminated (8), a conspicuous effect on C18:2 biosynthesis was the major change observed, this compound increased 11 times with respect to a similar growth temperature but with continuous aeration and illumination. In absolute, under growth conditions 8, the decrease of desaturation was more pronounced.

The cyanobacterium strain isolated from Pantelleria hard sand is a filamentous-small-sized strain; therefore, according to literature data, it belongs to *Spirulina* genus (Staley et al., 1989; Rippka and Herdman, 1993), and thus should not contain C18:3 fatty acids, typical instead of *Arthrospira* genus (Cohen et al., 1995). Our strain, in fact, possessed only C18:2 as poly-unsaturated acyl chain in all tested growth conditions, and according to the observations of Cohen et al. (1995), it did not accomodate in any of the four groups of cyanobacteria, previously defined on the basis of fatty acid profiles (Kenyon et al., 1972). The C18:2 acyl chain was also found in some strains of *S. subsalsa* (Cohen et al., 1995) and these strains as well as our "pantelleria" cyanobacterium can be positioned in the fifth group that contains C18 poly-unsaturated fatty acids with not more than two double bonds. The genetic molecular approaches will definitively clustered them.

Living cells subjected to any deleterious environmental change respond in many different ways by reducing their growth rate, by producing polymers such as polysaccharides and/or polyhydroxyalkanoates, by chemical changes such as modification of lipids in order to always maintain the critical degree of membrane fluidity. Although the molecular bases of the stress response phenomenon is poorly documented in cyanobacteria so far, there exists increasing evidence for the synthesis of stress proteins upon cell exposure to changes in temperature, salinity, nutrient supply, light intensity, etc. (Tandeau de Marsac et al., 1993).

The most studied effects were those due to temperature on lipid composition, and they were extensively studied for cyanobacteria (Sato and Murata, 1982; Quoc and Dubacq, 1997). These studies, in particular, were concerned with the *Arthrospira–Spirulina* genus, in order to optimize the productivity of γ-linolenic acid for commercial purposes. Generally speaking, high temperatures largely favor the less desaturated acyl chains. Such a feature is also observed in our strain, in which by lowering the temperature to 4°C, the saturated acyl chains were less biosynthesized, thus lowering the ratio of saturated/unsaturated acyl chains from 1.22 to 0.9 at 26°C (Table 1) (Cohen et al., 1987; Funteu et al., 1997). Moreover, the di-unsaturated/mono-unsaturated ratio increased from 0.03 to 0.30; the increasing biosynthesis of shorter chain with respect to longer one, could also be a right answer to maintain at a lower temperature appropriate membrane functionality. Some paper reported changes also at the level of lipid classes by lowering temperature (Quoc and Dubacq, 1997); these authors observed that in *S. platensis*, by lowering growth temperature from 35 to 24°C, the MGDG decreased and this was compensated by an increase of SQDG, while DGDG and PG appeared more stable. In our case, this behavior was not observed and that only a little increase of DGDG was noted.

Numerous studies on chemical and/or metabolism changes in cyanobacteria induced by light, nitrogen, or other nutrients have been reported (Tandeau de Marsac et al., 1993). In contrast, despite the crucial role of phosphate in cell metabolism, structure and regulation, little attention has been paid to the effects of deprivation in this nutrient in cyanobacteria (Tandeau de Marsac et al., 1993). Some authors reported that *S. platensis* is highly resistant to phosphorus deficiency (Funteu et al., 1997). We tested the deprivation of such an ion on "pantelleria" strain in two different experiments: (a) culture subjected simultaneously to nitrogen starvation (3), and (b) culture also subjected to the deprivation of aeration (6). In both cases, the phosphorus deprivation did not markedly change the fatty acid profile obtained with a phosphate-containing culture medium.

Previous paper reported that *S. platensis*, although highly resistant to phosphorus deprivation, is sensitive to nitrogen or sulfur absence, which rapidly inhibited growth (Funteu et al., 1997). These authors reported that a normal lipid and fatty acid composition is maintained during nitrogen starvation (Funteu et al., 1997). In our experiments in which nitrogen was reduced to one fifth of the normal value, we observed a quite similar lipid pattern with a low decrease of PG. At the level of fatty acids, the reduction of nitrogen gave an increase of unsaturation of acyl chains mainly as C16:1 that increased ca. 1.5 fold. Some papers reported the effect of different concentrations and sources of nitrogen on fatty acid profiles of *S. platensis* mainly on γ-linolenic species (Mahajan and Kamat, 1995), but the incubation conditions were different from the present ones and the data were difficult to compare.

The effect of light intensity on the degree of fatty acid unsaturation of algae cannot be generalized, and conflicting data have been reported for different species (Cohen et al., 1987). Pantelleria strain was incubated with light/dark cycle (8) and, under these conditions concerning light duration, a slight increase in the

degree of unsaturation was observed. Moreover, a large increment of C18:2, as was also described in some other species of algae, grown under different light intensity, was found (Cohen et al., 1987). The absence of aeration (4) did not result in large changes in fatty acid profiles: a little increase of ratio between saturated and unsaturated acyl chains together with a preferential biosynthesis of longer (C18:0) acyl chains, and a low decrease of C18:2. This behavior was opposite to that usually observed when temperature decreased, as if the aeration influenced the fatty acid composition in a similar way as when temperature increases.

The results thus obtained on lipid composition, by modulating the growth conditions of the "pantelleria" strain, suggest that the membrane, by modulating the lipid composition, maintains its correct fluidity to ensure a correct functionality under different tested growth conditions.

