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Adapting and Expanding Genetic Toolkits for Industrially and Environmentally Relevant Chassis Organisms: *Bacillus subtilis*

by Monica Chu, Sean Halper, Jimmy Gollihar, Randy Hughes, Jeanet Mante, and Randi M. Pullen

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Adapting and Expanding Genetic Toolkits for Industrially and Environmentally Relevant Chassis Organisms: *Bacillus subtilis*

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14. ABSTRACT The U.S. Army operates in diverse environments around the world. Sustaining operations requires fortifying our materials and equipping our Warfighters to persist in sometimes austere environments. Microbes live in every ecological niche on the planet and have evolved defenses and biochemical capabilities to survive and thrive. ARL is unlocking the potential of diverse and sometimes novel organisms as new biotechnology chassis organisms for engineering. The collection of genetically tractable microbes serves as a repository for biotechnology endeavors touching upon applications space such as sensing, biomanufacturing, sustainable operations, and assured provisioning. We present the design and validation of a genetic toolkit for <i>Bacillus subtilis</i> , a soil-dwelling, Gram-positive bacterium that is also an important microbial cell factory for biomanufacturing. This organism was selected for further toolkit development based on its genetic tractability, ability to form spores, complete genome annotation, and heavy interest in both environmental and industrial applications.					
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1. Introduction

Biotechnology for the U.S. Army involves careful design of biological systems for optimal material properties. The single-nucleotide resolution attainable through genetic engineering provides fine control over various material characteristics such as reactivity, tensile strength, elasticity, and conductivity, to name a few. In addition, the ability to manipulate material properties on such a high resolution unlocks an entire landscape of properties that was previously not accessible through traditional chemical synthesis. Building genetic toolkits for microbial species that have been selected for their capacity to produce materials of Army relevance requires validating the function of all parts that are domesticated into the standard framework, building a Department of War (DoW) repository of validated parts, and ensuring parts and their data are compliant with automation and database standards for future automation and ML projects.

Motivated by the urgent need for a standardized genetic toolkit for *Bacillus subtilis*, U.S. Army Combat Capabilities Development Command (DEVCOM) Army Research Laboratory (ARL) researchers performed literature and database searches, used predictive calculators, performed genome mining, and otherwise rationally designed genetic parts. All parts were designed to be compatible with a standard cloning framework.¹ Standardized frameworks are critical to supporting automation infrastructure, rapid prototyping, building repositories, and implementing ML algorithms.² Strong data repositories and advanced analytical tools support the DoW vision for responsible AI.³ Transitioning toward state-of-the-art and streamlined workflows in biotechnology eliminates data siloing, fosters greater collaboration, and shortens the time on each Design–Build–Test–Learn cycle (Figure 1). The capability to rapidly pivot to the Army’s emerging needs and learn from large, complex, data repositories will position the Army to have overmatch in the domain of biotechnology.

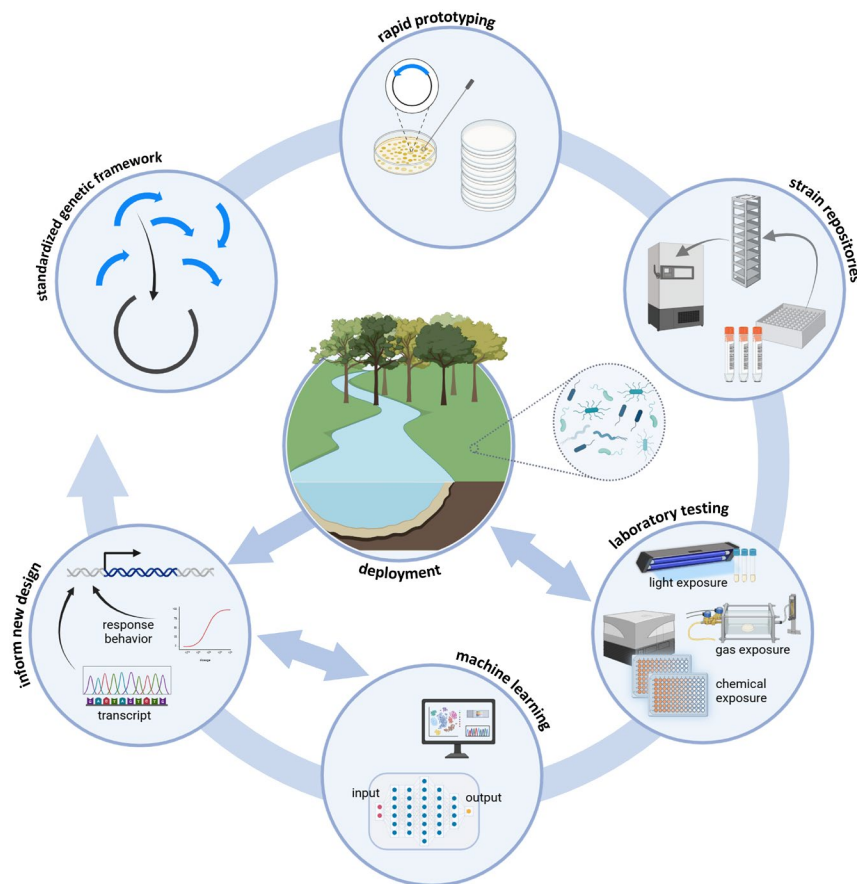


Figure 1. Design-Build-Test-Learn cycle integrated into the Army's framework for fielding biotechnology.

1.1 Engineering *B. subtilis*

B. subtilis is a Gram-positive soil bacterium known for its genetic tractability, well-described protein secretion pathways, and ability to form endospores to survive harsh environments.⁴ Thus, *B. subtilis* is a model organism for studies in molecular mechanisms of genetic recombination and widely used as an industrial chassis for large-scale heterologous production of enzymes. Its versatility has been demonstrated in applications in brewing biocontrol agents for agriculture, textile production, and medicine.^{5,6} Improvements in the fidelity and efficiency of DNA synthesis have enabled use of *B. subtilis* as a tool for directed evolution of enzymatic libraries.⁷ Its secretion system has been well characterized and is leveraged for biomanufacturing and biotechnology projects requiring the secretion of proteins.^{8–10} Moreover, recent efforts in *B. subtilis* genome reduction have paved the way for improved secretion and translation in industrial processes.^{11,12}

Historically, genetic transformation of *B. subtilis* has been demonstrated through means of electroporation, chemical competence, conjugation, and transfection (in

rare instances).¹³ In this report, we focus on specific genetic tools designed for transformation of *B. subtilis* via two methods: chemical competence and conjugation. Chemical competence is the most common mode of transformation for *B. subtilis*, often employing circularized or linearized DNA.¹⁴ Although *B. subtilis* is capable of both replicating and naturally integrating exogenous DNA, the primary genetic tools described in this work will be for targeted genomic editing of *B. subtilis* but designed with simple adaptability for engineering replicating plasmids if needed. The second method, conjugation, is a naturally encoded process that enables horizontal gene transfer of integrative and conjugative elements (ICEs) to recipient firmicutes that may not be amenable to chemical methods of transformation.¹⁵ This process has been well-studied in *B. subtilis* JH642. As such, previous efforts to engineer the ICE system in *B. subtilis* have also been described.¹⁶ All genetic elements presented in this report are reuseable in Golden Gate-compatible vectors to be used in ectopic expression or genomic integrations.

