Glutamine synthetase from the marine cyanobacteria *Prochlorococcus* spp.: characterization, phylogeny and response to nutrient limitation

Sabah El Alaoui,† Jesús Diez,† Fermín Toribio,‡ Guadalupe Gómez-Baena,§ Alexis Dufresne∥ and Jose M. García-Fernández*†

1Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Campus de Rabanales, 14071-Córdoba, Spain.
2Station Biologique, UMR 7127, CNRS, INSU et Université Pierre et Marie Curie, BP74, 29682-Roscoff Cx, France.

Summary

The regulation of glutamine synthetase (EC 6.3.1.2) from *Prochlorococcus* was previously shown to exhibit unusual features: it is not upregulated by nitrogen starvation and it is not inactivated by darkness (El Alaoui *et al.* 2001 Appl Environ Microbiol 67: 2202–2207). These are probably caused by adaptations to oligotrophic environments, as confirmed in this work by the marked decrease in the enzymatic activity when cultures were subjected to iron or phosphorus starvation. In order to further understand the adaptive features of ammonium assimilation in this cyanobacterium, glutamine synthetase was purified from two *Prochlorococcus* strains: PCC 9511 (high-light adapted) and SS120 (low-light adapted). We obtained ~100-fold purified samples of glutamine synthetase electrophoretically homogeneous, with a yield of ~30%. The estimated molecular mass of the subunits was roughly the same for both strains: 48.3 kDa. The apparent $K_m$ constants for the biosynthetic activity were 0.30 mM for ammonium, 1.29 mM for glutamate and 1.35 mM for ATP; the optimum pH was 8.0. Optimal temperature was surprisingly high (55°C). Phylogenetic analysis of glnA from three *Prochlorococcus* strains (MED4, MIT9313 and SS120) showed they group closely with marine *Synechococcus* isolates, in good agreement with other studies based on 16 S RNA sequences. All of our results suggest that the structure and kinetics of glutamine synthetase in *Prochlorococcus* have not been significantly modified during the evolution within the cyanobacterial radiation, in sharp contrast with its regulatory properties.

Introduction

*Prochlorococcus* is a marine cyanobacterium that has become a major model in marine ecological studies. Although it was rather recently discovered (Chisholm *et al.*, 1988), its importance was rapidly evidenced because of many outstanding properties (Partensky *et al.*, 1999a): it is the smallest (Chisholm *et al.*, 1992) and most abundant photosynthetic organism in the oceans, and presumably on Earth (Partensky *et al.*, 1999b); it contributes significantly to the total primary production in wide oligotrophic regions of the oceans (Goericke and Welschmeyer, 1993; Vaulot *et al.*, 1995); it shows a high degree of genetic diversity (Scanlan *et al.*, 1996; Laloui *et al.*, 2002; Rocap *et al.*, 2002), with different ecotypes co-existing along the water column (Moore *et al.*, 1998); it is able to grow down to 200 m depth, using a photosynthetic apparatus with a remarkable pigment complement (Hess *et al.*, 1996; La Roche *et al.*, 1996; Partensky *et al.*, 1997; Garzarek *et al.*, 2000). A large number of studies have been published on the above mentioned fields (for review, see Partensky *et al.*, 1999a,b; Partensky and Garzarek, 2003; Scanlan and West, 2002; Ting *et al.*, 2002), leading to a profound reassessment in the traditional view of the picoplanktonic community structure and significance in the oceans.

Although a main characteristic of *Prochlorococcus* is its success in very oligotrophic regions of the oceans, where it usually outcompetes co-existent *Synechococcus* populations, the basic knowledge of nutrient assimilation in *Prochlorococcus* is still very scarce. The rather difficult cultivation of *Prochlorococcus* in the laboratory (El Alaoui *et al.*, 2001) and the availability of only two axenic strains, PCC 9511 (Rippka *et al.*, 2000) and MED4-Ax (Saito *et al.*, 2002), which correspond to the same genotype (MED4; Rippka *et al.*, 2000), have certainly played against detailed studies on nutrient assimilation by *Prochlorococcus*.

