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A Suppression Subtractive Hybridization Approach Reveals Niche-Specific Genes That May Be Involved in Predator Avoidance in Marine *Synechococcus* Isolates

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Picocyanobacteria of the genus *Synechococcus* are important contributors to marine primary production and are ubiquitous in the world's oceans. This genus is genetically diverse, and at least 10 discrete lineages or clades have been identified phylogenetically. However, little if anything is known about the genetic attributes which characterize particular lineages or are unique to specific strains. Here, we used a suppression subtractive hybridization (SSH) approach to identify strain- and clade-specific genes in two well-characterized laboratory strains, *Synechococcus* sp. strain WH8103 (clade III) and *Synechococcus* sp. strain WH7803 (clade V). Among the genes that were identified as potentially unique to each strain were genes encoding proteins that may be involved in specific predator avoidance, including a glycosyltransferase in strain WH8103 and a permease component of an ABC-type polysaccharide/polyol phosphate export system in WH7803. During this work the genome of one of these strains, WH7803, became available. This allowed assessment of the number of false-positive sequences (i.e., sequences present in the tester genome) present among the SSH-enriched sequences. We found that approximately 9% of the WH8103 sequences were potential false-positive sequences, which demonstrated that caution should be used when this technology is used to assess genomic differences in genetically similar bacterial strains.

Marine cyanobacteria of the genus *Synechococcus* are ubiquitous in the world's oceans and are major contributors to marine primary production (18, 30). This genus is genetically diverse, with at least 10 specific lineages or clades in the marine cluster A *Synechococcus* group already identified from phylogenetic analyses of 16S rRNA, internal transcribed spacer, and *rpoC1* gene sequences (13, 32, 39). It has been suggested that the successful colonization of the marine ecosystem by this genus likely reflects the adaptation of these specific lineages to particular niches (36), which gives rise to a competitive advantage for members of a lineage under a given suite of environmental conditions. Consistent with this idea is the fact that molecular ecological data obtained by using specific oligonucleotides or antibodies have shown that there are obvious differences in the relative abundance of these lineages in particular oceanic areas (7, 8, 13, 14, 40; N. J. Fuller, S. Mazard, and D. J. Scanlan, unpublished data). Furthermore, in these genetic lineages phenotypic traits, such as motility (41) and possibly chromatic adaptation in nonmotile strains (27), show phylogenetic coherence, suggesting that some traits are clade specific. Underlying these phenotypic differences are genomic differences in either single genes or gene clusters, which, combined with differential expression of common genes in strains belonging to different clades, allow specific marine *Synechococcus* lineages to adapt to different niches.

Genome sequencing has highlighted some of the underlying genomic differences that underpin the physiology of specific niche-adapted groups in the closely related genus *Prochlorococcus*, in which there are lineages that are adapted to niches in the surface or depths of a water column (12, 33), but much less is known about the more widespread genus *Synechococcus*. In order to begin to define more specifically differences at the genomic level that have allowed marine *Synechococcus* lineages to adapt to specific niches, we utilized a suppression subtractive hybridization (SSH) approach to identify genes that might be considered clade or strain specific.

SSH was developed and has been mostly used to identify genetic differences between virulent and avirulent strains of various pathogens (2, 11, 17, 29, 45), but more recently it has also been used to identify genes unique to the saxitoxin-producing cyanobacterium *Anabaena circinalis* (31), as well as to identify genetic diversity in an environmental metagenome (15). In this technique pools of genomic DNA from a bacterial strain of interest (tester) are depleted, by hybridization and PCR, by sequences that are also in a reference strain (driver). The remaining highly enriched fraction of tester-specific sequences is then cloned and sequenced. We chose this technique over, for example, DNA-DNA hybridization using a microarray, because the latter technique identifies only genes that are present in the reference genome and not genes that are absent.

In order to gauge the utility of the SSH approach for assessing genomic differences in the marine genus *Synechococcus*, *Synechococcus* strains WH8103 (clade III) and WH7803 (clade V) were used as the tester and driver (and vice versa), respectively, to identify genes "unique" to each strain. Strain WH8103 is closely related to another clade III strain, strain WH8102. The complete genome of *Synechococcus* sp. strain WH8102 was recently published (28). Hence, our SSH approach should also identify the genes that are potentially clade specific (i.e., present in both WH8103 and WH8102 but not in WH7803). *Synechococcus* sp.

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strains WH7803 and WH8103 are axenic, well-characterized laboratory strains that represent different *Synechococcus* clades (13, 44). These strains have very similar DNA base ratios, which helps rule out genomic differences due to G+C content. Furthermore, several physiological differences between these strains with regard to the composition of the light-harvesting pigments and the ability to swim or use urea have already been identified (9, 44). Together, these features should allow rigorous testing of the SSH technique for identifying genomic differences which ultimately could define niche-specific genes (i.e., genes important in open-ocean regions or in coastal or up-welling regions) in these important photoautotrophs.

MATERIALS AND METHODS

Genomic DNA isolation. Genomic DNA was isolated from *Synechococcus* sp. strains WH8103 and WH7803 by using a previously described protocol (13).

