A lower isoelectric point increases signal sequence–mediated secretion of recombinant proteins through a bacterial ABC transporter

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Efficient protein production for industrial and academic purposes often involves engineering microorganisms to produce and secrete target proteins into the culture. Pseudomonas fluorescens has a TliDEF ATP-binding cassette transporter, a type I secretion system, which recognizes C-terminal LARD3 signal sequence of thermostable lipase TliA. Many proteins are secreted by TliDEF in vivo when recombined with LARD3, but there are still others that cannot be secreted by TliDEF even when LARD3 is attached. However, the factors that determine whether or not a recombinant protein can be secreted through TliDEF are still unknown. Here, we recombined LARD3 with several proteins and examined their secretion through TliDEF. We found that the proteins secreted via LARD3 are highly negatively charged with highly-acidic isoelectric points (pI) lower than 5.5. Attaching oligo-aspartate to lower the pI of negatively-charged recombinant proteins improved their secretion, and attaching oligo-arginine to negatively-charged proteins blocked their secretion by LARD3. In addition, negatively supercharged green fluorescent protein (GFP) showed improved secretion, whereas positively supercharged GFP did not secrete. These results disclosed that proteins’ acidic pI and net negative charge are major factors that determine their secretion through TliDEF. Homology modeling for TliDEF revealed that TliD dimer forms evolutionary-removed positively-charged clusters in its pore and substrate entrance site, which also partially explains the pI dependence of the TliDEF-dependent secretions. In conclusion, lowering the isoelectric point improved LARD3-mediated protein secretion, both widening the range of protein targets for efficient production via secretion and signifying an important aspect of ABC transporter–mediated secretions.

As demand grows for protein products in clinical, industrial, and academic applications, more methods are developed to achieve an efficient mass production of proteins from microorganisms (1–4). Some of these methods involve engineering microorganisms to produce and secrete target proteins into the culture broth, effectively removing the need to refold the proteins that are produced and to engage in intensive protein purification to isolate the target protein from cellular proteins (5, 6). Pseudomonas fluorescens, a Gram-negative bacterium, is considered one of the best candidates for protein-production engineering through secretion due to its generally non-pathogenic nature against humans (7) and resilience against fermenting conditions because it does not accumulate acetate (8).

P. fluorescens has an ATP-binding cassette (ABC)2 transporter, TliDEF, that belongs to the type I secretion system (T1SS). TliDEF is composed of three different protein multimers: TliD, TliE, and TliF, where TliD is an ABC; TliE is a membrane fusion protein; and TliF is an outer membrane factor. In wild-type P. fluorescens, TliDEF secretes thermostable lipase TliA and metalloprotease PrtA (9). In previous studies, the lipid ABC transporter recognition domain 3 (LARD3) on the C terminus of TliA was conjugated to recombinant proteins, namely green fluorescent protein (GFP) and epidermal growth factor (EGF) (10). These proteins were produced and effectively secreted out to the extracellular space when introduced into the P. fluorescensΔtliA ΔprtA strain, with conjugated LARD3 to their C terminus.

Many proteins, including those mentioned above, were well-secreted when conjugated with LARD3, but other proteins were only localized in the cytoplasm. A key factor for this phenomenon remains to be determined. Is there any critical factor that determines whether or not a given signal-attached protein can be secreted by the ABC transporter? To investigate the difference between these proteins, we have conjugated various protein genes to LARD3 and introduced them to P. fluorescens. We analyzed both the supernatant and the cell pellet culture of various proteins to determine which proteins can be secreted, which proteins cannot, and what differences they have.

In this study, we took advantage of the pDART plasmid vector we developed in our previous study to conveniently attach

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This article contains supplemental Figs. S1–S5, Table S1, and Section A.

