

Divergent evolution of a bifunctional *de novo* protein

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Abstract: Primordial proteins, the evolutionary ancestors of modern sequences, are presumed to have been minimally active and nonspecific. Following eons of selective pressure, these early progenitors evolved into highly active and specific proteins. While evolutionary trajectories from poorly active and multifunctional generalists toward highly active specialists likely occurred many times in evolutionary history, such pathways are difficult to reconstruct in natural systems, where primordial sequences are lost to time. To test the hypothesis that selection for enhanced activity leads to a loss of promiscuity, we evolved a *de novo* designed bifunctional protein. The parental protein, denoted Syn-IF, was chosen from a library of binary patterned 4-helix bundles. Syn-IF was shown previously to rescue two different auxotrophic strains of *E. coli*: $\Delta ilvA$ and Δfes . These two strains contain deletions for proteins with very different biochemical functions; *IlvA* is involved in isoleucine biosynthesis, while *Fes* is involved in iron assimilation. In two separate experiments, Syn-IF, was evolved for faster rescue of either $\Delta ilvA$ or Δfes . Following multiple rounds of mutagenesis, two new proteins were selected, each capable of rescuing the selected function significantly faster than the parental protein. In each case, the evolved protein also lost the ability to rescue the unselected function. In both evolutionary trajectories, the original bifunctional generalist was evolved into a monofunctional specialist with enhanced activity.

Keywords: protein design; polar/nonpolar patterning; functional promiscuity; binary code; four helix bundle; directed evolution; bifunctional protein

Introduction

Natural proteins have been shaped by evolution to be efficient catalysts with high levels of activity and

substrate specificity. In most cases, these functional sequences are “specialists”: They perform a single function, and they do it well. Because of this specialization, many proteins—an entire proteome of specialists—are required to provide the full complement of functions necessary to sustain a living organism.

Abbreviations: IPTG, isopropyl β -D-1-thiogalactopyranoside; Syn-F4, synthetic protein rescues Δfes , fourth generation; Syn-I3, synthetic protein rescues $\Delta ilvA$, third generation.

Additional Supporting Information may be found in the online version of this article.

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Unlike their modern day descendants, primordial proteins were probably generalists, not specialists.¹ Although they were inefficient at any given reaction, they were capable of multitasking, with low-level activities in a range of reactions. These multifunctional generalists would have enhanced “the catalytic versatility of an ancestral cell that functioned with limited enzyme resources.”² It has been suggested that selection for greater levels of activity and specificity caused these ancestral sequences to lose their ability to multitask, and led them to evolve from generalists to specialists.²

Although it seems reasonable to speculate that natural selection drove marginally active generalists to evolve into highly active specialists, hypotheses

about molecular evolution in natural biological systems are difficult to test when primordial sequences are lost to time and cannot be assayed for activity and specificity. Recently, however, non-natural biological systems have become available for such studies, as advances in synthetic biology and *de novo* protein design produce novel sequences that never existed in nature.^{3,4} Many of these novel proteins fold and function. Moreover, they can be evolved—both *in vitro* and *in vivo*. In contrast to primordial natural sequences, these novel sequences are not lost to history; they are readily available for testing hypotheses about structure, function, and molecular evolution.

To experimentally investigate the evolution of generalists to specialists, we focused on a *de novo* protein drawn from a library of 10⁶ novel protein sequences designed via polar/nonpolar patterning to fold into 4-helix bundles.⁵ Previous work demonstrated that many proteins from this and similar libraries are able to bind cofactors and other small molecules.^{6–8} Moreover, a number of these proteins exhibit rudimentary catalytic activities.⁸ Most importantly, several novel proteins from this library function *in vivo*: They enable the growth of auxotrophic *E. coli*, strains in which a gene essential for growth on minimal medium had been deleted. In initial studies, four different auxotrophs were found to be rescued by *de novo* proteins. $\Delta serB$, $\Delta gltA$, and $\Delta ilvA$ lack enzymes in the biosynthesis of serine, glutamate, and isoleucine, respectively, and Δfes has impaired iron assimilation.⁹

