

The cyanate utilization capacity of marine unicellular Cyanobacteria

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Abstract

Cyanate, a by-product of urea decomposition, is a potential nitrogen (N) source in marine environments, but to date it has received scant attention. Cyanobacteria presumably acquire this compound via a substrate-specific ABC-type transporter (*cynABD*), and they convert it to ammonium and carbon dioxide by cyanase (*cynS*) activity. Participation of cyanate utilization genes in N-stress responses in cyanobacteria has been implied previously, but its ecological context has not been studied. We employed polymerase chain reaction protocols for *cynA* amplification to examine the potential for cyanate recruitment in the Gulf of Aqaba, northern Red Sea. We also monitored growth of cyanobacterial strains with different *cynABDS* complements on cyanate-containing media. We showed that cyanate is utilized as the sole N source by strains with the full gene complement, and residual growth, fed by natural decay of cyanate to ammonium, was observed in strains lacking any of these genes. Natural abundance of *cynA* products in the oligotrophic Gulf of Aqaba indicates that cyanate constitutes an essential N source for *Prochlorococcus*, but not for *Synechococcus* populations. Noncyanobacterial *cynA* sequences indicate cyanate utilization by a variety of other phototrophic microorganisms. We hypothesize that in stratified water bodies, cyanate utilization is confined to the N-deplete upper photic zone, where it plays a role in “regenerated” primary production.

Cyanate is probably the simplest organic nitrogen (N) compound known for any organism. Cells produce it as a by-product of glutamine metabolism, with carbamoyl phosphate as the intermediate, and of arginine degradation via the urea cycle. Cyanate is potentially toxic to the cell, and neutralization of cyanate occurs via the activity of cyanate lyase (EC 4.2.1.104; synonyms—cyanase, cyanate hydratase). Cyanate lyase is a cytoplasmic holoenzyme composed of 15-kDa monomers and has an approximate 150,000-kDa molecular weight. The enzyme is found in bacteria, fungi, algae, higher plants, and animals. The properties of *Escherichia coli* cyanate lyase have been reviewed by Anderson and Little (1986). Cyanate lyase

efficiently degrades cyanate to ammonium and carbon dioxide. *E. coli* not only neutralizes cyanate generated intracellularly, it was also reported to take up cyanate from the environment. A cyanate-specific permease was identified, and the knock-out mutants produced were incapable of satisfying their N requirements when cyanate was the sole N source (Sung and Fuchs 1989). Recently, Espie et al. (2007) reported an abundance of cyanate transport genes among bacteria. An ABC-type transporter for cyanate was identified in the freshwater cyanobacteria *Synechococcus elongatus* strain PCC7942 (Harano et al. 1997) and *Synechococcus* sp. strain PCC6301. This transporter showed a high degree of similarity to ABC-type transporters for nitrate and bicarbonate uptake in cyanobacteria. The cyanobacterial ability to utilize cyanate has never been evaluated in an ecological context, and nothing is known about cyanate as an N source for cyanobacterial productivity in aquatic systems.

Unicellular, non-nitrogen-fixing cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* form an abundant fraction of marine phytoplankton, and they contribute up to 65% of primary production in oligotrophic ocean waters (Partensky et al. 1999). They often dominate in N-deplete surface waters, where they engage in regenerated production fueled by urea and ammonium as the most conspicuous N sources. Utilization of these compounds has been characterized for both *Synechococcus* and *Prochlorococcus* (Collier et al. 1999; Moore et al. 2002). Ammonium

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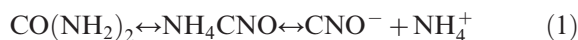
Acknowledgments

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and urea transport systems (encoded by the *amt* and *urt* genes, respectively) were first identified in *Synechocystis* PCC6803 (Montesinos et al. 1998; Valladares et al. 2002). Orthologs of these genes were found in genome sequences of unicellular marine cyanobacteria (Palenik et al. 2003; Rocap et al. 2003).

The spontaneous transformation of urea to ammonium cyanate in aqueous solution and then consecutively on to cyanate has long been known (Walker and Hambly 1895):



Since urea excretion by zooplankton is well documented (Parsons and Harrison 1983), cyanate may thus form an N source that accumulates in the marine environment. Putative genes for cyanate transport (*cynABD*) and its subsequent conversion to ammonium (*cynS*) were identified in *Synechococcus* WH8102 and in *Prochlorococcus* MED4 (Palenik et al. 2003; Rocap et al. 2003). This observation led García-Fernández et al. (2004) to consider that the growth of these species may be supported by cyanate as the sole source of external N. However, very little is known about the ability of marine cyanobacteria to acquire and metabolize cyanate. Even less is known about the availability of cyanate in the ocean environment. Presence of the *cynABD* orthologs in genomes of the two major oceanic primary producers—*Prochlorococcus* and *Synechococcus*—indicated an importance of cyanate utilization in the N-limited environments. Here we present a hypothesis regarding the role of cyanate in the N cycling in the marine environment based on experimental observations from culture studies, detection of environmental *cynA* and *urtA* sequences, and theoretical considerations.

Methods

Bacterial strains and growth conditions—*Prochlorococcus* spp. strains MED4 and MIT9313 were grown in PRO99 medium (Moore et al. 2002) buffered with 0.5 mmol L⁻¹ 4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid. *Synechococcus* spp. strains WH8102 and WH7803 were grown on artificial seawater medium (Moore et al. 2002). Both media were supplemented with 0.8 mmol L⁻¹ ammonium chloride (NH₄Cl; J. T. Baker). Cultures were maintained at 25°C ± 1°C with gentle agitation at 80–90 rpm on an orbital shaker at a light intensity of ~10 μmol Q m⁻² s⁻¹ for the *Prochlorococcus* MIT9313 and ~20–25 μmol Q m⁻² s⁻¹ for other strains. For nitrogen nutrition experiments NH₄Cl was replaced with variable concentrations of freshly prepared sodium cyanate (NaOCN; Aldrich), 0.4 mmol L⁻¹ urea (Amresco), or 0.8 mmol L⁻¹ sodium chloride to produce N-free medium. The growth was determined from either daily increment in optical density (OD₇₅₀) using a Spectronic® 20 Genesys™ spectrophotometer or in vivo fluorescence at 680 nm using a 10-AU Fluorometer (Turner Design).