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2 The abbreviations used are: ABC, ATP-binding cassette; T1SS, type I secretion system; MCS, multiple-cloning site; PDB, Protein Data Bank; Trx, thioredoxin; NBD, nucleotide-binding domain; TMD, transmembrane domain; MBP, maltose-binding protein; MAP, mussel adhesion protein.
LARD3 (11). The pDART plasmid has a multiple-cloning site (MCS) directly followed by an in-frame LARD3 gene. Thus, genes inserted into the multiple-cloning site of pDART are expressed with LARD3 attached to their C termini. These attached LARD3 sequences are recognized by the TliDEF ABC transporter, letting the recombinant protein be secreted by the TliDEF complex. pDART also includes a kanamycin resistance gene for clonal selection; broad host range origin of replication, TliDEF complex. pDART also includes a kanamycin resistance transporter, letting the recombinant protein be secreted by the expressed with LARD3 attached to their C termini. These genes inserted into the multiple-cloning site of pDART are all of them (with one exception, CTP-TliA) were above ~5.5, being either positively or less negatively charged. In contrast, the secreted proteins were relatively acidic and highly negatively charged with a pl that does not exceed 5.5 (Fig. 2A and supplemental Table S1). Similarly intriguing, other reports of recombinant protein secretion through ABC transporters often, if not always, involve acidic, negatively-charged target proteins: mannanase (13), phospholipase A1,3 alkaline phosphatase (11), and epidermal growth factor (10). We also added the pl values of these proteins in Fig. 2A. The secretion of NKC-TliA and CTP-TliA decreased dramatically from that of original TliA (Figs. 1B and 2B). These are derivatives of TliA with an N-terminally attached short, extremely positively-charged sequence (supplemental section A). CTP-TliA was not secreted at all. Note that CTP has nine consecutive residues composed solely of arginine with only one exception, alanine (RRARRRRRR), as described in supplemental section A. Then, after quantification, the percentage of secreted protein versus the total amount of expressed protein was plotted. The obtained % secretion values were then plotted against the proteins’ pl value (Fig. 2B). There seemed to be a weak negative correlation between protein pl and their secretion efficiency, but there were also a few exceptions.

**Results**

**Cross-analyzing the secretion of recombinant proteins and their pl**

Thirteen genes (Table 1) that code for proteins of different sizes, flexibility, bulkiness, charges, etc. were introduced to *P. fluorescens* ΔtliA ΔprtA via pDART, where they are attached to a C-terminal LARD3 signal sequence. The supernatant and cell pellet were analyzed via Western blotting (Fig. 1). Mannanase, MBP, NKC-TliA, EglV, GFP, and thioredoxin were both detectable in the cell pellet and the supernatant, showing successful expression and secretion out to the extracellular media. However, MAP, cutinase, chitinase, capsid, Hsp40, and CTP-TliA were not detected in the supernatant despite being detected in the cell pellet, signifying that they were not secreted. These non-secreted proteins have a relatively high theoretical pl; all of them (with one exception, CTP-TliA) were above ~5.5, being either positively or less negatively charged. In contrast, the secreted proteins were relatively acidic and highly negatively charged with a pl that does not exceed 5.5 (Fig. 2A and supplemental Table S1). Similarly intriguing, other reports of recombinant protein secretion through ABC transporters often, if not always, involve acidic, negatively-charged target proteins: mannanase (13), phospholipase A1,3 alkaline phosphatase (11), and epidermal growth factor (10). We also added the pl values of these proteins in Fig. 2A. The secretion of NKC-TliA and CTP-TliA decreased dramatically from that of original TliA (Figs. 1B and 2B). These are derivatives of TliA with an N-terminally attached short, extremely positively-charged sequence (supplemental section A). CTP-TliA was not secreted at all. Note that CTP has nine consecutive residues composed solely of arginine with only one exception, alanine (RRARRRRRR), as described in supplemental section A (14). Then, after quantification, the percentage of secreted protein versus the total amount of expressed protein was plotted. The obtained % secretion values were then plotted against the proteins’ pl value (Fig. 2B). There seemed to be a weak negative correlation between protein pl and their secretion efficiency, but there were also a few exceptions.

**Analysis of lunasin and its derivatives**

Lunasin is an anticancer polypeptide from soybean Glycine max (15). It has a unique feature of nine consecutive aspartic acid residues at its C terminus (16). We have constructed multiple derivatives of lunasin with different lengths of the oligo-aspartate tails. Lunasin and its derivatives were introduced to *P. fluorescens* via pDART, and their expression and secretion were observed via Western blotting (Fig. 3A). The original lunasin showed that the highest secretion and relative amount of secreted protein declined as the length of the oligo-aspartate tail decreased. We have also observed decreased secretion and expression levels in lunasin-D15 (lunasin with 15 Asp at tail).