1.2 Golden Gate Framework

Since many legacy shuttle vectors used in *Bacillus* are derivatives of established commercial vectors used in *Escherichia coli* (*E. coli*), they often contain long stretches of extraneous DNA or unneeded cloning scars, which can have unpredictable and unintended consequences when expressed in *Bacillus*. We adapt an existing modular cloning framework to minimize extraneous sequences, standardize our shuttle vectors, eliminate potential leaky expression, and enable reusability of parts.

Modular cloning genetic toolkits such as BioBricks 1.0,¹⁷ BioBricks 2.0,¹⁸ BacilloFlex,¹⁹ ProUSER2.0,²⁰ and SubtiToolKit²¹ allow for the construction of multi-transcriptional unit (TU) plasmids for metabolic engineering in *B. subtilis* cells. By reducing the variability in genetic construct design and allowing high resolution control that allows precise genetic elements to vary, modular cloning toolkits permit the direct comparison of populations with differing genetic machinery. We chose to adapt the Yeast Toolkit (YTK)¹ modular cloning framework. This decision was based on the need to have a standard cloning framework that could apply to both the bacteria and eukaryotes in the ARL organism repository. The YTK provides a framework of *E. coli* shuttle vectors with elements that can be used in the target organism of interest and leverage Golden Gate-style cloning for assembly. Golden Gate cloning is a method that uses a defined set of Type II restriction enzymes and a fixed catalog of “zip codes” that define each type of part (Table 1). The “zip codes” are the overhangs or sticky ends generated from digestion by the respective restriction enzyme. Because each type of part is defined by its sticky ends, the ends therefore act as the instructions

controlling the arrangement of all the parts in the final assembly (FA). ARL has previously published reports that focused on building toolkits for other microorganisms using the same standard cloning framework.^{22–25}

Table 1. Types of parts built for the *Bacillus* toolkit.

Type	Name
Type 2	Promoter + ribosomal binding site
Type 2a	Promoter
Type 2b	Ribosomal binding site
Type 3	Open reading frame
Type 3a	Signal peptide
Type 3b	Open reading frame for signal peptide
Type 4	Transcriptional terminator
Type 4a	Signal peptide or epitope tag
Type 4b	Transcriptional terminator for tag or peptide
Type 6	Antibiotic resistance cassette
Type 7	5' homology arm
Type 8a	Replication origin
Type 8b	3' homology arm

1.3 Assays and Characterization

As part of our methodology for onboarding organisms into the ARL genetic engineering framework shown in Figure 2, we perform several characterization studies: 1) growth studies, 2) gene expression studies, and 3) assay development for phenotypic screens.

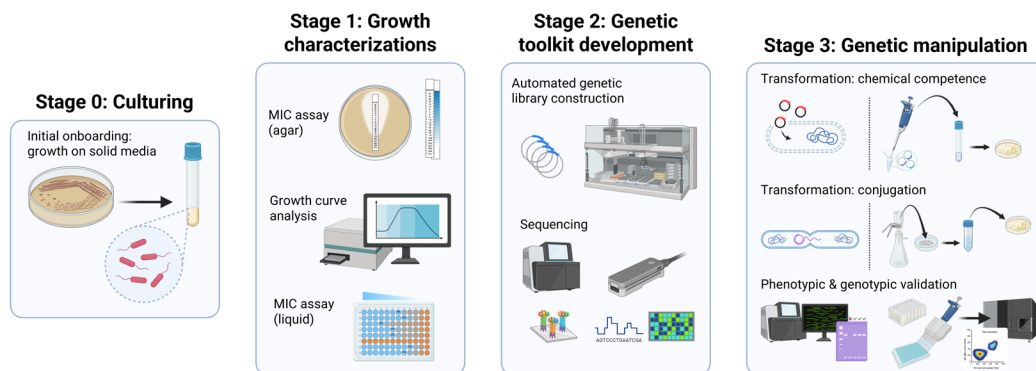


Figure 2. Summary of *B. subtilis* onboarding workflow.

The first characterization method we perform is a series of growth studies. Minimal inhibitory concentration (MIC) screenings are necessary to determine what the appropriate antibiotic concentration in each media should be when screening for transformants. This is done on both plate-based and liquid-based assays. All the shuttle vectors in this work contain antibiotic cassettes for both *E. coli* and *B. subtilis*. The antibiotic cassette in the shuttle vector that becomes integrated into the

recipient genome is encoded between the homology arms (this usually contains spectinomycin, tetracycline, erythromycin, or chloramphenicol resistance), whereas the antibiotic cassette (kanamycin) for selection in *E. coli* remains outside. The MIC studies are only conducted for the antibiotic resistance gene that becomes integrated in the genome. Additional growth studies can be carried out to determine the appropriate concentrations for antibiotic selection on transformants such that selective pressure can be retained for the recombinant plasmid in passaged cultures. In this report, we perform growth studies in shake flasks as well as on a Beckman Coulter BioLector microbioreactor. Microbioreactors approximate the conditions, and therefore growth is more reflective of that which would be experienced by the culture in a batch fermentation or scaled-up fermentation process.

The second characterization method that we perform is a series of studies on gene expression, evaluating a range of de novo-designed and putative promoter and terminator sequences. Most commonly, fluorophores are used as reporter genes in these plasmids to screen for the gene constructs that best promote target functions. As reporter proteins are produced and expressed, plate reader and flow cytometry techniques can be used to quantitatively assess enzyme production, in batch or in each cell, respectively. Fluorescence data from fluorescence-activated cell sorting (FACS) and absorbance data from the BioTek3 Plate Reader are part of our high-throughput workflow to validate gene expression.

There are numerous factors that can influence the transformation efficiency of bacteria including the structure of the recombinant DNA (linear/circular), the growth phase of the host, and the targeted chromosomal integration site for integrative plasmids.²⁶⁻²⁹ Other genetic elements from the toolkit that require specialized assays include genetic integration site validation, secretion tag screening, and spore display screening. The methods used for validating these types of parts are discussed in the Methods section.

Here, we describe a modular cloning framework designed to enable the facile interchangeability of genetic parts such as promoters, ribosome binding sites (RBSs), coding regions, guide RNA (gRNA) encoding regions, C-terminus and N-terminus tags, homology arms for integration, antibiotic resistance cassettes, and replicative origins for simple cloning into the *B. subtilis* 168 wild-type strain. This toolkit will enable rapid prototyping with a wide range of tunable expression control.

