PROTEIN EVOLUTION

Characterizing the landscape of evolvability

A framework to experimentally traverse the large space of functionally neutral variants in a toxin–antitoxin protein complex reveals insights on evolvability and entrenchment of molecular interactions.

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Molecular evolution is a complex dynamical process in which multiple factors contribute to the composition of proteins and nucleic acids. Such factors include selective pressures on thermodynamic stability, three-dimensional folds, translation rates, functional interactions, enzymatic activities and recognition specificity. Neutral evolution and drift also have an important role by maintaining diversity, which can potentially lead to neofunctionalization or allow evolutionary pathways that were previously blocked by energetic or functional constraints. This expansion of potential evolutionary pathways is often called evolvability[1]. Evolvability is difficult to observe in real time or to infer from phylogenetic datasets. A more tractable approach is to quantify and predict molecular evolutionary changes that might lead to specific phenotypes such as disruption, maintenance or enhancement of function or stability, especially for interactions between proteins.

Writing in *Nature Ecology & Evolution*, Ding et al.[2] present an experimental and computational framework to assess evolvability in the interaction between the toxin ParE3 and the antitoxin ParD3. To obtain a comprehensive picture of how the mutational landscape influences functional outputs in this interaction, the authors used deep mutational scanning combined with a high-throughput co-expression system. Because a failed antitoxin–toxin complex becomes deleterious for cell proliferation when co-expressed in *Escherichia coli*, the effect of mutations can be investigated using growth assays.

The authors use this approach to identify single-point mutants of ParE3 and ParD3 that retain function through complex binding. They also tested for single-point mutants in ParE3 that could rescue disruptive mutations in ParD3, and also showed that nonspecific, neutral mutations could lead to novel functional interfaces after further evolution.

In an initial experiment (Fig. 1a), the authors created a combinatorial library for the 93-amino-acid ParD3 antitoxin. About 2,000 variants of ParD3 were tested in combination with the wild-type ParE3 toxin. This experiment allowed the authors to estimate how robust the antitoxin is to mutations and to isolate the most relevant amino acid sites for complex formation. They found that oligomerization regions are important, as are (as expected) sites located at the interface with the toxin. These same interface sites also emerge from coevolutionary analysis as being strongly coupled. Coevolutionary analysis creates a global statistical model of amino acid interactions from multiple sequence alignments of the families of both proteins. Residue sites are considered strongly coupled when information about the amino