SSH. Genes unique to either *Synechococcus* sp. strain WH8103 or *Synechococcus* sp. strain WH7803 were identified by SSH using first WH8103 as the tester and WH7803 as the driver (to identify WH8103-specific sequences) and then WH7803 as the tester and WH8103 as the driver (to identify WH7803-specific sequences). SSH was performed using a CLONTECH PCR-Select bacterial genome subtraction kit (CLONTECH Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions but with the following modifications.

A combination of AluI and HaeIII was used to digest *Synechococcus* sp. strain WH8103 and *Synechococcus* sp. strain WH7803 chromosomal DNAs, which yielded fragments that were less than 1 kb long. Adaptor ligation was performed according to the kit protocol, and this was followed by addition of 1 μl T4 DNA ligase and reincubation at 15°C overnight to ensure sufficient ligation of adaptors.

To determine the efficiency of adapter ligation when *Synechococcus* sp. strain WH8103 was used as the tester, PCR primers were designed to amplify a 1,146-bp fragment of *nirA* (accession number AF065403), a gene encoding nitrate reductase (5), containing no AluI or HaeIII recognition sites. The gene-specific primers were SSHLEW8103F (5′-CGA CAT CAC CAC AAG GCA AA-3′) and SSHLEWH8103R (5′-TGA CCA ATA GTT GGG TTG CG-3′), while SSHPRIMER1 (5′-CTA ATA CGA CTC ACT ATA GGG C-3′) was the adapter-specific primer. PCRs were carried out in 25-ml mixtures containing each deoxynucleoside triphosphate at a concentration of 200 μM, 2 mM $MgCl_2$, 10 pmol of each primer, 1 μl of template (prepared as instructed in the kit manual), and 0.75 U of *Taq* polymerase in 1× enzyme buffer (Invitrogen, Carlsbad, CA). The amplification conditions comprised a denaturation step of 94°C for 3 min; 80°C for 1 min, at which time *Taq* polymerase was added; and 72°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min and a final extension for 5 min at 72°C. The reaction mixtures were stored at 4°C prior to analysis. Products (10 μl of 25 μl) were resolved by gel electrophoresis on a 1.5% (wt/vol) agarose gel at 100 V. DNA was stained with ethidium bromide (0.5 μg ml^{-1}), visualized under short-wavelength UV, and photographed using a gel documentation system (UVP Inc., Upland, CA).

When *Synechococcus* sp. strain WH7803 was used as the tester, a 307-bp fragment of *napA* (accession number AAG45172), a gene encoding nitrate permease (A. F. Post, D. Lindell, A. Moyal, S. Solomon, and Q. Wang, unpublished data), containing no AluI or HaeIII recognition sites was used to perform a ligation efficiency analysis. The primers used were SSHLEW7803F (5′-GCT TGG CGA ACT TTG GTC ATT T-3′) and SSHLEWH7803R (5′-CTG ATT GAA GTC CTG AGC AGA T-3′). PCRs were performed as described above, but the amplification conditions were 94°C for 3 min; 75°C for 1 min, at which time *Taq* polymerase was added; and 72°C for 2 min, followed by 27 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min and a final extension for 2 min at 68°C.

For the primary PCR using the adapter-specific primer SSHPRIMER1 (5′-CTA ATA CGA CTC ACT ATA GGG C-3′), PCRs were performed as described above, but the amplification conditions were 94°C for 3 min; 75°C for 1 min, at which time *Taq* polymerase was added; and 72°C for 5 min, followed by 30 cycles of 94°C for 1 min, 66°C for 1 min, and 72°C for 1.5 min.

For the nested PCR, primers SSHnestpri1 (5′-TCG AGC GGC CGC CCG GGC AGG T-3′) and SSHnestpri2 (5′-AGC GTG GTC GCG GCC GAG GT-3′) were used, and PCRs were again performed as described above, except that the template DNA was 1 μl of a 1:40 dilution of the PCR products from the primary PCR. The amplification conditions consisted of 94°C for 3 min, 75°C for 1 min, at which time *Taq* polymerase was added, and 72°C for 5 min, followed by 15 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1.5 min.

DNA sequencing. The nested PCR products were TA cloned into the pCR2.1TOPO vector, and plasmid DNA was extracted using a QIAprep Spin Miniprep kit (QIAGEN GmbH, Hilden, Germany). PCR products were sequenced bidirectionally at the Warwick University sequencing facility using Big Dye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, Calif.) and were examined with a 3100 genetic analyzer. The M13R primer (5′-CAG GAA ACA GCT ATG AC-3′), which annealed to the plasmid 74 bp upstream of the insert, was used as the sequencing primer.

Computer analysis. SSH-enriched sequences from *Synechococcus* sp. strains WH8103 and WH7803 were initially analyzed by BLASTX searches (3) against the closed WH7803 genome (F. Partensky, personal communication). Sequences derived from WH8103 were aligned with the complete genome sequence of WH7803 using Washington University BLASTN (W. Gish, 1996 to 2004; http://BLAST.wustl.edu) with gapped alignments. Sequences with more than 85% nucleotide identity to the WH7803 sequence were considered false positives and were removed from the WH8103-specific set. The rest of the specific sequences were analyzed by BLASTX against the GenBank nonredundant database (http://www.ncbi.nlm.nih.gov). Analyses of WH7803 sequences were completed with the aid of the draft annotation of the WH7803 genome based on Glimmer, Critica, and GeneMarks software predictions with manual annotation. All of the WH7803-specific sequences exhibited 100% identity with the WH7803 genome sequence.