2. Methods, Assumptions, and Procedures

2.1 Golden Gate Design and Assembly

Genetic parts can be designed as gBlocks or eBlocks (Integrated DNA Technologies) to have BsmBI and BsaI sites corresponding to the YTK.¹ For this toolkit, the entry vector YTK001, Type 1 connectors, Type 5 connectors, and Type 8a origins of replication were taken directly from the YTK. Figure 3 describes the components that we adapted to generate the *Bacillus* toolkit in this work. For adaptation into *B. subtilis*, we generated organism-specific Type 3 coding sequences (CDSs), Type 4 terminators, Type 6 resistance markers, and Type 7/8b homology arms with corresponding YTK part types and overhangs. Promoter-5'-UTR pairs were designed as Type 2 parts and separately designed as Type 2a promoters or Type 2b 5'-UTRs. For facile design and synthesis, we used the templates described in Appendix Table A-1, where the desired DNA sequence is inserted between the flanking regions.

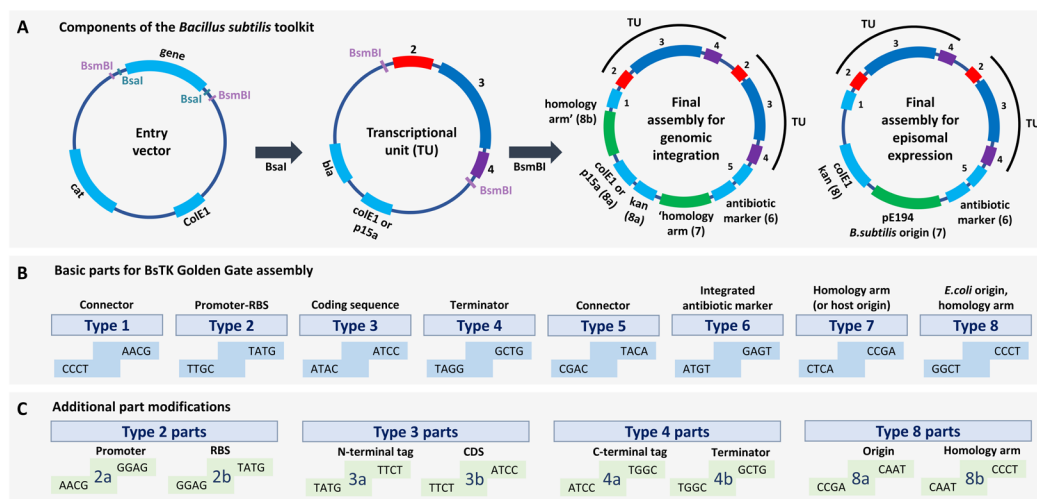


Figure 3. A) Hierarchical assembly of genetic parts in the *B. subtilis* toolkit follows a hierarchical workflow in which individual parts vectors (e.g., promoters, CDSs, and terminators) can be assembled into TUs. B) The toolkit is composed of eight major part types. C) Four of the eight major part types may optionally be split into subparts.

2.2 Primer Design for Type 1–8 Parts and Domestication into Entry Vectors

We provide methods for two possible case scenarios that will allow genetic parts to become domesticated into the toolkit.

- Case 1: DNA was ordered in the form of an IDT eBlock with overhangs already attached. The eBlock is directly domesticated into the entry vector backbone (YTK001) using the entry vector Golden Gate protocol described in Section 2.3.
- Case 2: Template DNA exists without Type IIS overhangs. Once the sequence of interest has been designed with the correct flanking regions, primers are designed to perform extension polymerase chain reaction (PCR) from the template DNA to obtain a double-stranded, linear DNA with the correct overhangs. The PCR product is then purified and domesticated into the entry vector backbone (YTK001) using the entry vector Golden Gate protocol described in Section 2.3.

Primers are designed with the following parameters: contain at least 18 base pairs (bp) of homology (annealing region to the template DNA); have a melting temperature of 60 °C for primers with higher guanine-cytosine (GC) content (>60%); and use GC enhancer with PCR reaction.

Once the Golden Gate entry vector has been transformed into *E. coli*, colony PCR for entry vector transformants can be performed with these primers: forward, 5'-[GGTATCTTTATAGTCCTGTCGG]-3'; reverse, 5'-[AGTACTGCGATGAGTGGCAG]-3'.

2.3 Golden Gate Cloning into *E. coli*: TUs and FAs

In this section, we describe the general cloning procedure for generating *B. subtilis* integrative and replicative vectors (Figures 4A–D). Gene inserts for entry vectors can be synthesized as an 1) IDT eblock or gblock or 2) PCR amplified from an existing plasmid (see Figure 4A). Domestication of genes into entry vector format using Golden Gate reactions is shown in Figure 4B. The presence of BsaI restriction sites on the entry vectors enables one-pot assemblies of *Bacillus* shuttle vectors without the need for intermediate TU vectors (see Figure 4C). The general workflow for the BsTK Golden Gate toolkit established in this work is shown in Figure 4D as follows:

- (I) PCR amplification of gene of interest, and domestication of genes into entry vector format;
- (II) Entry vectors can be assembled into TU vectors (III), or alternatively assembled into integration vectors (IV, FA3), or part-specific dropout (DO) vector (IV, FA2);
- (IV) Multiple TU vectors can be assembled into FAs (FA1) for multi-TU plasmids;

- (V) FAs can be sequenced using the universal ConS and ConE primers described in Section 3.

Due to the high concentration of DNA needed for *B. subtilis* transformation, direct transformation of Golden Gate reaction products (with or without purification) is not recommended. Therefore, Golden Gate reaction products are first transformed into *E. coli*, plasmid purified, and transformed into *B. subtilis* using the chemical competence protocol described in Section 3.

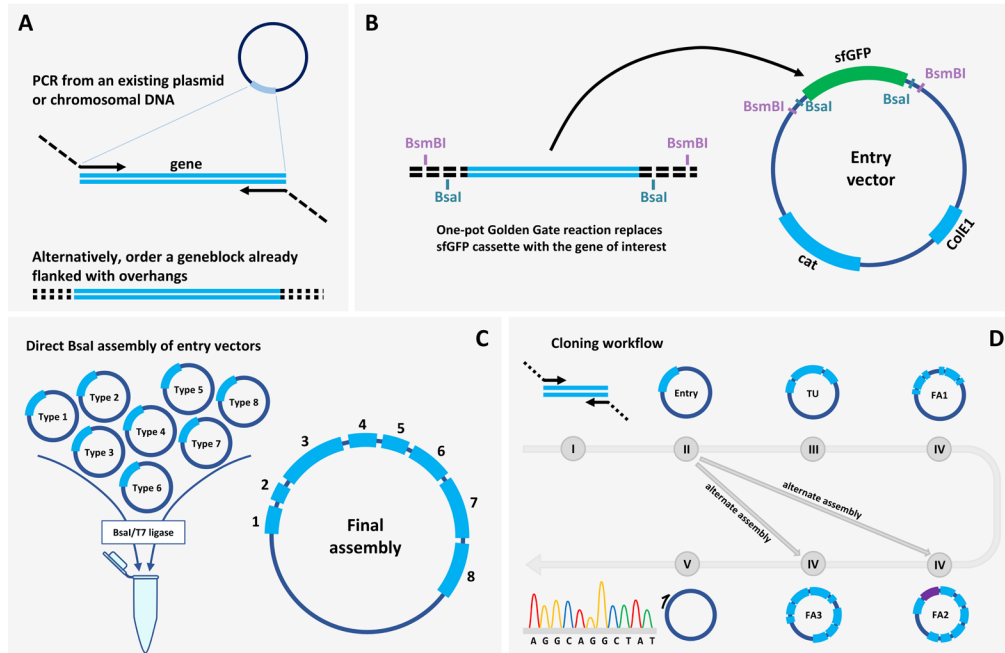


Figure 4. Workflow for plasmid construction starting from entry vector to FAs for genome integration. **A)** Genetic elements of interest are amplified from existing plasmids or ordered as gene blocks. **B)** The genetic element has complimentary overhangs to allow one step integration into an entry vector. **C)** Full plasmids are generated from parts 1–8 with complimentary overhangs from Type II restriction enzyme digest using Bsal and T7 DNA ligase. **D)** Multi-level assembly can be accomplished by various cloning schemes.