Cyanate and urea decomposition experiments—A surface seawater sample was rendered particle free by passage over a 0.2-μm Supor® polyethersulfone membrane filter

(Nalgene) and was heat sterilized at 120°C for 20 min. Three hundred fifty-milliliter solutions of 20 μmol L⁻¹ cyanate or urea were freshly prepared in sterile seawater, divided over two 1000-mL Nalgeware flasks, and incubated at 25°C ± 1°C at constant light of 25 μmol Q m⁻² s⁻¹ and at 37°C ± 3°C for 5–7 d in daylight. Concentrations of ammonium resulting from urea or cyanate decomposition were measured daily, employing the orthophthaldialdehyde method with fluorescence detection on a Hoeffer DyNA Quant TM 200 fluorometer using an internal standard (Holmes et al. 1999). Linear approximation was applied for decomposition rate calculations.

Deoxyribonucleic acid (DNA) extraction—Seawater samples (20 liters) originating from 0-m and 80-m depths at Sta. A (29°28'N, 34°56'E) in the Gulf of Aqaba were filtered onto 0.45-μm Gelman SUPOR filters, immersed in storage buffer, and stored at -80°C until nucleic acid extraction. Following disruption of the cells with lysozyme, sodium dodecyl sulfate and proteinase K DNA extraction was performed by the phenol-chloroform method according to the protocol of Penno et al. (2006). DNA concentrations were determined photometrically (NanoDrop).

DNA analyses—To obtain products of the cyanobacterial *cynA* gene, polymerase chain reaction (PCR) was performed using five degenerate primer sets (Table 1). The PCR was performed using 1–2 ng environmental DNA, 0.5 μmol L⁻¹ of each primer, 0.25 mmol L⁻¹ each deoxyribonucleotide triphosphate, 1.25 units (U) of *Taq* DNA polymerase (PEQLAB), and 10× PCR buffer containing 15 mmol L⁻¹ MgCl₂ in a final volume of 50 μL. The reaction was preincubated at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 45 s; primer annealing at 45–52°C for 30 s; and elongation at 72°C for 30–45 s. A final elongation step at 72°C was performed for 5 min. The bands of expected size were excised and purified from 1.5% agarose gels with the Wizard® SV for Gel and PCR kit (Promega). To obtain products of the cyanobacterial *urtA* gene, PCR reactions were performed using the *urtA*-F2/R1 primer combination. A specific forward primer, *urtA*-SF, was employed for *Synechococcus urtA* amplification (Table 1). PCR reactions were run with ReddyMix™ (ABgene) in a final volume of 25 μL using 1–2 ng environmental DNA and 1 μmol L⁻¹ of each primer. Reactions were denatured at 94°C for 5 min followed by 41 cycles of 1-min denaturation at 94°C, 1 min of annealing at 49–52°C, and 1 min of elongation at 72°C, followed by a final elongation step at 72°C for 5 min. For both *cynA* and *urtA*, the products were cloned into the pGEM®-T Easy Vector System II (Promega). Chemically competent cells of *E. coli* DH5α were transformed with ligated plasmids by heat shock at 42°C for 45 s. The cells were grown overnight on the Luria–Bertani agar plates containing 100 μg mL⁻¹ carbenicillin, 80 μg mL⁻¹ X-gal, and 0.5 mmol L⁻¹ isopropyl-beta-D-thiogalactopyranoside. According to the blue–white screening, plasmid mini-preps were carried out on relevant colonies. The *cynA* PCR products were verified by restriction endonuclease cleavage using EcoRI (BioLabs) or by colony PCR.

Table 1. Degenerate primer (F and SF, forward; R, reverse) sequences for PCR amplification of *cynA* and *urtA* products from environmental DNA templates with their annealing temperatures and expected size of PCR products in *Prochlorococcus* strain MED4.*

	Primer	DNA target	Sequence (5'–3')	Temperature (°C)	Size (bp)
cynA	cynA F	General cyanobacteria	GARYTNGAYGCNTAYCAYATGC	44–50	705
	cynA R	General cyanobacteria	TGDATCCARTGNGMRAANGAYTGCC	45	
	PcynA R	<i>Prochlorococcus</i> specific	YTGWATCCAATGWSWAAAWSWYTGCC	44–45	
	ScynA R	<i>Synechococcus</i> specific	YTGRATCCAATGSSWAAAWSWYTGCC	45–50	
	cynA-2 F	Cyanobacteria specific	GAYGCNTAYCAYATGC	45	423
	cynA-2 R	Cyanobacteria specific	GGNCAAYCCNTGYTGYGCNTT	45	
	cAdc F	Cyanobacteria specific	SCH KCR GAY ATG AAA GGM TTY	52	517
urtA	cAdc-long R	Cyanobacteria specific	GYT TAA ART CDA TTC KGT CDG G	52	
	urtA-F2	General cyanobacteria	THTTYGGYGGHTGGACHTC	49–52	655
	urtA-SF	<i>Synechococcus</i> specific	CSGACTACGTSWYCCSCGYACSKCK	49–52	439
	urtA-R1	General cyanobacteria	ACCATGTTRTADGCMGAYTC	49–52	

* Nucleotide abbreviation according to the IUPAC code: N, G,A,T,C; V, G,A,C; B, G,T,C; H, A,T,C; D, G,A,T; K, G,T; S, G,C; W, A,T; M, A,C; Y, C,T; R, A,G; bp, base pairs.

