

Mechanisms Driving Genome Reduction of a Novel *Roseobacter* Lineage Showing Vitamin B₁₂ Auxotrophy

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Keywords: *Roseobacter*, CHUG, genome reduction, vitamin B₁₂ auxotrophy

28 **Summary**

29 Members of the marine *Roseobacter* group are key players in the global carbon and
30 sulfur cycles. While over 300 species have been described, only 2% possess reduced
31 genomes (mostly 3-3.5 Mbp) compared to an average roseobacter (>4 Mbp). These
32 taxonomic minorities are phylogenetically diverse but form a Pelagic *Roseobacter* Cluster
33 (PRC) at the genome content level. Here, we cultivated eight isolates constituting a novel
34 *Roseobacter* lineage which we named 'CHUG'. Metagenomic and metatranscriptomic read
35 recruitment analyses showed that CHUG members were globally distributed and active in
36 marine environments. CHUG members possess some of the smallest genomes (~2.52 Mb)
37 among all known roseobacters, but they do not exhibit canonical features of genome
38 streamlining like higher coding density or fewer paralogues and pseudogenes compared to
39 their sister lineages. While CHUG members are clustered with traditional PRC members at
40 the genome content level, they show important differences. Unlike other PRC members,
41 neither the relative abundances of CHUG members nor their gene expression levels are
42 correlated with chlorophyll a concentration across the global samples. Moreover, CHUG
43 members cannot synthesize vitamin B₁₂, a key metabolite made by most roseobacters but not
44 by many phytoplankton species and thus thought to mediate the roseobacter-phytoplankton
45 interactions. This combination of features is evidence for the hypothesis that CHUG members
46 may have evolved a free-living lifestyle decoupled from phytoplankton. This ecological
47 transition was accompanied by the loss of signature genes involved in
48 roseobacter-phytoplankton symbiosis, suggesting that relaxation of purifying selection is
49 likely an important driver of genome reduction in CHUG.

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52 Introduction

53 The marine *Roseobacter* group is a subfamily-level lineage in the *Alphaproteobacteria*
 54 and plays an important role in global carbon and sulfur cycling (1, 2). It is highly abundant in
 55 the coastal environments, accounting for up to 20% of all bacterial cells (3–5). Over 300
 56 species and 100 genera have been described (6), the vast majority of which harbor large and
 57 variable genomes and grow readily on nutrient-rich solid media which are not representative
 58 of the niches found in the oligotrophic oceans. Early culture-independent 16S rRNA gene
 59 surveys showed that the oceanic roseobacters are represented by a few uncultivated lineages
 60 (1, 7). Recently, novel cultivation techniques and single-cell genomics have made available
 61 (partial) genome sequences of several previously uncultivated lineages including NAC11-7
 62 (8), DC5-80-3 (also called RCA) (9, 10) and CHAB-I-5 (11, 12). Although these lineages are
 63 spottily distributed throughout the *Roseobacter* phylogeny, they together form a pelagic
 64 *Roseobacter* cluster (PRC). The PRC members consistently harbor smaller genomes and
 65 show more similar genome content compared to other roseobacters (11). Learning their
 66 evolutionary histories is essential to understand how the genetic and metabolic diversity of
 67 the pelagic *Roseobacter* lineages has formed, which in turn helps appreciate their roles in
 68 oceanic carbon and sulfur cycles. However, most PRC members form orphan branches and
 69 lack closely related reference genomes, which hampers our further understanding of their
 70 evolutionary trajectories.

71 Here, we isolated eight closely related roseobacters from several ocean regions that
 72 consistently possessed some of the smallest genomes (~2.52 Mb) among all known
 73 roseobacters. They together formed a novel *Roseobacter* lineage which we named ‘CHUG’
 74 (Clade Hidden and Underappreciated Globally) that was abundant and active in global oceans.
 75 Unlike other PRC members, the global distribution of CHUG members was uncorrelated with
 76 chlorophyll a (Chl-a) concentration and they cannot *de novo* synthesize vitamin B₁₂, which is

often the metabolite roseobacters supply to phytoplankton during their symbiosis (2, 13–15).
Therefore, the reductive evolution of CHUG may also indicate a dissociation with
phytoplankton, a feature so far unique to CHUG among pelagic roseobacters.

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81 **Materials and Methods**

Detailed methods are described in Supplementary Text 1. Briefly, samples were collected
from surface water of the South China Sea, the East China Sea and the northern Gulf of
Mexico. Over 20 CHUG isolates were retrieved following different dilution cultivation
procedures, and genomes of eight isolates from the three ocean regions were sequenced with
Illumina platforms, assembled with SPAdes (16) and annotated with Prokka (17). Among
these, the isolate HKCCA1288 was further sequenced with PacBio Sequel platform to obtain
a complete and closed genome. The average nucleotide identity (ANI) between genomes was
calculated using fastANI (18). The assembled genome size, gene number, coding density and
GC content of each genome were obtained using CheckM (19), whereas the estimated
genome size was adjusted as $(\text{assembled genome size})/(\text{completeness} + \text{contamination})$
(20). Pseudogenes were predicted following our recent study (21), and other genomic features
were summarized using custom scripts
(<https://github.com/luolab-cuhk/CHUG-genome-reduction-project>). The phylogenetic
ANOVA analyses were performed to compare the analyzed genomic traits while controlling
for the evolutionary history of those traits using the ‘phylANOVA’ function of the ‘phytools’
R package (22).

The *TARA* Ocean metagenomic and metatranscriptomic sequencing data with size
fractions up to 3 μm (prokaryote-enriched) (23, 24) and metagenomic sequencing data with
size fraction of 5-20 μm (nanoplankton-enriched) (25) were mapped to all 79 roseobacters
studied here using bowtie (26) and BLASTN (27). Only reads sharing >95% similarity

and >80% alignment to their best hit were kept for relative abundance and activity calculation. The correlation analysis was performed using the ‘rcorr’ function in the ‘Hmisc’ R package (28), and the significance level was adjusted using stringent Bonferroni correction for multiple comparisons.

The *Roseobacter* phylogeny was constructed based on 120 bacterial marker genes (29), and the reference *Roseobacter* genomes included in the phylogeny followed a previous study (30). The orthologous gene families were predicted with OrthoFinder (31), and a binary matrix of the presence and absence pattern of orthologous gene families were used to construct the genome content dendrogram. The gene copy number of each orthologous family was further used to estimate the ancestral genome content for CHUG, its sister group and the outgroup using BadiRate (32).

Results

The CHUG diversity

Eight strains constituting a novel lineage (Fig. 1A) within the *Roseobacter* group, which we named Clade Hidden and Underappreciated Globally (CHUG), were isolated from the coastal waters of the South China Sea, the East China Sea, and the northern Gulf of Mexico (Table S1). Their genomes shared $\geq 99.7\%$ 16S rRNA gene identity and $\geq 93\%$ whole-genome average nucleotide identity (ANI). The CHUG lineage further exhibited $\geq 98.2\%$ 16S rRNA gene identity when sequences of a few uncultivated members were added (Fig. S1), which was comparable to other pelagic *Roseobacter* lineages (98% (10) for DC5-80-3 and 96% (33) or 98% (7) for CHAB-I-5). CHUG genomes had $\leq 96.5\%$ 16S rRNA gene identity and $\leq 71\%$ ANI compared to the sister group members (Fig. 1A). All CHUG isolates were sampled exclusively from pelagic environments, whereas members of their sister group and the outgroup inhabit highly diverse salty environments including pelagic ocean, saline lake, algal

culture and coastal sediment (Table S1).

We also constructed a dendrogram based on the presence/absence pattern of orthologous gene families (Fig. 1B). Although not monophyletic in the phylogeny based on shared genes (Fig. 1A), CHUG and seven other genomes from taxa previously sampled from pelagic environments formed a coherent group called the Pelagic *Roseobacter* Cluster (PRC) (11). One previously identified PRC member, *Roseobacter* sp. R2A57 (4.13 Mb), was not affiliated with PRC in this study. To facilitate our analysis and discussion, we divided the 79 roseobacters used in the present study into five groups: CHUG (eight genomes), its sister group (five genomes), the outgroup of CHUG and its sister group (six genomes), other PRC members (seven genomes) and other reference roseobacters (53 genomes).

Genomic features

Among the eight CHUG genomes, one (HKCCA1288) was closed with 2.66 Mb and the remaining draft genomes were nearly complete ($\geq 98.5\%$) according to CheckM predictions (Table S1). Among other roseobacter genomes under comparison, at least 17 genomes were closed and the remaining ones were nearly complete ($\geq 96.5\%$) (Table S1). Based on the assembled genome sizes, CHUG members possessed much smaller genomes (2.52 ± 0.07 Mb, Fig. 2A) than an average roseobacter (4.16 ± 0.68 Mb). Further, their genome sizes were comparable to those of the NAC11-7 cluster represented by the strain HTCC2255 (estimated complete size to be 2.34 Mb), which is a basal roseobacter with the smallest genome among all known roseobacters (34). As in HTCC2255, no plasmids were found in the CHUG genomes. However, the coding density of CHUG ($91.7 \pm 0.5\%$, Fig. 2B) showed no significant difference with its sister group and the outgroup ($90.7 \pm 0.7\%$ and $90.7 \pm 0.6\%$, respectively) based on the phylogenetic ANOVA analysis ($p > 0.05$, ‘phylANOVA’ in the ‘phytools’ R package; the same test used below unless stated otherwise), which performs a

simulation-based comparison while taking into account the influence of phylogeny on the trait evolution (22). CHUG genomes had a lower genomic GC content ($55.4 \pm 0.8\%$, Fig. 2C) compared to their sister group ($63.5 \pm 1.6\%$, $p < 0.05$), although no significant difference was identified compared to the outgroup ($63.8 \pm 2.6\%$). In terms of pseudogenes, the number (99 ± 24 , Fig. 2D) and ratio ($3.9 \pm 0.9\%$, Fig. 2E) in CHUG members were not significantly different from those of the sister group (128 ± 51 ; $3.3 \pm 1.1\%$) and outgroup (148 ± 37 ; $3.7 \pm 0.9\%$). The seven other PRC members also had smaller genomes (3.26 ± 0.51 Mb, Fig. 2A) and a reduced GC content ($49.6 \pm 5.5\%$, Fig. 2C) compared to the 53 other reference roseobacters (genome size: 4.32 ± 0.64 Mb, GC content: $61.9 \pm 4.1\%$; $p < 0.01$), but there was no significant difference between the two groups in terms of the coding density ($90.4 \pm 0.9\%$ for seven PRC members versus $89.3 \pm 1.5\%$ for other roseobacters, Fig. 2B), or the number (108 ± 49 for seven PRC members versus 205 ± 134 for other roseobacters, Fig. 2D) and ratio of pseudogenes ($3.2 \pm 1.4\%$ for seven PRC members versus $4.7 \pm 2.4\%$ for other roseobacters, Fig. 2E).

CHUG genomes showed increased use of carbon atoms per amino-acid-residue side chain (C-ARSC, 2.833 ± 0.005 , Fig. 2F) compared to the sister group (2.799 ± 0.004 , $p < 0.05$). However, no significant difference was found in CHUG members in the use of C-ARSC compared to the outgroup (2.803 ± 0.011), nor that of nitrogen atoms per amino-acid-residue side chain (N-ARSC, 0.345 ± 0.002 , Fig. 2G) compared to the sister group (0.344 ± 0.008) or the outgroup (0.346 ± 0.006). Likewise, the seven other PRC genomes had significantly higher C-ARSC (2.879 ± 0.031 , Fig. 2F) than the 53 other reference roseobacters (2.817 ± 0.026 , $p < 0.01$), but there was no significant difference between their N-ARSC (0.336 ± 0.004 for seven PRC members versus 0.348 ± 0.009 for other roseobacters, Fig. 2G).

We further investigated the codon usage and amino acid usage patterns in these lineages.