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3 J. Park, J. Y. Oh, J. H. Park, S. C. Kim, J. K. Song, and J. H. Ahn, submitted for publication.
Lunasin-D20 was not expressed in either the cell or supernatant. The exact sequence of the lunasin polypeptide and its derivatives is given in Fig. 3B. Based on this experiment, we determined that the optimal length of the oligo-aspartate sequence would be approximately nine, and we set up the experiments below.

**Addition of aspartate oligomers, pFD10 and pBD10 construction**

Among the 20 most common amino acids, aspartic acid has the lowest side chain pK\(_a\) value (17). Inspired by the lunasin protein sequence, we developed two plasmids that add the oligo-aspartate sequence to the inserted proteins as well as the LARD3 signal sequence. We have synthesized an aspartate–decamer-coding DNA sequence (D10) based on the DNA sequence of the lunasin gene’s oligo-aspartic acid tail. After the synthesis, we conjugated D10 to the pDART plasmid, creating two types of plasmid that either add D10 to the N terminus of the cargo polypeptide (pFD10) or to the C-terminal side (pBD10) of the MCS of pDART, where the target genes are inserted (Fig. 4). Then, selected proteins were inserted into both of the newly created plasmids, pFD10 and pBD10. These

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**Figure 1. Secretion of selected proteins.** A and B, Western blotting image showing the expression and secretion of the target proteins. The cell samples show the amount of the protein that remains in the cytoplasm, and the supernatant samples represent the amount of protein that is localized to the extracellular space. For comparison, equivalent amounts of cell extract and culture supernatant (16 \(\mu\)l) were loaded onto the gel and were analyzed via Western blotting. 50 ng of TlIA was loaded in the middle of the gel as a reference. Two other Western blottings were obtained from different culture samples. All of the unpresented results exhibit similar patterns. Below the images, there are Western blottings of the same samples but with primary antibody against cytosolic Neo, the neomycin/kanamycin phosphotransferase 2 protein. The nonspecific lysis or leakage is minimal in all samples except capsid.

**Figure 2. Correlation between % secretion of the target proteins and their pI values.** A, protein isoelectric point and secretion. The pI value of the target proteins is calculated from the sequence, including the attached LARD3, as provided in supplemental section A. The proteins that have not been secreted have their bars colored red. AP, EGF, and PLA1 are proven to be secreted in previous studies and are added in this figure. B, secretion percentage and pI. The percent secretion values were calculated by supernatant signal divided by the sum of supernatant and cell signals. Three different biological replicates (independent culture samples) of the experiment in Fig. 1 were used for the quantitative analysis. Two highly basic outlier proteins that were not secreted, MAP (pI = 9.61) and capsid (pI = 9.25), were excluded from the plot. There was a negative correlation between the protein pI and their % secretion.

**Figure 3. Secretion of lunasin and its derivatives.** Lunasin and its derivatives with different lengths of oligo-aspartic acid tail were expressed and secreted via LARD3 attachment to determine the optimal length of the oligo-aspartic acid sequence in P. fluorescens expression and secretion system. A, expression of lunasin and its derivatives in the cell and supernatant were detected via Western blotting. 36-\(\mu\)l eq of cell extract and culture supernatant were loaded onto the gel and were analyzed via Western blotting. B, protein sequence and domain structure of lunasin and its derivatives whose length of the aspartic acid tail is modified. They were named as lunasin-D0, lunasin-D5, original lunasin (D9), lunasin-D15, and lunasin-D20.

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pFD10 or pBD10-cloned recombinant proteins were introduced to *P. fluorescens* alongside their pDART-cloned counterparts, and the secretion efficiency was analyzed via Western blot analysis.

**Insertion of TliA-derived recombinant proteins into pFD10 and pBD10**

NKC-TliA and CTP-TliA are both derivatives of TliA, each with an N-terminal basic-peptide attachment. Their secretion efficiency through TliDEF is significantly smaller than wild-type TliA (Fig. 1A and supplemental Fig. S1). However, the oligo-aspartate attachment on them by pFD10 or pBD10 greatly increases their secretion. In terms of the secretion ratio (secreted protein intracellular protein), NKC-TliA shows a dramatic increase in secretion after the addition of either an upstream or downstream D10 sequence (Fig. 5, A and B). CTP-TliA also shows a drastic increase in secretion in both the Western blotting and activity plate assays when a downstream D10 sequence was added by pBD10 (Fig. 5, C and D). In enzyme plate activity assays, the halo sizes of NKC-TliA and CTP-TliA in pDART or pBD10 are generally consistent with the band strength of the supernatant samples in their respective Western blotting results. However, pFD10 has a slightly smaller halo than expected from their band strength, indicating the possibility of a reduced enzymatic activity.