In subsequent studies, a *de novo* protein that rescued the auxotrophic strain $\Delta ilvA$ was shown to also rescue Δfes .¹⁰ Because of its ability to rescue both functions, this protein is denoted as Syn-IF (Synthetic protein rescues $\Delta ilvA$ and Δfes). [Syn-IF was originally called Syn-IlvA1 in Ref. [9].] The two deleted genes encode dramatically different enzymes: IlvA encodes threonine deaminase, the first enzyme in the biosynthetic pathway from threonine to isoleucine, whereas Fes encodes ferric enterobactin esterase, which cleaves the enterobactin siderophore in an iron-acquisition pathway.^{11,12} The bifunctional activity of the *de novo* protein Syn-IF is reminiscent of the generalist proteins hypothesized to be progenitors of modern-day enzymes. However, in contrast to natural ancestors, which are no longer available, Syn-IF is readily available and can be evolved in the laboratory to probe whether selecting for higher levels of a single activity causes a loss of the unselected function, thereby leading to a transition from generalist to specialist.

In separate experiments, we selected mutants of Syn-IF that enabled faster growth in either $\Delta ilvA$ or Δfes cells. After several rounds of evolution, descendants that were more active in rescuing $\Delta ilvA$ were tested for their ability to rescue Δfes ,

and vice versa. We found that enhanced ability to rescue one function was accompanied by a loss of ability to rescue the other function. Although the single parental sequence rescued both functions in the double knockout strain, the evolved progeny sequences were able to rescue the double knockout only when both were provided simultaneously. Thus, selecting for increased activity caused one generalist progenitor to evolve into two specialist progeny.

Results

The parental *de novo* sequence, Syn-IF, rescues the deletions of two different functions

The *de novo* protein Syn-IF was originally selected for its ability to rescue an auxotrophic strain of *E. coli* containing the deletion of *ilvA*.⁹ Subsequently, Syn-IF was found to rescue another auxotrophic strain, one in which *fes* was deleted.¹⁰ These initial findings were confirmed by testing the growth rate of cells expressing Syn-IF in each strain. Expression of Syn-IF in $\Delta ilvA$ cells on minimal plates produced visible colonies in 2 days. Expression of Syn-IF in Δfes cells grown on minimal plates produced visible colonies in 7 days. Therefore, Syn-IF is a bifunctional protein with primary and secondary functions. We deemed this sequence a suitable starting point to test whether a *de novo* protein—which did not evolve in nature—could be evolved from a specialist to a generalist in response to selective pressure in a laboratory setting. Specifically, we used directed evolution to attempt to improve, and/or switch, the primary function of Syn-IF.

Evolution of Syn-IF toward faster rescue of each deletion

Evolution toward faster rescue of Δfes . Syn-IF rescues Δfes cells on minimal plates in 7 days, considerably slower than the native *fes* enzyme, which enables growth in less than 2 days under the same conditions. We looked to improve this secondary function of Syn-IF through directed evolution in the Δfes strain. Four generations of mutagenesis and selection produced a sequence with seven mutations (Fig. 1). This sequence, named Syn-F4 (Synthetic protein rescues Δfes , fourth generation), allowed Δfes cells to produce colonies on minimal plates in less than 2 days [Fig. 2(A)], significantly faster than the parental protein.

Evolution toward faster rescue of $\Delta ilvA$. Using standard conditions for induction (0.05 mM isopropyl β -D-1-thiogalactopyranoside [IPTG]), Syn-IF rescues the growth of $\Delta ilvA$ cells on minimal plates in nearly the same time as native IlvA, producing colonies in only 2 days. Because it seemed unlikely that a mutant of Syn-IF would promote faster growth than

