

The Ocean as a Global Reservoir of Antibiotic Resistance Genes

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Recent studies of natural environments have revealed vast genetic reservoirs of antibiotic resistance (AR) genes. Soil bacteria and human pathogens share AR genes, and AR genes have been discovered in a variety of habitats. However, there is little knowledge about the presence and diversity of AR genes in marine environments and which organisms host AR genes. To address this, we identified the diversity of genes conferring resistance to ampicillin, tetracycline, nitrofurantoin, and sulfadimethoxine in diverse marine environments using functional metagenomics (the cloning and screening of random DNA fragments). Marine environments were host to a diversity of AR-conferring genes. Antibiotic-resistant clones were found at all sites, with 28% of the genes identified as known AR genes (encoding beta-lactamases, bicyclomycin resistance pumps, etc.). However, the majority of AR genes were not previously classified as such but had products similar to proteins such as transport pumps, oxidoreductases, and hydrolases. Furthermore, 44% of the genes conferring antibiotic resistance were found in abundant marine taxa (e.g., *Pelagibacter*, *Prochlorococcus*, and *Vibrio*). Therefore, we uncovered a previously unknown diversity of genes that conferred an AR phenotype among marine environments, which makes the ocean a global reservoir of both clinically relevant and potentially novel AR genes.

The spread of antibiotic resistance (AR) is critically important to human health. Past research has focused on resistance in clinical environments (e.g., hospitals), but the rise of community-acquired infections of resistant bacteria has fueled interest in AR genes in natural environments (1–3). Natural environments can be important, as they can act as reservoirs of AR genes (1, 2). Such environments include soils (4, 5), glaciers (6), and animals (7–9). Additionally, the frequency of AR in human hosts is also higher than previously thought (10, 11), and the AR genes found in soil bacteria have also been found in clinical pathogens (2). One set of environments that has received little attention is marine environments. Oceans are dilute systems, and hence, there may be little selection for antibiotic production, as compounds can rapidly diffuse away from the producer (12).

However, there are three possible mechanisms that can lead to the occurrence of AR in marine environments. One is through coastal runoff of AR bacteria from terrestrial sources. In this case, we expect to find AR genes in bacterial taxa nonnative to marine environments. The second mechanism is through selection for AR due to anthropogenic antibiotic runoff, which challenges native bacteria to become resistant. The third is selection for resistance in response to antibiotic production in marine environments. Antagonistic microbial interactions can occur on marine snow (13) or in small parcels of seawater (14, 15). These interactions may include the production of antibiotics and subsequent selection for resistance.

Despite the large expanse of the oceans, we currently have little understanding of the presence, diversity of organisms, or types of genes responsible for AR in the marine environment. To address this limitation in our understanding of AR in natural environments, and to test the two mechanisms above, we used functional metagenomics (i.e., the cloning and functional screening of DNA fragments from communities) to identify genes conferring resistance to specific antibiotics in marine waters. We specifically asked the following: (i) what is the frequency of resistance to different antibiotics in specific marine environments, (ii) what is the diver-

sity of marine AR genes, and (iii) are these genes harbored by marine bacteria?

MATERIALS AND METHODS

Sample collection. Two replicate seawater samples were collected from five sample sites: Agua Hedionda Lagoon (21 December 2010) (33°8′44.1″N, 117°20′35.8″W), which contains an aquaculture facility; Newport Bay (5 April 2011) (33°37′ 29.8″N, 117°53′35.2″W), a natural bay that has freshwater influence from San Diego Creek and Delhi Channel; Los Angeles (LA) Harbor (21 January 2012) (33°42′37.0″N, 118°15′23.5″W); the San Pedro Ocean Time Series (21 January 2012) (33°33′ 00″N, 118°24′00″W), an open-ocean site that has coastal influence; and the Hawaii Ocean Time Series (HOT) (15 June 2013) (22°45′ 00″N, 158°00′00″W), which is an open-ocean site. These locations were chosen because they represented a range of marine environments with different proximities to the coast. Eight to 16 liters of seawater was collected in replicate and prefiltered through 2.7- μ m glass microfiber filters (Whatman GF/D, Pittsburgh, PA) and then collected on 0.22- μ m polyestersulfone Sterivex filters (Millipore, Billerica, MA).

DNA was extracted from the Sterivex filters using a protocol modified from that of Boström and colleagues (16). We added 1,620 μ l of Tris-EDTA-sucrose buffer to each filter and froze the filters for at least 24 h. The filters were then thawed, 180 μ l of lysozyme buffer was added, and the filters were incubated at 37°C for 30 min. We then added 180 μ l of proteinase K and 100 μ l of sodium dodecyl sulfate and incubated the filters at 55°C overnight. Sodium acetate and cold isopropanol were added to precipitate macromolecules, and the solution was left at –20°C for at least

Received 4 March 2015 Accepted 20 August 2015

Accepted manuscript posted online 21 August 2015

Citation Hatosy SM, Martiny AC. 2015. The ocean as a global reservoir of antibiotic resistance genes. *Appl Environ Microbiol* 81:7593–7599. doi:10.1128/AEM.00736-15.

