Title

Functional Unknomics of the SAR11 clade using bioinformatics approaches

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Abstract (249 words)

A substantial fraction of the genes in marine bacteria lack detectable sequence similarity to genes with known functions. These functionally uncharacterized genes— collectively referred to as the "unknome"—represent a largely unexplored genetic repertoire harboring insights into marine bacterial ecology. In this study, we explored the function of the unknome of SAR11 clade, the most abundant bacterial lineage in the ocean, with a particular focus on genes that provide insight into their ecology. Based on the COG classification, approximately 58% of SAR11 ortholog groups were classified as unknome. Among the SAR11 unknome, we successfully inferred the functions of 69 ortholog groups that are conserved in SAR11 clade by protein structure similarity searches and genomic context analyses. These ortholog groups include putative transporter components, supporting the current ecological understanding that SAR11 clade is specialized in substrate uptake to adapt to oligotrophic marine environments. Furthermore, structural analysis indicated that the DUF2237-containing protein, enriched in marine environments, has potential interactions with purine nucleotide-containing compounds. This may suggest the existence of unique nucleotide utilization mechanisms in marine bacteria. In addition, we found candidates of virus defense systems within the unknome, which demonstrates that diverse defense systems are present at least in one-third of the cultured SAR11 strains. The conservation of these viral defense systems, even within streamlined SAR11 genomes, suggests that they confer significant ecological advantages. Our exploration provided insights into the genetic basis of bottom-up processes (adaptation to oligotrophic environments) and top-down processes (antiviral defense strategy) contributing to ecological success of SAR11.

Introduction (607 words)

The SAR11 clade, also known as *Candidatus* Pelagibacterales, is the most abundant group of heterotrophic bacteria in marine environments, accounting for approximately 25% of all bacterioplankton cells in the ocean photic zone (1). Due to its high abundance, members of SAR11 clade play crucial roles in marine ecosystems and material cycles (2,3). The notable characteristics of SAR11 clade are their slow growth rate and highly streamlined genome, which typically lacks accessory genes such as antiviral defense systems and, occasionally, essential biosynthetic pathways (3–6). Due to their poor growth under laboratory conditions, culture-based studies of SAR11 clade are limited in achieving comprehensiveness and throughput, and the reasons for their ecological dominance are still not fully resolved.

The ecological success of SAR11 has been explored from both bottom-up and top-down perspectives. On the bottom-up side, their extremely resource-efficient lifestyle is thought to provide a strong advantage in oligotrophic marine environments. Several observations supports this view, including the genome streamlining (5,7), presence of multifunctional transporters and highly expressed solute binding-proteins (8–10), loss of motility and transcriptional regulation (5,7,11), and light-utilization by proteorhodopsin (12,13). In contrast, from a top-down perspective, SAR11 are considered to be less affected by predation and lysis, in line with the "Kill-the-Winner" hypothesis. According

to the hypothesis, fast-growing species are expected to be more susceptible to viral

infections, implying that the slow-growing SAR11 strains are less affected by viral

infections (14,15). However, the isolation of Pelagiphages (16) and evidence of their

frequent infections to SAR11 (17–20) indicate that virus-host interactions are more

complex than initially assumed. The "King of the Mountain" (KoM) hypothesis offers

one possible explanation, proposing that the high abundance of SAR11 leads to increased

contact frequency, promoting frequent genetic recombination, which could influence viral

susceptibility (21). The KoM hypothesis is supported by the high intraspecific

recombination rate and the presence of highly diverse genomic regions that encode

membrane proteins that could act as viral receptors, variation that may help SAR11

populations evade viral infection (22–24). However, there is still a lack of direct evidence

for the presence of antiviral defense systems and their frequent horizontal gene transfer

among SAR11 (3,20).

Currently, genome-based approaches play indispensable roles in microbiology,

particularly for lineages that are difficult to cultivate, such as the SAR11 clade. However,

a growing challenge in the field is the discovery of a large number of genes with no

similarity to any functionally characterized sequences (25,26). The issue is particularly

acute in marine metagenomes (25,27), suggesting that ecologically important genes may

remain overlooked and annotated as hypothetical proteins. For example, the recent

discoveries of diverse antiviral defense systems originating form previously

uncharacterized genes (28) suggest that many important functions await discovery within

this uncharacterized gene space. To overcome the limitation of sequence-based

annotation, an increasing number of studies are adopting bioinformatics approaches that

go beyond sequence similarity, including protein structure prediction and genomic

context analysis (29–31). However, these cutting-edge approaches that could

substantially enhance functional annotation have not been systematically applied to

SAR11 clade.