Glutamine synthetase (GS) is the enzyme that catalyses the incorporation of ammonium into glutamate to produce glutamine. It is a key enzyme of nitrogen assimilation,
integrating the nitrogen and carbon metabolic processes; therefore it is finely regulated through mechanisms that have been particularly well studied in E. coli, involving complex cascades of adenylylation (Stadtman, 1990). Because of its status as a model enzyme, glutamine synthetase from cyanobacteria has also been the subject of a number of studies (for review, see Flores and Herrero, 1994; Florencio and Reyes, 2002); cyanobacterial glutamine synthetase activity is not regulated by covalent modification; furthermore, a novel regulatory process by protein–protein interaction has been recently described in Synechocystis PCC 6803 (García-Domínguez et al., 1999; 2000; Florencio and Reyes, 2002). On the other hand, transcriptional control of glnA expression is exerted through the global regulator of nitrogen assimilation in cyanobacteria, NtcA (Herrero et al., 2001).

In spite of the extraordinary ecological importance of marine cyanobacteria, there are few studies dealing with glutamine synthetase in these organisms (Warr et al., 1984; Carpenter et al., 1992; Wyman, 1999); furthermore, although several cyanobacterial GSs have been purified (Stacey et al., 1977; Sampaio et al., 1979; Orr et al., 1981; Florencio and Ramos, 1985; Mérida et al., 1990; Alhama et al., 1992; Yuan et al., 2001), no study has attempted to date a detailed characterization of the enzyme in marine cyanobacteria. We have previously studied the physiological regulation of glutamine synthetase in Prochlorococcus (El Alaoui et al., 2001), and its inactivation by metal-catalyzed oxidative systems (Gómez-Baena et al., 2001). We observed several important differences on the regulation of this enzyme when compared with GSs from other cyanobacteria; namely, the enzymatic activity did not decrease under darkness, and it was not upregulated in the light (at depth) occurring in the oligotrophic regions where it is most abundant.

In this paper, we further addressed this issue, with the aim of analysing whether the adaptive modifications occurred during the evolution of the Prochlorococcus ecotypes could also affect the structure and/or kinetics of glutamine synthetase. To this end, we purified and characterized the enzyme from two ecotypes, PCC 9511 and SS120, representative of the high- and low-light adapted populations of Prochlorococcus respectively. Besides, we performed phylogenetic analysis of the proteins encoded by glnA in Prochlorococcus MED4, MIT9313 (whose genomes have been sequenced by the American Department of Energy-Joint Genome Institute) and SS120 (sequenced by the Genoscope, France). We discuss our results with regard to glutamine synthetases from other cyanobacteria, both from marine and freshwater environments.

Results

Purification of GS from Prochlorococcus strains PCC 9511 and SS120

Cell-free extracts from 30 l of Prochlorococcus PCC 9511 cultures were utilized for GS purification; all the manipulations were carried out at 4°C. Preliminary studies showed that addition of the protease inhibitor PMSF at 1 mM concentration to buffers inhibited GS activity and consequently it was discarded. A buffer containing 2 mM MnCl2 in Tris-HCl pH 7.5 (buffer A) was used throughout the purification procedure. Once cells were resuspended in this buffer, as described in Experimental procedures, 20% ammonium sulphate was added to extracts under continuous stirring during 30 min. After centrifugation at 48,000 g for 45 min, all GS activity was detected in the supernatant. Attempts at introducing a second step of ammonium precipitation provoked a strong inactivation of the enzyme, and were therefore avoided. The supernatant was extensively dialysed overnight in Spectra/Por MWCO 3500 bags, with 5 l of cold buffer A containing 0.1 M NaCl for 12 h, to remove ammonium sulphate from samples before the chromatographic steps. Although the precipitation with ammonium sulphate induced a marked inactivation of the enzyme (75%), the dialysis resulted in recovery of almost 80% of the initial GS activity (see Table 1).

The sample thus obtained was applied to a Q-Hyper D cationic interchange column (quaternary ammonium as the interacting group; Amersham Biosciences) in a BioSys 2,000 FPLC system (Beckman), at a flow of 60 ml h−1, recovering samples of 1 ml. Once the absorbance at 280 nm decreased to the baseline, GS was eluted using a linear NaCl concentration gradient (from 0.1 to 0.4 M).

Table 1. Purification of GS from Prochlorococcus PCC 9511.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Yield (%)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>39.1</td>
<td>36.8</td>
<td>100</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>20% (NH4)2SO4</td>
<td>35.5</td>
<td>10.0</td>
<td>27.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Dialysis</td>
<td>29.2</td>
<td>30.0</td>
<td>81.5</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Q-HyperD</td>
<td>0.1</td>
<td>11.0</td>
<td>30</td>
<td>110</td>
<td>117</td>
</tr>
</tbody>
</table>

© 2003 Society for Applied Microbiology and Blackwell Publishing Ltd, Environmental Microbiology, 5, 412–423
This gradient eluted a main GS activity peak, corresponding to ~30% of the initial activity (Fig. 1A). A second, minor GS activity peak was eluted after the gradient was finished, by a step of 0.8 M NaCl in buffer A constant until the end of the chromatography.