Syn-IF MYGKLNQLFHNLEIVEDLNKNWHRESRNLHDFEDELHQLVKHFHFMQGHKNEGKLDIVKQLDKLFRDLNHLQRKDDTVHHLHHQLNKLLEQLDNLVHR
 Syn-I3 MYGKLNQLFHNLEIVEDLNKNWHRESRNLHDFEDELHQLVKHFHFMQGHKNEGKLDIVKQLDKLFRDLNHLQRKDDTVHHLHHQLNKLLEQLDNLVHR
 Syn-F4 MYGKLNQLFHNLEIVEDLNKNWHRESRNLHDFEDELHQLVKHFHFMQGHKNEGKLDIVKQLDKLFRDLNHLQRKDDTVHHLHHQLNKLLEQLDNLVHR
 1 10 20 30 40 50 60 70 80 90 100

Figure 1. Sequences of the parental protein Syn-IF, and the evolved proteins, Syn-I3 and Syn-F4. Residues that differ from the original sequence are highlighted and underlined. Sequences and properties of the evolutionary intermediates can be found in the Supporting Information.

the natural IlvA protein, we chose to evolve Syn-IF for $\Delta ilvA$ rescue under more stringent conditions. Lowering the concentration of IPTG to 0.01 mM slowed the rescue of $\Delta ilvA$ by Syn-IF to 6 days. (The native IlvA protein on the same vector still rescued in 2 days.) Three generations of mutagenesis and selection for faster growth led to the isolation of a sequence containing three mutations (Fig. 1). This sequence, named Syn-I3 (Synthetic protein rescues $\Delta ilvA$, third generation), allowed $\Delta ilvA$ cells to produce colonies on minimal plates (containing 0.01 mM IPTG) in only 2 days [Fig. 2(C)].

Evolution toward faster rescue of one function leads to loss of the other function

In addition to assessing the selected function in each strain, we also monitored how the unselected function was affected. After three rounds of selection, the evolved sequence Syn-I3 had lost its secondary function and no longer rescued Δfes cells [Fig. 2(D)]. Perhaps more surprisingly, the evolved sequence Syn-F4 had lost the primary function of the parental Syn-IF, and no longer rescued $\Delta ilvA$ [Fig. 2(B)]. Thus, in both evolutionary trajectories, selection for increased activity in one function led to a loss of the other function.

