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Molecular Classification of Commercial *Spirulina* Strains and Identification of Their Sulfolipid Biosynthesis Genes

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Cyanobacterial strains of the genus *Spirulina* have recently been identified as an excellent source of sulfolipids, some of which possess anti-HIV properties. Thus, to investigate the distribution of sufolipid biosynthesis pathways in *Spirulina*, a genetic screening/phylogentic study was performed. Five different strains of *Spirulina* [*Spirulina* (Jiangmen), *Spirulina* sp., *S. platensis*, *S. maxima*, and *Spirulina* seawater] sourced from different locations were initially classified *via* 16S rDNA sequencing, and then screened for the presence of the sulfolipid biosynthesis genes *sqdB* and *sqdX via* a PCR. To assess the suitability of these strains for human consumption and safe therapeutic use, the strains were also screened for the presence of genes encoding nonribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs), which are often associated with toxin pathways in cyanobacteria. The results of the 16S rDNA analysis and phylogenetic study indicated that *Spirulina* sp. is closely related to *Halospirulina*, whereas the other four *Spirulina* strains are closely related to *Arthrospira*. Homologs of *sqdB* and *sqdX* were identified in *Spirulina* (Jiangmen), *Spirulina* sp., *S. platensis*, and the *Spirulina* seawater. None of the *Spirulina* strains screened in this study tested positive for NRPS or PKS genes, suggesting that these strains do not produce NRP or PK toxins.

Keywords: *Spirulina*, nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS), sulfolipid, anti-HIV, molecular phylogeny

Cyanobacteria belonging to the genus *Spirulina,* previously collectively grouped within the genus *Arthrospira* [33], are a valuable source of natural products with a variety of

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structures and biological activities. *Spirulina* is widely used as a human health supplement and also as animal feed, owing to its high protein content and high concentration of essential amino acids, vitamins, minerals, and fatty acids. In addition, *Spirulina* has been shown to possess a range of therapeutic properties [11]. These therapeutic properties have been attributed (at least in part) to the presence of sulfoquinovosyldiacylglyceride (SQDG), a natural sulfolipid that is also produced by a range of other photosynthetic organisms. This compound has been reported to possess anti-HIV activity [9, 15, 16, 21, 22, 25], antitumor activity [26, 29], and anti-inflammatory activity [32].

The first sulfolipid biosynthesis operon to be genetically characterized was that of the purple bacterium *Rhodobacter sphaeroides* [3]. A number of studies on photosynthetic organisms, such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*, have subsequently identified other sulfolipid synthetase genes, including those responsible for the final steps in sulfolipid assembly; *sqd1* (*sqdB*) and *sqd2* (*sqdX*) [27, 34]. In 2001, Blinkova *et al.* [4] demonstrated that sulfolipid extracted from *Spirulina platensis* inhibited the activity of HIV, thereby revealing cyanobacteria as a potential source of therapeutic sulfolipids. However, cyanobacteria are also notorious for their production of potent scary metabolite toxins. Therefore, care must be taken when selecting strains for human consumption and therapeutic use.

Cyanotoxins are frequently produced nonribosomally by nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) [2, 7, 18, 20, 30]. Several studies suggest that NRP and PKS genes confer an evolutionary advantage to the cyanobacteria that possess them. However, certain species appear to lack these genes altogether, and to date they have not been identified in *Spirulina*.

Despite the wide use of *Spirulina* in the health food industry, classification of the genus remains unclear. Products

marketed as *Spirulina* may in fact belong to the genus *Arthrospira* and vice versa. Thus, in an attempt to shed some light on this subject, this study examined the 16S rDNA sequences of several commercial strains marketed as *Spirulina*. Furthermore, to assess the potential therapeutic value of these strains in treating diseases such as HIV, they were also screened for sulfolipid biosynthesis genes. Finally, the potential toxicity of these strains was examined by screening for NRPS- and PKS-encoding genes.

