Association of lanthipeptide genes with TnpA\textsubscript{REP} transposases in marine picocyanobacteria

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ABSTRACT

Lanthipeptides are a family of ribosomally synthesized, post-translationally modified peptides that are widespread among bacteria, typically functioning as antibacterials. The marine picocyanobacteria \textit{Prochlorococcus} and \textit{Synechococcus} produce an unusual and diverse set of lanthipeptides of unknown function called prochlorosins. While well-studied model bacteria produce one or two different molecules of this type, a single picocyanobacterium can produce as many as 80; the community of picocyanobacteria in a single milliliter of seawater can collectively encode up to 10,000 prochlorosins. The molecular events that led to this expansion and diversification of the lanthipeptide repertoire in picocyanobacteria – the numerically dominant photosynthesizers in the oceans – is unknown.

We present evidence for an unusual association between prochlorosin genes with a single-stranded DNA transposase belonging to the TnpA\textsubscript{REP} family. The genes co-occur and co-localize across the phylogeny of marine picocyanobacteria forming a distinct association pattern within genomes, most likely resulting from the transposase activity. Given the role of TnpA\textsubscript{REP} homologs in other bacteria, we propose - based on genomic structures - that they contribute to the creation of the prochlorosin structural diversity through a diversifying recombination mechanism.

IMPORTANCE

Only a few mechanisms have been described that promote the diversification of a targeted gene region in bacteria. We present indirect evidence that the TnpA\textsubscript{REP} transposases associated with
prochlorosins in picocyanobacteria could represent a novel such mechanism, and explain the extreme expansion and diversification of prochlorosins in this abundant marine microbe.

**OBSERVATION**

Cubillos-Ruiz et al. [1] revealed that prochlorosins – lanthipeptides produced by marine picocyanobacteria – represent an extreme expansion and diversification of the lanthipeptide family described in other bacteria [2]. While typical model bacteria encode 1 or 2 of these compounds, select strains of picocyanobacteria encode up to 80 different ones – representing an extensive diversity of cyclic peptide structures. The unusual abundance of these genes in the otherwise very streamlined genomes of these tiny cells suggests a strong selection pressure to maintain and diversify them [1]. Their biological function in the ecosystem is unknown.

Prochlorosin biosynthesis involves the production of precursor peptides (ProcA) which are matured by a promiscuous modifying enzyme (ProcM). ProcA peptides are composed of an N-terminal signal peptide – the “leader peptide” – directing the sequence to ProcM which cyclizes the C-terminal “core peptide” by adding intramolecular lanthionine bonds, the defining feature of lanthipeptides [2]. The leader peptide is subsequently cleaved, yielding the final cyclic prochlorosin. While the leader peptide-encoding sequence is well-conserved across picocyanobacteria, the core peptide-encoding sequence is responsible for the immense diversity of prochlorosins, through the accumulation of small insertion/deletion events [1]. The precise molecular events leading to this unique expansion and diversification has remained elusive.

A likely mechanism for the expansion of prochlorosins might be diversifying recombination – a process by which a localized region of DNA (with respect to a given gene) is modified through a dedicated recombination mechanism, leading to a hotspot of sequence diversity across closely related bacterial cells. Diversification can lead to variability in gene expression, or structural changes in a particular protein domain – and the selective advantage it provides is often (but not exclusively) related to ‘camouflage’, like avoiding infection by phages or avoiding detection by the immune system. Precise mechanisms of diversifying recombination that have been described in other bacteria are: 1) localized homologous recombination (bacteriocins, like colicins [3], [4] or syringacins [5], capsule serotypes [6], adhesins [7], or restriction-modification systems [8]); 2) invertase-mediated shuffling (pilins [9], capsule serotypes and various surface-exposed antigens [10], [11]; 3) diversity-generating retroelements (transcriptional regulators, pilin-like proteins and signaling proteins [12]); and 4) integrons (shuffling of genes cassettes including antibiotic resistance and other virulence genes [13], [14]. We conducted BLAST searches of a vast prochlorosin database for the machinery operating in these four other bacterial systems and found nothing similar.