Synechococcus urtA fragments were identified by PvuII digestion of PCR products. PCR-positive plasmid clones containing right size inserts were sent out for double-strand DNA sequencing. The sequence data were deposited in the NCBI GenBank database under the accession numbers EU430123–EU430208, EU430210–EU430228, and EO435014–EO435016. DNA sequences were aligned using the ClustalW multiple sequence alignment tool in BioEdit (version 7.0.0) editor, and phylogenetic trees were constructed by maximum likelihood analysis in TreePuzzle 5.2. Accession numbers of the individual sequences used for phylogenetic studies are listed in Table 2.

Results

Genetic potential for urea and cyanate utilization in cyanobacterial genomes—Since no reports were available regarding the occurrence and utilization of cyanate in ocean waters, we commenced our study with an inventory of the genetic potential for urea, cyanate, and ammonium utilization deduced from genome sequences of marine cyanobacteria. Twenty-three available genome sequences for marine *Synechococcus* and *Prochlorococcus* show that all carry the *ntcA* and *amt* genes required for the adaptive response to ammonium limitation and its acquisition via a high-affinity permease, respectively (Table 3). Three *Prochlorococcus* strains (MIT9211, MIT9515, and SS120) and one *Synechococcus* strain (WH7803) lack the genes encoding urea transport (with the exception of a divergent *urtA* copy also found in strain WH7805) and urease, and they are thus incapable of utilizing urea (see also Moore et al. 2002), regardless of whether it is generated intracellularly or is available in the environment. *Synechococcus* strains from the MC-A group are capable of cyanate degradation, as indicated by the presence of the *cynS* gene, whereas the MC-B type WH5701 lacked this gene (Table 3). Among *Prochlorococcus* only the genomes of high light–adapted (HL) strain MED4 and the low light–adapted (LL) strains NATL1A and NATL2A carry *cynS*. The *cynABD* genes required for cyanate acquisition were found only in *Synechococcus* WH8102 and *Prochlorococcus* MED4, two oceanic strains associated with oligotrophic

surface layers. Genomes of both strains carry the *cynA* gene (PMM0370 and SYNW2487, respectively), which encodes the periplasmic substrate-binding component of the transporter distinct from other anion ABC-type transporters (Omata et al. 2002; Espie et al. 2007). The deduced amino sequences predict proteins with a considerably longer N-terminal region than that found in *Synechococcus* PCC7942 and in orthologs present in the genomes of some photosynthetic bacteria, *Rhodospseudomonas palustris*, *Methylibium petroleiphilum* (synonym—*Rubrivivax gelatinosus* strain PM1), and *Roseovarius*. A screen of the GenBank database showed that *cynA*-like sequences are found in α -, β -, and γ -proteobacteria, chlamydiae, and cyanobacteria (Fig. 1A). Orthologs of *cynA* are abundant among photosynthetic bacteria (15 out of 28 total), particularly among α -proteobacteria and cyanobacteria (Fig. 1B). In most cases, bacterial genomes with *cynA* orthologs also contained *nrtA* orthologs, a substrate binding component of the nitrate transporter. Phylogenetic analysis (not shown) grouped *cynA* sequences away from *nrtA* with 100% bootstrap values. Orthologs of *cmpA*, another member of this transporter family encoding the substrate binding subunit for bicarbonate, were found exclusively in cyanobacteria. Interestingly, the current list contains only gram-negative bacteria, and to date *cynA*-like genes have not been identified in gram-positive bacteria.

Genetic potential for urea and cyanate utilization in the marine environment—In accordance with the genome screening we found that cyanobacterial populations from the Gulf of Aqaba showed an abundant presence of the *urtA* gene, indicating that urea utilization is widespread in this community. PCR amplification of 439–base pair products (see Table 1) from environmental DNA sampled at different depths and during different seasons routinely indicated the presence of *urtA*. Phylogenetic analysis of *urtA* sequences showed that *Prochlorococcus* formed a cluster distinct from *Synechococcus*. The latter group showed a degree of polytomy in the branching pattern, in part caused by the deep branching of the divergent *urtA* copies found in WH7805 and WH7803. The divergent *urtA* are not part of the *urt-ure* operon in these strains, and as

Table 2. Accession numbers of the individual sequences used for comparative and phylogenetic studies. All accession numbers are of genome sequences unless stated otherwise.

