

Optimized Hemolysin Type 1 Secretion System in *Escherichia coli* by Directed Evolution of the Hly Enhancer Fragment and Including a Terminator Region

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Type 1 secretion systems (T1SS) have a relatively simple architecture compared to other classes of secretion systems and therefore, are attractive to be optimized by protein engineering. Here, we report a KnowVolution campaign for the hemolysin (Hly) enhancer fragment, an untranslated region upstream of the *hlyA* gene, of the hemolysin T1SS of *Escherichia coli* to enhance its secretion efficiency. The best performing variant of the Hly enhancer fragment contained five nucleotide mutations at five positions (A30U, A36U, A54G, A81U, and A116U) resulted

in a 2-fold increase in the secretion level of a model lipase fused to the secretion carrier HlyA1. Computational analysis suggested that altered affinity to the generated enhancer fragment towards the S1 ribosomal protein contributes to the enhanced secretion levels. Furthermore, we demonstrate that involving a native terminator region along with the generated Hly enhancer fragment increased the secretion levels of the Hly system up to 5-fold.

Introduction

Recombinant protein production at high titers in functional form and at reduced costs has been one of the central issues in biotechnology for decades.^[1] *Escherichia coli* (*E. coli*) has been continuously implemented in recombinant protein production^[2] and is often still the preferred production host in research laboratories^[3] and used in industrial scales.^[4,5] Recombinant protein secretion is beneficial over intracellular production

strategies to meet biotechnological purposes because of various reasons such as simplifying downstream processes, reducing risk of cytosolic degradation, higher quality and solubility of secreted protein, avoidance of inclusion body formation, and reduced costs of production processes.^[5–7]

So far, at least eight different secretion systems for Gram-negative bacteria, including *E. coli*, have been elucidated that secrete a wide range of transport-substrates.^[7,8] Successful secretion of recombinant proteins by five of these secretion systems has been reported so far, but often with low titers and for a narrow range of substrates. Attempts to achieve reasonable secretion levels for recombinant protein secretion have been continuously made commonly with limited success, presumably due to the complexity of the multi-component secretion machineries.^[6,7,9]

Type 1 secretion systems (T1SS) possess a relatively simple architecture consisting of only three membrane proteins, providing a secretory pathway for Gram-negative bacteria to secrete substrates in a single step and in an unfolded state.^[10,11] The first and probably the best studied T1SS is the HlyA secretion system of uropathogenic *E. coli*.^[10,12] The substrate of this system is HlyA, an enzyme of 110 kDa molecular weight which belongs to the RTX (repeats-in-toxins) family. Members of this family exhibit as a common feature a consensus glycine-rich motif (GGxGxDxUx), where x can be any amino acid and U refers to a large hydrophobic amino acid.^[13]

The secretion signal of HlyA is located within the 50–60 C-terminal amino acid residues.^[10] The secretion signal of HlyA along with three conserved glycine-rich motifs is known as HlyA1 which has a molecular weight of 24 kDa. HlyA1 has been employed as a carrier for secretion of heterologous proteins. Despite a long list of successfully secreted heterologous proteins, HlyA1 is still far away from being an universal secretion platform, due to a narrow range of possible substrates

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as well as low yields of secretion.^[14–17] It should be noted that an engineered T1SS has recently been developed to allow efficient production of peptides and small proteins.^[18]

The HlyA secretion system forms a tripartite double-membrane-spanning channel through association of an inner membrane ATP-binding cassette transporter (HlyB), a membrane fusion protein (HlyD), and an outer membrane protein (TolC).^[19] The next component of the HlyA system is HlyC. Though not essential for secretion, it acylates HlyA at two internal lysine residues (Lys564 and Lys690) prior to secretion. This modification turns the substrate into an active toxin which is able to lyse the membranes, for example, of human erythrocytes.^[11,20]

The encoding genes of the Hly system, except of the *tolC* gene, are located within the *hly* operon (Figure 1).^[10] Interestingly, a terminator region is located between the genes *hlyA* and *hlyB*, and therefore, the *hly* operon requires an anti-terminator factor RfaH to read through the terminator region. It was reported that the expression of *hly* operon is reduced in the absence of the RfaH factor and the expression of *hlyC* and *hlyA* are uncoupled from *hlyB* and *hlyD* genes.^[21]

Recently, it was demonstrated that the presence of a 5'-untranslated region upstream of the *hlyA* gene resulted in a multiple fold enhanced secretion level of heterologous proteins through the Hly system. This 5'-untranslated region, known as Hly enhancer fragment, is rich in uracil and adenine bases, and represents the C-terminal part of the *hlyC* gene. Interestingly, the established vector harboring the Hly enhancer fragment enabled secretion of fast-folding fusion proteins to HlyA1 that could not be previously secreted.^[20] We thus envisaged to subject this enhancer fragment to directed evolution attempting to optimize the secretion efficiency of the Hly system. It should be noted here that other parts of the Hly system might also be targeted by directed evolution when attempting to optimize this system.

Enhancer fragments, known to be involved in the translation initiation process, have an influence on the amount of proteins translated.^[22–24] Protein translation is initiated by association of the ribosome to mRNA, which is considered the rate-limiting step in protein synthesis. This step of translation is facilitated by interaction of Shine-Dalgarno (SD)/anti-SD sequences as well as the S1 ribosomal protein/enhancer fragment.^[23] Subsequently, the ribosome should dissociate from the initiation complex to slide over the mRNA for translating the downstream codons. It has been demonstrated that the presence of an enhancer fragment upstream of the SD

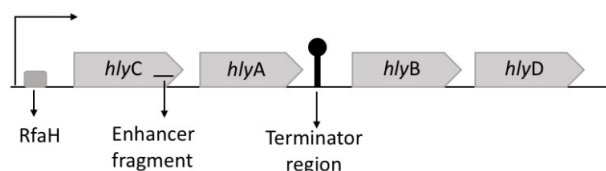


Figure 1. The *E. coli hly* operon. RfaH encodes the anti-terminator factor that is required for reading through the terminator region between *hlyA* and *hlyB* genes.

sequence causes an increased dissociation rate and destabilizes the initiation complex.^[23]

Until now only little attention has been paid to engineering of the Hly secretion system that may stem from lack of an elucidative structure and derived secretion mechanism. Directed evolution may represent a promising approach to engineer this system as it does not require any knowledge of the molecular structures to identify protein variants with optimized performance.^[25–27]

The KnowVolution strategy was published in 2015 as an integrative protein engineering approach.^[28,29] It combines directed evolution with computational analysis in order to obtain maximized improvements with minimized experimental efforts. A KnowVolution campaign consists of four phases: Phase (I) beneficial positions are identified through random mutagenesis and screening; Phase (II) potentially beneficial positions are subjected to site-saturation mutagenesis (SSM) in the wildtype enzyme to explore the full natural diversity and eliminate non-contributing positions. Sequencing of beneficial SSM libraries generates a molecular understanding of each amino acid exchange; Phase (III) comprises of a computationally assisted analysis in which beneficial amino acid substitutions are analyzed and grouped (amino acid substitutions that might interact with each other and independent ones). The latter analysis enables to further identify beneficial amino acid positions that interact with the identified ones. Clustered/interacting positions are recombined in the final phase to yield final variants with optimized performance. Various examples of successful KnowVolution campaigns have been published, *e.g.*, with glucose oxidases, phytases, proteases, cellulases, laccases, aryl sulfotransferases, or polymer-binding peptides.^[7,30–34]

Here, we report on a KnowVolution campaign, according to the published procedure^[35] with slight modifications, to optimize the 5'-untranslated region of the HlyA enhancer fragment. To this end, we have applied a high-throughput screening system, for the first time for directed evolution of the HlyA system, based on a lipase (lip).^[36] Notably, all the previously published studies that have applied KnowVolution campaign have dealt with catalytic or adhesion promoting proteins, whereas in the current study an untranslated region, the Hly enhancer fragment, was subjected to a KnowVolution campaign.

