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The use of *psbB* for the identification of cyanobacterial lichen symbionts

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Introduction

A lichen symbiotic association may be either bipartite, where the photobiont is a green alga or a cyanobacterium, or tripartite where all three organisms are present (Rai *et al*., 2000). In bipartite cyanolichens the cyanobacteria provide both fixed carbon and nitrogen to the mycobionts, whereas in tripartite lichens the cyanobiont's role is predominately restricted to nitrogen fixation. The lichen genus *Pseudocyphellaria* contains large foliose species with either green algal or cyanobacterial photobionts. In New Zealand, species are found in tussock grassland and rainforest biomes where they are able to exploit both low and high light levels for photosynthesis. In this study we undertook the isolation of cyanobacteria from four cyanolichen species and used *psbB* as a marker to distinguish between cyanobionts and epiphytes or commensals. The *psbB* gene is specific to photosynthetic organisms and exists as a single copy that can be easily amplified with degenerate PCR primers (Urbach *et al.,* 1998). A comparison of *psbB* from isolates and lichen samples identified two cyanobacteria with sequences that were identical to the lichens from which they were cultured; whereas, three isolates had different sequences from their respective lichens. In addition, the use of non-coding regions of *psbB* to distinguish between cyanobionts and commensals has been investigated.

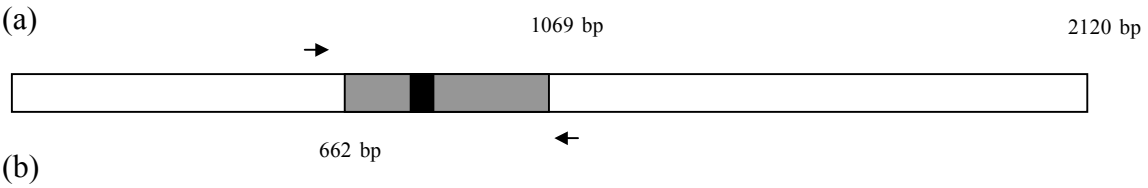
Materials and methods

Lichen specimens of four bipartite cyanobacterial species of the genus *Pseudocyphellaria* were collected from different sites in the South Island of New Zealand: *Pseudocyphellaria crocata* (L.) Vain., found in exposed habitats; *P. maculata* D. J. Galloway, from moist shaded environments; *P. murrayi* D. J. Galloway, located in moderate to dense shade niches and *P. neglecta* (Müll. Arg.) H. Magn., from low humidity locations exposed to high light. These samples were identified according to Galloway (1988). The DNA was extracted from isolated cultures using a modified protocol from Arteaga-Nieto *et al.* (2000) and from lichen thalli as described in Cubero *et al.* (1999). Degenerate primers to amplify *psbB* were designed using the CODEHOP program (Rose *et al.*, 1998). The RNA was extracted from cyanobacteria using the protocol of Bhaya (1999). Upstream *psbB* cDNA sequence was obtained using 5' RACE.

Results

To investigate whether the isolated cyanobacteria represented the major photosynthetic symbiont, as opposed to a minor symbiont, epiphyte or commensal, a region of the *psbB* gene, represented in Fig. 1 (a), was amplified. The *psbB* gene sequences corresponding to isolates and lichen thalli have been compared in Fig. 1 (b). The number of nucleotide differences between the isolates and thalli varied. In the 408 bp region of *psbB* that was aligned the *P. crocata* isolate IsoA, and the *P. crocata* thallus, differed by 20 bases; the *P. maculata* isolate

and thallus varied by 26 bases, and the *P. murrayi* isolate and thallus had 16 nucleotide differences. All sequences obtained from PCR products of the lichen thalli resulted in electropherograms with unambiguous peaks indicating the presence of one genotype, and therefore a single photobiont (Kroken and Taylor, 2000). Accordingly none of the above isolates represented major cyanobionts. However, the isolates *P. crocata* IsoB and *P. neglecta* IsoB had identical sequences to the lichens from which they were cultured, thus these isolates were considered to be potential major photobionts. A *P. neglecta* isolate, IsoA, was also obtained but was found to be a green alga (data not shown).