The CHUG genomes tended to comprise codons with more adenine/thymine (A/T) and less guanine/cytosine (G/C) for 11 amino acids compared to the sister group and for 12 amino acids compared to the outgroup, respectively ($p < 0.05$, Fig. S2 and Supplementary Text 2.1). Furthermore, CHUG members possessed a higher fraction of isoleucine and lysine in their proteomes but a lower fraction of glycine, proline, valine and tryptophan when compared to the sister group or outgroup ($p < 0.05$, Fig. S3), which may be partially affected by the differences of nitrogen (N) use in their corresponding codons (Supplementary Text 2.1).

Consistent with their genome size differences, CHUG genomes contained a significantly smaller number of coding genes ($2,486 \pm 78$, Fig. 2H) compared to the outgroup ($3,939 \pm 214$, $p < 0.01$) and the seven other PRC genomes ($3,253 \pm 545$ genes, $p < 0.05$). The CHUG genomes contained $2,215 \pm 70$ orthologous gene families (Fig. 2I) with 1.12 ± 0.01 gene copy per family (Fig. 2J). By comparison, the outgroup genomes contained $3,259 \pm 130$ families ($p < 0.01$) orthologous gene families with 1.20 ± 0.04 ($p > 0.05$) gene copy per family, while the seven other PRC genomes possessed $2,678 \pm 398$ families ($p > 0.05$) orthologous gene families with 1.21 ± 0.05 ($p < 0.01$) gene copy per family. No significant difference occurred between CHUG and the sister group ($3,865 \pm 591$ genes, $3,197 \pm 345$ gene families and 1.20 ± 0.05 copy per family). Additionally, while the number of genes and number of gene copies per family of the seven other PRC genomes was not significantly different from those in the 53 other reference roseobacters ($4,199 \pm 644$ genes and 1.25 ± 0.12 copy per family, Fig. 2H,J), the seven other PRC genomes had fewer orthologous families compared to the 53 other reference roseobacters ($3,362 \pm 362$, $p < 0.01$, Fig. 2I).

Global distribution and ecological drivers

We used recruitment analysis with the global-scale *TARA* Ocean metagenomic and metatranscriptomic datasets with size fractions up to $3 \mu\text{m}$ (prokaryote-enriched) (23, 24) to

quantify the global distribution of CHUG and other PRC members. The eight CHUG members recruited 0.0005% and 0.0008% of all metagenomic (Fig. 3A) and metatranscriptomic (Fig. 3B) reads, respectively. The CHUG members appeared to be less abundant and less active than other PRC representatives such as *Rhodobacterales* bacterium HTCC2255 (NAC11-7), *Rhodobacteraceae* bacterium SB2 (CHAB-I-5) and *Planktomarina temperata* RCA23 (RCA or DC5-80-3) (Welch's t-test, $p < 0.01$ for each). A similar pattern was also found using *TARA* Ocean metagenomic sequencing data with the size fraction of 5-20 μm (nanoplankton-enriched; Fig. 3C) (25). The CHUG members further represented 1.165% of the total reads from the nutrient perturbation experiments in mesocosm situated in the Red Sea (Fig. S4A) (35), and they also showed seasonality, as they recruited 0.007%, 0.032% and 1.623% of the total reads sampled at Kwangyang bay ocean (36) in February, May and August 2015, respectively (Fig. S4B).

Next, we sought to identify the ecological factors that may drive the global distribution and activity of the CHUG members, and to compare it to seven other PRC members using the *TARA* Ocean metagenomic (Fig. 3D) and metatranscriptomic (Fig. 3E) samples. The relative abundance and activity of CHUG members and the PRC member *Rhodobacteraceae* bacterium HIMB11 were not correlated with other PRC members, chlorophyll a (Chl-a) concentration, or the total carbon (Fig. 3D,E; Bonferroni corrected $p < 0.05$). On the other hand, the relative abundances of other PRC members were mutually positively correlated with each other, with Chl-a concentration, and with total carbon in both metagenomic and metatranscriptomic samples (Fig. 3D,E; Bonferroni corrected $p < 0.05$). In addition, the activity of CHUG genomes was positively correlated with nitrate and depth (Fig. 3E; Bonferroni corrected $p < 0.05$). From a gene-centric perspective, $58.6\% \pm 1.2\%$ and $88.3\% \pm 12.7\%$ genes from the eight CHUG genomes and seven other PRC members recruited *TARA* metatranscriptomic reads, respectively. Among the most expressed gene families (top 5%),

many were housekeeping genes involved in transcription, translation, cell cycle, respiration, the tricarboxylic acid cycle (TCA) cycle, and the biosynthesis of amino acids, chaperones, cell wall, and capsule (Fig. 4). Both CHUG and other PRC members also had highly expressed genes for light utilization (e.g. the photosynthesis gene cluster or proteorhodopsin) and nutrient (e.g. carbohydrates and amino acid) transport. Additional highly expressed genes among CHUG members included those involved in zinc transport, the cytochrome *cbb₃*-type oxidase, acetate transporters, and genes for mercury homeostasis, among which the latter two were exclusively found in CHUG members (Fig. 4). On the other hand, some highly expressed orthologous gene families specific to the seven other PRC members were related to phosphonate transport and taurine degradation.

Genome reduction and vitamin B₁₂ auxotrophy

Since CHUG has a well-supported sister group and outgroup (Fig. 1A), we reconstructed the gene gain and loss events that were associated with the origin of the CHUG cluster (Fig. 5A). The last common ancestor (LCA) of the CHUG cluster was estimated to have 2,320 genes, 2,134 orthologous gene families (1.09 gene copy per family), and a genome size of 2.35 Mb. There were 172 families (185 genes) gained and 406 families (425 genes) lost on the ancestral branch leading to the LCA of CHUG, while 28 and 52 families (30 and 79 genes) underwent copy number increase and decrease, respectively. Compared to its sister group and the outgroup, CHUG members lost 412 Kb (9.8%) on the ancestral branch leading to its LCA (filled triangle in Fig. 5A).

We further compared the metabolic potential between CHUG (Fig. 5A), the reconstructed ancestors (Fig. 5B), seven other PRC genomes (Fig. 5C), and other reference roseobacters (Table S2). Since the CHUG genomes experienced net DNA and gene losses, we explored whether metabolic auxotrophies (i.e., inability to synthesize a compound

required for the growth) arose as a result of these losses. Among the sequenced CHUG members, the genome of the strain HKCCA1288 was closed, which improved our auxotrophy inference. CHUG genomes harbored the complete pathways for the synthesis of all 20 amino acids, many of which, such as the synthesis of lysine (*dapD*) and methionine (*metH* and *ahcY*), were under active expression in the wild (Fig. 4). They further encoded the key genes for thiamine (vitamin B₁) synthesis (thiamine-phosphate pyrophosphorylase, *thiE*) and pyridoxine (vitamin B₆) synthesis (pyridoxamine 5'-phosphate oxidase, *pdxH*). Nevertheless, the key gene for biotin (vitamin B₇) synthesis (biotin synthase, *bioB*) was not found in CHUG nor in the sister group and the outgroup, suggesting that the biotin auxotrophy in CHUG was not part of their net gene losses.

Intriguingly, CHUG was auxotrophic for cobalamin (vitamin B₁₂) biosynthesis, which can be synthesized by most roseobacters (2). This was validated using a growth assay, in which CHUG strain HKCCA1288 did not grow in the defined medium lacking vitamin B₁₂ but grew well with the supplement of vitamin B₁₂ (Fig. 6A). As a comparison, the model roseobacter *Ruegeria pomeroyi* DSS-3, which is equipped with the *cobG* route, grew equally well in the presence or absence of vitamin B₁₂ (Fig. 6B). Mapping of the vitamin B₁₂ *de novo* synthesis to the phylogeny (Fig. 1A) indicates that the loss of this capability was most likely associated with the genome reduction leading to the LCA of the CHUG lineage. On the other hand, no genome content changes were inferred related to vitamin B₁₂ synthesis by the ancestral genome reconstruction (Fig. 5B). This controversy can be ascribed to the fact that the *de novo* synthesis of cobinamide has two non-homologous pathways (i.e., aerobic and anaerobic synthesis of cobinamide, the key precursor of vitamin B₁₂, via key genes *cobG* and *cbiX*, respectively), and distinct pathways are maintained in the CHUG sister lineages (Fig. 1A). The ancestral genome reconstruction further inferred that the loss of vitamin B₁₂ *de novo* synthesis capability was compensated with the coincidental gain of a putative vitamin B₁₂

transporter (Fig. 5B), which was absent from all other PRC members capable of *de novo* vitamin B₁₂ synthesis (Fig. 5C). Taken together, the loss of *de novo* synthesis capability and the gain of a putative transporter indicates that CHUG may have to acquire vitamin B₁₂ or its precursor from the environment.

Metabolic potential for community interaction

Besides the loss of genes for *de novo* vitamin B₁₂ synthesis, the CHUG members have also lost genes for chemotaxis (*cheAB*) and flagellar assembly (*fliC*). These genes were essential to mediate roseobacter-phytoplankton interactions (37), but may become dispensable when switching to a planktonic lifestyle (38). Consistent with this, the quorum-sensing (QS) system (*luxR*), type IV secretion system (*virB*), and type VI secretion system (*vasKF*) involved in organismal interactions were rarely found in the CHUG genomes (Fig. 5A). CHUG members also lost the gene cluster encoding gene transfer agent (GTA), which resembles small double-stranded DNA (dsDNA) bacteriophages that increase horizontal gene transfer (HGT) and metabolic flexibility at high population density (39).

Metabolic potential for nutrient acquisition

Nitrogen (N) is a primarily limiting nutrient in surface oceans (40). Genes encoding the nitrogen regulatory protein P-II (*glnBD*) were highly expressed in the wild CHUG populations (Fig. 4). Genes encoding the high-affinity ammonium transporter (*amtB*) and nitrogen regulation two-component system (*ntrBC* and *ntrXY*) were found in the CHUG genomes. Genes encoding urease (*ureABC*) were also identified in CHUG members, though the urea transport system (*urtABCDE*) was not in any CHUG genomes. It is possible that urea was assimilated via passive diffusion across the cell membrane in CHUG as shown in other bacteria (41), or that urea was taken up by another promiscuous transporter. The genes

encoding the transporter for nitrate/nitrite assimilation (*nrtABC*) were also missing in CHUG genomes. CHUG members retained the genes for the spermidine/putrescine transporter (*potABCD* and *ABC.SP*) (Table S2), and the latter was among the most highly expressed genes in the oceanic CHUG populations (Fig. 4). However, the CHUG members did not carry genes for other polyamine transport systems (*oocMQT* for octopine/nopaline and *potFGHI* for putrescine). The CHUG also retained and highly expressed *aapJMPQ* for the general L-amino acid transporter (Fig. 4), but lost genes encoding the polar amino acid transport system *ABC.PA*, which was prevalent in all other roseobacters studied here. CHUG further had a reduced number of genes (only one copy) encoding the branched-chain amino acid transport system (*livFGHKM*) compared to its sister group (at least three copies), the outgroup (at least three copies) and other PRC members (at least two copies; Table S2). Overall, fewer genes involved in the acquisition of amino acids were found in CHUG (Table S2), but they may remain efficient due to the high expression level of the retained genes.

Phosphorus (P) is often a co-limiting nutrient in surface oceans (40). To deal with P limitation, the CHUG members may be assisted by the essential regulatory and metabolic pathways known to be induced by P-limitation including the two-component regulatory system (*phoBR*), the high-affinity phosphate transporter (*pstABCS*) for phosphate acquisition and the C-P lyase (*phnGHIJKLM*) for phosphonate utilization. However, they have lost the *phoX* encoding an alkaline phosphatase for phosphodiester utilization (42) during the genome reduction process (Fig. 5A,B). A notable evolutionary innovation upon the emergence of the CHUG lineage was a gain of the gene encoding phospholipase C (*plcP*) (Fig. 5A,B), which was missing from all the seven other PRC members (Fig. 5C). The *plcP* is the key gene of the pathway for phospholipid substitution with non-phospholipids in response to P starvation, and was prevalently found in marine bacterioplankton (43).