The same procedure was used to purify GS from Prochlorococcus SS120, the low-light model strain. Only minor differences were observed when comparing results from both strains, the main one being the appearance of a more prominent second GS peak in the chromatographic Q-Hyper D profile corresponding to the SS120 strain (Fig. 1B). This difference could be due to the presence of contaminants in cultures of this strain.

Table 1 summarizes the obtained results for the strain PCC 9511. The purification yielded ~40 ml of homogenous GS samples, with a total enzymatic activity of 11 U. It is worth noting that the whole purification procedure was performed in less than 24 h, allowing to quickly obtain purified GS samples for characterization.

Analysis of the different samples obtained during the purification process by SDS-PAGE (Fig. 2) demonstrated that the eluted peak contained an homogenous protein with a molecular mass of ~48.6 kDa, closely corresponding to the one expected for GS from Prochlorococcus MED4 as estimated from the MED4 glnA sequence (51.877 kDa). The estimated molecular mass of the purified enzyme from the SS120 strain was very similar (not shown). These GS preparations were aliquoted, frozen and kept at -20°C to be used for the characterization described hereafter.

**Characterization of purified GS from Prochlorococcus PCC 9511**

Different physico-chemical and kinetic parameters of the enzyme were studied (Table 2). The optimal pH were 7.5 and 8.0 for the transferase and biosynthetic activities respectively. Interestingly, the optimal temperatures for both activities were found to be 55°C, a rather unexpected result as Prochlorococcus cultures have an optimal temperature for growth of 24°C (Moore et al., 1995). The GS transferase activity was quite stable at 37°C, maintaining more than 95% of the initial activity after 60 min at such temperature (not shown). Similarly, the biosynthetic activity remained almost constant for several days when samples were kept at 4°C. However, freezing of samples induced an almost complete loss of biosynthetic activity (not shown). Accordingly, all samples utilized for biosynthetic assays were kept at 4°C after purification. K_\text{m} constants of the transferase and biosynthetic activities were calculated utilizing the Lineweaver–Burk plot method. As previously observed in other studies...
Characterization and phylogeny of glutamine synthetase from Prochlorococcus

(García-Fernández et al., 1997), Km value for ADP could not be calculated for the transferase activity, since all tested concentrations produced similar results (not shown). The determined Km values for the rest of substrates were comparable to those described in other cyanobacteria (Table 2). This fact is particularly interesting in the case of the biosynthetic Km values for ammonium and ATP (see Discussion).

Western blotting of GS from HL and LL-adapted Prochlorococcus strains

In order to assess a possible change in the molecular mass of GS from Prochlorococcus (as reported for urease; Palinska et al., 2000), crude extracts from the strains PCC 9511, MED4, TAK9803-2 (high light adapted) and SS120, NATL1A, NATL2A (low light adapted) were prepared as indicated in Experimental procedures, but fourfold concentrated to improve the immunochemical detection of GS (Gómez-Baena et al., 2001). Western blotting was carried out (Fig. 3) using antibodies anti-GS from Synechocystis PCC 6803. We detected immunoreacting bands in the six strains, all of them showing a molecular mass similar to that of the purified GS from Prochlorococcus PCC 9511 (~ 48 kDa; Figs 2 and 3). In the case of NATL1A, the corresponding band showed a weaker intensity compared with the other strains, although the amount of total protein loaded into each well was equivalent for all of them.

Effect of nutrient limitation in vivo on the GS activity from Prochlorococcus PCC 9511 and SS120

Prochlorococcus populations are almost ubiquitous in the oceans, but are specially thriving in oligotrophic regions, where they outcompete co-existent Synechococcus (Partensky et al., 1999a,b). This fact suggests that Prochlorococcus is particularly well adapted to the oligotrophy found in such regions. Thus, we analysed the in vivo effect of limitation in three of the main nutrients: nitrogen, phosphorus and iron, on the glutamine synthetase activity in cultures of Prochlorococcus PCC 9511 (axenic, HL) MED4 (non-axenic, HL) and SS120 (non-axenic, LL) (Fig. 4A), after 120 h of transfer to the corresponding media. In the case of PCC 9511, nitrogen starvation did not induce an upregulation of GS, whereas phosphorus starvation provoked a marked decrease in this activity. The results were rather different

