ACS SyntheticBiology

Research Article

pubs.acs.org/synthbio

Universal Genetic Assay for Engineering Extracellular Protein Expression

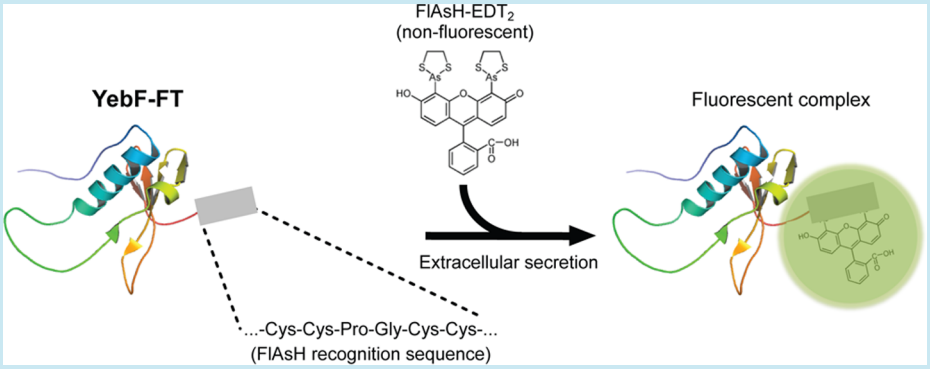
Charles H. Haitjema,[†] Jason T. Boock,[‡] Aravind Natarajan,[†] Miguel A. Dominguez,[§] Jeffrey G. Gardner,[§] David H. Keating,[§] Sydnor T. Withers,[§] and Matthew P. DeLisa*[,†,‡]

[†]Department of Microbiology, Cornell University, Ithaca, New York 14853, United States

[‡]School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York 14853, United States

[§]Great Lakes Bioenergy Research Center, Madison, Wisconsin 53706, United States

Supporting Information



ABSTRACT: A variety of strategies now exist for the extracellular expression of recombinant proteins using laboratory strains of *Escherichia coli*. However, secreted proteins often accumulate in the culture medium at levels that are too low to be practically useful for most synthetic biology and metabolic engineering applications. The situation is compounded by the lack of generalized screening tools for optimizing the secretion process. To address this challenge, we developed a genetic approach for studying and engineering protein-secretion pathways in *E. coli*. Using the YebF pathway as a model, we demonstrate that direct fluorescent labeling of tetracysteine-motif-tagged secretory proteins with the biarsenical compound FlAsH is possible *in situ* without the need to recover the cell-free supernatant. High-throughput screening of a bacterial strain library yielded superior YebF expression hosts capable of secreting higher titers of YebF and YebF-fusion proteins into the culture medium. We also show that the method can be easily extended to other secretory pathways, including type II and type III secretion, directly in *E. coli*. Thus, our FlAsH-tetracysteine-based genetic assay provides a convenient, high-throughput tool that can be applied generally to diverse secretory pathways. This platform should help to shed light on poorly understood aspects of these processes as well as to further assist in the construction of engineered *E. coli* strains for efficient secretory-protein production.

KEYWORDS: *bacterial expression, biarsenical FlAsH labeling, protein folding and export, secretion-pathway engineering, type II and type III protein secretion, YebF*

Expression of recombinant proteins into the culture medium is highly desirable owing to the ease of recovering extracellular proteins, the increased likelihood of correct protein folding, the avoidance of proteolytic degradation and toxicity to the production host, and the possibility of chemistries that can only take place outside of the cell (i.e., degradation of impermeable substrates). Although *Escherichia coli* remains one of the most widely used hosts for making soluble proteins in the cytoplasmic and periplasmic compartments,[1,2] it has historically been passed over for applications requiring secretion. This can be attributed to the longstanding belief that laboratory strains of *E. coli* (e.g., B and K12 strains) lack dedicated secretion systems and do not secrete proteins into the extracellular medium under normal growth conditions.[3]

In the last 10 years, this paradigm has been overturned with the discovery of endogenous proteins, such as YebF and the osmotically inducible protein Y (OsmY), that are naturally secreted by laboratory *E. coli* strains.[4,5] Both of these proteins are first localized into the periplasmic compartment via the Sec pathway and, in the case of YebF, then translocated across the outer membrane in a process that appears to involve OmpF/C.[6] The mechanism by which OsmY transits the outer membrane remains a mystery. Importantly, both YebF and OsmY can function as carriers for delivering 'passenger' proteins linked to their C-termini into the culture medium

Received: September 16, 2013
Published: November 7, 2013

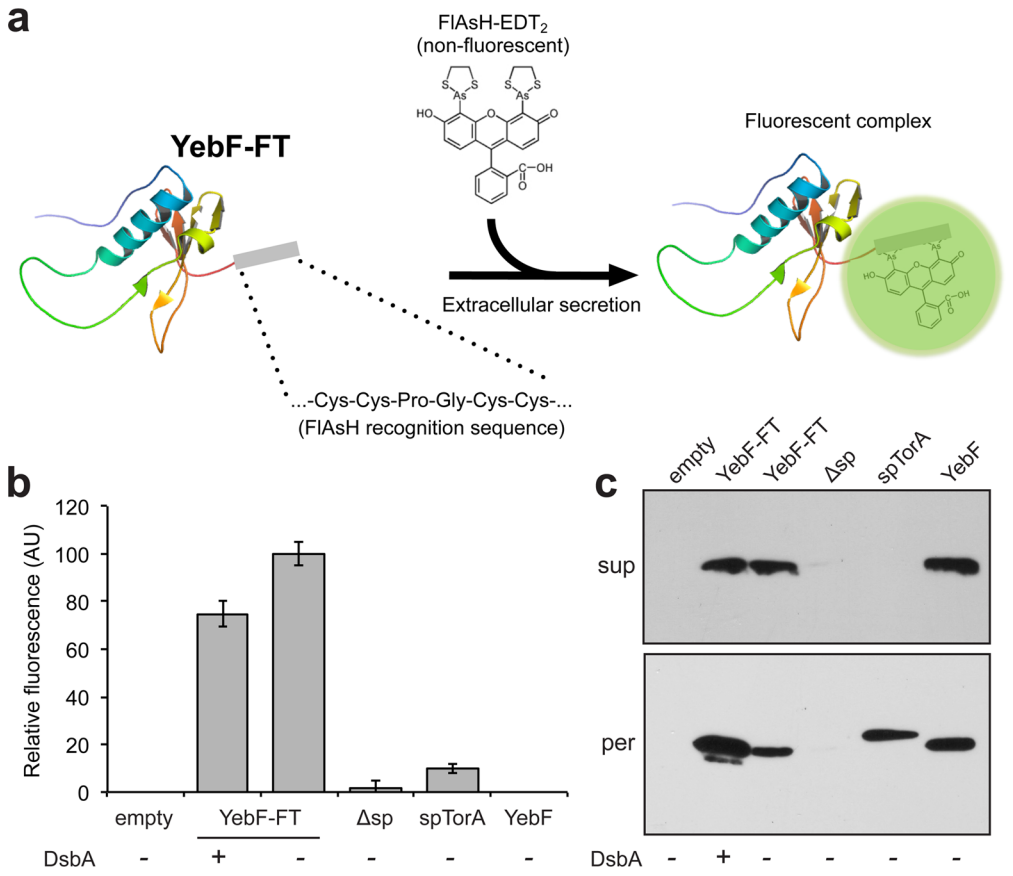


Figure 1. High-throughput screen for extracellular protein expression. (a) Schematic of the FlAsH-based genetic screen for protein secretion. The secretory target protein YebF is modified with a C-terminal tetracysteine epitope, or "FlAsH-tag" (FT), which binds specifically to FlAsH-EDT$_2$, creating a fluorescent complex. (b) FlAsH labeling of wt BW25113 (+) and BW25113 Δ*dsbA* (−) cells expressing full-length YebF-FT and derivatives lacking the signal peptide (Δsp), lacking the FT (YebF), or with the signal peptide replaced with that of *E. coli* TorA (spTorA). Data was normalized to the signal from BW25113 Δ*dsbA* cells expressing full-length YebF-FT and is expressed as the mean ± SEM of biological triplicates. (c) Western blot analysis of periplasmic (per) and supernatant (sup) fractions derived from the same cells in panel b. Blots were probed using anti-His antibodies.

