Cryptogamie, Algol., 2006, 27 (2): 185-198

Analyses of ribosomal sequences and biotechnological potential as sources of C-phycocyanin in one Chilean strain of *Spirulina* and two foreign strains of *Arthrospira* (Cyanophyceae)

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(Received 9 March 2005, accepted 13 October 2005)

Abstract – We used Amplified Ribosomal DNA Restriction Analysis (ARDRA) and sequencing to genetically characterize one Chilean strain of *Spirulina* (*S. subsalsa* CONC-050) and two foreign strains of *Arthrospira* (*A. maxima* CONC-040 and *A. platensis* M2). The potential of the strains as a source of the pigment C-phycocyanin (C-PC) was also evaluated. Restriction fragment profiles of the ribosomal internal transcribed spacer (ITS) from *A. maxima* CONC-040 matched those of the one previously well characterized *Arthrospira* clade (clade II) of Scheldeman *et al.* (1999). The ITS sequence of *Spirulina* was interrupted by the $tRNA^{Ile}$ gene while the ITS regions of *Arthrospira* were interrupted by both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes. Phylogenetic analysis, including ITS sequences from other strains deposited in GenBank, showed that the ITS region of *S. subsalsa* CONC-050 is almost identical to the previously sequenced *S. subsalsa* FACHB351. In relation to the *Arthrospira* group, *A. maxima* CONC-040 was reconfirmed as a member of cluster II, while *A. platensis* M2 was the most divergent and did not group with any other *Arthrospira* strain. C-PC content was significantly higher in *S. subsalsa* CONC-050. Antioxidant capacity was evaluated in aqueous extracts containing the same quantity of C-PC. The most protective extract was the one from *A. maxima* CONC-040, which was the strain that was less productive in terms of C-PC per culture volume.

***Spirulina* / *Arthrospira* / Ribosomal sequences / ARDRA / Phylogenetic analysis / C-Phycocyanin**

Résumé – Analyses des séquences ribosomales et du potentiel biologique comme sources de phycocyanine-C d'une lignée chilienne de *Spirulina* et de deux lignées étrangères d'*Arthrospira* (Cyanophyceae). Une lignée chilienne de *Spirulina* (*S. subsalsa* CONC-050) et deux lignées étrangères d'*Arthrospira* (*A. maxima* CONC-040 et *A. platensis* M2) ont été caractérisées à l'aide de la technique ARDRA (Amplified Ribosomal DNA

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Communicating editor: John Beardall

Restriction Analysis), leurs séquences ont été analysées. En outre, leur potentiel comme source de phycocyanine-C (C-PC) a été évalué. Les profils des fragments de restriction des espaceurs ribosomaux internes transcrits (ITS) de l'*Arthrospira maxima* CONC-040, s'apparentent à ceux de l'*Arthrospira* clade (clade II) de Scheldeman *et al.* (1999). Les séquences ITS de la *Spirulina* ont été interrompues par le gène $ARNt^{Ile}$ tandis que les régions ITS de l'*Arthrospira* ont été interrompues par les gènes $ARNt^{Ile}$ et $ARNt^{Ala}$. L'analyse phylogénique, incluant les séquences ITS chez d'autres lignées présentes dans GenBank, a montré que la région ITS du *S. subsalsa* CONC-050 est presque identique à celle du *S. subsalsa* FACHB351, déjà décrite. Au sein des *Arthrospira*, l'*A. maxima* CONC-040 a été re-confirmé comme un membre du cluster II, tandis que l'*A. platensis* M2 se montre très divergent, en ne se regroupant pas avec une autre lignée d'*Arthrospira*. Le contenu de C-PC a été significativement élevé dans le *S. subsalsa* CONC-050. La capacité anti-oxydante a été évaluée à partir d'extraits aqueux contenant la même quantité de C-PC. L'extrait le plus protecteur a été celui provenant de l'*A. maxima* CONC-040, qui a été la lignée moins productive en termes de C-PC par volume de culture.

***Spirulina* / *Arthrospira* / Séquences ribosomales / ARDRA / analyse phylogénique / C-Phycocyanine.**

INTRODUCTION

The cyanobacterial genera *Spirulina* Turpin and *Arthrospira* Stizenberger are both characterized by being non-heterocystous, helical, sheath-less filaments that are generally motile. The taxonomy of these genera is controversial because many of the strains cultivated and commercially used as a source of proteins and cited as *Spirulina* belong, in fact, to the genus *Arthrospira* (Jeeji Bai, 1999; Scheldeman *et al*., 1999; Baurain *et al*., 2002).