Bacterial strain	Accession No.
<i>Acaryochloris marina</i> MBIC11017	CP000828
<i>Agrobacterium tumefaciens</i> str.C58	AE007870*
<i>Alkalilimnicola ehrlichei</i> MLHE-1	CP000453
<i>Anabaena variabilis</i> ATCC 29413	CP000117
<i>Azorhizobium caulinodans</i> ORS 571	AP009384
<i>Bradyrhizobium</i> sp. BTAi1	CP000494
<i>Bradyrhizobium</i> sp.ORS278	CU234118
<i>Bradyrhizobium japonicum</i> USDA 110	BA000040
<i>Caulobacter crescentus</i> CB15	AE005673
<i>Cyanothece</i> sp. CCY0110	AAXW00000000
<i>Cyanothece</i> sp. PCC8801	AAQ23048†
<i>Gleobacter violaceus</i> PCC 7421	BA000045
<i>Lentisphaera araneosa</i> HTCC2155	ABCK00000000
<i>Limnobacter</i> sp. MED105	ABCT00000000
<i>Magnetospirillum gryphiswaldense</i> MSR-1	CU459004
<i>Magnetospirillum magnetotacticum</i> MS-1	AAAP00000000
<i>Mesorhizobium loti</i> MAFF303099	BA000012
<i>Methylobacterium</i> sp. 4-46	ABAY00000000
<i>Methylbium petroleiphilum</i> PM1	CP000555
<i>Methylobacillus flagellatus</i> KT	CP000284
<i>Methylobacterium chloromethanicum</i> CM4	ABEX00000000
<i>Methylobacterium extorquens</i> PA1	CP000908
<i>Methylobacterium populi</i> BJ001	ABFR00000000
<i>Microcystis aeruginosa</i> PCC 7806	CAO88017*, EF115423‡, CAO87745§, CAO88252†
<i>Nostoc</i> sp. PCC7120	BA000019
<i>Phormidium laminosum</i>	Z19598*, AY158222‡
<i>Polaromonas</i> sp. JS666	CP000316
<i>Polynucleobacter</i> sp. QLW-P1DMWA-1	CP000655
<i>Prochlorococcus</i> sp. AS9601	CP000551
<i>Prochlorococcus</i> sp. MED4	BX548174
<i>Prochlorococcus</i> sp. MIT9215	CP000840
<i>Prochlorococcus</i> sp. MIT9211	CP000878
<i>Prochlorococcus</i> sp. MIT9301	CP000095
<i>Prochlorococcus</i> sp. MIT9303	CP000554
<i>Prochlorococcus</i> sp. MIT9312	CP000111
<i>Prochlorococcus</i> sp. MIT9313	BX548175
<i>Prochlorococcus</i> sp. MIT9515	CP000552
<i>Prochlorococcus</i> sp. NATL1A	CP000553
<i>Prochlorococcus</i> sp. NATL2A	CP000095
<i>Prochlorococcus</i> sp. SS120	AE017126
<i>Pseudomonas stutzeri</i> A1501	CP000304
<i>Rhizobium etli</i> CFN 42	CP000133
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> 3841	AM236080
<i>Rhodobacter capsulatus</i>	AY273169*
<i>Rhodoferrax ferrireducens</i> T118	CP000267
<i>Rhodopseudomonas palustris</i> BisA53	CP000463
<i>Rhodopseudomonas palustris</i> CGA009	BX571963
<i>Rhodopseudomonas palustris</i> HaA2	CP000250
<i>Roseovarius</i> sp.217	AAMV00000000
<i>Roseovarius</i> sp. TM1035	ABCL00000000
<i>Sinorhizobium meliloti</i> 1021	AL591688
<i>Synechococcus</i> sp. BL107	AATZ00000000
<i>Synechococcus</i> sp. CC9311	CP000435
<i>Synechococcus</i> sp. CC9605	CP000110
<i>Synechococcus</i> sp. CC9902	CP000097
<i>Synechococcus</i> sp. JA-3-3Ab	CP000239
<i>Synechococcus</i> sp. JA-2-3B'a(2-13)	CP000240
<i>Synechococcus</i> sp. PCC6803	BA000022
<i>Synechococcus</i> sp. PCC7942	CP000100
<i>Synechococcus</i> sp. RCC307	CT978603
<i>Synechococcus</i> sp. RS9916	AAUA00000000

Table 2. Continued.

Bacterial strain	Accession No.
<i>Synechococcus</i> sp. RS9917	AANP00000000
<i>Synechococcus</i> sp. WH5701	AANO00000000
<i>Synechococcus</i> sp. WH7803	CT971583
<i>Synechococcus</i> sp. WH7805	AAOK00000000
<i>Synechococcus</i> sp. WH8102	BX548020
<i>Thermosynechococcus elongatus</i> BP-1	BA000039
<i>Verminophrobacter eiseniae</i> EF01-2	CP000542
<i>Pseudomonas aeruginosa</i> 2192	AAKW00000000
<i>Xanthobacter autotrophicus</i> Py2	CP000781

* *nrtA*.† *cnpA*.‡ *ntcA*.§ *amt1*.

yet there is no putative function assigned to these homologs. Natural communities in the Gulf of Aqaba revealed that *urtA* is found among the most abundant cyanobacterial genotypes, those of the *Synechococcus* clades II and III and of the *Prochlorococcus* HLII clade (Fig. 2). This finding indicates that urea may be an important N source for both populations.

Genome screening indicates that cyanate acquisition potential within the cluster of marine cyanobacteria is restricted to a few genotypes of open-ocean picocyanobacteria in the MC-A clade. PCR products of *cynA* were amplified from environmental templates of the Gulf of Aqaba originating from the upper parts of the photic zone

in summer, when waters are stratified and inorganic N sources are at or below detection (Lindell et al. 2005). Environmental samples ($n = 5$) taken during winter mixing or during the spring bloom (nitrogen-replete conditions) failed to yield any PCR product. Environmental *cynA* sequences clustered mostly with *Prochlorococcus* and with only a single sequence aligning with *Synechococcus* (Fig. 3). Interestingly, the *Prochlorococcus*-like sequences branched away from strain MED4, indicating that they are associated with HLII-type *Prochlorococcus*, the abundant genotype encountered in the Red Sea (Penno et al. 2006, and references therein). The *cynA*-2 primer set (see Table 1) failed to amplify cyanobacterial *cynA* but yielded products

Table 3. Survey of genomic databases for the genetic ability of marine *Synechococcus* and *Prochlorococcus* for nitrogen-stress responses (*ntcA*), ammonium uptake (*amt*), urea conversion and uptake (*ure* and *urt* genes), cyanate conversion, and uptake (*cyn*).