Editor: H. Nojiri

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00736-15>.

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1 h. The precipitate was pelleted by centrifugation at $15,000 \times g$ for 30 min. The supernatant was decanted and the pellet was resuspended in Tris buffer using a 37°C water bath for 30 min. DNA was then purified using a genomic DNA Clean and Concentrator kit (Zymo Corp., Irvine, CA).

Library construction. At least 2 μg of DNA from each replicate was sonically sheared to 3 kb using a S2 Focused Acoustic Shearer (Covaris Inc., Woburn, MA). Fragments of 2 to 4 kb were gel extracted using a Zymo gel extraction kit (Zymo Corp.). DNA was treated with an End-It end repair kit (Epicentre, Madison, WI) to create blunt ends on the DNA fragments. Fragments were then ligated into the pZE-21 plasmid (11, 17) using a Fast-Link ligation kit (Epicentre, Madison, WI). The ligation reaction was then purified using a DNA Clean and Concentrator-5 kit (Zymo Corp.). We added 4 μl of ligation buffer to 50 μl of Lucigen (Middleton, WI) electrocompetent Ecloni cells in a 2-mm electroporation cuvette. The cells were electroporated at 1,800 V, 250 Ω , and 50 μF and immediately transferred to 975 μl of recovery medium (Lucigen). Transformed cells were allowed to recover at 37°C for 1 h. After 1 h, the cultures were diluted to 1:10 and 1:100, and 1 μl of each was plated on a plate containing LB plus kanamycin (50 $\mu\text{g}/\text{ml}$) and incubated overnight to determine the initial titer. Three milliliters of LB and 50 $\mu\text{g}/\text{ml}$ of kanamycin were added to the cultures and incubated at 37°C for 1.5 h. These cultures were diluted 1:100, and 1 μl was plated on LB plus kanamycin (50 $\mu\text{g}/\text{ml}$) and incubated overnight at 37°C to determine the titer of successful transformants. Libraries were then preserved with 10% glycerol and stored at -80°C .

Approximately 10^6 cells in glycerol stock were plated on LB plates with antibiotics. The antibiotic concentrations used inhibited growth of Ecloni cells transformed with empty pZE-21 plasmid and included ampicillin (60 $\mu\text{g}/\text{ml}$) (a semisynthetic antibiotic that arrests cell wall synthesis), tetracycline (8 $\mu\text{g}/\text{ml}$) (a naturally produced antibiotic that inhibits protein synthesis), sulfadimethoxine (700 $\mu\text{g}/\text{ml}$) (a synthetic antibiotic that inhibits folic acid synthesis), or nitrofurantoin (5 $\mu\text{g}/\text{ml}$) (a synthetic antibiotic that damages intracellular macromolecules). Antibiotic-resistant clones were picked and grown in 200 μl of LB plus kanamycin (50 $\mu\text{g}/\text{ml}$) in a 96-well plate overnight at 37°C. Plates were sent for Sanger sequencing to Beckman Coulter, where approximately 800 nucleotides were sequenced from each end of the insert.

Identification of antibiotic resistance genes. We compared sequences to the Antibiotic Resistance Genes Database (ARDB) (18) and the nonredundant (NR) database from GenBank using BLAST. Nucleotide sequences were compared to an amino acid database (blastx) using the BLOSUM62 substitution matrix and an E value cutoff of 10. Genes that were previously classified as AR genes from either the ARDB or GenBank were labeled as known AR genes. Genes that were not known AR genes were classified as previously unclassified AR genes. In addition, we attempted to assemble overlapping regions of paired-end sequences to form contiguous sequences.

Identifying marine versus nonmarine taxa. We compared sequences to the NR database from NCBI using BLAST. We then took the top hit for each sequence and searched the taxa against the EnvDB (19). In order to differentiate between bacteria that were transported through effluent (nonmarine) and native marine bacteria (marine), records for each taxon were then identified as either marine or nonmarine. A record was considered marine if the environment of that record was saline water, saline sediment, marine host, freshwater-saline water interface, soil-saline water interface, or hydrothermal. If more than 50% of the environmental records were marine, then that taxon was classified as marine. The taxon was labeled as unknown if the identification was broad (e.g., *Alphaproteobacteria*). We also determined the functional type of protein from these hits (e.g., oxidoreductase or transporter). Using the top hit from GenBank, we searched UniProt for molecular functions. If the UniProt record identified an Enzyme Commission number or a molecular function (transport, DNA binding, etc.), we assigned that function to the gene. If there was no Enzyme Commission number or molecular function, we identified that type as unknown.