327 Metabolic potential for energy acquisition

328 Members of the CHUG cluster maintained some energy conservation strategies that are
 329 commonly found in other roseobacters. One example was the acquisition of light energy. The
 330 complete photosynthesis gene cluster underlying the aerobic anoxygenic photosynthesis
 331 (AAnP) were identified in all CHUG members, five of the seven other PRC genomes, and 21
 332 of the 64 non-PRC genomes (Table S2). Other light energy acquisition mechanisms including
 333 genes encoding proteorhodopsin and xanthorhodopsin were only found in the PRC member
 334 HTCC2255 and in the two *Octadecabacter* genomes, respectively. Two marker genes (*pufAB*)
 335 of the photosynthesis gene cluster were among the most highly expressed genes in oceanic
 336 CHUG and other PRC members, and the proteorhodopsin in *Rhodobacterales* bacterium
 337 HTCC2255 was also highly expressed (Fig. 4). In total, the potential for light utilization was
 338 found in 14 of the 15 PRC members, but in only 23 of 64 non-PRC roseobacters (Table S2).
 339 The association of the light acquisition trait with the PRC members was significant, which
 340 remains when the biased phylogenetic distribution of this trait was under control as shown by
 341 the binaryPGLMM analysis ($p < 0.05$) (44). This result indicates that light utilization may
 342 facilitate their survival under nutrient-depleted pelagic environments (45, 46). However, it is
 343 not clear why the reduced CHUG genomes employ the photosynthesis gene cluster rather
 344 than a rhodopsin system for light acquisition, considering that the photosynthesis gene cluster
 345 consists of about 40 genes (46) whereas a rhodopsin system requires only the rhodopsin gene
 346 and an associated chromophore retinal gene (47). In fact, the possibility of an evolutionary
 347 replacement of photosynthesis gene cluster with proteorhodopsin remains open, because
 348 proteorhodopsin and photosynthesis gene cluster occur in two closely related ecotypes of
 349 DC5-80-3 (also called RCA), respectively (48), suggesting that the replacement of one
 350 phototrophic type with the other could happen rapidly.

351 Another example for energy conservation is the oxidation of reduced inorganic

compounds. The CHUG carried genes for the oxidation of carbon monoxide (CO) and sulfide/thiosulfate as energy sources. Most roseobacters encode type II carbon monoxide dehydrogenase (*codh*), but only those with type I CODH may perform CO oxidation (49). Four of the eight CHUG genomes possessed type I CODH (*coxL*) and thus may oxidize CO (Fig. 5). This gene was further identified in three genomes from its sister group, three genomes from the outgroup, three PRC genomes and 18 other reference genomes (Table S2). All CHUG members possessed the sulfide:quinone oxidoreductase (*sqr*) for the oxidation of sulfide to zero valence sulfur (S^0) (50), the persulfide dioxygenase (*pdo*) for the oxidation of S^0 to sulfite which could spontaneous react with S^0 to generate thiosulfate (50), and the complete *sox* pathway for the oxidation of thiosulfate to sulfate (51) (Fig. 5). The *sqr* and *pdo* were also found in four other PRC genomes and 32 of the 64 non-PRC genomes, while the *sox* pathway was found in all seven PRC genomes and 42 non-PRC genomes (Table S2). Unlike the capability of light utilization, no uneven distribution was identified for *coxL*, *sox*, *sqr* and *pdo* between PRC and non-PRC roseobacters (χ^2 test for *coxL* and binaryPGLMM analysis for the remaining genes; $p > 0.05$).

CHUG cannot perform nitrate/nitrite reduction for energy conservation due to the lack of genes involved in nitrate reduction to nitrite (nitrate reductase, periplasmic *napAB* or membrane-bound *narGHI*), nitrite reduction to ammonium (nitrite reductase, *nirBD*) or nitrite reduction to nitric oxide (NO-forming nitrite reductase, copper-containing *nirK* or haem-containing *nirS*) (2). The *narGHI* and *nirBD* were identified in some genomes affiliated with the sister group and the outgroup (Fig. 5A). These genes were also missing from other PRC genomes, but were found in some reference *Roseobacter* genomes (Table S2).

376 Other important metabolic pathways relevant to *Roseobacter* ecology

377 Among the major pathways for glycolysis, all CHUG members maintained the key gene
 378 encoding phosphogluconate dehydratase (*edd*) for the Entner-Doudoroff (ED) pathway, but
 379 none of them contained the key gene for phosphofructokinase (*pfk*) in the
 380 Embden-Meyerhof-Parnas (EMP) pathway (Fig. 5). Both pathways were prevalent in the
 381 sister group and the outgroup. Ancestral genome content reconstruction inferred that the EMP
 382 pathway was lost at the LCA of the CHUG lineage (Fig. 5B) as a result of genome reduction.
 383 Interestingly, the seven other PRC genomes held an identical pattern to CHUG, in which the
 384 ED pathway was universally preserved but the EMP pathway was missing. Although
 385 generating less ATP and NADH, the ED pathway can provide NADPH and may accompany
 386 increased resistance to oxidative stress compared with the EMP pathway (52). This likely
 387 confers an important benefit to these pelagic roseobacters inhabiting the surface ocean where
 388 reactive oxygen species (ROS) production is intensive (53). The catabolic product of the ED
 389 pathway, pyruvate, can be further degraded through the tricarboxylic acid cycle (TCA) cycle,
 390 the genes of which were highly expressed in environmental CHUG members (Fig. 4).

391 Many roseobacters can degrade aromatic compounds through the aerobic ring-cleaving
 392 pathways (54). All CHUG members harbored the protocatechuate ring cleavage pathway
 393 (protocatechuate 3,4-dioxygenase, *pcaGH*), which is one of the most common pathways for
 394 the degradation of monoaromatic compounds among roseobacters (55). However, they did
 395 not carry *paaABCDE* encoding ring-1,2-phenylacetyl-CoA epoxidase (key enzyme for the
 396 phenylacetate ring cleavage pathway) or *hmgA* encoding homogentisate 1,2-dioxygenase (key
 397 enzyme for the homogenisate ring cleavage pathway) (54). As these two pathways were
 398 inferred to be present in the LCA shared by CHUG and its sister group (filled circle in Fig.
 399 5A), we hypothesize that their absence from CHUG resulted from genome reduction. All the
 400 three ring cleavage pathways were common in the seven other PRC genomes (Table S2).

Methylated compounds are important substrates for roseobacters (56). Briefly, the CHUG members possessed the metabolic potential to utilize dimethylsulfoniopropionate (DMSP) via both demethylation (DMSP demethylase, *dmdA*) and cleavage (*dddD* or *dddL*) pathway. However, genes encoding trimethylamine dehydrogenase (*tmd*) and trimethylamine monooxygenase (*tmm*) involved in trimethylamine N-oxide (TMAO) and trimethylamine (TMA) degradation, respectively, were not identified in the CHUG genomes, nor in most genomes affiliated with their sister group and the outgroup. However, these genes were identified in some other PRC members. Genes involved in taurine transport (*tauABC*) and degradation (*xsc*) were not found in CHUG members, but they were present, and the latter was highly expressed, in seven other PRC members (Fig. 4).

Discussion

The CHUG population dynamics are uncoupled from phytoplankton abundance

Though the novel lineage CHUG and the previously known Pelagic *Roseobacter* Cluster (PRC) members all reach high global abundance and activity, the ecological factors driving their global distribution are different. DC5-80-3 and NAC11-7 abundances were previously shown to be positively correlated with phytoplankton blooms (1, 4, 57–60) and their abundance and activity were both found to be significantly correlated with Chl-a abundance here (Fig. 3D,E). In the PRC lineage CHAB-I-5, a few members carry signature genes mediating organismal interactions (e.g., type VI secretion system and quorum sensing) (12), and thus may also explore microenvironments such as phytoplankton and organic particles. In fact, CHAB-I-5 abundance and activity was also positively correlated with Chl-a across the global ocean samples (Fig. 3D,E), though such a correlation was not found in a previous study with a more limited sampling effort (11). In the case of CHUG, no significant correlation with Chl-a was identified (Fig. 3D,E). Indeed, when the *TARA Ocean*

metagenomic sequencing reads at the nanoplankton-enriched size fraction (5-20 μm) were recruited, CHUG members exhibited a lower relative abundance than the other PRC representatives by approximately one order of magnitude (Fig. 3C). Together, these data support our hypothesis that members of the CHUG lineage evolved a free-living lifestyle decoupled from phytoplankton.

The possible contrasting roles of CHUG versus other pelagic roseobacters in relationship to phytoplankton were further supported by the absence of the *de novo* vitamin B₁₂ synthesis in all CHUG members but its presence in all other PRC members. The auxotrophy for vitamin B₁₂ was also validated for HKCCA1288 - for which we generated a complete genome sequence - in a growth assay (Fig. 6). The marine eukaryotic algae are predominantly vitamin B₁₂ auxotrophs (61), whereas most roseobacters have the potential to synthesize vitamin B₁₂ (2). This complementarity is one of the major mechanisms that facilitate mutualistic interactions between roseobacters and phytoplankton (2, 13–15), which also helps explain why roseobacters are often among the most abundant bacteria associated with marine eukaryotic phytoplankton (62–64). The loss of vitamin B₁₂ synthesis in CHUG is unusual because members of the *Roseobacter* group are known as the dominant bacterial lineages associated with marine phytoplankton groups (65) and their evolutionary history was likely correlated with phytoplankton diversification (2, 66). They usually benefit from the fixed carbon or other excretions released by phytoplankton and, in return, produce secondary metabolites (e.g. vitamins, indole-3-acetic acid) to promote phytoplankton growth (15, 67, 68). These interactions likely occur in microzones immediately surrounding phytoplankton cells, which may create gene flow barriers and facilitate population differentiation of associated roseobacters (69). Therefore, the ecology and evolution of the *Roseobacter* group in the pelagic ocean are generally shaped by marine phytoplankton, making the possible separation from this ecological pattern in the CHUG lineage unique.

451

452 Potential evolutionary forces driving genome reduction of the CHUG roseobacters

453 The most abundant marine bacterioplankton, such as the *Pelagibacterales* (also called
454 the SAR11 clade) in the Alphaproteobacteria and the *Prochlorococcus* in Cyanobacteria, are
455 often equipped with very small genomes (70). The evolutionary mechanisms driving their
456 genome reduction have been discussed extensively. Among these, positive selection for
457 metabolic efficiency (i.e., ‘genome streamlining’) has been theorized as the dominant force
458 driving their genome reduction (70, 71). Although CHUG members possessed smaller
459 genomes and lower GC content compared to the sister group and the outgroup, they did not
460 show other features of genome streamlining, such as higher coding density, fewer paralogues,
461 or a lower proportion of pseudogenes (70, 72). Therefore, the genome reduction process of
462 CHUG members did not meet the canonical definition of ‘genome streamlining’.

463 Other important evidence against the genome streamlining explanation for CHUG
464 genome reduction was from the genomic proxies for nutrient acquisition and saving strategies
465 used by marine bacterioplankton. Among the selective factors that may drive
466 bacterioplankton genome reduction in the pelagic ocean, N limitation is considered as the
467 dominant factor (34, 70, 73, 74). Although the relative abundance of gene transcripts (but not
468 the genes) in the wild CHUG populations was positively correlated with the nitrate
469 concentration (Fig. 3E), which provides marginal evidence for a role of N limitation, other
470 key evidence was missing. For example, we did not observe a reduced use of N in the amino
471 acid sequences (approximated by N-ARSC) in CHUG compared to the sister group and the
472 outgroup. Similar observation was used as evidence against the hypothesis that N limitation is
473 a strong driver of genome streamlining in other marine bacterioplankton lineages (75, 76). A
474 second potential ecological factor driving genome streamlining is P limitation (77), though
475 this theory has been debated (78). Genome reduction likely leads to a sizable decrease in

cellular P requirement and thus may confer a competitive advantage in the P-limited marine environments (79). Although a few important genes for P acquisition (*pst* for high-affinity phosphate transporter and *phn* for C-P lyase) were retained during the CHUG genome reduction and a gene encoding phospholipase C (*plcP*) responsible for cell membrane phospholipid substitution for non-phosphorus lipids (43) was even acquired, the key P scavenging gene encoding PhoX alkaline phosphatase was lost (Fig. 5). Therefore, available evidence for either N or P limitation as a driver of CHUG genome reduction was self-contradictory.

