The marine cyanobacterium *Synechococcus* sp. WH7805 requires urease (urea amidohydrolase, EC 3.5.1.5) to utilize urea as a nitrogen source: molecular-genetic and biochemical analysis of the enzyme

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Cyanobacteria assigned to the genus *Synechococcus* are an important component of oligotrophic marine ecosystems, where their growth may be constrained by low availability of fixed nitrogen. Urea appears to be a major nitrogen resource in the sea, but little molecular information exists about its utilization by marine organisms, including *Synechococcus*. Oligonucleotide primers were used to amplify a conserved fragment of the urease (urea amidohydrolase, EC 3.5.1.5) coding region from cyanobacteria. A 5.7 kbp region of the genome of the unicellular marine cyanobacterium *Synechococcus* sp. strain WH7805 was then cloned, and genes encoding three urease structural subunits and four urease accessory proteins were sequenced and identified by homology. The WH7805 urease had a predicted subunit composition typical of bacterial ureases, but the organization of the WH7805 urease genes was unique. Biochemical characteristics of the WH7805 urease enzyme were consistent with the predictions of the sequence data. Physiological data and sequence analysis both suggested that the urease operon may be nitrogen-regulated by the *ntcA* system in WH7805. Inactivation of the large subunit of urease, *ureC*, prevented WH7805 and *Synechococcus* WH8102 from growing on urea, demonstrating that the urease genes cloned are essential to the ability of these cyanobacteria to utilize urea as a nitrogen source.

Keywords: nitrogen assimilation, cyanobacterium, ammonium, nitrate, phytoplankton

INTRODUCTION

The unicellular cyanobacteria assigned to the genus *Synechococcus* (Waterbury & Rippka, 1989) are an important component of the phytoplankton in many marine and freshwater planktonic ecosystems (Fogg, 1987; Waterbury *et al.*, 1986). They contribute most significantly to total primary production in their most oligotrophic habitats (Chisholm, 1992; Fogg, 1987;

Joint, 1986), such as those large oceanic regions where nitrogen availability is thought to limit primary productivity. The nitrogen utilized by the cyanobacteria and other primary producers in these regions appears to derive largely from the planktonic food-web-driven recycling of nitrogen from biomass into ammonium and urea (Dugdale & Goering, 1967; Fogg, 1987; Hayward, 1991; Price & Harrison, 1988). Urea, which is generally present in marine waters at levels of 0.1–1 µM (Antia *et al.*, 1991), is formed by bacterial degradation of nucleic and amino acids and excreted by many animals as a waste product (McCarthy, 1980; Walsh, 1997). Urea is also the single dominant component of the diverse group of organic nitrogenous compounds known collectively as dissolved organic nitrogen that is present in the oligotrophic oceans at a total concentration of about 5 µM (Antia *et al.*, 1991).

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Abbreviations: GS, glutamine synthetase; RUBISCO, ribulose biphosphate carboxylase-oxygenase.

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For all phytoplankton that have been examined except the Chlorophytes, urease is the enzyme responsible for decomposing urea (Leftley & Syrett, 1973). Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-requiring metalloenzyme that catalyses the hydrolysis of urea, releasing two molecules of ammonia and one of carbonic acid from each urea molecule. Urease enzymes may be composed of one polypeptide (840 amino acid residues long in jack bean), or of two (238 and 569 amino acid residues long in *Helicobacter* strains) or three (approx. 100, 100, and 600 amino acid residues long in most bacteria) separate subunits. Whatever the subunit distribution, the sequences of urease structural genes are highly conserved (Mobley *et al.*, 1995). In most bacteria, *ureA*, *ureB* and *ureC* encode, respectively, the two small and one large structural subunits of the urease enzyme. The production of active urease also requires four accessory genes that are usually named *ureD*, *ureE*, *ureF* and *ureG*. These genes encode proteins required for the assembly of the nickel metallocentre found at the urease active site (Mobley *et al.*, 1995; Moncrief & Hausinger, 1997). *UreG* sequences are almost as highly conserved as the urease structural genes, but *UreD*, *UreE* and *UreF* are more variable. All seven of the polypeptides required to produce active urease are usually found in one cluster in bacteria (Mobley *et al.*, 1995). Urease is generally a cytoplasmic enzyme (Mobley & Hausinger, 1989; Mobley *et al.*, 1995), and the ammonium it releases from urea can therefore be assimilated directly by the organism. Urease may be expressed constitutively, or may be under many forms of regulation including global nitrogen status (ammonium repression), urea induction, or control by cell growth stage or by environmental factors such as pH (de Koning-Ward & Robins-Browne, 1997; Mobley & Hausinger, 1989).

The development of techniques to monitor the expression of urease in natural populations of marine phytoplankton would help to clarify the contribution that urea makes to their overall nitrogen nutrition, and improve our understanding of the role that urea plays in marine planktonic ecosystems. We have begun to approach this goal by investigating the genetic and biochemical basis of the utilization of urea as a nitrogen source by a model marine picophytoplankton, the unicellular cyanobacterium *Synechococcus* sp. strain WH7805.

METHODS

Strains and culture conditions. Marine *Synechococcus* isolate WH7805 was grown at 25 °C with about 50 µmol photons m⁻² s⁻¹ light from cool white fluorescent tubes on a shaking table in either modified SN or modified AN medium (Waterbury & Willey, 1988). Six other axenic strains from the Woods Hole culture collection (WH7803, WH8102, WH8103, WH8011, WH8112 and WH8113; Waterbury *et al.*, 1986) along with 15 new nonaxenic marine *Synechococcus* strains isolated from the California Current by B. Palenik (Toledo & Palenik, 1997) were also examined. Both AN and SN were modified by substituting FeCl₃ for ferric ammonium citrate, adding only vitamin B₁₂ in place of the Va vitamin mix, and adding NiCl₂