Golden Gate reactions are mixed in 10- μ L volumes using 1 μ L of 10-mM ATP, 0.5 μ L of T7 Ligase (NEB), 0.5 μ L of Type IIS restriction enzyme (Thermo Fisher Eco31I for BsaI digests and Thermo Fisher Esp3I for BsmBI digests), and 1 μ L of Type IIS restriction enzyme buffer (Thermo Fisher Buffer G for BsaI digests and Thermo Fisher Buffer Tango for BsmBI digests). Part plasmids were diluted to maintain a total DNA concentration of <200 fmols per Golden Gate reaction.

Genetic parts are first cloned into YTK001 as entry vectors using a BsmBI Golden Gate reaction. This ligation is directly transformed into *E. coli* 10 β cells, glycerol stocked, plasmid prepped, and sequenced.

The assembled entry vectors are combined in a BsaI Golden Gate reaction to produce intermediate TU assemblies or FAs (Table 2). Specifically, DO plasmids, which include the essential homology arms and resistance cassettes, are generated by predigesting YTK008, YTK047, YTK073, YTK090, the desired Type 6 resistance marker, Type 7 3' homology arm, and Type 8b 5' homology arm parts with Eco31I and subsequently ligating with T7 ligase.

Table 2. Golden Gate protocols: thermocycler conditions.

Entry vector			TU vector			FA vector		
Temp (deg)	Time (min)	Cycles	Temp (deg)	Time (min)	Cycles	Temp (deg)	Time (min)	Cycles
37	1	No.	37	1	30	37	5	30
16	1		16	1		16	5	
37	30		37	30		37	30	
85	15		16	30		85	15	

2.4 Designing sfGFP DO Plasmids

One of the advantages to engineering *B. subtilis* is its natural competency and amenability to genomic integrations. As part of this toolkit, we have designed several Type 7 and Type 8b parts (serving as homology arms) to enable facile Golden Gate assemblies of shuttle vectors for double homologous recombination in the *B. subtilis* genome. A list of the types of parts in the toolkit is given in Table 3. Some examples of homology arms include *amyE* locus, *bglS* locus, and *thrC* locus. Sequences for these parts are included in the Appendix.

Table 3. Building a sfGFP DO vector requires a seven-part assembly.

Type	Plasmid source	Description
Type 1	YTK008	ConS
Type 2-3-4	YTK047	sfGFP
Type 5	YTK073	ConE
Type 6	This study	Antibiotic resistance cassette (e.g., spectinomycin, erythromycin, chloramphenicol)
Type 7	This study	Downstream homology arm
Type 8a	YTK090	<i>E. coli</i> origin and kanamycin resistance cassette
Type 8b	This study	Upstream homology arm

2.5 Designing Part-Specific DOs for Libraries

To make this toolkit compatible with contemporary library cloning methods, we developed a generalized library cloning workflow (Figure 5), compatible with both traditional³⁰ and high-throughput cloning and characterization methods.^{31,32} First, the Type 2-3-4 YTK047 DO sequence is amplified with primers specific to the part

type of the target library (Figure 5A). The part library is amplified directly (more commonly done with large libraries/oligopools) or cloned into an entry vector (more commonly done with individual parts or a large library/oligopool that requires additional functional DNA³³) (Figure 5B). The amplified DO sequence is cloned into an FA vector with other required parts via a multipart Golden Gate assembly, producing a part-specific DO plasmid with conserved sequences outside of the excisable region (Figure 5C). The library is then cloned into the part-specific DO using a high-efficiency, two-part Golden Gate assembly (Figure 5D). The final plasmid library can then be characterized via high-throughput sequencing, FACS, or other appropriate means.

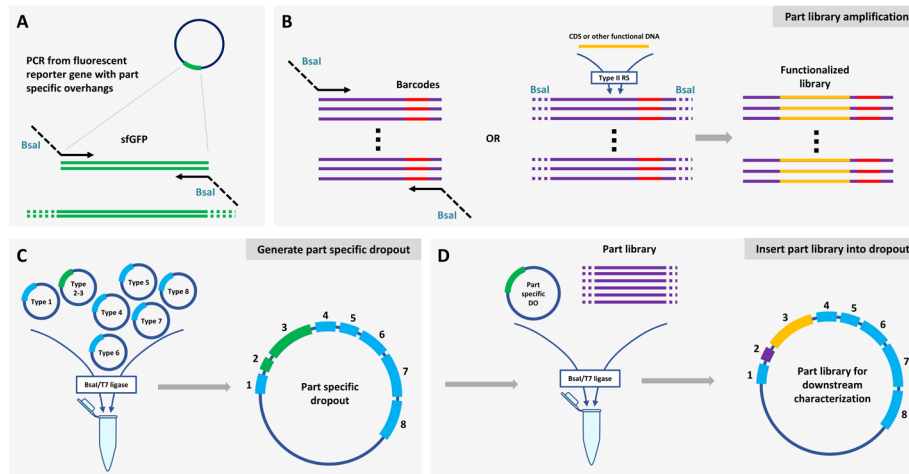


Figure 5. A generalized library cloning workflow using the Golden Gate toolkit: A) generating BsaI overhangs; B) cloning a library; C) a part-specific DO; and D) inserting a library into a DO.

In this section, we describe three specific applications of library cloning: 1) a novel RBS library specifically for *B. subtilis*; 2) the construction of a library of secretion tags, which can be used to test tagged enzyme combinations; and 3) the construction of a spore display system.

2.5.1 Application 1: Constructing an RBS Library for *B. subtilis*

Two libraries of Type 2b 5'UTRs were designed to include RiboJ as an insulating ribozyme upstream of the RBS. The first synthetic RBS library was designed using RBS Library Calculator 2.1³⁴⁻³⁷ to uniformly vary translation initiation rates between 39 and 567k when expressing the mKate2 fluorescent protein. The second RBS library was designed to maximize translation rate with design constraints consistent with an existing *Bacillus* toolbox.³⁸ These RBS sequences can be found in Appendix Table A-1.

To enable expedited cloning of these library sequences, we developed two Type 2b part-specific DOs for integrating into the *amyE* locus: one with the strong constitutive P_{veg} promoter³⁹ and one with the weak P_{liaG} promoter.⁴⁰ Individual RBSs were assembled into these DOs and subsequently transformed into *B. subtilis*.