In the process of our detailed exploration of prochlorosin genomic structure and context, however, we observed the association of prochlorosin genes with a single-stranded DNA
transposase from the TnpA<sub>REP</sub> family [15]–[18] (Fig. 1). TnpA<sub>REP</sub> transposases are single-stranded DNA recombinases that – unlike the closely-related IS200-like transposases – are considered domesticated in the sense that they do not move across genomes in a typical transposon structure, but rather are involved in creating large repeat regions containing 'REPs' (Repetitive Extragenic Palindromes, motifs that form DNA hairpin structures) [15], [18]–[20]. For example, there are 600 REP repeats in <i>E. coli</i>, representing ~1% of its genome [21]. Importantly, <i>tnpA<sub>REP</sub></i> genes are physically associated with REP repeat regions (usually flanking them), and several studies have confirmed a biochemical link between TnpA<sub>REP</sub> and REP motifs, supporting their role as the primary driver for the expansion and diversification of REP regions in bacterial genomes [16], [17], [22], [23], a feature that is reminiscent with the pattern of sustained expansion, diversification, and elimination of proc<i>A</i> genes we have observed in marine picocyanobacteria [1].

We found a number of TnpA<sub>REP</sub> family transposase homologs in marine picocyanobacteria, all of them forming a monophyletic group that appears to be descended from TnpA<sub>REP</sub> in other cyanobacteria such as <i>Trichodesmium erythraeum</i> (Sup Fig 1, 2). They belong to a subclass of TnpA<sub>REP</sub> (RAYT group 1 in [18], or subclass 2.1 in [24]) which possess an additional conserved domain in the C-terminal region, likely involved in DNA-binding [24]. In marine picocyanobacteria, the <i>tnpA<sub>REP</sub></i> genes tend to co-localize with clusters or individual proc<i>A</i> genes, which encode for the peptides that are cyclized by ProcM to become the final product: prochlorosins (Fig. 1A). Out of the 136 <i>tnpA<sub>REP</sub></i> sequences (including full-length copies and fragments) found in marine picocyanobacteria, 32% are within 200 bp of a proc<i>A</i> gene (likely an underestimate because repeat-containing regions of the genome are difficult to assemble and contigs often end next to transposases genes, Sup Fig 3). Full-length transposase copies – as well as numerous copies of truncated fragments (often corresponding to the C-terminal extremity of TnpA<sub>REP</sub>, Sup Fig 4) – are interspersed with proc<i>A</i> gene clusters (Fig. 1A, Sup Fig 5). In addition, the co-occurrence of <i>tnpA<sub>REP</sub></i> genes in a genome with proc<i>A</i> genes is nearly universal. Among the 618 genomes of <i>Prochlorococcus</i>, the TnpA<sub>REP</sub> are only found within the LLIV clade (28 genomes), like prochlorosins [1]. Except for a few exceptions, likely due to genome incompleteness, if a genome encodes prochlorosins it has <i>tnpA<sub>REP</sub></i> genes, and vice versa (Fig 1B).
FIG 1 (A) Example of co-localization of tnpA<sub>REP</sub> and procA genes in several prochlorosin clusters in the Prochlorococcus MIT9313 genome. Prochlorosin genes in blue were annotated following [1]. Brackets indicate the repeated arrangement pattern of procA genes facing tnpA<sub>REP</sub> genes. (B) Co-occurrence of procA and tnpA<sub>REP</sub> genes in marine picocyanobacteria. The phylogenetic distance tree was built using the built-in IMG-Proportal phylogeny tool and the tree was annotated using the Interactive Tree Of Life ( iTOL). Prochlorococcus genomes are restricted to the LLIV clade, as all other clades are lacking both tnpA<sub>REP</sub> genes (not shown) and Prochlorosins [1]. The bar diagram on the tree indicates the number of individual procA genes (blue) and individual tnpA<sub>REP</sub> genes (only ‘full-length’ genes encoding for > 200 amino acids TnpA<sub>REP</sub>, in red) within a given strain (no bar indicates the absence of the genes). Bracketed numbers on the red and blue bars indicate scale.