Table 2. Physico-chemical and kinetic parameters of GS purified from Prochlorococcus PCC 9511 compared with those of other cyanobacterial GSs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Apparent molecular mass of subunit (kDa)</th>
<th>Optimal temp (°C)</th>
<th>Optimal pH</th>
<th>Apparent Km (mM)</th>
<th>Apparent Kₘ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transferase</td>
<td>Biosynthetic</td>
<td>Transferase</td>
<td>Biosynthetic</td>
<td>Gln</td>
</tr>
<tr>
<td>Prochlorococcus PCC 9511</td>
<td>48</td>
<td>55</td>
<td>55</td>
<td>7.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Synechocystis PCC 6803</td>
<td>52</td>
<td>34</td>
<td>35</td>
<td>6.5</td>
<td>7.2</td>
</tr>
<tr>
<td>Synechococcus PCC 6301</td>
<td>47</td>
<td>35</td>
<td>38</td>
<td>6.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Synechococcus RF-1</td>
<td>56</td>
<td>–</td>
<td>37</td>
<td>8.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Calothrix PCC 7601</td>
<td>49</td>
<td>40</td>
<td>37</td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Nostoc PCC 7120</td>
<td>50</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.6</td>
</tr>
</tbody>
</table>

a. Data from this work.
b. Data from reference Mérida et al. (1990).
d. Data from reference Yuan et al. (2001).
e. Data from reference Mérida et al. (1990).
f. Data from reference Orr et al. (1981).
followed by a very marked decline in both the cell concentration (not shown) and GS activity, and eventually death of all cells in the starved cultures.

We further studied the effect of nutrient limitation in Prochlorococcus, by analysing MED4 cultures starved for a long time (Fig. 5). Thus we carried out experiments similar to those presented in Fig. 4A, but harvesting the cells after 312 h growing in the corresponding nutrient-deprived media. We measured GS transferase activity, total protein concentration, chlorophyll concentration and GS protein concentration in the obtained samples. The Fig. 5A shows the changes in protein concentration and GS activity expressed relative to protein concentration; the Fig. 5B shows the changes in chlorophyll concentration and GS activity expressed relative to chlorophyll concentration.

For MED4 and SS120: nitrogen starvation induced a clear increase in GS activity (more than twofold), whereas iron- and phosphorus-starvation provoked a decrease, stronger in the case of iron. However, because these strains contain heterotrophic bacteria, the interpretation of these results could be controversial (see Discussion).

The effect of iron starvation on PCC 9511 was remarkable, as in all experimental attempts, Prochlorococcus cells were dead after 120 h growing on media without iron (not shown). Thus we conducted time-course experiments in order to follow the changes in specific GS activity from Fe-starved Prochlorococcus PCC 9511 cells (Fig. 4B). We observed a decrease of the GS activity after only 8 h,
concentration. Nitrogen starvation induced a decrease (more than 50%) in both the protein and chlorophyll concentration, also detected to a lesser extent in phosphorus and iron-starved cultures. Regardless of the parameter used to standardize GS activity in these long-term starved cultures, we observed that it increased clearly in nitrogen-limited cultures (threefold when considering U/mg prot, and fourfold when considering U/mg chl), whereas no significant changes were observed under phosphorus or iron depletion (Fig. 5A and B). In addition, Western blotting of the starved samples showed that the GS protein concentration remained fairly unchanged when compared with the control (Fig. 5C). These results are particularly interesting in the case of nitrogen limitation, which induced a marked increase of the GS activity.

**Phylogeny of glnA from Prochlorococcus**

The glnA genes from *Prochlorococcus* encode predicted GS subunits of 52 876.58 Da (MED4), 52 728.41 (SS120) and 52 687.78 Da (MIT9313), with an isoelectric point of 4.887, 5.230, and 5.050 respectively. As observed with the purified GS subunit size estimated by SDS-PAGE (Fig. 2), these values are analogous to that deduced for the GS from the model freshwater strain *Synechocystis* PCC 6803 (molecular mass of 52 998.93 Da, isoelectric point of 4.894).