(b)

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P. crocata          GGCCGGGGATTTGGGTTTCTGACGCTTATGGAATAACTGGGGCTGCCCAGGGAGTAGCACCGGA
P. crocata IsoA     ----------A--------------------TG-T-----C--C--A----C---------A--
P. crocata IsoB     ------------------------------------------------------------

P. maculata         GGCCGGGGATTTGGGTTTCTGACGCTTATGGAATAACTGGGGCTGCCCAGGGAGTAGCACCGGA
P. maculata IsoA    -C--A--A--G------A-----C-C-----T--------T--C--G----C------------

P. murrayi          GGCCGGGGATTTGGGTTTCTGACGCTTATGGAATAACTGGTGCTGCCCAAGGAGTAGCACCGGA
P. murrayi IsoA     -C--A-----A--------------------T--------G--------G-----------A--

P. neglecta         GGCCGGGGATTTGGGTTTCTGACGCTTATGGAATAACTGGGGCTGCCCAAGGCGTAGCACCGGA
P. neglecta IsoB    ------------------------------------------------------------
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Fig. 1. The *psbB* gene. (a) *Anabaena* PCC 7120 *psbB* gene. Black arrows indicate primers dpsbBf (ttcctggctgccgtntggcaytgg) and rdpsbB (ggtggttgcgttgccrtaccacat) used to generate PCR products for sequencing. The grey area shows the region of the gene for which the sequences were aligned. The black region, representing nucleotides 789-854, is shown in Fig.1 (b). (b) Sequence alignment of a 66 bp region of *psbB* from lichen thalli and corresponding isolates (identified as IsoA or IsoB). A dash represents a cyanobacterial isolate nucleotide that is identical to the sequence of the lichen from which it was isolated.

Similarly, a sequence comparison of a 426 bp region of the 16S rRNA gene of the cyanobacterial isolates and lichen thalli confirmed that only the two isolates *P. crocata* IsoB and *P. neglecta* IsoB were candidate symbionts (data not shown). Interestingly, these two isolates, which exhibited different morphologies, had identical 16S rRNA gene sequences, but their *psbB* sequences differed by six nucleotides.

Table 1. Percent similarity of *psbB* and 16S rRNA gene sequences of *P. neglecta* IsoB, *Nostoc* PCC 73102, *Anabaena* PCC 7120 and *Synechocystis* PCC 6803.

Cyanobacterial strains	non-coding[a]	coding[b]	16S rRNA[c]
P. neglecta IsoB/*Nostoc* PCC 73102	93.6	96.4	97.6
P. neglecta IsoB/*Anabaena* PCC 7120	68.9	86.8	94.1
Nostoc PCC 73102/*Anabaena* PCC 7120	67.1	87.0	95.7
Nostoc PCC 73102/*Synechocystis* PCC 6803	42.3	76.7	87.7

[a]5' *psbB* corresponding to *Anabaena* PCC 7120 bases 71-302; [b]partial *psbB* coding region corresponding to *Anabaena* PCC 7120 bases 303-1069; [c]partial 16S rRNA corresponding to *Anabaena* PCC 7120 bases 336-746. The *psbB* sequences are: *Nostoc* PCC 73102 from http://genome.ornl.gov/microbial/npun/; *Anabaena* PCC 7120 accession no. X58847, and *Synechocystis* PCC 6803 accession no. M17109. The 16S rRNA sequences are: *Nostoc* PCC 73102 accession no. AF027655; *Anabaena* PCC 7120 accession no. X59559, and *Synechocystis* PCC 6803 from http://www.kazusa.or.jp/cyano.