MATERIALS AND METHODS

Spirulina Strains and Culturing
The *Spirulina* sp. culture was obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO). The *Spirulina* (Jiangmen), *S. platensis*, *S. maxima*, and *Spirulina* seawater were provided as lyophilized pellets by Yue Jian Biology Engineering Co. Ltd. (Jiangmen, China), Elken (Malaysia), OxyMin (Australia), and the South China Sea Institute of Oceanology (SCSIO) (Guangzhou, China), respectively. The *Spirulina* sp. was cultured in an MLA medium [5] plus seawater, pH 7−7.5. The seawater was obtained from the South Australian Research and Development Institute (SARDI), and the culturing performed in 1-l Erlenmeyer flasks containing 400 ml of the culture medium. The cultures were grown in an orbital mixer incubator (70 rpm, 25°C) under cool white light (ca. 1,500 lux) on a 12:12 light:dark cycle.

DNA Extraction
Chromosomal DNA was extracted from the *Spirulina* samples using the XS DNA extraction protocol [8]. Briefly, the samples were suspended in an XS lysis buffer and incubated at 65°C for 3 h. After the lysis was completed, the samples were incubated on ice for 10 min. The DNA was then extracted using a phenol−chloroform−isoamyl alcohol solution and precipitated *via* the addition of 50 ml of 3 M NaAc and 1 ml of ice-cold ethanol. The precipitated DNA was collected *via* centrifugation, air-dried, and then resuspended in a TE buffer. The purity and concentration of the DNA extracts were determined spectrophotometrically at 260 nm and 280 nm.

16S rDNA Amplification
The five strains of *Spirulina* were identified by 16S rDNA amplification and sequencing using the cyanobacterial specific primers 27F [19] and 809R [10] according to the methods described by Gehringer *et al.* [8]. The thermal cycling for 16S rRNA gene amplification was performed using a GeneAmp PCR system 2400 Thermocycler (Perkin Elmer, Norwalk, USA) and consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, strand extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. All the primers used in this study were supplied by Sigma Genosys. The sequencing was performed using a PRISM BigDye Terminator V3.1 cycle sequencing system (Applied Biosystems, Foster City, CA, U.S.A.) and analyzed using an ABI 3730 Capillary Sequencer.

NRPS and PKS Gene Amplification
The genes encoding NRPSs and PKSs were amplified *via* a PCR using the degenerate oligonucleotide primer pairs MTF2/MTR [18] and DKF/DKR [17], respectively. The thermal cycling conditions for the NRPS gene amplifications consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 10 s, primer annealing at 52°C for 30 s, strand extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The thermal cycling conditions for the PKS gene amplifications were identical to those described for the NRPS gene amplifications, except the primer annealing was performed at 55°C.

Sulfolipid Biosynthesis Gene (*sqdB* and *sqdX*) Amplification
The degenerate oligonucleotide primer pairs dsqdX1F (5'-GGATYCAYGTKGYBAAYCCDGC-3') and dsqdX1R (5'-CCNGCBGCCATNGCYTC-3'), and dsqdBF (5'-GAYGGNTAYTGYGGNTGG-3') and dsqdBR (5'-GGCGTRAAYTGRTTRAANAC-3') were designed to amplify conserved regions within the cyanobacterial *sqdX* and *sqdB* genes, respectively. The thermal cycling conditions for the *sqdX* and *sqdB* amplification were initiated with a denaturation step at 94°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 10 s, primer annealing at 54 or 55°C for *sqdX* and *sqdB*, respectively, for 5 s, strand extension at 72°C for 5 s, and a final extension step at 72°C for 5 min.

Purification of DNA
When the PCR resulted in the amplification of a single PCR product, the product was purified *via* ethanol precipitation as follows: 2 volumes of ice-cold absolute ethanol were added to the completed PCR reaction, which was then vortexed, and incubated on ice for 15 min. The precipitated PCR products were collected *via* centrifugation at 16,000 ×*g* for 15 min. Following removal of the supernatant, the pellets were washed with 190 ml of 70% ethanol. The final DNA pellet was air-dried at room temperature then resuspended in a TE buffer.