In the Gomont monograph (Gomont, 1892), which is the starting point for the nomenclature of the family Oscillatoriaceae, *Arthrospira* and *Spirulina* are recognized as two distinct genera. Later, the genus *Arthrospira* was subsumed into *Spirulina* by Geitler (1932). At present, differences in DNA base composition, ultrastructural and biochemical characteristics, and the comparison of 16SrRNA gene sequences strongly support the argument that the two genera are indeed quite distinct (Jeeji Bai, 1999).

There is doubt among taxonomists about the reliability of using morphology as a taxonomic criterion in *Arthrospira*. For example, the only criterion utilized to separate *A. fusiformis* from *A. maxima* was the degree to which their trichomes are coiled (tightly in *A. fusiformis* and loosely in *A. maxima*). Li *et al*. (2001) demonstrated that this is an unreliable taxonomic character and suggested that *A. fusiformis* and *A. maxima* should be considered the same species based on the reversible transformation of their coiled trichomes (from tightly to loosely coiled and vice versa) and their identical 16S rRNA gene sequences.

In most eubacteria, the genes for rRNA are organized in operons with the genes encoding 16S, 23S and 5S rRNAs separated by internal transcribed spacer (ITS) regions. The ITSs have fewer mutational constraints than the neighboring genes; nevertheless, they contain several motifs that prevent

premature transcription termination and have a role in holding the secondary structure of the nascent rRNA for processing into mature rRNAs. The spacer between the 16S and 23S rRNA genes can be interrupted by 0, 1 or 2 tRNA genes. ITS exhibits great variation in length and sequence, and it has been used in many bacterial groups to delineate closely related strains (Normand *et al*., 1996; Boyer *et al*., 2001).

Nelissen *et al*. (1994) and Li *et al*. (2001) studied the phylogenetic relationship among strains of the genera *Arthrospira* and *Spirulina* based on 16S rRNA gene sequences. These authors demonstrated that the *Arthrospira* strains are not closely related to the *Spirulina* strains: while the *Arthrospira* strains grouped within the filamentous genera of cyanobacteria, the *Spirulina* grouped within the unicellular genera. Additionally, studies using RFLP and sequence analyses of the ITS revealed the presence of different clusters within the *Arthrospira* clade (Scheldeman *et al*., 1999; Baurain *et al*., 2002).

There are also differential structural features in the ribosomal ITS spacer of *Spirulina* and *Arthrospira*: thus, the ITS of the two *Arthrospira* strains sequenced by Nelissen *et al*. (1994) contains $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, which is the most common configuration in cyanobacterial RNA operons, whereas the ITS of the *Spirulina* strain contains only the $tRNA^{Ile}$ gene.

Recognition of markers to discriminate strains of *Spirulina* and *Arthrospira* utilized in the bioindustry is an imperative, and ITS sequences appear to present sufficient variability to be considered as potentially useful tools (Nelissen *et al*., 1994). Additionally, the ITS sequence data may also be useful to unravel the genetic diversity among the strains of *Spirulina* and *Arthrospira*, which will help to solve their taxonomic affiliation. Genetic diversity knowledge also offers the possibility of genetic improvement through simple strain selection.

Even though a large number of *Spirulina* and *Arthrospira* species and strains have been described, the focus has been mainly on taxonomic and phylogenetic aspects (Scheldeman *et al*., 1999; Baurain *et al*., 2002; Li *et al*., 2001; Nelissen *et al*., 1994; Margheri *et al*., 2003), and almost no information exists on their comparative biotechnological potential.

C-Phycocyanin (C-PC), a water-soluble blue pigment, is one of the major constituents of the *Spirulina* and *Arthrospira* cells. Phycocyanin is used as a colorant in food (chewing gums, dairy products, ice creams, jellies, soft drinks, desserts, etc.) and cosmetics (http://www.cftri.com/tech/phyco.html, http://www.spirulinasource.com/earthfoodch5c.html). Dainippon Ink and Chemicals (Japan) has developed a blue food color from *Spirulina* called "Lina-Blue", which is an extract of phycocyanin (Dainippon Ink and Chemicals, 1985). Due to its fluorescence properties, phycocyanin has also become important as a marker for immunodiagnostics. In addition, phycocyanin is of therapeutic value due to its significant antioxidant, radical scavenger, anti-inflammatory, hepatoprotective, anti-arthritic and anti-cancer properties demonstrated in both *in vitro* and *in vivo* experimental models (Sarada *et al*., 1999; Belay, 2002).