Here, we focused on the functionally unknown genes, referred to as the 'functional

unknome' (26), within SAR11 genomes and sought to predict their functions by

combining bioinformatics approaches. We placed particular emphasis on genes that may

be ecologically important for the SAR11 clade, including those related to substrate uptake,

phototrophy, and virus-host interactions. By combining structure-based predictions,

genomic context information, and environmental distribution patterns, we inferred

potential functions for conserved components of the SAR11 unknome. Further, we

systematically explored viral defense systems within the SAR11 unknome. Our findings

demonstrate the importance of re-annotating genes with unknown functions to uncover

hidden aspects of non-model marine lineages.

Materials and Methods (1212 words)

Collection and evaluation of the SAR11 genomes

We compiled the SAR11 genomes that were analyzed by Haro-Moreno et al. (2020)

(32) and expanded the dataset with genomes from freshwater, brackish water, Eastern

Pacific, and polar environments to better represent the under-sampled clades (**Table S1**)

(33–36). The final dataset includes 321 genomes, mainly from cultured strains and single-

amplified genomes (SAGs), as metagenome-assembled genomes (MAGs) are difficult to

reconstruct for SAR11 due to high genomic diversity (Fig. S1) (37). Genome quality was

assessed by using CheckM2 v1.0.2 (**Fig. S1A**) (38). Estimated genome size was

calculated using Completeness and Contamination scores following the previous study

(36), and GC content was calculated using Seqkit v2.4.0 (**Fig. S1B**) (39). Among the 253

genomes with >50% completeness and <10% contamination, we identified at least 184

species based on <95% ANI, as calculated using FastANI v1.34 (40).

Phylogenomic classification of the SAR11 genomes

The concatenated alignment of 120 bacterial single-copy genes (5,036 sites;) was

constructed using GTDB-Tk v2.1.0 classify-wf (41). Eight genomes were excluded

because they had <10% of the alignment sites covered. The *Rickettsia bellii* genome

(GCA_002078315.1) served as the outgroup following a previous study (32). The

maximum-likelihood tree was inferred with IQ-TREE2 v2.2.0.3 using ModelFinder-

selected Q.insect+F+I+R9 model, with 1,000 ultrafast bootstrap and 1,000 SH-aLRT

replicates (42,43). The tree was visualized in R using ggplot2, ggtree, and ggtreeExtra

(44–46). Subclades were assigned based on tree topology following the previous

classifications (6,3,32,36); genomes falling outside known subclades remained

unassigned. Although the phylogenetic positions of subclades IV and V are debated and

are not classified within the *Ca.* Pelagibacterales in several previous studies (32,47–49),

we treated them as part of SAR11 clade, considering their shared genomic and ecological

characteristics (3).

Functional annotation of ortholog groups and definition of the unknome

A total of 306,416 protein-coding genes in all 321 SAR11 genomes were

predicted with prokka v1.14.6 (50), and clustered into ortholog groups (OGs) using

SonicParanoid2 v2.0.3 (51). Transmembrane regions were predicted using TMHMM 2.0

(52), and proteins were classified as transmembrane (TM) if they met either of the

following criteria: (i) ExpAA >10 aa and PredHel >1, or (ii) ExpAA >10 aa, PredHel = 1,

and First60 <10. All coding sequences were annotated with COG-2020 and KEGG

Orthology (KO) using COGclassifier v1.0.5 (https://github.com/moshi4/COGclassifier)

and KofamScan v1.3.0 (53), respectively. The unknome genes were defined as those were

not annotated by COGclassifier or assigned COGs belonging to category S (Function

unknown), category R (General function prediction only); unknome OGs were those with

>50% unknome members. The OGs distributions among each subclade were visualized

with the UpSetR (54). Environmental distribution data were retrieved from Global

Microbial Gene Catalog (GMGC) v1.0 for bacteriorhodopsin and core unknome OGs

based on their COG IDs (55). The core unknome was defined as unknome OGs occurring

in more than half of the SAR11 genomes. Only GMGC entries annotated with both habitat

categories (e.g., marine, freshwater and human gut) and COG IDs were analyzed. Habitat

proportions were calculated per OG, counting multiple habitat annotations as independent

occurrences. To simplify classification, "human skin," "human gut," "human nose,"

"human vagina", and "human oral" were grouped as "human-associated".

Neighborhood OG analysis

Genomic contexts of all coding sequences were analyzed using GFF files

generated by the prokka pipeline. Two genes were considered neighbors if they were

located within five genes of each other on the same contig. Neighboring pairs that showed

a reciprocal co-localization probability of ≥75% across at least ten genomes were retained

and visualized as a network using igraph (56) and ggnetwork (57). Since our analysis

focused only on a phylogenetically close lineage, we adopted a more conservative

threshold than the previous study (29) to avoid false positives due to phylogenetic

constraints. Note that pairs with highly unequal occurrence frequencies may not meet this

criterion, even if the rarer OG always co-occurs with the more prevalent one. Neighboring

pairs containing unknome OGs are manually checked using predicted structures by

AlphaFold2, and the complex formation was evaluated by pDockQ and pDockQ2 scores

(58–60).