The sequences of glutamine synthetase proteins deduced from the available cyanobacterial *glnA* genes (including those from *Prochlorococcus* strains MED4, MIT 9313 and SS120) were aligned (Fig. 6A) using the Jotun-Hein method from the MEGALIGN 4.0 software, and the corresponding phylogenetic tree is shown in Fig. 6B. Sequences from *Synechococcus* WH8103, *Synechococcus* WH5701, *Trichodesmium* IMS101 and *Trichodesmium* theiobiauti are incomplete, and consequently were omitted from the phylogenetic tree in order to avoid any bias in their position. Several interesting features can be drawn from this tree: the marine *Synechococcus* and *Prochlorococcus* sequences are grouped together, separated from freshwater model strains; the GSs from *Nostoc* are rather different from the rest and more early branching; besides, *glnA* from *Prochlorococcus* MIT9313 appears more closely related to the sequence from *Synechococcus* WH7803 and WH8102 than to *Prochlorococcus* MED4 or SS120.

Many blocks are conserved throughout this alignment (Fig. 6A), including 12 amino acid residues involved in the biosynthetic reaction of glutamine synthetase (Crespo *et al.*, 1999). Altogether, the *Prochlorococcus* sequences show a remarkable similarity with those from well studied strains as *Synechocystis* PCC 6803 or *Synechococcus* PCC 7942.

**Discussion**

The central ecological significance of several marine cyanobacterial groups as primary producers (namely *Prochlorococcus*, *Synechococcus*, and *N₂*-fixing *Synechocystis* and *Trichodesmium*) is widely accepted nowadays (Vaulot *et al.*, 2002). Because a main feature of the natural habitat of *Prochlorococcus* is the limitation in nutrients (Partensky *et al.*, 1999a), the occurrence of mechanisms to optimize nutrient uptake and/or utilization by *Prochlorococcus* has been postulated (Partensky *et al.*, 1999a; Scanlan and West, 2002). Our group has observed some striking traits concerning the in vivo regulation of GS in the strain PCC 9511 (El Alaoui *et al.*, 2001) and the inability of different *Prochlorococcus* strains to assimilate nitrate (El Alaoui *et al.*, 2001; López-Lozano *et al.*, 2002). In this study, we further explored the possible evolutive modifications of glutamine synthetase from *Prochlorococcus*. We hypothesized that some of these changes could represent key ecological advantages in very oligotrophic environments.

We have established a protocol that allowed the purification of homogeneous samples of GS from *Prochlorococcus* in less than 24 h; this short time was an important parameter, because we managed to avoid any modification of the enzyme due to artifacts from the purification procedure itself. Besides, the purifications were started at the beginning of the week, allowing to complete a series of characterization studies during the following days without breaks.

The molecular mass of purified GS subunits from *Prochlorococcus* PCC 9511 (Fig. 2) and SS120 (not shown), as estimated by SDS-PAGE, were very similar to those described for other cyanobacteria (Table 2). This was confirmed by Western blotting (Fig. 3), where no significant differences among the six studied strains, including both HL- and LL-adapted ecotypes, could be detected. Thus apparently there are no changes in the size of glutamine synthetase from *Prochlorococcus*; this is in contrast with urease from the same organism (Palinska *et al.*, 1999), which shows the smallest size among all cyanobacterial ureases. The characterization of GS transferase activity from *Prochlorococcus* PCC 9511 showed that also optimal pH and K_m constants are comparable with those from other cyanobacteria (Table 2). A surprising result was the optimal temperature for the glutamine synthetase activity (55°C), strikingly different from those of *Synechocystis* PCC 6803, *Synechococcus* PCC 6301 or *Nostoc* PCC 7120, which is between 34 and 40°C. This result seems not to be an artifact, as the optimal temperature for the biosynthetic activity (determined by independent, chromatographic methods) was found to be the same. Given that *Prochlorococcus* can not grow above 28°C (Moore *et al.*, 1995), it is difficult to imagine physio-
logical explanations for such a high optimal GS temperature.

In the case of the biosynthetic activity, we were particularly interested in the $K_m$ constants for ammonium or ATP because of its possible physiological relevance in the adaptation to marine environments limited in nitrogen and/or light. Yet, the determined values (Table 2) are in the same order of that from other cyanobacteria, such as Synechocystis PCC 6803. Therefore, the affinity of GS from Prochlorococcus for their physiological substrates is apparently unaffected by significant evolutionary changes.