In this study, we used Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the 16S+ITS and ITS to genetically characterize one Chilean strain of *Spirulina* and two foreign strains of *Arthrospira*. ITS sequencing was used for a more exhaustive genetic characterization and phylogenetic analysis of the strains. In order to estimate their biotechnological potential as phycocyanin sources, the phycocyanin content and the antioxidant capacity of the crude extracts of the strains were also evaluated. Taxonomic affiliation, phylogenetic position and biotechnological potential of the strains are discussed.

MATERIAL AND METHODS

Organisms and culture conditions

Three cyanobacterial strains were included in this study: *Arthrospira maxima* strain CONC-040 whose geographic origin is unknown, obtained from CINVESTAV, Mexico*; Arthrospira platensis* strain M2 from the Mombolo Lake, Chad and *Spirulina subsalsa* strain CONC-050 isolated from a small brackish lagoon in Antofagasta, Northern Chile. The three strains were kindly donated by Ernesto Retamales, Universidad de Antofagasta, Chile and are now maintained in the Culture Collection of Microalgae at the Universidad de Concepción, Concepción, Chile. Clonal cultures of each strain were obtained by isolation of single filaments using a capillary pipette. All the strains were cultivated in 250-ml Erlenmeyer flasks with 140 ml of *Spirulina* medium (Starr & Zeikus, 1987) under a continuous photon flux density of 110 µmol m^{-2} s^{-1} and 28 ± 2ºC. For C-phycocyanin and antioxidant capacity determinations, five replicates of each strain were established. One-way analysis of variance (ANOVA) and Tukey test were used for statistic analysis of data. Differences were considered significant at a probability of 5% ($P \leq 0.05$). The computational program used was STATISTICA version 6.0.

DNA extraction and PCR amplification of ribosomal sequences

Total DNA was extracted from 5 ml of exponentially growing cultures of each strain, according to Gómez & González (2001). 16SrDNA+ITS and ITS fragments were PCR amplified by using primers 16S5'F and 23S5'R (for 16SrDNA+ITS) and 16S3'F and 23S5'R (for ITS) of Scheldeman *et al*. (1999). Amplifications were done in 25 µl containing *ca*. 10 ng DNA. PCR reaction composition and amplification program were performed according to Scheldeman *et al*. (1999). PCR products were analyzed by electrophoresis of 2 µl of the reaction mixture through 2% agarose gel and visualized with ethidium bromide staining.

ARDRA of the 16S+ITS and ITS fragments

For 16SrDNA+ITS and ITS fragments, 6 and 8 µl of the PCR product, respectively, were digested without further purification with 10 U of the restriction enzymes *Eco*RV, *Hha*I, *Hinf*I and *Mse*I according to manufacturer's recommendations (BioLabs New England). The digestion products were separated on 3 % agarose gels and visualized by ethidium bromide staining.

Sequencing of ITS, alignment and phylogenetic analysis

Prior to sequencing, ITS fragments were gel purified using the kit Wizard PCR Purification System (Promega, Madison, WI). Each purified PCR fragment was sequenced on both strands by the primers 16S3'F (annealing at positions 1522-1541 of the 16SrDNA) and 23S5'R (annealing at positions 26-44 of the 23SrDNA) on an ABI Prism 377 DNA sequencer (Centro de Síntesis y Análisis de Biomoléculas, Universidad de Chile, Chile).

The ITS sequences of the three cyanobacterial strains were aligned to the ITS sequences of *Spirulina* and *Arthrospira* strains available in GenBank. The alignment was done using ClustalW software available online (http://www.ebi.ac.uk/clustalw/) and improved using secondary structure information on conserved functional domains in cyanobacterial ITS regions (Iteman *et al*., 2000).

The resulting alignment was cladistically analyzed with both distance-based [neighbor-joining (NJ)] and character state-based [maximum parsimony (MP) and maximum likelihood (ML)] tree building methods performed with PAUP* program version 4.0b8 (Swofford, 1999). The analyses employed coding (tRNAs) and non-coding (ISRs = intergenic spacer regions) sequences in the ITS regions of the strains. All sites were treated as independent, unordered and equally weighted characters. The analyses included 18 taxa of *Arthrospira* and 2 taxa of *Spirulina*; while a strain of the genus *Limnothrix* was included as an outgroup. Other ITS sequences from strains of *Arthrospira* available in the Genbank were excluded because their sequences were completely identical to the already selected strains. All the trees were performed by heuristic searches. NJ and ML analyses were based on the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985). Bootstrapping the data with 1000 and 100 resamplings was used to test the robustness of each branch in MP and ML trees, respectively.

C-Phycocyanin and antioxidant capacity determinations

The C-Phycocyanin (C-PC) content and the antioxidant capacity of aqueous extracts of the three cyanobacterial strains were determined in crude extracts from 18 day-old cultures (during the linear phase of growth).