(final concentration 50 nM), Na₂SeO₃ (final concentration 10 nM) and H₃BO₃ (final concentration 16 µM) to the trace metals mix. Both types of media were prepared without any fixed nitrogen and sterilized by autoclaving. Routine monitoring by plating on 6 g l⁻¹ agar-solidified medium enriched with 5 g glucose l⁻¹ and 0.5 g tryptone l⁻¹ ensured the maintenance of axenic cultures of the WH strains. To provide a nitrogen source, either 1 mM NaNO₃, 1 mM NH₄Cl, or 0.5 mM urea was added from a filter-sterilized stock solution before each culture was inoculated. Stock cultures (10 ml) were maintained in 18 mm culture tubes. Fifty-millilitre serial batch cultures for growth rate and urease regulation experiments were grown in 125 ml Erlenmeyer flasks plugged with cotton stoppers. These cultures were kept in exponential phase growth through six transfers by diluting 1 ml of a culture nearing the end of exponential growth (less than 10⁸ cells ml⁻¹) into 50 ml fresh medium. Concentrations of ammonium, nitrate and urea in the cultures were determined by direct assays (Grasshoff *et al.*, 1983). One-litre cultures for urease purification were grown in 2.8-litre Fernbach flasks. Cell densities were determined by epifluorescent cell counts in a hemacytometer at 400× magnification using a Zeiss Axioskop. Growth rates were also determined from the increase in chlorophyll absorbance at 680 nm (OD₆₈₀), corrected for scattering at 750 nm (OD₇₅₀) measured on a Perkin-Elmer Lambda 3B spectrophotometer.

Urease assay. Because WH7805 urease was somewhat unstable under conditions optimal for its activity, the enzyme was kept at suboptimal pH (buffer A; 25 mM sodium/potassium phosphate, pH 7.3, plus 1 mM Na₂EDTA) until it was assayed. Urease activity was measured by following the release of ammonium from urea. Generally 50 µl crude cell extracts or purification fractions were added to 950 µl assay buffer (100 mM Na₂HPO₄) with 50 µl 10 mM urea (500 µM urea in the assay; final pH approximately 8.6) and incubated at 25 °C unless otherwise stated. At various times, 100 µl was removed, added to 900 µl freshly prepared MilliQ water, and the ammonium concentration in this sample determined by the indophenol method (Grasshoff *et al.*, 1983). Urease activities were normalized to the total protein concentration of each sample, estimated using the Bio-Rad DC protein assay in microtitre plate format, and are reported here as 1 unit = 1 nmol urea degraded min⁻¹ (mg total protein)⁻¹. Whether urease was assayed in or purified from fresh cell pellets or from cells that were stored as pellets at -80 °C made no obvious difference to its properties or activity.

Purification of urease. Two types of localization experiments were performed on freshly harvested cells. Osmotic shock treatment was performed according to the procedure of Laudenbach *et al.* (1991). The high-EDTA treatment used to remove the outer membrane was performed as described by Brahamsha (1996a). For the purification of urease, 12 litres of exponential-phase WH7805 grown on nitrate was collected and stored at -80 °C. The cell pellets were resuspended in 28 ml buffer A and disrupted by three passes through a French pressure cell (Spectronic Instruments) at 20000 p.s.i. (138 MPa) to produce the crude extract. Insoluble material and unbroken cells were removed by centrifugation at 50000 g for 45 min at 4 °C to produce the 50000 g supernatant. The 50000 g supernatant was then heated to 60 °C for 45 min with gentle mixing in a rotisserie hybridization oven. Heat-insoluble material was removed by centrifugation at 50000 g for 30 min at 4 °C, producing the heat supernatant. After storage at 4 °C overnight, the volume of the heat supernatant was adjusted to 100 ml with buffer A, and the fraction precipitating between 20% and 35% saturation ((NH₄)₂SO₄

was collected, dissolved in 8 ml buffer A, and dialysed overnight against two 1-litre volumes of buffer A to produce the ammonium sulfate cut fraction. The sample was then adjusted to 0.2 M NaCl in buffer A and loaded onto a 1 ml Pharmacia HiTrap Q Sepharose column by hand. The column was placed onto a GradiFrac and the salt concentration was increased linearly from 0.2 M to 0.4 M NaCl in buffer A over 2 h at a flow rate of 1 ml min⁻¹. Ten microlitres of each 1 ml fraction was assayed to locate the peak of urease activity. Sixteen fractions containing the peak were combined and concentrated using Amicon Centricon-3 centrifugal concentrators to a volume of 1 ml to produce the Q column peak fraction. This sample was then loaded onto a Pharmacia Sephacryl S-300 HR column running at a flow rate of 0.5 ml min⁻¹. The five 1 ml fractions (assayed as above) containing the peak of urease activity were combined and concentrated to a final volume of 1 ml, producing the S300 column peak. The S-300 column was calibrated using the Pharmacia gel filtration HMW calibration kit. Fractions were analysed by SDS-PAGE on Novex 4–20% polyacrylamide Tris-Glycine gels stained either with the Novex Colloidal Coomassie Staining Kit or with the Bio-Rad Silver Stain Plus Kit.

Cloning and sequencing. The deduced amino acid sequences of the 15 urease gene sequences present in GenBank in March 1994 were aligned using CLUSTAL V (Higgins *et al.*, 1992). Oligonucleotides were designed to match the highly conserved regions found at residues 217–223 (KLHEDWG) and 315–322 (MLMVCHHL) of UreC in the similar alignment presented by Mobley *et al.* (1995). The sequences of the two primers were 5' aagstscacgaggactgggg 3' and 5' aggtgtggcasaccatsagcat 3'. These primers should allow PCR-based amplification of a 317 bp portion of the urease coding region from most ureases sequenced to date. A 100 µl reaction containing 1 µM each primer, 2 mM MgCl₂, 200 µM each dNTP, 0.5 µg genomic DNA isolated from appropriate strains, 2 units *Taq* polymerase (Perkin-Elmer) and 1× *Taq* buffer was cycled 35 times with denaturing at 92 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. The 317 bp fragments produced from WH7805 and *Anabaena* sp. strain PCC 7120 by these primers were cloned using the Invitrogen TA cloning system and sequenced, confirming by their extensive homology with other ureases that they arose from a urease coding region. The sequence of the *Anabaena* PCC 7120 product has been deposited in GenBank under accession number AF-065139. The WH7805 PCR fragment was then used as a probe for Southern analyses using the Boehringer Mannheim Genius system to identify overlapping 4.8 kbp *Eco*RI and 5.4 kbp *Pst*I fragments covering a 9 kbp region of the genome of WH7805 surrounding the PCR product. These fragments were cloned from size-selected pUC118 plasmid libraries via colony-lift hybridization to the PCR product (Sambrook *et al.*, 1989), yielding plasmids pJC107 and pJC104, respectively. For sequencing, nested deletions were produced from these clones and from subclones in both orientations using the Promega Erase-a-Base kit. Gaps were filled in by sequencing from oligonucleotides synthesized commercially to serve as internal primers. Sequencing was performed using the ABI Prism cycle sequencing kit and protocol and an ABI 373 autosequencer. The entire 9 kbp region was sequenced completely on both strands, sequences were proofread by hand, and no ambiguities remained. The 5.7 kbp portion of the sequence reported here has been deposited in GenBank under accession number AF056189.

