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ScienceDirect
Biochemical and Biophysical Research Communications 362 (2007) 81–87
BBRC
www.elsevier.com/locate/ybbrc

Desaturase genes in a psychrotolerant *Nostoc* sp. are constitutively expressed at low temperature

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Received 25 July 2007
Available online 7 August 2007

Abstract

Antarctic psychrotolerant, *Nostoc* sp. (SO-36), when grown at 25 °C and then shifted to 10 °C, showed an increase in the tri-unsaturated fatty acid [$C_{18:3(9,12,15)}$] at the expense of mono- [$C_{18:1(9)}$] and di-unsaturated [$C_{18:2(9,12)}$] fatty acids. These results indicate that the activities of the enzymes DesA and DesB are up-regulated, when cultures were grown at 10 °C or shifted to 10 °C from 25 °C. However, RT-PCR studies indicated a constitutive expression of *desA*, *desB*, *desC,* and *desC2* genes when cultures grown at 25 °C were shifted to 10 °C. This constitutive expression of *des* genes is in contrast to that observed in mesophilic cyanobacteria, in which desA and *desB* are transcriptionally up-regulated in response to lowering of growth temperature.

Keywords: Cyanobacteria; *Nostoc* sp.; Desaturases; *Des* genes; Constitutive expression; Antarctica; Psychrotolerant; Fatty acid composition; RT-PCR

In cyanobacteria the synthesis of polyunsaturated fatty acids is catalyzed by fatty acyl lipid desaturases namely DesA, DesB, DesC, and DesD which introduce a double bond at Δ12, Δ15 (ω3), Δ9, and Δ6 positions of the C18 fatty acids, respectively [1–3]. Subsequent studies on *Synechocystis* sp. PCC 6803 revealed that the desturase genes namely *desA*, *desB*, and *desD* genes but not *des*C are up-regulated at the transcriptional level, when cells are transferred to a lower growth temperature; i.e., from 34 °C to 22 °C [4–6] and simultaneously, the activities of the enzymes, DesA, DesB, and DesD are increased, while that of DesC remained unchanged [6]. Subsequent experiments demonstrated that cyanobacterial mutants defective in *desA*, *desB,* and *desD* are cold sensitive, thus providing crucial evidence that fatty acid unsaturation is essential for low temperature tolerance [4,7–9].

In contrast to mesophilic cyanobacteria, psychrotolerant cyanobacteria grow optimally at 25 °C and are also capable of growing at 10 °C, a temperature at which the mesophilic cyanobacteria barely grow. Therefore, it is predicted that the psychrotolerant cyanobacteria, would probably differ in their response to low temperature, with respect to both the fatty acid changes and gene expression as compared to mesophilic cyanobacteria. In the present study it is demonstrated that psychrotolerant *Nostoc* sp. (SO-36) grown at 10 °C or when the cells grown at 25 °C were transferred to 10 °C, show an increase in $C_{18:3(9,12,15)}$. But, this increase was not reflected in the levels of the transcripts of *desA* and *desB*, implying that, unlike mesophilic cyanobacteria, *desA* and *desB* genes are constitutively expressed at low temperatures in the psychrotolerant *Nostoc* sp. (SO-36). This is the first study on the cloning

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doi:10.1016/j.bbrc.2007.07.150

and expression of desaturase genes of a psychrotolerant cyanobacterium.

Materials and methods

Bacterial strains and growth conditions. Psychrotolerant *Nostoc* sp. strain SO-36 (here after referred to as *Nostoc* sp.) isolated from a water sample from an Antarctic lake [10] and which grows between 10 and 30 °C was cultured in BG-11 medium [10]. *Escherichia coli* DH10B, which served as the host for cloning, was grown at 37 °C in Luria–Bertani (LB) medium of pH 7.2 [10].