2.5.2 Application 2: Constructing a Secretion Tag Library

We expanded the utility of our toolkit to build specific libraries for secretion in *B. subtilis* because this opens avenues of applications in cell-free sensing and biomolecule production. *B. subtilis* is known to effectively secrete peptides through the Tat pathway and Sec-SRP pathway.⁴¹ Intrinsic to the nature of Gram-positive bacteria, *B. subtilis* lacks an outer membrane, which streamlines the secretion process because it does not require an additional transport system to pass proteins through a second lipid bilayer. Owing to its simplicity and feasibility, *B. subtilis* has been industrially used in the secretion of enzymes such as amylases⁴² and xylanases.⁴³

2.5.1.1 Overview of *B. subtilis* Secretion Mechanisms

In general, the well-characterized Sec-dependent secretion pathway^{44,45} in *B. subtilis* involves several major processes: 1) the transport of unfolded protein to the translocation channel SecYEG, which is facilitated by signal recognition particle (SRP) and its receptor (FtsY); 2) translocation of the unfolded protein with signal peptide (SP) powered by translocase motor, SecA, which is a peripheral ATPase that binds to both the SRP complex and the SecYEG/SecDF-YajC complex; and 3) signal cleaving of the protein facilitated by SipS/SipW along with SP peptidases (e.g., SppA and TepA) that degrade remnants of the SPs post-cleavage.^{46,47} These SP peptidases typically contain a Ser/Lys catalytic dyad that enables cleavage.⁴⁸

The Tat-dependent secretion pathway is different from the Sec-dependent pathway because it can transport folded proteins across its membrane. Thus, utilization of this pathway is a favorable alternative to proteins that are found to be incompatible with the Sec pathway. The Tat pathway is specific to SPs that contain a twin-arginine motif.⁴⁹ In previous studies, researchers have identified proteins such as PhoD⁵⁰ and YwbN⁴⁹ to be exported through the Tat pathway. The proteins, TatAdCd and TatAyCy, function as translocases in this pathway.⁴⁵

2.5.1.2 Engineering High-Throughput Secretion Tag Screening for This Toolkit

We utilized our toolkit to generate a library of secretion tags as Type 3a parts. These genetic parts were generated using a PCR extension protocol and domesticated into entry vectors as described by Townsel et al.⁵¹ As a hypothetical example, we

designed two Type 3a DOs (Figure 6A). The 3a *amyE* DO is designed to screen the efficacy of SPs fused to the amylase enzyme and is phenotypically evaluated using clearance zones on starch plates. Similarly, the 3a *bglS* DO is designed to screen the efficacy of β -glucanase secretion. β -glucanase activity can be phenotypically evaluated using clearance zones on lichenin plates. The phenotypic assays for amylase and β -glucanase are further described in Figure 6B and Section 3.3.

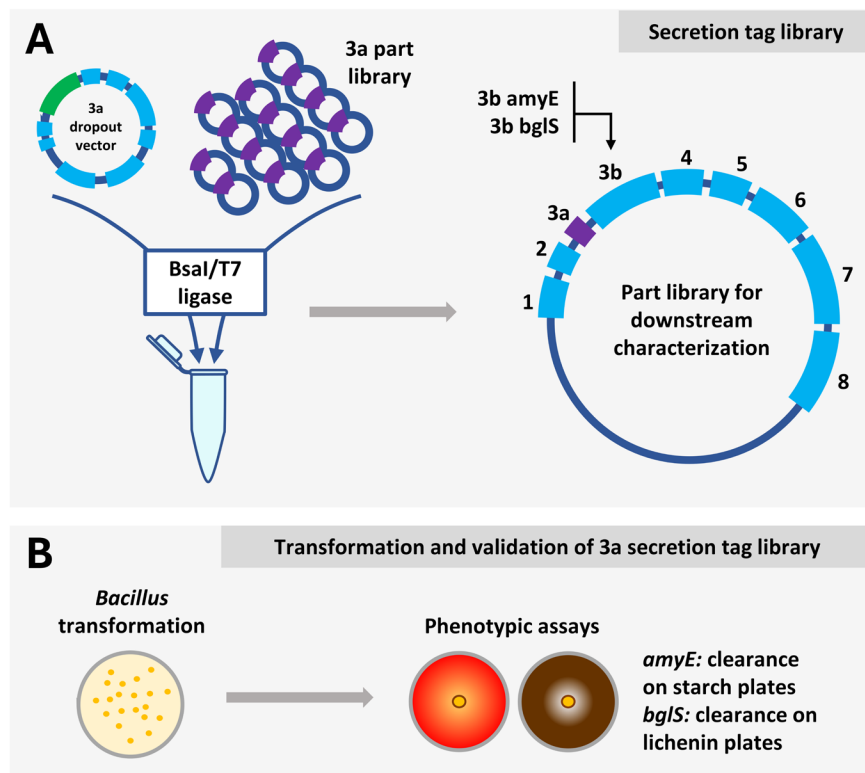


Figure 6. Workflow for secretion tag library generation using the Golden Gate-compatible DO vectors: A) secretion tag library and B) phenotypic assays validating genomic integration.

2.5.3 Application 3: Constructing a Spore Display System

Spore surface display is a unique capability that has been leveraged in *B. subtilis* as a modality of rapid sensing without the need for live, metabolically active cells. In addition, spores are resistant to many abiotic environmental stresses such as heat, drought, chemicals, and radiation.⁵² *Bacillus* spores have been utilized for heterologous expression of a variety of peptides and catalytic enzymes. As early as 2001, Isticato et al.⁵³ demonstrated spore surface display of the tetanus toxin by fusing *cotB* (spore coat protein) to the C-terminal fragment of the tetanus toxin. Since then, many advances in *Bacillus* spore display have been made, such as the surface display of nitrilase,⁵⁴ lipase,^{55,56} esterase,⁵⁷ laccase⁵⁸ and decarboxylase.⁵⁹

Bartels et al.⁶⁰ previously demonstrated a spore display system with a suite of 12 vectors, designed using plasmid backbones from the *Bacillus* BioBrick Box and the

six crust proteins located on the endospore: CotV, CotW, CotX, CotY, CotZ, and CgeA. By fusing the various crust proteins to a reporter, they were able to functionalize endospores and perform rapid evaluations of surface-displayed proteins.

In this work, we designed a library of spore display components as both Type 3a parts (N-terminal fusion) and Type 3b parts (C-terminal fusion) (shown in Figure 7A). Similar to the secretion tag library described in the previous section, we constructed two DO vectors to enable rapid Golden Gate cloning of the Type 3a and 3b libraries containing spore display tags.

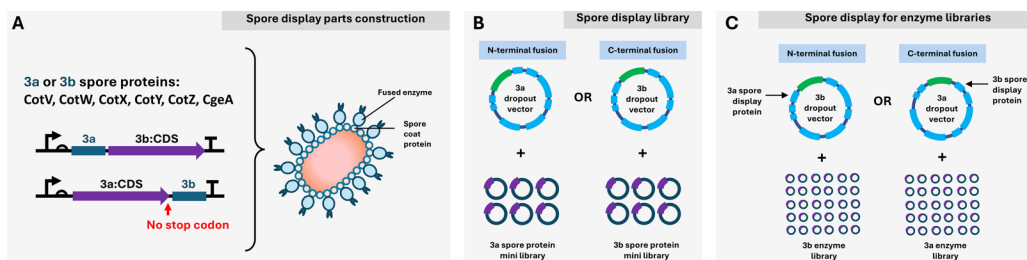


Figure 7. Cloning framework of spore coat protein fusions: A) spore display parts construction; B) spore display library; and C) spore display for enzyme libraries.