The response of the GS activity from Prochlorococcus to the starvation of three main nutrients in the oceans (nitrogen, phosphorus and iron) was unexpected. We previously reported that starvation of phosphorus was more stressing for GS activity than starvation of nitrogen in the
strain PCC 9511 (El Alaoui et al., 2001). In this study, we further studied this subject by analysing two additional strains (MED4, non-axenic, HL-adapted, and SS120, non-axenic and LL-adapted), and another key element in the ocean: iron (Figs 4 and 5). These results confirm our previous findings in the case of PCC 9511; the results obtained with MED4 are quite interesting, as MED4 corresponds to the very same genotype as PCC 9511 (Rippka et al., 2000), but it is non-axenic. Consequently, different results from identical experiments performed with PCC 9511 and MED4 are presumably caused by the contaminant heterobacteria growing in MED4 cultures. In this view, the opposite results produced by nitrogen starvation on PCC 9511 versus MED4 (i.e. slight decrease versus 2.5-fold increase; Fig. 4A) suggest that the heterobacteria from MED4 show a typical response in bacteria subjected to N limitation: upregulation of the GS activity (observed as well in long-term experiments; Fig. 5A).

Concerning P starvation in MED4, we observed also a decrease (25%) when compared with the control culture (Fig. 4A), although not so clear as the one observed in PCC 9511. Finally, iron starvation proved to be the more traumatic on GS activity, as it provoked a 80% decrease in MED4 (Fig. 4A). Furthermore, iron-starved cultures of PCC 9511 were unable to stand the 120 h considered in these experiments; under these conditions, we observed a 80% decrease (25%) when compared with the control culture (Fig. 4A); however, only experiments performed on LL axenic strains will confirm this hypothesis. Whatever the case, it can be concluded that the early effect of iron starvation on the GS of the studied cultures is stronger than that of N- or P-limitation. This fact could suggest that natural, co-existent populations of cyanobacteria and heterotrophic bacteria in the oceans are rather responsive to the presence/absence of iron, as previously reported after large-scale experiments to study the effect of iron limitation in the field (Coale et al., 1996), and also by studies focused on the iron stress in Prochlorococcus (Garcia-Fernández et al., 1998; Mann and Chisholm, 2000).

Studies on glnA from cyanobacteria (Fig. 6B) showed a phylogenetic tree in good agreement with those previously reported for rpoC1 (Toledo et al., 1999), 16S RNA (West et al., 2001) or 16S-23S rDNA ITS (Laloui et al., 2002; Rocap et al., 2002): Prochlorococcus is closely related to marine Synechococcus strains, in a clade of marine pico-phytoplankton clearly separated from other cyanobacteria (West et al., 2001). This separation is consistent with previously described cyanobacterial phylogenies based on 16S RNA (Honda et al., 1999) and the hli gene family (Bhaya et al., 2002). The higher similarity of GS from Prochlorococcus MIT9313 to that of Synechococcus WH7803 or WH8102 than to Prochlorococcus MED4 or SS120 could be an additional indication of the process that, presumably, induced the appearance of Prochlorococcus from ancient marine Synechococcus-like organisms. In this model, low-light adapted Prochlorococcus strains (such as MIT9313; genome size of 2.4Mbp) evolved from marine Synechococcus (such as WH8102; genome size of 2.72 Mbp) by losing some unessential genes (Hess et al., 1996; 2001; Strehl et al., 1999; Rippka et al., 2000; López-Lozano et al., 2002; Moore et al., 2002; Ting et al., 2002), leading to a progressive compaction of the genome (to strains like LL-adapted SS120–1.75 Mbp-, and eventually HL-adapted MED4–1.66 Mbp), that has been proposed as one of the advantages of Prochlorococcus to adapt to very oligotrophic marine conditions (Strehl et al., 1999). This model would explain the higher similarity between GS from Prochlorococcus SS120 and MED4 than SS120 and MIT9313 (both LL-adapted strains) observed in Fig. 5B.

The alignment of the deduced GS sequences from the available glnA genes from cyanobacteria (Fig. 6A) showed a very similar size (~53 000 Da) for the GS subunits, suggesting that the typical dodecameric structure of prokaryotic GS is conserved among all cyanobacteria, and consequently a molecular mass for the native enzyme of ~636 000 Da. Our results in Prochlorococcus (Figs 1 and 2) are in good agreement with this idea. All the amino acid residues described to be involved in the biosynthetic reaction centre in E. coli (Fig. 6A) are fully conserved in...
the cyanobacterial GSs, including the studied Prochlorococcus strains. If we consider these results together with the previous ones on physico-chemical and kinetic properties, it seems that no major modifications on the GS protein from Prochlorococcus could be detected. Its regulation, though, has been shown to possess several remarkable differences when compared with the other cyanobacteria (El Alaoui et al., 2001); thus we can conclude that within the cyanobacterial radiation leading to the appearance of Prochlorococcus (which is currently accepted as a modern organism in evolutionary terms), and as far as GS is concerned, the natural selection played mainly on its regulatory mechanisms and not on its structure or kinetic properties. A related situation has also recently been pointed out in the phosphorus metabolism in Prochlorococcus (Scanlan and West, 2002): the genomic comparison of Prochlorococcus MED4 versus MIT9313 showed that the phoR gene from MIT9313 (encoding a histidine kinase involved in P sensing and transcriptional control of P uptake through PhoB) contains a stop codon and frameshift within the coding sequence. The consequence is an inability to regulate the P assimilation machinery in this strain, probably reflecting the relatively high P concentrations found in deep waters, the habitat of Prochlorococcus MIT9313. Further comparative analysis of the three Prochlorococcus genomes in combination with more detailed physiological studies will probably help to explain the unusual regulatory features of glutamine synthetase in this organism; i.e. the small number of histidine kinases and response regulators observed in Prochlorococcus (Scanlan and West, 2002) could provoke simplified regulatory networks.