Cloning of des genes. In *Nostoc* sp. the conversion of the C_{18} fatty acid to $C_{18:3(9,12,15)}$ is catalyzed in a step wise manner by DesC, DesA, and DesB, respectively, and C_{16} is converted to to $C_{16:1(9)}$ by DesC2 [10]. These desaturases are coded by the genes *desC*, *desA*, *desB,* and *desC2,* respectively. Cloning of *desC* and *desC2*, from *Nostoc* sp. was reported in our recent paper [10]. The strategy used for cloning *desA* was similar to that described for *desC* [10] except that the genomic DNA of *Nostoc* sp. was digested with ClaI and XbaI, and hybridized with a 120 bp *desA* fragment identical to the partial sequence of *desA* from *Anabaena* sp. PCC 7120. The *desA* fragment was amplified from the genomic DNA of *Nostoc* sp. using primers designed based on the conserved regions of *desA* of cyanobacteria (Table 1). The 120 bp fragment hybridized to a 4.5 kb genomic DNA fragment that was ligated to pBluescript II KS (+) (Stratagene, La Jolla, CA) and used to transform *E. coli* DH10B by electroporation. Transformed cells were screened by colony hybridization using the 120 bp partial PCR product of *desA* as a probe [11]. A single positive clone (pC36AB) was obtained. Sequencing of the insert was done as earlier [10] and it confirmed the full-length sequence of both *desA* and *desB*.

RNA preparation and RT-PCR. Cells at a given time point were collected and RNA was isolated using the RNEasy kit (Qiagen, Hilden, Germany) and quantitated spectrophotometrically at 260 nm. Reverse transcription (RT) was performed using commercially synthesized reverse primers of the *des* genes designed based on the sequence of *des* genes of *Nostoc* sp. as determined in the present [*desA* (AJ621245), *desB* (AJ621246)] and in the previous study [*desC* (AJ621244) and *desC2* (AJ621247)] (Table 1). The RT-PCR procedure was essentially as described by Sambrook et al. [11]. The RT product was used for PCR of each of the genes. PCR was performed for 25 cycles, with 30 s for denaturation, annealing, and elongation in each cycle. 16S ribosomal RNA gene served as an internal control for quantitation in these experiments.

Analysis of fatty acid composition in the total lipids and individual lipids of Nostoc sp. *(SO-36).* Total cell lipids were extracted using the procedure of Bligh and Dyer [12] and the four individual lipid fractions namely DGDG (digalactosyl diacylglycerol), MGDG (monogalactosyl diacylglycerol), PG (phosphatidyl glycerol), and SQDG (sulfoquinovosyl diacylglycerol) were separated on TLC plates (Merck No. 5721, 20 × 20 cm) [13]. Fatty acid analysis was done essentially according to the method of Sato and Murata [13] as described earlier [10].

Results and discussion

Unique features of Nostoc sp. (SO-36)

Psychrotolerant *Nostoc* sp., which contains $C_{16:0}$, $C_{16:1(9)}$, $C_{18:1(9)}$, $C_{18:2(9,12)}$, and $C_{18:3(9,12,15)}$ fatty acids (Table 2), is a Group 2 cyanobacterium [10]. Generally, in cyanobacteria, temperature shift-down in growth temperature brings about an increase in desaturation of fatty acids [2]. For instance, in Group 2 cyanobacteria (e.g. *Anabaena variabilis*) conversion of $C_{16:0}$–$C_{16:1(9)}$, $C_{16:1(9)}$–$C_{16:2(9,12)}$, $C_{18:0}$–$C_{18:1(9)}$, $C_{18:1(9)}$–$C_{18:2(9,12)}$, and $C_{18:2(9,12)}$–$C_{18:3(9,12,15)}$ occurs [2,14]. *Nostoc* sp., like *Anabaena variabilis* also exhibits increases in the levels of $C_{18:3(9,12,15)}$ (Table 2 and Fig. 1) in temperature shift-down experiments, but differs in that it did not show any increase in the level of $C_{16:1(9)}$ and also lacks $C_{16:2(9,12)}$ (Table 2 and Fig. 1). In Group 2 cyanobacteria (e.g. *Anabaena variabilis* and *Synechococcus* PCC 7002), the ratio of C_{18}:C_{16} fatty acids is about 0.9 [3], whereas, in psychrotolerant *Nostoc* sp., the ratio is 1.5 (60:40) (Table 2), implying a preponderance of C_{18} fatty acids. Another prominent feature of Nostoc sp., grown either at 10 °C or 25 °C, was the presence of high mol% (86%) of unsaturated fatty acids ($C_{16:1(9)}$,

Table 1
Primers used for cloning of desA and desB, for reverse transcription of des genes and amplification of 16S rRNA gene from Nostoc sp. (SO-36)