When the PCR resulted in the amplification of multiple PCR products, they were isolated and purified using a MoBio Ultra Clean (gel purification) kit according to the manufacturer's instructions.

Sequence Analysis
The DNA sequences were viewed and analyzed using the ABI PRISM-Autoassembler program, and multiple sequence alignments were compiled and analyzed using Bioedit. A BLASTn search was used to identify the most closely related sequences in the NCBI database. The phylogenetic analysis was performed using CLUSTALX2 for protein alignments. The settings used in the multiple alignments were a 10.0 gap opening, 0.2 gap extensions, and 0.5 DNA transition weight. The phylogenetic trees were constructed using a neighbor-joining and bootstrap analysis [14] and viewed using NJplot [23].

GenBank Accession Numbers
The sequences presented in this study are available under the following GenBank accession numbers: 16S rDNA HQ008224−HQ008228, *sqdB* HQ008229−HQ008232, and *sqdX* HQ008233−HQ008236.

RESULTS

16S rDNA Amplification
16S rDNA gene fragments were successfully amplified from all five *Spirulina* strains. The resulting PCR products

Table 1. Similarity of 16S rDNA to the closest relatives in GenBank.

Species	Designation	SOURCE	Closest relative in GenBank	% Identity	Accession Number
Spirulina platensis	*Spirulina platensis*	Western Australia, Australia	*Arthospira platensis* PCC 9223	100	DQ393285.1
Spirulina	*Spirulina* seawater	Queensland, Australia	*Arthospira platensis* PCC 9223	100	DQ393285.1
Spirulina	*Spirulina* (Jiangmen)	Kuala Lumpur, Malaysia	*Arthospira platensis* PCC 9223	99	DQ393285.1
Spirulina sp.	*Spirulina* sp.	Jiangmen, China	*Halospirulina* sp. 'CCC Baja-95 C1.3'	99	Y18790.1
Spirulina maxima	*Spirulina maxima*	Guangzhou, China	*Arthospira platensis* MMG-9	99	FJ839360.1

from the *Spirulina* sp., *Spirulina* (Jiangmen), *S. platensis*, and *Spirulina* seawater were 1.425, 1.369, 1.363, and 1.402 kb, respectively. In contrast, it was only possible to amplify 0.640 kb from the *S. maxima* template DNA. A subsequent sequence analysis revealed a 100% similarity between the *Spirulina platensis* and *Spirulina* seawater sequences and that of *Arthrospira platensis* (Table 1). The *S. maxima* sequence was also 99% similar to *A. platensis,* while the *Spirulina* (Jiangmen) sequence was 99% similar to *A. platensis* (Table 1). The 16S rDNA sequence of the Australian strain, *Spirulina* sp., was 99% similar to that of *Halospirulina* sp. (Table 1).

NRPS and PKS Gene Amplification
The five *Spirulina* strains were screened for NRPS and PKS genes *via* a PCR. However, PCR products were only amplified from the *Spirulina* sp. DNA template (Fig. 1). These fragments were smaller than expected and did not share a high sequence homology with known NRPS/PKS

Fig. 1. Results of NRPS and PKS PCR amplifications from *Spirulina* sp.
Lanes 1–6 correspond to NRPS PCR amplicons (*Spirulina* sp.), NRPS PCR positive control (*Microcystis aeruginosa* PCC 7806), NRPS PCR negative control, PKS PCR amplicons (*Spirulina* sp.), PKS PCR positive control (*Microcystis aeruginosa* PCC 7806), and PKS PCR negative control. L indicates the molecular weight markers (Fermentas).