Moreover, the arrangement of tnpA<sub>REP</sub> (full-length and truncated fragments) and procA genes shows a clear pattern of association: First, the genes are always facing each other in opposite
directions (see brackets in Fig 1A, Fig 2), while procA genes are always in cis with each other, and often regularly spaced in clusters [1]. The distance between the facing \textit{tnpA\textsubscript{REP}} genes and the facing procA is extremely well conserved across distantly related strains (Fig 2B, Sup Fig 5). This conservation of intergenic distance is unlikely to happen by chance and suggests a mechanism physically linking the genes, possibly through mobilization by the transposase. Second, the intergenic sequence between the facing \textit{tnpA\textsubscript{REP}} and procA genes is well-conserved among marine \textit{Synechococcus} to \textit{Prochlorococcus} genomes, as well conserved as the leader peptide-encoding sequence (Fig 2A), suggesting that it plays a role in the association.

To begin to unravel the mechanism of association we searched for potential TnpA\textsubscript{REP} recognition targets. Of note, marine picocyanobacteria belong to a TnpA\textsubscript{REP} subgroup that was not found to associate with REP-like sequences, but rather with inverted repeats (consensus sequence \textit{GGGG[AT][CG]A[CG]}) [24], [25]. We could not find any sign of association with inverted repeats, but we did detect motifs forming DNA secondary structures: a hairpin structure (Fig 2A) in the intergenic sequence, and a Guanine quadruplex (G4) structure (Fig 2A) (see methods for details); both of these structure types have been shown to be potential recognition targets for TnpA\textsubscript{REP} transposases [16], [17], [23]. Without further clues, the hairpin structure could simply correspond to conserved rho-independent terminators, which are common in cyanobacteria. The G4 structures are more conspicuous as they form around a highly conserved GGC\textsubscript{CGG} motif which delineates the transition from the leader to the core peptide. This site serves as an anchor point for the ProcM modifying enzyme [2]. Despite being a feature of only some of the procA genes (G4 is predicted to form in 25 to 45\% of procA genes according to different prediction software, see methods for details), the conservation of occurrence at this particular site suggests that it could play a role. Interestingly, G4 structures have been shown to participate in pilin antigenic variation - one of the best described diversifying recombination mechanisms - in different proteobacteria such as \textit{N. gonorrhoeae} [26]. However, the configuration in the latter is significantly different, as there the G4 is placed a few hundred base pairs upstream of the recombination hotspot, and recombination occurs by swapping a domain of the active pilin locus with the domain of idle pilin loci in the genome. In contrast, prochlorosins don’t result from domain swapping, but rather from indels in the core peptide region [1].
Fig 2 (A) Alignment of selected genomic regions containing full-length *tnpA*<sub>REP</sub> (red) facing *procA* genes (blue) across the phylogeny of marine picocyanobacteria. The upper panel shows the percentage of nucleotide sequence identity (colors for each given position are green for 100% identity, yellow between 30 - 100%, red below 30%). The border between leader to core peptide region is indicated (triangle at the end of the leader peptide-encoding region). As previously described [1] the leader peptide-encoded region of *procA* is conserved across strains while the core peptide-encoding region is highly variable and contains large numbers of indels. Interestingly, in the case of facing *tnpA*<sub>REP</sub> and *procA* genes, the *tnpA*<sub>REP</sub> and the intergenic region are equally well conserved, showing that diversity is precisely focused on the core peptide-encoding region as the gene pair evolves. Two types of DNA secondary structures are predicted to form (see methods for detail): a ‘hairpin’ structure in the intergenic region, and a guanine quadruplex (G4) structure exactly at the end of the leader peptide region. (B) Distance between *tnpA*<sub>REP</sub> and *procA* gene pairs in all 26 instances in the data set (see Sup Fig 5) where the genes face each other. Since the core peptide-encoding region tends to vary in length, both distance from the tip of *tnpA*<sub>REP</sub> to the tip of facing *procA* (purple) and to the end of the leader peptide of facing *procA* (turquoise) are shown.
Overall, these results show that \textit{tnpA\textsubscript{REP}} and \textit{procA} genes are physically linked in picocyanobacterial genomes in a non-random pattern, and this linkage is most probably the result of the TnpA\textsubscript{REP} enzyme activity. Importantly, the closest TnpA\textsubscript{REP} relatives in cyanobacteria (Sup Fig 1) do not colocalize with lanthipeptide-related genes, suggesting that this association is unique to marine picocyanobacteria resulting in the unique multiplication and diversification of lanthipeptide genes. Thus, we propose that similar to how TnpA\textsubscript{REP} drives the expansion and diversification of the REP sequences repertoire in other bacterial genomes, the TnpA\textsubscript{REP} homologs in marine picocyanobacteria genomes have associated with lanthipeptide genes, contributing to their expansion and diversification and resulting in the extreme diversity we see among prochlorosins. We suggest that the \textit{tnpA\textsubscript{REP}} / \textit{procA} association enables a powerful diversifying recombination mechanism, precisely focused on modifying the core peptide encoding sequence, while maintaining the leader peptide sequence unchanged. We note that another model for the driving force of prochlorosin diversity has been proposed by Cubillos-Ruiz (2015) [27]. In that model, a putative \textit{chi} site within the precursor peptide is postulated to serve as a hotspot for localized homologous recombination, mediated by the RecBCD DNA repair pathway. The two models are not necessarily mutually exclusive and could work in concert.

