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Spotlight

Hallmarks of icosahedral virus capsids emerged during laboratory evolution of a bacterial enzyme

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Viruses are fascinating molecular machines that inspire many therapeutic design efforts. Tetter, Terasaka, Steinauer *et al.* recently reported the laboratory evolution of a synthetic nucleocapsid from a bacterial enzyme. Their work sheds light on the potential origin of viruses and points the way to improved nanotechnology platforms.

At the turn of the 20th century, scientists discovered submicroscopic biological particles capable of infecting living organisms and transmitting disease [1]. Intensive study during the 20th century revealed the central architectural elements of these infectious agents: a nucleic acid genome surrounded by a protein shell [2]. We now know these remarkable molecular machines, viruses, to be the most abundant biological entities on Earth and key players in the evolution of life over the past 3.8 billion years. Understanding how viruses first arose is fundamental to understanding evolution, preventing and treating disease, and inspiring novel therapeutic technologies. However, the details of how viruses originated remain elusive and it is difficult to obtain evidence supporting any specific viral origin hypothesis. One leading theory posits that virus precursors were initially formed by repurposing host cell proteins [3]. In a recent publication, Tetter, Terasaka, Steinauer *et al.* provide ground-up evidence supporting this hypothesis by evolving a bacterial enzyme into a synthetic nucleocapsid (NC) that possesses several of the structural hallmarks of viruses [4]. Beyond offering important evolutionary insights, this work highlights promising avenues for future nanotherapeutic research.

Not all container-like protein assemblies package a genome: many non-viral proteins assemble into nanocompartments that function as storage vessels, enzymatic reaction chambers, or protein-bounded organelles. For example, lumazine synthase is a pentameric bacterial enzyme that in some species further assembles into 60-subunit nanocompartments, making it an ideal candidate for developing a capsid-like architecture [5]. In previous work, the Hilvert group engineered *Aquifex aeolicus* lumazine synthase (AaLS) to selectively package its own encoding mRNA by rewiring the AaLS monomer to display RNA-binding peptides on the nanocompartment interior that bind specific mRNA stem-loops, termed BoxB tags [6] (Figure 1A). The resulting mRNA-packaging nanocompartment, or NC, contains the fundamental biological relationship essential for evolution: the linkage between genotype (encapsidated nucleic acid) and phenotype (biological functions and interactions). Terasaka *et al.* evolved the NC in two stages, from NC-1 to NC-3, which ultimately packaged its full-length mRNA genome at a rate of one genome per eight capsids [6] (Figure 1C). Tetter, Terasaka, Steinauer *et al.* have now further optimized NC-3 through additional rounds of directed evolution (Figure 1B). The group generated a library of NC variants via error-prone PCR and subjected them to increasingly stringent selection by incubation with progressively smaller nucleases for longer periods of time. The encapsidation efficiency of the best-performing mutant, NC-4, was 2.5 full-length mRNA genomes per capsid. Although other synthetic NCs have previously been designed [7], to our knowledge, encapsidation capacities this high have not been reported. Together, the keen choice of starting nanocontainer and the clever evolution scheme enabled drastic improvements in production yield, particle uniformity, packaging efficiency, and nuclease resistance from NC-1 to NC-4.

Tetter, Terasaka, Steinauer *et al.* used cryo-electron microscopy to show that the evolution from AaLS to NC-4 was accompanied by dramatic changes in the structure of the protein shell. These changes strikingly recapitulate several structural hallmarks of virus capsids. First, NC-3 and NC-4 protect their genetic material by forming large, nonporous structures that prevent nuclease access to the encapsidated RNA genome (Figure 1D). This feature, universally shared by known nonenveloped viruses, did not evolve in a single large evolutionary jump, but in several gradual steps from AaLS to NC-4. Second, NC-4 is formed from a single type of protein subunit that occupies four slightly distinct positions in the capsid, each of which makes subtly different interactions with neighboring subunits. This phenomenon, termed quasi-equivalence [2], enables the generation of large protein shells from a small number of gene products and is a defining characteristic of many icosahedral virus capsids. Finally, and most remarkably, the NC-4 protein achieves quasi-equivalence in precisely the same way as many virus capsid proteins: through domain swapping and subtle changes in backbone conformation. A hinge region between the swapped domains that emerged during evolution and the engineered loop introduced during circular permutation both adopt slightly different conformations in each of the four quasi-equivalent subunits. The emergence of the architectural principles of icosahedral viruses during the evolution of NC-4 powerfully supports the notion that viruses may have originated from cellular proteins that evolved to package nucleic acid. It also suggests that quasi-equivalence