Collection and comparison of predicted protein structures

All protein structures of *Ca.* Pelagibacter ubique HTCC1062 strain and *Ca.*

Pelagibacter sp. IMCC9063 strain were downloaded from the AlphaFold Protein

Structure Database (61). To improve the structural prediction of the conserved 108 core

unknome OGs, the structures were predicted with AlphaFold2 based on the core unknome

proteins of HTCC1062 excluding the signal peptide regions predicted by DeepTMHMM

ver 1.0.24 (62). Structural similarity search for the unknome proteins were performed

using Foldseek version 7.04e0ec8 (63) against the Protein Data Bank (PDB), which was

downloaded on September 4, 2023 (64).

Meta-transcriptome data analysis of SAR11 genes

Sequence reads of the 509 meta-transcriptomes obtained in the Tara Ocean

samples were mapped onto the 321 SAR11 genomes (**Table S2**) (65). We downloaded

paired-end sequence data from NCBI SRA by SRA toolkit (https://github.com/ncbi/sra-

tools), and then quality control was performed by using fastp with options -q 20 -n 10 -l

60 (66). The quality-controlled reads were mapped via bwa-mem2 (67). Transcripts per

million (TPM) were calculated using a script from a previous study

(https://github.com/yosuken/CountMappedReads2) (68).

Analysis of DUF2237 domain-containing proteins

A total of 7,968 DUF2237 domain-containing proteins were retrieved from the

Pfam database on April 21, 2023. These sequences were annotated with domain

information using pfam_scan (69) and aligned using MAFFT v7.508 (70) in the auto

mode. The alignment was visualized on the predicted monomer structure of the

DUF2237-containing protein of *Ca.* P. ubique HTCC1062 strain using ConSurf-DB (71)

and PyMOL v2.5.4 (72). The Predicted Aligned Error plot was visualized using a Python

package (https://github.com/cddlab/alphafold3_tools).

The OceanDNA MAG catalog v1 (73) and the Pfam database were utilized to assess

the phylogenetic distribution of DUF2237 genes. Genes annotated as COG3651 and

COG5524 were identified as DUF2237-containing proteins and proteorhodopsin,

respectively. A multiple sequence alignment of 120 marker genes from 7,696

representative MAGs was generated with the GTDB-Tk classify-wf. The resulting

alignment contained 5,036 sites. Subsequently, a maximum likelihood phylogenetic tree

was inferred with IQ-TREE2 (v2.2.0.3) using ModelFinder-selected Q.yeast+R10 model,

with 1,000 SH-aLRT replicates. The phylogenetic distribution of DUF2237 and

proteorhodopsin was visualized on the phylogenetic tree using ggplot2, ggtree, and

ggtreeExtra (44–46).

The structure of the *Ca. P.* ubique HTCC1062 strain's DUF2237 protein, predicted

by AlphaFold2, was prepared in PDBQT format using MGL Tools v1.5.7 (74) for

docking simulations. A ligand library was constructed by converting 258 compounds with

biological roles from the KEGG Compound database (br8001) and seven nucleotide-

containing molecules from the Protein Data Bank Japan (NAI, PO4, NDP, FAD, COA,

C2E, NAD) into PDBQT format using Open Babel v3.1.0 (75) and MGL Tools **(Table**

S3). The grid box was defined as 25 × 20 × 20 Å to the pocket site of the DUF2237

protein predicted by PrankWeb 3 (76). All docking simulations were performed using

Autodock vina v1.2.5 (77) with an exhaustiveness value of 100. The possibility of binding

to ATP was evaluated using ATPbind (78).

Defense system annotation

DefenseFinder (updated on June 5, 2024) was used to identify potential antiviral

defense genes (79). To check for the completeness of defense systems, neighboring genes

of each defense gene found by DefenseFinder were manually annotated and compared to

known defense genes registered to DefenseFinder wiki (80) by genomic context,

sequence alignment using MAFFT, and structure similarity search using AlphaFold2 and

Foldseek.

Result (2024 words)

Collection and phylogenomic analysis of SAR11 genomes

We collected 321 SAR11 genomes representing five subclades (**Table S1**). Subclade

classification based on a maximum-likelihood tree inferred from GTDB marker genes

was largely consistent with those inferred from 16S rRNA gene and 232 conserved single-

copy genes in previous studies (3,6,32) **(Fig. 1A)**. Subclade V exhibited larger genome

size than other subclades **(Fig. S1B)**, consistent with its higher metabolic potential (49).