In conclusion, the morphology (Cohen et al., 1995) and the fatty acid profiles lead to assign the "pantelleria" strain to *Spirulina* genus. In particular, the fatty acid profile is very similar to that of *S. subsalsa*, while the C + G mol% of DNA is ca. 8% higher than that of type strain PCC 6313. The genetic molecular approaches will definitively classify the "pantelleria" isolate and permit the elucidation of the taxonomy of this genus of Oscillatoriales. Finally, *Spirulina* from Pantelleria is able to produce exopolysaccharides (EPS) (Nicolaus et al., 1999). This is the first report for the EPS production from *Spirulina* species; in fact, the species studied before for EPS production, designed as *Spirulina*, belonged to the genus *Arthrospira* (Filali Mouhim et al., 1993; Hayashi and Hayashi, 1996).

The *Spirulina* from Pantelleria has been deposited in 1995 in Pasteur Culture Collection, Paris (PCC 9445).

## 3. Experimental

### 3.1. Study site and sampling

Samples were collected during the 1993 summer into sterile (Falcons) tubes, from the hard sand of the volcanic lake of Venere, close to the seashore in the island of Pantelleria in the south of Sicily (Italy).

### 3.2. Media and culture conditions

Enriched alkaline medium (named AO) contained the following components (g $l^{-1}$): NaCl 1.0, $CaCl_2$ 0.04, $MgSO_4 \times 7H_2O$ 0.097, $NaNO_3$ 2.5, $K_2SO_4$ 1.0, EDTA 0.08, $FeSO_4$ 0.01, $K_2HPO_4 \times 3H_2O$ 0.67, $Na_2CO_3$ 4.0, $NaHCO_3$ 13.0. $K_2HPO_4$, $Na_2CO_3$ and $NaHCO_3$ were autoclaved separately. The pH of the medium came to 9.5.

Solid media were prepared by addition of 2% agar. The growth of *Spirulina* strain "pantelleria" was tested in the following media: BG11, $BG11_0$, ASN III, MN (Staley et al., 1989) and medium named A that contained (g $l^{-1}$): $KNO_3$ 2.0, $K_2HPO_4$ 0.2, $MgSO_4 \times 7H_2O$ 0.1; $CaSO_4$ 20, $FeSO_4 \times 7H_2O$ 1.4, EDTA 1.6. *Spirulina major*, obtained from Pasteur Culture Collection, Paris, France (PCC 6313), was grown in all tested media for *Spirulina* species from Pantelleria island.

*Spirulina* "pantelleria" was purified by repeated dilution and streaking on the solid alkaline medium (AO). It was cultivated in 990 ml alkaline medium in 1 l bottle gassed continuously by a stream of air (250 l $h^{-1}$), at 30°C and under constant illumination with a ring-like cool white fluorescent lamp (32 W) providing 1100 lx for at least 7 days. Culture used was not bacteria free (bacterial counts not exceeding 70 colonies $ml^{-1}$, as measured by plating samples of the culture on nutrient agar plates containing alkaline medium). The cells, grown exponentially under the different experimental conditions, were harvested by centrifugation at 9850 *g*. The pellet was washed twice with an iso-osmotic saline solution, collected by centrifugation and then lyophilized. Cell yields ranged between 0.3 and 0.4 g of dry wt. *Spirulina major* (PCC 6343) was usually grown in BG11 medium, unless otherwise stated, according to Rippka and Herdman (1993). *Spirulina* "pantelleria" was also cultivated under different growth conditions of temperature, aeration and nutrient content.

Examination for growth with glucose in the presence of DCMU in light, was performed according to the method of Kenyon et al. (1972), with the sole exception that the media used were AO and nitrogen deprived AO.

Growth was monitored both by weighing dry cells and by measurement of chlorophyll content according to Tandeau de Marsac and Houmard (1988).

### 3.3. Microscopy

Cell morphology was determined by phase-contrast microscopy 40 times (Zeiss). Colony morphology was determined by Leica M8 stereomicroscope.

### 3.4. Lipid analysis

Freeze-dried samples of cyanobacteria were extracted with $CHCl_3$/*i*-PrOH (1:1) at room temperature. Lipid extracts (ca. 14–15% of dry cells) were analyzed by TLC eluted with $CHCl_3$–MeOH–$H_2O$ (65:25:4) according to Nicolaus et al. (1995). Fatty acid methyl esters were obtained and analyzed by CG–MS as previously reported (Nicolaus et al., 1995).

The data shown are mean values of at least two

independent samples, each analyzed in duplicate, and they represent mean values with a range of $<3\%$ for major ($>10\%$ of fatty acids) peaks and 5% for minor peaks.

### 3.5. DNA extraction and GC % content

The cells were freezed and thawed several times for lisys. DNA was extracted and purified according to Stam and Stulp (1988).

Direct analysis of DNA base composition was done by HPLC (Waters 600 E system controller) after digestion of DNA with nuclease $P_1$ and bacterial alkaline phosphatase (Tamaoka and Komagota, 1984). The column was a Lichrocart 100 RP 18 installed in LCD analytical 4000 SM (programmable wavelength detectors), and a Gilson data system. The nucleosides were eluted by a mixture of 0.06 M $NH_4H_2PO_4$ (pH 4.0) and MeCN (20:1), at a flow rate of 1 ml $min^{-1}$ at room temperature. Each nucleoside was detected by its UV absorbance at 270 nm. The mol% of C + G was determined according to Mesbah et al. (1989) by using DNA of *Sulfolobus solfataricus* as standard.

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