P. neglecta IsoB ACATTCTCATTTTT-GCTTGGCGGTGGCTACCCTACAAGGTAAACCTCCCGAAGCGAGTAAGCATTGCCAAACCACCCACGCTGTCTGACTTATAAGATTAAGCTGTGGTAAAAC-TC
Nostoc PCC 73102 ACATTCTCATTTTTTGCTTGGCGGTGGCTACCCTACAAGGTAAACCTCCCGAAGCGAGTAAGCATTGCCAAAGCACCCACCCTGTCTGACTTATAAGACTAA-CTGTGGTAGTAC-TC
Anabaena PCC 7120 ACATTCTCATTTTT-ACTTGGCGATCGCCACCCTCTAGGGACACAATCCCAAAACGGTTGAGTGTCGCCAAATAATCCACTCTCCGAACTTAATAAAATTCGGGTGTGGTAAAGGATC

P. neglecta IsoB TGGCTCTGGCAATAACAAAAAATAAGAGACAGTTTTCTCGAGTGGCGA-CTTCGTTTCTCAGAATCTCAGT--AAGAAAATTGCTTTGTAAAC-TAACTCATCGAGGAGGAGCGTAGTCA
Nostoc PCC 73102 TGGCTCTGGCAATAACAAAAAACACAAGAGAGAGTTTTCTCGTGGCGAATTTCGTTCCTTAGAATCTCAGT--AAGAAAATTGCTTTGTAAAC-TAACTCATCGAGGAGGAGCGTAGTCG
Anabaena PCC 7120 AGACTCTGGCAATAAAAAAAAATTTCAGAGAAG---TCCGAGGTGAGG-CTTCCAACCTTGGAACCTTAACTAAAGAAAATTGTTTTGTAAACATAACTCATCGAGGAGGAGCGTAGTCG

P. neglecta IsoB ATGGGACTACCCTGGTACCGAGTACATACAGTCGTTCTGAATGATCCAGGGCGGCTGATTTCTGTACACCTGATGCACACAGCCTTAGTAGCAGGCTGGGCTGGTTCGATGGCACTGAT
M G L P W Y R V H T V V L N D P G R L I S V H L M H T A L V A G W A G S M A L Y

Nostoc PCC 73102 ATGGGACTACCCTGGTACCGAGTACATACAGTCGTTCTGAATGATCCAGGGCGACTGATTTCTGTACACCTGATGCACACAGCCTTAGTAGCAGGCTGGGCTGGTTCGATGGCACTGAT
M G L P W Y R V H T V V L N D P G R L I S V H L M H T A L V A G W A G S M A L Y

Anabaena PCC 7120 ATGGGACTACCCTGGTACCGAGTACATACAGTAGTTCTGAATGACCCAGGGCGACTGATTTCTGTACACTTGATGCACACAGCCCTGGTGGCAGGTTGGGCTGGTTCGATGGCACTGAT
M G L P W Y R V H T V V L N D P G R L I S V H L M H T A L V A G W A G S M A L Y

Fig. 2. Comparison of *P. neglecta* IsoB, *Nostoc* PCC 73102 and *Anabaena* PCC 7102 *psbB* 5' non-coding region and the start of the coding region together with the corresponding amino acid translation. The positions where nucleotide differences occur are shown in bold.

The 5' sequence of the *psbB* cDNA was obtained using RACE to determine whether the non-coding region of the gene would be a more informative marker in distinguishing between different *Nostoc* strains. Partial non-coding and coding regions of the *psbB* genes of *P. neglecta* isolate IsoB, *Nostoc* PCC 73102 and *Anabaena* PCC 7120 have been compared in Fig. 2. Table 1 shows the percentage of similarity, calculated by the Homologies computer program, between the *psbB* sequences of the different cyanobacteria and between part of the 16S rRNA gene of the same cyanobacteria. The percent similarity between the non-coding and coding regions showed little variation when comparing the *P. neglecta* IsoB isolate and *Nostoc* PCC 73102. In addition, the 16S rRNA gene sequences of these two cyanobacteria gave a similar percent identity as the *psbB* sequences. However, the percent similarity between either of the two *Nostoc* strains and *Anabaena* PCC 7120 was much higher for the coding region than the non-coding region, and was even higher for the partial 16S rRNA gene sequence. These results indicated that the non-coding and coding regions of the *psbB* gene were as informative as the partial 16S rRNA gene sequences in distinguishing the closely related *P. neglecta* IsoB and *Nostoc* PCC 73102 cyanobacterial strains. In contrast, for less closely related strains the non-coding region of *psbB* may be the most informative of the three sequences. This was also indicated in the comparison of the *Nostoc* PCC 73102 and *Synechocystis* PCC 6803 sequences in Table 1.