Results

Secretion of lipase through the Hly secretion system using a one-plasmid system

A plasmid containing multiple genes essential for secretion via the Hly system was successfully constructed that included the regions encoding for the Hly enhancer fragment (untranslated region consisting of a 159 base pairs), the lipase fused to HlyA1, and the proteins HlyB and HlyD. The gene encoding TolC, the OM protein of the Hly secretion system, is endogenous and thus not present on the plasmid. Instead of a two-plasmid system, a one-plasmid system as constructed in this study was

used because it results in a lower deviation between clones which generally should be avoided for directed evolution studies.

The constructed plasmid was introduced into chemically competent *E. coli* BL21-Gold (DE3) and the cells were grown on tributyrin agar plates. Halo formation around single cell clones indicated successful secretion of functional lipase-HlyA1 (Figure 2a). Cell clones harboring single plasmid either pSU2627-*EF*(159bp)-*lipA-hlyA1* or pK184-*hlyBD* did not form halos (Figure 2b and 2c), indicating that lipase-HlyA1 secretion is dependent on the Hly secretion system. Test expression of clones secreting lipase-HlyA1 was performed in shaking flasks directly employing the supernatant of cultures (see SDS-PAGE analysis in Figure 2d).

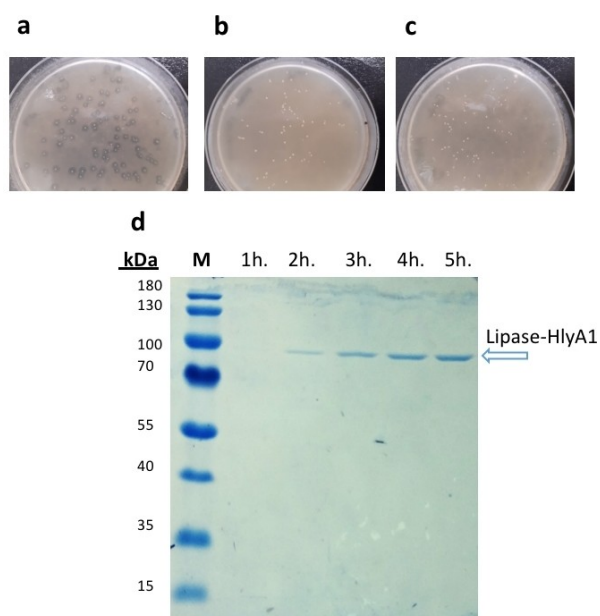


Figure 2. Secretion of lipase-HlyA1 through the Hly secretion system. Lipolytic activity of secreted lipase-HlyA1 was analyzed on tributyrin agar plates. Cells harboring the plasmid a) pK184-*EF-lipA-hlyA1BD*, b) pSU2627-*EF*(159bp)-*lipA-hlyA1*, and c) pK184-*hlyBD*. d) SDS-PAGE analysis of supernatant (unconcentrated) of clones secreting lipase-HlyA1 through the Hly secretion system. M, marker proteins; the molecular weight of the marker proteins is given on the left; (xh.) unconcentrated supernatant of culture, where x denotes the number of hours after induction.

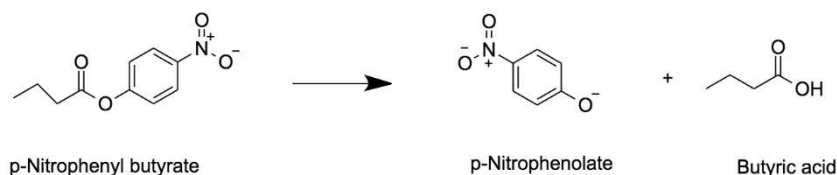


Figure 3. Hydrolysis of the substrate *p*NPB by secreted lipase. The production of *p*-nitrophenolate was monitored spectrophotometrically at 410 nm for 8 min. The figure was generated using the ChemDraw software.

MTP-based screening system for secreted lipase

Lipase activity was determined through hydrolysis of *p*-Nitrophenyl butyrate (*p*NPB) to butyric acid and yellow *p*-nitrophenolate (Figure 3). The production of *p*-nitrophenolate was continuously monitored spectrophotometrically at 410 nm. The *p*NPB-based screening system in 96-well microtiter plates was reported previously.^[37,38] Here, the amount of the secreted lipase was monitored by determination of lipase activity in supernatants of cultures grown for 16 h. The amount of *p*-nitrophenolate formed within 8 minutes of *p*NPB-lipase assay is correlated to the amount of secreted lipase in the supernatant.

Directed evolution of the Hly enhancer fragment through the KnowVolution strategy

Phase I (identification of beneficial positions): A random mutagenesis library of the Hly enhancer fragment was generated through random mutagenesis *in vitro*, employing error-prone PCR and a low-fidelity DNA polymerase in a buffer with 0.9 mM MnCl₂ resulting in an average of 8.5 mutations per enhancer fragment (159 bp). Hence, the calculated mutation load was 40.28 mutations per kb. The generated library with a size of 1408 clones was screened in a 96-well MTP format using the *p*NPB screening assay to identify possible beneficial variants. After screening of the random mutant library, rescreening of promising variants was repeated for five times. Promising variants showing an increased secretion level compared to the WT clone were selected and sequenced. Alignment of the enhancer fragment of the promising variants indicated that those variants harbored mutations repeatedly in 5 positions, including: +30, +36, +54, +81, +116.

Phase II and Phase III (determination and recombination): All five positions were subjected to site saturation mutagenesis to explore the natural full diversity. Notably, KnowVolution campaigns have been used so far for protein evolution only, whereas we have now used this evolution strategy to optimize the function of a DNA sequence, which in our case is not translated into a protein sequence. Saturation mutagenesis and recombination of possible beneficial positions were performed in parallel. Thereby, a library with a size of 1232 clones was generated at these 5 positions and screened using the *p*NPB screening system. The rescreening of promising variants was performed and led to the selection of seven clones that showed a 1.5 to 2-fold increase of secreted lipase-HlyA1 protein