without affecting outer-cell-membrane integrity.[4,5,7] To date, YebF and OsmY have been used to localize a wide range of structurally diverse proteins into the growth medium including *E. coli* alkaline phosphatase, *Bacillus subtilis* α-amylase, numerous human proteins (e.g., interleukin-2, leptin, and osteopontin)[4,5,7] and even post-translationally modified proteins.[8,9] More recently, YebF and OsmY have been used for delivering therapeutic proteins from probiotic vehicles such as *E. coli* Nissle 1917[10] and for consolidated bioprocessing whereby secretion of cellulolytic enzymes is coordinated with hydrocarbon-producing pathways in a single microorganism.[11−13] Despite the ability of YebF and OsmY to direct proteins into the growth medium in biologically active forms, the relatively low yields achieved to date have limited the utility of these pathways. For example, the conversion of xylan to biodiesel by engineered *E. coli* was limited by the suboptimal extracellular activity of OsmY-xylanase fusions.[11] Hence, new methods are needed for engineering microbial hosts with enhanced secretion capacity.

Genetic approaches based on fluorescent assays and high-throughput screening technologies constitute an extremely powerful strategy for the rapid isolation of mutants that confer complex phenotypes of interest.[14] Unfortunately, genetic assays for protein secretion that can be applied generally to any pathway of interest independently of the phenotype of the pathway or of its specific substrates do not exist. To address this shortcoming, we have developed a universal genetic assay for bacterial protein secretion whereby modification of a secreted protein of interest with a C-terminal tetracysteine motif permits specific labeling of the secreted protein with biarsenical fluorescent compounds (e.g., FlAsH). Because the outer membrane of *E. coli* is naturally impermeable to FlAsH,[15] undesired labeling of intracellular proteins is avoided, rendering the assay highly selective for secreted proteins. With tetracysteine-tagged YebF as the secretion substrate, we screened an *E. coli* transposon library and identified several insertional knockout mutations that resulted in a large increase in extracellular fluorescence and a dramatic enhancement in YebF secretion. When YebF chimeras composed of C-terminally fused cellulolytic enzymes were expressed in these knockout strains, increases in fusion-protein secretion were similarly observed. Finally, our FlAsH-based strategy was easily adapted to other protein-secretion pathways, confirming the universality of the approach and providing a tool to facilitate the engineering of virtually any secretory pathway in *E. coli* for efficient extracellular protein expression.

RESULTS AND DISCUSSION

FlAsH-Tetracysteine Assay for Extracellular Protein Expression. To develop a general assay that could be applied

dx.doi.org/10.1021/sb400142b | *ACS Synth. Biol.* 2014, 3, 74−82

to many diverse pathways, we sought a readily identifiable phenotype that was independent of the specific secretion pathway or substrate and compatible with high-throughput screening. We hypothesized that the FlAsH-tetracysteine system[16] could be adapted for this purpose (Figure 1a). To test this notion, a "FlAsH tag" (-FLNCCPGCCMEP-) containing a tetracysteine motif within a sequence that was previously optimized for dye affinity and fluorescence[17] was appended to the C-terminus of YebF along with a 6×-His tag, yielding YebF-FT. *In situ* labeling of wild-type (wt) *E. coli* strain BW25113 expressing YebF-FT with the biarsenical dye FlAsH-EDT_2, which is nonfluorescent until it binds with high affinity and specificity to the tetracysteine motif, produced significant fluorescence above background (Figure 1b). This fluorescence corresponded with the appearance of YebF-FT in the supernatant (Figure 1c). The fluorescence was slightly enhanced in cells lacking the periplasmic disulfide oxidase DsbA (Figure 1b), presumably because of less periplasmic oxidation of the cysteine residues in the FlAsH tag. Cells expressing YebF without a FlAsH epitope appeared as dim as empty vector control cells (Figure 1b); however, accumulation of this construct in the supernatant was comparable to YebF-FT (Figure 1c), indicating that the FlAsH epitope was not inhibitory to secretion.

Although FlAsH-EDT_2 is permeable to mammalian cell membranes,[16,17] it does not easily permeate the *E. coli* membrane.[15] Indeed, virtually no fluorescence was measured when the 21-residue Sec-dependent N-terminal signal peptide was removed from YebF-FT (Δsp; Figure 1b), a modification that causes YebF to accumulate in the cytoplasm.[8] This lack of fluorescence was corroborated by the absence of YebF-FT in the periplasmic and supernatant fractions (Figure 1c). Interestingly, when the Sec signal peptide of YebF-FT was replaced with the signal peptide from *E. coli* trimethylamine N-oxide reductase (spTorA), which is specific for the twin-arginine-translocation (Tat) pathway, it accumulated in the periplasm but was not secreted into the culture medium (Figure 1c). Cells expressing this construct exhibited weak fluorescence that was barely above background (Figure 1b). Taken together, these results indicate that FlAsH-EDT_2 selectively labels extracellular YebF-FT but not intracellular precursors of the same protein.

Isolation of Highly Fluorescent Transposon Mutants. We next sought to determine if our genetic assay could be used to isolate *E. coli* mutants with enhanced secretion activity. Specifically, *E. coli* strain EC100 was subjected to *in vitro* transposon mutagenesis with the EZ-Tn5™ <R6Kγ*ori*/KAN-2> Tnp transposon. The library of transposon mutations was transduced in BW25113 Δ*dsbA* cells carrying the YebF-FT plasmid using a high-throughput P1 phage transduction method.[18] The resulting transposon library was labeled with FlAsH-EDT_2 in a microtiter plate format, and labeled clones were screened for fluorescence. The fluorescence activity in each well was used to calculate a B-score.[19] Eight hits with statistically significant increases in fluorescence (B-score > 5) were isolated (Figure 2a). The isolated mutants contained transposon insertions in the following nonessential genes: *entC*, *entE*, *envZ*, *mzrA*, *nlpD*, *ompR*, *tnaA*, and *yihF*. To confirm these hits as *bona fide* hypersecretors, BW25113 cells with precisely defined, single-gene deletions corresponding to these hits were obtained from the Keio collection[20] and each was freshly transformed with the YebF-FT plasmid. In each case, the fluorescence generated from each knockout strain was greater than that measured for wt BW25113 cells expressing YebF-FT, with some mutants (e.g., *envZ* and *nlpD*) exhibiting as much as a 3-fold enhancement in fluorescence (Figure 2b). Importantly, the single-gene-deletion strains yielded fluorescence signals that were nearly identical to the corresponding transposon insertion