S. No	Primer[a]	Sequence	Position	Gene
1	RTDAF	ACGCTTGGCATCCAATCAGA	407–426	*desA*
2	RTDAR	AATGGAACCTACCCACCAGAG	513–493	*desA*
3	RTDBF	ACTGCGGACACCAATCTTTTTC	278–298	*desB*
4	RTDBR	TGTGACTAATCCGCCAACCAT	385–365	*desB*
5	RTDCF	ACGCTGATGTTCCTCGTTTCAC	395–416	*desC*
6	RTDCR	GCAATACGCCTAGAGCAACCTG	496–475	*desC*
7	RTDC2F	TGGCTGGATCGCTACTTCTT	475–494	*desC2*
8	RTDC2R	CAGCATCAAAGGTGCGATAA	649–630	*desC2*
9	desAF	TA(T/C)CA(T/C)TT(T/C)TGGATGAGT	688–705	DesA
10	desAR	AC(A/C)TT(A/G/T)AT(A/G)TC(A/G)TG(A/G(CA)	848–832	DesA
11	16S1 (F)	GAGTTTGATCCTGGCTCAG	9–27	16S rRNA
12	16S2 (R)	ACGGCTACCTTGTTACGACTT	1477–1498	16S rRNA

[a] Primers used for reverse transcription are designated as RTDXXX. F and R identify the forward and reverse primers.

Table 2
Fatty acid composition of the total lipids of Antarctic Nostoc sp. (SO-36) grown at 10 °C and 25 °C

Growth temperature (°C)	Fatty acid (mol%) (Mean ± SD)						
	$C_{16:0}$	$C_{16:1(9)}$	$C_{18:0}$	$C_{18:1(9)}$	$C_{18:1\ (11)}$	$C_{18:2\ (9,12)}$	$C_{18:3\ (9,12,15)}$
10	12.0 ± 0.3	27.7 ± 0.4	1.0 ± 0.3	15.4 ± 0.3	1.8 ± 0.2	21.6 ± 0.5	20.6 ± 0.5
25	12.0 ± 0.3	30.0 ± 0.4	4.0 ± 0.1	17.0 ± 0.3	1.0 ± 0.3	29.0 ± 0.5	7.0 ± 0.3

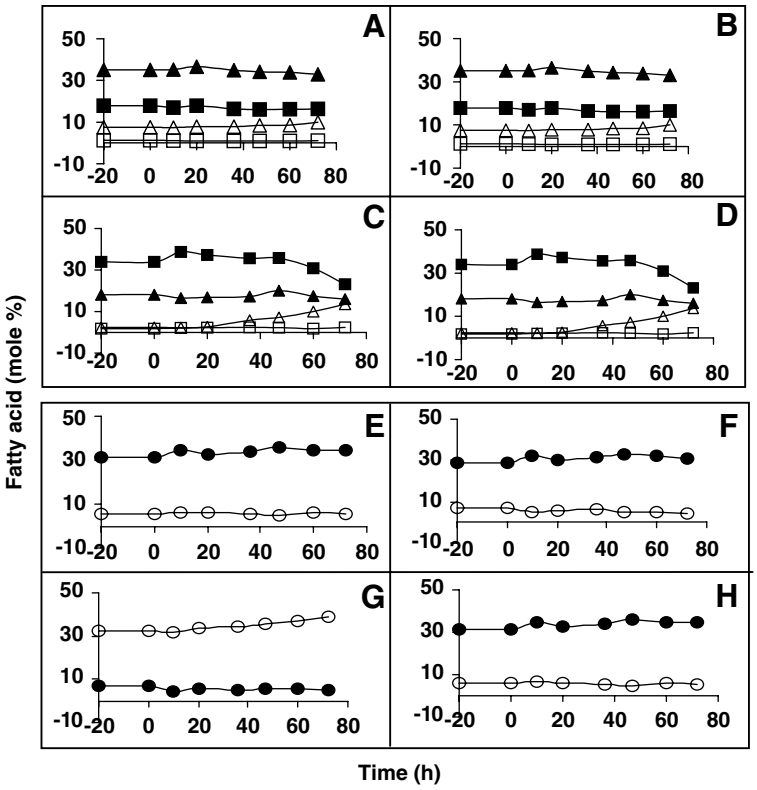

Fig. 1. (A–H) Changes in the C18 (A–D) and C_{16} fatty acids (E–H) of MGDG (A, E), DGDG (B, F), SQDG (C, G) and PG (D, H) in Nostoc sp. (SO-36) with time in response to a temperature shift from 25 °C to 10 °C. The values are the averages of at least two experiments. The deviation was not more than ± 2%. $C_{18:0}$ (□); $C_{18:1(9)}$ (■); $C_{18:2(9,12)}$ (▲); $C_{18:3(9,12,15)}$ (△); $C_{16:0}$ (○), and $C_{16:1}(9)$ (●).