In addition, photosynthetic activity was compared between *P. neglecta* IsoB and *Nostoc* PCC 73102 cells by measuring oxygen evolution in the presence of 15 mM $NaHCO_3$. The cell lines were cultured in 24 h light at 20°C for five weeks before the measurements in Fig. 3 were recorded. The major distinguishing feature of these data was the rate of oxygen consumption in the *P. neglecta* IsoB cells before the actinic light was turned on. However, it is possible that the rate of respiration in the *P. neglecta* IsoB sample contained a component arising from non-photosynthetic bacteria as the isolate was not axenic. Nevertheless, the two strains appeared to have adapted differently to the growth conditions.

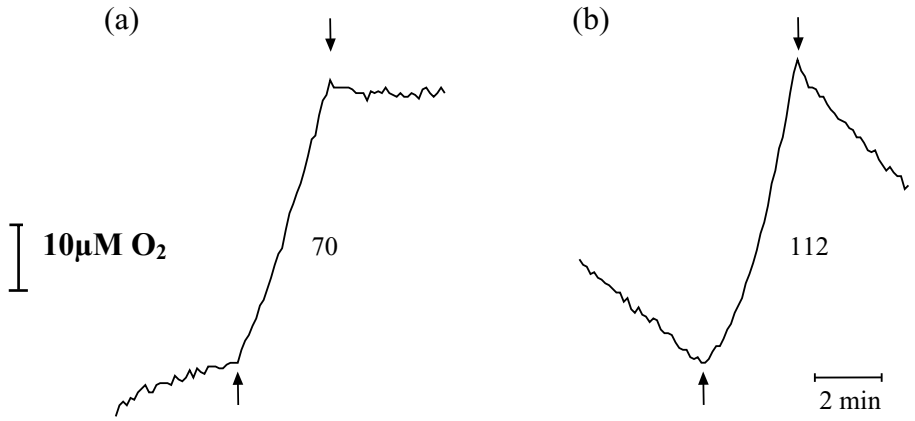

Fig. 3. Traces of oxygen concentration from cyanobacterial cells as determined with a Clark electrode measured at a light intensity of 4000 µE.m^{-2}.s^{-1} at 20°C in unbuffered Bold's basal zero nitrogen medium with 15 mM $NaHCO_3$ as the electron acceptor. The chlorophyll concentrations were 12 µg/ml for *Nostoc* PCC 73102 and 14 µg/ml for the *P. neglecta* IsoB isolate. Arrows indicate light on and off. (a) *Nostoc* PCC 73102 and (b) *P. neglecta* isolate, IsoB. The rates indicated in the figure are in µmole O_2.(mg of chlorophyll)$^{-1}$.h^{-1}. In additional measurements the rates varied from 45 to 78 µmole O_2.(mg of chlorophyll)$^{-1}$.$h^{-1}$for *Nostoc* PCC 73102 and from 85 to 113 µmole O_2.(mg of chlorophyll)$^{-1}$.$h^{-1}$for the *P. neglecta* IsoB isolate.

Discussion

Cyanobacteria were isolated from lichen thalli and *psbB* gene sequences were used to identify two potential symbionts. The 16S rRNA gene sequences of these two cyanobacterial isolates were also identical to lichen thalli from which they were cultured. However, while the two isolates had identical partial 16S rRNA gene sequences they could be distinguished by the sequences of their coding region of *psbB*. Interestingly, the coding and non-coding regions of *psbB* were equally able to distinguish between closely related species but the non-coding region was more successful at distinguishing between more distantly related cyanobacteria.

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