Because evidence for genome streamlining was weak in this lineage, we examined neutral evolutionary forces as potential explanations for CHUG genome reduction. In fact, neutral mechanisms have recently been considered to play an important role in driving genome reduction of marine bacterioplankton lineages (80–82). Most of the prior studies focused on *Prochlorococcus* (see references cited in the following paragraphs). While some extended their discussions to *Pelagibacteriales* (81, 83), knowledge on the evolutionary mechanisms driving genome reduction of most other marine bacterioplankton lineages is rather limited.

One potentially important neutral driver is random genetic drift due to a reduction of effective population size (N_e). A previous study showed that the major genome reduction event coincided with an accelerated rate of accumulating deleterious mutations in the early evolution of *Prochlorococcus*, providing important evidence that genetic drift was likely the primary mechanism of genome reduction in this lineage (81). Specifically, the power of genetic drift (i.e., the inverse of N_e) of an ancestral lineage (e.g., the ancestral branch underlying the ancient genomic events) can be approximated by the ratio of the radical nonsynonymous nucleotide substitutions per radical nonsynonymous site (d_R) to the conservative nonsynonymous nucleotide substitutions per conservative nonsynonymous site

(d_C) (81). Because the replacement by a physicochemically dissimilar amino acid (or radical change) is likely to be more deleterious than the replacement by a similar amino acid (or conservative change) (84, 85), the elevated d_R/d_C ratio is evidence for genetic drift acting to accumulate the deleterious type of mutations (i.e., the radical changes) in excess. In terms of the CHUG, the d_R/d_C ratio was not significantly elevated compared to its sister group (Fig. S5A) under two independent methods for biochemical classification of the 20 amino acids (Fig. S5B,C), suggesting that the deleterious type of mutations was not accumulated in excess at the ancestral branch leading to the LCA of the CHUG lineage (filled triangle in Fig. 5A). Since this ancestral branch corresponds to the time when major genome reduction occurred for CHUG, we can conclude that genetic drift was unlikely to be an important driver of CHUG genome reduction.

A second potentially important neutral driver of prokaryotic genome reduction is increased mutation rate, which was also proposed to explain *Prochlorococcus* genome reduction (86). Mathematical modeling predicts that not all auxiliary genes can be maintained by purifying selection when mutation rate is increased, and that an increase of 10 fold in mutation rate may lead to a 30% decrease in genome size (87). More recently, this hypothesis was supported with empirical data from comparative genomics analyses (82), though whether increased mutation rate is a truly important driver of prokaryotic genome reduction is debated (88). Given the potentially important role of increased mutation rate in driving prokaryotic genome reduction, determining the unbiased spontaneous mutation rate of the CHUG and the sister lineage using the mutation accumulation experiment followed by whole genome sequencing of the mutant lines becomes an urgent research need.

One more potentially important but rarely discussed neutral force leading to genome reduction is the loss of the genes that were important in the initial habitat but became dispensable after the bacteria switched to a new environment. This neutral loss mechanism,

termed relaxation of purifying selection, may also have contributed to genome reduction in *Prochlorococcus* (89). Importantly, the loss of dispensable genes under this mechanism is not related to the change of N_e but results instead from a change of habitat or lifestyle. Unlike other pelagic roseobacter members, CHUG members do not exhibit a correlative pattern between their global distributions and Chl-a (Fig. 3D,E), which can be used as a proxy for phytoplankton abundances (90). This is supported by evidence at the molecular and physiological level, in which the *de novo* synthesis of vitamin B₁₂, a fundamental metabolite roseobacters produce and supply to phytoplankton, was missing from all CHUG members but present in all other pelagic roseobacters (Fig. 1A, Fig. 6). Once the capability of *de novo* vitamin B₁₂ synthesis was lost, the CHUG ancestor may have lost its ability to establish symbiosis with phytoplankton and subsequently undergone a major shift of its planktonic lifestyle, namely from phytoplankton-associated to free-living. Given that phytoplankton cell surfaces can be more densely populated compared to the bulk seawater (65), genes contributing to roseobacter-phytoplankton symbiosis (e.g., motility and chemotaxis), depending on population density and involved in interactions with other bacteria (e.g., quorum sensing, gene transfer agent), may have become dispensable during this transition (38). Indeed, the loss of these signature genes contributed to the genome reduction of CHUG (Fig. 5). We therefore propose that the relaxation of purifying selection may be one of the primary evolutionary forces leading to the major genome reduction of CHUG.

Data availability

Genomic sequences of the eight CHUG genomes are available at the NCBI GenBank database under the accession number PRJNA574877.

Code availability

The custom scripts used in this study are available in the online repository (<https://github.com/luolab-cuhk/CHUG-genome-reduction-project>).

Acknowledgments

This research was funded by the National Science Foundation of China (41776129), the Hong Kong Research Grants Council General Research Fund (14163917), the Hong Kong Research Grants Council Area of Excellence Scheme (AoE/M-403/16), and the Direct Grant of CUHK (4053257 & 3132809). The research was also supported by a Louisiana Board of Regents grant (LEQSF(2014-17)-RD-A-06) and a Simons Early Career Investigator in Marine Microbial Ecology and Evolution Award to JCT.

Conflict of Interest

The authors declare no competing interests concerning the submitted work.

References

1. Buchan A, González JM, Moran MA. Overview of the marine *Roseobacter* lineage. Appl Environ Microbiol 2005; 71(10):5665–77.
2. Luo H, Moran MA. Evolutionary ecology of the marine *Roseobacter* clade. Microbiol Mol Biol Rev 2014; 78(4):573–87.
3. Moran MA, Belas R, Schell MA, González JM, Sun F, Sun S et al. Ecological genomics of marine Roseobacters. Appl Environ Microbiol 2007; 73(14):4559–69.
4. Giebel H-A, Kalhoefer D, Lemke A, Thole S, Gahl-Janssen R, Simon M et al. Distribution of *Roseobacter* RCA and SAR11 lineages in the North Sea and characteristics of an abundant RCA isolate. ISME J 2011; 5(1):8–19.
5. Wemheuer B, Wemheuer F, Hollensteiner J, Meyer F-D, Voget S, Daniel R. The green

- 577 impact: bacterioplankton response toward a phytoplankton spring bloom in the southern
578 North Sea assessed by comparative metagenomic and metatranscriptomic approaches. *Front*
579 *Microbiol* 2015; 6:805.
- 580 6. Pujalte MJ, Lucena T, Ruvira MA, Arahal DR, Macián MC. The Family
581 *Rhodobacteraceae*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F,
582 editors. *The Prokaryotes*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014. p. 439–512.
- 583 7. Buchan A, Hadden M, Suzuki MT. Development and application of quantitative-PCR tools
584 for subgroups of the *Roseobacter* clade. *Appl Environ Microbiol* 2009; 75(23):7542–7.
- 585 8. Luo H, Swan BK, Stepanauskas R, Hughes AL, Moran MA. Comparing effective
586 population sizes of dominant marine alphaproteobacteria lineages. *Environ Microbiol Rep*
587 2014; 6(2):167–72.
- 588 9. Giebel H-A, Kalhoefer D, Gahl-Janssen R, Choo Y-J, Lee K, Cho J-C et al. *Planktomarina*
589 *temperata* gen. nov., sp. nov., belonging to the globally distributed RCA cluster of the marine
590 *Roseobacter* clade, isolated from the German Wadden Sea. *Int J Syst Evol Microbiol* 2013;
591 63(Pt 11):4207–17.
- 592 10. Voget S, Wemheuer B, Brinkhoff T, Vollmers J, Dietrich S, Giebel H-A et al. Adaptation
593 of an abundant *Roseobacter* RCA organism to pelagic systems revealed by genomic and
594 transcriptomic analyses. *ISME J* 2015; 9(2):371–84.
- 595 11. Billerbeck S, Wemheuer B, Voget S, Poehlein A, Giebel H-A, Brinkhoff T et al.
596 Biogeography and environmental genomics of the *Roseobacter*-affiliated pelagic CHAB-I-5
597 lineage. *Nat Microbiol* 2016; 1(7):16063.
- 598 12. Zhang Y, Sun Y, Jiao N, Stepanauskas R, Luo H. Ecological genomics of the
599 uncultivated marine *Roseobacter* lineage CHAB-I-5. *Appl Environ Microbiol* 2016;
600 82(7):2100–11.
- 601 13. Wagner-Döbler I, Ballhausen B, Berger M, Brinkhoff T, Buchholz I, Bunk B et al. The
602 complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's
603 guide to life in the sea. *ISME J* 2010; 4(1):61–77.
- 604 14. Durham BP, Sharma S, Luo H, Smith CB, Amin SA, Bender SJ et al. Cryptic carbon and
605 sulfur cycling between surface ocean plankton. *Proc Natl Acad Sci U S A* 2015;
606 112(2):453–7.
- 607 15. Cooper MB, Kazamia E, Helliwell KE, Kudahl UJ, Sayer A, Wheeler GL et al.

608 Cross-exchange of B-vitamins underpins a mutualistic interaction between *Ostreococcus*
609 *tauri* and *Dinoroseobacter shibae*. ISME J 2018.

610 16. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS et al. SPAdes:
611 a new genome assembly algorithm and its applications to single-cell sequencing. J Comput
612 Biol 2012; 19(5):455–77.

613 17. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;
614 30(14):2068–9.

615 18. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput
616 ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat Commun
617 2018; 9(1):5114.

618 19. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the
619 quality of microbial genomes recovered from isolates, single cells, and metagenomes.
620 Genome Res 2015; 25(7):1043–55.

621 20. Parks DH, Rinke C, Chuvochina M, Chaumeil P-A, Woodcroft BJ, Evans PN et al.
622 Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of
623 life. Nat Microbiol 2017; 2(11):1533–42.

624 21. Chu X, Li S, Wang S, Luo D, Luo H. Gene loss through pseudogenization contributes to
625 the ecological diversification of a generalist *Roseobacter* lineage. ISME J 2020.

626 22. Revell LJ. phytools: an R package for phylogenetic comparative biology (and other
627 things). Methods in Ecology and Evolution 2012; 3(2):217–23.

628 23. Sunagawa S, Coelho LP, Chaffron S, Kultima JR, Labadie K, Salazar G et al. Ocean
629 plankton. Structure and function of the global ocean microbiome. Science 2015;
630 348(6237):1261359.

631 24. Salazar G, Paoli L, Alberti A, Huerta-Cepas J, Ruscheweyh H-J, Cuenca M et al. Gene
632 expression changes and community turnover differentially shape the global ocean
633 metatranscriptome. Cell 2019; 179(5):1068-1083.e21.

634 25. Vargas C de, Audic S, Henry N, Decelle J, Mahé F, Logares R et al. Eukaryotic plankton
635 diversity in the sunlit ocean. Science 2015; 348(6237):1261605.

636 26. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012;
637 9(4):357–9.