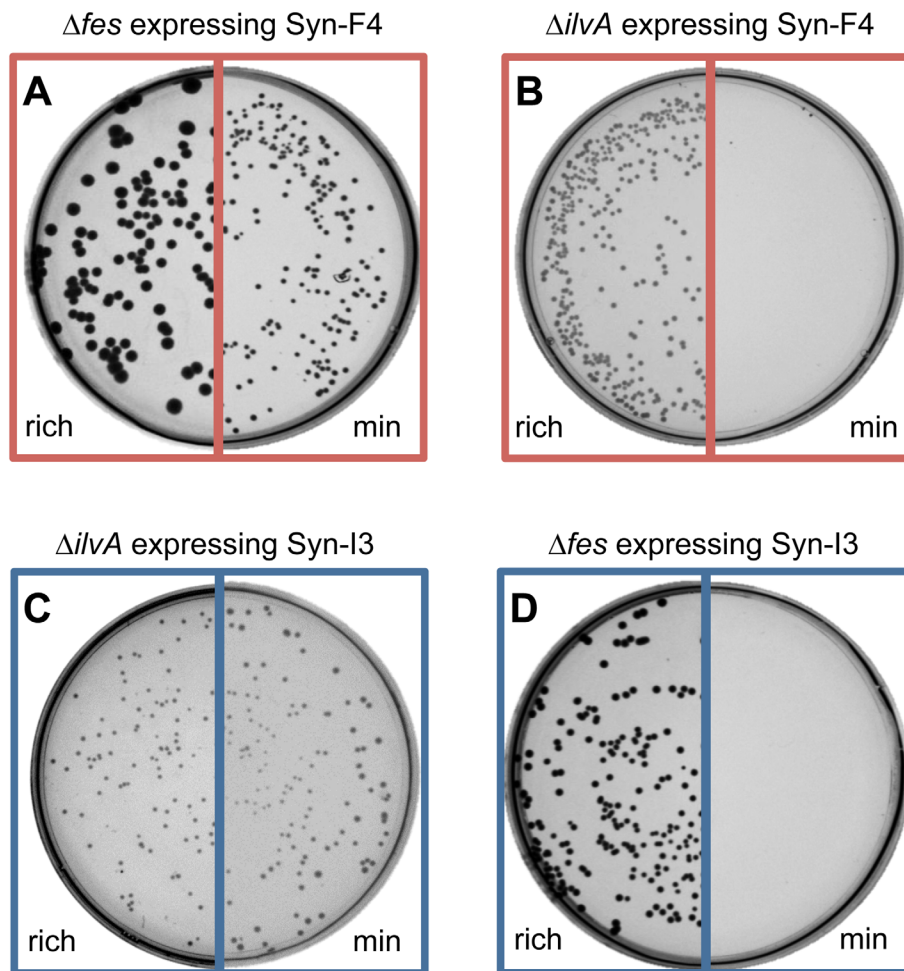


Figure 2. Rescue of $\Delta ilvA$ and Δfes cells by evolved proteins Syn-I3 and Syn-F4. In each panel, the left side shows growth on rich plates supplemented with IPTG. Growth on rich plates is a control demonstrating transformation of the appropriate plasmid into the host. The right side of each panel shows transformants from the same experiment plated on minimal media supplemented with the same amount of IPTG. Growth on minimal plates demonstrates the ability of the *de novo* protein to rescue the conditionally essential function deleted in the host strain. Growth was observed on rich plates after 1 day. On minimal plates, growth was observed after 2 days for Δfes /Syn-F4 and $\Delta ilvA$ /Syn-I3 (image taken after 6 days). No growth was observed on minimal plates with Δfes /Syn-I3 or $\Delta ilvA$ /Syn-F4 after incubation for 7 days.

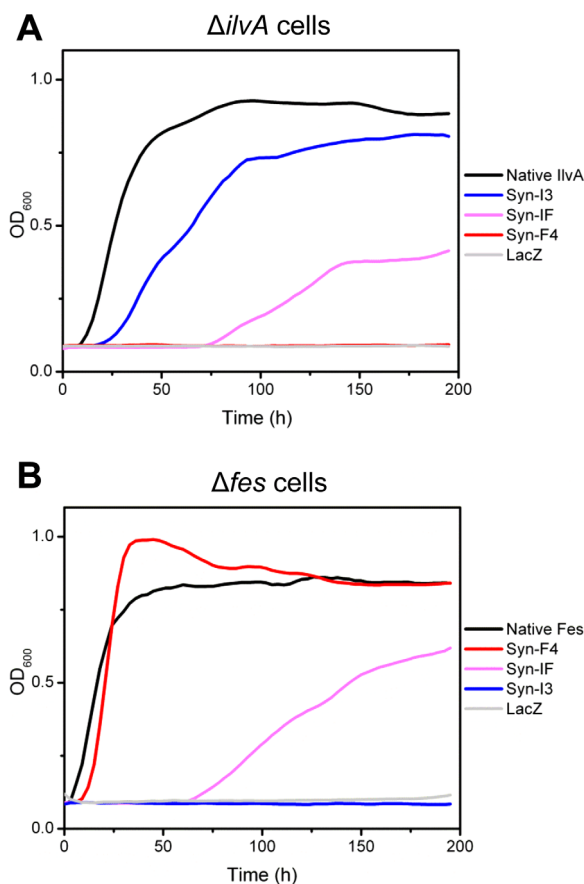


Figure 3. Growth rates in liquid minimal media of $\Delta ilvA$ cells (panel A) and Δfes cells (panel B) harboring plasmids expressing the indicated proteins. Δfes cells were grown in 0.05 mM IPTG, and $\Delta ilvA$ cells were grown in 0.01 mM IPTG (see text).