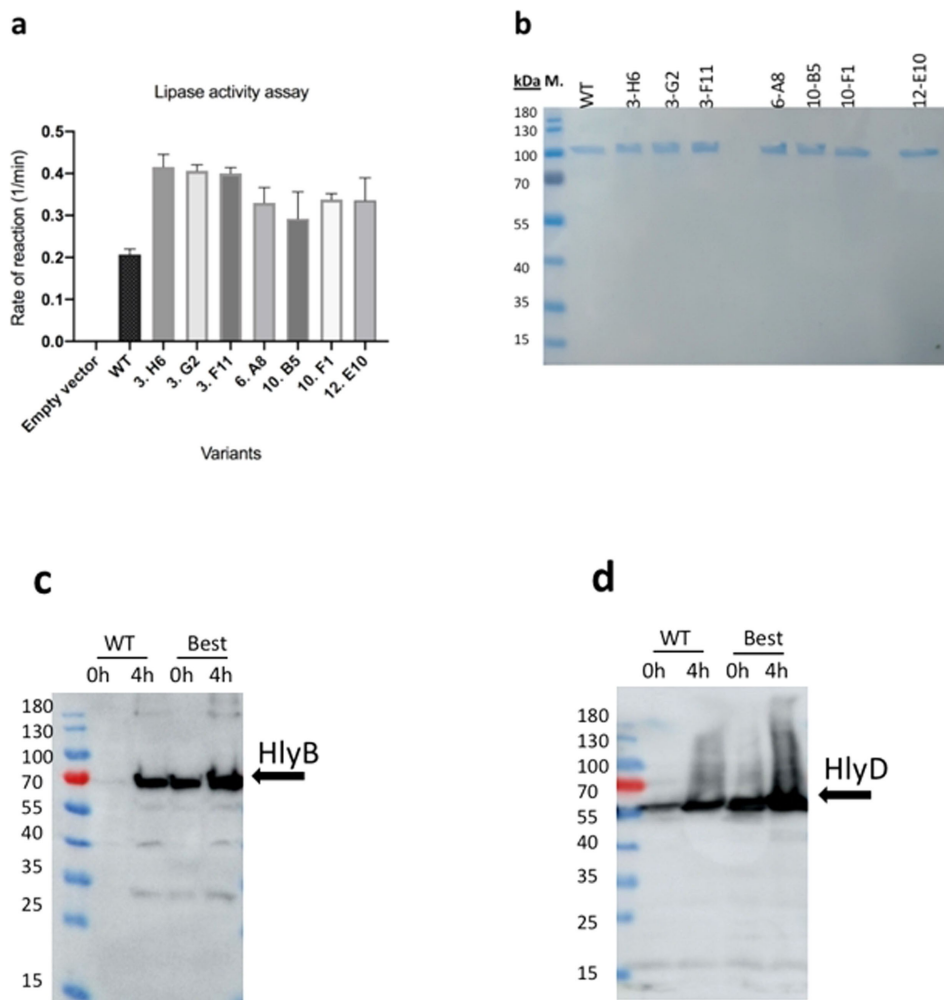


Figure 4. Secretion of lipase-HlyA1 through the Hly secretion system using enhancer fragment variants. a) Rate of lipase activity of the wildtype and the improved variants. The reported values and calculated error bars are based on at least three independent replicates. b) SDS-PAGE of unconcentrated supernatant of wildtype and the improved variants. Western blot analysis of *E. coli* cells demonstrated that the expression level of c) HlyB and d) HlyD were higher for cells having the “Best” enhancer fragment in comparison to the cells having WT enhancer fragment. The pLac promoter on plasmid pK184 is known to be leaky, therefore, HlyB and HlyD are expressed even before induction at 0 h.

compared to the WT (Figure 4a). The nucleotide mutations of each variant in the enhancer fragment are presented in Table 1.

Next, the secretion levels of lipase-HlyA1 of the seven improved variants and the WT clone were compared via test expressions in 100 mL Erlenmeyer flasks. The supernatant of the cultures (unconcentrated) was analyzed by SDS-PAGE (Fig-

Table 1. Nucleotide mutations in enhancer fragment – untranslated region – of the improved variants.

Variants	Nucleotide and mutations
WT	A30, A36, A54, A81, A116
3-H6	A30U, A36U, A54G, A81U, A116U
10-F1	A30G, A36A, A54G, A81U, A116U
3-F11	A30U, A36A, A54G, A81U, A116C
6-A8	A30A, A36A, A54G, A81U, A116U
12-E10	A30U, A36U, A54G, A81U, A116U
3-G2	A30G, A36U, A54G, A81G, A116A
10-B5	A30C, A36C, A54G, A81U, A116A

ure 4b). Among the improved variants, variant 3-H6 seems to be the best as determined by either the pNPB lipase activity assay or test expression. The respective enhancer fragment was termed “Best” enhancer fragment and was used for further experiments. Furthermore, the level of secretion of HlyB (Figure 4c) and HlyD (Figure 4d) was analyzed by western blot for *E. coli* cells having either the WT or “Best” enhancer fragment. The analysis of western blot of three biological replicates confirmed that the expression level of both proteins was higher in cells having “Best” enhancer fragment as compared to cells having the WT one.

RNA folding simulation and 3D structure prediction of the enhancer fragments

It is known that the enhancer fragment, upstream of the SD sequence, plays an important role in promoting protein trans-

lation via interaction with the S1 ribosomal protein,^[22,23] albeit not being translated into a protein sequence. We have previously reported that the HlyA enhancer fragment interacts with the S1 ribosomal protein.^[20]

In order to explain the structural effects of the nucleotide mutations of the “Best” enhancer fragment and their consequences on the increased secretion level of lipase-HlyA1, the following computational analysis was performed. First, RNA folding simulation studies on both enhancer fragment variants (WT and “Best”) were performed. As shown in Figure 5a, the “Best” enhancer showed a significant conformational change compared to WT, indicating that the introduced mutations affected mRNA folding. The structural angle change of the “Best” enhancer compared to WT is shown in Figure 5a. Subsequently, the modified mRNA’s structure resulted in changes in docking pose upon interaction between S1 ribosomal protein and the enhancer fragment (Figure 5b and 5c). The WT/S1 ribosomal protein complex had a docking score

of -7858708.37 while “Best”-S1 complex showed a reduced docking score of -6379871.3 suggesting that the binding interactions between the “Best” enhancer and S1 is not as strong as in the WT. Interestingly, three out of the five determined beneficial positions were located on the interface between ribosomal protein S1 and mRNA (*i.e.*, positions +30, +36, +81).

Ribosomal protein S1 binds with different affinities to the enhancer fragment variants

The above computational analysis indicated differences in interactions of the “Best” enhancer fragment with the S1 ribosomal protein compared to the WT fragment. This observation was experimentally tested by a RNA pull-down assay. RNA molecules of both “Best” and WT enhancer fragments were synthesized and labeled with 3'-Biotin TEG.