genes in the database. The sequence obtained for the NRPS PCR was most similar (38%) to the biotin-(acetyl-CoA-carboxylase) ligase of *Denitrovibrio acetiphilus*, whereas the sequence obtained for the PKS PCR was most similar (73%) to an unknown protein from the *Oryza sativa* Japonica Group. It is likely that these amplifications were the result of nonspecific primer binding.
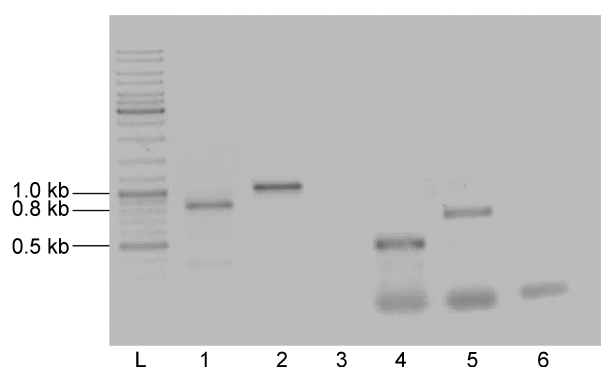
Sulfolipid Biosynthesis Gene (*sqdB* and *sqdX*) Amplification
The five *Spirulina* strains were screened for *sqdB* and *sqdX* genes *via* a PCR. Unique amplicons of the expected sizes (0.900 kb for *sqdB* and 0.600 kb for *sqdX*) were obtained from both PCRs when the *S. platensis*, *Spirulina* (Jiangmen), and *Spirulina* seawater template DNAs were used. In addition to the 0.900 kb fragment, a sec dominant amplicon (0.700 kb) was obtained for the *sqdB* PCR when the *Spirulina* sp. template DNA was used. Conversely, no amplicons were obtained from the *S. maxima* template DNA. Some nonspecific amplification products were obtained from the *sqdB* and *sqdX* PCRs when the *Synechocystis* PCC 6803 template DNA (positive control) was used (Fig. 2).
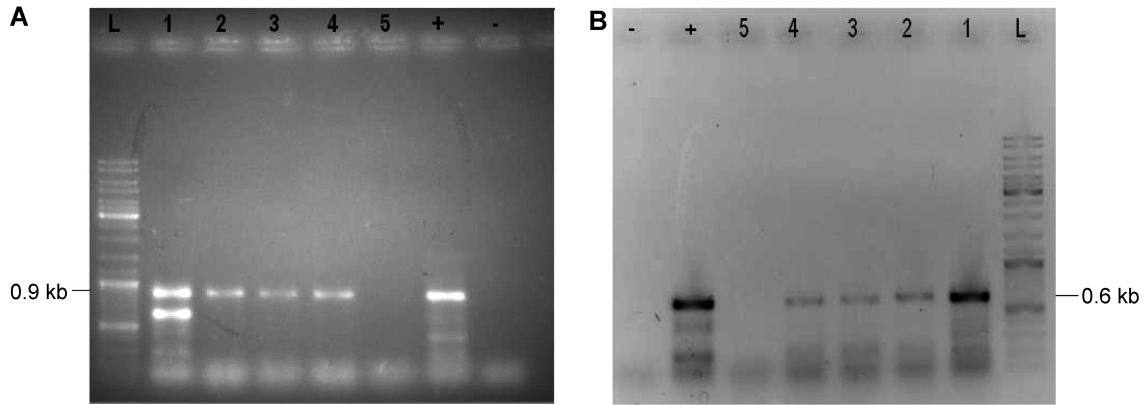
Phylogenetic Analysis
Two separate phylogenetic analyses were conducted using the *sqdB* and *sqdX* homologs identified in this study plus several reference sequences (including cyanobacterial, archaeal, plant, and algal sequences) obtained from the NCBI database. Thus, phylogenetic analyses of sulfolipid biosynthetic pathway genes and their evolutionary rate variations were studied.

The results of the *sqdB*-based analysis are presented in Fig. 3, which shows that the *Spirulina* (Jiangmen) sequence was clustered together on the same branch as the *Arthrospira maxima* sequence, whereas the *S. platensis* and *Spirulina* seawater sequences belonged to another lineage with the *Arthrospira maxima* sequence. Only the *Spirulina* sp. sequence was clustered tightly with the *Crocosphaera watsonii* reference sequences. Interestingly, the reference