Testing the \textit{chi} site hypothesis would involve demonstrating the \textit{chi} site activity, whereas validation of the model we propose would require experimental assays determining the recognition sequence and catalytic activity of a \textit{Prochlorococcus} TnpA\textsubscript{REP} transposase as performed for the TnpA\textsubscript{REP} homologs in \textit{E. coli} [16], [17].
MATERIALS AND METHODS

Dataset. The genomes of Prochlorococcus LLIV clade and marine Synechococcus were taken from the IMG/ProPortal database (https://img.jgi.doe.gov/cgi-bin/proportal/main.cgi) hosted within the JGI Integrated Microbial Genomes system. A few additional Synechococcus genomes from National Center for Biotechnology Information (NCBI) database were added to the dataset: WH8101, UW179A, UW105, BS56D, and the metagenome-assembled genome EAC657. The set of Prochlorococcus and Synechococcus genomes containing tnpA<sub>REP</sub> and/or procA genes that were used in this study are listed in Table S1.

Gene annotation, sequence alignments, and phylogenetic tree construction. Prochlorosin annotations were taken from [1], and TnpA<sub>REP</sub> from [18], [24]. Prediction of procA and tnpA<sub>REP</sub> genes was expanded to new strains using BLASTP (E value of 1e-5) with ProcA or TnpA<sub>REP</sub> protein sequence query. For Prochlorosins, the end of the leader peptide was predicted by searching the motif GCTGG[TAGC]GG in the procA sequence. For strain phylogeny, we used the built-in IMG-Proportal phylogeny tool and annotated the tree (Fig 1B) using the Interactive Tree Of Life (iTOL). The facing procA-tnpA<sub>REP</sub> nucleotide sequence alignment (Fig 2A) was performed in Geneious Prime 2020.0.5 using the geneious aligner. The TnpA<sub>REP</sub> protein sequence alignment was performed using MUSCLE and the tree (Sup Fig 1) was built using the Geneious tree builder (Neighbour-Joining); Only the TnpA<sub>REP</sub> sequences above 200 amino acids were kept for the alignment. The Prochlorococcus MIT9313 TnpA<sub>REP</sub> fragments protein sequence alignment (Sup Fig 4) was performed using the Geneious aligner.