Rescue and growth rates in liquid media

To confirm and quantify the improvement in rescue, the evolved proteins Syn-I3 and Syn-F4 were expressed in either $\Delta ilvA$ or Δfes cells, and their growth rates in liquid minimal media were compared to the same cells expressing the parental protein Syn-IF. They were also compared to positive controls expressing the appropriate native enzyme and negative controls expressing LacZ. Consistent with the evolution on solid media described above, Δfes cells were grown in the presence of 0.05 mM IPTG, and $\Delta ilvA$ cells were grown in the presence of 0.01 mM IPTG.

$\Delta ilvA$ cells expressing the control proteins produced the expected results: the negative control, LacZ, did not support growth, while native IlvA supported faster growth than any of the *de novo* proteins [Fig. 3(A)]. Most importantly, $\Delta ilvA$ cells expressing the evolved protein, Syn-I3, grew much faster than the same cells expressing the parental protein, Syn-IF. (As shown in Figure 3(A), $\Delta ilvA$ cells containing Syn-I3 showed growth in 25 h, while those containing Syn-IF did not show growth until 75 h had elapsed.) Meanwhile, $\Delta ilvA$ cells expressing

Syn-F4 failed to grow at all. Thus, Syn-I3, which had been evolved to rescue $\Delta ilvA$ provided faster rescue of $\Delta ilvA$, while Syn-F4, which had been evolved to rescue Δfes , did not rescue $\Delta ilvA$ [Fig. 3(A)].

In Δfes cells, as expected, the negative control (LacZ) failed to support growth, while the positive control, native Fes, supported rapid growth [Fig. 3(B)]. The evolved protein, Syn-F4, showed significant growth in 18 h, much faster than the parental protein Syn-IF, which did not show growth until nearly 75 h had elapsed. Indeed, the evolved protein enabled Δfes cells to grow at a similar rate to cells expressing the natural Fes protein from the same vector [Fig. 3(B)]. Notably, Syn-I3, which was evolved to rescue $\Delta ilvA$, failed to support the growth of Δfes cells.

The growth curves shown in Figure 3 confirm the results observed on solid media: selection for higher activity caused the evolution from one generalist to two specialists.

The *de novo* proteins express at similar levels

The altered abilities of the evolved proteins to rescue the deletion strains could, in principal, result from altered levels of expression. To test this possibility, we assessed the expression levels of Syn-IF, Syn-I3, and Syn-F4 in each deletion strain. Protein expression was induced, cultures were normalized for cell density, and cell lysates were analyzed by SDS-PAGE. As shown in Figure 4, the three proteins express at similar levels in each of the strains. Thus, dramatic changes in expression do not account

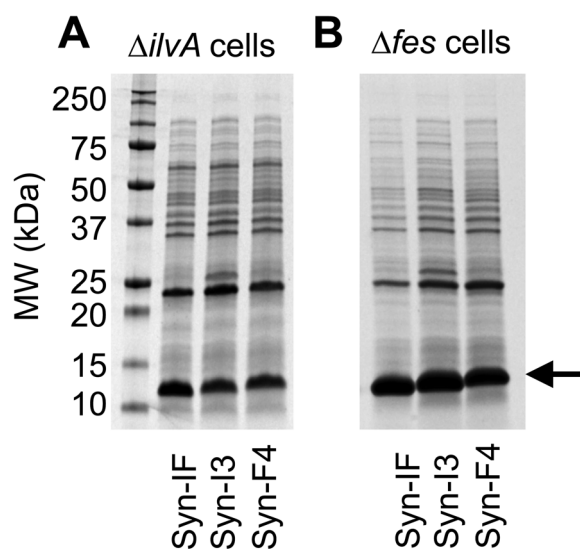


Figure 4. Expression of synthetic proteins in deletion strains. Cells were grown in rich liquid media at 33°C supplemented with either 0.01 mM ($\Delta ilvA$) or 0.05 mM (Δfes) IPTG. The *de novo* proteins migrate at the expected molecular weights of ~12.5 kDa marked by the arrow.