Strain genomic libraries. Control libraries were constructed similarly to the functional metagenomic libraries described above. Cultures of antibiotic-sensitive *Escherichia coli* and *Synechococcus* strain WH8102 were grown to a density of 10^6 cells ml^{-1} and syringe filtered through a Sterivex filter. DNA was extracted and clone libraries were constructed as described above for the seawater samples. Clones resistant to ampicillin, tetracycline, and nitrofurantoin were paired-end sequenced at Beckman Coulter.

Statistical analyses. The number of sequenced genes found at each location was resampled to obtain rarefaction curves at each environment. The number of resistant positive clones for each site and antibiotic was normalized to the number of transformants for that site and antibiotic to obtain the frequency of positive clones. Differences in the frequency of positive clones, the frequency of known AR genes, and the frequency of marine taxa between locations and between antibiotics were calculated using the Kruskal-Wallis test in the R core package (20). Differences in composition of known AR genes and unclassified AR genes between locations and antibiotics were calculated using permutational analysis of variance (ANOVA) from the vegan package in R (21) using 999 permutations.

Nucleotide sequence accession numbers. Insert sequences reported in this article were deposited in GenBank under accession numbers [KS307228](#) to [KS308058](#).

RESULTS

In order to quantify the extent and diversity of AR genes in marine environments, we applied functional metagenomics to screen DNA from five marine sites against four antibiotics (ampicillin, tetracycline, sulfadimethoxine, and nitrofurantoin), which differed in their modes of activity (see Table S1 in the supplemental material). The average length of paired-end sequences was 772 nucleotides, and no contiguous sequences could be assembled. We found resistant clones at all sites with mean frequencies ranging from 1.6×10^{-6} to 8.7×10^{-5} AR positives per transformant (Fig. 1 and Table 1; see also Table S2). Environments significantly differed in their frequencies ($P = 0.004$), with Los Angeles (LA) Harbor having the highest average frequency of resistant clones (8.7×10^{-5}) (Fig. 1; see also Table S2). The environment with the second highest frequency of resistant clones was the open-ocean site at the Hawaiian Ocean Time Series (HOT) (7.2×10^{-5}), and the lowest frequency was observed at the Agua Hedionda Lagoon, Carlsbad, CA (1.6×10^{-6}).

Frequencies of resistance also varied between antibiotics ($P = 0.03$) (Fig. 1; see also Table S2). Nitrofurantoin had the highest frequency of resistant clones (mean = 9.7×10^{-5} per transformant), while sulfadimethoxine had the lowest frequency (mean = 9.4×10^{-6} per transformant). This is noteworthy, as both sulfadimethoxine and nitrofurantoin are fully synthetic antibiotics and thus not produced by microorganisms.

We next examined the diversity of resistance genes. We divided the genes into two main categories: previously known and unclassified AR genes (Fig. 2; see also Table S3 in the supplemental material). The sites did not differ significantly in their overall frequencies of known AR gene types ($P = 0.1$) (see Table S2). However, sites did vary in the composition of known AR genes based on a permutational ANOVA ($P = 0.01$) (see Table S2), so they each contained a different set of AR genes. For instance, the Agua Hedionda lagoon was characterized by an abundance of bicyclomycin pumps (*bcr*), while the open-ocean HOT had mostly TEM1 beta-lactamases (*bl2b_tem1*). In addition, the frequency of known AR genes did not differ by antibiotic ($P = 0.08$), but there was a difference in the composition of known AR genes for each antibiotic ($P = 0.009$) (Fig. 2; see also Table S2). Tetracycline