Inactivation of *ureC* in *Synechococcus* WH7805 and WH8102. *ureC* was insertionally inactivated using a strategy described by Brahamsha (1996a). The 477 bp *Sph*I–*Eco*RI fragment of

the WH7805 *ureC* gene, which is completely internal to *ureC*, was subcloned from pJC107 using *Sph*I (the resulting *Sph*I fragment is 522 bp in length because the second *Sph*I site is in the pUC118 polylinker, which also introduces a *Pst*I site into the construct), and ligated into the *Sph*I site of pMUT100. The resulting plasmid, pMUTjc1, was mobilized into *Synechococcus* sp. strains WH7805 and WH8102 as a suicide vector by conjugation using methods previously described (Brahamsha, 1996b). Exconjugants were selected on SN pour plates containing kanamycin (25 µg ml⁻¹) and were screened by Southern blot hybridization to confirm that the restriction pattern was indicative of integration of pMUTjc1 at the site of homology. Urease activity levels and the ability of the transconjugants to utilize nitrate (5 mM), ammonium (2 mM) or urea (1 mM) as sole nitrogen source were assessed as described above and compared to the wild-type strains WH7805 and WH8102.

RESULTS

Growth of WH7805 on nitrate, ammonium and urea

Urea, ammonium and nitrate could each be utilized as the sole nitrogen source supporting growth of *Synechococcus* WH7805 (Fig. 1). The long-term suitability of these nitrogen sources was confirmed by the maintenance of stock WH7805 cultures for more than 12 passages on media containing only one of the three compounds as sole nitrogen source. Each of the three nitrogen sources also supported the growth of the 15 nonaxenic marine *Synechococcus* isolates from the California Current (Toledo & Palenik, 1997) tested, as well as five of the six other axenic isolates from the WH culture collection (Waterbury *et al.*, 1986) tested. The single exception was *Synechococcus* sp. strain WH7803, which could use either ammonium or nitrate but not urea. Closer inspection of the growth rate of WH7805 in serial batch cultures, which were kept in exponential phase through six transfers and never allowed to exhaust

Fig. 1. Growth of *Synechococcus* WH7805 cultures utilizing different nitrogen sources. Changes in the chlorophyll absorbance (680 nm) of representative batch cultures that started with 1 mM nitrate (○), 1 mM ammonium (□) or 0.5 mM urea (△) as the sole nitrogen source and were allowed to grow to stationary phase are shown.

Fig. 2. SDS-PAGE analysis of the partial purification of WH7805 urease. M, markers (molecular masses to the left and right of the figure); 1, crude extract; 2, 50000 g supernatant; 3, heat supernatant; 4, ammonium sulfate cut; 5, Q column peak; 6, S300 column peak. The gel on the left was stained with Coomassie blue, and both gels on the right were silver-stained. For fractions 1, 2, 3 and 4, 0.03% of the urease activity was loaded; for fractions 5 and 6, 1.5% and 4.5% (50 and 150 times more than the initial fractions, respectively) of the urease activity was loaded. The arrows indicate the putative 11 and 60 kDa urease subunit bands (see text).

Table 1. Summary of the partial purification of urease from WH7805

Activity is given in units of nmol urea hydrolysed min⁻¹. See text for a detailed description of each step in the procedure.

Purification step	Total protein (mg)	Total activity (units)	Recovery (% initial activity)	Specific activity [units (mg total protein) ⁻¹]	Purification factor
Crude extract	139.6	2308	100	16.5	1
50000 g supernatant	61.2	1883	81.6	30.8	1.9
Heat supernatant	47.1	1829	79.2	38.8	2.4
Ammonium sulfate cut	18.9	992	43	52.5	3.2
Q column peak	0.28	312	13.5	1132	68.6
S300 column peak	0.11	165	7.1	1300	78.8

their supply of nitrogen to less than 0.2 mM, showed that the three nitrogen sources were not entirely equivalent. For the last two transfers (which were representative of the first four), ammonium-grown cells exhibited doubling times of 18.7 and 19.5 h, with urease activities of 3.2 and 3.5 units, respectively. Urea-grown cells had similar growth rates, doubling in 18.3 and 20.2 h, but 50% higher urease activity levels of 5.7 and 4.3 units, respectively. Nitrate-grown cells exhibited 15% slower growth, doubling in 21.0 and 22.8 h, along

with fivefold higher urease activity levels of 22 and 14 units, respectively.

Biochemical characterization of WH7805 urease activity

Experiments performed during development of the purification protocol described below defined several biochemical characteristics of the WH7805 urease. Urease activity was generally higher in disrupted than in

Fig. 3. Cloning of the urease genes from WH7805. (a) PCR amplification using *ureC*-directed degenerate primers against genomic DNA isolated from marine *Synechococcus* strains WH7803 and WH7805, freshwater *Synechococcus* PCC 7942, filamentous *Anabaena* PCC 7120, and a urease-negative *E. coli* (DH5 α). The size of the expected PCR product from *ureC* is indicated by arrows, and the sizes of molecular mass markers are indicated to the right. (b) Southern analysis using the WH7805 PCR product as a probe against genomic DNA from marine *Synechococcus* WH7803 and WH7805 digested with either *EcoRI* (E) or *PstI* (P). Sizes of molecular mass markers are indicated to the right. (c) Sequences of the PCR product from *Anabaena* 7120 (this study), and the PCR product regions from *Synechococcus* WH7805 (this study) and *Synechocystis* PCC 6803 (GenBank accession number D90903), compared to the consensus of 15 other *UreC* sequences available in February 1994. The consensus is majority-rule (Z = I, L, V, or T X = no consensus); asterisks (*) mark perfectly conserved residues, and the primer target sites are within the first seven and last eight residues. (d) Map of the urease region from WH7805. Restriction sites are indicated by vertical lines and letters: E,