$C_{18:1(9)}$, $C_{18:2(9,12)}$, and $C_{18:3(9,12,15)}$) (Table 2), compared to other mesophilic Group 2 cyanobacteria. These differences are probably a reflection of the differences in the acclimative capability of the Antarctic cyanobacterium *Nostoc* sp., which is a psychrotolerant strain (capable of growth at 10 °C but not at 35 °C), unlike the mesophilic cyanobacteria, which are capable of growth between 20 °C and 40 °C.

Increased activity of DesA and DesB in Antarctic Nostoc sp. (SO-36)

Nostoc sp., grown isothermally at 10 °C or when grown at 25 °C and then transferred to 10 °C, showed an increase in mol% of $C_{18:3(9,12,15)}$ at the expense of $C_{18:1(9)}$ and (or) $C_{18:2(9,12)}$, implying an increase in the activity of DesA and DesB, a phenomenon commonly observed in Group 2, Group 3 and Group 4 cyanobacteria [3,15]. In *Nostoc* sp., $C_{18:3(9,12,15)}$ increased in MGDG and PG following down-shift in temperature from 25 °C to 10 °C whereas desaturation of $C_{16:0}$–$C_{16:1(9)}$ did not vary in all the four lipid fractions (Fig. 1). These results are in contrast to the changes observed in *Anabaena variabilis*, where $C_{18:3(9,12,15)}$ increased in all the four individual lipids (MGDG, DGDG, SQDG, and PG) and $C_{16:1(9)}$ in SQDG, and $C_{16:2(9,12)}$ in MGDG and DGDG following down-shift in temperature [16]. Further these changes in *Anabaena variabilis* occurred 10 h after down-shift in temperature, whereas in *Nostoc* sp., it was observed after 20 h (Fig. 1). This differential response observed between *Nostoc* sp. and *Anabaena variabilis* [16] may indicate that the modes of acclimation in a mesophilic and a psychrotolerant cyanobacterium are not identical.

Cloning and sequence analysis of desA and desB of Nostoc sp. (SO-36)

The cloning and characteristics of *desC* (AJ621244) and its isoform *desC2* (AJ621247) from *Nostoc* sp. have been reported in a recent study by us [10]. In this study, the two other *des* genes namely *desA* (AJ621245) and *desB* (AJ621246) of *Nostoc* sp. have been cloned from a partial genomic library of *Nostoc* sp. (for details see Materials and methods). Alignment of the sequences revealed that *desA*, *desB,* and *des*C are in tandem (Fig. 1A, Supplementary data) and have an ORF of 822 bp, 1053 bp, and 1080 bp, respectively, and are separated by intergenic regions (Fig. 1A, Supplementary data). The three genes do not form an operon, since sequence analysis at the BDGP Neural Network Promoter Prediction site (http://www.fruitfly.org/seq_tools/promoter.html) revealed putative promoter sequences for all the three genes (Fig. 1A, Supplementary data). The −10 and −35 sequences, upstream of the transcription initiation sites are identified for the three genes. *desB* from *Nostoc* sp. seems to be falling into the rare category of bacterial genes that are translated

from a GTG instead of ATG. *desC2* has an ORF of 858 bp and the putative promoter, −10 and −35 sequences have also been identified for *desC2* (Fig. 1B, Supplementary data). Diad symmetries, which indicate putative *rho* dependent termination sites are shown by all the four genes (Figs. 1A and B, Supplementary data).