Species	Strain	<i>ntcA</i>	<i>amt</i>	<i>ureABCDEFG</i>	<i>urtABCDE</i>	<i>cynS</i>	<i>cynABD</i>
<i>Synechococcus</i> sp.	BL107*†	+	+	+	+	+	
	CC9311‡	+	+	+	+	+	
	CC9605*†	+	+	+	+	+	
	CC9902*†	+	+	+	+	+	
	RCC307*§	+	+	+	+	+	
	RS9916*†	+	+	+	+	+	
	RS9917*†	+	+	+	+	+	
	WH8102*	+	+	+	+	+	+
	WH7803*§	+	+			+	
	WH7805*†	+	+	+	+	+	
	WH5701*†	+	+	+	+		
	AS9601*†	+	+	+	+		
	MED4*	+	+	+	+	+	+
	MIT9215*	+	+	+	+		
MIT9301†	+	+	+	+			
<i>Prochlorococcus</i> sp.	MIT9303*†	+	+	+	+		
	MIT9312*	+	+	+	+		
	MIT9313*	+	+	+	+		
	MIT9515*†	+	+				
	MIT9211†	+	+				
	NATL1A*†	+	+	+	+	+	
	NATL2A*	+	+	+	+	+	
	SS120§	+	+				

* NCBI GenBank.

† Venter Institute.

‡ Tigr Institute.

§ Genoscope.

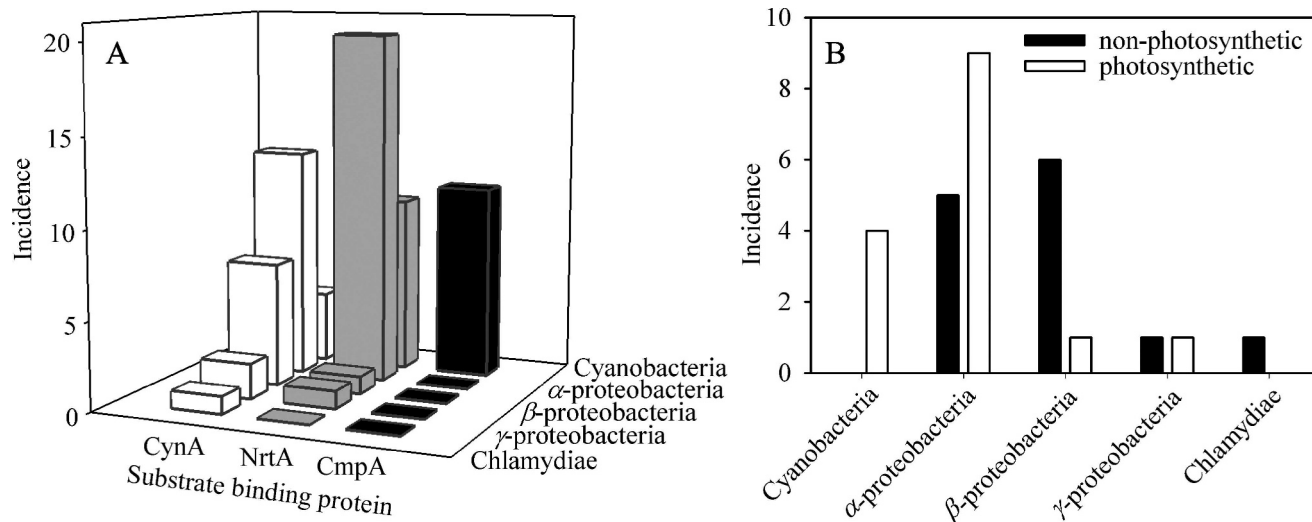


Fig. 1. (A) Distribution of cyanate (CynA), nitrate (NrtA), and bicarbonate (CmpA) substrate-binding proteins of ABC transporters, as identified in genome sequences among gram-negative bacteria. (B) Assignment of the cyanate substrate-binding proteins to photosynthetic and nonphotosynthetic representatives among different divisions of gram-negative bacteria.

with a high sequence similarity to the cyanate transport gene of *Roseovarius* sp. 217, a photosynthetic proteobacterium (Fig. 3). Whereas the significance of a cyanate utilization capacity in this group is not understood, the finding underlines that cyanate may constitute an N source to various picoplankton groups, including anoxygenic phototrophs.

Urea and cyanate nutrition in marine cyanobacteria—To examine the abilities of marine cyanobacteria to utilize urea, *Synechococcus* strains WH8102 (*urtABCDE⁺ureABCDEF⁺*) and WH7803 (*urtABCDE⁻ureABCDEF⁻*) were grown in medium containing urea as a sole nitrogen source along with control cultures grown on ammonium or non-nitrogen-containing medium. Culture density, fluorescence, and chlorophyll *a* (Chl *a*) content monitored growth. After a short lag phase, the WH8102 strain possessing a genetic potential for urea utilization grew at approximately the same rate as the control culture (0.47 d⁻¹ and 0.48 d⁻¹, respectively). The WH7803 (*urtABCDE⁻ureABCDEF⁻*) strain showed a low but constant growth rate of 0.194 d⁻¹ after 48 h lag phase, in contrast to an N-deprived control culture, which showed no growth at all.

The functional assignment of the above-mentioned *cynA* sequences as the substrate binding protein of the cyanate transporter was based on sequence similarity. The role of this gene in cyanate utilization by marine cyanobacteria (and, by extension, by anoxygenic phototrophs and nonphotosynthetic bacteria) has not been shown to date. We selected strains that differed in their ability to acquire cyanate (*cynABD⁺*) and to convert this compound to ammonium and carbon dioxide (*cynS⁺*): *Prochlorococcus* MED4 (*cynABD⁺S⁺*) along with *Synechococcus* WH8102 (*cynABD⁺S⁺*) are predicted to utilize both internally generated and externally supplied cyanate, as opposed to *Synechococcus* WH7803 (*cynABD⁻S⁺*), which lacks the genes for cyanate uptake. *Prochlorococcus* MIT9313

(*cynABD⁻S⁻*) was chosen as an organism incapable of cyanate metabolism. Growth experiments with cyanate as the sole nitrogen source were run alongside reference cultures grown on NH₄⁺ and -N media. Both (*cynABD⁺S⁺*) strains grew successfully on cyanate at a growth rate similar to that of ammonium-grown cells. The amount of cyanate added correlated with the cell densities when cultures entered stationary phase. Surprisingly, *Synechococcus* WH7803 showed a short lag phase and then a moderate growth (specific growth rate of 0.51 d⁻¹, compared to 0.74 d⁻¹ for ammonium-grown control cultures), indicating the ability of the (*cynABD⁻S⁺*) strain to satisfy its nitrogen requirements from cyanate despite the apparent lack of an uptake system. Moreover, *Prochlorococcus* strain MIT9313, which lacks the genetic potential to utilize cyanate altogether, was able to survive in the cyanate-based medium.