**Insertion of negatively-charged proteins to pFD10 and pBD10**

Genes for GFP, mannanase, MBP, and Trx were introduced to pDART, pFD10, and pBD10. The resulting recombinant plasmids were used to transform *P. fluorescens* ΔtliA ΔprtA, where the genes were expressed, and the produced proteins were secreted by the TliIDEF transporter. GFP showed the most dramatic increase. A comparison of the band strength of pDART and pBD10-inserted GFP showed a remarkable change in the supernatant versus intracellular protein. pFD10-GFP also exhibited some improvement in terms of the ratio between the supernatant and the cell pellet (Fig. 6A). The case of mannanase was somewhat vague, but we could conclude that pBD10-mannanase exhibits a better secretion than pDART-mannanase. Although the absolute expression itself decreased, there was a small improvement in the ratio when an upstream D10 sequence was added by pFD10 (Fig. 6B). The secretion of MBP improved in both pFD10 and pBD10 in terms of the supernatant/cell ratio, compared with pDART (Fig. 6C). In the case of Trx, the supernatant/cell ratio improved in pFD10 and pBD10 (Fig. 6D).

**Addition of positively charged amino acid oligomers, construction and analysis of pBR10**

We constructed an additional plasmid that closely resembles pBD10, but with one difference: the D10 sequence, the DNA sequence that codes for aspartate oligomer, was replaced with R10 (codes for arginine oligomer). We inserted the TliA and GFP gene to pDART, pBD10, and pBR10 plasmids and examined their secretion by enzyme plate assay (TliA only) and Western blotting (Fig. 7). In Western blotting of TliA, pDART
Protein isoelectric point and ABC transporter secretion

Figure 7. Secretion of TliA and GFP in pDART, pBD10, and pBR10. Two negatively-charged proteins, TliA and GFP, were inserted in the plasmids that attach nothing except the signal sequence (pDART), oligo-aspartate (pBD10), and oligo-arginine (pBR10). A, Western blot of TliA in these plasmids. TliA in pDART and pBD10 shows good secretion. However, the secretion was blocked when R10 was attached. B, enzyme blot assay of TliA in these plasmids. TliA was blocked when it was inserted to pBR10. C, Western blot of GFP in these plasmids. Similarly, pDART and pBD10 showed good secretion, although secretion was blocked when R10 was attached.

and pBD10 exhibited good secretion efficiency. pBR10, however, blocked the secretion (Fig. 7A). Similar patterns were observed in enzyme assay; pBR10 did not exhibit halo, but the others did (Fig. 7B). In Western blotting of GFP, both pDART and pBD10 showed good secretion. However, the secretion was blocked when R10 was attached. pBD10 exhibited good secretion, whereas secretion was blocked when R10 was attached.

Western blot analysis of supercharged proteins

GFP and its two supercharged derivatives, GFP (−30) and GFP (+36), were recombined with LARD3 and introduced to P. fluorescens ΔtliA ΔsptA. The samples were analyzed via Western blotting (Fig. 8). Both GFP and GFP (−30) were detected in the cell pellet and the supernatant, indicating that they were effectively expressed and secreted to the extracellular space. Note that GFP (−30) was more strongly localized to the supernatant than the original GFP. In contrast, GFP (+36) is heavily expressed but localized in the cell pellet and is not secreted to the extracellular space. The pl values for these recombinant proteins are 4.64 for GFP (−30), 5.36 for unmodified GFP, and 10.42 for GFP (+36).

Discussion

Altogether, these results indicate that the secretion rate is higher in more negatively-charged proteins in the TliDEF ABC transporter. There was a qualitative correlation between the protein pl value and whether it could be secreted by the TliDEF ABC transporter; highly-acidic and negatively-charged proteins were secreted by the TliDEF ABC transporter, whereas proteins with a less negative charge and higher pl value were hardly secreted. This finding is also consistent with the meta-analysis by Delepelaire, which states that the proteins naturally transported by the ABC transporters are very acidic, “all of them are (with only one exception) very acidic proteins with pl around 4”, at least for the proteins he had meta-analyzed (18).