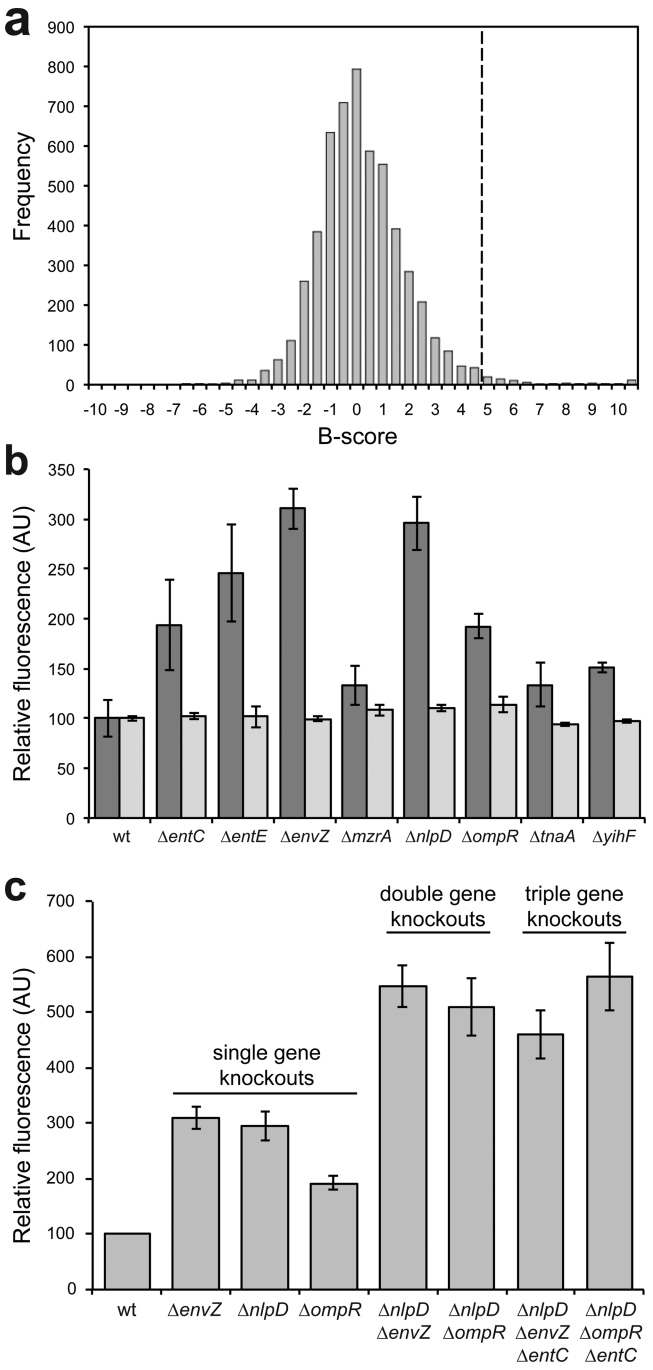


Figure 2. Isolation of genetic mutants with markedly enhanced fluorescence. (a) B-score analysis of fluorescence data generated from FlAsH-labeled transposon library members expressing YebF-FT. B-scores of 5 or higher (dashed line) were chosen for further characterization. (b) FlAsH labeling of mutant strains identified in high-throughput screening (dark gray) or the same strains complemented with a plasmid-encoded copy of the deleted gene (light gray). (c) FlAsH labeling of single-, double-, or triple-mutant strains expressing YebF-FT. All fluorescence data was normalized to the signal from wt BW25113 cells expressing YebF-FT and is expressed as the mean ± SEM of biological triplicates.

dx.doi.org/10.1021/sb400142b | *ACS Synth. Biol.* 2014, 3, 74−82

mutants, indicating that the Tn5 gene disruption was solely responsible for the enhanced secretion phenotype. Genetic complementation of each BW25113 mutant strain carrying the YebF-FT plasmid with plasmid-encoded copies of the deleted gene resulted in basal levels of YebF-FT secretion (Figure 2b). It is also worth mentioning that higher secretion levels could be obtained by combining deletions of these eight loci. For example, BW25113 Δ*nlpD* Δ*envZ* cells, which combined the two best single-gene deletions, exhibited a >5-fold enhancement in fluorescence compared to wt cells (Figure 2c). It should be noted, however, that deletion of additional loci in this strain (e.g., Δ*nlpD* Δ*envZ* Δ*entC*) did not result in any further improvement.

Western blot analysis revealed that all mutants accumulated significantly greater amounts of YebF-FT in the culture medium compared to the parental wt strain (Supporting Information Figure S1a), confirming that the FlAsH-tetracysteine assay reliably uncovers gain-of-function mutations in the target secretory pathway. The higher levels of YebF secretion in the mutant strains had little to no impact on cell viability (Figure S1b). The amount of YebF purified from the supernatant of one of the best mutant strains, namely, BW25113 Δ*nlpD*, increased up to 3.2-fold relative to wt BW25113 cells (1.2 versus 0.4 mg/L, respectively; Figure S1c), consistent with the fluorescence and western blot results. It is noteworthy that even higher overall titers were achieved when YebF was expressed from a strong T7 promoter, yielding 4.4 mg/L in wt BL21(DE3) cells and 10−13 mg/L in BL21(DE3)-derived knockout mutants (Figure S1c). Taken together, these results indicate that protein secretion is an engineerable phenotype in *E. coli*. Moreover, the gain-of-function afforded by the knockouts is transferable between different *E. coli* strains and can be combined to achieve even higher levels of secretion.

Mutants Have Stable Membranes and Secrete More YebF into Culture Medium. To gain potential insight into the mechanism by which the isolated gene deletions aid the accumulation of secreted YebF, we characterized the envelope stability of each mutant strain. This involved assaying for detergent sensitivity and leakage of cytoplasmic and periplasmic components into the supernatant. As reported previously,[21] Δ*ompR* cells exhibited leakage of cytoplasmic protein RNase I and periplasmic proteins maltose-binding protein (MBP) and DsbA. Additionally, this strain had increased detergent sensitivity (Supporting Information Table S1). Cells lacking *envZ* gave a similar phenotype to Δ*ompR* cells in these analyses. Thus, the increased fluorescence associated with the Δ*envZ* and Δ*ompR* strains may be due in part to increased membrane instability, resulting in passive diffusion of cellular proteins into the growth medium. The remaining six mutants showed no signs of membrane instability or permeability.