**Experimental procedures**

**Chemicals**

All chemicals were of reagent grade, obtained from Merck or Sigma. Standard proteins for determination of the molecular mass of GS subunits were purchased from Sigma and Bio-Rad.

**Prochlorococcus strains and growth conditions**

Prochlorococcus strains PCC 9511 (high-irradiance adapted, axenic), MED4 (high-irradiance adapted, non-axenic), TAK9803-2 (high-irradiance adapted, non-axenic), SS120 (low-irradiance adapted, non-axenic) NATL1A and NATL2A (low-irradiance adapted, non-axenic) were routinely cultured in Polycarbonate Nalgene flasks (10 l) using PCR-S11 medium as described (Rippka et al., 2000). The sea water used as basis for this medium was kindly provided by the Instituto Español de Oceanografía (Spain). Cells were grown in a culture room set at 24°C under continuous blue irradiances (40 and 4 μE m⁻² s⁻¹ for HL- and LL-adapted strains, respectively). Cells were collected during the exponential phase of growth. Growth was determined by measuring the absorbance of cultures at 674 nm.

**Cell collection**

Cells were centrifuged at 30 100 g for 5 min in an Avanti J-25 Beckman centrifuge equipped with a JA-14 rotor. After pouring most of the supernatant and carefully pipetting out the remaining medium, the pellet was directly resuspended in 500 μl of cold buffer containing 2 mM MnCl₂ in 50 mM Tris-HCl pH 7.5, and immediately frozen at −20°C until used for enzymatic analysis.

In the experiments studying nutrient starvation, cultures were diluted fivefold with the appropriate media (standard PCR-S11 for controls, or media lacking either nitrogen, phosphorus, or iron). Two aliquots were prepared from each culture (control and nutrient-depleted), which were subjected to standard conditions of light and temperature. Samples were taken at the indicated time following the protocol described above.

**Enzymatic assays**

Glutamine synthetase transferase activity was determined as previously described (El Alaoui et al., 2001), during 30 min at 37°C. The reaction mixture contained: 100 mM glutamine, 10 mM sodium hydroxylamine, 50 μM manganese chloride, 10 μM ADP and 50 mM sodium arseniate in 0.2 M MOPS pH 7. The GS biosynthetic activity was determined basically as described (Marqués et al., 1989). The reaction mixture contained: 22.5 mM glutamate, 9 mM ammonium chloride, 22.5 mM magnesium chloride and 6 mM ATP in 40.5 mM Hepes pH 7. One unit of the activity is the amount of enzyme that transforms 1 μmol of substrate min⁻¹.

After thawing, the samples were centrifuged at 16 100 g for 5 min, and the supernatants were used for the GS assay. Protein concentration was determined using the Bio-Rad Protein Assay kit, based on the method described by Bradford (Bradford, 1976). Chlorophyll concentration was determined as previously described (Mackinney, 1941). Both parameters were used to standardize the enzymatic activities. Results shown in Figs 4 and 5 correspond to the average of three independent determinations; error bars correspond to the standard deviations.

**Determination of Km constants for the transferase and biosynthetic GS activities**

The method of Lineweaver–Burk was utilized, by plotting the double inverses of enzymatic activities versus substrate concentrations, with constant concentrations of the other substrates. Standard concentration for each substrate has been described above.