Characteristics of the desaturases of Nostoc sp. (SO-36)

The four desaturases of *Nostoc* sp., like all the known cyanobacterial desaturases, possessed the transmembrane domains and conserved histidine cluster motifs characteristic of desaturases (Table 3 and Fig. 2, Supplementary

Table 3
Characteristics of the four acyl-lipid desaturases of Antarctic Nostoc sp. (SO-36)[a]

	desA	*desB*	*desC*	*desC2*
Amino acids	350	359	273	285
Molecular mass (kDa)	41.2	41.8	31.3	33.2
Specificity				
Position	sn-1	sn-1	sn-1	sn-2
Fatty acid	$C_{18:1(9)}$	$C_{18:2\ (9,12)}$	$C_{18:0}$, $C_{16:0}$	$C_{18:0}$, $C_{16:0}$
Head group				
MGDG	+	+	+	+
DGDG	+	+	+	?
SQDG	+	+	+	?
PG	+	+	+	?
Predicted transmembrane domains (amino acid numbers)	41–63, 67–89, 199–221	43–65, 71–93, 200–222, 231–253	12–34, 40–62, 158–180	17–39, 48–70, 75–97, 172–194, 249–271
Cluster1 (HXXXH)	HDCGH	HDCGH	HRLVTH	HRLLHH
Cluster 2 (HXXHH)	HNYHH	HRTHH	HRIHH	HRQHH
Cluster 3 (HXXHH)	HVPHH	HVAHH	HNNHH	HNNHH

+, indicates the action of the enzyme; ?, indicates uncertainty.
[a] The above data are based on the ORFs of desA and desB cloned in the present study and desC and desC2 cloned by us in an earlier study [10].

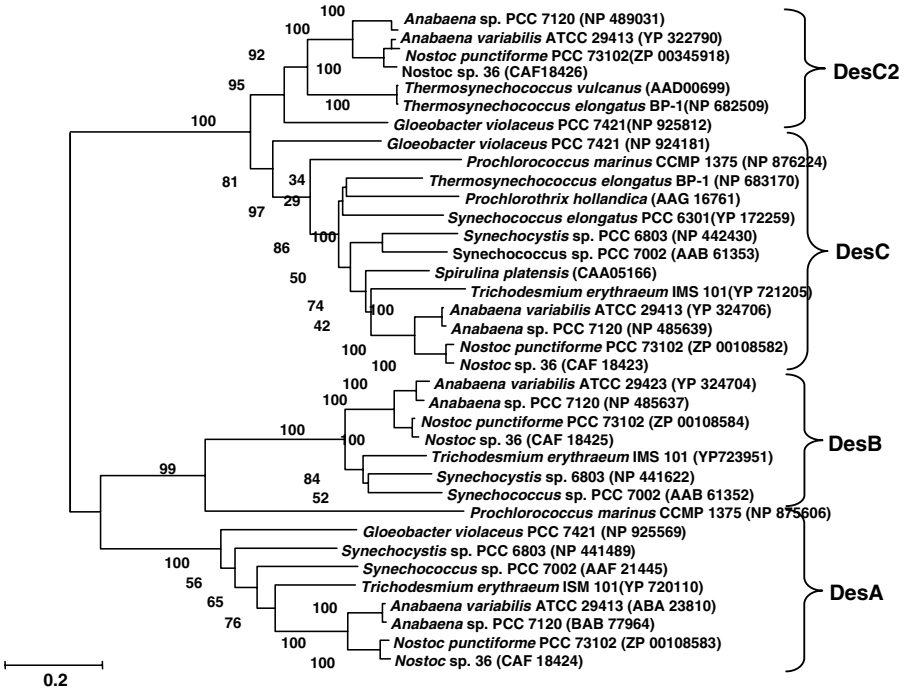

Fig. 2. Neighbor-joining phylogenetic tree based on the deduced amino acid sequences of desA, desB, desC, and desC2 homologs of cyanobacteria. The amino acids sequences corresponding to the des genes in cyanobacteria were obtained from databases (GenBank/EMBL/DDBJ) and the sequences were aligned with CLUSTAL W ver. 1.83. The phylogenetic tree was drawn with Njjplot (http://pbil.univ-lyon1.fr/software/njplot.html). The accession number of each Des homolog is indicated beside the name of the corresponding cyanobacterium. Bootstrap values (>50%) expressed relative to 1000 replications are given at the respective nodes.

data). The hydropathy profiles clearly revealed clusters of hydrophobic regions that define the putative membrane-spanning domains in all the desaturases (Fig. 2, Supplementary data and Table 3).