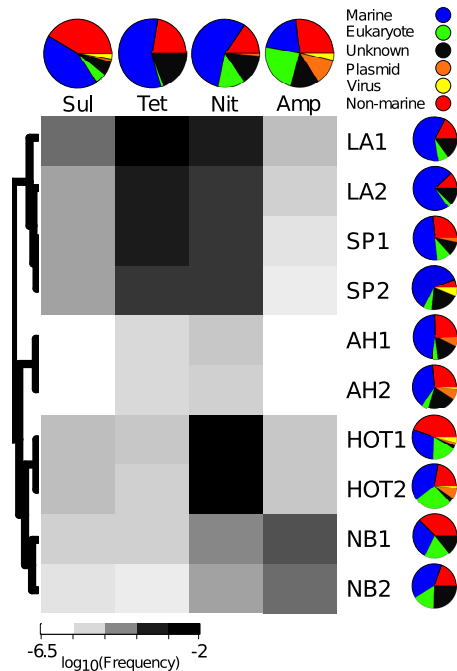


FIG 1 Frequencies of antibiotic-resistant clones across samples and antibiotics. White squares represent low frequency, and darker squares represent higher frequency. Rows represent sample sites: Los Angeles Harbor (LA), San Pedro Channel (SP), Hawaii Ocean Time Series (HOT), Newport Bay (NB), and Agua Hedionda Lagoon (AH). Numbers next to sample sites represent replicate samples. Columns represent antibiotics on which clones were screened: sulfadimethoxine (Sul), tetracycline (Tet), nitrofurantoin (Nit), and ampicillin (Amp). “Unknown” taxa represent bacterial sequences that could not be assigned as marine or nonmarine because the taxonomic designation was too broad (e.g., *Alphaproteobacteria*).

screens had a high frequency of bicyclomycin pump genes (69%), followed by the tetracycline efflux pump gene *tetA1* (11%). Ampicillin screens mostly contained beta-lactamase genes, of which the majority was TEM1 (bl2b_tem1) (65%). Other beta-lactamases were present, but at lower frequencies (2.5 to 3% per gene). There were few known AR genes isolated on the synthetic antibiotics, sulfadimethoxine and nitrofurantoin (Fig. 2). Those isolated on sulfadimethoxine included the genes for target-modified dihydropterate synthase (*sul1*, *sul2*, and *sul3*) and target-modified dihydrofolate reductase (*dhfrA24*). Three types of known resistance genes were found on nitrofurantoin: the ABC transporter gene *bcrA*, the MFS transporter gene *rosB*, and the penicillin binding protein gene *pbp2*. Each of these genes represented 33% of the known AR genes found on nitrofurantoin. Known genes made up 28% of the sequenced clones, and their products ranged between 10 and 100% in amino acid similarity to those of other AR genes (Fig. 2B). Of the known AR genes, the majority were identified as multidrug efflux pump genes (*bcr*; 36%) or beta-lactamase genes (bl2b_tem1; 29%) (Fig. 2A). TEM1 beta-lactamase (bl2b_tem1) shared the highest similarity to sequences in the Antibiotic Resistance Genes Database (ARDB) (>80%) (Fig. 2B). The multidrug efflux pump gene *bcr* was represented by two groups differing by their similarity to other AR genes (Fig. 2B). In addition, the genes that conferred resistance to nitrofurantoin and sulfadimethoxine all shared less than 75% sequence similarity to other AR genes (Fig. 2B).

The majority of genes identified (72%) did not match known AR genes in either ARDB or GenBank (see Table S3 in the supplemental material). We therefore grouped these genes by function (oxidoreductases, ligases, transport pump protein, etc.). Similar to the case with known AR genes, we observed differences in the composition of gene functions present between locations ($P = 0.004$) (see Table S2). Genes of unknown function formed the plurality at HOT (47%), Newport Bay (25%), and Agua Hedionda Lagoon (19%), while they represented 13% and 14% of the genes at San Pedro Channel and LA Harbor, respectively. Beyond these unknown types, HOT and Newport Bay were characterized by hydrolases, which comprised 12% and 16% of the gene functions identified there, respectively. Newport Bay was also dominated by ligases (19% of gene functions). LA Harbor and San Pedro were both defined by the same gene functions, which included oxidoreductases (19% and 31%, respectively), ligases (14% and 15%, respectively), and transferases (14% and 11%, respectively). Agua Hedionda Lagoon was characterized by oxidoreductases, DNA binding proteins, and transporters (15%, 12%, and 10% of the functional types, respectively). Additionally, we found that across environments we did not capture the entire diversity of genes that confer resistance (see Fig. S1).

Across antibiotics, the majority of unclassified AR genes were detected on tetracycline (44%), followed by ampicillin (27%) and then nitrofurantoin and sulfadimethoxine (both 14%). Over half of resistance (54%) to ampicillin was conferred by genes of unknown function. Resistance to tetracycline was predominately conferred by genes for oxidoreductases, ligases, DNA binding proteins, and regulatory proteins. Transferases and hydrolases were the dominant functional types isolated on sulfadimethoxine, and nitrofurantoin resistance was predominately conferred by genes for oxidoreductases and ligases.