Aside from leakiness in the case of Δ*envZ* and Δ*ompR*, the reason for enhanced YebF secretion in the absence of these other genes remains undetermined. In some cases, the identified genes serve to maintain the integrity of the bacterial envelope and to provide a favorable environment for proper membrane protein folding, possibly by inducing a fine-tuned stress response and/or by modifying the composition of the bacterial cell envelope. For instance, *mzrA* is a known modulator of the EnvZ/OmpR two-component signal-transduction system and reduces envelope stress, in part, through reduction of outer-membrane-protein (OMP) synthesis and upregulation of OMP degradation.[22] Hence, we speculate that overexpression of YebF may activate an MzrA-mediated homeostasis loop that reduces envelope stress by degrading YebF. In the absence of MzrA (or EnvZ/OmpR), this loop is interrupted, and higher levels of YebF accumulation can be reached. The *nlpD* gene is located in an operon with *rpoS* whose gene product, RpoS (σ^S or σ^{38}), controls a large array of genes that are expressed during stationary phase and under various stress conditions in *E. coli* and other bacteria. NlpD itself is an outer membrane lipoprotein that remodels the cell wall matrix by stimulating peptidoglycan hydrolysis.[23] It is possible, therefore, that the absence of NlpD results in an altered cell wall composition that favors YebF secretion. TnaA catalyzes the β-elimination of L-tryptophan to produce indole, and its absence results in decreased extracellular expression of proteins by the type III secretion system.[24] Our results similarly link *tnaA* deletion and altered indole signaling with the export of extracellular proteins. Finally, *entC* and *entE* are part of an operon that encodes enzymes necessary for biosynthesis of the siderophore enterobactin. It is possible that YebF is somehow associated with iron transport and that interruption of enterobactin synthesis relieves this association so that YebF can be hyper-secreted. However, an equally plausible explanation is that YebF secretion simply benefits from disabling another secretory pathway (e.g., enterochelin or indole secretion).

Enhanced Extracellular Expression of YebF-Cellulase Fusions. To investigate whether these mutations could also improve the secretion of different passenger proteins, we created fusions between YebF and four cellulolytic enzymes from *Cellvibrio japonicus*, a gram-negative bacterium known for plant cell wall degradation.[25] The selected target enzymes were Cel3A, Cel5B, Cel6A, and Cel9A, a predicted β-glucosidase, endoglucanase, exoglucanase, and endoglucanase, respectively. When these YebF fusions were expressed individually or coexpressed polycistronically in wt BW25113 cells, accumulation of each enzyme alone or all four enzymes together was observed in the extracellular medium (Figure S2a,b).

Next, we transformed each of the BW25113 mutant strains with plasmids containing individual *C. japonicus* cellulolytic enzymes each fused to YebF. Western blot analysis of the supernatant fractions revealed that each mutant strain was able to promote enhanced secretion of at least one YebF chimera compared to wt cells (Figure 3a). In particular, the Δ*nlpD* strain enhanced the secretion of all enzymes except for Cel9A, whereas the Δ*entE* and Δ*tnaA* strains enhanced the secretion of all enzymes. Greater accumulation of these enzymes was accompanied by increased endoglucanase activity in the culture medium, with Cel5B activity increasing by as much as 3-fold and Cel9A activity increasing greater than 2.5-fold in the supernatants of Δ*nlpD* and Δ*entE* cells, respectively, compared to wt cells (Figure 3b).

Given the ability of the Δ*nlpD*, Δ*entE*, and Δ*tnaA* strains to improve secretion of several cellulases, we also investigated whether these mutations similarly enhanced the concomitant cosecretion of these enzymes. Previous studies have demonstrated that cosecretion of multiple cellulolytic enzymes can support growth of *E. coli* cells on model cellulosic and hemicellulosic substrates.[12,13] Hence, we tested the extent to which coexpression of all four *C. japonicus* enzyme fusions bestowed cells with the ability to digest carboxymethyl cellulose (CMC), a soluble derivative of cellulose. Whereas control cells carrying an empty plasmid did not grow in liquid M9 minimal medium with CMC as the sole carbon source, wt cells cosecreting the four YebF-cellulase fusions were able to grow

dx.doi.org/10.1021/sb400142b | *ACS Synth. Biol.* 2014, 3, 74−82

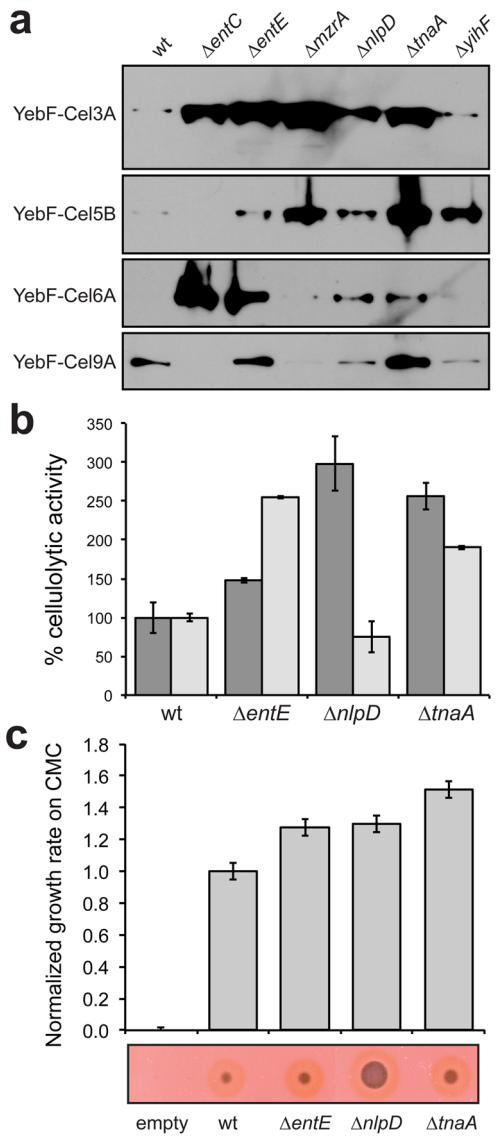


Figure 3. Enhanced extracellular expression of YebF and YebF-cellulase fusions. (a) Western blot analysis of cell-free supernatant fractions from wt BW25113 cells or mutant strains expressing YebF-Cel3A, YebF-Cel5B, YebF-Cel6A, and YebF-Cel9A. All blots were probed using anti-His antibodies. (b) Endoglucanase activity of cell-free supernatant fractions from wt BW25113 cells or mutant strains expressing YebF-Cel5B (dark gray) or YebF-Cel9A (light gray). Percent cellulolytic activity was determined by normalizing the endoglucanase activity of each sample by the activity measured in the cell-free supernatant from wt BW25113 cells, where the activity for Cel5B and Cel9A was 23.0 and 24.9 mU/mL, respectively. Data is expressed as the mean ± SEM of biological triplicates. (c) Growth of wt BW25113 cells or mutant strains coexpressing all four YebF-cellulase fusion proteins in liquid M9 minimal medium or on solid M9 agar, each supplemented with 1% CMC. Relative growth rate in liquid culture was determined by dividing the specific growth rate of each sample by the specific growth rate of wt BW25113 cells (μ = 0.024 h^{-1}). Growth rate data is expressed as the mean ± SEM of biological triplicates. M9 agar plates were flooded with Congo red to visualize zones of CMC clearing surrounding the colonies.

under these conditions (Figure 3c). When the four YebF-cellulase fusions were coexpressed in the Δ*nlpD*, Δ*entE*, and Δ*tnaA* strains, digestion of CMC was more efficient, as evidenced by a 25−50% increase in the specific growth rate of the mutants compared to wt cells (Figure 3c). In addition, a visible enhancement of growth and CMC hydrolysis was observed, as indicated by zones of clearing surrounding colonies when CMC-containing agar plates were flooded with Congo red (Figure 3c). Importantly, when grown on glucose as the sole carbon source, all strains grew comparably to each other as well as to wt cells cosecreting the YebF fusions, indicating that neither the mutations nor the cosecretion of four enzymes had any notable impact on cell growth.