Phylogenetic relationship between desaturases of cyanobacteria

Based on the deduced protein sequence it appears that the four *des* genes are very well conserved in cyanobacteria. The four genes exhibit high similarity with *Nostoc punctiforme* PCC 73102 (92–97%), *Anaebaena variabilis* ATCC 29413 (80–87%), and *Anabena* sp. PCC 7120 (80–87%), which are members of *Nostocales* (Table 4). The similarity with other cyanobacteria was less than 70% (Table 4). The four desaturases thus seem to be conserved across genera in cyanobacteria and also in plants like *Zea mays*, *Oryza sativa*, *Arabidopsis thaliana*, *Nicotiana tabacum,* etc. with similarities ranging from 27% to 51% (Table 4).

Phylogenetic analysis based on the amino acid sequence indicated that DesA, DesB, DesC, and DesC2 from cyanobacteria form four distinct clades (Fig. 3). The only exception observed was that of DesA of *Prochlorococcus marinus* CCMP 1375 (NP 875606) which appears to form a clade with DesB rather than DesA. The clade of DesC is closely related to the clade of DesC2 whereas that of DesA is closely related to the clade of DesB. Further, within each clade the desaturases of *Nostoc* sp., *Nostoc punctiforme, Anabaena* sp. PCC 7120, and *Anabaena variabilis* form a robust clade with high bootstrap values (>95%) (Fig. 3) clearly indicating that they are phylogenetically closely related.

Expression of the desaturase genes of Nostoc sp. (SO-36)

Increase in the mol% of $C_{18:3(9,12,15)}$ in psychrotolerant *Nostoc* sp., either in cells grown at 10 °C or in cells shifted to 10 °C, in comparison to cells grown at 25 °C, may be due to the up-regulation of the expression of the *des* genes. To evaluate this hypothesis, cells were grown at 25 °C to an OD of 0.5, shifted to 10 °C and cells were collected at 0, 15, 30, 45, 60, 120, and 150 min and processed for RT-PCR. The results clearly indicated that the transcripts of *desA*, *desB*, *desC,* and *desC2* did not show any up- or down-regulation of the expression of desaturase genes, even 150 min after shifting the culture from 25 °C to 10 °C (Fig. 3). The RNA used in these studies was free of DNA, as evidenced from the fact that when RNA was used as the template, PCR amplification of the des genes was not observed (data not shown).

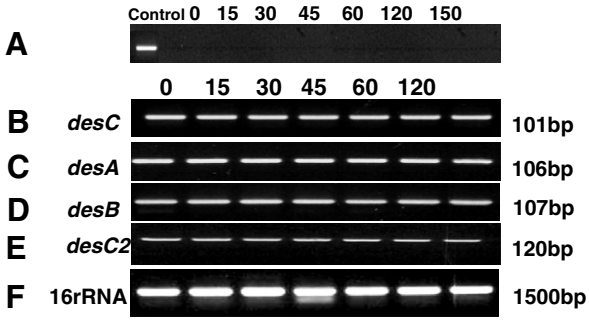

Fig. 3. Expression of desaturase genes of Nostoc sp. (SO-36) as monitored by RT-PCR. Nostoc sp. (SO-36) was initially grown at 25 °C to an OD730 of 0.5, shifted to 10 °C and then aliquoted between 0 and 150 min and used for the preparation of total RNA, to be used for RT-PCR. (A) RNA isolated from Nostoc sp. (SO-36) at different time points after a shift from 25 °C to 10 °C showed absence of DNA contamination in the RNA as judged by PCR using primers of desA. First lane is PCR with genomic DNA. (B–E) Transcript levels of desC, desA, desB, and desC2 of Nostoc sp. (SO-36) at different time points after a shift from 25 °C to 10 °C. (F) PCR product of 16S rRNA gene using cDNA as the template and 16S rRNA primers indicating equal amount of RNA used.