Urea and cyanate stability in seawater—Aqueous solutions of urea decompose to ammonium cyanate at room temperature. In its turn, ammonium-cyanate may spontaneously degrade to ammonium and carbon dioxide (Dirnhuber and Schütz 1948). The expected product of both partial and complete urea decomposition would thus be ammonium. To evaluate this phenomenon in a marine environment, we measured ammonium accumulation in seawater amended with urea at 25°C and 37°C (Fig. 4). The dissociation rate of urea was estimated at 8.3 and 9.0 nmol ammonium L⁻¹ h⁻¹ at the respective temperatures. Thus, in marine environments this process may contribute significantly to the pool of free ammonium.

Cyanate is known to decompose to ammonium and carbon dioxide in both neutral and slightly acidic aqueous solutions (up to pH 7.8), while temperature and ionic strength affect the decomposition rate constant (Lister 1955). Subsequently, we studied whether cyanate decomposition occurs in seawater (pH 8.2–8.3, ionic strength

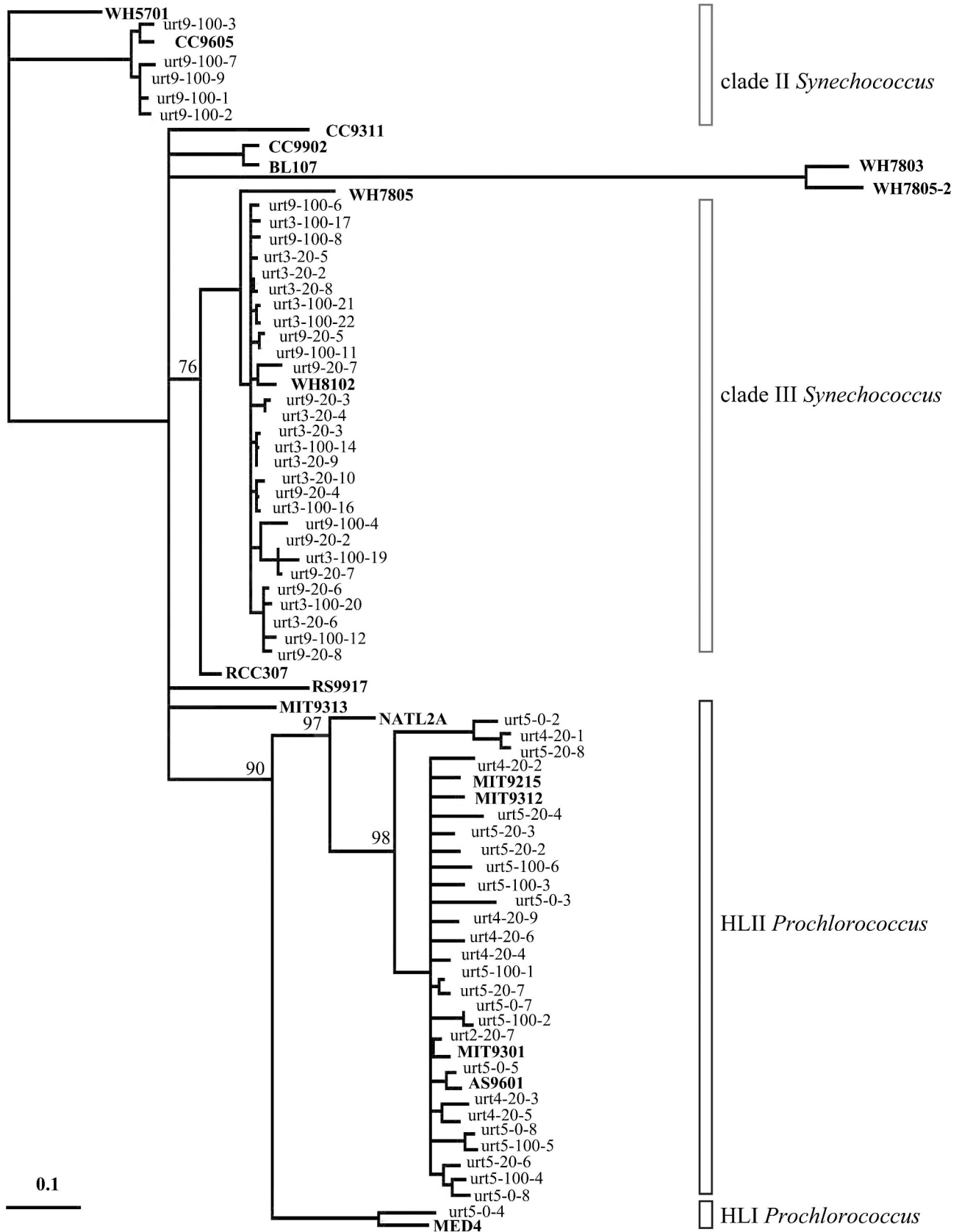


Fig. 2. Maximum likelihood analysis of environmental sequences of the urea transport gene *urtA* alongside sequences derived from cyanobacterial genomes. PCR products were obtained from environmental DNA templates from the photic zone in the northern Gulf of Aqaba. The scale bar provides a distance measure for 0.1 substitutions per 100 nucleotides between sequences.

