Small Biomolecules for Big Applications

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Leveraging directed evolution methodologies to engineer unnatural RiPP natural products.

Directed evolution has seen significant use in academia and industry for the functional optimization of peptides and proteins. By screening libraries of mutants for the most desirable phenotypes, variants of the original protein or peptide can be discovered that are optimal for any given application. Presently, directed evolution is limited to products that are directly encoded by DNA, which imparts the essential properties of replicability and mutability. As such, the approach can only be applied to large biomolecules. The current paper by the van der Donk group brings us one step closer to the goal of leveraging directed evolution for the development of novel small molecules with pharmaceutical importance.1

The molecules of interest to Hetrick et al. are a class of natural products known as ribosomally synthesized and post-translationally modified peptides, or RiPPs.1−3 Unlike other classes, the substrates for RiPPs are directly encoded by a “substrate” gene, making them ideal as subjects of evolutionary methods. Once translated, these linear precursors are modified by one or more enzymes and proteolytically cleaved to produce the final, mature product of the biosynthetic pathway. The cyclization motifs commonly observed in RiPPs provide structural rigidity, impart function, and protect against proteolysis. These modifications turn otherwise unassuming linear peptides into potent small molecules.

RiPPs are a particularly good class of biomolecules for the application of directed evolution because of their broad range of shapes, sizes, and bioactivities.1−4 They can be as small as the enzyme cofactor pyrroloquinoline quinone (PQQ), a mashup of two amino acids, or larger like nisin, the 34 amino acid peptide antibiotic used in the present study. The structural organization of the precursor peptide is also favorable for in vitro evolution: the portion of the precursor peptide that is modified eventually to afford the mature RiPP is known as the “core,” usually located at the C-terminus. The N-terminal portion, or the “leader” sequence, generally serves as the recognition element for the modification enzymes.5 By separating the recognition element from the sequence that is modified, RiPPs are highly tolerant of mutations within the core peptide region. In a hypothetical 10mer RiPP where only two amino acids are strictly required for cyclization, randomly mutating the other eight positions can lead to a possible ~10^10 RiPPs, each with a unique structure and, possibly, biological activity. The ability to generate such chemical diversity is a powerful strategy for creating new bioactive molecules.

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To demonstrate the possibility of evolving RiPPs for specific functions, the authors turned to two well-established platforms for directed evolution: yeast surface display and phage display.1,4 Yeast display offers the advantage of simple sorting using flow cytometry, but as the authors note, yeast may not have all the necessary cofactors needed to produce particularly complex RiPPs.1 Phage display, on the other hand, allows for a broader range of enzymatic machineries with which to carry out post-translational modifications, but lacks the same ease of selecting desirable mutants. The authors focus on lanthipeptides, a subfamily of RiPPs characterized by one or more thioether-based cyclization motifs (Figure 1A).

In the first use of yeast surface display to evolve a bacterial RiPP, Hetrick et al. successfully impart a new function to the antibacterial molecule lacticin 481. By selecting for mutants that bind to αvβ3 integrin, they recapitulate the known RGD amino acid binding motif and generate, within two...
generations, novel integrin ligands with affinities similar to other engineered ligands, and only an order of magnitude weaker than the best ligands available (Figure 1B). Notably, this was accomplished by randomizing only one of the macrocycles (the C-ring) in lacticin 481. It is conceivable that randomization of other rings in lacticin 481 can also deliver integrin binders, among other bioactivities.

The use of phage display for RiPP evolution is substantially more complicated than yeast display, requiring a pull-down assay and subsequent reinfection into *Escherichia coli* prior to sequencing. Despite these challenges, access to certain cofactors may make phage display a more versatile platform in the long run, as demonstrated by the directed evolution of nisin A, which requires a more complex biosynthetic pathway compared to lacticin 481.1,4 The authors randomized the sequences of two macrocycles in nisin A (the A- and B-rings) and, using phage display, demonstrated efficient selection for lipid II binders, the natural target of nisin (Figure 1B). This new molecule lacks the antibiotic

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Figure 1. Directed evolution of lanthipeptides. (A) Structure of the lanthionine (R = H) or methylanthionine (R = CH3) thioether cross-link, a hallmark of lanthipeptides. (B) Yeast surface display or phage display of a library of lanthipeptides randomized at the core sequence allows for selection of variants that exhibit tight binding to desired targets.1 See text for further details.

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activity of the native sequence, possibly due to a different binding mode compared to wild-type, and reflective of the limitation to target molecules suitable for a pull-down assay.

The molecules and targets chosen by the authors have been thoroughly examined, making them ideal candidates for a proof-of-concept study. Even so, the study required overcoming several hurdles: surface display requires expression of a fusion construct, with a yeast or phage protein attached to the N- or C-terminus of the RiPP precursor peptide. Any fusions with the precursor peptide could potentially prevent the necessary post-translational modifications. Indeed, this drawback was hinted at in the authors’ expansion upon the initial work of Urban and co-workers, who successfully fused lanthipeptide precursors to the C-terminus of a phage minor coat protein. Nisin’s activity is dependent upon binding of the N-terminal portion of the molecule to lipid II, and even one additional N-terminal residue can abolish its affinity. The authors worked nicely around this problem by placing the precursor peptide at the N-terminus of the phage protein. However, it is likely that some systems will not tolerate fusions at all.

The ability to generate and select from large libraries of RiPPs, as demonstrated by the authors, opens new lines of research, which will surely be explored in the future. Intriguingly, the approach may be applied to additional RiPP families, which contain unique cyclization motifs, thereby expanding the practical use of other RiPPs discovered thus far.

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