The 306,416 protein-coding genes were clustered into 5,603 OGs using SonicParanoid2

(81). To estimate the unknome fraction, all genes were annotated using the KO and the

COG databases. As a result, 173,549 and 257,412 genes were assigned to KO and COG,

respectively **(Fig. 1B)**. At the OG level, 1,286 OGs (23%) and 2,683 OGs (48%) were

assigned KO and COG annotation, respectively **(Fig. 1CD and Table S4)**.

We next classified these OGs according to their conservation within the five SAR11

subclades. The largest set combination corresponded to OGs conserved across all

subclades (968 OGs), exceeding those unique to any single subclade **(Fig. S2A)**. This

pattern is consistent with the streamlined SAR11 genomes, in which the core genome

constitutes a large proportion (5,6). In contrast, most OGs with unknown functions were

specific to clade I or clade V **(**790 and 557 OGs; **Fig. S2B)**, reflecting diversity of clade

I and the large genome size of clade V (49). Additionaly, 192 unknown-function OGs

specific to clade III, a brackish and freshwater clade, may contribute to the low-salinity

adaptation **(Fig. S2B)**.

Among the COG-assigned genes, categories E (amino acid transport and metabolism,

271 OGs), M (cell wall and plasma membrane biosynthesis, 252 OGs), C (energy

production, 236 OGs), and J (translation and ribosome-related, 236 OGs) and were the

most frequently observed in the SAR11 clade **(Fig. 1D)**. The ordering of these categories,

such as category M exceeding category K (transcription), contrasts with that of *E. coli*

(82). This pattern reflects their reduced transcriptional regulation, a feature of genome

streamlining (5–7). KO-assigned genes were fewer than COG-assigned genes **(Fig. 1BC)**

and included functionally unknown genes, such as those containing domains of unknown

function (DUF).

Definition and characteristics of the 'unknome'

Because some KOs lack functional annotation, we defined the SAR11 unknome

based on COG classifications. It comprised category S, R, and OGs without COG

assignment **(Fig. 1B)**, totaling 3,255 OGs (58.1%) **(Fig. 1D)**. The unknome genes are

typically short and AT-rich **(Fig. S3AB)**. As in *E. coli* (83), these unknome genes show a

slightly higher proportion of TM proteins than functionally known genes **(Fig. 1C,**

Fisher's exact test p < 2.2e-16). Tara Oceans metatranscriptomes confirmed the

unknome OGs are expressed in situ, with only slightly lower expression than functionally

known genes **(Fig. S3C and Fig. S4, Wilcoxon rank sum test p < 2.2e-16)**.

Functional prediction of the unknome based on neighboring gene functions and

predicted protein structures.

Next, we predict the functions of the unknome using two bioinformatics approaches.

The first was neighborhood gene analysis, which predicts gene function from the genomic

context because functionally related genes in prokaryotes often occur in close proximity

in prokaryotic genomes (e.g., in operons or syntenic regions) (84,85). We defined

neighboring OG pairs as those located within five genes of each other (details in Methods).

We then constructed a neighboring OG network containing 474 OGs and 620 edges **(Fig.**

2A). Functionally related OGs formed connected components (**Fig. 2A**), the largest of

which corresponded to the ribosomal protein operon.

Focusing on the connected components that include the unknome OGs (gray nodes),

OG1814, a DUF4340-containing OG without a COG assignment, was conserved near the

genes annotated as ABC transporter components, such as OG 1815, OG1772, and OG688.

In HIMB59 (GCA_000299115.1), proteins from these four OGs were predicted to form

a transporter complex with a pDcokQ2 score of 0.268, greater than the confidence

threshold of 0.23 **(Fig. 2BC and Fig. S5AB, pDockQ2 > 0.268)**, suggesting that OG1814

function as a novel transporter subunit. Similarly, OG1249 (COG4696, a DUF2139-

containing gene) was conserved within the type IV pilus operon, indicating a potential

role as a minor pilus component (**Fig. 2D**). In total, 38 components containing both

unknome and characterized OGs were identified. Although detailed functions remain

unresolved for many, these patterns reveal functional links within the SAR11 clade—

between unknome and known OGs, and among the unknome themselves.

We next applied protein structure prediction and structure similarity searches. Using

Ca. P. ubique HTCC1062 (subclade Ia.3) and LD12 IMCC9063 (subclade IIIa.2) as the

representatives, we compared predicted protein structures to those in the PDB. In

HTCC1062, 1,212/1,328 proteins (91%) showed >90% probability of being homologous

to a PDB entry, and 237 of these shared <20% sequence identity with their best hits (**Fig.**

2E). In IMCC9063 strain, 1,216/1,382 proteins (88%) showed high probability of

structural homologs, 221 of them having <20% sequence identity **(Fig. S6)**. Among

unknome proteins, structurally similar proteins were found for 154/242 (67%) in

HTCC1062 and 164/293 (56%) in IMCC9063. These results demonstrate that structural

similarity searches effectively detect remote homologs within the SAR11 genomes.