- 638 27. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
639 tool. J Mol Biol 1990; 215(3):403–10.
- 640 28. Harrell Jr FE. Package ‘Hmisc’. CRAN2018 2019; 2019:235–6.
- 641 29. Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil P-A et al. A
642 standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of
643 life. Nat Biotechnol 2018; 36(10):996–1004.
- 644 30. Simon M, Scheuner C, Meier-Kolthoff JP, Brinkhoff T, Wagner-Döbler I, Ulbrich M et al.
645 Phylogenomics of *Rhodobacteraceae* reveals evolutionary adaptation to marine and
646 non-marine habitats. ISME J 2017; 11(6):1483–99.
- 647 31. Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative
648 genomics. Genome Biol 2019; 20(1):238.
- 649 32. Librado P, Vieira FG, Rozas J. BadiRate: estimating family turnover rates by
650 likelihood-based methods. Bioinformatics 2012; 28(2):279–81.
- 651 33. Lekunberri I, Gasol JM, Acinas SG, Gómez-Consarnau L, Crespo BG, Casamayor EO et
652 al. The phylogenetic and ecological context of cultured and whole genome-sequenced
653 planktonic bacteria from the coastal NW Mediterranean Sea. Syst Appl Microbiol 2014;
654 37(3):216–28.
- 655 34. Luo H, Swan BK, Stepanauskas R, Hughes AL, Moran MA. Evolutionary analysis of a
656 streamlined lineage of surface ocean *Roseobacters*. ISME J 2014; 8(7):1428–39.
- 657 35. Coello-Camba A, Diaz-Rua R, Duarte CM, Irigoien X, Pearman JK, Alam IS et al.
658 Picocyanobacteria community and cyanophage infection responses to nutrient enrichment in
659 a mesocosms experiment in oligotrophic waters. Front. Microbiol. 2020; 11.
- 660 36. Kim Y, Jeon J, Kwak MS, Kim GH, Koh I, Rho M. Photosynthetic functions of
661 *Synechococcus* in the ocean microbiomes of diverse salinity and seasons. PLoS ONE 2018;
662 13(1):e0190266.
- 663 37. Geng H, Belas R. Molecular mechanisms underlying *Roseobacter* phytoplankton
664 symbioses. Curr Opin Biotechnol 2010; 21(3):332–8.
- 665 38. Luo H, Moran MA. How do divergent ecological strategies emerge among marine
666 bacterioplankton lineages? Trends Microbiol 2015; 23(9):577–84.
- 667 39. Biers EJ, Wang K, Pennington C, Belas R, Chen F, Moran MA. Occurrence and

668 expression of gene transfer agent genes in marine bacterioplankton. Appl Environ Microbiol
669 2008; 74(10):2933–9.

670 40. Moore CM, Mills MM, Arrigo KR, Berman-Frank I, Bopp L, Boyd PW et al. Processes
671 and patterns of oceanic nutrient limitation. Nature Geosci 2013; 6(9):701–10.

672 41. Veaudor T, Cassier-Chauvat C, Chauvat F. Genomics of urea transport and catabolism in
673 Cyanobacteria: biotechnological implications. Front Microbiol 2019; 10:2052.

674 42. Luo H, Benner R, Long RA, Hu J. Subcellular localization of marine bacterial alkaline
675 phosphatases. Proc Natl Acad Sci U S A 2009; 106(50):21219–23.

676 43. Sebastián M, Smith AF, González JM, Fredricks HF, van Mooy B, Koblížek M et al.
677 Lipid remodelling is a widespread strategy in marine heterotrophic bacteria upon phosphorus
678 deficiency. ISME J 2016; 10(4):968–78.

679 44. Paradis E, Schliep K. ape 5.0: an environment for modern phylogenetics and evolutionary
680 analyses in R. Bioinformatics 2019; 35(3):526–8.

681 45. Yooseph S, Nealson KH, Rusch DB, McCrow JP, Dupont CL, Kim M et al. Genomic and
682 functional adaptation in surface ocean planktonic prokaryotes. Nature 2010; 468(7320):60–6.

683 46. Brinkmann H, Göker M, Koblížek M, Wagner-Döbler I, Petersen J. Horizontal operon
684 transfer, plasmids, and the evolution of photosynthesis in *Rhodobacteraceae*. ISME J 2018;
685 12(8):1994–2010.

686 47. Pinhassi J, DeLong EF, Béjà O, González JM, Pedrós-Alió C. Marine Bacterial and
687 Archaeal Ion-Pumping Rhodopsins: Genetic Diversity, Physiology, and Ecology. Microbiol
688 Mol Biol Rev 2016; 80(4):929–54.

689 48. Sun Y, Zhang Y, Hollibaugh JT, Luo H. Ecotype diversification of an abundant
690 *Roseobacter* lineage. Environ Microbiol 2017; 19(4):1625–38.

691 49. Cunliffe M. Correlating carbon monoxide oxidation with cox genes in the abundant
692 Marine *Roseobacter* Clade. ISME J 2011; 5(4):685–91.

693 50. Xin Y, Liu H, Cui F, Liu H, Xun L. Recombinant *Escherichia coli* with sulfide:quinone
694 oxidoreductase and persulfide dioxygenase rapidly oxidises sulfide to sulfite and thiosulfate
695 via a new pathway. Environ Microbiol 2016; 18(12):5123–36.

696 51. Friedrich CG, Quentmeier A, Bardischewsky F, Rother D, Kraft R, Kostka S et al. Novel
697 genes coding for lithotrophic sulfur oxidation of *Paracoccus pantotrophus* GB17. J. Bacteriol.

698 2000; 182(17):4677–87.

699 52. Klingner A, Bartsch A, Dogs M, Wagner-Döbler I, Jahn D, Simon M et al. Large-Scale
700 ¹³C flux profiling reveals conservation of the Entner-Doudoroff pathway as a glycolytic
701 strategy among marine bacteria that use glucose. *Appl Environ Microbiol* 2015;
702 81(7):2408–22.

703 53. Diaz JM, Hansel CM, Voelker BM, Mendes CM, Andeer PF, Zhang T. Widespread
704 production of extracellular superoxide by heterotrophic bacteria. *Science* 2013;
705 340(6137):1223–6.

706 54. Gulvik CA, Buchan A. Simultaneous catabolism of plant-derived aromatic compounds
707 results in enhanced growth for members of the *Roseobacter* lineage. *Appl Environ Microbiol*
708 2013; 79(12):3716–23.

709 55. Alejandro-Marín CM, Bosch R, Nogales B. Comparative genomics of the protocatechuate
710 branch of the β -ketoadipate pathway in the *Roseobacter* lineage. *Marine Genomics* 2014;
711 17:25–33.

712 56. Chen Y. Comparative genomics of methylated amine utilization by marine *Roseobacter*
713 clade bacteria and development of functional gene markers (*tmm*, *gmaS*). *Environ Microbiol*
714 2012; 14(9):2308–22.

715 57. Wagner-Döbler I, Biebl H. Environmental biology of the marine *Roseobacter* lineage.
716 *Annu Rev Microbiol* 2006; 60:255–80.

717 58. West NJ, Obernosterer I, Zemb O, Lebaron P. Major differences of bacterial diversity and
718 activity inside and outside of a natural iron-fertilized phytoplankton bloom in the Southern
719 Ocean. *Environ Microbiol* 2008; 10(3):738–56.

720 59. Rich VI, Pham VD, Eppley J, Shi Y, DeLong EF. Time-series analyses of Monterey Bay
721 coastal microbial picoplankton using a ‘genome proxy’ microarray. *Environ Microbiol* 2011;
722 13(1):116–34.

723 60. Landa M, Blain S, Christaki U, Monchy S, Obernosterer I. Shifts in bacterial community
724 composition associated with increased carbon cycling in a mosaic of phytoplankton blooms.
725 *ISME J* 2016; 10(1):39–50.

726 61. Helliwell KE. The roles of B vitamins in phytoplankton nutrition: new perspectives and
727 prospects. *New Phytol* 2017; 216(1):62–8.

728 62. González JM, Simó R, Massana R, Covert JS, Casamayor EO, Pedrós-Alió C et al.
729 Bacterial community structure associated with a dimethylsulfoniopropionate-producing North
730 Atlantic algal bloom. *Appl Environ Microbiol* 2000; 66(10):4237–46.

731 63. Amin SA, Parker MS, Armbrust EV. Interactions between diatoms and bacteria.
732 *Microbiol Mol Biol Rev* 2012; 76(3):667–84.

733 64. Li S, Chen M, Chen Y, Tong J, Wang L, Xu Y et al. Epibiotic bacterial community
734 composition in red-tide dinoflagellate *Akashiwo sanguinea* culture under various growth
735 conditions. *FEMS Microbiol Ecol* 2019; 95(5).

736 65. Seymour JR, Amin SA, Raina J-B, Stocker R. Zooming in on the phycosphere: the
737 ecological interface for phytoplankton-bacteria relationships. *Nat Microbiol* 2017; 2:17065.

738 66. Luo H, Csuros M, Hughes AL, Moran MA. Evolution of divergent life history strategies
739 in marine alphaproteobacteria. *MBio* 2013; 4(4).

740 67. Durham BP, Dearth SP, Sharma S, Amin SA, Smith CB, Campagna SR et al. Recognition
741 cascade and metabolite transfer in a marine bacteria-phytoplankton model system. *Environ*
742 *Microbiol* 2017:3500–13.

743 68. Shibl AA, Isaac A, Ochsenkühn MA, Cárdenas A, Fei C, Behringer G et al. Diatom
744 modulation of select bacteria through use of two unique secondary metabolites. *Proc Natl*
745 *Acad Sci U S A* 2020; 117(44):27445–55.

746 69. Wang X, Zhang Y, Ren M, Xia T, Chu X, Liu C et al. Cryptic speciation of a pelagic
747 *Roseobacter* population varying at a few thousand nucleotide sites. *ISME J* 2020.

748 70. Giovannoni SJ, Cameron Thrash J, Temperton B. Implications of streamlining theory for
749 microbial ecology. *ISME J* 2014; 8(8):1553–65.

750 71. Giovannoni SJ, Tripp HJ, Givan S, Podar M, Vergin KL, Baptista D et al. Genome
751 streamlining in a cosmopolitan oceanic bacterium. *Science* 2005; 309(5738):1242–5.

752 72. Swan BK, Tupper B, Sczyrba A, Lauro FM, Martinez-Garcia M, González JM et al.
753 Prevalent genome streamlining and latitudinal divergence of planktonic bacteria in the
754 surface ocean. *Proc Natl Acad Sci U S A* 2013; 110(28):11463–8.

755 73. Luo H, Thompson LR, Stingl U, Hughes AL. Selection maintains low genomic GC
756 content in marine SAR11 lineages. *Mol Biol Evol* 2015; 32(10):2738–48.

757 74. Mende DR, Bryant JA, Aylward FO, Eppley JM, Nielsen T, Karl DM et al.

758 Environmental drivers of a microbial genomic transition zone in the ocean's interior. Nat
759 Microbiol 2017; 2(10):1367–73.

760 75. Grzyski JJ, Dussaq AM. The significance of nitrogen cost minimization in proteomes of
761 marine microorganisms. ISME J 2012; 6(1):71–80.

762 76. Lee MD, Ahlgren NA, Kling JD, Walworth NG, Rocap G, Saito MA et al. Marine
763 *Synechococcus* isolates representing globally abundant genomic lineages demonstrate a
764 unique evolutionary path of genome reduction without a decrease in GC content. Environ
765 Microbiol 2019; 21(5):1677–86.

766 77. Hessen DO, Jeyasingh PD, Neiman M, Weider LJ. Genome streamlining and the
767 elemental costs of growth. Trends Ecol Evol (Amst) 2010; 25(2):75–80.

768 78. Vieira-Silva S, Touchon M, Rocha EPC. No evidence for elemental-based streamlining of
769 prokaryotic genomes. Trends Ecol Evol (Amst) 2010; 25(6):319-20; author reply 320-1.

770 79. Thingstad T, Rassoulzadegan F. Conceptual models for the biogeochemical role of the
771 photic zone microbial food web, with particular reference to the Mediterranean Sea. Progress
772 in Oceanography 1999; 44(1-3):271–86.

773 80. Batut B, Knibbe C, Marais G, Daubin V. Reductive genome evolution at both ends of the
774 bacterial population size spectrum. Nat Rev Microbiol 2014; 12(12):841–50.