We next asked which taxa hosted the genes and whether the organisms were native to marine environments. Each sample contained a mixture of putative marine bacteria (44%), nonmarine bacteria (25%), eukaryotes (10%), vectors (4%), viruses (1%), and unassigned taxa (15%) (Fig. 1, 2B, and 3). The majority of

TABLE 1 Numbers of transformants and resistant clones sequenced from each replicate at each sample location

Location ^a	Replicate	No. of transformants (CFU/μl)	No. of clones resistant to ^b :			
			Amp	Tet	Nit	Sul
AH	1	26,100	26	166	291	14
	2	35,100	27	213	226	10
HOT	1	20,600	24	4	1,040	4
	2	22,400	18	4	1,321	4
LA	1	5,290	10	269	122	34
	2	8,590	5	294	163	22
NB	1	3,900	303	13	56	12
	2	5,000	160	4	90	6
SP	1	5,520	1	153	98	17
	2	7,070	1	153	121	21

^a AH, Agua Hedionda Lagoon; HOT, Hawaii Ocean Time Series; LA, Los Angeles Harbor; NB, Newport Bay; SP, San Pedro Channel.

^b Amp, ampicillin; Tet, tetracycline; Nit, nitrofurantoin; Sul, sulfadimethoxine.

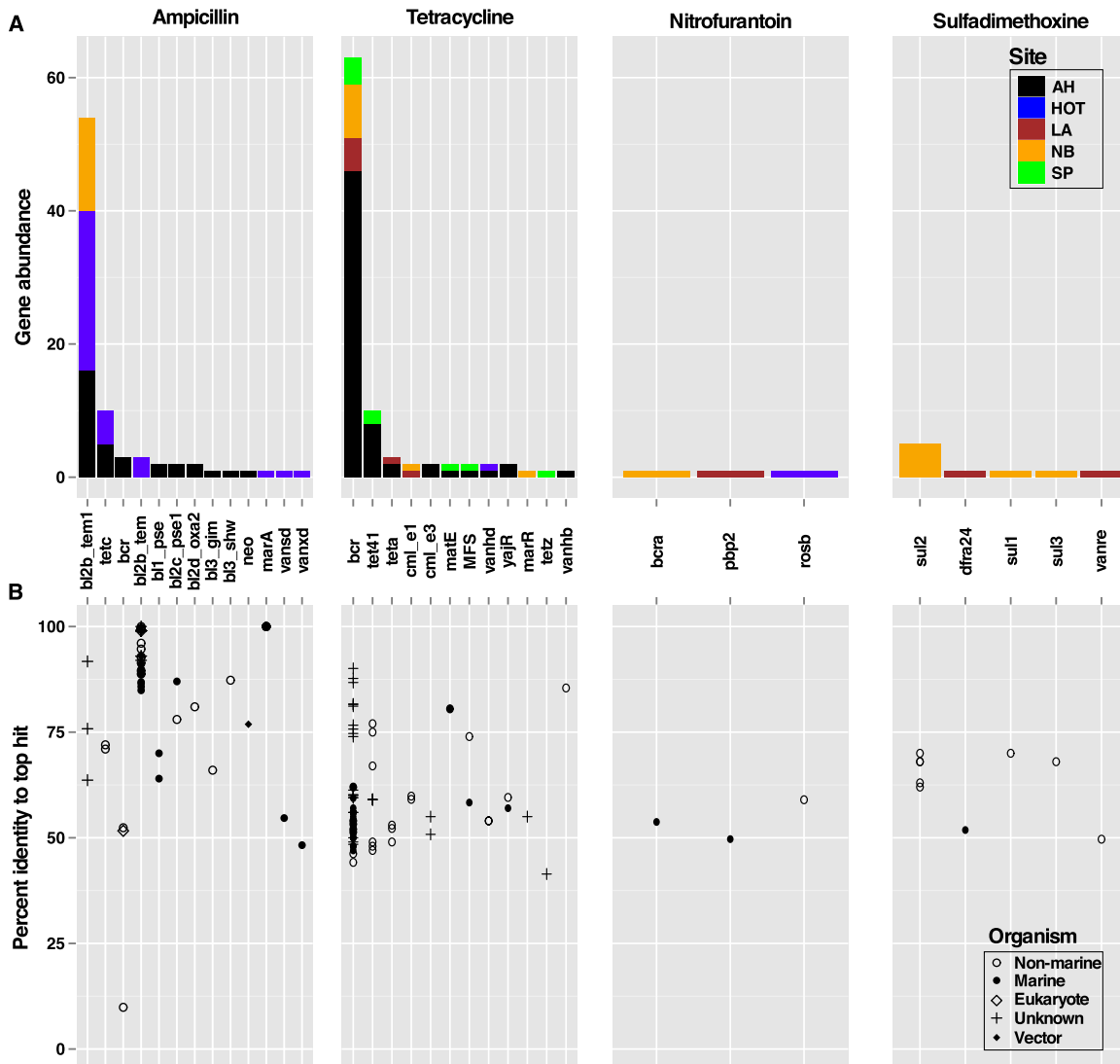


FIG 2 Abundance and percent amino acid identity to known AR genes. (A) Abundance of known AR genes across sample sites. (B) Percent identity between AR genes from marine environments and the Antibiotic Resistance Gene Database or GenBank. Each symbol represents one sequence, and symbols represent the host organism the gene was in, identified using GenBank and EnvDB.