The filaments were collected from 4 ml of culture by centrifugation and the pellet was re-suspended in 3 ml of sodium acetate buffer (20 mM pH 5.5). C-PC was extracted by two freezing-and-thawing cycles. The released C-PC was estimated spectrophotometrically using the formula PC (mg PC/ml crude extract) = $(A_{620} - 0.7 \times A_{650})/7.38$ (Boussiba & Richmond, 1979). The antioxidant capacity of the crude extracts, containing C-PC, was determined with the deoxyribose assay by detecting hydroxyl radicals coupled with the thiobarbituric test (Aruoma, 1994; Piñero-Estrada *et al*., 2001). All the reactions were prepared containing the same quantity of C-PC, namely 13 µg. A control tube containing 13 µg of pure C-PC from cyanobacteria (Sigma Co.) was also included. Estimates of the percentage of antioxidant protection in the presence of C-PC were performed with respect to a control tube containing phosphate buffer (100% damaged) instead of crude or pure C-PC.

RESULTS AND DISCUSSION

Size analysis of PCR products

Operons containing the ribosomal genes are normally present in multiple copies in the bacterial genome. The presence of multiple non/identical rRNA operons has been previously reported in cyanobacteria (Boyer *et al*., 2001; Iteman *et al*., 2000). We consistently obtained a single band from the PCR amplification of 16S+ITS and ITS fragments in the three strains studied, which suggests that each one has a single or multiple identical in size rRNA operons (Fig. 1).

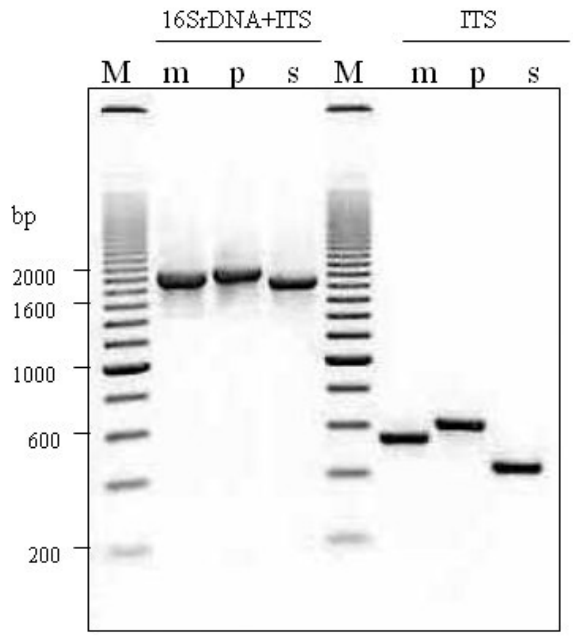

Fig. 1. Agarose electrophoresis gel of PCR amplified 16SrDNA+ ITS and ITS fragments of the three cyanobacterial strains, *Arthrospira maxima* CONC-040 (m), *Arthrospira platensis* M2 (p) and *Spirulina subsalsa* CONC-050 (s). M: molecular size marker (200 bp ladder).

Scheldeman *et al.* (1999) did not find any length variability when they amplified the 16S + ITS region or ITS alone in 37 strains of *Arthrospira* belonging to several species. On the contrary, we found remarkable variability in the size of 16S+ITS and ITS fragments among the strains, mainly due to differences in their ITS regions. 16S+ITS fragments sizes from *A. platensis* M2, *A. maxima* CONC-040 and *S. subsalsa* CONC-050 were 2110, 1920 and 2010 bp, respectively, while their ITS products were 610, 540 and 440 bp, respectively (Fig. 1). The ITS of *A. platensis* M2 was atypically longer than the ITS reported for other strains of the genus (Scheldeman *et al.*, 1999; Baurain *et al.*, 2002).

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Although the 16S+ITS fragments from the three strains analyzed were not so different in size (Fig. 1), ARDRA analysis revealed remarkable differences in their internal sequences. Even though the ITS region should account for most of the variability, the 16SrRNA gene also exhibited polymorphism that could be detected by the restriction enzymes used here (Fig. 2). Thus, 16S+ITS fragment digestion with some enzymes showed a higher number of fragments than the ones generated from ITS fragment digestion for the same strain with the same enzyme. For example, the ITS fragment of *A. maxima* CONC-040 has just one cut site for *Eco*RV, and two restriction fragments are generated from this region (Fig. 3);