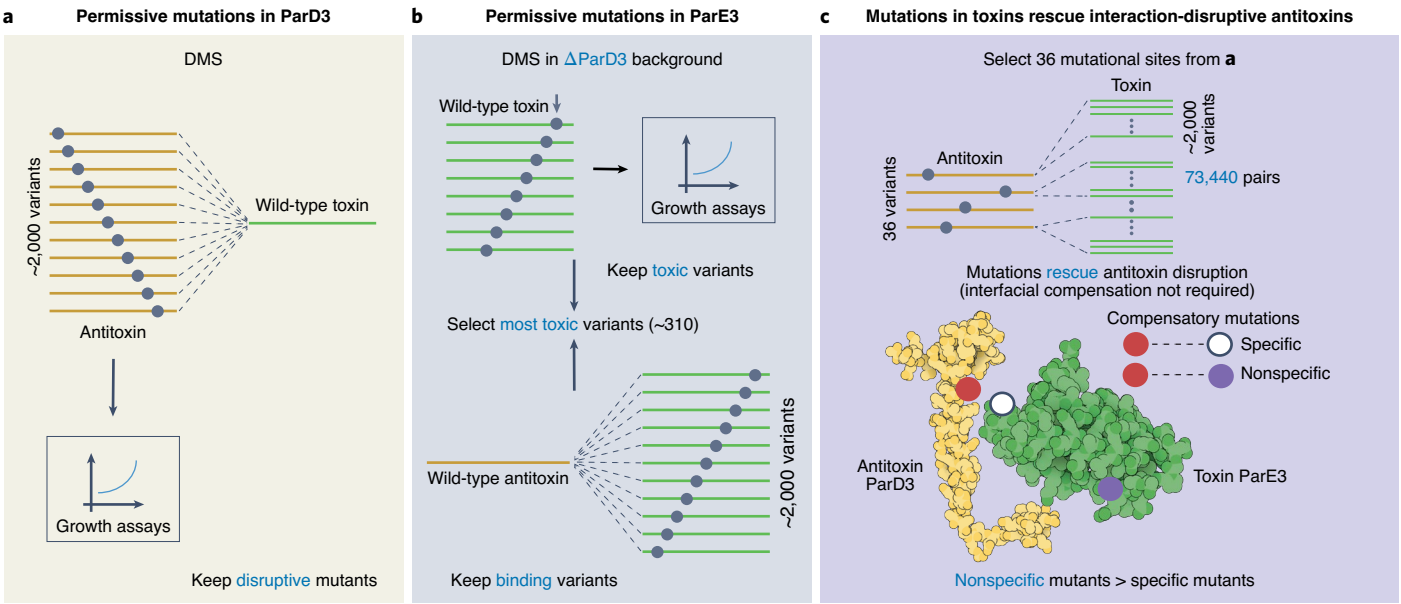

Fig. 1 | Mutational analysis of toxin–antitoxin binding. a–c, A series of deep mutational scanning experiments, along with high-throughput growth assays and modelling, were used to identify antitoxin variants that disrupt function (**a**); toxin variants that preserve toxicity and binding (**b**), and a combination of mutants that rescue disrupted binding in the antitoxin background (**c**). Many of these toxin compensatory mutants did not directly interact at the interface and were recognized by multiple antitoxin variants. Protein structures rendered with Protein Imager[11].

acid composition in one site is predictive of the second site, even after phylogenetic sampling effects have been reduced[3]. This result is consistent with previous work that has shown that an accurate ParD3–ParE3 interface can be computationally inferred[4].

The authors then performed a second set of deep mutational scanning experiments (Fig. 1b) to identify mutations in ParE3 that maintain toxicity in an environment removed from the antitoxin, and another set of experiments to identify toxin variants that also retain binding. Taken together, they were able to isolate the most toxic variants (about 310) out of thousands of possibilities. Finally, the authors used the results of the first two experiments to assess mutations in the toxin that enable binding to antitoxins that possess interaction-disrupting mutations (Fig. 1c). They did this by selecting 36 antitoxin variants from their first experiment that include sites that strongly covary, as well as sites with amino acids commonly found in ParD3 antitoxin homologues. These 36 variants were paired with the library of toxin variants from their second experiment to evaluate more than 73,000 protein pairs and identify whether some of these toxins could reinstate binding. They found that for the set of 310 most toxic variants, a total of 11 variants improved growth in ParD3(W59T) antitoxin mutants that previously abrogated binding. Interestingly, they saw that rescue mutants in the toxin were distributed across the protein, with only a minority providing specific compensation at the interface and near the mutated site. Instead, the majority of such mutants were found on locations distant from the mutated site — and in several cases, outside of the interface. In fact, they also observed this trend in the rest of the antitoxin binding-disruptive mutants. They concluded that there exists a subset of toxin mutants that preserve a toxicity similar to the wild type, but also allow the antitoxin to evolve towards variants that would otherwise be disruptive. Examples such as this of evolvability (in which neutral mutations in the toxin allow future changes in the antitoxin to remain functional) and entrenchment (in which reverting mutations in the toxin that were previously neutral become deleterious if the antitoxin mutates in the future) have been discussed in evolutionary theory[5–7] but have previously only been shown experimentally in anecdotal cases.

Finally, Ding and colleagues analysed whether such pairs that reestablish function can be explained via coevolutionary analysis, which has been useful for identifying physically interacting amino acid sites that coevolve[3,8,9]. In principle, if such mutants are able to restore functional outcomes, then they should be correlated and might show a high degree of coupling. However, the authors did not observe this; instead, pairs that re-established function were indistinguishable from random selection of coupled pairs. There are two possible explanations for this observation. The first is that those particular compensatory changes have never occurred in the evolutionary history of the toxin–antitoxin families and therefore the statistical model does not have any samples of covariance among those sites. A second view is that the restorative effects observed in the work of Ding et al.[2] cannot be explained by a small number of strong interacting pairs: instead, the compensation is the result of a collective phenomenon that involves the additive effect of smaller couplings, reminiscent of allostery. A similar effect has been observed in a study[10] that reestablished function in chimeric proteins built with a mixture of domains that do not have a large number of contacting residues. A score based on the collection of couplings — even if those were for noncontacting sites — allowed the design of mutants that brought back functional activity[10].

A remarkable result of the work of Ding and colleagues is the ability to measure, in a systematic way, the number of cases of both evolvability and entrenchment. These data provide a road map for understanding the branching possibilities in the evolution of protein interactions. Their work might have a wide range of applications in amino acid coevolution, ranging from protein complex design to the ability to predict evolutionary trajectories of relevant biomolecules — a topic of relevance in our current pandemic era. ❐

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Competing interests

The author declares no competing interests.