775 81. Luo H, Huang Y, Stepanauskas R, Tang J. Excess of non-conservative amino acid
776 changes in marine bacterioplankton lineages with reduced genomes. Nat Microbiol 2017;
777 2:17091.

778 82. Bourguignon T, Kinjo Y, Villa-Martín P, Coleman NV, Tang Q, Arab DA et al. Increased
779 mutation rate is linked to genome reduction in prokaryotes. Curr Biol 2020;
780 30(19):3848-3855.e4.

781 83. Viklund J, Ettema TJG, Andersson SGE. Independent genome reduction and
782 phylogenetic reclassification of the oceanic SAR11 clade. Mol Biol Evol 2012;
783 29(2):599–615.

784 84. Zuckerkandl E, Pauling L, Bryson V, Vogel HJ. Evolving genes and proteins. Science
785 1965:68–71.

786 85. Dayhoff MO. Atlas of protein sequence and structure. National Biomedical Research
787 Foundation; 1972.

788 86. Dufresne A, Garczarek L, Partensky F. Accelerated evolution associated with genome
789 reduction in a free-living prokaryote. *Genome Biol* 2005; 6(2):R14.

790 87. Marais GAB, Calteau A, Tenaillon O. Mutation rate and genome reduction in
791 endosymbiotic and free-living bacteria. *Genetica* 2008; 134(2):205–10.

792 88. Gu J, Wang X, Ma X, Sun Y, Xiao X, Luo H. Unexpectedly high mutation rate of a
793 deep-sea hyperthermophilic anaerobic archaeon. *ISME J* 2021; In press.

794 89. Luo H, Friedman R, Tang J, Hughes AL. Genome reduction by deletion of paralogs in the
795 marine cyanobacterium *Prochlorococcus*. *Mol Biol Evol* 2011; 28(10):2751–60.

796 90. Roesler C, Uitz J, Claustre H, Boss E, Xing X, Organelli E et al. Recommendations for
797 obtaining unbiased chlorophyll estimates from in situ chlorophyll fluorometers: A global
798 analysis of WET Labs ECO sensors. *Limnol. Oceanogr. Methods* 2017; 15(6):572–85.

799 91. Nguyen L-T, Schmidt HA, Haeseler A von, Minh BQ. IQ-TREE: a fast and effective
800 stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 2015;
801 32(1):268–74.

Figure legend

Fig. 1. (A) Maximum likelihood phylogenomic tree showing the position of CHUG in the *Roseobacter* group. The phylogeny was inferred using IQ-TREE (91) based on a concatenation of 45,904 amino acid sites over 120 conserved bacterial proteins (29). Solid circles in the phylogeny indicate nodes with bootstrap values >95%. The potential of aerobic (key gene *cobG*, red) and anaerobic (key gene *chiX*, green) cobinamide synthesis (the first stage of Vitamin B₁₂ synthesis) is labeled at the tips. Subclades of the *Roseobacter* group are marked according to a recent study (30). **(B)** Dendrogram of the same *Roseobacter* genomes based on the presence/absence pattern of orthologous gene families.

Fig. 2. Genomic feature comparisons between CHUG, their sister group, the outgroup, seven other PRC members, and other reference roseobacters. The significance level in genomic features between CHUG and the other four groups are shown in red, while that between seven other PRC members and the remaining groups are shown in blue. The markers * and ** denote $p < 0.05$ and $p < 0.01$ (phylANOVA analysis (22)), respectively. Abbreviations: C-ARSC, carbon atoms per amino-acid-residue side chain; N-ARSC, nitrogen atoms per amino-acid-residue side chain.

Fig. 3. The global distribution of CHUG and its ecological correlation with environmental factors. **(A, B & C)** The relative abundance of CHUG and other PRC members in the bacterial communities based on recruitment analysis using the metagenomic *TARA* Ocean sequencing samples with size fractions up to 3 μ m (A), and metatranscriptomic sequencing samples with size fractions up to 3 μ m (B), and metagenomic sequencing samples with size fraction of 5-20 μ m (C). **(D & E)** Correlation analysis between the relative abundance of CHUG and other PRC members and environmental parameters measured in the *TARA* Ocean metagenomic (D) and metatranscriptomic (E) samples. The p value is adjusted using stringent Bonferroni correction. Nonsignificant correlations are indicated by crosses for

$p > 0.05$ after adjusting. Abbreviations: AO, Arctic Ocean; NAO, North Atlantic Ocean; SAO, South Atlantic Ocean; IO, Indian Ocean; MS, Mediterranean Sea; NPO, North Pacific Ocean; SPO, South Pacific Ocean; RS, Red Sea; SO, Southern Ocean; fCDOM, fluorescence, colored dissolved organic matter.

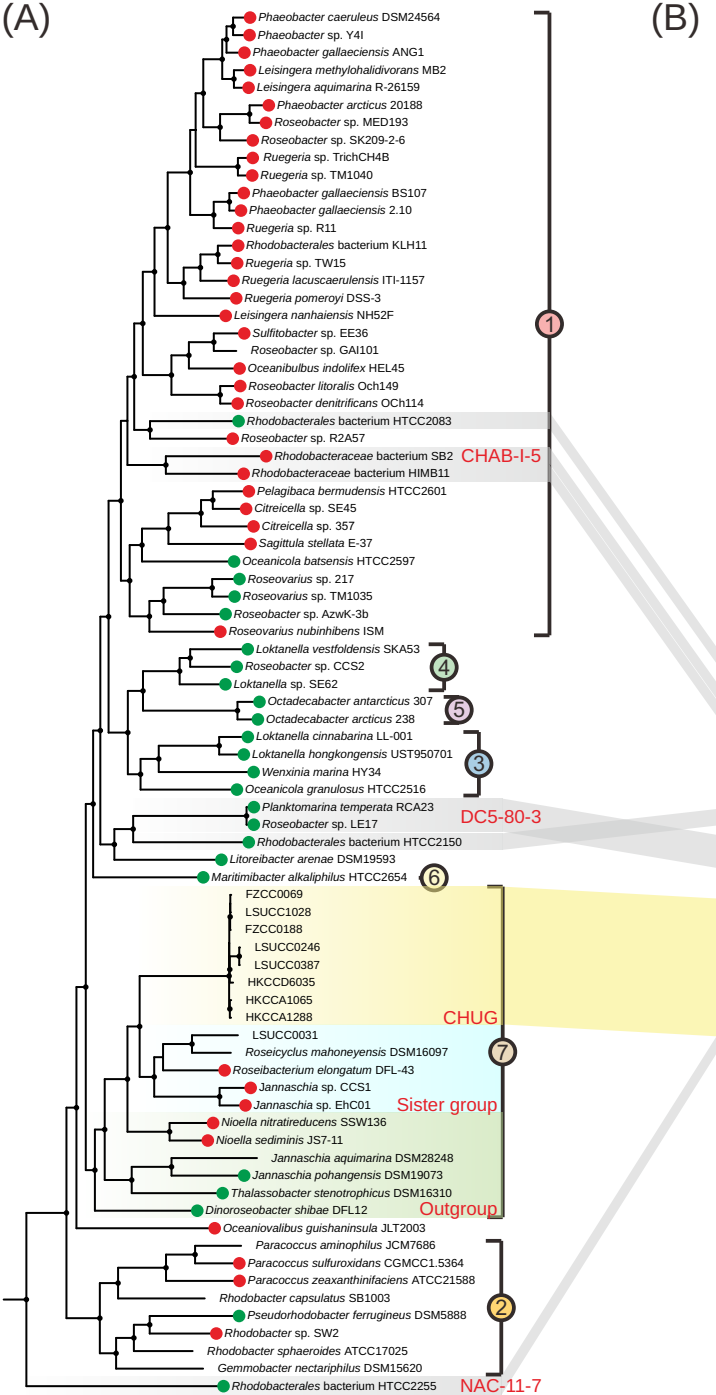
Fig. 4. The average expression level of gene families in CHUG and seven other PRC members. Gene families with their gene expression level at top 5% found exclusively in CHUG members, exclusively in seven other PRC members, and shared by CHUG and other PRC members are shown in magenta, orange, and red dots, respectively. The remaining gene families are shown in gray dots. Gene families specific to CHUG and seven other PRC members are shown in the upper and right panel, respectively. Abbreviations: RPKM, Reads Per Kilobase per Million mapped reads; *aapJ*, general L-amino acid transport system; ABC.MS, multiple sugar transport system; ABC.PA, polar amino acid transport system; ABC.SP, spermidine/putrescine transport system; *acnB*, aconitate hydratase 2; *actP*, acetate permease; *ahcY*, adenosylhomocysteinase; *ccoO*, cytochrome *cbb₃*-type oxidase; *dapD*, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase; *glnB*, nitrogen regulatory protein P-II; *gmd*, GDPmannose 4,6-dehydratase; *icd*, isocitrate dehydrogenase; *ilvC*, ketol-acid reductoisomerase; *katG*, catalase-peroxidase; *leuD*, 3-isopropylmalate/(R)-2-methylmalate dehydratase; *merA*, mercuric reductase; *merC*, *merP* and *merT*, mercuric ion transport system; *metH*, 5-methyltetrahydrofolate--homocysteine methyltransferase; *phnA*, phosphonoacetate hydrolase; *phnD*, phosphonate transport system; *potD*, spermidine/putrescine transport system; *pufA* and *pufB*, light-harvesting complex 1; *rbsB*, ribose transport system; *rhaS*, rhamnose transport system; *ureJ*, urease; *wza*, polysaccharide biosynthesis/export protein; *xsc*, sulfoacetaldehyde acetyltransferase; *yaeT*, Outer membrane protein assembly factor; *zntA*, lead, cadmium, zinc and mercury transporting ATPase; *zupT*, zinc transporter.

Fig. 5. The phyletic pattern of select genes. The solid and open circles in the right panel represent the presence/absence of the genes, respectively. **(A)** The phyletic pattern in the CHUG, its sister group and its outgroup. The phylogenomic tree shown in the left panel is pruned from the full phylogenomic tree shown in Fig. 1A, and branch length is ignored for better visualization. The ancestral genome reconstruction was performed with BadiRate (32). Each ancestral and leaf node is associated with three numbers, representing the total number of orthologous gene families at this node, and the number of orthologous gene families gained and lost on the branch leading to this node. The LCA of CHUG, the LCA shared by CHUG and its sister group, and the LCA shared by CHUG, its sister group and the outgroup are marked with a filled triangle, a filled circle, and a filled star, respectively. **(B)** The estimated phyletic pattern of the above-mentioned three LCAs. **(C)** The gene presence and absence pattern in the CHUG and other seven PRC genomes. The dendrogram in the left panel is pruned from that shown in Fig. 1B. Abbreviations: *thiE*, thiamine-phosphate pyrophosphorylase; *pdxH*, pyridoxamine 5'-phosphate oxidase; *bioB*, biotin synthase; *cobG*, precorrin-3B synthase; *cblX*, sirohydrochlorin cobaltochelatase; *cobV*, adenosylcobinamide-GDP ribazoletransferase; *btuB*, vitamin B12 transporter; *amtB*, ammonium transport system; nitrogen regulatory protein P-II (*glnBD*); *ntrBC*, nitrogen regulation two-component system; *ntrXY*, nitrogen regulation two-component system; *ureABC*, urease; *urtABCDE*, urea transport system; *nrtABC*, nitrate/nitrite transport system; *phoBR*, two-component phosphate regulatory system; *pstABCS*, phosphate transport system (high affinity); *phnGHIJKLM*, carbon-phosphorus (C-P) lyase; *phoX*, alkaline phosphatase; *plcP*, phospholipase C; PGC, photosynthesis gene cluster; *coxL*, carbon monoxide dehydrogenase (type I forming); *sqr*, sulfide quinone oxidoreductase; *pdo*, persulfide dioxygenase; *sox*, thiosulfate oxidizing SOX complex; *napAB*, nitrate reductase (periplasmic); *narGHI*, nitrate reductase (membrane-bound); *nirBD*, nitrite reductase; *nirK*,