intact cells, suggesting that the urease was localized intracellularly rather than on the cell surface. In experiments performed to yield optimal cell breakage, no detectable activity was found in the high-speed membrane pellet from cells disrupted by treatment with lysozyme followed by sonication, and less than 1% of the initial urease activity was found in the high-speed membrane pellet of cells disrupted by passage through a French pressure cell, showing that the urease activity of WH7805 was soluble. Two experiments suggested that the urease activity of WH7805 was cytoplasmic rather than periplasmic. After osmotic shock, only 14% of the initial activity was released while 86% remained with the cells, and after removal of the outer cell membrane by treatment with EDTA (Brahamsha, 1996a) no detectable activity was released from the cells. Only one peak of urease activity, with an apparent molecular mass of 420 kDa, was identified in extracts subjected to size-exclusion chromatography on both Sephacryl S-300 and S-400 resins. The pH optimum for urea degradation in crude extracts of WH7805 was found to be 8.6, with less than half of maximal activity remaining at pH 7.6 or 9.6. No preference for Na⁺ or K⁺ counterions in the phosphate buffer system used was detected, and no other buffer systems tested (including carbonate and Tris) gave different results or higher activities. The initial rates of urea degradation were measured at urea concentrations in the range 10–1000 μ M, and suggested that the WH7805 urease activity displayed simple Michaelis–Menten saturation kinetics with a K_m of 232 μ M ($r^2 = 0.997$).

Most organisms use the enzyme urease to degrade urea, but Chlorophytes and some yeasts are known to use instead an ATP- and biotin-dependent urea amidolyase pathway (EC 3.5.1.45). The two activities may be distinguished by several biochemical characteristics, including differential sensitivity to inhibitors and different requirements for optimal activity (Leftley & Syrett, 1973). Two lines of evidence supported the hypothesis that the urea-degrading activity measured in WH7805 was due to the presence of urease rather than the urea amidolyase pathway. First, only a moderate (less than 50%) stimulation of urea-degrading activity was observed in the presence of ATP at concentrations up to 100 μ M, and a decline in activity at higher ATP levels was observed (data not shown). A similar moderate stimulation was also observed in the presence of up to 1 mM EDTA, suggesting that in both cases chelation of inhibitory metal ions might be responsible for the stimulation of urease activity, as has been suggested previously for algal ureases (Leftley & Syrett, 1973). Second, as with most ureases (Mobley & Hausinger, 1989), the urease activity of WH7805 was moderately

EcoRI; S, *SphI*; P, *PstI*; B, *BamHI*. The locations and orientations of the urease genes are indicated by arrows labelled with the gene name. The location of the 317 bp fragment of *ureC* amplified by PCR is marked by the dotted box. The portions of the region originally cloned in pJC107 and pJC104 are indicated below the map.

(a)

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ureF/ureG
849 GCTCCCCATCAGCTTCGGAATAA 871
<ureG< S G M      • S R F L<ureF<

ureE/ureF
1520 GGAGGTCATGGCGTTTCATG 1539
<ureF< S T M      * P T E H<ureE<

ureD/ureA
3116 ACACACTGATAACGACCGAGCCAGTCCATGCAT 3148
>ureD> T H *                      M H >ureA>

ureA/ureB
3434 CCCATTCGTTGAGGCTGAACCGATGGCACCC 3464
>ureA> P I R *                      M A P >ureB>

ureB/ureC
3765 CTCGACGCCTGACCCATGCCCTAT 3788
>ureB> L D A *                      M P Y >ureC>

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(b)

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<ureE< M
2050 CATCGCGTGTGCTGCAAGGCTGCTCCCTTTCACCCTGAG

2091 CCTGCCAGCCGTCTGCCTTGATTGGTGTGATTGCTACA

2132 GGTCATCGCAGCTCGAGAACCGAGCGTGGGGAATG 2167
                      M >ureD>

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Fig. 4. Sequences between the *ure* open reading frames in WH7805. (a) Regions between the closely spaced and overlapping *ure* genes. (b) The putative divergent promoter region between *ureE* and *ureD*. Nucleotide sequences are given from 5' to 3' in the same orientation and with the same numbering system as used in the map in Fig. 3(d) and in GenBank accession number AF056189. Potential ribosome-binding sites are underlined and the potential NtcA-binding site is double underlined.

heat stable. It had a broad temperature optimum from 40 to 55 °C, where it was over twice as active as it was near the WH7805 growth temperature optimum of 20 to 25 °C. The WH7805 urease was inactivated by temperatures higher than 55 °C; it was stable for about 2 h at 60 °C, but lost half of its activity within 30 min at 70 °C and within 10 min at 80 °C.

Partial purification of the WH7805 urease enzyme

A five-step purification protocol resulted in an 80-fold purification of WH7805 urease activity (Table 1, Fig. 2). Ribulose biphosphate carboxylase-oxygenase (RUBISCO) and glutamine synthetase (GS), both identified by N-terminal sequencing, were two of the most difficult contaminants to remove from urease. The heat treatment was most effective in the removal of RUBISCO, while size-exclusion chromatography was most effective in the removal of GS (approx. 50 kDa bands in samples 2 versus 3 and 5 versus 6, respectively, in Fig. 2). The phycobiliproteins were effectively removed by the ion-

exchange chromatography step (samples 4 versus 5, Fig. 2). Significant amounts of urease protein or activity or both were lost in each of the last three steps. In the S300 column peak, 7% of the total urease activity was recovered with 0.08% of the initial protein. This final fraction produced a number of bands on a silver-stained SDS-PAGE gel (sample 6, Fig. 2).