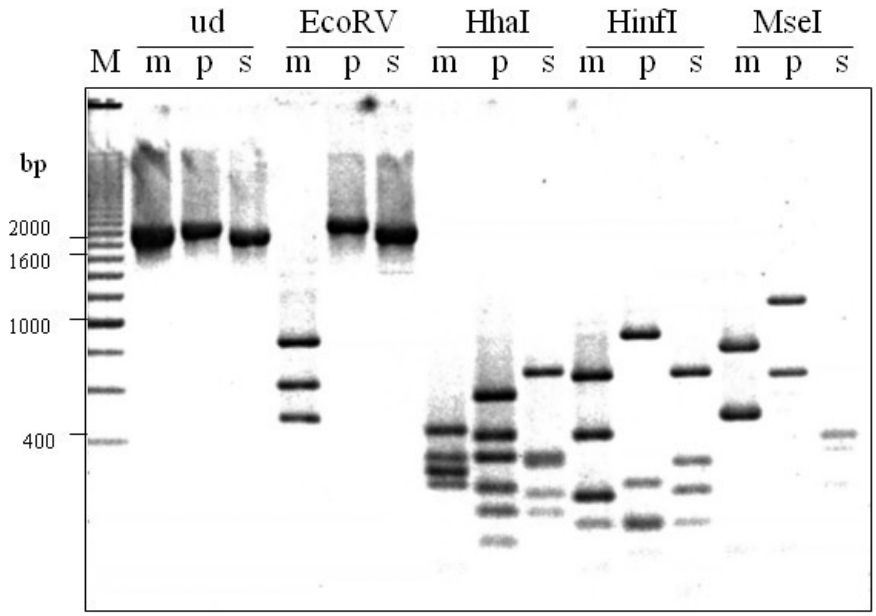

Fig. 2. Agarose electrophoresis gel of PCR amplified 16SrDNA+ITS and RFLP patterns of the three cyanobacterial strains *Arthrospira maxima* CONC-040 (m), *Arthrospira platensis* M2 (p) and *Spirulina subsalsa* CONC-050 (s) with the enzymes EcoRV, HhaI, HinfI and MseI. M: molecular size marker (200 bp ladder). ud: undigested 16SrDNA+ITS fragment.

however, digestion of the 16S+ITS fragment from this strain generated at least 3 detectable fragments, which means that at least one more cut site for *Eco*RV is present in the 16SrRNA gene of *A. maxima* CONC-040; this cut site is not present in any of the 16SrRNA genes of the rest of the strains (Fig. 2).

Scheldeman *et al.* (1999) used ARDRA to analyze the ITS region of 51 strains of *Arthrospira* with 4 restriction enzymes. Cluster analysis showed the separation of all the strains into two main clusters, named I and II, with cluster I additionally subdivided into IA and IB. We compared the ARDRA patterns of ITS obtained for the three strains analyzed with the same enzymes used by Scheldeman *et al.* (1999). The lengths of fragments, longer than 50 bp, for *A. maxima* CONC-040 were for *Hinf*I: 281, 261; *Eco*RV: 499; *Mse*I: 490, 52; *Hha*I: 292, 113, 88. ARDRA patterns of this strain with the four enzymes exactly matched those of cluster II of Scheldeman *et al.* (1999). However, the ARDRA patterns of *A. platensis* M2 did not fall in any of the two genotypic clusters found by the above authors. Again, *A. platensis* M2 shows atypical attributes that do not permit its unequivocal taxonomic corroboration.

Lu *et al.* (1997) had already reported that analysis of length polymorphism and ARDRA patterns of ribosomal ITS are sufficient to discriminate between different genera and species in unicellular and filamentous cyanobacteria. Coincidentally, we found remarkable differences in length and ARDRA patterns of the ribosomal ITS among the three strains analyzed, data that support their different taxonomic genus and species affiliation.