Universal Reporter of Diverse Secretory Mechanisms. Given that our genetic assay required only minor modification of the target substrate with a short epitope tag for specific interaction with the FlAsH reagent, we hypothesized that the approach could be readily adapted to other secretory mechanisms. To test this notion, we first introduced C-terminal FTs to other *E. coli* proteins, namely, OmpA, OmpF, and OsmY, that were previously observed to accumulate in the culture medium in a specific manner without affecting outer-cell-membrane integrity, albeit by unknown mechanisms.[5,7,26] FlAsH labeling resulted in measurable fluorescence for OsmY-FT that was approximately 2-fold lower than the signal from YebF-FT (Figure 4a). No signal was observed for OmpA-FT and OmpF-FT under the conditions tested here. Western blot analysis corroborated the FlAsH labeling, revealing that YebF-FT was secreted at significantly higher levels than OsmY-FT and also that OmpA-FT and OmpF-FT were not detectable in the supernatant (Figure 4b). We suspect that the lack of secretion for OmpA-FT and OmpF-FT is because they remained associated with the outer membrane under the conditions tested.

Next, we examined if the FlAsH-tetracysteine strategy could be extended to the endogenous type II secretion (T2S) system of *E. coli*. Transcription of the two operons in the chromosomal *gsp* locus, encoding the majority of the *E. coli* T2S components, is silenced by the nucleoid-structuring protein H-NS.[27] However, mutants lacking H-NS and carrying the *gsp* genes cloned on a moderate-copy-number plasmid can efficiently secrete the endogenous endochitinase, ChiA. Here, we cotransformed H-NS-deficient *E. coli* cells with a plasmid harboring the complete *gsp* locus and a second plasmid encoding the target substrate ChiA with a C-terminal FT (ChiA-FT). These cells secreted appreciable amounts of ChiA-FT, as evidenced by a strong fluorescence signal above background (Figure 4c). As expected, there was no measurable fluorescence when ChiA-FT was replaced with AvrPto-FT, an FT-tagged substrate of the *Pseudomonas syringae* type III secretion (T3S) system (Figure 4c). A similar lack of fluorescence was observed following expression of an FT-tagged version of pectate lyate (PelB-FT), a T2S substrate from *Dickeya dadantii* (formerly *Erwinia chrsyanthemi*). This latter result is consistent with the stringent species specificity among gram-negative T2S systems[28] and demonstrates the ability of the assay to provide mechanistic insight into biologically relevant phenomena.

Besides endogenous secretion mechanisms, *E. coli* can be recombinantly endowed with secretion systems from other gram-negative bacteria. For example, the T2S system encoded by the *out* gene cluster of *D. dadantii* can be functionally transferred to *E. coli*,[29] enabling these cells to selectively secrete heterologous proteins, among them, PelB. When *E. coli* cells coexpressed the *out* genes along with PelB-FT, a strong fluorescent signal was observed that was significantly brighter than cells expressing PelB-FT in the absence of the *out* genes or

dx.doi.org/10.1021/sb400142b | *ACS Synth. Biol.* 2014, 3, 74−82

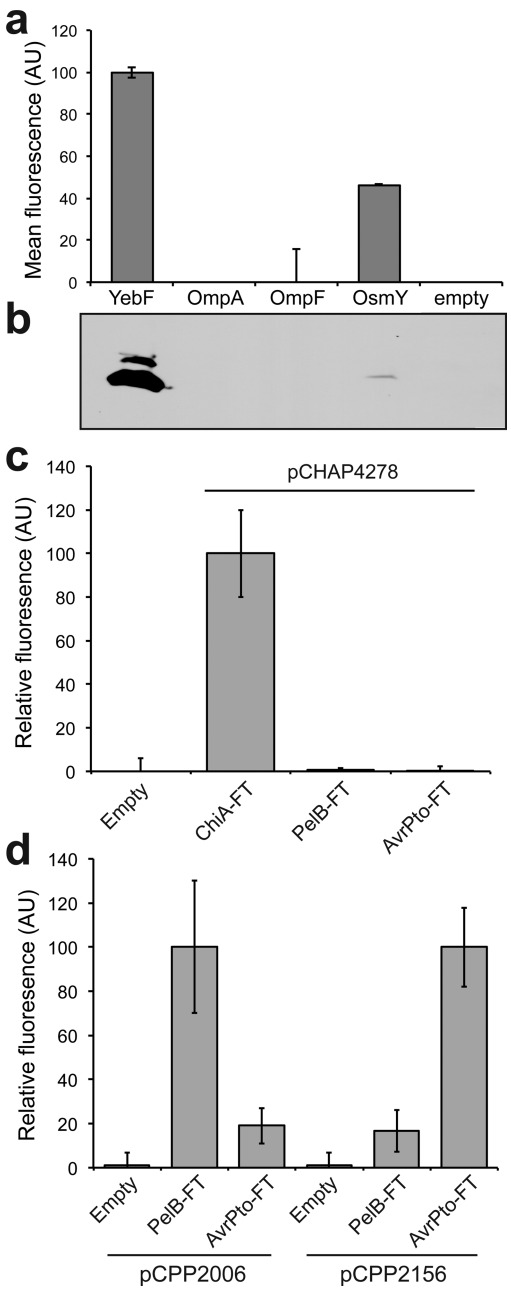


Figure 4. FlAsH-tetracysteine assay is a universal reporter of protein secretion. (a) FlAsH labeling of wt BW25113 cells expressing YebF-FT, OmpA-FT, OmpF-FT, and OsmY-FT. Because OmpA and OmpF are also known to be associated with the outer membrane, FlAsH analysis was performed in the cell-free supernatant fraction to avoid labeling proteins in the outer membrane. Data was normalized to the signal from wt BW25113 cells expressing YebF-FT and is expressed as the mean ± SEM of biological triplicates. (b) Western blot analysis of cell-free supernatant fractions from same cells as in panel a. Blots were probed with anti-His antibodies. (c) FlAsH labeling of MC4100 Δ*hns* cells expressing ChiA-FT, AvrPto-FT, or PelB-FT in the presence of plasmid pCHAP4278, which encodes the *E. coli gsp* gene cluster. Data was normalized to the signal from cells expressing ChiA-FT and is expressed as the mean ± SEM of biological triplicates. (d) FlAsH labeling of MC4100 wt cells expressing AvrPto-FT or PelB-FT in the presence of plasmid pCPP2006, which encodes the *D. dadantii out* gene cluster, or plasmid pCPP2156, which encodes the *D. dadantii* Hrp system. Data was normalized to the signal from cells expressing PelB-FT (for pCPP2006 cells) or AvrPto-FT (for pCPP2156 cells) and is expressed as the mean ± SEM of biological triplicates.

the T3S substrate AvrPto-FT in the presence of the *out* genes (Figure 4d). Along similar lines, the T3S system encoded by the *hrp* gene cluster from *D. dadantii* enables *E. coli* to secrete T3S substrates, including the *P. syringae* Avr proteins, into the culture supernatant.[30] Following coexpression of the *hrp* genes and AvrPto-FT, labeled *E. coli* cells were significantly more fluorescent than AvrPto-FT-expressing cells lacking the *hrp* genes or PelB-FT-expressing cells harboring the *hrp* genes (Figure 4d). Collectively, these data indicate that the FlAsH-tetracysteine strategy is a versatile genetic reporter that is compliant with diverse secretory mechanisms and faithfully reports known biological specificity.