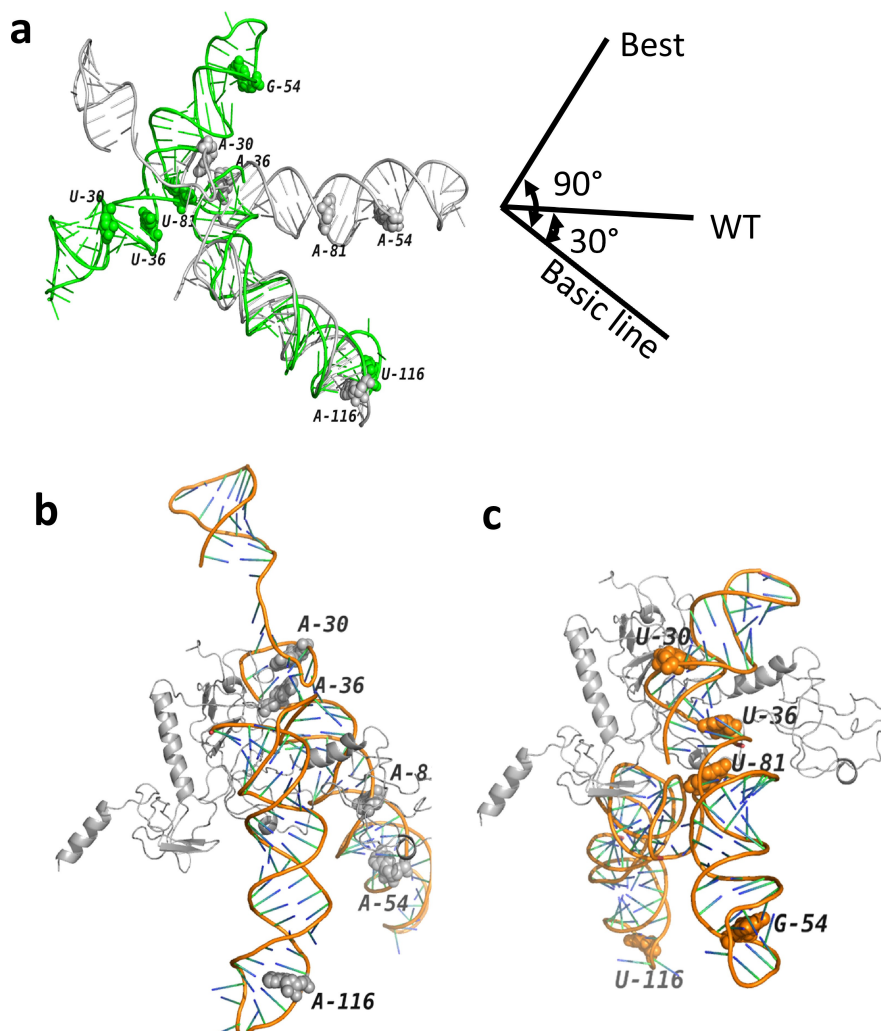


Figure 5. RNA folding simulation and docking analysis. a) The comparison between WT and the “Best” mRNA enhancer fragments. The “Best” enhancer is shown in green and WT enhancer is depicted in grey. The right panel shows a schematic diagram of the structural angle change of the “Best” enhancer compared to WT. 3D model of the interaction of S1 ribosomal protein with enhancer fragment b) WT and c) the “Best” variant. The S1 ribosomal protein is displayed as grey cartoon. The substituted nucleotides are highlighted with spheres in grey for WT and in green/orange for the “Best” enhancer. The docking pose was obtained from the NP-dock molecular docking server.

Next, both enhancer fragment variants were immobilized on magnetic beads and thereafter, incubated with *E. coli* cell extract. The amount of bound protein on the beads carrying either the “Best” enhancer fragment or the WT enhancer fragment was analyzed by SDS-PAGE (Figure 6a). The two strong pull-down bands around 70 and 100 kDa on the SDS-gel were cut out and the proteins were identified by liquid chromatography-tandem mass spectrometry. The dedicated band around 100 kDa was identified as aldehyde-alcohol dehydrogenase from *E. coli*, while the band around 70 kDa with 58% coverage of peptide sequences was identified as *E. coli* S1 ribosomal protein. The semi-quantification of SDS-PAGE using the ImageJ indicated that in comparison to the RNA molecule of WT enhancer fragment, about 50% less S1 ribosomal protein was pulled down by the RNA molecule of the “Best” enhancer fragment (Figure 6b). This data supports the interpretation of the computational analysis suggesting that the interaction of S1 ribosomal protein with the “Best” enhancer fragment is weaker than with the WT fragment. These findings suggest that in the presence of the “Best” enhancer fragment, the translation of lipase-HlyA1 might increase which leads to a higher level of lipase secretion.

Enhanced secretion of cutinase-HlyA1

To test whether the “Best” Hly enhancer fragment could also improve the secretion of other fusion proteins, a fusion between the gene encoding cutinase from *Fusarium solani pisi*^[39] was constructed either with the “Best” or WT enhancer fragment. The test expression for both constructs was conducted and the supernatant was used for western blot analysis as well as pNPB hydrolysis assay. Western blot analysis of the supernatant revealed that the “Best” enhancer fragment was also able to promote the secretion of the cutinase-HlyA1 (Figure 7a). Furthermore, the pNPB assay confirmed more than 2-fold secretion of cutinase as compared to the WT (Figure 7b).

This observation indicates that the “Best” enhancer fragment variant increases the secretion efficiency of the Hly secretion system not only for lipase-HlyA1 but also for the target protein, cutinase-HlyA1.

Introduction of a terminator region between *hlyA1* and *hlyB* genes

Given the ability of the “Best” enhancer fragment to improve the secretion of two tested HlyA1 fusion proteins, *i.e.*, lipase and cutinase, we also investigated whether employing the Hly terminator region between *hlyA1* and *hlyB* genes would further improve the secretion of the Hly secretion system. Previous studies have demonstrated that the anti-terminator factor RfaH is required for reading through the terminator signal located between *hlyA* and *hlyB* genes in the *hly* operon and increases the transcriptional elongation as well as the steady-state level of *hly* mRNA.^[21]

In this study, the *hly* terminator, a stem loop between *hlyA1* and *hlyB* gene, was introduced on the plasmids encoding either lipase or cutinase and along with the “Best” enhancer fragment. The presence of the terminator resulted in enhanced secretion of both target hydrolases. A higher level of lipase-HlyA1 secretion was achieved in the strain carrying the plasmid containing the “Best” enhancer fragment along with the terminator region compared to the strain having either the WT plasmid or the “Best” plasmid only (Figure 8a). Estimation of the amount of secreted protein by SDS-PAGE showed an increase of more than 3.5-fold for lipase-HlyA1 with the newly constructed plasmid. In the presence of the “Best” enhancer fragment and terminator region, successive increase in the amount of secreted lipase-HlyA1 in the supernatant was observed. This increase only after four hours of induction led to formation of enzyme oligomers (observed as white particles) in the supernatant culture, which mostly contained lipase-HlyA1. Based on the known concentration of purified HlyA, concentration of secreted lipase-HlyA1 in the supernatant was