When the genes were introduced to pFD10, some proteins exhibited an increased secretion. But in general, their expressions tended to be reduced relative to pDART. This may be due to a reduction in mRNA stability or translation rate due to alterations in its secondary structure. In addition, the target genes that were inserted into pFD10 were expressed with D10 immediately following the start codon methionine, with an extremely negatively charged N-terminal region. This may reduce the protein half-life if they are targeted by cellular degradation mechanisms. For pBD10, this is not the case because the D10 sequence was followed by LARD3, and thus the charged region is not located at the terminus in translations of pBD10 (Fig. 4C). More specific reasons are not yet understood.

The combined results of the pFD10, pBD10 experiments, and secretion patterns of supercharged proteins indicate that proteins engineered to carry more negative charges are secreted better. We expect that this reduces the energy required to overcome the electric potential energy barrier between the cytoplasm and the extracellular space. In general, Gram-negative bacteria maintain an inner membrane potential that is roughly around −150 mV, with the cytoplasmic side more negatively charged than the periplasmic space (19, 20). This polarized charge distribution is maintained by various cellular mechanisms, including active proton transport across the membranes. The outer membrane potential is also negative, with the periplasm being more negatively charged than the extracellular space due to negatively-charged membrane-derived oligosaccharides that are localized there (21). However, the magnitude is significantly smaller, typically less than −30 mV, due to the pore-rich nature of the outer membrane (22, 23). Considering all these facts, it is energetically more favorable to secrete highly negatively-charged proteins, affecting the general equilibrium of secretion. The significance of the membrane potential on the protein alignment has already been studied in many previous studies; charged residues determine the orientation of the polypeptide during the interaction with the membrane and affect protein translocation through membrane (24–26). As seen from these biological phenomena, the membrane potential is quite powerful at the biochemical level, and it has a significant impact on the change in free energy during translocation through ABC transporters. Moving a polypeptide across the inner membrane with a −150-mV potential requires an energy of ∼3.5 kcal/mol per charge that the polypeptide carries. The calculation under constant pressure, temperature, and concentration is presented in Equation 1,

\[ w = -nFV = 14.47n \text{kJ/mol} = 3.5n \text{cal/mol} \] (Eq. 1)

where \( n \) is the total charge of the polypeptide, and \( F \) is the Faraday constant. Thus, if a protein has 10 positive net charges...
Protein isoelectric point and ABC transporter secretion

(n = +10), then w = +35 kcal/mol and secretion becomes that much more unfavorable. A typical change in free energy ($\Delta G$) during ATP hydrolysis under in vivo concentrations is $-11.4$ kcal/mol (27). The models suggested for the ABC transporter mechanism indicate that the ABC protein operates through continuous switching between the “inward-facing” and “outward-facing” conformation coupled to ATP hydrolysis (28, 29).

According to this model, one of the major forces that drive translocation in ABC transporters is the force of this “power stroke” (30). The negative membrane potential applies an electrostatic force that either helps (for negative charges) or opposes (for positives charges) the force created by this power stroke, kinetically accelerating or hampering the translocation process and ultimately affecting the secretion equilibrium.

This “membrane potential” hypothesis is further supported by a number of previous studies spanning various secretion types. A positive-charge inducing mutation on an E. coli lipoprotein is reported to have reduced its secretion by interrupting its folding near the membrane in both prokaryotes and eukaryotes (31, 32). Either neutralizing or reversing the net negative charge of the passenger domain of E. coli autotransporter type (Va secretion system) halts the transportation across the outer membrane that also has an “inside-negative” charge configuration across the membrane (33).