Table 4
Protein sequence similarity of desaturases of Nostoc sp. (SO-36) with desaturases of other cyanobacteria[a]

Organism	Similarity (%) with desaturases of Nostoc sp. (SO-36)			
	desA (CAF18424)	*desB* (CAF18425)	*desC* (CAF18423)	*desC2* (CAF18426)
Nostoc punctiforme PCC 73102	95, (ZP00108583)	94, (ZP00108584)	97, (ZP00108582)	92, (ZP00345918)
Anabaena variabilis ATCC 29413	81, (ABA23810)	85, (YP324704)	87, (YP324706)	80, (YP322790)
Anabaena sp. PCC 7120	80, (BAB77964)	87, (NP485637)	85, (NP485639)	80, (NP489031)
Synechocystis sp. PCC 6803	61, (NP441489)	67, (NP441622)	63, (NP442430)	NA
Trichodesmium erythraeum IMS101	60, (YP720110)	68, (YP723951)	70, (YP721205)	NA
Synechococcus sp. PCC 7002	54, (AAF21445)	65, (AAB61352)	63, (AAB61353)	NA
Synechococcus elongatus PCC 6301	NA	NA	64, (YP172259)	
Thermosynechococcus vulcanus	NA	NA	NA	65, (AAD00699)
Gloeobacter violaceus PCC 7421	52, (NP925569)	NA	55, (NP924181)	60, (NP925812)
Prochlorococcus marinus CCMP 1375	26, (NP875606)	NA	53, (NP876224)	NA
Thermosynechococcus elongatus BP-1	NA	NA	66, (NP683170)	65, (NP682509)
Prochlorothrix hollandica	NA	NA	60, (AAG16761)	NA
Spirulina platensis	NA	NA	74, (ADD00996)	NA
Zea mays	27, (BAE93382)	50, (BAA22441)	NA	NA
Oryza sativa	27, (BAD09176)	50, (ABF95395)	NA	NA
Arabidopsis thaliana	25, (AAA32782)	51, (NP196177)	41, (BAC42216)	NA
Nicotiana tabacum	NA	51, (BAA1475)	NA	NA

[a] Sequences were retrieved from the NCBI database and the accession numbers of the genes are indicated in parentheses.

These results clearly indicate that the desaturase genes in the Antarctic *Nostoc* sp., which is cold tolerant, are constitutively expressed, unlike the mesophilic cyanobacteria where some of the desaturase genes are transcriptionally activated at lower temperatures of growth. For instance, low temperature up-regulates transcription of *desB* and *desC* in *Synechococcus* sp. PCC 7002 [17] and *desA*, *desB,* and *desD* in *Synechocystis* sp. PCC 6803 [5,6]. Further, in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. strain PCC 7002, the enhanced levels of *desA*, *desB,* and *desD* mRNAs resulted from both induction of gene expression and enhancement of mRNA stability [6,18]. Although the mRNA levels for the desaturase genes in these systems increased very rapidly, the fatty acid composition of membrane lipids changed only very slowly during a 10–12 h period following the temperature shift-down. Taken together, these results would imply that the rate-limiting step for increasing the desaturation of membrane lipids at low temperature in psychrotolerant cyanobacteria (as in Antarctic *Nostoc* sp.), is post-transcriptional whereas in mesophilic cyanobacteria up-regulation of the desaturase genes at low temperature appears to be a common acclimative response to lower temperature. Post-transcriptional mechanisms for upregulating the activity of the required gene products for cold adaptation has been observed in many other organisms. For example, β-tubulin from an Antarctic microbe, *Euplote focardii*, is heavily phosphorylated [19] at low temperatures; ω3 fatty acid desaturase in wheat roots is temperature dependent and is translationally regulated [20]; maize plants exposed to lower temperatures respond by phosphorylating a minor chlorophyll a/b protein [20].

Conclusions

It may be concluded that the desaturase genes, which are involved in modulating the fatty acid composition differ in their gene expression in that they are low temperature-inducible in mesophiles, whereas in psychrotolerant cyanobacteria, they are constitutively expressed and this mode of expression may be essential for low temperature growth and acclimation. To the best of our knowledge, this is the first report on the expression of the desaturase genes in a psychrotolerant cyanobacterium.

Acknowledgments

This work was supported by a grant from the India–Japan Cooperative Science Program of the Department of Science and Technology, Government of India, and the Japanese Society for the Promotion of Science to I.S., N.M., and S.S. and by the Program for Cooperative Research on Stress Tolerance of Plants of the National Institute for Basic Biology, Japan. S.C. thanks the University Grants Commission, New Delhi, Government of India, for a Junior and a Senior Research Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.150.

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