Cloning and sequencing of the WH7805 urease genes

Two highly conserved regions around the urease active site were chosen as targets for oligonucleotides (see Methods). PCR using these degenerate oligonucleotides as primers yielded a fragment of the predicted size of 317 bp from WH7805 and *Anabaena* sp. strain PCC 7120 genomic DNA (Fig. 3a), as well as from all other urealytic marine *Synechococcus* later tested (data not shown). No amplification products were observed from non-urealytic organisms such as the freshwater cyanobacterium *Synechococcus* sp. strain PCC 7942 or *Escherichia coli* DH5 α (Fig. 3a). The non-urealytic marine *Synechococcus* sp. strain WH7803 produced a larger product than expected (Fig. 3a); sequencing of the WH7803 PCR product revealed that it did not arise from urease, and its amplification was subsequently eliminated by modifying the conditions of the PCR reaction. Southern analysis using the PCR product from WH7805 as a probe suggested that the PCR product from WH7805 arose from a single-copy target in its genome and also showed that a similar region was lacking from the WH7803 genome (Fig. 3b). The sequences of the PCR products from WH7805 and from the heterocystous, dinitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 confirmed that these products did arise from urease (Fig. 3c).

The WH7805 PCR product was used as a probe to identify and clone overlapping 5.4 kbp *Pst*I and 4.8 kbp *Eco*RI fragments from the genome of WH7805 (Fig. 3b), generating pUC118-based plasmids pJC104 and pJC107, respectively. A map of the entire *Eco*RI fragment plus the adjacent 900 bp of the *Pst*I fragment is shown in Fig. 3(d). Seven open reading frames in this region were identified by homology to urease structural subunits and accessory genes already sequenced from a variety of bacteria, and this region appeared to encode all of the functions needed to produce active urease (Mobley *et al.*, 1995). *ureA*, *ureB* and *ureC* encoded the structural subunits of the urease enzyme, and were similar in sequence and arrangement to the urease structural subunits of most other bacteria. All of the residues thought to be essential for urease activity (Jabri *et al.*, 1995) were present in the WH7805 *ureABC* genes. The predicted molecular masses of *UreA* and *UreB* were both 11 kDa, and that of *UreC* was 60 kDa. These predicted subunit sizes corresponded to the estimated molecular masses of two bands enriched during the partial purification of WH7805 urease (Fig. 2). *ureD*, *ureE*, *ureF* and *ureG* were predicted to encode proteins that are similar in both size (35, 19, 24 and 22 kDa, respectively) and sequence to previously analysed homologues

(Mobley *et al.*, 1995). A P-loop motif thought to be essential to the function of UreG (Moncrief & Hausinger, 1997) was conserved in WH7805. The histidine-rich region at the C-terminus of UreE thought to be important for nickel binding (Brayman & Hausinger, 1996) was of moderate length in WH7805. As in many other bacteria, *ureE*, *ureF* and *ureG* were contiguous, as were *ureA*, *ureB* and *ureC*. However, in WH7805 these two groups of genes were uniquely arranged, with *ureEFG* in the opposite orientation from *ureDABC*. The coding regions of *ureE* and *ureF* as well as *ureF* and *ureG* overlapped (Fig. 4a), suggesting that these accessory proteins were translationally coupled and formed an operon. While the coding regions of *ureD* and *ureA*, *ureA* and *ureB*, and *ureB* and *ureC* did not overlap, they were closely spaced (Fig. 4a), suggesting that they may also form an operon. The two groups of genes were separated by a small region that may contain a divergent promoter and an NtcA-binding site imparting global nitrogen regulatory control to the urease genes (Fig. 4b). No known urease-related genes were identified in the 3.3 kbp sequenced downstream of *ureC* (data not shown); the region downstream of *ureG* has not yet been explored.

Codon usage in the urease genes of WH7805 taken together (*ureABCDEFG*) was generally similar to that reported for eight genes from the closely related strain *Synechococcus* WH7803 (Table 2, and Carr & Mann, 1994). Among the WH7805 *ure* genes, *ureA*, *ureB* and *ureC* exhibited third-codon position preference in the order C > G > T ≥ A and had a third-position G + C content of 77.2, 77.6 and 81.9 mol %, respectively; *ureE*, *ureF* and *ureG* exhibited third-codon preference in the order G > C > T > A and had a third-position G + C content of 68.2, 70.5 and 69.7 mol %, respectively. *ureD* did not clearly belong to either group, exhibiting third-codon position preference C > G > A > T and a third-position G + C content of 72.8 mol %. Table 2 summarizes the codon usage for *ureABC*, *ureEFG* and *ureD*. Differences in codon usage between *ureABC* and *ureEFG* occurred in several cases (e.g. Phe, Pro, Ala, Asp, Glu, Cys); in some cases *ureD* was more similar to *ureABC* (e.g. Ala, Cys, Glu), but in others it was more like *ureEFG* (e.g. Asp) or distinct from either group (e.g. Phe, Pro). Differences in codon bias and nucleotide content among the urease genes of WH7805 could reflect differences in their expression levels (Berg & Silva, 1997; Wilbanks & Glazer, 1993), although there is no direct evidence of the relative expression levels of the urease genes in WH7805. Differences in base composition might also reflect genome-scale influences driven by the opposite orientation of *ureDABC* versus *ureEFG* (Freeman *et al.*, 1998).

Inactivation of *ureC*

To determine whether the urease structural genes identified in this study were required for the ability of marine *Synechococcus* to utilize urea as sole nitrogen source, the *ureC* gene was inactivated in two strains,

WH7805 and WH8102, by interrupting its coding sequence via homologous recombination between the chromosome and a completely internal *ureC* fragment from WH7805 carried by a suicide vector, pMUTjc1 (see Methods). To verify that integration had taken place at the target site and that no wild-type copies of *ureC* remained in the transconjugants, chromosomal DNA isolated from the mutants was digested with *Pst*I and probed with the 522 bp *Sph*I fragment of pMUTjc1. The 5.4 kbp *Pst*I fragment present in wild-type WH7805 was completely absent in the transconjugants (Fig. 5), even following overexposure of the Southern blot. It was replaced by two hybridizing fragments of 6.3 and 1.35 kbp, which agreed well with the fragment sizes of 6.1 and 1.3 kbp expected to result from integration of the suicide vector, carrying a new *Pst*I site, at the region of homology on the chromosome. Similarly, in WH8102 the wild-type 2.7 kbp fragment was completely replaced in the mutants by two hybridizing fragments of 1.35 and 3.3 kbp (Fig. 5).