The assembly method demonstrated in Figure 7B describes 12 possible options for spore coat protein fusions to a single enzyme (or any protein sequence of desire). This mode of assembly should be applied for evaluating the efficacy of fusing different coat proteins to the same enzyme. For more optimized models, where the spore coat protein that is most optimal for the fusion has already been selected, the DO vector should be assembled as described in Figure 7C, where the fusion protein is encoded in backbone and the “dropout” component of the backbone is a Type 3a or Type 3b sfGFP part that is exchanged for a Type 3a/3b enzyme sequence.

3. Experimental Protocol

3.1 Strains

B. subtilis 168 wild-type was used to test all constructs described in this work. This organism lends itself well to genetic modification due to its natural competence. Chemically competent *B. subtilis* cells can be made using MNGE media as described in the media formulations in Section 3.2. All intermediate cloning was performed in *E. coli* 10 β cells from New England Biolabs. Finished plasmids were transformed into *E. coli* MC1061, a strain routinely used for propagation and maintenance of shuttle vectors, used here to increase integration efficiency of plasmids in *B. subtilis*.

3.2 Media Formulations

The media formulations are comprised as follows:

- MNGE media contains 59 mM $\text{K}_2\text{HPO}_4 \times 3 \text{ H}_2\text{O}$, 44 mM KH_2PO_4 , 3.4 mM sodium citrate dihydrate, 90 mM glucose, 10 mM monosodium glutamate, 42 μM ferric ammonium citrate, 245 μM L-tryptophan, and 30 mM $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$;
- M9 media contains 47.7 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 8.5 mM NaCl, 18.7 mM NH_4Cl , 100 μM $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$, and 2 mM $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$;
- AS media contains 7.56 mM $(\text{NH}_4)_2\text{SO}_4$, 11.76 mM NaNO_3 , 7.04 mM K_2HPO_4 , 13.4 mM KCl, and 10 mg/L yeast extract.

3.3 Transformation of Integrative Plasmids into *B. subtilis* 168

Foundational approaches to transformation, which date as far back as the 1950s, increase the permeability of cellular membranes for the introduction of exogenous DNA. *B. subtilis*-competent cells are typically prepared chemically, using glucose minimal mediums and a mixture of amino acids.^{61,62} Otherwise, competency can be induced by electroporation, wherein an electric field is pulsed in a controlled environment.^{63–65} When cells recover in the appropriate media and are plated, a transformation efficiency of approximately 10^3 – 10^4 and approximately 10^6 are generally observed for chemical competence and electroporation protocols, respectively.⁷ However, both methods of transformation are often applied to a select few plasmid conditions, involve large culture volumes, and are time consuming. Here, we optimized the transformation of *B. subtilis* cells and developed a protocol that maintains at least a 2-week shelf-life of competent cells (Figure 8). The transformation efficiencies of chemical competent cells were ultimately compared with that of electro-competent cells. Approaches to making freezer stocks follow the standard chemical competence protocol but involve the replacement of MNGE media with a glycerol medium and using liquid nitrogen to snap-freeze aliquots. We explored beyond standard media components and conditions to circumvent the anticipated twofold decrease in transformation efficiency after a few weeks to 1 month of storage.

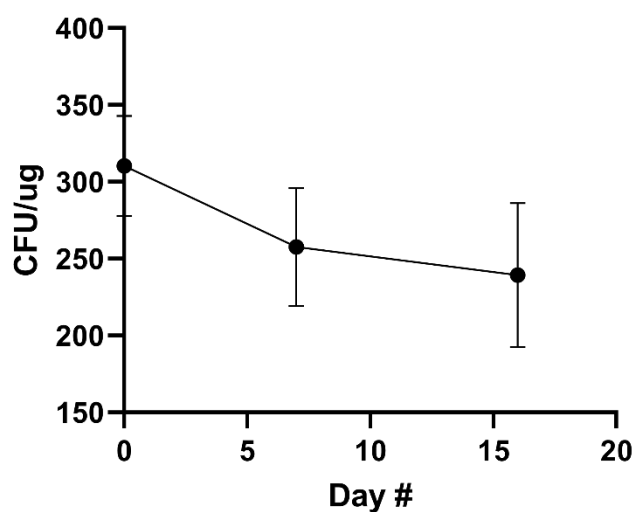


Figure 8. Evaluation of competent cell longevity in -80°C . Transformation efficiency (colony-forming unit [CFU]/ μg) was evaluated on day 0, 7, and 16 of freezer storage in -80°C .

3.4 Phenotypic Screening and Colony PCR

For insertion mutants at the *amyE* locus (interruption in the amylase gene), starch–iodine phenotypic screens could be performed to ascertain the loss of amylase function. In this assay, antibiotic-selected colonies were restructured onto agar plates with 1% soluble starch and incubated overnight at approximately 16 h. The following day, starch plates with grown colonies were flooded with iodine and subsequently assessed for zones of clearance surrounding the colonies, which would indicate amylase activity. If there was no zone of clearance, then this would indicate successful interruption in the amylase gene (Figure 9, left panel).

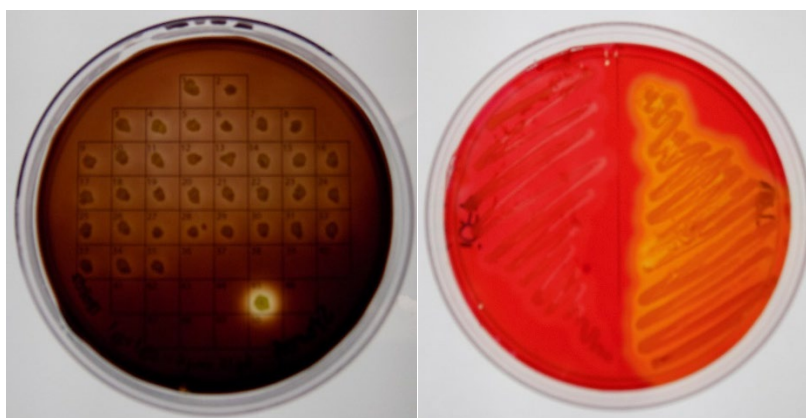


Figure 9. Starch plate assays for *amyE* and *bgIS* integration.