**Electrophoresis and determination of molecular mass of purified GS**

Denaturing SDS electrophoresis was performed as described (Laemmli, 1970) on a MiniProtean II system (Bio-
Rad) utilizing 12% acrylamide gels, with 40 µl samples from the different purification stages. Electrophoresis was carried out for 60 min at 200 V (120 mA). Gels were stained in a 40% methanol, 10% acetic acid solution containing 0.1% Coomasie brilliant blue. The subunit molecular mass of the purified GS was estimated by comparing the mobility of the enzyme by polyacrylamide gel electrophoresis in the presence of SDS with that of proteins of known molecular mass.

Western blotting

Samples subjected to SDS electrophoresis as described above were transferred to a nitrocellulose membrane (Sigma) utilizing a semidy Trans-Blot SD system (Bio-Rad). Transfer was performed for 30 min at 20 V (150 mA). After transfer, the membrane was treated as follows: washing for 15 min with TBS-T buffer (20 mM Tris-HCl pH 7.5 supplemented with 155 mM NaCl and 0.1% Tween 20); blocking with TBS-T containing 1% bovine serum albumin for 1 h; incubation with primary antibody (anti-GS from Synechocystis PCC 6803 produced in rabbit) diluted 1 : 5000 in TBS-T for 1 h; washing threefold for 5 min with TBS-T buffer; incubation with secondary antibody (anti-immunoglobulin from rabbit produced in goat, linked with alkaline phosphatase) diluted 1 : 4000 in TBS-T for 15 min; washing threefold for 5 min with TBS-T buffer. Then, the immunoreacting material was detected by addition to the membrane of a solution containing the substrates for alkaline phosphatase: 120 ml of a nitroblue-tetrazolium solution (50 mg in 1 ml of 70% dimethylformamide) and 120 ml of a 5-bromo-4-chloro-3-indolylphosphate solution (25 mg in 1 ml dimethylformamide) in 20 ml of 0.1 M ethanalamine pH 9.3. The enzymatic reaction was stopped by addition of excess volume of distilled water, in order to avoid excessive background.

Prochlorococcus genomic sequences and phylogenetic analysis

Preliminary sequence data on the Synchococcus WH8102, Nostoc ATCC 29133, Trichodesmium erythraeum and Prochlorococcus (strains MED4 and MIT9313) genomes were obtained from the JGI-DOE web site (http://www.jgi.doe.gov/JGI_microbialhtml/index.html). This data has been provided freely by the US DOE Joint Genome Institute for use in this publication only. Preliminary sequence data on the Thermosynchococcus elongatus BP-1 genome were obtained from the Kazusa Institute web site (http://www.kazusa.or.jp/cyano,Thermo/index.html). Preliminary sequence data on the Prochlorococcus SS120 genome was obtained courtesy of Dr F. Partensky (Station Biologique de Roscoff, France) and the Genoscope (http://www.sb-roscoff.fr/Phyto/ProSS120).

All available cyanobacterial glnA sequences (as of September 4, 2002) were retrieved from GenBank, with the following accession numbers: Anabaena azollae, AJ249658; Calothrix PCC 7801, L05689; Nostoc PCC 7120, X00147; Synechococcus PCC 7002, Z13965; Synechococcus PCC 7942, AF031129; Synechococcus WH 5701, AF027612; Synechococcus WH 7803, AF026393; Synechococcus WH 8103, AF027611; Synechocystis PCC 6803, X69199; Thalassiosirium thiebautii, U30820; Trichodesmium IMS 101, AF169960. Molecular masses and pl values of the corresponding GS subunits were calculated using the software EditSeq 4.0 (Lasergene software package, DNAsStar Inc). Alignment of the deduced protein sequences and phylogenetic tree were obtained using the Jotun-Hein method with MegAlign 4.0 (Lasergene package).

Acknowledgements

We thank Dr F. Partensky for providing strains, early access to Prochlorococcus SS120 genome sequences, stimulating discussions and critical reading of the manuscript; Dr R. Rippka for providing the axenic PCC 9511 strain; and Dr P. Candau for providing antibodies anti-GS from Synechocystis PCC 6803. We acknowledge the kind collaboration of Carlos Massó de Ariza and the Instituto Español de Oceanografía for supplying the seawater. This work was supported by doctoral fellowships to S.E.A. (Agencia Española de Cooperación Internacional), G.G.-B. (Junta de Andalucía) and A.D. (Région Bretagne), a postdoc fellowship of the University of Córdoba to J.M.G.-F., the E. U. program MASTIII (MA3-CT97-0128), the University of Córdoba (Programa Propio de Investigación), and the Junta de Andalucía (III Plan Andaluz de Investigación).

References


Characterization and phylogeny of glutamine synthetase from Prochlorococcus 421