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database under the following accession numbers: for WH8103 SSH-enriched fragments, DU635204 to DU635313 and DU635372; and for WH7803 SSH-enriched fragments, DU635093 to DU635203.

RESULTS AND DISCUSSION

Genomic subtraction between *Synechococcus* sp. strains WH8103 and WH7803. (i) Genes specific to WH8103 or to WH8103 and WH8102. Plasmid DNA was extracted from 174 white or light blue colonies obtained from the nested PCR product cloning; 60 of the clones provided insufficient sequence data or had no insert. Of the 114 sequences obtained, 10 were defined as false positives and another 25 were found to be duplicates (i.e., identical to the sequence[s] of another fragment[s]), which left 79 unique sequences for analysis (Table 1). The lengths of the fragments sequenced ranged from 45 bp to 708 bp. The WH8103 fragment sequences obtained were identified by BLASTN comparison to the complete genome sequence of the very closely related organism *Synechococcus* sp. strain WH8102 (accession number NC_005070) (28). Five fragments (79, 140, 281, 313, and 363 nucleotides) exhibited no homology with the *Synechococcus* sp. strain WH8102 genome. Either these fragments contained numerous stop codons in all reading frames and hence were likely intergenic regions in WH8103, or the open reading frames exhibited no significant similarity to sequences encoding database proteins.

The remaining fragments were initially considered to be absent from the WH7803 genome, or at least the level of homology was below the level which allowed DNA-DNA hybridization during the SSH procedure. However, the availability during this work of the complete *Synechococcus* strain WH7803 genome allowed verification by more rigorous bioinformatic analysis of the absence of these fragments in this strain. Hence, we were able to identify fragments which might be false positives arising from linear amplification of driver-derived DNA, as well as fragments which we defined as false positives because they exhibited more than 85% nucleotide identity with the WH7803 sequence (see below).

Fifty-seven of the remaining WH8103 and WH8102 enriched fragments had significant hits with gene sequences in