resistance genes came from marine taxa at Los Angeles Harbor (66% marine and 15% nonmarine), San Pedro Channel (56% marine, 16% nonmarine), and Agua Hedionda Lagoon (42% marine and 26% nonmarine), while the distribution was more evenly split for Newport Bay (35% marine and 29% nonmarine) and Hawaii (34% marine and 32% nonmarine). The marine bacterial taxa included abundant lineages, like *Pelagibacter*, *Prochlorococcus*, and *Roseobacter* (Fig. 3). The putative nonmarine taxa were characterized by *Escherichia*, *Parvibaculum*, *Flavobacterium*, and *Rhodobacteracea* (Fig. 3). Across environments, marine bacteria made up the majority of resistant clones, except for HOT, which was characterized by an even distribution of marine and nonmarine taxa (Fig. 1). The resistant marine bacteria at HOT were dominated by *Prochlorococcus* (29%), *Pelagibacter* (21%), and *Vibrio* (18%), whereas sites closer to shore, like Newport Bay and LA Harbor, were dominated by *Ruegeria* (19% and 15%, respectively). The San Pedro Channel was dominated by *Pelagibacter*

(22%) and *Roseovarius* (17%), and Agua Hedionda Lagoon was dominated by *Octadecabacter* (16%).

The composition of resistant taxa also varied across antibiotics. Resistance to tetracycline and nitrofurantoin was identified predominately in marine taxa (57% and 56%, respectively). Sulfadimethoxine resistance was found in both marine and nonmarine taxa (43% and 42%, respectively), and we saw a similar distribution in resistance to ampicillin (26% nonmarine and 20% marine). Among the marine taxa, resistance to ampicillin was primarily observed in *Vibrio* (19%) and *Prochlorococcus* (12%). Resistance to tetracycline was predominately found in *Octadecabacter* (13%), *Roseovarius* (12%), and *Ruegeria* (12%). Resistance to nitrofurantoin and sulfadimethoxine was mostly observed in *Pelagibacter* (30% and 18%, respectively), while resistance to sulfadimethoxine was also found in *Puniceispirillum* (15%).

Both known and unclassified AR genes were found in marine

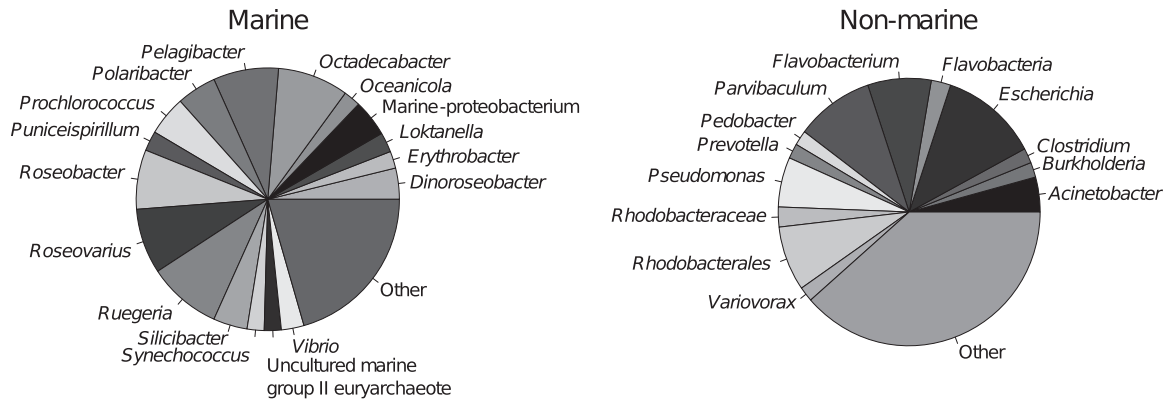


FIG 3 Relative abundances of marine and nonmarine bacterial taxa within all samples. “Other” represents groupings of taxa that had less than 2% relative abundance.

taxa (see Tables S4 and S5 in the supplemental material). Overall, the dominant AR gene type was the bicyclomycin pump gene (*bcr*). This gene was predominantly found in *Octadecabacter* and *Dinoroseobacter* (see Table S4). The next abundant known AR gene was the TEM1 beta-lactamase gene, which was found only in *Vibrio* (see Table S4). Organisms such as *Prochlorococcus* contained genes like *rosB* (encoding a putative potassium antiporter), *vanSD*, and *vanXD* (D-alanine–D-alanine ligase activity) (see Table S4). Among the marine taxa, the functions of previously unclassified AR genes were predominately ligases (18%), oxidoreductases (15%), and DNA binding proteins (15%) (see Table S5). *Ruegeria* contained the most unclassified AR genes (13% of the total), with *Pelagibacter*, *Roseovarius*, and *Roseobacter* each hosting 9% of the total unclassified AR genes (see Table S5).