Fig. 2. Amplification of *sqdB* (**A**) and *sqdX* (**B**) homologs from *Spirulina* strains.
Lanes 1–5 correspond to *Spirulina* sp., *S. platensis*, *Spirulina* (Jiangmen), *Spirulina* seawater, and *S. maxima* PCR products, respectively. + indicates the positive control (*Synechocystis* PCC 6803). - indicates the negative control. L indicates the molecular weight markers (Fermentas).

sequences of *Synechococcus* sp. PCC 7942 and *Prochlorococcus marinus*, previously identified as highly significant lineages unique to alpha-proteobacteria that do not form a phylogenetic group, were divided into two groups. Thus, the phylogenetic tree suggested that the *Spirulina* sequences that were clustered in the same lineage as *Synechocystis* sp. PCC 6803 may have the same function in the photosystem II complex.

The results of the *sqdX*-based analysis are presented in Fig. 4. The *Spirulina* (Jiangmen), *S. platensis*, and *Spirulina* seawater sequences were clustered together on the same branch as the *Arthrospira maxima* sequence. Once again, the *Spirulina* sp. sequence was clustered tightly with the *Crocosphaera watsonii* reference sequence. When the

Spirulina sequences were aligned with other cyanobacterial reference sequences, they were found to form a different branch within the alpha-proteobacteria, plant, and algae reference sequences. This branch further justified that *sqdX* associated with the *sqdB* gene may export a similar compound, SQDG. In addition, both of the phylogenetic trees showed that green algae, plants, and cyanobacteria originated from the same cyanobacterial ancestor.

Discussion

The recent discovery that *Spirulina* may be an excellent source of therapeutic compounds, as well as a rich source
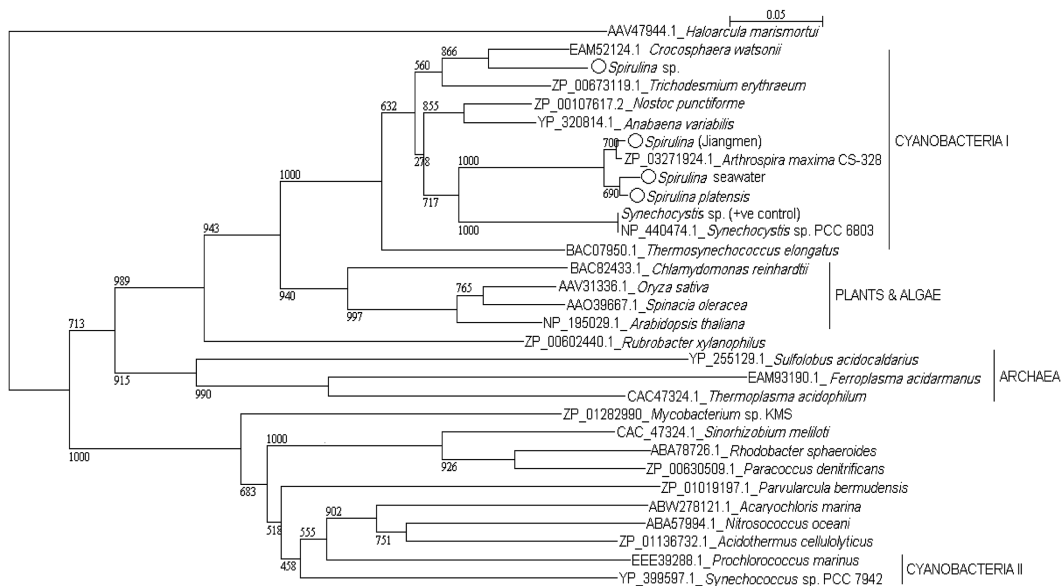
Fig. 3. Phylogenetic tree based on *sqdB* homologs.
Sequences determined in this study are preceded by an open circle. Bootstrap values (1,000 resampling events) are shown for key branches.
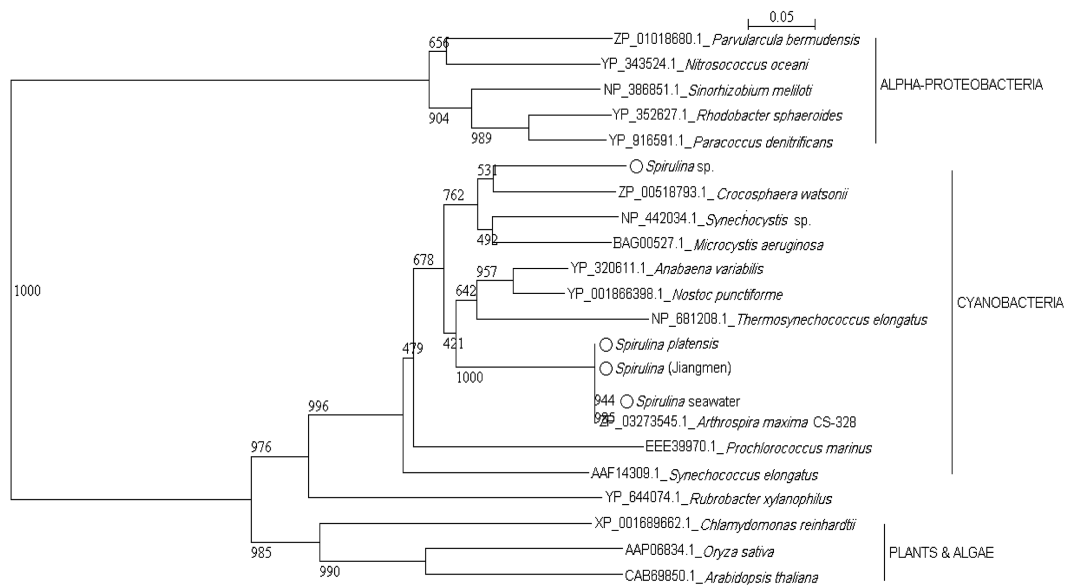
Fig. 4. Phylogenetic tree based on *sqdX* homologs.
Sequences determined in this study are preceded by an open circle. Bootstrap values (1,000 resampling events) are shown for key branches.