TABLE 1. Summary of *Synechococcus* sp. strain WH8103 SSH-enriched DNA fragments

No. of sequences	Fragment[a]	Length (bp)	Homology to predicted encoded protein[b]	e value[c]	Identity[d]	Homolog accession no.	Gene or organism
1	A001	376	Hypothetical protein	6e-36	72/72	NP_897845	SYNW1754
			Hypothetical protein	6e-09	29/29	NP_897846	SYNW1755
2	A002	708	Conserved hypothetical protein	1e-80	140/236	NP_897078	SYNW0985
	A003	597		2e-44	93/98		
1	A005	301	Putative ABC transporter, ATP binding component	3e-54[e]	100/100	NP_897205	SYNW1112
3	A006	277	Hypothetical protein	6e-06	28/63 (38/63)	NP_896979	SYNW0886
1	A010	462	Hypothetical protein	4e-61	109/110	NP_896450	SYNW0355
2	A012	274	Putative asparagine synthetase protein	2e-47	89/90	NP_898542	SYNW2453
5	A014	340	ABC transporter component, possibly zinc transport	7e-50	97/102	NP_898568	SYNW2479
	A143	437		2e-32	80/101		
	A155	317		1e-09	42/66		
	A051	339		6e-48	98/102		
1	A018[f]	174	Conserved hypothetical protein	2e-27	57/57	NP_897305	SYNW1212
7	A020[f]	338	ABC phosphate transporter, ATP binding component, PstB	2e-59	111/112	NP_897365	SYNW1270
	A077[f]	336		3e-57	110/112		
1	A022[f]	278	Conserved hypothetical protein	2e-20	47/48	NP_896965	SYNW0872
3	A024	310	Putative chromate transport protein CHR family	2e-7	21/21	NP_897416	SYNW1323
1	A025[f]	207	Glutathione *S*-transferase domain protein	5e-36	68/68	NP_897024	SYNW0931
1	A026[f]	285	Inositol monophosphate family protein	8e-16	43/44	NP_898277	SYNW2186
4	A029	292	Putative urea ABC transporter, urea binding protein	9e-55	97/97	NP_898531	SYNW2442
	A055	116		8e-17	36/37		
	A097	295		2e-29	61/81		
1	A030	228	ABC transporter, nitrate/sulfonate/bicarbonate-like substrate binding protein	1e-38	75/76	NP_897508	SYNW1415
1	A033[f]	129	Conserved hypothetical protein	2e-15	35/36	NP_896807	SYNW0714
1	A039[f]	132	FtsH ATP-dependent protease homolog	5e-18	44/44	NP_897393	SYNW1300
1	A040[f]	187	Photolyase family protein	4e-29	62/62	NP_897337	SYNW1244
1	A045[f]	136	Probable exodeoxyribonuclease V, beta subunit RecB	7c-18	43/44	NP_897010	SYNW0917
2	A046[f]	237	Putative photosystem II oxygen-evolving complex 23,000-Da protein PsbP	2e-37	77/78	NP_897020	SYNW0927
6	A048	135	SwmB repeated sequence	5e-18	42/44	NP_897046	SYNW0953
	A062	158		1e-24	52/52		
	A113	522		9e-51	65/73		
	A130	463		1e-22	59/94		
	A146	426		2e-40	89/100		
	A169	425		7e-73	140/141		
1	A049[f]	120	ABC transporter substrate binding protein, phosphate	5e-14	39/39	NP_897906	SYNW1815
1	A052	120	Carbamoyl phosphate synthase, large subunit	5e-15	39/39	NP_896923	SYNW0830
1	A059	213	Hypothetical protein	2e-9	26/26	NP_896473	SYNW0378
2	A061	58	Hypothetical protein	4e-6	18/18	NP_897910	SYNW1819
1	A065	190	Putative 4′-phosphopantetheinyl transferase	3e-17	37/40	NP_898252	SYNW2161
1	A070	371	Hypothetical protein	2e-66	121/122	NP_897657	SYNW1564
1	A075	146	Possible DnaJ domain	2e-8	34/48	NP_897323	SYNW1230
1	A078[f]	339	Putative deacetylase sulfotransferase	2e-58	107/110	NP_896178	SYNW0083
1	A079[f]	163	Cell division protein FtsH4	2e-17	46/54	NP_897304	SYNW1211
3	A080	100	Putative ATPase, AAA family	3e-14	31/33	NP_898249	SYNW2158
1	A088[f]	172	Glutathione peroxidase	2e-9	32/33	NP_896194	SYNW0099
2	A094	352	Hypothetical protein	9e-26	48/48	NP_896484	SYNW0389
	A095	300		1e-22	48/48		
1	A099[f]	237	Apocytochrome b6	8e-11	35/48	NP_892444	PMT1649
1	A106	409	Hypothetical protein	2e-10	18/22	NP_897994	SYNW1903
1	A118	83	Porin homolog	5e-9	27/27	NP_898317	SYNW2228
3	A121	103	Adenylosuccinate synthase	2e-9	31/33	NP_897864	SYNW1773
1	A135[f]	108	Possible ferredoxin	2e-13	35/35	NP_897859	SYNW1768
1	A136	381	COG2089; sialic acid synthase	3e-8	27/47	ZP_00128881	*Desulfovibrio desulfuricans* G20
1	A141	176	Hypothetical protein	4e-19	30/36	NP_898389	SYNW2300
1	A144	211	Possible glycosyltransferase	1e-31	55/69	NP_896511	SYNW0416
2	A150	445	Hypothetical protein	3e-40	82/83	NP_897748	SYNW1655
	A166	283		9e-48	90/93		
1	A171	112	Putative succinate dehydrogenase flavoprotein subunit	5e-17	35/36	NP_896684	SYNW0591

[a] For A004, A009, A021, A023, A027, A028, A035, A036, A042, A050, A056, A063, A091, A098, A102, A103, A105, A114, A115, A129, A134, and A148 the e value was >0.005 or the level of identity was <50% and there were no significant hits.

[b] Where it is known, the product of the gene is indicated. The products of genes without a known product were designated conserved hypothetical if they had a homolog(s) in a different organism(s) or hypothetical if they had no homolog.

[c] The e value indicates the probability of the match. A match with an e value of <0.05 or >50% identity over the entire length of the sequence was considered significant.

[d] Where the level of identity was <50% but there was significant similarity, the percentages of positives are indicated in parentheses.

[e] The e value for WH7803 was 9.9e-36, but since the fragment encodes an ABC transporter, we considered this difference significant in this gene category.

[f] False positives were defined as sequences with >85% nucleotide identity with WH7803 as determined by WU-BLASTN with gapped alignment (Gish, 1996 to 2004; http://blast.wustl.eduref).

the database, and 55 of the hits were best hits in WH8102. Another 22 sequences had no significant hits when tBLASTX was used, indicating that they were derived from intergenic spacer regions or were entirely novel sequences. Thus, the majority of the differences found correspond to genes rather than noncoding DNA. The WH8103 and WH8102 enriched fragments were found to be evenly distributed around the *Synechococcus* sp. strain WH8102 genome, as shown in Fig. 1.