Both parental strains, *Synechococcus* WH7805 and WH8102, were capable of growth with 1 mM urea as sole nitrogen source, but transconjugant strains 23-A, -B, -C and -D derived from WH7805 as well as 23-1 and -2 derived from WH8102 were not able to use urea as sole nitrogen source. The ability of these strains to grow utilizing either ammonium or nitrate as sole nitrogen source was unimpaired. Additionally, urease activity was normal in the parental strains but undetectable in the transconjugant strains. These results indicate that *ureC* is required both for urease activity and for growth on urea by *Synechococcus* sp. strains WH7805 and WH8102, and that the ability to degrade urea is not required for growth on either nitrate or ammonium. Furthermore, these results indicate that despite differences in restriction fragment patterns in the wild-type strains, recombination can occur between the *ureC* genes of these two strains of marine *Synechococcus*.

DISCUSSION

Urea provides a useable nitrogen source for many, but not all, cyanobacteria (Berns *et al.*, 1966; Kapp *et al.*, 1975; Kratz & Myers, 1955; McLachlan & Gorham, 1962). Among the marine *Synechococcus* surveyed in this study, only WH7803, which otherwise appears closely related to WH7805 (Palenik, 1994; Waterbury *et al.*, 1986), was unable to utilize urea. Contrary to a previous report (Waterbury *et al.*, 1986), WH8113, WH8011 and WH8103 each grew well with urea as the sole nitrogen source under the growth conditions used here. The region of *ureC* targeted for PCR amplification was present in all urea-utilizing cyanobacterial strains tested, but was lacking in the two strains (WH7803 and the freshwater *Synechococcus* strain PCC 7942) that could not use urea. Southern blots hybridized with a 1.25 kbp fragment from the opposite end of pJC104 (leaving a 2 kbp gap between the C-terminus of *ureC* and the 1.25 kbp probe) revealed that WH7803 lacked not only the small region of *ureC* targeted by our PCR

Table 2. Codon usage in the urease-encoding (*ureABC*) and accessory (*ureDEFG*) genes of *Synechococcus* WH7805

For each combination of coding regions, the raw number of occurrences of each codon is given, followed by the percentage utilization of that codon among its synonyms.

Amino acid	Codon	<i>ureABCDEFG</i>		<i>ureABC</i>		<i>ureD</i>		<i>ureEFG</i>	
		No.	%	No.	%	No.	%	No.	%
Phe	TTT	14	30.4	3	11.5	3	37.5	8	66.7
	TTC	32	69.6	23	88.5	5	62.5	4	33.3
Leu	TTA	3	1.4	0	0.0	1	2.3	2	2.0
	TTG	48	22.7	7	10.1	5	11.6	36	36.4
	CTT	21	10.0	7	10.1	2	4.7	12	12.1
	CTC	41	19.4	23	33.3	8	18.6	10	10.1
	CTA	8	3.8	0	0.0	4	9.3	4	4.0
	CTG	90	42.7	32	46.4	23	53.5	35	35.4
Ile	ATT	18	24.0	11	21.6	1	14.3	6	35.3
	ATC	56	74.4	39	76.5	6	85.7	11	64.7
	ATA	1	1.3	1	2.0	0	0.0	0	0.0
Met	ATG	31	100	18	100	3	100	10	100
Val	GTT	7	6.2	3	5.7	1	5.9	3	7.0
	GTC	18	15.9	8	15.1	5	29.4	5	11.6
	GTA	1	0.9	0	0.0	1	5.9	0	0.0
	GTG	87	77.0	42	79.2	10	58.8	35	81.4
	TCT	4	3.9	1	3.0	0	0.0	3	6.5
Ser	TCC	21	20.4	8	24.2	4	16.7	9	19.6
	TCA	6	5.8	0	0.0	2	8.3	4	8.7
	TCG	14	13.6	4	12.1	2	8.3	8	17.4
	AGT	12	11.7	3	9.1	2	8.3	7	15.2
	AGC	46	44.7	17	51.5	14	58.3	15	32.6
	CCT	8	8.3	2	4.4	3	16.7	3	9.1
Pro	CCC	43	44.8	30	66.7	5	27.8	8	24.2
	CCA	11	11.5	0	0.0	5	27.8	6	18.2
	CCG	34	35.4	13	28.9	5	27.8	16	48.5
Thr	ACT	6	6.7	3	5.4	1	7.1	2	10.0
	ACC	56	62.2	41	73.2	5	35.7	10	50.0
	ACA	8	8.9	2	3.6	5	35.7	1	5.0
Ala	ACG	20	22.2	10	17.9	3	21.4	7	35.0
	GCT	39	21.8	11	13.8	6	19.4	22	32.4
	GCC	92	51.4	57	71.3	18	58.1	17	25.0
	GCA	15	8.4	6	7.5	2	6.5	7	10.3
Tyr	GCG	33	18.4	6	7.5	5	16.1	22	32.4
	TAT	7	36.8	2	25.0	2	66.7	3	37.5
	TAC	12	63.2	6	75.0	1	33.3	5	62.5
Stop	TAA	0	0.0	0	0.0	0	0.0	0	0.0
	TAG	0	0.0	0	0.0	0	0.0	0	0.0
His	TGA	7	100	3	100	1	100	3	100
	CAT	16	30.8	6	25.0	3	27.3	7	41.2
Gln	CAC	36	69.2	18	75.0	8	72.7	10	58.8
	CAA	20	27.8	4	15.4	9	42.9	7	28
Asn	CAG	52	72.2	22	84.6	12	57.1	18	72
	AAT	14	31.1	8	34.8	1	9.1	5	45.5
Lys	AAC	31	68.9	15	65.2	10	90.9	6	54.5
	AAA	16	38.1	10	37.0	3	50.0	3	33.3
Asp	AAG	26	61.9	17	63.0	3	50.0	6	66.7
	GAT	32	36.4	8	17.8	6	50.0	18	58.1
	GAC	56	63.6	37	82.2	6	50.0	13	41.9

Table 2. (cont.)