of nutrients has attracted the attention of both the pharmaceutical and health food industries. However, the taxonomic status of *Spirulina* remains unclear, partially due to the erroneous group classification of these organisms as *Arthrospira* by Geitler since 1932 [33]. Furthermore, relatively few phylogenetic studies have since been attempted to remedy the ambiguous taxonomic status of *Spirulina* despite the widespread use of these organisms for human consumption. Accordingly, this study examined the 16S rDNA phylogenetic relationship between five different *Spirulina* strains obtained from various geographical locations around the globe. Four of these commercial strains of *Spirulina* [*i.e.*, *S. platensis*, *S. maxima*, *Spirulina* (Jiangmen), and *Spirulina* seawater] widely available as health food supplements were found to be closely related to *Arthrospira* strains listed in the database. The taxonomic status of these strains of *Spirulina* is also further supported by chemotaxonomic evidence. Kwei *et al.* [13] previously determined that the fatty acid patterns in these strains of *Spirulina* had a substantial variance. For example, *Spirulina* sp. does not contain gamma linolenic acid, thereby deviating from the fatty acid composition of other strains of *Spirulina*. Thus, it would seem that these strains may be misclassified as *Spirulina* when they are in fact *Arthrospira*. Meanwhile, the remaining strain investigated in this study, *Spirulina* sp., was found to be closely related to *Halospirulina* strains listed in the database. Previous studies have also shown that this cyanobacterium has the same phenotypes as *Spirulina subsalsa* and is characterized with a low nutritional value of polyunsaturated fatty acids; moreover, it cannot be economically produced in outdoor conditions

because of its low productivity [12, 31]. Therefore, the taxonomic results varied according to the strain, and the exploitation of *Spirulina* sp. would seem to have a lower potential as a food supplement. Thus, the classification of *Spirulina* strains should include other criteria, such as morphological and life-cycle information. However, these criteria cannot always be assessed, as some strains of *Spirulina* have been pelletized for commercial food supplements.