$\Delta ilvA$ MYGKLNQLFHNLNEIVEDLNKNWHRESRNLDHFEDELHQIVKHPHFHQGHKNEGKLDIVKQLDKLFRLDNLQKDDTVHHLHHQLNKLLEQLDNEVHR
 Δfes MYGKLNQLFHNLNEIVEDLNKNWHRESRNLDHFEDELHQIVKHPHFHQGHKNEGKLDIVKQLDKLFRLDNLQKDDTVHHLHHQLNKLLEQLDNEVHR
 1 10 20 30 40 50 60 70 80 90 100

Figure 5. Alanine scanning mutagenesis of Syn-IF. All residues in the sequence (except the initiator methionine) were mutated individually to alanine. Each singly substituted sequence was then tested for its ability to support the growth of either $\Delta ilvA$ or Δfes cells on minimal plates with 0.05 mM IPTG. Positions where the alanine mutant supported growth at a rate similar to or faster than the parental Syn-IF are highlighted green. Positions where the alanine mutant slowed rescue are yellow, and those that eliminated rescue are red.

for the abilities of the evolved proteins to rescue $\Delta ilvA$ or Δfes .

Different sequences are responsible for different functions

Comparing the mutations that gave rise to the evolved proteins Syn-I3 and Syn-F4 shows that different parts of the sequence are important for each of the two different functions (Fig. 1). This led us to question whether different parts of the parental sequence Syn-IF are responsible for the two activities of the original bifunctional protein. To investigate the relation between sequence and function, we mutated every residue in Syn-IF to alanine and monitored the effect of each mutation on each of the two functions.

As shown in Figure 5, most of the mutations have different impacts on the rescue of each deletion. Many alanine mutants destroy the ability of Syn-IF to rescue $\Delta ilvA$ cells (red in top line of Fig. 5), however, the majority of these same mutants still rescue Δfes cells (green in bottom line of Fig. 5). The locations of critical residues differ for the two functions: Most of the alanine mutations that abrogate rescue of $\Delta ilvA$ had no effect on the rescue of Δfes , while many of the mutations that knocked out (or slowed) the rescue of Δfes had little or no effect on the rescue of $\Delta ilvA$. In particular, nearly all residues from 32 to 52 were important for the rescue of $\Delta ilvA$, yet mutations in this region rarely affected the rescue of Δfes .

These results suggest that the ability of the parental Syn-IF sequence to rescue the two deletion strains is not accomplished using the same active site. Instead, it appears that the parental protein, Syn-IF, is indeed bifunctional, with different primary and secondary functions, and key residues required for each function are spatially distinct.

Two evolved specialists are required to provide two essential functions

In natural systems, the evolution of new functions is thought to arise by gene duplication, followed by the divergent evolution of one ancestral sequence toward two sequences capable of performing two different functions.² If both functions are required for viability, and if the evolved sequences are specialists that each perform only one function, then both of the

evolved sequences will be required to sustain cell growth.

In our synthetic biological system, the parental bifunctional protein, Syn-IF, was duplicated and then evolved toward different functions in two different selective environments (i.e., two different deletion strains grown on minimal plates). As described earlier, and shown in Figures 2 and 3, each of the evolved sequences is a specialist and rescues only the function for which it was selected. Therefore, in an environment where both functions are essential for viability, both sequences should be required to support cell growth. We tested this expectation by transforming individual plasmids expressing either Syn-I3 or Syn-F4, or both plasmids simultaneously, into the double deletion strain, $\Delta ilvA\Delta fes$, and testing for growth on minimal plates. As shown in Figure 6, the expected result was observed: neither sequence alone enabled growth, however, the cotransformation produced abundant colonies.