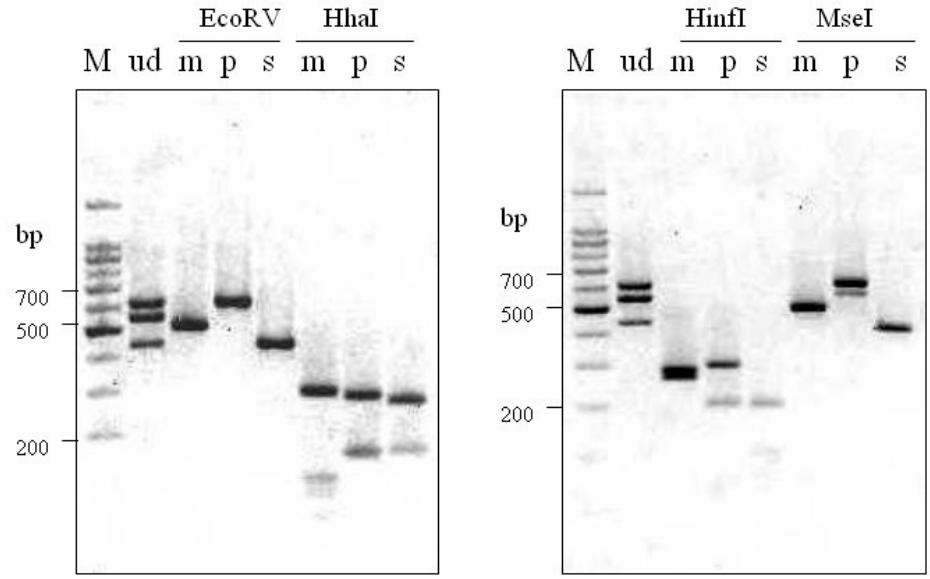

Fig. 3. Agarose electrophoresis gel of PCR amplified ITS and RFLP patterns of the three cyanobacterial strains, *Arthrospira maxima* CONC-040 (m), *Arthrospira platensis* M2(p) and *Spirulina subsalsa* CONC-050 (s) with the enzymes EcoRV, HhaI, HinfI and MseI. M: molecular size marker (100 bp ladder). ud: a mix of undigested ITS fragments from the three cyanobacterial strains.

ITS organizational patterns

The use of ribosomal ITS region in taxonomical studies, population genetics and phylogeny is a potentially powerful tool; however it is important to keep in mind the potential problems imposed by the possibility of multiple non/identical rRNA operons. We sequenced both strands of the ITS amplified by PCR and the same sequence was obtained; furthermore, we did not find ambiguities in any position of the sequences (data not shown). Therefore**,** we can assume that unique or multiple identical ribosomal operons are present in the strains analyzed.

Among the cyanobacteria, the most common matched pattern of ITS configuration is the presence of two tRNA sequences ($tRNA^{Ala}$ and $tRNA^{Ile}$) (Boyer *et al*., 2001). The absence of $tRNA^{Ala}$ in *Spirulina*'s ITS region was the first report of this configuration for a cyanobacterial ITS region (Nelissen *et al*., 1994).

Both ITS organization patterns were detected among the strains studied here: the two *Arthrospira* contained both $tRNA^{Ala}$ and $tRNA^{Ile}$, while *Spirulina* contained just $tRNA^{Ile}$ (Fig. 4). This was in agreement with the previous configurations reported for these genera (Baurain *et al*., 2002; Nelissen *et al*., 1994).

Even when ITS sequences from both *Arthrospira* strains showed the same overall configuration pattern, the sizes of the individual intergenic spacer regions (ISRs) within their ITS regions differed remarkably. The size difference

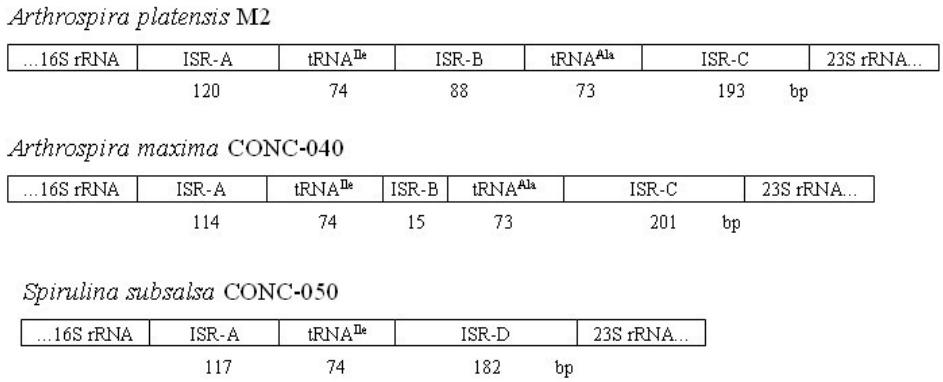

Fig. 4. Scheme of the ribosomal ITS regions from the three cyanobacterial strains included in this study, showing configurations and relative sizes of the intergenic spacer regions (ISRs).

is particularly noticeable in ISR-B, which contains 88 bp in *A. platensis* M2 and 15 bp in *A. maxima* CONC-040 (Fig. 4). In fact, most of the *Arthrospira* strains sequenced until now exhibit ISR-B regions as short as 15 bp (see Genbank Accession numbers indicated in Fig. 5). This result is another peculiar trait of *A. platensis* M2.

Taxonomic affiliation and phylogenetic positions of the studied strains

Iteman *et al.* (2000) determined that both the tRNA-lacking and the tRNA-containing ribosomal ITS regions contain the sequences necessary for proper folding and processing to release functional rRNA and tRNA in cyanobacteria. We detected the presence of almost all structural features of the ITS identified by Iteman *et al.* (2000) in each of the studied strains, and this information was used for improving the initial alignment.