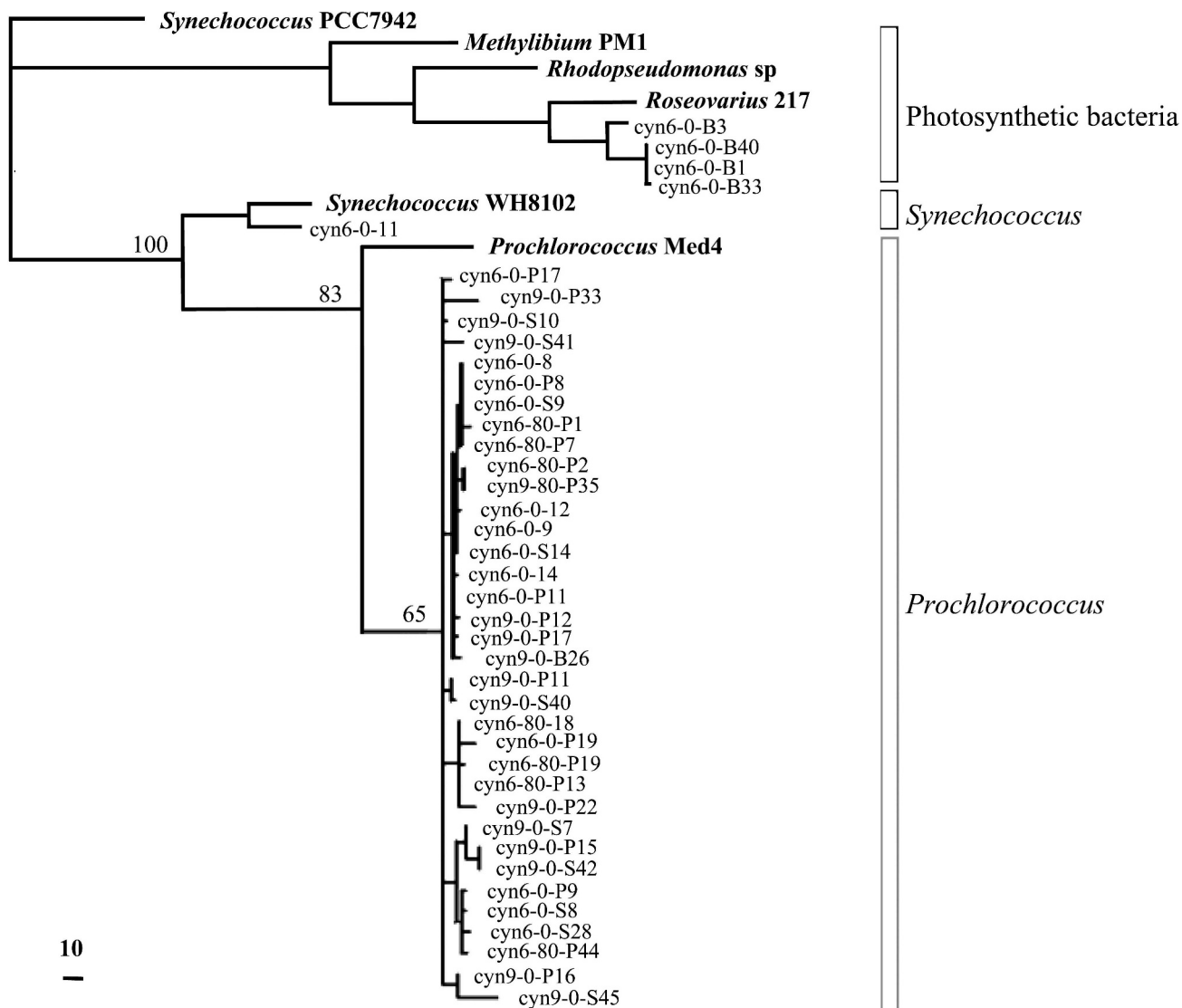


Fig. 3. Maximum likelihood analysis for environmental DNA sequences (657 nucleotides) from the Gulf of Aqaba, northern Red Sea, aligned with known sequences of bacterial *cynA*, encoding the periplasmic component of a cyanate ABC transporter. The scale bar provides a distance measure for 10 substitutions per 100 nucleotides between sequences.

0.7 mol L⁻¹) with its high inorganic carbon concentrations and significant buffering capacity. Ammonium accumulation in seawater amended with cyanate was measured over a 5-d period (Fig. 4). Cyanate produced 19.3 nmol ammonium L⁻¹ h⁻¹ at 25°C, while at the higher temperature this rate rose by more than 50% to 46.6 nmol ammonium L⁻¹ h⁻¹.

Discussion

The screening of cyanobacterial genome sequences indicates that utilization of urea, available either intracellularly or extracellularly, is a common trait among marine *Synechococcus* (exception, strain WH7803) and *Prochlorococcus* (exception, strains SS120, MIT9515, MIT9211). The operons for both urea acquisition (*urtABCDE*) and urea hydrolysis (*ureABCDEFGH*) are present in all of these strains, indicating that utilization of urea as an external N

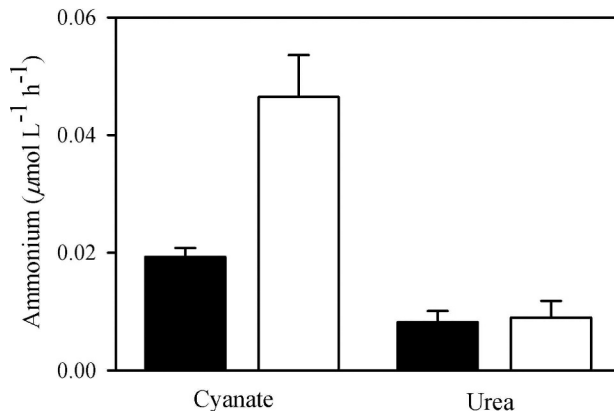


Fig. 4. The rate of ammonium accumulation resulting from spontaneous decomposition of 10 μmol L⁻¹ urea and 10 μmol L⁻¹ cyanate in sterile, particle-free seawater at 25°C (black bars) and 37°C (white bars).