DNA secondary structure prediction. Hairpin structure formation was predicted using RNAstructure [28]. The consensus sequence ACTCAAGCCCTTGCAATTAGCAGGGGCTTTTTATT in the Fig 2A alignment intergenic region is predicted to form a hairpin structure (bold nucleotides) with a free energy of -10.3 kcal/mol. Guanine quadruplexes (G4) were assessed using the dataset of 2509 distinct procA genes (Operating Prochlorosin Units, OPU) from [1]. In total, the G4-PREDICTOR V.2 software predicts G4 structures in 45% of OPUs, while the G4hunter software predicts G4 structures in 25.7% of OPUs.

3D structure modeling. The in silico prediction of Prochlorococcus MIT9313 PMT_0830 TnpA<sub>REP</sub> 3D structure was performed using SWISS-MODEL, with the E. coli TnpA<sub>REP</sub> (PDB_ID 4ER8) serving as the template for homology modeling.

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COMPETING INTERESTS

The authors declare no conflict of interest.
REFERENCES

[1] A. Cubillos-Ruiz, J. W. Berta-Thompson, J. W. Becker, W. A. van der Donk, and S. W. Chisholm, “Evolutionary radiation of lanthipeptides in marine cyanobacteria,” Proc. Natl. Acad. Sci. U. S. A., vol. 114, no. 27, pp. E5424–E5433, Jul. 2017.

[2] L. M. Repka, J. R. Chekan, S. K. Nair, and W. A. van der Donk, “Mechanistic Understanding of Lanthipeptide Biosynthetic Enzymes,” Chem. Rev., vol. 117, no. 8, pp. 5457–5520, Apr. 2017.

[3] M. A. Riley and J. E. Wertz, “Bacteriocin diversity: ecological and evolutionary perspectives,” Biochimie, vol. 84, no. 5–6, pp. 357–364, May 2002.

[4] M. G. K. Ghequire, S. K. Buchanan, and R. De Mot, “The ColM Family, Polymorphic Toxins Breaching the Bacterial Cell Wall,” MBio, vol. 9, no. 1, pp. e02267–17–11, Feb. 2018.

[5] D. A. Baltrus, M. Clark, C. Smith, and K. L. Hockett, “Localized recombination drives diversification of killing spectra for phage-derived syringacins,” ISME J., vol. 13, no. 2, pp. 1–13, Dec. 2018.

[6] R. D. Waite, J. K. Struthers, and C. G. Dowson, “Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation,” Mol. Microbiol., vol. 42, no. 5, pp. 1223–1232, Dec. 2001.

[7] N. Bilek, C. A. Ison, and B. G. Spratt, “Relative Contributions of Recombination and Mutation to the Diversification of the opa Gene Repertoire of Neisseria gonorrhoeae,” J. Bacteriol., vol. 191, no. 6, pp. 1878–1890, Feb. 2009.

[8] Y. N. Srikhanta et al., “Phasevarions mediate random switching of gene expression in pathogenic Neisseria,” PLoS Pathog., vol. 5, no. 4, p. e1000400, Apr. 2009.

[9] A. Gyohda, N. Furuya, A. Ishiwa, S. Zhu, and T. Komano, “Structure and function of the shufflon in plasmid R64,” Adv. Biophys., vol. 38, no. Complete, pp. 183–213, 2004.

[10] C. M. Krinos, M. J. Coyne, K. G. Weinacht, A. O. Tzianabos, D. L. Kasper, and L. E. Comstock, “Extensive surface diversity of a commensal microorganism by multiple DNA inversions,” Nature, vol. 414, no. 6863, pp. 555–558, Nov. 2001.

[11] R. C. Johnson, “Site-specific DNA Inversion by Serine Recombinases,” Microbiology spectrum, vol. 3, no. 3, pp. 1–36, Feb. 2015.

[12] B. G. Paul et al., “Retroelement-guided protein diversification abounds in vast lineages of Bacteria and Archaea,” Nature Microbiology, vol. 2, no. 6, pp. 1–7, Apr. 2017.