Discussion

Models for the evolution of protein activity assume that the earliest functional polypeptides were

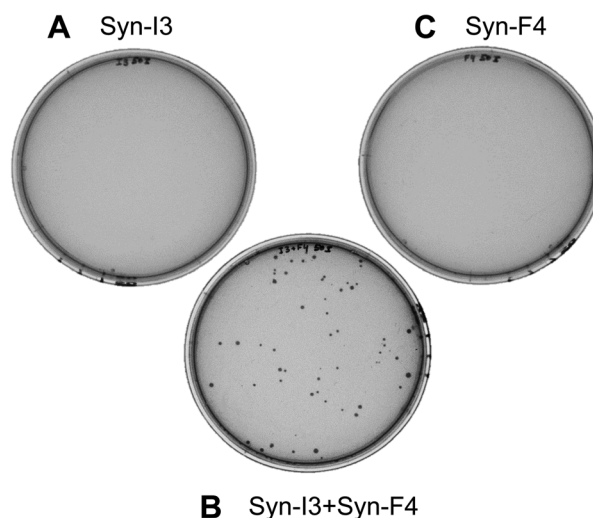


Figure 6. Cotransformation of Syn-I3 and Syn-F4 enables $\Delta ilvA\Delta fes$ cells to grow on minimal plates. Transformed alone, neither Syn-I3 nor Syn-F4 supports the growth of the double knockout $\Delta ilvA\Delta fes$, as seen in panels A and C. However, when transformed with both plasmids (panel B), $\Delta ilvA\Delta fes$ cells produce colonies on minimal plates containing 0.05 mM IPTG and appropriate antibiotics. Images were taken following incubation for 10 days.

weakly catalytic and multifunctional. Over time, these generalists evolved into specialists, as nature selected for the efficiency and specificity required to sustain fully developed metabolic pathways.² Although the primordial generalists are gone, replaced by modern specialists, traces of secondary activity can still be seen in some modern proteins. For example, an extensive study by Patrick *et al.* showed that many deletion mutants of *E. coli* can be rescued by overexpressing other *E. coli* proteins, which have secondary or promiscuous activities.¹³

Traditional studies of molecular evolution rely on phylogenetic and bioinformatic analyses to infer how primordial sequences may have evolved into modern proteins. However, as early ancestral sequences are not available, it is not possible to delineate the complete evolutionary trajectories that led to modern proteins. In contrast to gazing back at evolutionary history, recent advances have made it possible to direct evolution forward in controlled laboratory experiments. Indeed, evolution *in vitro* has led to numerous proteins with real-world applications.^{14–17} However, these studies invariably rely on starting materials (natural proteins) that evolved from unknown primordial ancestors. The possibility of “restarting” evolution *de novo* from known sequences that are not descended from natural progenitors has become feasible only recently, as advances in synthetic biology and protein design facilitate the production of primordial-like *de novo* sequences that are not biased by evolutionary history.

In this study, we initiated a novel evolutionary trajectory starting from a naïve *de novo* sequence, Syn-IF. This protein was not explicitly designed for any function, and was only biased for structure by specifying a polar/nonpolar sequence pattern consistent with a 4-helix bundle.^{5,9} Nonetheless, Syn-IF was able to rescue two auxotrophic *E. coli* strains, *ΔilvA* and *Δfes*, which were deleted for enzymes with two unrelated functions. In separate experiments, we subjected Syn-IF to two different selective pressures. Strikingly, as each individual function improved, the resulting descendant protein lost the ability to perform the unselected function. In both evolutionary trajectories, the bifunctional generalist evolved into a monofunctional specialist.

With only seven mutations in its 102-residue sequence, Syn-IF evolved into Syn-F4, and its primary activity switched from rescuing *ΔilvA* cells to rescuing *Δfes* cells. This sort of functional change is reminiscent of a process that presumably occurred many times in nature, following the duplication of an ancestral gene encoding a multifunctional protein. One copy of the gene could evolve toward higher efficiency of its primary activity, while the other copy could evolve toward increased levels of a secondary activity. In our laboratory studies, the two gene copies were evolved in separate cells, while in

natural evolution this could have occurred in the same cell or organism.