In order to situate the studied strains in a phylogenetic scenario, we analyzed their ribosomal ITS sequences using distance-based and character state-based tree building methods. We included in the analysis ITS sequences deposited in the GenBank for other strains of *Arthrospira* and *Spirulina*. All the phylogenetic trees (distance, MP, and ML) consistently separated *Spirulina* and *Arthrospira* into two main clades, where the *Spirulina* separation was more strongly supported (bootstrap value 100%) than the *Arthrospira* main clade (bootstrap value 77-73%) (Fig. 5). This result was expected considering the absence of a complete gene (tRNA^{Ala}) in the *Spirulina* genus.

Spirulina subsalsa CONC-050 presented an almost identical ITS region to *S. subsalsa* FACHB351, a strain deposited in the GenBank with the code AF329394. The only differences between both sequences are one insertion/deletion (position 527) and one substitution (position 576).

With regard to the *Arthrospira* group, the strain *A. platensis* M2 was the most divergent; in fact, this strain fell out of the two clusters grouping all of the *Arthrospira* whose ITS have been sequenced until now (clusters I and II described first by Scheldeman *et al.* (1999) and then by Baurain *et al.* (2002)) (Fig. 5).

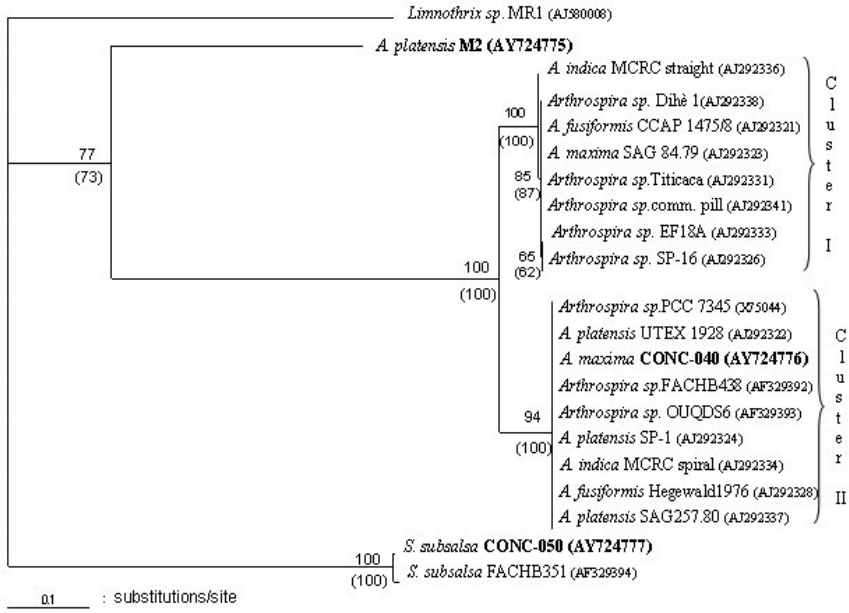

Fig. 5. Phylogram based on maximum likelihood (ML) analysis of the entire ITS region (635 characters) of 18 taxa of *Arthrospira* and 2 taxa of *Spirulina*. A strain of the oscillatorial genus *Limnothrix* was included as outgroup. In bold, the strains whose ITS regions were sequenced in this study. Genbank accession numbers are indicated in parenthesis for each strain. Bootstrap values are indicated to the left of each node: ML value above the line and MP value in parenthesis under the line.

The strain *A. platensis* M2 has been intensely used in physiological and mass culture studies (Qiang *et al*., 1996, 1998; Tredici & Chini Zittelli, 1998; De Oliveira *et al*., 1999; Carlozzi, 2003), and our results demonstrate that, at least from a genetic point of view, this is not a typical strain of *Arthrospira*. This is a very interesting result from an applied perspective, since assumptions about the biotechnological potential of this strain could have been incorrectly extrapolated to the entire species (or even genus). Genetic characterization of microalgal strains used in the bioindustry is, therefore, not trivial, especially when considering that it is probable that genotypic divergences are reflected in physiological and biochemical differences.

The remaining strains of *Arthrospira* (most of which were already studied by Baurain *et al*., 2002) clearly belong to cluster I or II with each branching supported by maximal bootstrap values (Fig. 5). Strains in cluster I and II could be additionally situated in sub-clusters IA or IB and IIA or IIB, respectively, as was reported by Scheldeman *et al*. (1999), based on ARDRA analysis, and then by Baurain *et al.* (2002), based on sequencing analysis of ribosomal ITS regions. Our research did not clearly detect these sub-clusters, probably due to the inclusion of other genera (*Spirulina* and *Limnothrix*), unlike previous publications, in the phylogenetic analyses.