This study and others have demonstrated that structurally diverse recombinant proteins synthesized in *E. coli* can be targeted to the extracellular environment when fused C-terminally to the YebF protein. In fact, we show that the YebF domain can be used to cosecrete at least four recombinant enzymes from a single host cell. However, although YebF and other comparable carrier proteins (e.g., OsmY) can deliver biologically active proteins into the culture medium, the amounts of secreted proteins that accumulate in the medium using these systems are often too low to be practically useful.[4,7,11] This problem is exacerbated by a lack of generalizable screens and selections for genetic dissection or laboratory evolution of secretory pathways. As a result, optimization efforts have been limited to tedious and low-throughput experiments (i.e., one-gene-at-a-time approaches such as the deletion of *lpp* or overexpression of *dsbA*) that often lead to little or no improvement in target protein secretion.[13]

To address this shortcoming, we developed a versatile assay for protein secretion based on the covalent labeling of secreted proteins with FlAsH-EDT_2. The FlAsH system was first described over a decade ago as a tool for labeling intracellular proteins in eukaryotic cells.[16] Since that time, few studies have successfully demonstrated labeling of intracellular proteins in bacteria, most likely because of outer-membrane impermeability to the FlAsH-EDT_2 compound.[15] Although this may be a limitation for certain applications, we exploited this impermeability to rapidly label secreted proteins in the extracellular environment without the need to first isolate the cell-free supernatant. This feature is particularly useful for automated screening of large genetic libraries. For example, using our assay, we isolated gain-of-function mutants from a transposon library and identified the genetic lesions that were responsible for the enhanced secretory phenotype. Specifically, we identified six mutations that led to marked increases in the secretion of YebF as well as YebF fusions without loss of membrane integrity. Although the specific role of these mutations in the YebF pathway, if any, remains to be determined, these alleles may eventually provide mechanistic clues about the YebF mechanism and/or insight for the further optimization of protein production using YebF fusions.

A drawback of our system is that the diffusion of fluorescently labeled secretory substrates into the extracellular milieu precludes the use of higher-throughput, single-cell techniques such as flow cytometry; hence, only a limited number of clones ($<10^5$) can be screened with our assay in its current format (i.e., 384-well plates screened using robotic automation). One possible approach for increasing the throughput would be to encapsulate bacterial cell libraries in water−oil−water double emulsions,[31] allowing the entrapment of otherwise diffusible fluorescent biomolecules such as FlAsH-labeled secretory proteins within the microcompartment. The

dx.doi.org/10.1021/sb400142b | *ACS Synth. Biol.* 2014, 3, 74−82

emulsions could then be analyzed directly by flow cytometry, enabling larger libraries (>10^6 members) to be screened.

Besides its ability to assist in the construction of genetically engineered *E. coli* strains for efficient extracellular protein production, our FlAsH-tetracysteine assay should also help open the door to useful and unanticipated mechanistic insights on protein secretion. For example, an interesting observation from our studies is that YebF targeted to the Tat pathway was not transported across the outer membrane but accumulated in the periplasm. The YebF protein is likely in a folded state when it reaches the periplasm because the intrinsic quality control mechanism of Tat transport dictates that substrate proteins are fully folded in the cytoplasmic compartment prior to export.[32] Thus, the retention of ssTorA-YebF-FT in the periplasm suggests that the folded conformation is not compatible with outer-membrane translocation and provides experimental support for the current model whereby YebF is threaded through an OmpF/C outer-membrane channel in a linear, unfolded conformation.[6] Beyond YebF, the universality of our genetic assay should permit genetic dissection of numerous diverse secretory pathways including, but not limited to, the pathways tested here (e.g., YebF, OsmY, T2S, and T3S). We anticipate that application of this screening tool will improve our understanding of these important biological processes and will also enable optimization of these systems, leading to the design of superior expression systems capable of secreting recombinant proteins to the culture medium at high titers.

METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* strain BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) and single-gene knockout mutants of BW25113 from the Keio collection[20] were used for high-throughput screening and related secretion experiments. Double- and triple-gene knockout mutants of BW25113 were made by P1 transduction using the corresponding BW25113 single-gene knockout mutants as donor and plasmid pCP20[20] to remove the Kan marker as needed. For comparison, BL21(DE3) and isogenic mutants were also used. Mutants of BL21(DE3) were made by P1 transduction using the corresponding BW25113 single-gene knockout mutants as donor. *E. coli* strains MC4100 and MC4100 Δhns[27] were used for the T3S and T2S experiments, respectively. All strains were routinely grown aerobically at 37 °C in Luria−Bertani (LB) medium, and antibiotics were supplemented at the following concentrations: ampicillin (Amp, 100 μg/mL), kanamycin (Kan, 50 μg/mL), and chloramphenicol (Cm, 20 μg/mL). Protein synthesis was induced when the cells reached an absorbance at 600 nm (A_{600}) of ∼0.5 by adding 0.2% arabinose and/or 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the media. *E. coli* strains were grown on M9 minimal medium supplemented with $MgSO_4$ (1 mM) and $CaCl_2$ (0.1 mM). Glucose (0.2% w/v) and CMC (1% w/v) were used as the sole carbon sources for cells grown in minimal medium. Congo red staining was performed as described previously.[33]

Plasmid Construction. All plasmids used in this study are listed in Table S2. Plasmid pTrc-YebF was created by insertion of DNA encoding *yebF* between the *Sac*I and *Xba*I sites of pTrc99A. *Cellvibrio japonicus* genes *cel3A*, *cel5B*, *cel6A*, and *cel9A* were codon-optimized and cloned between the *Xba*I and *Hin*dIII sites of pTrc-YebF to create pTrc-YebF-Cel3A, pTrc-YebF-Cel5B, pTrc-YebF-Cel6A, and pTrc-YebF-Cel9A, respectively. A C-terminal 6×-His tag was included in each plasmid. To create plasmid pYebF-Cel4, DNA encoding each of the YebF-cellulase fusions was PCR-amplified with C-terminal 6×-His, HA, FLAG, and c-Myc epitope tags, respectively, and cloned into an expression plasmid as a polycistron. To create pTrc-YebF-FT and pTrc-ΔspYebF-FT, DNA encoding YebF and YebF lacking its 21-residue signal peptide was PCR-amplified using a reverse primer containing the FT and 6×-His sequence and inserted into the *Sac*I and *Xba*I sites of pTrc99A. Plasmid pTrc-spTorA-YebF-FT was created by inserting spTorA between the *Nco*I and *Sac*I sites of pTrc-ΔspYebF-FT. Plasmids pTrc-AvrPto-FT, pTrc-ChiA-FT, and pTrc-PelB-FT were constructed in a similar manner as pTrc-YebF-FT. Plasmids expressing *entC*, *entE*, *envZ*, *mzrA*, *nlpD*, *ompR*, *tnaA*, and *yihH* were generated by inserting the genes in the *Sac*I and *Xba*I sites following a Shine−Delgarno sequence in pBAD33. Plasmid pET-21a-YebF was constructed by PCR-amplifying YebF and cloning the resulting PCR product between the *Sac*I and *Hin*dIII sites of pET-21a, which includes a 6×-His tag but no FlAsH sequence. All primers were synthesized by Integrated DNA Technologies, and each plasmid was verified by DNA sequencing.