[13] D. Mazel, “Integrons: agents of bacterial evolution,” Nat. Rev. Microbiol., vol. 4, no. 8, pp. 608–620, Aug. 2006.

[14] J. A. Escudero, C. Loot, and D. Mazel, “Integrons as Adaptive Devices,” in Molecular Mechanisms of Microbial Evolution, vol. 39, 1 vols., P. H. Rampelotto, Ed. Cham: Springer International Publishing, 2018, pp. 199–239.

[15] J. Nunvar, T. Huckova, and I. Licha, “Identification and characterization of repetitive extragenic palindromes (REP)-associated tyrosine transposases: Implications for REP evolution and dynamics in bacterial genomes,” BMC Genomics, vol. 11, no. 1, p. 44, Jan. 2010.

[16] B. Ton-Hoang et al., “Structuring the bacterial genome: Y1-transposases associated with REP-BIME sequences †,” Nucleic Acids Res., vol. 40, no. 8, pp. 3596–3609, Dec. 2011.

[17] S. A. J. Messing et al., “The processing of repetitive extragenic palindromes: the structure of a repetitive extragenic palindrome bound to its associated nuclease,” Nucleic Acids Res., vol. 40, no. 19, pp. 9964–9979, Aug. 2012.

[18] F. Bertels, J. Gallie, and P. B. Rainey, “Identification and Characterization of Domesticated
Bacterial Transposases,” *Genome Biol. Evol.*, vol. 9, no. 8, pp. 2110–2121, Aug. 2017.

[19] J. Nunvar, I. Licha, and B. Schneider, “Evolution of REP diversity: a comparative study,” *BMC Genomics*, vol. 14, p. 385, Jun. 2013.

[20] F. Bertels and P. B. Rainey, “Within-Genome Evolution of REPINs: a New Family of Miniature Mobile DNA in Bacteria,” *PLoS Genet.*, vol. 7, no. 6, pp. e1002132–13, Jun. 2011.

[21] S. Bachellier, J. M. Clément, and M. Hofnung, “Short palindromic repetitive DNA elements in enterobacteria: a survey,” *Res. Microbiol.*, vol. 150, no. 9–10, pp. 627–639, Nov. 1999.

[22] M. Bocková, T. Špringer, I. Nečasová, J. Nunvar, B. Schneider, and J. Homola, “Monitoring RAYT activity by surface plasmon resonance biosensor,” *Anal. Bioanal. Chem.*, vol. 407, no. 14, pp. 3985–3993, Jan. 2015.

[23] T. Charmavets, J. Nunvar, I. Nečasová, J. Völker, K. J. Breslauer, and B. Schneider, “Conformational diversity of single-stranded DNA from bacterial repetitive extragenic palindromes: Implications for the DNA recognition elements of transposases,” *Biopolymers*, vol. 103, no. 10, pp. 585–596, Jul. 2015.

[24] Y. Quentin, P. Siguier, M. Chandler, and G. Fichant, “Single-strand DNA processing: phylogenomics and sequence diversity of a superfamily of potential prokaryotic HuH endonucleases,” *BMC Genomics*, vol. 19, no. 1, pp. 1–20, Jun. 2018.

[25] P. P. Di Nocera, E. De Gregorio, and F. Rocco, “GTAG- and CGTC-tagged palindromic DNA repeats in prokaryotes,” *BMC Genomics*, vol. 14, p. 522, Jul. 2013.

[26] L. A. Cahoon and H. S. Seifert, “Focusing homologous recombination: pilin antigenic variation in the pathogenic *Neisseria*,” *Mol. Microbiol.*, vol. 81, no. 5, pp. 1136–1143, Sep. 2011.

[27] A. F. Cubillos-Ruiz, “Ecology and evolution of lanthipeptides in marine picocyanobacteria,” Massachusetts Institute of Technology, 2015.

[28] J. S. Reuter and D. H. Mathews, “RNAstructure: software for RNA secondary structure prediction and analysis,” *BMC Bioinformatics*, vol. 11, p. 129, Mar. 2010.