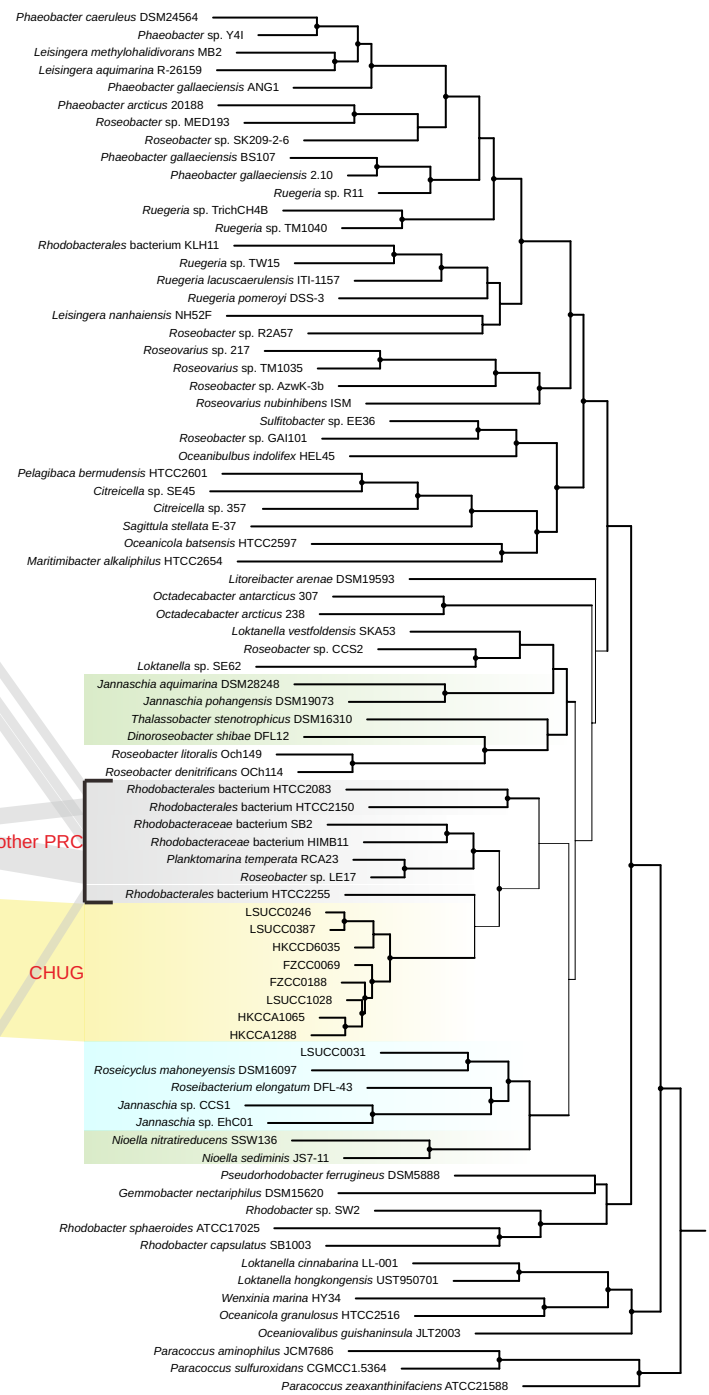
copper-containing NO-forming nitrite reductase; *nirS*, haem-containing NO-forming nitrite reductase; *pfk*, phosphofructokinase; *edd*, phosphogluconate dehydratase; *pcaGH*, protocatechuate 3,4-dioxygenase; *paaABCDE*, ring-1,2-phenylacetyl-CoA epoxidase; *hmgA*, homogentisate 1,2-dioxygenase; *cheAB*, chemotaxis family protein; *fliC*, flagellin; *luxR*, quorum-sensing system regulator; *virB*, type IV secretion system protein; *vasKF*, type VI secretion system protein; GTA, gene transfer agent; *dmdA*, DMSP demethylase; *dddD*, DMSP acyl-CoA transferase; *dddL*, dimethylpropiothetin dethiomethylase; *tmd*, trimethylamine dehydrogenase; *tmm*, trimethylamine monooxygenase; *tauABC*, taurine transport system; *xsc*, sulfoacetaldehyde acetyltransferase.

Fig. 6. Growth assay of (A) CHUG strain HKCCA1288 and (B) model roseobacter *Ruegeria pomeroyi* DSS-3. Strains cultured on defined marine ammonium mineral salts (MAMS) medium with and without vitamin B₁₂ were plotted in red and blue, respectively. Three triplicates were performed for each condition and error bars denote standard deviation.

(A)



(B)



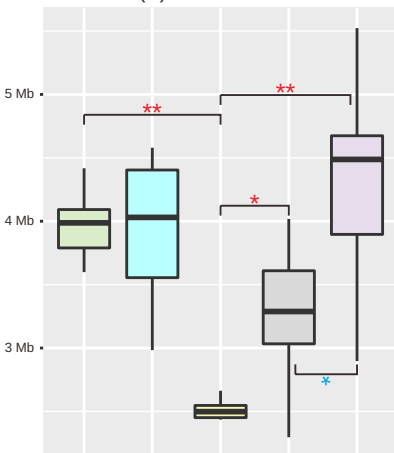
● *de novo* VB₁₂ synthesis (cobG pathway)

● *de novo* VB₁₂ synthesis (cbiX pathway)

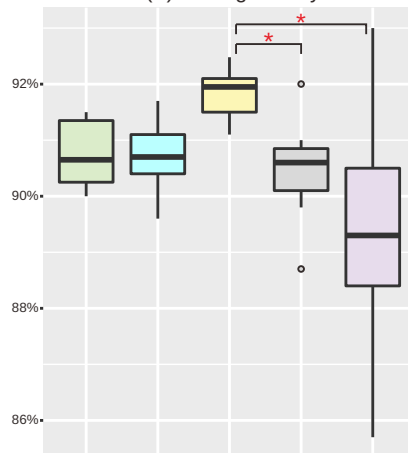
— Tree scale: 0.1

— Tree scale: 0.01

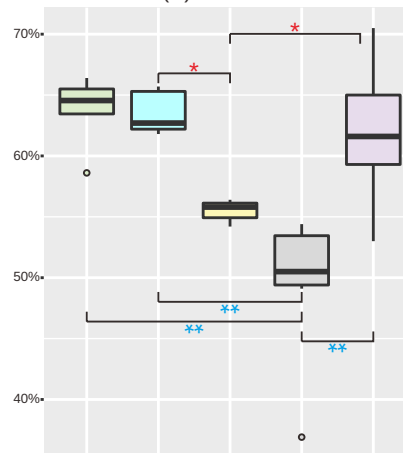
(A) Genome Size



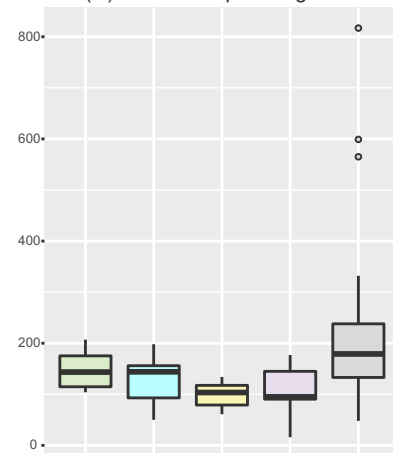
(B) Coding density



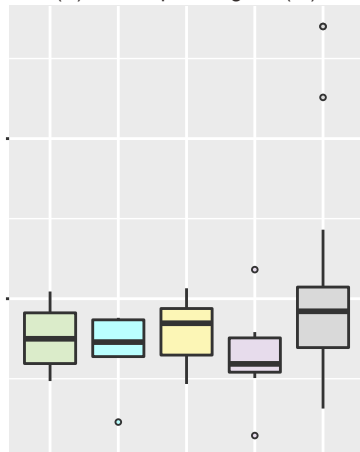
(C) GC content



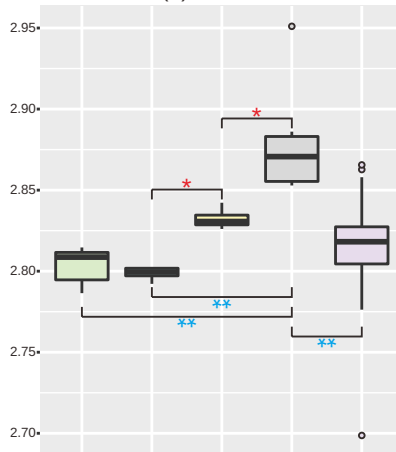
(D) Number of pseudogenes



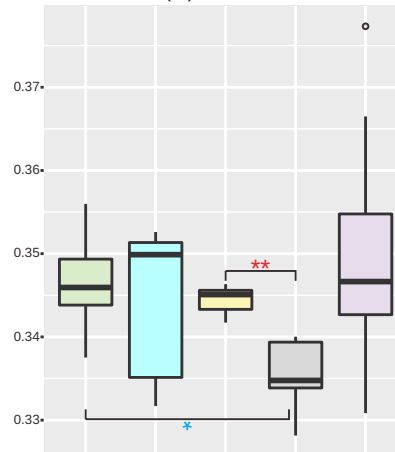
(E) ratio of pseudogene (%)



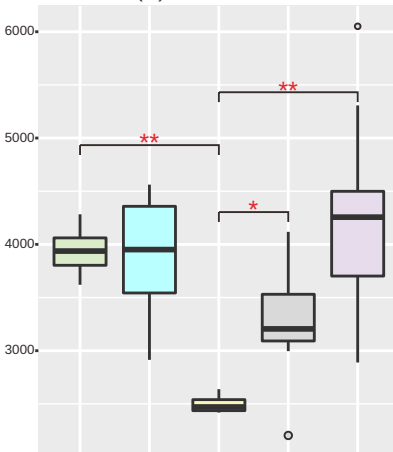
(F) C-ARSC



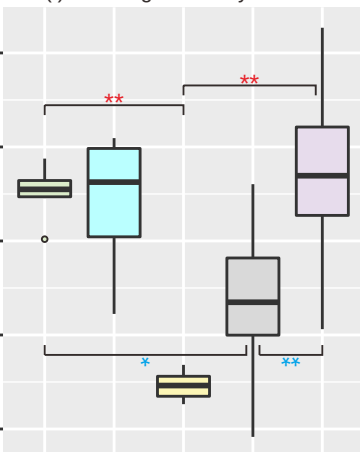
(G) N-ARSC



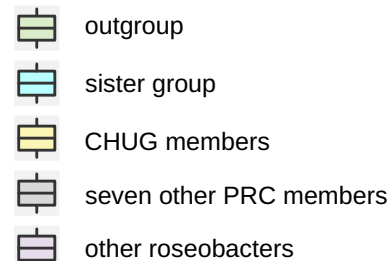
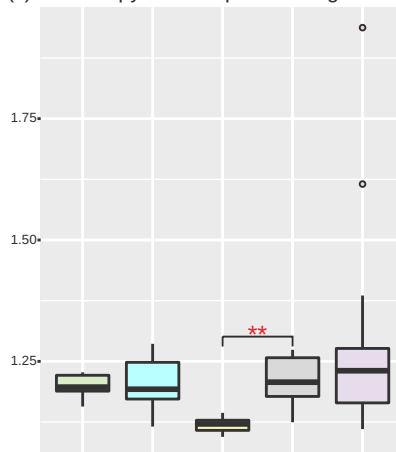
(H) Gene number