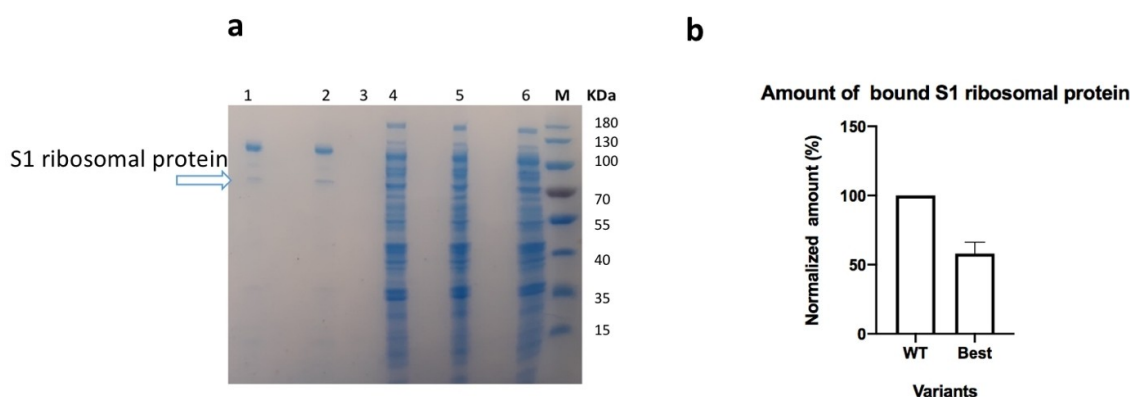


Figure 6. RNA pull-down assay. a) SDS-PAGE of eluted proteins by immobilized RNA molecules of the “Best” enhancer fragment (1), WT enhancer fragment (2), without RNA (3) and washing flow through of washing of the beads “Best” (4), WT (5), and without RNA (6). The position of the S1 ribosomal protein is indicated by an arrow. b) Semi quantification of SDS-PAGE via ImageJ software. The reported values and calculated error bars are based on three independent replicates. In each replicate, the reported value of the “Best” enhancer fragment was normalized based on the value of the WT.

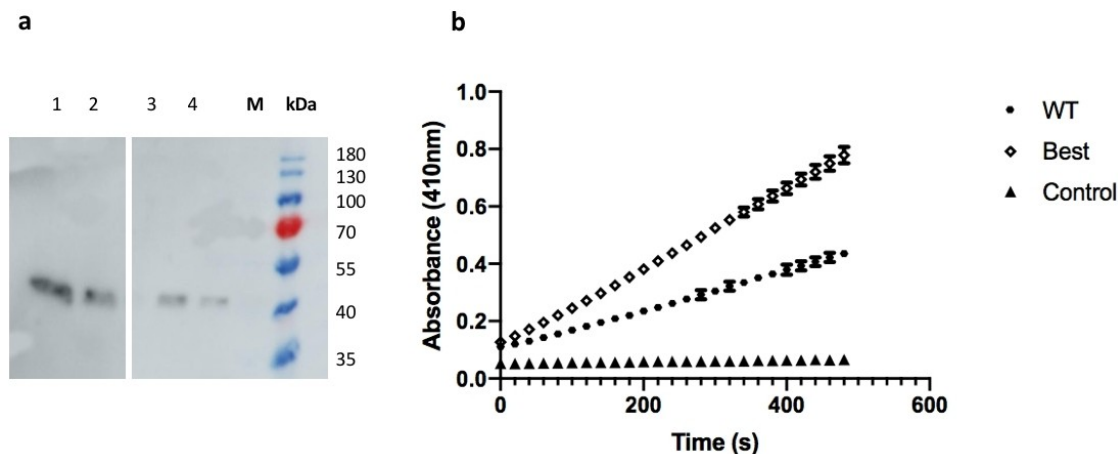


Figure 7. Enhanced secretion of cutinase-HlyA1 by the “Best” enhancer fragment. a) Western blot analysis of supernatant fractions from the “Best” enhancer fragment (1 and 2) and the WT (3 and 4) cultures. b) Activity of the cutinase-HlyA1 in the supernatant of the culture dedicated to either the “Best” or WT enhancer fragment using the pNPB assay. The reported absorbance at 410 nm and the error bars correspond to average values of three replicates. The control is the absorbance of the buffer and substrate without addition of culture supernatant.

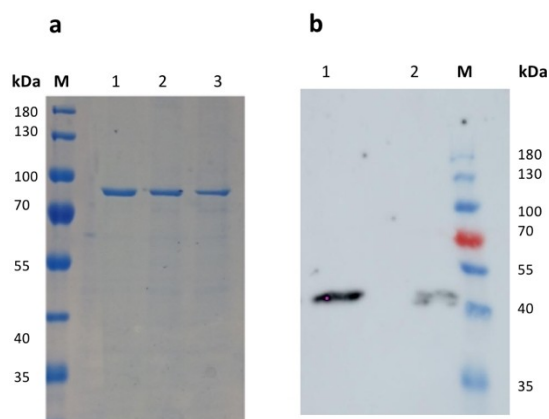


Figure 8. Influence of the HlyA terminator on the amount of secreted proteins. a) Secreted lipase-HlyA1; SDS-PAGE of supernatant fractions from terminator with the “Best” enhancer fragment (1), the “Best” enhancer fragment only (2), and the WT (3) strains; stained with Coomassie Brilliant Blue G-250. b) Secreted cutinase-HlyA1; western blot analysis of supernatant fractions from terminator along with the “Best” enhancer fragment (1) and the WT (2) strains.

quantified as about 180 mg per liter (corresponding to 60 mg/OD₆₀₀). Furthermore, the amount of lipase-HlyA1 (wet weight) in collected white particles from 25 mL culture was estimated to be above 1.5 mg (~60 mg/L).

In case of cutinase, Western blot analysis revealed that the presence of the terminator region along with the “Best” enhancer fragment secreted significantly higher amounts of cutinase-HlyA1 in the culture medium compared to the WT plasmid (Figure 8b). Semi quantification of the Western blot analysis with ImageJ software estimated an increase of around 5-fold increase for the cutinase-HlyA1 when expressed using the newly constructed plasmid.

Discussion

For heterologous protein production, secretion of proteins is beneficial compared to cytosolic expression, especially in terms of costs and the quality of produced proteins. Secretion offers various advantages for protein production, such as higher solubility and stability of target protein, reduced risk of protein degradation by cytosolic proteases, and easy purification process.

Although, *E. coli* is the most preferred producer host for research laboratories,^[3] its potential uses as a secretory host is limited since the expression of recombinant proteins mostly takes place either in the cytoplasm or periplasm and if in the supernatant, in low titers. For secreted proteins, *Bacillus* species are the expression hosts of choice with a secreted product yield of 20 g/L.^[4,6,9,40] In this regard, investigating the secretion of recombinant proteins by *E. coli* is a continuous topic of interest and therefore, tremendous efforts have been made to investigate further improvements in the secretion efficiencies of *E. coli* to make it more competitive, as both laboratory and industrial strain.^[6,9,41,42]

Among the secretion systems of Gram-negative bacteria including *E. coli*, T1SS, T2SS, T3SS, T5SS, and T8SS have been used for biotechnological purposes.^[6] Among them, T1SS has the most minimalistic architecture and is therefore an attractive target for further improvements.

This study set out with the aim of improving the secretion efficiency of the Hly secretion system of *E. coli*. As a member of the T1SS, the Hly system consists of only three compartment proteins^[10] that makes the plasmid-expression of this system in the production hosts easy.^[6] Recently, a 5'-untranslated region upstream of the *hlyA* gene, known as the Hly enhancer fragment, was identified that was able to increase the secretion level of HlyA1 fusion proteins by several folds.^[20] In the current study, this Hly enhancer fragment was subjected to optimization by directed evolution using a KnowVolution campaign.^[28,31]

Noteworthy, this study is the first case study that applies a KnowVolution campaign for evolution of an untranslated DNA region for further improvement of the HlyA secretion system of *E. coli*.