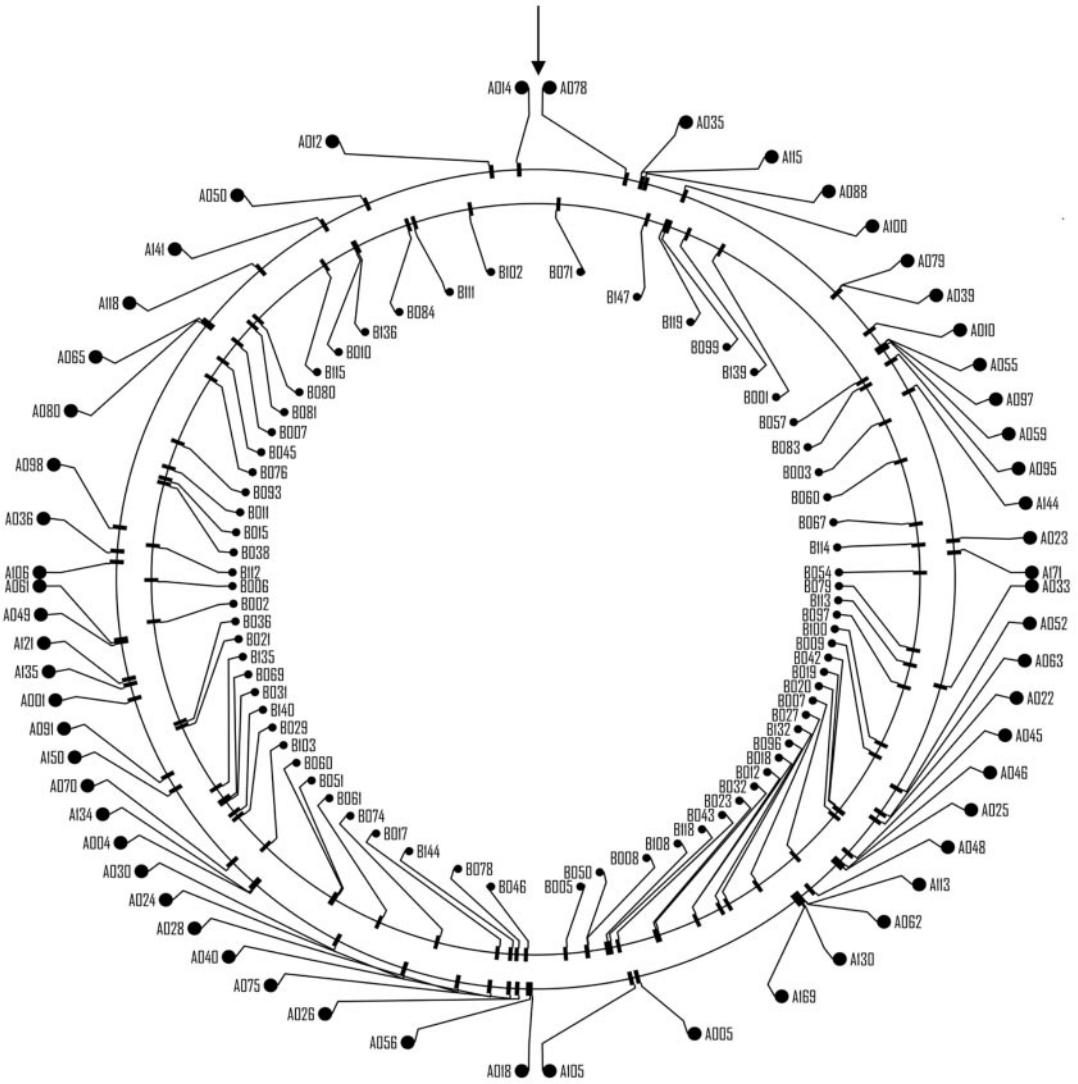

FIG. 1. Genomic distribution of SSH-enriched DNA fragments. SSH-enriched DNA sequences from *Synechococcus* sp. strain WH8103 were mapped on the complete genome of *Synechococcus* sp. strain WH8102 (outer circle), while SSH-enriched sequences from *Synechococcus* sp. strain WH7803 were mapped on the complete genome sequence of this strain (inner circle). The origin of both sequences is indicated by an arrow.

The genes to which the fragments correspond are shown in Table 1. Several of the DNA fragments exhibited homology with the sequence encoding the ABC urea transporter, SYNW2442 (fragments A029, A055, and A097), or the sequence encoding SwmB (fragments A048, A062, A113, A130, A146, and A169); the former is required for high-affinity urea transport (42), and the latter is required for swimming motility (20). Both of these physiological traits (i.e., urea utilization and swimming motility) are known to be absent in WH7803 (9, 41, 44). SYNW2442 appears to be a homolog of *urtA*, a component of the *urtABCDE* gene cluster, and hence this component of the high-affinity urea transport system can be added to the urease structural genes as missing genes required for urea utilization by this strain. The genes for several other potential ABC transporter components appear in these WH8103- and WH8102-specific sequences, including the gene encoding a potential nitrate-like substrate binding protein for an ABC transporter, SYNW1415. The presence of such a gene is interesting given the fact that WH8102 contains a reported deletion after nucleotide position 617 (resulting in a frameshift that produces a stop codon 26 codons downstream [see EMBL entry CAE08977]) in the *napA* (or *nrtP*) gene encoding a nitrate permease belonging to the major facilitator superfamily (5, 35, 43). Certainly, in WH7803 the *napA* product seems to be the sole nitrate transporter, since interposon mutagenesis of the gene revealed a strain that could not grow on nitrate (A. F. Post, D. Lindell, A. Moyal, S. Solomon, and Q. Wang, submitted for publication). This leads to speculation about whether nitrate uptake by the *napA* product is a functional system in *Synechococcus* sp. strain WH8102 and whether the SYNW1415 product is part of a functional nitrate transport system together with other ABC transporter components encoded by SYNW1416 and SYNW1417 adjacent to SYNW1415 on the genome. Cer-

tainly, thus far, nitrate transport via an ABC-type system, encoded by the *nrtABCD* genes, appears to be restricted to freshwater cyanobacteria (24, 25).