source is of at least equal importance to metabolism of internally generated urea, consistent with their N physiology in culture studies (Moore et al. 2002). The reactive cyanate anion NCO^- affects a wide range of biological compounds, changing their structure, charge, and function (Stark 1972; Kraus and Kraus 1998) and leading to cell death (Guilloton and Karst 1987). The detoxifying function of cyanate lyase in *E. coli* was first reported by Taussig (1960) and was confirmed for freshwater cyanobacteria *Synechocystis* PCC 6803, *Synechococcus* PCC6301 (UTEX 625), and PCC 7942 by Miller and Espie (1994) and Harano et al. (1997). Interestingly, *cynS* (cyanate lyase) is found in all MC-A *Synechococcus* strains sequenced so far but in only three *Prochlorococcus* genomes. This indicates a higher probability of internal cyanate generation through either urea cycle activity or carbamoyl phosphate decomposition in the former group. However, the active uptake of externally available cyanate is limited to a single *Synechococcus* and *Prochlorococcus* strain.

Interestingly, *cynA* was abundantly present during summer stratification in the Gulf of Aqaba (June–September), a period of the *Prochlorococcus* blooms in N-deplete waters (Lindell and Post 1995) with minor *Synechococcus* presence. With the exception of a single *Synechococcus*-like sequence, all cyanobacterial clones clustered as *Prochlorococcus*-like sequences, but on a separate branch, away from HLI strain MED4. Although the current lack of *cynA* in HLII *Prochlorococcus* strains hinders a definite assignment, we propose that these environmental *cynA* sequences actually derive from HLII genotypes. This would imply that the HLII genomes sequenced to date may not be fully representative of the HLII types found in the Gulf of Aqaba. Based on a variety of different molecular marker genes HLII genotypes were found to be overwhelmingly abundant in the Gulf of Aqaba (Fuller et al. 2005; Lindell et al. 2005), with only a single HLI genotype ever detected (Penno et al. 2006). Our primer sets permitted amplification of *cynA* from both cyanobacteria and anoxygenic phototrophs, but not from nonphotosynthetic bacteria. Likewise, no single LL *Prochlorococcus cynA* was identified in samples taken in the deep photic zone. Routine *cynA* PCR amplification was found in summer, but never in winter. Together, our PCR approach allows detection of *cynA* only in photosynthetic organisms occupying the shallow layers of the photic zone of a stratified N-deplete water column. Primary production in such waters is routinely characterized as depending on “regenerated production” (Harrison 1992) (i.e., primary production fueled by N sources such as ammonium, urea, and potentially cyanate, derived from heterotrophic activity). It is possible that *cynA*-carrying genotypes do not rise to abundance in mixed water columns, where nitrate drives “new” primary production.

As the shallow layers of the photic zone have the highest rates of primary production, they may form a localized source of urea. Urea originates from two main sources, decomposition of dissolved organic matter and zooplankton excretion (Bidigare 1983), but its concentrations measured in the open ocean are low ($<0.3 \mu\text{mol L}^{-1}$)

(Metzler et al. 2000). We have shown that in seawater at temperatures of 25°C and higher, urea decomposes spontaneously to cyanate and ammonium, a process known to occur in other systems as well (Beswick and Harding 1987, and references therein). Moreover, at low concentrations urea tends to transform to cyanate more quickly, since the thermodynamic equilibrium is shifted to ammonium cyanate (Dirnhuber and Schütz 1948). Cyanate in the ocean is thus likely produced via chemical rather than biological routes, mainly urea dissociation. Based on low ambient urea concentrations, and taking into account a limited chemical stability of cyanate, we presume that only traces may be present in the open ocean waters. Since spontaneous decomposition of cyanate occurs at physiologically significant rates, it is not surprising that strains lacking any combination of the cyanate utilization genes do still grow on media with cyanate as the sole N source. However, we showed here that such growth is much faster in strains carrying the cyanate utilization genes. Thus, a cyanate acquisition mechanism may be of added competitive advantage to specialized *Prochlorococcus* (and, to a lesser extent, *Synechococcus*) ecotypes occupying N-deplete niches in the surface ocean. Two marine strains (*Synechococcus* WH8102 and *Prochlorococcus* MED4) possess a cyanate acquisition mechanism, and both are associated with the surface layers (Palenik et al. 2003; Rocap et al. 2003). The *cynA* gene is strongly transcribed in N-depleted *Prochlorococcus* MED4 cells (Tolonen et al. 2006). Thus, *cynABD* expression may yield a cyanate “trap” that shifts the equilibrium of urea dissociation to ammonium cyanate (Dirnhuber and Schütz 1948).

Although cyanate utilization genes were identified in cyanobacteria (Palenik et al. 2003; Rocap et al. 2003) and their involvement in N-stress responses was implied (García-Fernández et al. 2004; Tolonen et al. 2006), no ecological implications were previously assigned. We showed for the first time that cyanate can be utilized as the main N source in marine cyanobacteria. Natural abundance of *cynA* products in the Gulf of Aqaba indicates that cyanate may actually constitute an essential N source in oligotrophic ocean environments, utilized by a variety of photosynthetic organisms, but utilized much less—if at all—by nonphotosynthetic organisms. We hypothesize that in stratified ocean water bodies cyanate utilization is confined to the N-deplete, upper photic zone, where it plays a role in “regenerated” primary production. Novel analytical methods are now required to assess the occurrence and distribution of cyanate in marine environments.

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