To initiate the translation in prokaryotic cells, the first and key step is binding the ribosome to the 5'-untranslated region of mRNA, which is followed by ribosome dissociation from the initiation complex to move to downstream codons of the mRNA for completion of translation. It has been suggested that the S1 ribosomal protein, the largest ribosomal protein of *E. coli* with a molecular weight of 68 kDa, interacts with mRNA within the enhancer region, upstream of the SD sequence, by an unknown mechanism.^[24,43,44] In an elegant set of experiments, it was demonstrated that the transition step from translation initiation to elongation takes effect by both interaction of SD/anti-SD sequences and enhancer/S1 by a negative allosteric effect. It is notable that the ribosome dissociation from initiation complex is the determining factor for translation efficiency.^[23]

Through KnowVolution campaign, two libraries with sizes of 1408 and 1232 clones, constructed by epPCR and site saturation mutagenesis, respectively, were screened. Screening a library size of 1000 to 2000 clones in which 40–60% of the generated enzymes variants are still active are commonly and successfully used in standard directed evolution campaigns.^[45] Iterative rounds of screening of a few thousand clones have proved to yield superior results than screening twenty to fifty thousand of clones since the number of “new” substitutions decreases rapidly with increased library size.^[46] The latter can be attributed to the strong mutational bias of polymerases.^[47] Therefore, superior strategies have been developed, e.g. iterative rounds of screening of small libraries (1000–2000 clones) with varied mutational biases, flow cytometry, and microfluidic screening systems that enable to screen millions of variants, and combined computational /experimental strategies which analyze and identify further beneficial positions based on the identified ones in the initial round of directed evolution (implemented if required in Phase III of a KnowVolution campaign).

All the mentioned findings are on a solid ground for enzyme properties such as activity, organic solvent resistance, pH or ionic liquid resistance, etc. In respect to expression/translocation improvement no datasets are available to link potential improvement possibilities to the screened library size. Therefore standard conditions of screening (number of clones) have been applied and combined in a KnowVolution campaign to identify key residues and to generate a molecular understanding and nature of mutations at identified key positions. In summary, the molecular understanding will be broadened through iterative rounds of screening of small libraries in the future, since alternatives, such as a beneficial screening that enables to screen millions of variants, are not available.

After screening of libraries, five beneficial positions (+30, +36, +54, +81, and +116) were identified in the Hly enhancer fragment. Mutations at these positions boosted the secretion levels of lipase and cutinase through the Hly system by 100%. It was somewhat surprising that adenine nucleotides were

present at all the five identified positions and were randomly substituted to uracil in the best performing enhancer variant, named as the “Best” enhancer fragment. It was already reported that uracil nucleotides of the enhancer fragment are beneficial for its interaction with the S1 ribosomal protein.^[23] Therefore, we reasoned that the flexibility of the interaction between S1 ribosomal protein and the enhancer fragment is increased in the “Best” enhancer fragment. Most likely, the presence of uracil at these positions may destabilize the initiation complex and increase the dissociation rate between S1 and mRNA. This was further supported by molecular docking studies and an RNA pull-down assay.

Furthermore, the results obtained from the secretion of cutinase from *Fusarium solani pisi* in the presence of the “Best” enhancer fragment indicated that this novel enhancer fragment is compliant with other secretory proteins in addition to lipase-HlyA1. Notably, it is the first time that cutinase protein from *Fusarium solani pisi* was tested as a secretory protein in the Hly secretion system, while lipase had been tested before.^[20] It also suggested a general applicability of the “Best” enhancer fragment.

Kondo *et al.* six described six genetic elements of *E. coli* which contribute to an increased gene expression levels and result in higher yields of the desired protein, namely: promoter, translation enhancer, Shine-Dalgarno sequence, spacer, encoding gene, and terminator of the gene.^[48] Our results additionally demonstrated that the presence of a terminator region along with the “Best” enhancer fragment can result in further improvement of secretion through the Hly secretion system. In *E. coli* cells, the anti-terminator factor RfaH reads through terminator regions. It was reported that RNA polymerase stops at so-called JUMPstart (just upstream of many polysaccharide-associated starts) sequences, where RfaH binds to RNA polymerase forming a complex able to read through the terminator region.^[20]

Conclusions

This study demonstrated that the secretion through the Hly secretion system can be improved significantly by engineering of genetic elements. *E. coli* cells expressing the optimized HlyA secretion system constructed in this study showed a secretion level of around 180 mg per liter for soluble lipase-HlyA1. Furthermore, a high protein content of above 1.5 mg of lipase-HlyA1 was estimated for the white particles in only 25 mL of culture. Additionally, the one-plasmid system developed in this study might offer an interesting alternative to two-vector systems, which have been used so far. This would also significantly reduce the costs of antibiotics when cells are cultivated at larger scale.

In addition, the employed evolutionary and genetic engineering strategies can generally be applied to all types of fusion proteins if required and provide an opportunity for optimizing the secretion titers by evolutionary approaches. Eventually, our approach is also applicable to those secretion systems that have been used in the past for biotechnological approaches

such as T2SS, T3SS, T5SS and T8SS, to significantly improve the *E. coli* secretion performance, thereby rendering *E. coli* a more competitive enzyme secreting host that might challenge the industrial applied host systems in the future.

Experimental Section

Materials

All chemicals used in this study were purchased from Sigma-Aldrich, Roche Diagnostics GmbH, or Applichem GmbH if not stated otherwise. Enzymes were purchased from New England Biolabs, except of polynucleotide kinase, which was provided from the SeSaM-Biotech (Aachen, Germany). All oligonucleotides were purchased from Eurofins MWG Operon and diluted in MilliQ-water. The commercially available kits NucleoSpin plasmid miniprep kit and PCR clean-up kit were purchased from Macherey Nagel (Dueren, Germany). DNA sequencing was done at either Eurofins MWG-Operon (Ebersberg, Germany) or Microsynth Seqlab (Göttingen, Germany). The Gibson assembly master mix kit as well as the Q5 site-directed mutagenesis kit were purchased from New England Biolabs.

Construction of a one-plasmid system for secretion

For cloning and library generation either *E. coli* DH5 α or *E. coli* BL21-Gold (DE3) were used. Plasmids and oligonucleotides used in this work are listed in Table 2 and Table 3, respectively. For all plasmids constructed in this study, pK184_hlyBD was used as the backbone vector. The *lac* promoter of plasmid pK184 is inducible with isopropyl β -D-1-thiogalactopyranoside (IPTG).

Table 2. Plasmids used in this study.

Backbone plasmid	Contained genes/fragments	Source
pK184	<i>hlyBD</i>	[20]
pSU2726	<i>EF*</i> (159bp)- <i>lipA-hlyA1</i>	[20]
pK184	<i>EF-lipA-hlyA1BD</i>	this study
pK184	<i>EF(Best)-lipA-hlyA1BD</i>	this study
pK184	<i>EF(Best)-lipA-hlyA1-Ter-hlyBD</i>	this study
pKEEx2	<i>NprE-cutinase</i>	[39]
pK184	<i>EF-cutinase-hlyA1BD</i>	this study
pK184	<i>EF(Best)-cutinase-hlyA1BD</i>	this study
pK184	<i>EF(Best)-cutinase-hlyA1-Ter^h-hlyBD</i>	this study

Table 3. Oligonucleotides used in this study.