Function prediction of the core unknome OGs

Given that OGs widely conserved throughout the SAR11 clade likely play critical

roles for their ecology, we further focused on the 108 core unknome OGs that are

conserved across all five clades and present in >162 genomes (>50% of genomes in the

dataset). By integrating information from neighboring genes, structurally similar proteins,

and KO assignments, 87 of 108 OGs had at least one line of supporting information **(Fig.**

S7). However, in many cases, the assigned KO showed no functional information, or the

functions of the similar structures or neighboring genes were also unknome

(**Supplemental Text**). Then, we performed an extensive literature search using gene and

domain names registered in the COG, KO, and STRING databases to capture functional

information not yet incorporated into current annotations. This search identified

functional insights for 10 OGs, including several characterize in *Caulobacter crescentus*,

320 a model alpha-proteobacterium (**Supplemental Text**). Overall, we found that 69 of 108

321 core unknome OGs have additional information or putative functions not registered in

322 COG database **(Table S5 and Supplemental Text)**. These include predicted components

323 of transporters, channels, or metabolic enzymes (e.g., OG228, OG305, OG365, OG448,

324 OG497, OG814, and OG824), which may support the SAR11 clade's adaptation to

325 nutrient-limited environments (**Fig S8 and Supplemental Text**).

327 **Functional analysis of DUF2237, the unknown gene with a skewed distribution in**

328 **the ocean**

329  We next examined ecological distribution to infer their potential functions of core

330 unknome OGs lacking functional information from any of the above analyses. We focused

331 on the OGs enriched in marine environment, as such genes often underpin ecological

332 success. A representative example is microbial rhodopsin (COG5524: bacteriorhodopsin)

333 (**Fig. 3A**), which function as a photoreceptor, enabling the capture and utilization of light

334 enagy in oligotrophic waters (86). The most marine-biased functionally unknown OG was

335 COG3908, which contains the DUF2093 domain **(Fig. 3A)**. Although no further

336 functional information was obtained, this OG was consistently located near the lipid

337 synthesis operon in SAR11 clade (**Fig. 2A**).

The second most marine-biased OG was annotated as COG3651, which contains

uncharacterized DUF2237 domain. DUF2237-containing genes have been reported to co-

occur with the proteorhodopsin, a light-driven proton pumps, in marine *Flavobacteriia*,

and a DUF2237 knockout in *Synechocystis* sp. PCC6803-P showed reduced phototaxis

(87). DUF2237 has been suggested to have a function related to light reception or

utilization. Although, three different structures of the DUF2237 protein had been

experimentally solved (PDB 2LQ3, 3USH, and 4IOH), the mechanistic link to phototaxis

remain unclear. Because light utilization is also ecologically important for SAR11 clade

(13,88,11), we further analyzed this unknome protein.

Initially, we re-evaluated the association between proteorhodopsin and DUF2237-

containing genes using OceanDNA MAG catalog (73) to validate the previous

observation in marine *Flavobacteriia*. As in the previous study, DUF2237 genes

significantly co-occurred with the proteorhodopsin **(Fisher's exact test, p < 2.2e-16).**

The high conservation of DUF2237 genes across most marine bacterial lineages, except

Patescibacteria and *Chloroflexota* **(Fig. 3B)**, indicates their broad ecological importance.

The predicted structure of the DUF2237-containing protein (SAR11_1247) indicates

dimeric assembly **(Fig. S9AB, pDockQ score = 0.314)**. Analysis of 7,968 DUF2237

domain-containing proteins in the Pfam database showed that this domain typically

occurs as a single-domain protein **(Fig. S10AB)**. Mapping sequence conservation from

these Pfam entries onto the structure revealed a highly conserved pocket region, likely a

concave ligand-binding site **(Fig. 3C)**. This suggests a potential enzymatic role for

DUF2237 in interacting with a specific ligand.

To identify potential ligands for the DUF2237 pocket, we performed docking

simulations using KEGG "Compounds with biological roles", excluding polysaccharides

to avoid including macromolecules. Nucleotide-containing ligands, including NADH, c-

di-GMP, and cAMP, showed the highest affinities for the DUF2237 protein from

HTCC1062 strain **(Fig. 3D, Table S3)**. ATP-binding prediction also supported nucleotide

interaction (78) (Probability = 0.85). Because NADH and ATP participate in key

metabolic pathways, and c-di-GMP and cAMP act as motility-related second messengers

(89–92), these results suggest that the reduced phototaxis observed in the DUF2237

knockout mutant may arise not from a defective light response but rather from altered

levels of small nucleotide-containing molecules that could mediate phototactic signaling.