Protein Analysis. Cells expressing recombinant proteins were harvested after overnight induction at 30 °C. Cell-free supernatant fractions were prepared by centrifugation at 5000*g* for 10 min, and the proteins were precipitated overnight with 1% TCA at 4 °C. After centrifugation at 13 000 rpm for 30 min, the pellet was washed once with ice-cold 100% acetone and dissolved in 1 M Tris-HCl, pH 8.0, and an equal volume of electrophoresis loading buffer. The periplasm was separated from the cytoplasm using the osmotic shock method.[32] All proteins samples were loaded in equal volumes for separation on an SDS-PAGE gel and then transferred to a nitrocellulose membrane for western blot analysis. The following primary antibodies were used: mouse anti-6×-His (1:5000; Abcam), mouse anti-FLAG (1:1000; Stratagene), rabbit anti-c-Myc (1:1000; Sigma), rabbit anti-HA (1:1000; Sigma), rabbit anti-DsbA (1:20 000; gifted); and mouse anti-MBP (1:2000, New England Biolabs).

Protein Purification. Following protein expression for 18 h at 30 °C, the supernatant fraction was collected by centrifugation at 5000*g* for 10 min followed by filtration through a 0.45 μm membrane. Protein purification was performed using an ÄKTApurifier system (GE Healthcare). Proteins were purified by IMAC using HisTrap-HP columns (GE Healthcare) followed by desalting the elution fraction into PBS using a PD 26/10 desalting column (GE Healthcare). Total protein amount was quantified using a BCA kit (Sigma) with a BSA standard curve.

FlAsH Labeling of Extracellular Proteins. Labeling of secreted proteins with FlAsH was performed following overnight induction with 0.1 mM IPTG at 30 °C. The following day, 1 μM FlAsH-EDT_2 and 1 mM DTT were added to each well in a 96-well plate (opaque sides, transparent bottom). Following incubation in the dark for 1 h at 37 °C, fluorescence was measured by 485 nm excitation and 528 nm emission, and each value was normalized by A_{600}. Data are expressed as the mean ± SEM of biological triplicates.

Transposon Insertion Library Generation. A genome-wide transposon Tn5 insertion library was generated in strain EC100 using the EZ-Tn5 <R6Kγori/KAN-2> Tnp transposome kit according to the manufacturer's instructions (Epicenter, Madison, WI). Following electroporation of the

dx.doi.org/10.1021/sb400142b | ACS Synth. Biol. 2014, 3, 74−82

transposome into the recipient strain, the outgrowth was plated on LB agar medium supplemented with 50 μg/mL of Kan. After overnight growth at 37 °C, the plates were flooded with LB medium, and any visible colonies were scraped off with a cell spreader. The initial Tn5 library was estimated to contain ~10^7 members. The cell suspension was supplemented with glycerol to a final concentration of 30% glycerol and stored at −80 °C. The Tn5 library frozen stock provided the donor strains for a P1 phage library of mutants. Overnight cultures inoculated with the Tn5 library frozen stock were grown in 5 mL of LB medium supplemented with 50 μg/mL of Kan. The overnight cultures were subcultured 1:25 in 50 mL of LB medium supplemented with 5 mM $CaCl_2$, 0.2% glucose, and 50 μg/mL of Kan. Following 1 h of incubation at 37 °C with shaking, 0.625 mL of P1 phage stock was added to the flask. The growth of the culture was monitored by A_{600}. After several hours, a precipitous drop in A_{600} was typically observed. The cultures were harvested 1 h after the drop in A_{600}. Twenty-five milliliters of culture was added to two 50 mL conical centrifuge tubes along with 0.635 mL of chloroform. Each tube was vortexed briefly and centrifuged at 10 000*g* for 5 min. The resulting P1 lysates from the supernatant were stored at 4 °C and used to produce strain libraries. Overnight culture (30 mL) of *E. coli* BW25113 Δ*dsbA* harboring pTrc-YebF-FT was centrifuged at 6000*g* for 5 min. The pellet was resuspended in 30 mL of LB medium supplemented with 100 mM $MgSO_4$ and 5 mM $CaCl_2$ and then incubated at 30 °C with 3 mL of P1 phage stock. After 30 min, 6 mL of 1 M sodium citrate was added to stop the reaction. The cells were again pelleted at 6000*g* for 5 min and resuspended in 10 mL of LB supplemented with 100 mM sodium citrate. The transduced cells were plated onto Omniwell plates (Nunc) containing LB agar medium supplemented with 50 μg/mL of Kan and 100 μg/mL of Amp. The resulting library was estimated to contain ~10^6 members.

Screening of Transposon Libraries. Individual clones (~10^4) were robotically picked into wells of 96-well bar-coded microtiter plates containing 100 μL of LB medium supplemented with 50 μg/mL of Kan and 100 μg/mL of Amp. The picked clones were incubated with shaking overnight at 30 °C and high humidity. The plates containing overnight cultures were loaded onto a Tecan Freedom EVO 200 liquid-handling robot, where all remaining processing was performed. The overnight cultures were subcultured (1:10) into clear-bottomed black microtiter plates with 180 μL of LB medium in each well supplemented with 0.5 mM IPTG, 50 μg/mL of Kan, and 100 μg/mL of Amp. The remaining overnight culture was supplemented with glycerol to a final concentration of 30% glycerol and stored at −80 °C. After 24 h incubation with shaking at 30 °C and high humidity, the subcultured plates were assayed for YebF secretion. Each culture was assayed for cell growth by measuring A_{600}, then 20 μL of concentrated assay cocktail containing 10 μM FlAsH-EDT_2 (Invitrogen) and 500 μM 2,3-dimercaptopropanol was added to each well. The plates were incubated with shaking for an additional 1 h before reading the culture fluorescence at 485 nm/528 nm.

Characterization of Outer Membrane Permeability. Assays for detergent sensitivity and RNase I, MBP, and DsbA leakage were performed essentially as described before.[21]

CMC Assay for Endoglucanase Activity. Cells were induced for protein expression overnight at 30 °C, and then 25 μL of cell-free supernatant was added to 25 μL of 2% CMC in 50 mM sodium acetate buffer (pH 5.5). Following incubation at 37 °C for 4 h or overnight, 100 μL of DNS solution[34] was added, and samples were boiled at 95 °C for 5 min. Then, 36 μL of the completed DNS reaction was added to 160 μL of water, and absorbance at 540 nm was measured. All samples were assayed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

Expression and purification of YebF, extracellular secretion of cellulases fused to YebF, characterization of outer membrane integrity and permeability, and plasmids used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: 607-254-8560; Fax: 607-255-9166; E-mail: md255@cornell.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Funding was provided by the DOE Great Lakes Bioenergy Research Center (GLBRC) project 3.2.8 and USDA NIFA award no. 2009-02202 (both to M.P.D.) as well as NSF GK12 award no. DGE-1045513 "Grass Roots: Advancing education in renewable energy and cleaner fuels through collaborative graduate fellow/teacher/grade-school student interactions" (to J.T.B.). We are grateful to Doug Weibel for the gift of FlAsH-EDT_2 and to Alan Collmer and Anthony Hay for generously supplying the T2S and T3S plasmids used in this study.

■ REFERENCES

(1) Georgiou, G., and Segatori, L. (2005) Preparative expression of secreted proteins in bacteria: Status report and future prospects. *Curr. Opin. Biotechnol. 16*, 538−545.

(2) Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol. 10*, 411−421.

(3) Pugsley, A. P., and Francetic, O. (1998) Protein secretion in *Escherichia coli* K-12: Dead or alive? *Cell. Mol. Life Sci. 54*, 347−352.