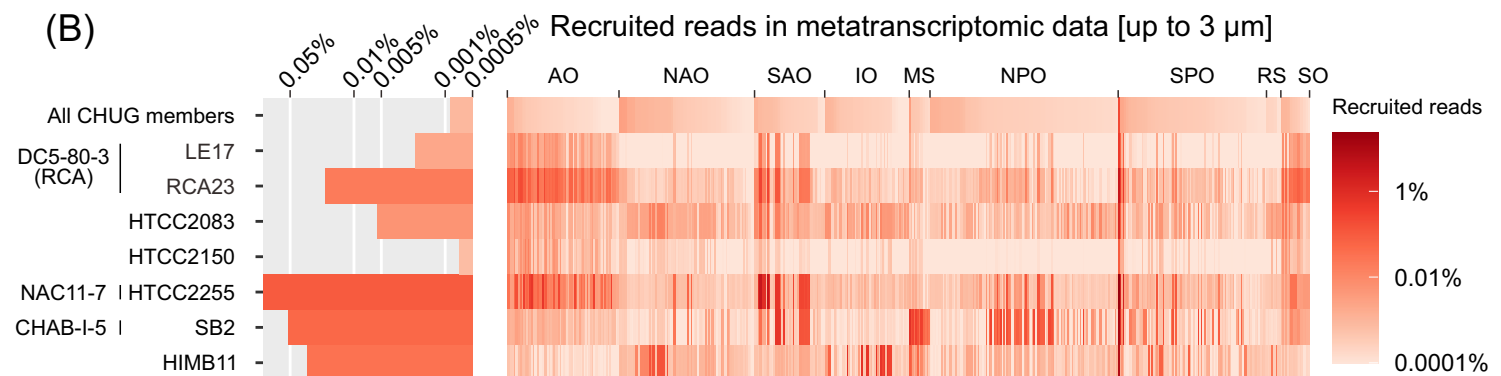
(I) Orthologous family number



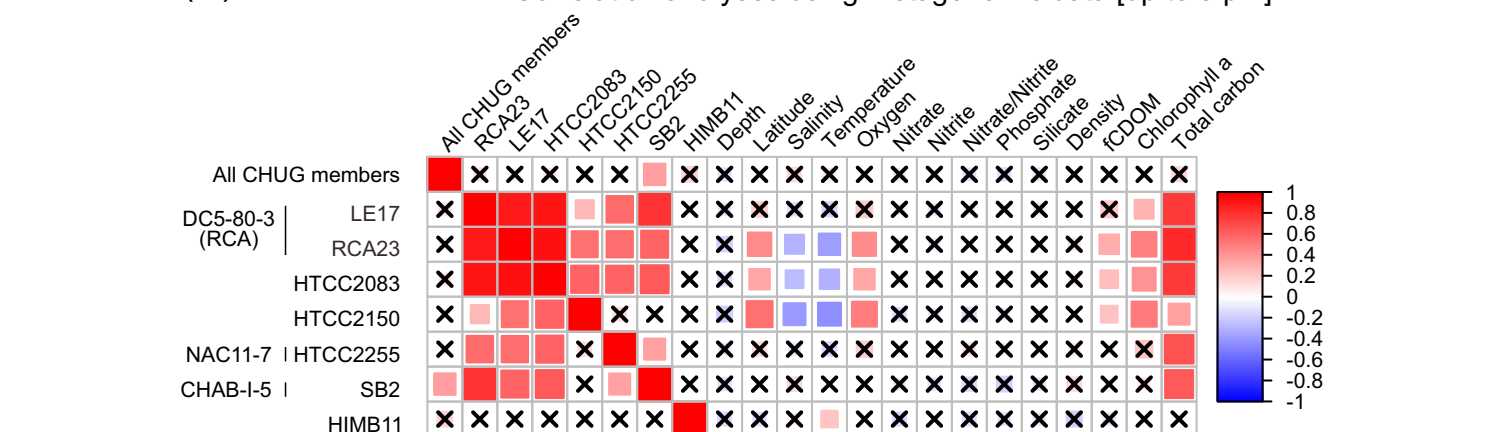
(J) Gene copy number per orthologous family



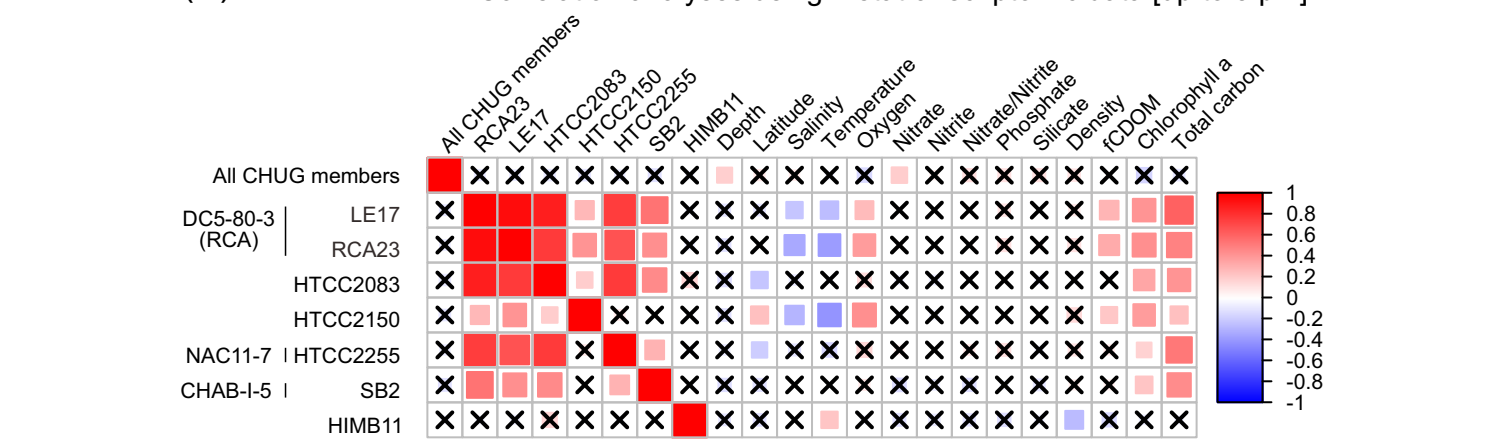
* $p < 0.05$, phylANOVA analysis
 ** $p < 0.01$, phylANOVA analysis



(D) Correlation analyses using metagenomic data [up to 3 μ m]

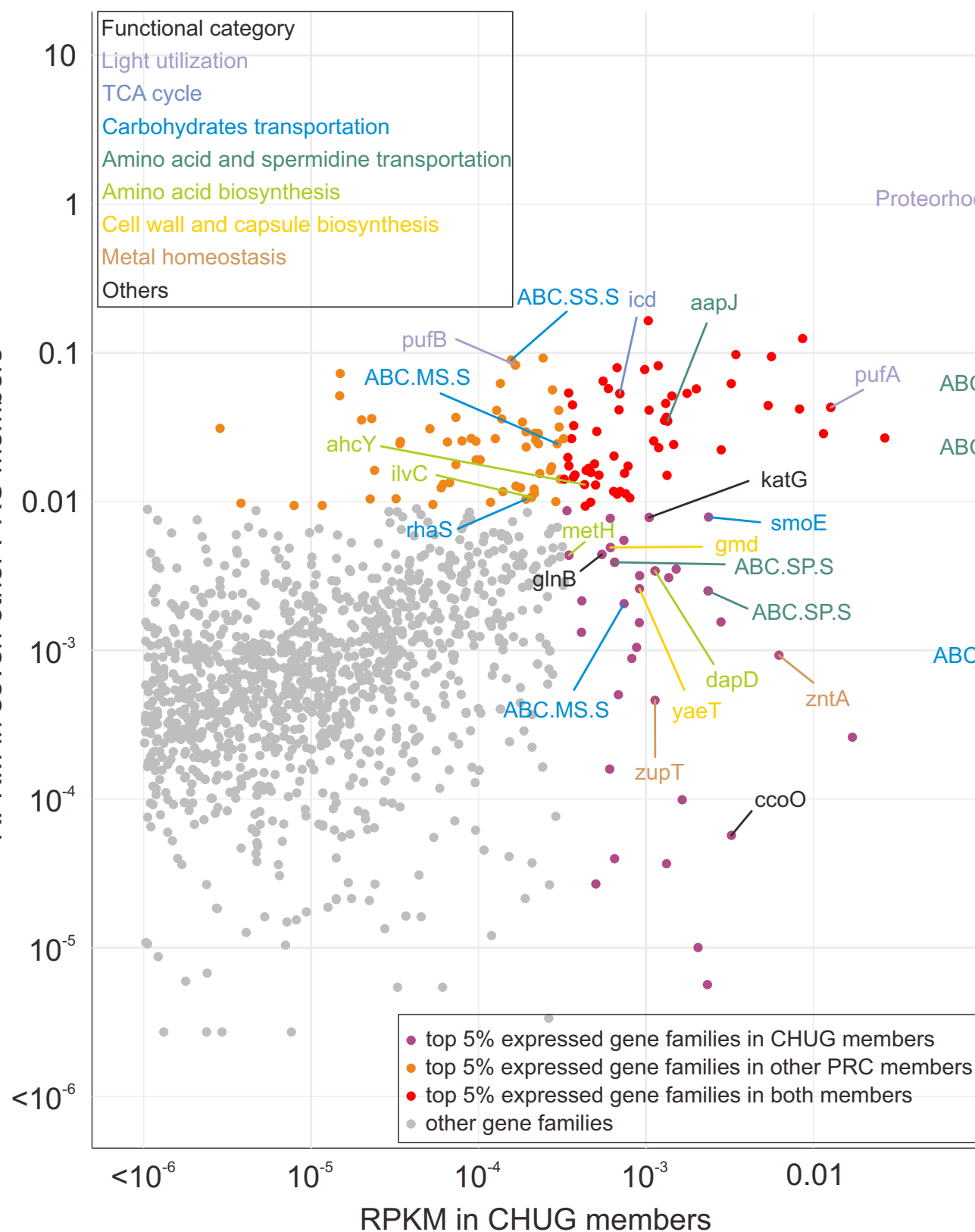


(E) Correlation analyses using metatranscriptomic data [up to 3 μ m]



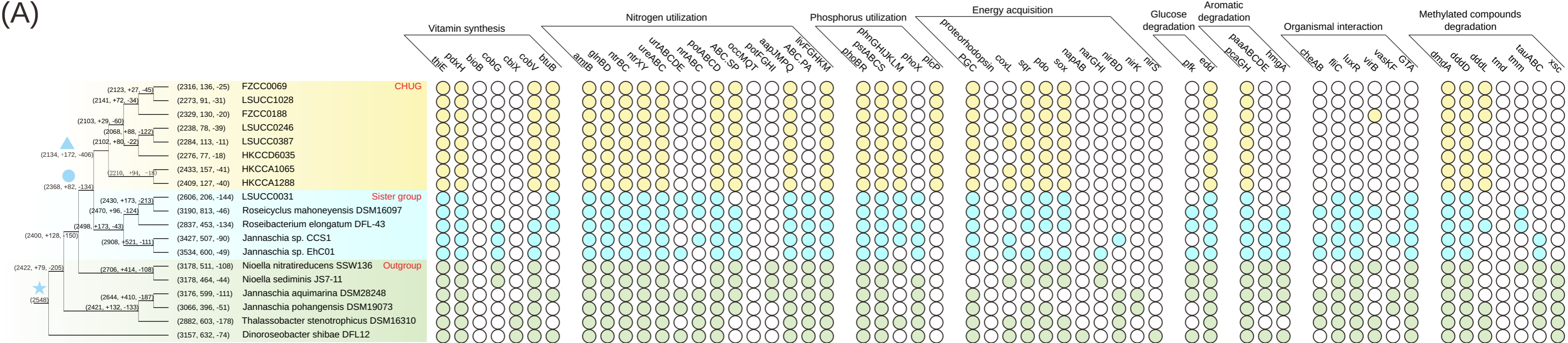
Orthologous gene families specific in CHUG members

RPKM in seven other PRC members



Orthologous gene families specific in seven other PRC members

(A)



(B)

LCA of CHUG ▲
 LCA of CHUG and its sister group ●
 LCA of CHUG, its sister group and the outgroup ★

(C)

