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 sulfolipid biosynthesis pathways in _Spirulina_, a genetic screening/phylologenic study was performed. Five different strains of _Spirulina_ [Spirulina (Jiangmen), _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 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 *Spirotrina* 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 *Arthrospira*. 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**

*Spirotrina* Strains and Culturing

The *Arthrospira* sp. culture was obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO). The *Sprirotrina* (Jiangmen), *S. platensis*, *S. maxima*, and *Sprirotrina* 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 *Sprirotrina* 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 *Spirotrina* 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 *Spirotrina* 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 3 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 59°C.

**Sulfolipid Biosynthesis Gene (sqdB and sqdX) Amplification**

The degenerate oligonucleotide primer pairs sqdxF (5'-GGATYCY AYGTKGYBAAAYCCDG-3') and sqdxaX (5'-CCNGCBGCCATN GCYT-3'), and sqdBF (5'-GAYGNNATYGGYGNTGG-3') and sqdBR (5'-GGCGTRAAYTGRTIAAANAC-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°C for sqdX and 72°C for sqdB, respectively, for 5 s, strand extension at 72°C for 5 s, and a final extension step at 84°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 CLUSTALX 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 *Spirotrina* strains. The resulting PCR products...