For insertion mutants at the *bgIS* locus (interruption in the β -glucanase gene), lichenin–congo red phenotypic screens can be performed to ascertain the loss of

glucanase function. In this assay, individual colonies are spotted onto Luria–Bertani (LB) agar plates containing the appropriate amount of antibiotic $\pm$ 0.05% w/v lichenin (0.125 g in 250 mL) then allowed to grow overnight at 37 °C. The next day the plates can be used to make glycerol stocks and tested for the presence or absence of lichenin digestion. The lichenin-containing plate is flooded with 5 mL of 0.1 % Congo Red dye dissolved in ddH₂O and left at room temperature for 30 min. The excess volume should be removed and the plate washed three times using 5 mL of 1 M NaCl for 10–15 min each. A clear halo around the streaks demonstrates the activity of the β -glucanase encoded by the *bglS* gene. The lack of a halo demonstrates that the *bglS* gene has been disrupted by integration of the gene constructs (Figure 9, right panel). Similarly, other genomic integration sites can be screened for the appropriate phenotype, such as threonine auxotrophy generated by disruption of the *thrC* locus.

3.5 Flow Cytometry and Analysis

Flow cytometry was run on a Sony SA3800 Spectral Analyzer. Compensation and gating were performed using the Sony SA3800 Software, with wild-type *B. subtilis* 168 as a negative control and *B. subtilis* amyE-Pveg-spovG-mKate2 as a positive control for establishing compensation spectral profiles. Ten thousand events with compatible forward scatter (FSC) and side scatter (SSC) were collected for each strain. The geometric mean of these events was calculated using Flowcal.⁶⁶

B. subtilis transformants were grown in LB media with the necessary antibiotic to an OD₆₀₀ > 1.0 before performing a dilution in MNGE minimal medium to final OD₆₀₀ < 0.1. The MNGE medium was supplemented with 500 μ g/mL glutamate, 40 μ g/mL uracil, and 50 μ g/mL threonine. After cells reached OD₆₀₀ approximately 1.0, aliquots of 5- μ L culture were diluted into 500 μ L of water for flow cytometry analysis. For early log-phase measurements, cell samples were taken at an OD₆₀₀ approximately 0.4. For late log-phase measurements, cell samples were taken at an OD₆₀₀ approximately 1.5. Flow cytometry was performed on the Sony SA3800 Spectral Analyzer with the following parameters: threshold FSC value 0.5%, FSC gain; 17 SSC voltage; 20% fluorescence PMT voltage; 69.8% acquisition of 10,000 events.

4. Results and Discussion

4.1 Strain-Specific Growth-Curve Characterizations in Minimal Media

We characterized *B. subtilis* growth in three types of minimal media: M9, AS, and SD media supplemented with either glucose or glycerol as the sole carbon source. *B. subtilis* shows optimal growth on M9 as a media background with glucose as a carbon source, exhibiting doubling times of approximately 22, 24, and 26 min on M9, SD, and AM media, respectively (Figure 10A). With glycerol as the sole carbon source, *B. subtilis* exhibited doubling times of approximately 27, 29, and 32 min on M9, SD, and AM media, respectively (Figure 10B).

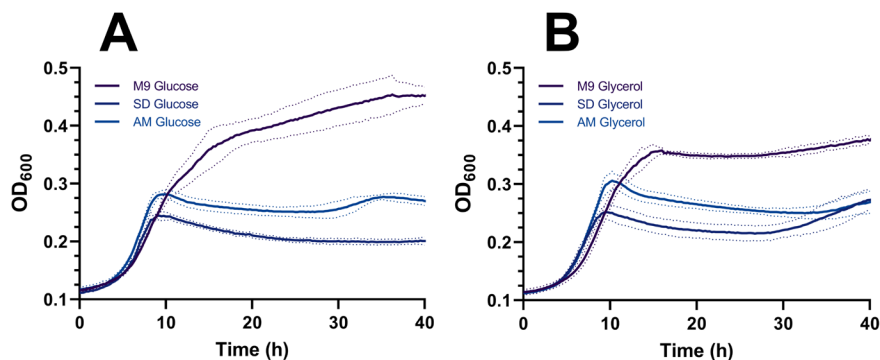


Figure 10. Growth curves for *B. subtilis* in minimal media (M9, SD, AM) as evaluated in kinetic mode on the BioTek plate reader. A) Growth curves in minimal media with glucose as a carbon source. B) Growth curves in minimal media with glycerol as a carbon source.

4.2 Simple Case Study: Applying a Random Forest Model to Optimize Media Components

To further characterize *B. subtilis* growth behavior in M9 media background, we titrated out each of the components of the media (glucose, CaCl₂, MgCl₂, NaCl, and NH₄Cl) and evaluated the growth in a microtiter plate. We modeled each of the growth curves using a modified Verhulst model with a Heaviside function, such that late-phase declines could also be accounted for (Figure 11). After fitting each growth curve to the equation, we extracted the coefficients to each of the equation parameters and used them as output features for the random forest model. The input features for the model were defined as the concentrations of each of the substrates present in the media. As expected, the model assigned the highest feature importance to glucose (Figure 12). Interestingly, the second highest feature importance was CaCl₂. As evident from the growth curves, increasing CaCl₂ in the media appeared to decrease the maximum carrying capacity of the culture.

Similarly, we utilized this same modeling framework to evaluate the influence of different carbon and amino acid supplements to the media for *B. subtilis* engineered for melanin synthesis, which is further outlined in Section 4.4.3.

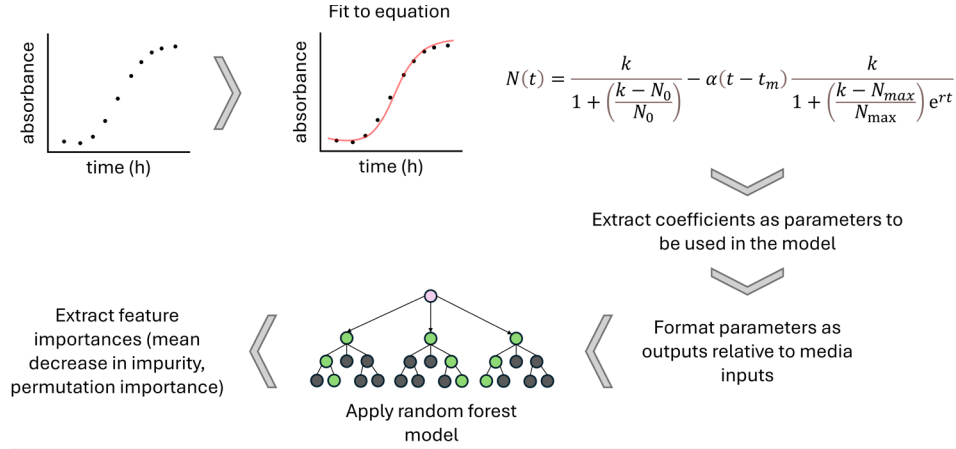


Figure 11. Workflow for preprocessing, fitting data, and preparing data for application in the random forest model.

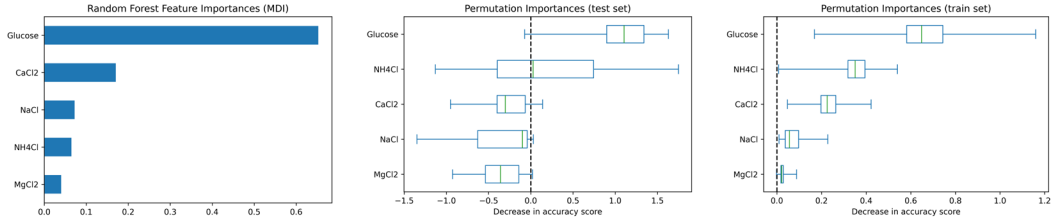


Figure 12. Random forest metrics for feature importance of each media component: glucose, CaCl₂, NaCl, NH₄Cl, and MgCl₂.