Antiviral defense genes on the SAR11 genomes

Given the numerous recent discoveries of defense genes within previously uncharacterized genes (28), we explored putative antiviral defense genes within the SAR11 genomes. Using DefenseFinder (80), we identified 43 defense systems, including restriction-modification (RM) and toxin-antitoxin systems **(Table S6)**. The RM systems were most common as previously reported (3,20). Excluding the well-characterized RM systems, 11 unknome genes showed putative defense roles **(Fig. S11)**. Furthermore, we examined genomic regions with only partial components of known systems and manually curated neighboring genes, as defense genes often cluster within specific loci.   This led to the identification of 8 additional putative defense systems, bringing the total to 51 putative defense systems across 46 SAR11 genomes **(Fig. 4A)**.

A previous study showed that only a few deep sea SAR11 genomes possess part of the CRISPR-Cas-like gene set (93). As in the previous study, Cas-like genes without CRISPR array were detected in two deep sea SAGs (AG-337-I02 and AG-410-B18). Three cultured-strains genomes and one MAG possess *dnd*, a dsDNA phosphorothioation (PT)-based defense system (94). In addition, we identified Ssp-like operons on two SAR11 genomes **(Fig. 4A)**. Same as the *dnd*, Ssp is a PT-dependent system, modifing DNA by SspBCD and cleaving foreign ssDNA by SspE (95,96). The SAR11 Ssp-like operons encode proteins structurally similar to the known SspBCD and an uncharacterized protein shorter than the known SspE. Although experimental validation

is needed, this finding indicates that PT-based modifications are used to distinguish self

from foreign DNA in the SAR11 clade.

We also identified Mza, DISARM, and ShosTA-like operons **(Fig. 4A)**. Mza

defends against ssDNA phages, although its mechanism remains unknown (97). The

DISARM-like operon in SAR11 encodes DrmAB, DrMII-type methyltransferase, and

helicases **(Fig. 4B)**, whereas DrmC, nonessential for defense, appears absent (98). The

distinct gene order suggests a divergent DISARM-like configuration. The ShosTA toxin-

antitoxin system was detected in three SAR11 genomes. The SAR11 ShosT protein is

approximately 100 amino acids shorter than the *E. coli* ShosT but retains the

phosphoribosyl transferase domain **(Fig. 4C)**. This suggests that the SAR11 clade may

have acquired a truncated but functional toxin, likely through genome reduction. AbiRb-

and AbiRc-like genes were also present as operons, but the AbiRc-like gene in SAR11

lacks its terminal domain **(Fig. 4D)**. Moreover, AbiRa is a sigma70 factor, yet the only

sigma70-type RNA polymerase subunit is present in the SAR11 genome, it is not located

within this operon. Therefore, it remains unclear whether this AbiR-like genes functions

as a known abortive infection system, and we did not include it as a defense system found

in this study.

Previous limited identifications of viral defense genes in SAR11 may be partly

due to the presence of these overlooked putative defense systems that slightly differ from

experimentally validated defense system components (**Fig. 4BCD**). When we focused on

high-quality genomes, defense systems were found in at least one-third of the cultured

strains and one-quarter of the SAGs. In contrast, defense systems were rarely detected

from the MAGs **(Fig. 4E)**. This result suggests that the SAR11 clade harbors more diverse

and abundant defense systems than previously inferred from the MAG-based analyses

(20).

Discussion (989 words)

As genomic data continue to expand exponentially, functionally unknown genes are

becoming more prevalent, and their study—termed Functional Unknomics (26)—is

gaining attention. Previous research on genes of unknown function has primarily focused

on model organisms (83,26,99) or large-scale sequence analyses of whole ecosystems,

involving MAGs and SAGs (25,29,30). This study represents the first systematic

investigation focused on genes of unknown function in the SAR11 clade as one of the

non-model organism lineages.