The use of cyanobacteria such as *Spirulina* as food supplements and a source of drugs raises numerous health concerns, as many cyanobacteria are known to produce harmful toxins. For example, certain strains of *Aphanizomenon flos-aquae*, a popular component of health food supplements, have been shown to produce cylindrospermopsin and possess NRPS genes, putatively involved in the synthesis of the toxin [24]. Although recent studies suggest that helically coiled cyanobacteria are more likely to possess NRPS genes than their nonspiral counterparts [6], there are several exceptions to this rule, as none of the *Spirulina* strains in this study contained any NRPS genes. To investigate the presence of putative toxin genes in *Spirulina*, five strains were screened for NRPS and PKS genes, and the results suggested that these unialgal cultures/samples do not contain NRPS/PKS genes and are therefore unlikely to produce NRP/PK toxins. Nonetheless, such findings do not preclude the need for rigorous biochemical/genetic screening regimes when bloom samples are destined for human consumption. Serious problems can arise when mixed bloom samples are used as raw materials. For example, *Aphanizomenon flos-aquae* production can be contaminated with microcystins, in which case *Microcystis*

has been determined as the responsible source. Therefore, this situation alerted researchers to be cautious in the production of food supplements.

One of the primary objectives of this study was to evaluate the presence of sulfolipid biosynthesis pathways in *Spirulina*. It is well known that most photosynthetic organisms can produce sulfolipids. However, sulfolipid production is not universal among cyanobacteria. For example, *Gleobacter violaceus* sp. PCC 7421 is unable to produce sulfolipid [28]. As there is a distinct lack of information regarding sulfolipid production in different strains of *Spirulina*, this study genetically screened five geographically distinct strains. All the strains investigated, except for *Spirulina maxima*, tested positive for *sqdB* and *sqdX* homologs, suggesting that these strains do in fact produce sulfolipid. A likely explanation for the lack of *sqdB* and *sqdX* amplicons obtained when using the *S. maxima* template was related to the quality of the purified DNA.

A phylogenetic analysis of the *Spirulina sqdB* and *sqdX* homologs demonstrated that these putative sulfolipid biosynthesis genes were highly conserved in *S. platensis*, *Spirulina* (Jiangmen), *Spirulina* seawater, and the reference strain *Arthrospira maxima* CS-328. The cyanobacterial reference sequences were shown to be phylogenetically separated and formed two groups, where the *Synechococcus elongates* reference sequence was positioned in the alpha-proteobacteria group, whereas *Synechocystis* sp. was compatible with green algae and higher plants. The role of sulfolipid in *Synechococcus* sp. PCC 7942 exemplifies that SQDG is bound specifically to the PS I complex, whereas in *Synechocystis* sp. PCC 6803 this compound has a deleterious effect on the photosystem II activity [1]. These two phylogenetic groups of cyanobacteria corresponding to *sqdB* can be explained based on two different rates of evolutionary change from anoxygenic to oxygenic photosynthetic prokaryotes [27]. The information encoded in the *Synechocystis* sp. sequence showed more genetic variation than that in the *Synechococcus elongates* sequence, which is why the *Synechocystis* sp. sequence was aligned with the higher plant and algae reference sequences. Therefore, these results suggested that the *Spirulina* sequences in the branch of *Synechocystis* sp. emphasized the necessity of *sqdB* in the photosystem II function, and that the role of *sqdB* differed with a species-specific function in cyanobacteria. Interestingly, the *sqdB* and *sqdX* homologs exhibited different phylogenetic distributions. The *sqdX* homologs demonstrated that this gene in these organisms partitioned primarily according to species relatedness. Furthermore, the geographical origins of these strains did not appear to influence the sequence of their putative sulfolipid biosynthesis genes.

In summary, the commercial *Spirulina* strains investigated in this study were found to belong to the genera *Arthrospira*.

The strains also appeared to lack NRPS/PKS genes and therefore do not produce known cyanobacterial NRP or PK toxins. All the strains investigated here possessed sulfolipid biosynthesis homologs (with the exception of *S. maxima*, where the *sqdB/X* status was inconclusive), making them a potentially good source of sulfolipids with possible therapeutic activity against HIV.

Acknowledgments

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