Amino acid	Codon	<i>ureABCDEFG</i>		<i>ureABC</i>		<i>ureD</i>		<i>ureEFG</i>	
		No.	%	No.	%	No.	%	No.	%
Glu	GAA	52	49.1	31	64.6	12	70.6	9	22
	GAG	54	50.9	17	35.4	5	29.4	32	78
Cys	TGT	8	30.8	2	20.0	1	14.3	5	55.6
	TGC	18	69.2	8	80.0	6	85.7	4	44.4
Trp	TGG	26	100	7	100	9	100	10	100
Arg	CGT	16	12.9	2	4.5	2	6.5	12	24.5
	CGC	65	52.4	28	63.6	19	61.3	18	36.7
	CGA	10	8.1	5	11.4	1	3.2	4	8.2
	CGG	26	21.0	6	13.6	7	22.6	13	26.5
	AGA	1	0.8	1	2.3	0	0.0	0	0.0
	AGG	6	4.8	2	4.5	2	6.5	2	4.1
Gly	GGT	24	15.5	11	13.4	3	11.5	10	21.3
	GGC	86	55.5	57	69.5	14	53.8	15	31.9
	GGA	17	11.0	4	4.9	4	15.4	9	19.1
	GGG	28	18.1	10	12.2	5	19.2	13	27.7
Sum		1706		778		320		608

Fig. 5. Southern analysis of *ureC*-inactivated *Synechococcus* strains. Genomic DNA isolated from the wild-type (WT) or transconjugants of pMUTjc1 in *Synechococcus* sp. strains WH7805 and WH8102 was digested with *Pst*I. The probe was the 522 bp *Sph*I fragment of pMUTjc1 comprising 477 bp of *ureC* and 45 bp of polylinker from pUC118, which includes a new *Pst*I site. The migration of size standards is indicated on the right.

primers, but also a region of several kbp downstream of the urease gene cluster. It is not known whether the absence of urease is characteristic of some wild cyanobacterial strains, or reflects changes in the genomes of strains such as PCC 7942 and WH7803 that have occurred since they were brought into culture. Similar to *Synechococcus* WH7805 and WH8102, wild-type *Synechococcus* sp. strain PCC 7002 can utilize urea or nitrate as its sole nitrogen source, but *ureC*-inactivated derivatives of PCC 7002 lose the ability to utilize urea while retaining the ability to grow on nitrate (Sakamoto *et al.*, 1998). Although urease appears to be nonessential for both naturally occurring (PCC 7942, WH7803) and genetically engineered (WH7805, WH8102, PCC 7002) urease-negative *Synechococcus* growing on nitrate or ammonium in culture, urease may play other roles in cellular metabolism, such as internal nitrogen recycling, that are beneficial to these organisms under natural conditions.

The urea-utilizing cyanobacteria examined to date have been reported to possess a nickel-requiring urea amidohydrolase (EC 3.5.1.5) that is soluble and cytoplasmic (Berns *et al.*, 1966; Ge *et al.*, 1990; Mackerras & Smith, 1986; Rai & Singh, 1987; Singh, 1990, 1991, 1993). The pH and temperature optima for WH7805 urease are toward the upper and lower ends, respectively, of the range reported for other cyanobacterial ureases (Table 3). The K_m value estimated here for WH7805 urease is within the range reported for most other cyanobacterial ureases (Table 3), which tend to have a higher affinity for urea than the ureases of many other bacteria (Mobley & Hausinger, 1989). Given that urea concentrations in the sea rarely exceed 1 μ M, and that urease in WH7805 is cytoplasmic, it is likely that

Table 3. Summary of cyanobacterial ureases

Organism	Native mol. mass (kDa)	Subunit mol. mass (kDa)*	K_m (μ M)	pH (and temp.) optimum	Crude specific activity (nU)†	Regulation‡	Reference
<i>Anabaena doliolum</i>	228	36	115	7 (40 °C)	240		Rai (1989)
			120	7–7.4 (40 °C)	3.8 (A) to 10.5 (U) 0.7 (A) to 7 (U)	GNR	Rai & Singh (1987) Singh (1990)
<i>Anabaena cylindrica</i>	197	32		8 (30 °C)	3.2		Argall <i>et al.</i> (1992)
			1300		16 to 58	C	Mackerras & Smith (1986) Carvajal <i>et al.</i> (1982)
<i>Spirulina maxima</i>	232	38	120	8.7	210		
<i>Leptolyngbya boryana</i>	220	66, 18, 11	250	8.2 (60 °C)	120	C	Jahns <i>et al.</i> (1995)
<i>Anabaena</i> PCC 7120	220	66, 14, 11	250	8.5 (65 °C)	80	C	Jahns <i>et al.</i> (1995)
<i>Anabaena variabilis</i>			46		0.8	C	Ge <i>et al.</i> (1990)
<i>Anacystis nidulans</i> ATCC 27144					25 (N) to 200 (–N)	GNR	Singh (1991)
			250, 1660 (biphasic)	7–7.4 (40 °C)	3.6 (A) to 10 (U)	GNR	Rai & Singh (1987)
<i>Synechococcus</i> WH7805	420	(60, 11, 11)	232	8.6 (45 °C)	3.5 (A) to 16.5 (N)	GNR	This study
<i>Synechocystis</i> PCC 6803		(61, 11, 11)					Kaneko <i>et al.</i> (1996)
<i>Plectonema calothricoides</i>					115		Berns <i>et al.</i> (1966)
<i>Synechococcus lividus</i>					75		Berns <i>et al.</i> (1966)
<i>Phormidium luridum</i>					30		Berns <i>et al.</i> (1966)
<i>Nostoc muscorum</i>					0.6 (A) to 9.8 (U)	GNR	Singh (1993)

* Values were determined biochemically except for those in parentheses, which were predicted from DNA sequences.

† Crude specific activity is given in nmol urea hydrolysed min⁻¹ (mg protein)⁻¹ (nU). (A), cultures grown with ammonium; (N), cultures grown with nitrate; (U), cultures grown with urea; (–N), nitrogen-deprived cultures.

‡ C, constitutive expression; GNR; global nitrogen regulation (ammonium; repression).