As was determined from ARDRA analysis of ITS, strain *A. maxima* CONC-040 fell into cluster II of Scheldeman *et al.* (1999) (Fig. 3); phylogenetic analysis of its ribosomal ITS sequence reconfirmed this classification (Fig. 5).

The phylogenetic ITS analyses indicate that different strains of *A. fusiformis*, *A. maxima* and *A. indica* fall in different clusters, which means that ambiguities still exist with respect to the taxonomic affiliation of these strains. Comparative morphological, physiological and other molecular studies should be done among these strains to solve this problem. Based on the high conservation of 16SrDNA and ITS sequences, Baurain *et al.* (2002) hypothesize that the *Arthrospira* strains studied until now could correspond to only one or two cosmopolitan species. While strain *A. maxima* CONC-040 confirms this assumption, the problem remains with *A. platensis* M2, which did not fit in any previously described *Arthrospira* cluster (Fig. 5). Does strain M2 belong to *Arthrospira*? Additional ribosomal ITS sequence data from other strains of *Arthrospira* would help to develop more representative information on the natural diversity of this genus.

C-Phycocyanin content and antioxidant capacity of the aqueous extracts

The characteristic blue color of phycocyanin makes it very attractive as a natural pigment for food and cosmetic industries. In this study, C-PC content in 18-day old cultures was significantly higher ($P < 0.05$) in *S. subsalsa* CONC-050 (28.5 μg ml^{-1} culture) than in the other strains (Tab. 1), at least in the culture conditions assayed. Unfortunately, there are no similar studies reported in the literature with which to compare these results. However, it is important to consider that this result might change since the physiological response of a strain is dependent on culture conditions.

The use of natural antioxidants as nutritional supplements has become increasingly important in the natural product industry. The antioxidant activity of C-PC as a scavenger of reactive oxygen radicals has been previously demonstrated (Varidaja & Madyastha, 2000), and C-PC would provide even 20 times more antioxidant activity than ascorbic acid (Romay & González, 2000). In fact, many of the therapeutic properties of C-PC are explained by means of its antioxidant activity (Belay, 2002).

Natural foods contain a complex nutrient mix that, acting as a whole, can exert a greater effect than when they are consumed individually (synergism). We compared the antioxidant capacity of the crude aqueous extracts of the strains

Table 1. C-Phycocyanin content and antioxidant capacity of aqueous crude extracts of 18 day- old cultures of the strains *Arthrospira maxima* CONC-040, *Arthrospira platensis* M2 and *Spirulina subsalsa* CONC-050 (n = 5 ± SE).

Strain	*C-PC content (μg ml^{-1} culture)*	*Percentage antioxidant protection (%)*
Arthrospira maxima CONC-040	1.5 ± 0.63	50.75 ± 3.92
Arthrospira platensis M2	5.64 ± 1.88	41.40 ± 2.75
Spirulina subsalsa CONC-050	28.5 ± 3.00	37.85 ± 2.85
C-Phycocyanin Sigma (13 μg)	—	5.49 ± 1.06

included in this study, containing the same quantity of C-PC (13 µg). We also included a sample containing 13 µg of highly purified C-PC from *A. platensis* (Sigma Co.). The percentage of antioxidant protection was significantly higher ($P < 0.05$) when C-PC is part of a crude extract than when it is highly purified (Tab. 1). These results could be explained by the presence of other antioxidant compounds in aqueous crude extracts and their synergistic interactions. The strain *A. maxima* CONC-040 exhibited the highest antioxidant protection (50.75%), followed by *A. platensis* M2 and *S. subsalsa* CONC-050, although the values of these latter were not significantly different ($P > 0.05$) (Tab. 1).

The biotechnological potential of a strain depend on what it will be used for. In this study, strain *S. subsalsa* CONC-050 was the most promising as a C-PC producer, which make it attractive for industries interested in natural pigments; whereas a strain with high antioxidant capacity like *A. maxima* CONC-040, would be of greater interest for the health food industry.

It is important to emphasize here that the biochemical composition of a microalgal strain is strongly dependent on the culture conditions and on the growing state of the alga when it is harvested. In order to exhaustively characterize these strains as PC sources, future studies should include analyses of their behavior under different culture conditions. Considering that they certainly represent different genetic entities, it is likely that the results of such studies will be very interesting.

Acknowledgments. This work was supported by a grant to P. I. Gómez from Universidad de Concepción, Chile (DIUC # 201.031.093-1.0).

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