Oligonucleotides	Sequence
P1	GTGAGCGGATAACAATTTGATTACGAATTCGAGC
P2	GACTGTTTCCTGTGTGATTATGCTGATGTGGTCAG
P3	TCACACAGGAACAGTCATG
P4	AATTGTTATCCGCTCACAATTC
P5	GGAAATTCCTTGCAAAAATG
P6	CATTTAATTACCTCTTAACCAG
P7	GGTTAAGAGGTAATTAATGGCGCCTACTAGTAAC
P8	TTTTTTGCAAGAGAATTTCCAGCAGAACCACGGAC
P9	AATTGTGAGCGGATAACAATTTG
P10	CTAAAGATGCCATTTAATTACC
P11	TAAATGGGCATCTTTAGCTATAAG
P12	GTTATCCGCTCACAATTCAC
P13	GTGCCACATAAGATTGCTATTTTTTTGGAGTCATAATGGCAATTCTGATTCTTG
P14	AGCCAGTAAGATTGCTATCATTTAAATTAATATATTATGCTGATGTGGTCAG

The one-plasmid system for the secretion of the *lipA* gene from *Serratia marcescens*^[36] was constructed as follows: plasmid pSU2726_hlyC(159bp)-*lipase-hlyA1* was used for the PCR amplification of *EF* (enhancer fragment 159bp)-*lipA-hlyA1* fragment using the primer sets P1 and P2, both containing an overhang region towards the backbone plasmid. The backbone plasmid, pK184_hlyBD, was linearized using the primer sets P3 and P4. Subsequently, both PCR products were digested with DpnI enzyme (20 U, 37 °C, overnight). Then, PCR products were purified with the PCR clean-up kit. The *EF-lipA-hlyA1* fragment was assembled into the pK184_hlyBD vector using the Gibson assembly kit, according to the instructions of the manufacturer. Subsequently, 2 μ L of the assembled product was transformed into chemically competent cells of *E. coli* DH5 α . The sequence of the constructed plasmid pK184_EF-*lipA-hlyA1BD* was confirmed via plasmid sequencing.

Generation of enhancer fragment mutant library by epPCR

Random mutagenesis of the enhancer fragment, with a size of 159 base pairs, was performed via error prone PCR with a high mutational load.^[46] To obtain the mutations in the first nucleotides of the enhancer fragment, the forward primer was designed to bind behind the *lac* promoter but upstream of the starting nucleotide of the enhancer fragment. The enhancer fragment was amplified using the primer sets P9 and P10. The epPCR mixture (50 μ L) contained: 50 ng of plasmid template pK184_EF-*lipA-hlyA1BD*, 1 \times ThermoPol buffer, 0.2 mM dNTP mix, 400 pM of each primer, 10 U Polymutase DNA polymerase, and 0.9 mM MnCl₂.

The vector template pK184_EF-*lipA-hlyA1BD* lacking the *EF* region was amplified using a high-fidelity polymerase, Q5 polymerase, and primer sets P11 and P12. The PCR mixture (50 μ L) contained: 50 ng of plasmid pK184_EF-*lipA-hlyA1BD* plasmid, 1 \times Q5 standard reaction buffer, 0.2 mM dNTP mix, 400 pM of each primer, and 1 U Q5 DNA Polymerase. Subsequently, the ep-PCR product of the enhancer fragment and the PCR product of the backbone vector were digested with DpnI enzyme, overnight, at 37 °C to remove any wildtype vector. The PCR products were purified using PCR clean-up kit. The whole plasmid construction of the epPCR product and the linear plasmid was performed via Gibson assembly according to the instructions of the manufacturer. Subsequently, 2 μ L of the assembled product was transformed into chemically competent cells of *E. coli* BL21-Gold (DE3). The transformed cells were cultured directly on tributyrin lysogeny broth (LB) agar plates (tryptone 10 g/L, yeast extract 15 g/L, NaCl 5 g/L, Agar 15 g/L, Gum Arabic 1.5 g/L, tributyrin 15 g/L) containing kanamycin 50 μ g/mL. Ten single cell clones of epPCR library were picked and sent for sequencing.

Cultivation in 96-well microtiter plates

For the cultivation of mutants from libraries, single clones showing lipase activity on tributyrin LB agar plates were cultured into 150 μL LB_{kan} in 96-well MTP (Microtiter plate, PS-F-bottom, Sarstedt, Germany). In each 96-well MTP plate, 4 wildtype clones and 4 empty vector clones were included as positive and negative clones, respectively. The plates were incubated for 24 hours (37 °C, 900 rpm, and 80% humidity) in a 96-well MTP shaker (SI505 MTP shaker incubator, Avantor). After cultivation, 50 μL of 50% glycerol (v/v) was added to each well and the plates, so called master plates, were stored at -80 °C until further experiments. For the expression of lipase-HlyA1, the master plates were used to inoculate the pre-culture in MTPs containing 150 μL LB_{kan} and cells were cultivated overnight (37 °C, 900 rpm, and 80% humidity). Subsequently, 2 μL of the pre-culture were used to inoculate the main culture MTPs (150 μL LB_{kan}) and the cells were grown for 3 hours (37 °C, 900 rpm, and 80% humidity) before induction with 1 mM of IPTG and 5 mM CaCl_2 . The cultures were further grown for 16 hours (37 °C, 900 rpm, and 80% humidity) in 96-well MTP shaker incubator. Afterwards, the MTPs were centrifuged (4 °C, 20 min, 3500 rpm) and the supernatants were harvested and used for the screening assay.

Site-saturation mutagenesis

Individual site-saturation mutagenesis at positions 30, 36, 54, 81, and 116 of the Hly enhancer fragment was performed to obtain all the possible mutations at these positions. Two reverse complementary oligonucleotides carrying degenerative nucleotides in all individual positions were designed and synthesized. Annealing of the oligos was performed according to the protocol mentioned by Sigma-Aldrich as follows. In short, oligos were dissolved in the annealing buffer containing 10 mM Tris pH 7.5, 50 mM NaCl, and 1 mM EDTA. Equal volumes of the equimolar oligonucleotides of both forward and reverse oligos were mixed and heated up to 95 °C for 2 min. Subsequently, the mixture was gradually cooled to 25 °C over a period of 45 min. The annealed oligos were used as mega primers in a Megawhop PCR in order to amplify the whole plasmid. Megawhop PCR mix (100 μL) contained: 0.3 mM dNTP, 500 ng annealed oligos, 2 U Q5 polymerase, 70 ng of the plasmid template pK184_EF-lipA-hlyA1BD, and 1 \times Q5 buffer. The PCR product was digested with DpnI enzyme, overnight, at 37 °C and purified using PCR clean-up kit. Subsequently, 2 μL of purified Megawhop PCR product was transformed into chemically competent cells of *E. coli* BL21-Gold (DE3). The transformed cells were cultured on tributyrin LB agar plates. Single cell clones were picked up and grown in 96-well MTP plates.