Glycosyltransferases are involved in carbohydrate modification of the cell envelope, and it has been suggested that they may be required for construction of the swimming motility apparatus in marine *Synechococcus*, since at least one of the components of this apparatus is glycosylated (6). The presence of a WH8103- and WH8102-specific fragment encoding a putative glycosyltransferase (SYNW0416) (Table 1) may support the hypothesis that this glycosyltransferase is involved in construction of the motility apparatus (28); alternatively, the enzyme may allow these organisms to modify their cell surface characteristics to help evade grazers and other predators, such as phage (28). Certainly, freshwater *Synechococcus* strains containing a highly glycosylated, paracrystalline surface layer have been shown to be ingested at lower rates than strains lacking this structure are ingested (23), and the same may be true in marine environments (22).

Succinate dehydrogenase (EC 1.3.99.1), encoded by *sdhA* (SYNW0591), a citric acid cycle enzyme, catalyzes the conversion between succinate and fumarate. It has been known for a long time that cyanobacteria have an incomplete citric acid cycle and lack α-ketoglutarate dehydrogenase (37). Interestingly, several other central carbon metabolism genes appear to be missing entirely in marine *Synechococcus* and *Prochlorococcus* genomes; these genes include genes encoding malate dehydrogenase, glucose-1-phosphate dehydrogenase, 6-phosphofructokinase, succinyl coenzyme A synthetase, and NAD- and NADP-dependent alcohol dehydrogenases (16). Succinate dehydrogenase appears to be an exception to this, however; the data presented here show that this enzyme is present in *Synechococcus* sp. strains WH8103 and WH8102 and *Prochlorococcus* spp. strains MIT9313 and SS120 but is not present in *Synechococcus* sp. strain WH7803 and *Prochlorococcus* sp. strain MED4.

Asparagine synthetase (EC 6.3.5.4), encoded by *asnB* (SYNW2453), is responsible for the following reaction, which results in the synthesis of asparagine: ATP + L-aspartate + L-glutamine → AMP + diphosphate + L-asparagine + L-glutamate. Several bacteria have been shown to possess a tRNA-dependent transamidation pathway in which aspartyl-tRNA^{Asn} is converted to asparaginyl-tRNA^{Asn} (4, 10). It has been demonstrated that in *Deinococcus radiodurans* (which lacks asparagine synthetase) this tRNA-dependent transamidation pathway is the sole route for asparagine synthesis (21). Roy et al. (34) have proposed that a truncated archaeal asparaginyl-tRNA synthetase that is responsible for transamidation of aspartyl-tRNA^{Asn} was introduced into bacteria via lateral gene transfer, where it became the evolutionary ancestor of bacterial asparagine synthetase. The presence of *asnB* (encoding asparagine synthetase) in *Synechococcus* sp. strain WH8103 but not in strain WH7803 suggests that *Synechococcus* sp. strain WH7803 is forced to rely on a tRNA-dependent transamidation pathway for the synthesis of asparagine, whereas *Synechococcus* sp. strain WH8103 possesses an alternative tRNA-independent pathway for asparagine synthesis.

(ii) Genes specific to WH7803. A total of 150 colonies were cloned into the pCR2.1TOPO vector, and 37 of the clones gave insufficient sequence data or had no insert. Of the remaining 113 sequences, 27 were found to be duplicates (i.e., they had sequences identical to the sequence[s] of another fragment[s]), which left 86 sequences for analysis (Table 2). The lengths of the fragments sequenced ranged from 114 bp to 897 bp. These sequences were identified by BLASTX comparison to the nonredundant database, as well as to the recently derived *Synechococcus* sp. strain WH7803 genome sequence (A. Dufresne, M. Ostrowski, P. Wincker, D. J. Scanlan, and F. Partensky, unpublished data). Forty of these sequences had significant hits to gene sequences in the database, and 20 had best hits to WH8102. These *Synechococcus* sp. strain WH7803-specific fragments were found to be evenly distributed around the genome (Fig. 1), suggesting that there are not distinct "islands" of genomic differences for both organisms. Similarly, only two of the fragments were found to consist of only noncoding sequences, while 14 fragments were a mixture of noncoding sequences and coding sequences and 35 fragments consisted of only coding sequences. Thus, the vast majority of the differences found corresponded to genes rather than to noncoding DNA. Many of the unique genes encode hypothetical or conserved hypothetical proteins, while 13 have an assigned function (Table 2). Among the "identifiable" strain-specific genes are genes that may be involved in deterring grazing or phage infection. For example, the fact that fragment B139 encodes a permease component of an ABC-type polysaccharide/polyol phosphate export system suggests that WH7803 may produce an extracellular polysaccharide, although this extracellular polysaccharide as well as being unpalatable to grazers may also be useful as a sink for excess carbon under nitrogen limitation conditions (26); alternatively, one of the fragments, fragment B114, potentially encodes a component of an ABC-type bacteriocin/antibiotic exporter, which may be important in alleviating bacterial competition via production of an antibacterial compound.