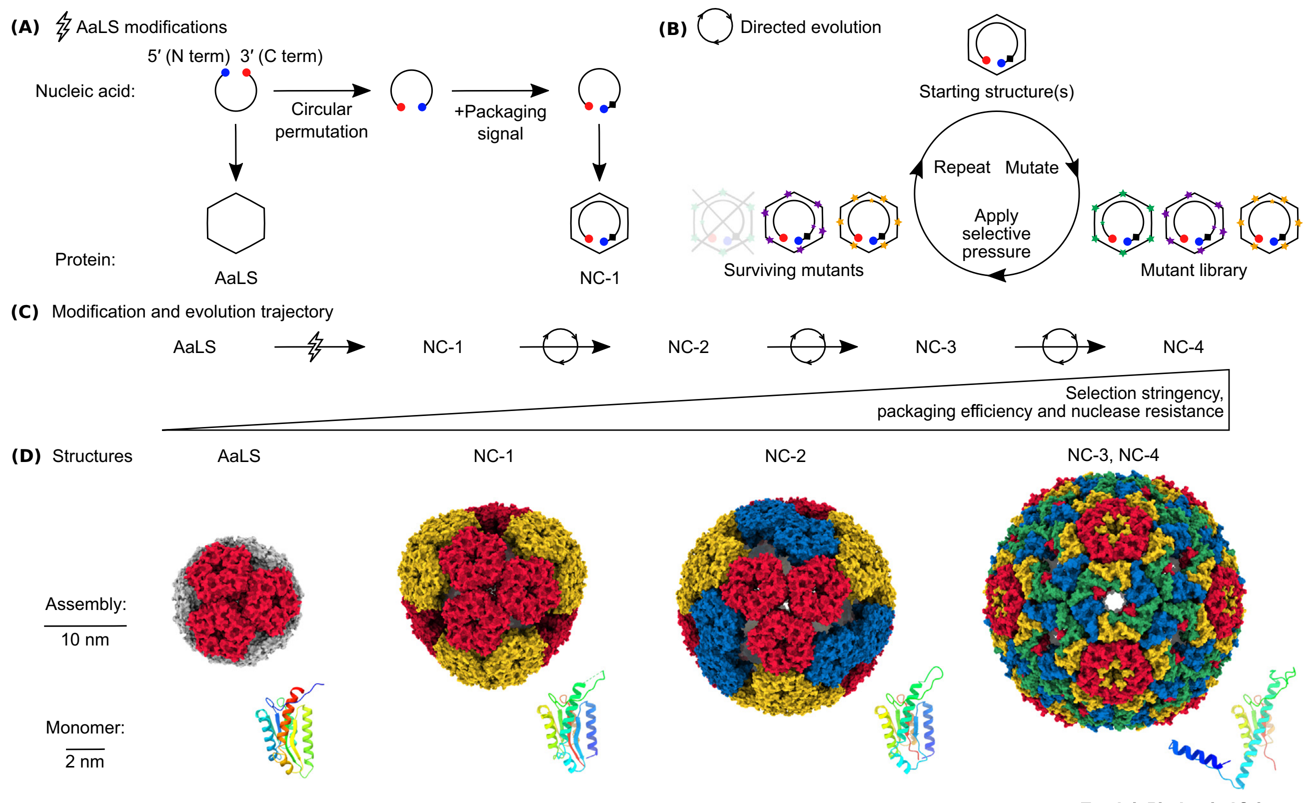

Figure 1. Modification and directed evolution from *Aquifex aeolicus* lumazine synthase (AaLS) to nucleocapsid-4 (NC-4). (A) AaLS was engineered to selectively package its mRNA genome by circularly permuting the protein chain for internal display of an RNA packaging signal. The corresponding mRNA is selectively encapsulated upon protein expression and assembly in *Escherichia coli*, making nucleocapsid-1 (NC-1). (B) The directed evolution strategy employed by Tetter, Terasaka, Steinauer *et al.* from NC-1 to NC-4 involved mutating the starting structure(s), applying a selective pressure (nuclease treatment), isolating the surviving mutant population, and repeating the process [4]. (C) Following the modification of AaLS to NC-1, Tetter, Terasaka, Steinauer *et al*. performed directed evolution with increasing stringency (decreasing nuclease size, increasing time), resulting in increased genome packaging efficiency and nuclease resistance. (D) Structures of protein assemblies (top row) and monomer subunits (bottom row, inset). Protein assemblies are colored according to pentamer–pentamer interactions. Monomer subunits (single chains) are colored as rainbows from blue (N terminus) to red (C terminus). Accession codes: PDB 1HQK, PDB 7A4F, PDB 7A4H, and PDB 7A4J, respectively.

arising from subtle backbone flexibility is the solution to large protein assembly formation that is most accessible to evolution.

In the final stage of directed evolution, Tetter, Terasaka, Steinauer *et al.* did not observe any NC structural changes, yet the fraction of total encapsidated RNA accounted for by the full-length genome increased from about 24 to 64% (Figure 1D, rightmost structure). Furthermore, an NC-4 variant lacking the RNA-binding peptide showed a two-fold reduction in production yield and notably increased structural heterogeneity. These observations led Tetter, Terasaka, Steinauer *et al.* to hypothesize that co-evolution of the protein and its encoding RNA occurred and that the evolution of additional packaging signals in the mRNA beyond the original engineered stem-loops drove the increase in encapsidation efficiency without altering protein structure. Evidence obtained from synchrotron X-ray footprinting, a technique used to map intra- and intermolecular interactions of RNA, shed light on the validity of this hypothesis. Tetter, Terasaka, Steinauer *et al.* observed that NC-3 and NC-4 both contain many mRNA packaging signals spread throughout their genomes. However, NC-4 uniquely contains a dense cluster of one especially strong packaging signal amongst several weaker packaging signals at the 5′ end of the genome, a pattern found in several viruses that appears to efficiently nucleate capsid assembly. Thus, NC-4 exhibited another hallmark of viral evolution: the co-occurrence of structural and genomic changes.

Although not a virus, NC-4 possesses several structural and functional characteristics that make it a convincing mimic. The work of Tetter, Terasaka, Steinauer *et al.* offers striking evidence for a cellular origin of viruses and showcases our growing ability to generate new nanotherapeutic

scaffolds through evolution or design. Viruses have evolved to efficiently perform many functions of biotechnological interest, including targeting specific cell types, crossing cellular membranes, delivering genomic information, and modulating the host immune system. More deeply understanding how these functions arose will improve our ability to both mimic and modify viruses for therapeutic applications, which remains challenging despite substantial recent progress [8–10]. Tetter, Terasaka, Steinauer *et al.* have shown us how to construct a capsid-like container that rivals the packaging efficiency of viruses; learning how to match their delivery capabilities with similar efficiency through evolution or rational design will usher in a new era in medicine.

Declaration of interests

The authors declare no conflicts of interest.

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