The other factor to consider is the charge state of TliD. TliD is the ABC protein, the inner membrane component, of the TliDEF transporter. It carries a nucleotide-binding domain (NBD) and a transmembrane domain (TMD), connected by a short inter-domain sequence (9). It is interesting that the TliD ABC protein has an extremely high theoretical pI value, especially around the TMD (pI 9.43) and inter-domain sequences (pI 8.14). Subsequent homology structural modeling of dimeric TliD, with Aquifex aeolicus PrtD (PDB code 5l22) (34) as a template (sequence identity 40.98%), revealed that it has positive charge distribution at the midpoint of its channel’s inner surface (Fig. 9, A and B). Moreover, a ConSurf homolog conservation analysis on TliD showed that these charges were indeed conserved, forming a positively charged sub-region at the midpoint of the channel (Fig. 9, C and D, yellow circles). In addition, on the kinked helix on the substrate entry window, there is a positively-charged residue that sticks out toward the pore of the window and blocks the window in ADP-bound state of TliD (34). The ConSurf results also verified that this residue was charge-conserved, as all of the 50 homologs had either arginine or lysine at this residue (Fig. 9C, black arrow). We expect that this positively-charged inner surface interacts with negatively-charged residues during protein transport, facilitating secretion (Fig. 9E).

Also, the positive charges in the inner surface of the channel and on the substrate entry window may repel positively-charged sections of polypeptides, blocking them from entering the channel and ultimately from being secreted, as can be seen from the results of oligo-arginine attachment. Here, we assumed that the cargo proteins unfolds (at least partially) during transport because the pore size of TliF, which is expected to have a very similar structure to E. coli TolC (PDB code 1tqq), has average interior diameter of 19.8 Å (35). This is apparently smaller than 20–30 Å, which is the average diameter of most globular proteins, including 24 Å of GFP’s barrel (36). TliF contains a relatively rigid β-barrel transmembrane structure, which means it is unlikely that it expands during transport.

Other ABC transporters, although their structures are not yet fully understood, have similarly positively-charged TMD in their ABC proteins. The E. coli hemolysin transporter, HlyB–HlyD–ToIC complex, also has a similar charge distribution with a significantly positive charge on the TMD of its ABC protein, HlyB, which is functionally homologous to TliD. The same is true for Dickeya dadantii PrtD. Along with the previously reported meta-analysis mentioned above (18), this fact strongly supports the charge dependence of the T1SS ABC transporter secretion mechanism.

In conclusion, only highly acidic proteins were capable of being transported through the ABC transporter. Either basic or only slightly acidic proteins could not be secreted through the ABC transporter. However, the secretion of recombinant proteins can improve when their pI value is artificially lowered through oligo-aspartate attachment or by negatively super-charging them. This result suggests that through simple pI inspection, it is possible to determine whether a protein of interest could be secreted by ABC transporters. Also, it is possible to increase the protein secretion by altering their charge, which can ultimately widen the range of targets to efficiently produce proteins via ABC transporter-dependent secretion.

Experimental procedures

Bacterial strains and growth media

Plasmid construction and genetic modifications were performed in E. coli XL1-BLUE. Protein expression and secretion were observed in the P. fluorescens ΔTliA ΔprtD strain, which is a double-deletion derivative of P. fluorescens SIK-W1 (37). Microorganisms were cultured in lysogeny broth (LB) with 30 µg/ml kanamycin. An enzyme plate assay for the target genes with lipase activity (TliA, NKC-TliA, and CTP-TliA) was prepared with LB agar containing blender-mixed 0.5% colloidal glycercly tributyrate. E. coli and P. fluorescens were incubated at 37 and 25 °C, respectively. E. coli transformation was performed following the standard heat-shock method, and P. fluorescens transformation was performed via electroporation at 2.5 kV, 125 ohms, and 50 microfarads, with electrocompetent cells prepared using a standard electroporation protocol (38). The transformed P. fluorescens were cultured in test tubes with 5 ml of liquid LB media, including 60 µg/ml kanamycin, and were incubated at 25 °C in an 180 rpm shaking incubator until the stationary phase was reached. The proteins were analyzed for both expression and secretion by seeding the transformed cells in liquid LB or streaking them on the solid-plate activity assay.