The results described demonstrate that evolution of Syn-IF into Syn-I3 and Syn-F4 generated proteins that produce dramatically different phenotypes. What biochemical activities are responsible for these different biological functions? Initial results suggest that these biochemical activities also differ dramatically, with Syn-F4 functioning as an enzyme and Syn-I3 as a regulatory protein. Research aimed at delineating these activities is underway and will be reported elsewhere.

Materials and Methods

Materials

Knockout strains of *E. coli* were obtained from the Keio collection.¹⁸ *De novo* and natural proteins were expressed from a modified pCA24N vector containing a chloramphenicol resistance gene, as described previously.⁹ Electrocompetent and chemically competent cells were made according to standard procedures.¹⁹ Rich (Luria Broth) and minimal (M9-glucose) media were supplemented with 30 μg/mL kanamycin (Kan) and 30 μg/mL chloramphenicol (Cam) for strain and plasmid selection, respectively. The working concentration of IPTG used was either 0.05 mM for *Δfes* experiments or 0.01 mM for *ΔilvA* experiments, unless otherwise noted.

Evolution

The plasmid containing the gene for Syn-IF was mutated using error prone PCR with 0.4 mM 8-oxo-dGTP as a mutagen and Pfu as the polymerase (Agilent Technologies). After each round of mutagenesis, the collection of mutated plasmids was transformed via electroporation into the appropriate deletion strain and grown on minimal plates supplemented with IPTG. Plates were incubated at 33°C, and the earliest colonies to appear were selected for further evaluation. The plasmids from these colonies were isolated, the genes recloned into fresh plasmid backbones and transformed into naïve cells to ensure that the phenotype correlated with the presence of the novel gene. The genes were sequenced, and the fastest rescuers were carried forward to the next round of mutagenesis.

Growth assays

Cells transformed with appropriate plasmids were grown overnight at 37°C in rich media. Cells were harvested by centrifugation, washed with 1× M9 salts (BD Difco), diluted in minimal media with IPTG to an OD of 0.001. Two hundred microliters of the diluted cells were added to individual wells on a 96 well plate and grown with shaking at 37°C in a Varioskan plate reader. Absorbance was measured at 600 nm every 3 h for a total of 200 h. Growth

assays were performed in triplicate and at least three independent experiments were conducted for each condition. Growth curves were plotted using five-point smoothing.

Protein expression

Cells were grown in rich media for 12 h, normalized to $OD_{600} = 0.2$, diluted by $1000\times$ in fresh media and grown until $OD_{600} = 0.5$, when they were induced with IPTG and allowed to express for 24 h at 33°C . Samples were centrifuged, suspended in $2\times$ Laemmli sample buffer (Bio-Rad) containing 5% β -mercapto ethanol, heated to 95°C for 5 min, and loaded onto SDS-PAGE (Bio-Rad). Gels were stained with Coomassie blue and imaged.

Alanine mutations

Alanine mutants were constructed using standard site-directed mutagenesis techniques and verified by DNA sequencing.²⁰ Plasmids were transformed into $\Delta ilvA$ and Δfes cells using standard heat-shock techniques. Equal amounts of the transformation were plated on rich and minimal plates supplemented with 0.05 mM IPTG and incubated at 33°C . Rich plates were incubated for 1–2 days. Minimal plates were incubated until colonies appeared, for a maximum of 7 days ($\Delta ilvA$ cells) or 10 days (Δfes cells). Each mutant gene was transformed into both strains at least twice.

Rescue of double deletion by two evolved proteins

The requirement for simultaneous expression of both evolved sequences, Syn-I3 and Syn-F4, to rescue the double knockout was tested by transforming plasmids encoding these sequences into the *E. coli* strain $\Delta ilvA\Delta fes$, which was constructed using procedures described previously.⁹ Transformations were performed using each plasmid separately or by cotransforming a mixture of the two plasmids. Transformed cells were plated on minimal plates with 0.05 mM IPTG and incubated at 33°C . For the cotransformation, growth was noted after 5 days. The individual Syn-I3 and Syn-F4 transformants exhibited no growth through 10 days.

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