Some marine taxa were host to several functional types. For example, *Roseobacter* was host to AR genes encoding DNA binding proteins, oxidoreductases, regulators, and transferases, and *Pelagibacter* was characterized by genes for transferases, ligases, lyases, and oxidoreductases (see Table S5). Some taxa predominately hosted one or two types, such as *Ruegeria*, which was characterized by ligases and DNA binding proteins and *Silicibacter*, which hosted AR genes encoding regulators (see Table S5).

It was unexpected to find a range of AR genes in abundant open-ocean bacteria like *Synechococcus*, *Prochlorococcus*, and *Pelagibacter*. To further examine this, we also analyzed a genome clone library of the antibiotic-sensitive marine cyanobacterium *Synechococcus* WH8102 as well as *Escherichia coli*. We then screened for the presence of resistance genes using the same procedure as with the environmental samples. Although the strains themselves were sensitive to antibiotics, we found multiple individual genes conferring resistance (Fig. 4). In *E. coli* we identified genes that were previously known (e.g., beta-lactamase genes) and unclassified AR genes (see Table S6 in the supplemental material). The majority of known AR genes were resistant to ampicillin, and they were identified as either the beta-lactamase gene *bl1_ec* or the multidrug efflux pump gene *ykkC* (see Table S6). The hits to *bl1_ec* had high amino acid similarity (>72%) to sequences in the ARDB, while hits to *ykkC* had lower amino acid similarity (<58%) (see Table S6). Resistance to tetracycline was associated with genes for known antibiotic efflux pumps, such as *bcr*, *carA*, *macB*, and *marA*, and known resistance to nitrofurantoin was conferred by the penicillin binding protein gene *pbp2* (see Table S6). In contrast, there were no known AR genes in the *Synechococcus* libraries.

The unclassified AR genes from *E. coli* included genes for hypothetical proteins, oxidoreductases, transport proteins, regulatory proteins, etc. (see Table S6). For *Synechococcus*, we did identify unclassified AR genes that conferred resistance to nitrofurantoin, which were a combination of hypothetical proteins, regulatory proteins, an oxidoreductase, a lyase, a transferase, a hydrolase, a ligase, and an isomerase (see Table S7). Thus, we clearly identified AR genes in antibiotic-sensitive strains of both marine *Synechococcus* and *E. coli*.

DISCUSSION

Using a functional metagenomic assay, we identified antibiotic resistance genes at multiple marine sites and resistance to four antibiotics. In addition, these genes were found in a range of marine bacteria, and while some of the genes were known antibiotic

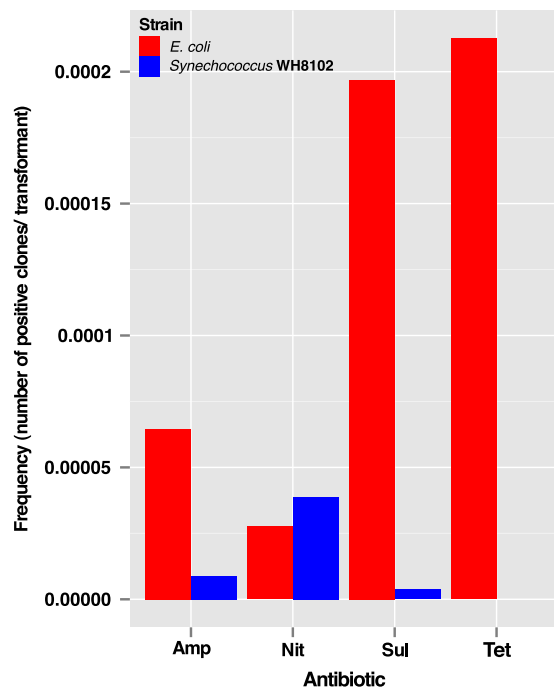


FIG 4 Frequency of antibiotic-resistant clones from the genomic libraries of *E. coli* and *Synechococcus* WH8102 on each of four antibiotics: ampicillin (Amp), nitrofurantoin (Nit), sulfadimethoxine (Sul), and tetracycline (Tet).

resistance genes, the majority were previously unclassified as such. Our results were consistent with past limited studies of marine samples, studies which were based on cell culture, PCR, and sequence-based metagenomics (22–24). However, this greatly expands our knowledge of the diversity of genes responsible for these resistance patterns by linking resistance phenotypes to genotypes and identifying potential novel resistance genes. While the estimated frequencies of resistance we found (up to 0.9% of cells) are less than estimated in gulls (up to 5% resistant cells) (9) and aquaculture sediments (up to 15% resistant cells) (25), this study highlights the sheer abundance and diversity of potential AR genes across marine sites.