WH7805 also possesses a high-affinity urea-transport system. Crude specific activities for cyanobacterial ureases range from 0.6 to 240 nmol urea hydrolysed min⁻¹ (mg protein)⁻¹ (Table 3). Assuming that each WH7805 cell contains approximately 50 fg nitrogen (Kana & Glibert, 1987) and 500 fg protein (Kramer & Morris, 1990), the level of urease activity measured at 25 °C in urea-grown WH7805 doubling approximately once per day is sufficient to supply its estimated nitrogen needs. In several cyanobacteria, urease activity is invariant during growth on different nitrogen sources, suggesting that urease may be constitutively expressed in these organisms (Table 3). In other cyanobacteria, urease activity varies during growth on different ni-

trogen sources in a manner suggesting that urease is subject to regulation by ammonium repression via the global nitrogen regulatory system, although ammonium-grown cells retain detectable urease activity (Table 3). The differences in urease activity levels reported here for WH7805 growing on different nitrogen sources suggest that urease expression in WH7805 may also exhibit (incomplete) ammonium repression via the global nitrogen regulatory system. Since ammonium concentrations in the sea rarely exceed 1 μ M, it appears likely that WH7805 would always possess some urease activity in its natural habitat. Whether this is characteristic of marine *Synechococcus* in general remains to be established.

In contrast to the general agreement among the biochemical properties of diverse cyanobacterial ureases, some data regarding their physical structures are in conflict. *Leptolyngbya boryanum* and *Anabaena* sp. strain PCC 7120 have been reported to possess typical microbial ureases with an alpha subunit of 66 kDa, a beta subunit of 14 or 18 kDa, and a gamma subunit of 11 kDa (Jahns *et al.*, 1995), similar to the subunit sizes reported here for *Synechococcus* sp. strain WH7805. The apparent native molecular mass of the *Leptolyngbya boryanum* and *Anabaena* PCC 7120 enzymes is 220 kDa, approximately 20% below the predicted value for a heterotrimeric (UreA₂UreB₂UreC)₃ quaternary structure similar to *Klebsiella aerogenes* (*K. pneumoniae*) urease (Jabri *et al.*, 1995; Mobley & Hausinger, 1989). The apparent native molecular mass of WH7805 urease was more consistent with a heterohexameric (UreA₂UreB₂UreC)₆ structure that yields a predicted native molecular mass of 497 kDa, 15% above the measured value of 420 kDa. Reports of both trimeric and hexameric structures for ureases are not unusual; both trimers and hexamers of jack bean urease are active (Mobley *et al.*, 1995), and other bacteria have been reported to possess hexameric ureases of similar native size to that from WH7805 (Mobley & Hausinger, 1989). More significant discrepancies have arisen with regard to the unique subunit structures reported for the ureases of the cyanobacteria *Spirulina maxima*, *Anabaena doliolum* and *Anabaena cylindrica* (Argall *et al.*, 1992; Carvajal *et al.*, 1982; Mackerras & Smith, 1986; Rai, 1989; Singh, 1990, 1991), which appear to comprise homohexamers of a single 32 to 38 kDa subunit (Table 3). Argall *et al.* (1992) were also unable to detect hybridization of a urease gene probe from *K. aerogenes* to genomic DNA of *Anabaena cylindrica*. However, given the difference in G + C content between these two organisms (60 versus 40 mol%, respectively), failure of this cross-hybridization does not provide conclusive evidence that amino acid level similarity between their urease genes is low. Although *Anabaena cylindrica* was not tested, the primers developed for this study were capable of amplifying the targeted fragment of *ureC* from *Anabaena* PCC 7120 (Fig. 3). Additionally, the sequence of the *Synechocystis* PCC 6803 genome (Kaneko *et al.*, 1996) shows that it possesses seven urease genes similar to those found in WH7805, and *Synechococcus* PCC 7002 possesses at least *ureC* (Sakamoto *et al.*, 1998). It appears likely that the incongruent biochemical data from *Spirulina maxima*, *Anabaena doliolum* and *Anabaena cylindrica* reflect the difficulties inherent in purifying some microbial ureases (the presence of a prominent 30 kDa band in Fig. 2 may represent just such an artifact), and that further work will confirm that all cyanobacteria do possess typical bacterial ureases like that found in WH7805.

Close spacing and overlapping of *ure* reading frames is also observed in other urease gene clusters (Jones & Mobley, 1989; Lee *et al.*, 1992; Maeda *et al.*, 1994). However, the split of WH7805 urease genes into one *ureDABC* cluster and a divergent *ureEFG* cluster is

unique among microbial ureases sequenced to date, which are more commonly grouped as *ureDABCEFG* or *ureABCEFGD* (Neyrolles *et al.*, 1996). *Proteus mirabilis* does have a divergent regulatory gene, *ureR* (Island & Mobley, 1995). Other cyanobacteria present even more unusual arrangements. The urease genes of *Synechocystis* PCC 6803 (Kaneko *et al.*, 1996) are completely unclustered and are scattered throughout its genome. Similarly, the *ureC* gene of *Synechococcus* PCC 7002 appears to be encoded separately from other urease genes (Sakamoto *et al.*, 1998). Closer examination of cyanobacterial ureases may reveal new models for the regulation and coordination of urease gene expression and enzyme assembly.

Although the divergent promoter region between *ureE* and *ureD* in WH7805 is novel among urease gene clusters, divergent promoters themselves are a common phenomenon (Beck & Warren, 1988), perhaps with important roles in providing control over the coordination and relative levels of expression of the genes on either side. The pattern of urease activity observed in WH7805 grown on different nitrogen sources suggests the possibility that urease is regulated by ammonium repression via *ntcA* as part of the global nitrogen regulatory system that is common among cyanobacteria (Frias *et al.*, 1993). The consensus sequence GTAN₈TAC is generally found in the -35 position relative to transcription initiation (Muro-Pastor *et al.*, 1996) in NtcA-regulated genes. Near the middle of the 112 bp separating the start codons of *ureE* and *ureD* in WH7805 is the sequence GTGN₈TAC, which is identical to a proposed NtcA-binding site located upstream of a nitrogen-regulated promoter for *ntcA* itself in the closely related strain *Synechococcus* sp. strain WH7803 (Lindell *et al.*, 1998). However, the WH7805 *ure* divergent promoter region may be too small to allow the position of this site to represent a -35 region for either or both of *ureE* and *ureD*; if it does represent an NtcA-binding site involved in regulating the expression of the WH7805 urease gene cluster, it may use a different mechanism. The sequence TTGATT is found inside of and just outside of the potential NtcA-binding site, and some short, complementary sequences [TCCCN₉GAGC versus GCTCN₁₄GGGGA] can be found upstream of each translation initiation site (Fig. 4b). The relevance of any of these sequences to the transcription, translation or regulation of the urease genes in WH7805 has yet to be established.

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