In the SAR11 clade, one of the most abundant marine bacterial lineages, a large

fraction of OGs remains functionally uncharacterized despite their crucial roles in marine

microbial ecology. To address this issue, we clustered 306,416 protein-coding genes in

321 SAR11 genomes into 5,603 OGs and classified 3,255 of them (58.1%) as unknome

based on the COG database **(Fig. 1CD)**. Utilizing genome context and predicted protein

structures, we successfully obtained functional information for 69 out of the 108 core

unknomes **(Table S5 and Supplemental Text).** These included many transporter genes,

supporting the strategy of SAR11 that specializes in substrate uptake, as proposed in

previous studies (3,9,10). Furthermore, our analysis confirmed that the utilization of

genomic context and predicted protein structures is valuable even for non-model

organisms. Especially, structural similarity search proved particularly effective in

functional annotation (**Fig. 2D, S7, S8 and Supplemental Text**). This may reflect both

the accumulation of neutral mutations during the divergence of SAR11 clade from well-

studied model organisms and shifts in genomic properties, including reduced GC content,

leading to the presence of many remote homologs (5). Although structural similarity

search can find distant relationships between SAR11 proteins and those in the PDB, the

latter also lack functional information, such as the DUF2237-containing protein. In this

study, we addressed this problem by manually examining the individual results. This

involved cross-referencing them with databases such as PDB, Pfam, COG, KO, Uniprot,

and STRING, and conducting literature searches for any reported functional insights

(Supplemental Text). This careful curation allowed us to focus on previously

uncharacterized genes potentially support ecological success of SAR11 clade

(Supplementary Text).

We found functionally unknown genes specifically related to the marine environment,

which are probably crucial for the ecology of the SAR11 clade **(Fig. 3A)**. Among them,

we found the DUF2237-containing proteins, which were previously thought to be related

to proteorhodopsin (87). The predicted protein structure and docking simulation results

suggested that DUF2237 is likely to be an enzyme associated with purine nucleotide-

containing compounds **(Fig. 3CDE)**. This result may indicate that the DUF2237 protein

binds to second messengers, such as c-di-GMP and cAMP, and is thus involved in

phototaxis. Alternatively, it might interact with NADH, indicating involvement in redox

processes. This finding collectively suggests that DUF2237 participates in fundamental

cellular processes, extending beyond a direct link to phototaxis. Although definitive

conclusions require further experimental validation, the possible binding of DUF2237 to

these compounds suggests that it represents a novel nucleotide-binding motif and it may

play a key role in marine bacterial ecosystems.

We explored the unknome genes to identify viral defense systems that may offer

insights into top-down factors contributing to ecological success of the SAR11 clade.

Previous studies suggested that SAR11 possesses only limited viral defense capacity

(3,20). However, our comprehensive genomic analysis revealed a scattered distribution

of diverse viral defense systems **(Fig. 4A)**. This pattern is consistent with the high

intraspecific recombination rates of SAR11 genes (22). While the previous hypothesis

proposed that a slow growth rate of SAR11 made it less vulnerable to phage lysis (15),

the discovery of antiviral defense systems indicates that SAR11 face viral pressure,

requiring more active resistance to viral infection than previously appreciated. The recent

discovery of anti-RM systems in the Pelagiphage genome further supports the existence

of the arms race between *Ca.* Pelagibacter and the Pelagiphage (80). The presence of

defense systems might potentially explain the unique ribosome-deprived phenomenon

known as 'zombie cells' in SAR11 upon viral infection, possibly resulting from abortive

infection (20). Moreover, the discovery of the Ssp and Mza systems in the SAR11

genomes, both of which are known to target ssDNA viruses, suggests the potential

existence of undiscovered ssDNA Pelagiphages. Given the high intraspecific

recombination rate of SAR11 genes (22), the sporadic distribution of defense systems

likely reflects population-level sharing of this repertoire, rather than frequent transfer

across distantly related taxa. Such population-level sharing may help SAR11 balance the

trade-off between genome streamlining and antiviral defense. This interpretation is

consistent with the concept of a bacterial pan-immune system, in which defense genes

are frequently gained and lost among closely related strains (100), and also consistent

with the KoM hypothesis, which proposes SAR11 maintains its dominance through high

diversity and efficient horizontal gene transfer driven by high contact frequency of closely

related strains (16,21). Collectively, these observations suggest a complex evolutionary

interplay between SAR11 and its phages, likely shaped by genome streamlining,

horizontal gene transfer, and diverse defense strategies.

Our results also underscore the importance of genome reconstruction methods in

evaluating the distribution of viral defense systems. Compared with SAGs and genomes

from cultured strains, viral defense genes were underrepresented in MAGs **(Fig. 4D)**,

which may have led to the previous observation that SAR11 possesses little viral defense

capacity (3,20). This underrepresentation likely stems from limitations in MAG assembly

and binning, reflecting the high variability of specific genomic regions (e.g., hyper

variable regions and metagenomic island) (6,23,24). Consequently, SAG analysis

provides a powerful approach to capture diversity of defense systems, as it circumvents

the challenges associated with MAG construction (37).

Looking forward, further advances in automated gene function annotation will be

essential to keep pace with the rapid expansion of microbial genomic data and to

complement meticulous but low-throughput manual annotation. By developing robust

analytical approaches and moving beyond model organisms, we can uncover novel

biological and ecological functions of microbes, providing deeper insight into the

diversity and ecological roles of microbial life in natural environments. Bioinformatics-

driven Functional Unknomics is poised to become a major frontier in microbial ecology,

particularly for lineages that remain difficult to culture.