(4) Zhang, G., Brokx, S., and Weiner, J. H. (2006) Extracellular accumulation of recombinant proteins fused to the carrier protein YebF in *Escherichia coli*. *Nat. Biotechnol. 24*, 100−104.

(5) Qian, Z. G., Xia, X. X., Choi, J. H., and Lee, S. Y. (2008) Proteome-based identification of fusion partner for high-level extracellular production of recombinant proteins in *Escherichia coli*. *Biotechnol. Bioeng. 101*, 587−601.

(6) Prehna, G., Zhang, G., Gong, X., Duszyk, M., Okon, M., McIntosh, L. P., Weiner, J. H., and Strynadka, N. C. (2012) A protein export pathway involving *Escherichia coli* porins. *Structure 20*, 1154−1166.

(7) Kotzsch, A., Vernet, E., Hammarstrom, M., Berthelsen, J., Weigelt, J., Graslund, S., and Sundstrom, M. (2011) A secretory system for bacterial production of high-profile protein targets. *Protein Sci. 20*, 597−609.

(8) Chen, N., Hong, F. L., Wang, H. H., Yuan, Q. H., Ma, W. Y., Gao, X. N., Shi, R., Zhang, R. J., Sun, C. S., and Wang, S. B. (2012) Modified recombinant proteins can be exported via the Sec pathway in *Escherichia coli*. *PLoS One 7*, e42519-1−e42519-8.

(9) Fisher, A. C., Haitjema, C. H., Guarino, C., Celik, E., Endicott, C. E., Reading, C. A., Merritt, J. H., Ptak, A. C., Zhang, S., and DeLisa, M. P. (2011) Production of secretory and extracellular N-linked glycoproteins in *Escherichia coli*. *Appl. Environ. Microbiol. 77*, 871−881.

(10) Seo, E. J., Weibel, S., Wehkamp, J., and Oelschlaeger, T. A. (2012) Construction of recombinant *E. coli* Nissle 1917 (EcN) strains

for the expression and secretion of defensins. *Int. J. Med. Microbiol. 302*, 276−287.
(11) Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., and Keasling, J. D. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature 463*, 559−562.
(12) Bokinsky, G., Peralta-Yahya, P. P., George, A., Holmes, B. M., Steen, E. J., Dietrich, J., Soon Lee, T., Tullman-Ercek, D., Voigt, C. A., Simmons, B. A., and Keasling, J. D. (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A. 108*, 19949−19954.
(13) Zheng, Z., Chen, T., Zhao, M., Wang, Z., and Zhao, X. (2012) Engineering *Escherichia coli* for succinate production from hemicellulose via consolidated bioprocessing. *Microb. Cell Fact. 11*, 37.
(14) Link, A. J., Jeong, K. J., and Georgiou, G. (2007) Beyond toothpicks: New methods for isolating mutant bacteria. *Nat. Rev. Microbiol. 5*, 680−688.
(15) Ignatova, Z., and Gierasch, L. M. (2004) Monitoring protein stability and aggregation in vivo by real-time fluorescent labeling. *Proc. Natl. Acad. Sci. U.S.A. 101*, 523−528.
(16) Griffin, B. A., Adams, S. R., and Tsien, R. Y. (1998) Specific covalent labeling of recombinant protein molecules inside live cells. *Science 281*, 269−272.
(17) Martin, B. R., Giepmans, B. N., Adams, S. R., and Tsien, R. Y. (2005) Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nat. Biotechnol. 23*, 1308−1314.
(18) Donath, M. J., 2nd, Dominguez, M. A., and Withers, S. T., 3rd. (2011) Development of an automated platform for high-throughput P1-phage transduction of *Escherichia coli*. *J. Lab. Autom. 16*, 141−147.
(19) Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J., and Nadon, R. (2006) Statistical practice in high-throughput screening data analysis. *Nat. Biotechnol. 24*, 167−175.
(20) Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol. 2*, 2006.0008-1−2006.0008-11.
(21) McBroom, A. J., Johnson, A. P., Vemulapalli, S., and Kuehn, M. J. (2006) Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *J. Bacteriol. 188*, 5385−5392.
(22) Gerken, H., Charlson, E. S., Cicirelli, E. M., Kenney, L. J., and Misra, R. (2009) MzrA: A novel modulator of the EnvZ/OmpR two-component regulon. *Mol. Microbiol. 72*, 1408−1422.
(23) Peters, N. T., Morlot, C., Yang, D. C., Uehara, T., Vernet, T., and Bernhardt, T. G. (2013) Structure-function analysis of the LytM domain of EnvC, an activator of cell wall remodelling at the *Escherichia coli* division site. *Mol. Microbiol. 89*, 690−701.
(24) Hirakawa, H., Kodama, T., Takumi-Kobayashi, A., Honda, T., and Yamaguchi, A. (2009) Secreted indole serves as a signal for expression of type III secretion system translocators in enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology 155*, 541−550.
(25) DeBoy, R. T., Mongodin, E. F., Fouts, D. E., Tailford, L. E., Khouri, H., Emerson, J. B., Mohamoud, Y., Watkins, K., Henrissat, B., Gilbert, H. J., and Nelson, K. E. (2008) Insights into plant cell wall degradation from the genome sequence of the soil bacterium *Cellvibrio japonicus*. *J. Bacteriol. 190*, 5455−5463.
(26) Xia, X. X., Han, M. J., Lee, S. Y., and Yoo, J. S. (2008) Comparison of the extracellular proteomes of *Escherichia coli* B and K-12 strains during high cell density cultivation. *Proteomics 8*, 2089−2103.
(27) Francetic, O., Belin, D., Badaut, C., and Pugsley, A. P. (2000) Expression of the endogenous type II secretion pathway in *Escherichia coli* leads to Chitinase secretion. *EMBO J. 19*, 6697−6703.
(28) Lindeberg, M., Salmond, G. P., and Collmer, A. (1996) Complementation of deletion mutations in a cloned functional cluster of *Erwinia chrysanthemi* out genes with Erwinia carotovora out homologues reveals OutC and OutD as candidate gatekeepers of species-specific secretion of proteins via the type II pathway. *Mol. Microbiol. 20*, 175−190.
(29) He, S. Y., Lindeberg, M., Chatterjee, A. K., and Collmer, A. (1991) Cloned *Erwinia chrysanthemi* out genes enable *Escherichia coli* to selectively secrete a diverse family of heterologous proteins to its milieu. *Proc. Natl. Acad. Sci. U.S.A. 88*, 1079−1083.
(30) Ham, J. H., Bauer, D. W., Fouts, D. E., and Collmer, A. (1998) A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. *Proc. Natl. Acad. Sci. U.S.A. 95*, 10206−10211.
(31) Aharoni, A., Amitai, G., Bernath, K., Magdassi, S., and Tawfik, D. S. (2005) High-throughput screening of enzyme libraries: Thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. *Chem. Biol. 12*, 1281−1289.
(32) DeLisa, M. P., Tullman, D., and Georgiou, G. (2003) Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. *Proc. Natl. Acad. Sci. U.S.A. 100*, 6115−6120.
(33) Gardner, J. G., and Keating, D. H. (2010) Requirement of the type II secretion system for utilization of cellulosic substrates by Cellvibrio japonicus. *Appl. Environ. Microbiol. 76*, 5079−5087.
(34) Xiao, Z., Storms, R., and Tsang, A. (2005) Microplate-based carboxymethylcellulose assay for endoglucanase activity. *Anal. Biochem. 342*, 176−178.