(iii) False positives. SSH-enriched fragments of *Synechococcus* sp. strain WH8103 contained no sequences that were false positives resulting from the linear amplification of driver-derived DNA. However, potential false positives that met the criterion of exhibiting more than 85% nucleotide identity with WH7803 (WH8103 enriched) sequences occurred at a rate of 9% of the total number of sequences. Without the complete genome sequence of WH8103 it is not possible to determine the number of false positives in the SSH-enriched WH7803 sequences. However, if we consider that SSH sequences conserved in at least five sequenced marine cyanobacterial genomes (MED4, SS120, MIT9313, WH8102, and WH7803) are potentially false positives, then there would be 15 candidates, a rate of 13% of the total (Table 2). Two examples are the clock gene *kaiC* (B006) and the ABC transporter component gene *pstB* (A020, A077), which appear to be present in all marine picocyanobacterial genomes analyzed so far. This apparently high number of false positives may simply reflect the criteria described above (i.e., the stringency used for determining matches and the assumption that a sequence conserved in at least five marine cyanobacterial genomes should also be conserved in WH8103). Alternatively, they may be a result of the fact that even small stretches of unique sequence within a fragment prevent suppression of common genes. Examples of the latter are likely the WH7803 SSH sequences derived from a possible porin gene (B007) and a periplasmic phosphate binding protein gene, *sphX*

TABLE 2. Summary of *Synechococcus* sp. strain WH7803 SSH-enriched DNA fragments

No. of sequences	Fragment[a]	Length (bp)	Homology to predicted encoded protein[b]	e value[c]	Identity[d]	Homolog accession no.	Gene or organism
3	B001	299	Rod shape determining protein	9e-12	36/38	NP_896217	SYNW0122
1	B003[e]	155	tRNA pseudouridine synthetase A	1e-10	30/44	NP_895584	PMT1757
4	B005[e]	143	Cobalamin biosynthetic protein CobN	3e-10	29/45	NP_894559	PMT0727
1	B006[e]	202	KaiC	6e-25	56/60	NP_896645	SYNW0550
3	B007	185	Possible porin	2e-16	35/58	NP_898219	SYNW2128
3	B008	196	Hypothetical protein	3e-9	30/54	NP_897352	SYNW1259
3	B010	160	Conserved hypothetical protein	2e-19	44/53	NP_894988	PMT1158
3	B012[e]	293	Putative methionyl-tRNA synthetase	2e-28	63/89	NP_894575	PMT0743
2	B017	250	Penicillin binding protein	2e-15	40/75	ZP_00165017	*S. elongatus* PCC7942
1	B022	225	Periplasmic phosphate binding protein SphX	2e-23	40/60	NP_897379	SYNW1286
2	B031	395	Possible neuromiedin U	3e-19	42/61	NP_895341	PMT1514
2	B036	450	Hypothetical protein	8e-18	49/119 (72/119)	NP_894091	PMT0258
2	B043[e]	169	Deoxyxylulose-5-phosphate synthase	8e-24	54/56	NP_875320	Pro0928
	B055	191		1e-23	54/56		
2	B045	166	Hypothetical protein	2e-17	44/47	NP_898287	SYNW2196
1	B050	169	Hypothetical protein	2e-11	34/49	NP_897352	SYNW1259
1	B051[e]	227	Diaminopimelate decarboxylase	2e-28	60/75	NP_897029	SYNW0936
1	B054[e]	210	Possible cystathione gamma-synthase	8e-08	26/32	NP_894058	PMT0225
1	B056	516	Glycyl-tRNA synthetase beta chain	1e-5	25/31	NP_924529	*Gloeobacter violaceus*
1	B057[e]	314	Conserved hypothetical protein	6e-49	91/99	NP_898241	SYNW2150
1	B058[e]	412	Conserved hypothetical protein	1e-12	40/80	NP_896449	SYNW0354
1	B060[e]	315	Coproporphyrinogen III oxidase	1e-41	73/85	NP_898131	SYNW2040
1	B067	312	Formamidopyrimidine-DNA glycolase	2e-32	61/94	NP_894019	PMT0186
1	B075	406	Hypothetical protein	5e-15	45/77	AAZ57900	PMN2A_0408
1	B076[e]	578	Conserved hypothetical protein	7e-27	60/100	NP_898274	SYNW2183
2	B079[e]	392	Putative glucosyl-glycerol-phosphate synthase	9e-27	54/67	NP_897374	SYNW1281
1	B080	333	Septum formation inhibitor-activating ATPase	2e-22	48/79	ZP_00108431	*Nostoc punctiforme*
2	B081	495	Catabolite gene activator and regulatory subunit of cAMP-dependent protein kinase	2e-26	42/85	ZP_00163669	*S. elongatus* PCC7942
1	B084	367	Small mechanosensitive ion channel, MscS family	1e-34	70/110	NP_895901	PMT2077
1	B096[e]	145	Putative modulator of DNA gyrase; TldD	8e-17	43/46	NP_897290	SYNW1197
1	B097	441	Conserved hypothetical protein	4e-19	45/58	NP_897620	SYNW1527
1	B102[e]	171	DAHP synthetase class I	1e-23	53/57	NP_896072	PMT2288
3	B103[e]	261	Putative ferredoxin like protein	1e-10	25/39	NP_896790	SYNW0697
1	B108	204	Putative glucosyl-glycerol-phosphate synthase	2e-27	54/67	NP_897374	SYNW1281
1	B110	159	Hypothetical protein	1e-18	44/52	NP_897620	SYNW1527
1	B112	140	Putative CaCA family calcium/proton exchanger	5e-10	30/35	NP_896602	SYNW0507
1	B113	415	Putative CO_2 hydration protein ChpX	2e-67	121/138	NP_897800	SYNW1709
1	B114	395	ABC-type bacteriocin/lantibiotic exporters, contain an N-terminal double-glycine peptidase domain	2e-8	27/80 (46/80)	ZP_00110567	*Nostoc punctiforme* PCC 731
1	B115	225	Hypothetical protein	2e-8	28/49	AAQ87420	*Rhizobium* sp. strain NGR234
1	B118[e]	292	ABC transporter, likely for sugar transport	2e-18	43/56	NP_894524	PMT0692
1	B139	310	ABC-type polysaccharide/polyol phosphate export systems, permease component	4e-07	53/102	ZP_00264217	*Pseudomonas fluorescens* PfO-1