Plasmid vector constructions

Plasmid pDART was used for the secretory production of different proteins (11). Plasmid vectors pFD10 and pBD10 were derivatives of pDART, constructed by adding codons for 10 aspartic acid residues to the target proteins in either the upstream or downstream position of MCS. The DNA sequence for 10 aspartic acids was amplified via PCR using synthesized G. max lunaasin gene (15) as a template. Two different PCR products were obtained, each for pFD10 and pBD10. One or
Figure 9. Charge distribution in the structure of TliD, the ABC protein of the TliDEF complex. A, electron repulsion surface of the TliD monomer. Colored according to its surface electric potential, from blue (+7 kT/e) to red (−7 kT/e). The inner surface of the central channel is circled yellow. Note that the circled inner surface is highly positively charged. B, TliD homodimer, with one of the monomers presented in the ribbon model. Substrate entry window is circled green. C, TliD, residues with conserved positive or negative charges are colored blue and red, respectively. The conserved positive charge cluster at the midpoint of the channel’s inner surface are circled yellow. The two α-helices that form substrate entry window are colored green. Among the two conserved positively-charged residues, Arg-316 (black arrow) sticks out to the pore. D, TliD dimer, seen from the periplasmic face. Positive charges are located in the middle of the channel (circled yellow), whereas negative charges are outside of the channel. E, schematic model of the TliD dimer, transporting a highly negatively charged recombinant polypeptide with the attached LARD3. The NBD and TMD of TliD are labeled accordingly. Note that the electric potential across the inner membrane (IM) is −150 mV, where the cytoplasm (CP) is more negative than the periplasm (PP). This potential difference also makes it more favorable to outward-transport negatively-charged proteins than positively-charged proteins. F, sequence mapping of the structures in C and D. Residues with high Bayesian conservation score (=7) are highlighted in gray. Among them, the charged residues are colored as blue (+ charged) and red (− charged). Green and yellow boxes represent the substrate entry window and the channel’s inner surface, respectively.
two arbitrary bases are inserted upstream or downstream of the primers to keep the translation in-frame, causing a slight size and pI difference between the pFD10- and pBD10-inserted proteins. Recombining the PCR product with pDART to construct pFD10 and pBD10 was accomplished with an In-Fusion cloning kit (Clontech In-Fusion HD cloning plus CE). To linearize pDART, it was digested with either XbaI (for pFD10 construction) or SacI (for pBD10). Then, the linearized pDART and the corresponding PCR products were digested with In-Fusion 3′-to-5′-exodeoxyribonuclease and re-ligated following the standard protocol of the In-Fusion kit. Ligation of these DNA fragments with complementary ~15-base 5′-overhangs resulted in pFD10 and pBD10 plasmid, ready for target gene insertions. pDART, pFD10, and pBD10 sequences near their MCSs are provided in supplemental section A.

**Construction of plasmids with inserted target genes**

Thirteen target genes were selected for pDART insertion. The full names and sources of these proteins are provided in Table 1. The genes were amplified with PCR from extracted genomic DNA samples (TliA, MBP, Trx, and Hsp40), total cDNA (EglIV), synthesized DNA products (NKC-TliA, CTP-TliA, MAP, lunasin, lunasin derivatives, GFP, and supercharged GFPs), or plasmids (other proteins), respectively. N-terminal signal peptides were detected with the SignalP web-based prediction algorithm (http://www.cbs.dtu.dk/services/SignalP) (39) and were excluded from secretory production in pDART, pFD10, or pBD10. From secretory proteins, the codons were optimized for either E. coli expression (supercharged GFPs) or P. fluorescens expression (TliA derivatives).

The lunasin gene was synthesized and amplified with PCR for pDART insertion. With various primers, we also synthesized its variations with differing lengths of the oligo-Asp sequence at their C terminus: lunasin-D0, lunasin-D5, lunasin-D15, and lunasin-D20 (Fig. 3B). These sequences were recombined with pDART for secretory production. NKC-TliA and CTP-TliA are derivatives of TliA. NKC is an antibiotic polypeptide developed in a previous study (40), and CTP is a cytoplasmic transduction peptide that was developed as a cellular import tag in a previous study (14). We have synthesized genes for these two, with codons optimized for P. fluorescens expression.

The supercharged variations of GFP, including negatively supercharged GFP (−30) and positively supercharged GFP (+36), were previously developed by replacing solvent-exposed residues of GFP with negatively or positively charged amino acids, respectively (12). We have completely synthesized genes that code for these two supercharged proteins, with codons optimized for E. coli expression.

The primers we used for PCR had restriction enzyme sites that were utilized to insert the target genes to the MCSs of the plasmids: pDART, pFD10, and pBD10. The PCR products and plasmid vectors were double-digested with two restriction enzymes for XbaI, Kpnl, SacI, and Spel (which is compatible with XbaI). The specific pair of enzymes used on each gene can be directly identified from the full sequences provided in supplemental section A. Then, they were ligated with T4 ligase. The constructed plasmids were then introduced into E. coli for cloning, and the cloned plasmids were first obtained using a standard plasmid purification method. The purified plasmids were then introduced to P. fluorescens, for which expression and secretion were analyzed.