There are several possible mechanisms for AR genes in the ocean. One is that resistant bacteria are transported into marine environments from terrestrial sources. However, we detected resistance genes in both marine and nonmarine taxa, and thus, dispersal from terrestrial sources cannot fully explain the results. A second possibility is that antibiotic resistance is selected for through anthropogenic antibiotic inputs. The resistance we found to nitrofurantoin and sulfadimethoxine could be the result of antibiotic effluents. Because nitrofurantoin and sulfadimethoxine are synthetic antibiotics, microbes would not experience these molecules in natural microbe-microbe interactions. Resistant bacteria have been isolated from marine environments in proximity to aquaculture facilities or waterways in proximity to human influence (23, 25–27). In addition, Port and colleagues (24) showed that frequencies of known antibiotic resistance genes were higher nearshore, so anthropogenic inputs can influence resistance patterns. Based on these observations, we would expect a lower frequency of AR genes in the open-ocean samples versus samples taken closer to shore. However, we found that frequencies of known AR genes were similar in offshore (e.g., HOT) and nearshore (e.g., LA Harbor) environments. Thus, it appears that human influence is not sufficient to explain our results, a pattern observed in soils (4, 5, 28). A third mechanism is that there are possible antagonistic *in situ* microbial interactions known to occur on particles (13). As we removed particles by prefiltering, particle-associated interactions are not likely to have been an important mechanism in our system. However, microbial interactions can possibly also occur in the water column using diffusible compounds (13, 14). If such interactions are common, this can lead to the widespread occurrence of AR genes, as observed in this study.

As the above-described mechanisms do not fully explain our results, we think a fourth mechanism, that proteins can be co-opted for resistance, is also at work. Past studies have shown that nonantibiotic efflux pumps can be used to transport antibiotics out of cells (10, 29, 30), and *E. coli* evolved to high temperature in antibiotic-free culture also evolved resistance to rifampin (31). In support of this, we found many genes that conferred resistance in our assay, and the functions of these genes are similar to those of known AR genes (e.g., transporters, oxidoreductases, and hydrolases), but their primary function may not be associated with resistance. We also found that genes from two antibiotic-sensitive strains could confer resistance when transformed into a host, and a co-opting mechanism may also explain the resistance we saw against synthetic antibiotics like sulfadimethoxine and nitrofurantoin. This finding was unexpected, but it may partly explain the high occurrence and diversity of AR genes in other environments, like the human gut (11). In addition, what we observed could also be a regulatory mechanism, in which the inserted gene was in a

new genomic context and induced resistance because of increased expression, protein-protein interactions, etc. Thus, organisms living in the oceans (and other environments) may harbor a large potential reservoir of AR genes that do not currently cause resistance but can be activated under the right circumstances (e.g., with the right promoter). And while beyond the scope of this initial survey, in order to understand how the unclassified AR genes directly cause resistance, further analysis of the previously unclassified AR genes is needed to identify the biochemical function of these genes. In addition, because we did not find contiguous sequences, we cannot rule out that the gene conferring resistance was not sequenced. It also is likely that this approach underestimates the frequency and diversity of AR-conferring genes in the ocean. First, our functional genomics assay identified only genes that are expressed in *E. coli*. Also, our insert size (2 to 4 kb) limits detection to simple genetic systems.

AR is no longer just a clinical issue (2). The discovery of natural reservoirs of resistance genes highlights the role natural environments play in the dissemination of AR genes. Here, we add oceans to the list. Covering 70% of Earth's surface area, oceans host on the order of 10^{29} bacterial cells (32). Given that oceans are prone to rapid vertical and horizontal transport of water masses and a key element to human commerce and recreation, this environment may be an important global reservoir of AR genes. While our method of identification, based on BLAST annotations, would identify fewer AR genes than more recent hidden Markov models (3), and our use of a single host (*E. coli*) limited us to finding genes that are expressed in *E. coli*, our results still highlight the oceans as a potential reservoir for AR genes in the proper selective environment.

ACKNOWLEDGMENTS

We thank Alyssa Kent for collecting seawater from the Hawaii Ocean Time Series, Andy Fields for helping with experimental work, and Jennifer Martiny for helpful comments on the manuscript.

In addition, we acknowledge funding from the Department of Education Graduate Assistance in Areas of National Need, fellowship P200A120144.

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