[a] For B002, B009, B011, B014, B015, B021, B023, B025, B026, B027, B028, B029, B033, B035, B038, B039, B041, B044, B046, B048, B053, B059, B061, B065, B066, B069, B071, B072, B074, B082, B083, B090, B093, B099, B101, B107, B110, B111, B132, B135, B136, B140, B144, B147, and B149 the e value was >0.005 or the level of identity was <50% and there were no significant hits (intergenic spacer or unique sequence).

[b] Where it is known, the product of the gene is indicated. The products of genes without a known product were designated conserved hypothetical if they had a homolog(s) in a different organism(s) or hypothetical if they had no homolog.

[c] The e value indicates the probability of the match. A match with an e value of <0.05 and >50% identity over the entire length of the sequence was considered significant.

[d] Where the level of identity was <50% but there was significant similarity, the percentages of positives are indicated in parentheses.

[e] Potential false positives were defined as an SSH sequence conserved in at least five sequenced marine cyanobacterial genomes (MED4, SS120, MIT9313, WH8102, and WH7803), using an e value of <0.05 and a level of identity of >50% at the nucleotide level.

(B022). Multiple copies of orthologues of each of these genes are maintained and randomly distributed throughout the genomes of sequenced cyanobacteria, including WH7803. Given that identification of false-positive sequences is critical for correct identification of clade- or strain-specific genes, in future SSH experiments workers should attempt to minimize these types of sequences. A potential approach to do this is to construct SSH libraries generated from a wider variety of restriction endonucleases than the restriction endonucleases used here (1) and to sequence a larger number of clones. With these

modifications unique SSH sequences representing truly unique genes would be returned with a higher frequency than false positives and sequences generated from short regions with low levels of identity.

Conclusions. We describe here identification of several interesting candidate genes that may represent clade-specific characteristics that allow occupation of a specific environmental niche by a particular marine *Synechococcus* lineage. Although we are cautious about the existence of a plethora of clade-specific traits, it would not be unexpected that some specific physiological traits are shared by clades, given that it is the total suite of traits that correlates with the environmental niche occupied.

Certainly, it is interesting that genes potentially related to predator avoidance have been identified using the SSH approach, and each marine *Synechococcus* strain apparently has distinct genetic predator avoidance capabilities. It is known that resistance to protist grazing can be influenced by a variety of factors, such as high motility, reduced or increased size, and cell surface masking (for a review see reference 19), while modification of the cell surface is also clearly important with respect to cyanophage infection. Hence, it is not unreasonable to suggest that the high numbers of cyanophage in marine waters (38) and also potentially the grazers themselves are involved in a "biological arms race" that results in strong selective pressures for the host cells to evolve specific mechanisms that make them resistant to predation.

Clearly, the SSH approach used here is a cheap and relatively fast approach to identify strain-specific genes in the marine genus *Synechococcus*. These organisms differ from most pathogenic organisms that have been targeted previously with the SSH approach because they lack defined "islands" of genomic differences. Certainly, the strain-specific sequences could augment information contained in microarrays, expanding the scope of gene content or gene expression analysis and allowing multistrain chips to be developed. Clearly, strain variation also provides valuable insights into evolutionary processes, while finding sequences that are variable but common in strains should facilitate more precise and reliable taxonomy beyond the 16S rRNA gene nomenclature, as has been initiated using *rpoC1* (39). Finally, mutagenesis studies (for an example, see reference 20) of the many conserved hypothetical genes and hypothetical genes identified here as strain specific should lead to a more fundamental understanding of the processes involved in niche adaptation.

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