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Author contributions

K.T., S.N., Y.N., and K.O. conceived and designed this study. S.N. collected data and

performed the bioinformatic analysis and data visualization throughout this study. K.T.

performed the meta-transcriptomic read mapping analysis. K.T., Y.N., K.O., T.D., K.H.,

and S.Y. provided critical interpretations of the data. S.N. drafted the manuscript. All

authors revised and approved the final version.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Data availability

534 The code and datasets generated and analyzed in this study are available in our Zenodo

535 repository, https://doi.org/10.5281/zenodo.16318636.

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Figure 1. Characterization of the Unknome from a phylogenetically comprehensive

SAR11 genome dataset.

(A) Maximum likelihood phylogenetic tree of SAR11 genomes. The color coding in the

phylogenetic tree indicates five subclade classifications. The colors below provides

more detailed subclade classifications and types of the genomes, that is MAG (red),

SAG (blue), or pure culture (light blue). The dots on the tree show UltraFast Bootstrap

values > 95%. **(B,C)** Sankey plots summarize the annotation profiles of all genes/OGs

and transmembrane region prediction results. Blue shows the unknome genes/OGs. TM

indicates transmembrane proteins predicted using TMHMM 2.0 (see Methods for

details). **(D)** COG annotation results for all OGs. NA denotes sequences not annotated

in the COG database. Blue bars show unknome genes in this study.

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Figure 2. Function prediction based on genomic context and protein structural similarity search.

(A) Summary of neighboring gene analysis. Network nodes represent each OGs, and colors indicate the COG functional categories. Pairs with a co-localization probability

$\geqq$ 75% within five genes upstream and downstream on the same contig are connected

by edges. Several known operons are highlighted with dashed outlines as examples. **(B)** Predicted protein complex structure of OG688, OG1772, OG1815, and OG1549 using AlphaFold-multimer. The complex forms an octameric assembly composed of four homodimers. The colors show the COG functional category. **(C, D)** Genome context around each network component in panel A. The genes outlined in bold were used for protein structure prediction in panel B. **(E)** Scatter plots of all *Ca.* P. ubique HTCC1062 strain proteins compared with known protein structures in the PDB. The dot lines show e-value = 1e-5 and amino acid identity = 20%, highlighting potential remote homologs

Figure 3. The core unknome distribution and functional analysis of DUF2237-containing protein.

(A) Environmental distribution of bacteriorhodopsin (COG5524) and COG-assigned core unknomes based on GMGC. Bar plot colors indicate habitat, and the COG ID color indicate whether function could be inferred using the bioinformatics approaches. **(B)** Phylogenetic distribution of the DUF2237-containing gene and proteorhodopsin genes on representative bacterial genomes in the OceanDNA MAG catalog. The heatmap below indicates phylum, and the presence of DUF2237-containing gene and proteorhodopsin gene. **(C)** The amino acid conservation of 7,963 DUF2237-containing genes in the Pfam database, mapped onto the predicted structure of the DUF2237

41   protein from *Ca.* P. ubique HTCC1062. Conservation is color-coded from blue (<10%),

42   to white (50%), to purple (>90%) based on the multiple sequence alignment. **(D)** Top

43   docking pose of *P. ubique* HTCC1062 DUF2237 protein (light blue) and NADH (red).

Figure 4. Defense genes identified in SAR11 genomes.

(A) Sporadic distribution of defense systems. Heatmap colors indicate genome
completeness and presence of defense systems. **(B)** Operon structure of known
DISARM systems and AG-447-N21 coding DISARM-like system. The colors indicate
function as follows: helicase (blue), methylase (yellow), DUF1998-containing protein
(green), gray (unknown), and PLD domain (white; not required for defense). **(C)**
Predicted structure comparison of the known ShosTA proteins from DefenseFinder wiki
(blue) and the ShosTA-like proteins in HTCC1002 (orange). Domain loss is highlighted
by the dotted box. **(D)** Predicted structure comparison of known AbiRbc proteins from
DefenseFinder wiki (blue) and AbiRbc-like proteins in AG-313-A04 (orange). **(E)** The
cumulative proportion of genomes with detected viral defense systems is plotted against
genome completeness. The cumulative proportion at each completeness value

58 represents the fraction of genomes at or below that completeness in which at least one
59 defense system (from panel A) was detected. Solid blue and red lines show the
60 cumulative proportion for SAGs and MAGs, respectively. The light blue dashed line
61 indicates the detection rate in 24 cultured genomes, serving as a reference.
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