Colorimetric screening system

The substrate used in this study to detect the amount of lipase-HlyA1 secretion was *p*-nitrophenyl butyrate (pNPB).^[49] Upon hydrolysis by lipase, *p*-nitrophenolate is formed, which can be detected spectrophotometrically at 410 nm. After cultivation and expression of the libraries, 10 μL supernatant of each single culture was transferred into a new 96-well MTP containing 90 μL of 50 mM TEA buffer (pH 7.4). The reaction was started by supplementing 100 μL of freshly prepared substrate solution containing 0.5 mM pNPB (dissolved in 10% v/v acetonitrile) in 50 mM TEA buffer (pH 7.4) in a final reaction volume of 200 μL . The amount of released *p*-nitrophenolate was recorded by measuring $A_{410\text{nm}}$ at 30 °C for 8 min with a kinetic interval of 20 seconds in a microtiter plate reader (Tecan infinite® M200 Pro Austria GmbH, Männedorf, Switzerland). The rate of conversion of pNPB to *p*-nitrophenolate (the differences of $A_{410\text{nm}}$ within 8 minutes) was calculated for each

reaction and used to identify clones with enhanced secretion of lipase-HlyA1 into the supernatant.

Construction of one-plasmid system for secretion

The one-plasmid system for secretion of cutinase from *Fusarium solani pisi* was constructed as follows: the plasmid vector pK184_EF-lipA-hlyA1BD was linearized, excepting the *lipA* gene, using the primer sets P5 and P6. The *cutinase* gene from plasmid pKEEx2_NprE-cutinase was amplified using the primer sets P7 and P8. Both PCR products were digested with DpnI enzyme overnight at 37 °C and purified using PCR clean-up kit. The *cutinase* gene was assembled into the linearized plasmid pK184_EF-hlyA1BD via Gibson assembly, according to the manufacturer's protocol. 2 μL of the assembled reaction was transformed into *E. coli* DH5 α competent cells and cultured on LB_{kan} agar plates. Cloning of the plasmid pK184_EF-cutinase-hlyA1BD was verified via sequence analysis.

Plasmid pK184_EF(Best)-cutinase-hlyA1BD was constructed in similar manner as it was already mentioned above, using plasmid pK184_EF(Best)-lipA-hlyA1BD as template.

To insert a terminator element between the *hlyA1* and *hlyB* genes on the above constructed one-plasmid systems for secretion, the sequence of this terminator element for the *hly* operon of uropathogenic *E. coli* UT189 was obtained from the National Center for Biotechnology Information (NCBI). Two non-overlapping primers P13 and P14 were designed based on the sequence of the terminator. The plasmids pK184_EF(Best)-lipase-hlyA1BD and pK184_EF(Best)-cutinase-hlyA1BD were used as backbone templates. The PCR amplification of the plasmids was performed using primer sets P13 and P14. The PCR products were separately incubated (5 min, room temperature) in the reaction buffer of the Q5-site directed mutagenesis kit based on the manufacturer's instruction (New England Labs, England). Subsequently, 2 μL of the resultant mixture was transformed into 50 μL of chemically competent cells of *E. coli* DH5 α . Sequencing data of single cell clones confirmed the insertion of the terminator sequence between the *hlyA1* and *hlyB* gene on the plasmids. The sequencing data of single cell clones confirmed the insertion of the terminator sequence between *hlyA1* and *hlyB* genes on the plasmids and thereby, successful construction of plasmids pK184_EF(Best)-lipA-hlyA1-Ter-hlyBD and pK184_EF(Best)-cutinase-hlyA1-Ter-hlyBD.

RNA pull-down assay

The 3'-Biotin TEG (triethylene glycol)-labeled RNA of wildtype enhancer fragment (5'-GAAUUUCAUG GAGGUAAAUA UGAUAAA-CAG UUAGCGAAUA AAAUUUUUAA ACAUAUCAC CACGAGUUAA UAACUGAAGU AAAAAGAAAG-3') and the "Best" enhancer fragment (5'-GUAUUUCUUG GAGGUAAAUA UGAUAGACAG UUAGCGAAUA AAAUUUUUAA ACUAUAUCAC CACGAGUUAA UAACUGAAGU AAAAAGAUAG-3') were synthesized by BioSpring (Germany). The RNA pull-down assay was performed using the Thermo Scientific Pierce magnetic RNA-protein pull-down kit (ThermoFisher Scientific, US) according to the manufacturer's instructions. Eluted proteins from the magnetic beads were run on a SDS-PAGE. Two SDS-PAGE bands depicting the bound proteins were extracted from the gel and analyzed by liquid chromatography-tandem mass spectrometry.

Expression and secretion experiments in shaker flasks

E. coli BL21-Gold (DE3) competent cells were transformed with the desired plasmids and grown on LB agar plates supplemented with

50 µg/mL kanamycin. A single colony was used to prepare a pre-culture and cultivated overnight (37 °C and 180 rpm). The overnight culture was used to inoculate 25 mL of 2YT medium supplemented with 50 µg/mL kanamycin at an OD₆₀₀ of 0.1 in 100 mL Erlenmeyer shaking flask. The cultures were cultivated at 37 °C and 180 rpm to an OD₆₀₀ of 0.4 to 0.6. Subsequently, the expression was induced with 1 mM IPTG and 5 mM CaCl₂. The culture was harvested after specific hours of expression by centrifugation and was analyzed either by SDS-PAGE^[50] or Western blot analysis. Staining of the SDS-gels was performed via Colloidal Coomassie G-250 Staining protocol.^[51] The intensity of the protein bands on the SDS-gels were quantified using ImageJ software (Image Processing and Analysis in Java). To determine the amount of secreted lipase-HlyA1, a series of purified HlyA solutions with known concentrations were also loaded on same SDS-PAGE. The amount of secreted lipase-HlyA1 of the supernatant was then compared to the HlyA solution through a calibration curve. Furthermore, to determine the amount of lipase-HlyA1 protein in the white particles of the 25 mL culture, those particles were isolated after four hours of induction from the supernatant and resuspended in water and SDS-sample buffer. The amount of protein was estimated as mentioned for lipase-HlyA1 of the supernatant.

Computational methods

The crystal structure of the S1 ribosomal protein (PDB ID: 6h4n, chain Y) was taken from PDB bank (<http://www.rcsb.org>).^[52] The online webserver SimRNA (<https://genesilico.pl/SimRNAweb>) was used to construct the 3D structure of the wild-type (WT) and "Best" enhancer fragments.^[53] The RNA sequence of enhancer fragments in a version with the residues A, C, G, and U only was used in Fasta format for the SimRNA modeling as described in the SimRNA user manual. The number of iterations was set to 500 steps of simulations with default parameters. After the simulation steps, 1% of the frames with the lowest energy were selected for clustering while the remaining frames were discarded. To compare RNA 3D structure of both fragment variants, their predicted structures were aligned using the RNA-align open-source algorithm (<https://zhanglab.ccmb.med.umich.edu/RNA-align>).^[54]

To model the plausible S1 ribosomal protein-Enhancer fragment interaction, docking of the enhancer fragments (WT, and the "Best") with the S1 ribosomal protein were performed using Nucleic Acid-Protein Dock (NPDock) web server (<http://genesilico.pl/NPDock>).^[55] Docking was carried out using the default parameters (1000 steps of simulation, initial temperature of 15,000 K). In total, 100 best scored models were used for clustering similar structures to obtain the final optimized structure. RMSD cut-off of 5 Å was used for clustering.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: directed evolution · Hly enhancer fragment · improved secretion efficiency · protein · protein secretion

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