**Western blotting conditions**

After 48 h of cell growth (secretion occurs during the entire growth), the liquid culture reached stationary growth phase, and the cell density reached ~1.5 × 10⁸ cells/ml (A₆₀₀ = 3). 400 μl of the liquid cultures were taken and centrifuged at 18,000 relative centrifugal force for 10 min to separate the supernatant and the cell pellet. 16 μl of culture (~0.048 OD) equivalents of the cell pellet extract and supernatant were each loaded onto 10% polyacrylamide gels. SDS-PAGE was used to separate the proteins according to their sizes. Then, the proteins were transferred to a nitrocellulose membrane (Amer sham Biosciences) for Western blotting. Polyclonal anti-LARD3 rabbit immunoglobulin G (IgG) and anti-neomycin phosphotransferase 2 (Abcam, ab33595) were utilized as the primary antibody with 1:3000 and 1:500 dilution each, and anti-rabbit recombinant goat IgG-peroxidase (anti-rIgG goat IgG-peroxidase) was used as the secondary antibody with 1:1000 dilution. The bands were then detected using a chemiluminescence agent (Advansta WesternBright Pico). Western blotting images were acquired using an Azure C600 automatic detecting system. All included Western blotting images are representative results from at least three different repeated experiments, starting over again from cell culturing with independent P. fluorescens colonies. After the images were obtained, we quantified the results of experiment 1 (Fig. 1) with ImageJ software. We calculated % secretion of the target proteins of this experiment. The % secretion was calculated as Equation 2,

\[
% \text{ secretion} = \frac{S_{\text{supernatant}}}{S_{\text{supernatant}} + S_{\text{cell}}} \times 100 \% \quad \text{(Eq. 2)}
\]

where S is the normalized signal strength of each bands in the Western blotting image, and the subscripts denote the sample type of the lanes.

**Computational analysis of polypeptide properties and protein structure**

The theoretical pl values of the target proteins were calculated using the ExPASy Compute pI/Mw tool (41–43). The entire sequences were used, and LARD3 and any additional sequences from the enzyme sites were included in the sequences for this purpose. The protein pl values are highly correlated with their charge per residue, and the correlation analysis of the protein pl values and their charge per residue is included in supplemental Fig. S2. SWISS-MODEL structural homology modeling (https://swissmodel.expasy.org/) was used to study the ABC transporter protein structures (44–47). We used A. aeolicus PrtD (PDB code 5122) (34) as a template, with sequence identity of 40.98%. Sequence alignment between TliD and the template is provided in supplemental Fig. S3. The model’s transmembrane helices were verified by DAS-TMfilter (http://mendel.imp.ac.at/sat/DAS/) (48), and the results are provided in supplemental Fig. S4. The surface of the obtained results was analyzed.
Protein isoelectric point and ABC transporter secretion

3D model was calculated and colored with Swiss PdbViewer (spdbv) (http://spdbv.vital-it.ch/).4 We used the ConSurf web server (http://consurf.tau.ac.il/2016/)7 to compare TliD with its homologs and to verify the structure prediction of TliD (49, 50). Specific parameters we put for the homolog searching are provided in supplemental material. The ConSurf homology analysis also approved our structural prediction in a sense that most of the transmembrane helices were highly conserved in the inner surface-facing residues; supplemental Fig. S5 contains further information on the conserved residues of TliD. Finally, we checked side-chain pKa values of the highly conserved arginine and lysine residues at the potentially important positions (Fig. 9, C, D and F) with the web-based PDB 2PQR server (http://nbcu-222.ucsd.edu/pdb2pqr_2.0.0/),4 using the predicted structure as an input. We visualized the results with the PyMOL software. All sequences that were used for the analysis are provided in supplemental section A.

Author contributions—J. H. A. conceived and coordinated the study. H. B. and J. P. designed and performed the experiments. H. B. wrote most of the paper. S. C. K. proposed target gene selections and provided key intellectual contents. All authors analyzed the results and approved the final version of the manuscript.

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