4.3 Determining MICs for Antibiotic Selection

We evaluated *B. subtilis* sensitivity to various antibiotics to determine MICs to use for selection. We tested ampicillin, chloramphenicol, kanamycin, spectinomycin, gentamicin, and tetracycline in agar-based medium and planktonic cultures. In agar-based assays, *B. subtilis* showed the least sensitivity to spectinomycin (~24 µg/mL) and the greatest sensitivity to gentamicin and erythromycin with MICs <1 µg/mL (Figure 13A). In planktonic cultures, *B. subtilis* demonstrated similar, but decreased sensitivity to all antibiotics tested (Figure 13B). Interestingly, we observed a marked decrease in sensitivity to erythromycin when testing cells in planktonic cultures. The MIC for erythromycin on plates was >0.25 µg/mL, whereas in planktonic cultures the MIC was >12.5 µg/mL.

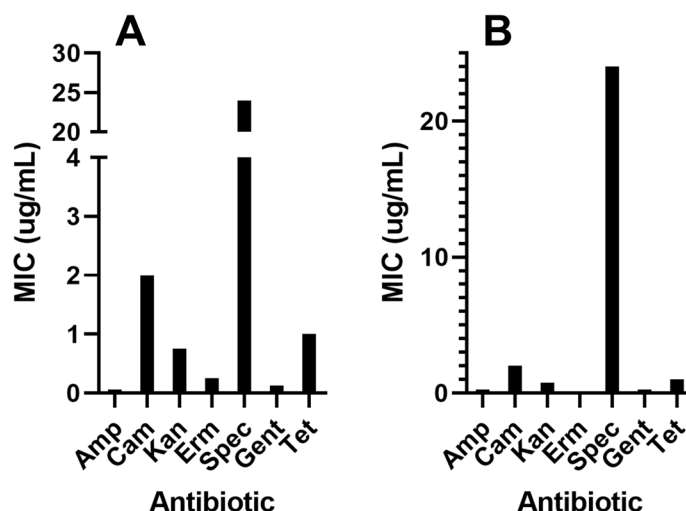


Figure 13. MICs for various antibiotics: A) plate-based assay; B) liquid assay (planktonic cultures).

4.4 Various Demonstrations of *Bacillus* Genetic Toolkit Utilization

4.4.1 Evaluating Recombination Efficiencies and Fluorescence Expression Levels at Various *B. subtilis* Integration Sites

Gene loci such as the *amyE*, *bglS*, *pyrD*, *sacA*, and *thrC* genes have been identified in literature to be feasible sites for genomic integration.^{67–69} Of these sites, the *amyE* locus and *bglS* locus can be easily screened with phenotypic selection, as discussed in Section 3. The *amyE* locus encodes an amylase gene, enabling phenotypic selection on starch plates stained with iodine. The *bglS* locus encodes a β -glucanase gene, enabling phenotypic selection on lichenin-containing plates stained with Congo red. Regarding the other loci, *pyrD* locus encodes a dihydroorotate dehydrogenase critical to pyrimidine metabolism, thus introducing a uracil auxotrophy. The *sacA* locus encodes a sucrose-6-phosphate hydrolase, inhibiting the bacillus transformant from metabolizing complex sugars. The *thrC* locus encodes a threonine synthase, which generates a threonine auxotrophy.

Utilizing the Golden Gate framework, we generated Type 7 and 8b homology arms for the construction of DO vectors as described in Section 2.4. The sequences are available upon request. To evaluate gene expression at all five of the chromosomal locations denoted above, we integrated a constitutively expressed mKate2 at each of the integration sites (Figure 14B, genome ring). The transformants are depicted in Figure 14A, and their respective transformation efficiencies are shown in Figure 14C. We observed similar transformation efficiencies across all integration sites (~1000 CFU/mL) except for *sacA* transformants that suffered from growth

deficiencies, which could be due to the interruption of a gene essential for processing complex sugars.

After evaluating the transformation efficiencies at each gene locus, we cultured the transformants and evaluated expression in a time course study via plate reader fluorescence measurements (Figure 15) and flow cytometry (Figure 14D). The flow cytometry revealed the highest expression to result from integrations at the *amyE* and *bglS* while observing diminished levels of expression when using integration sites such as the *pyrD* and *thrC* integration sites. This is not surprising given that genes closest to the origin of replication are expected to have higher expression (see Figure 11B). From the plate reader assays, we can also observe that differential expression at each gene locus is particularly evident at stationary phase when cultured in a rich media background.

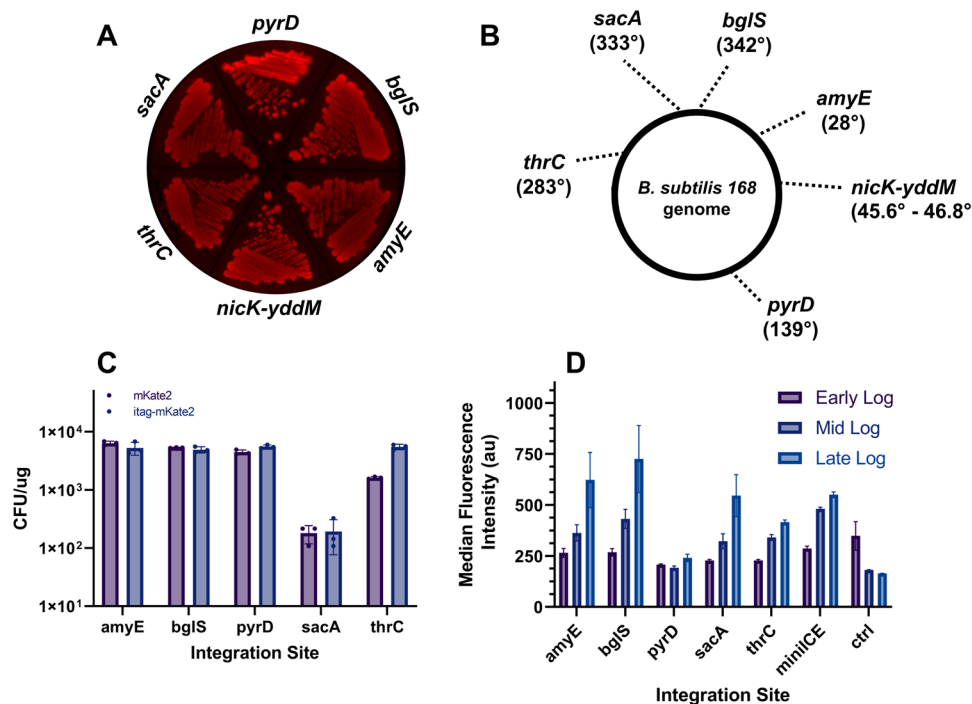


Figure 14. (A–D) Evaluation of fluorescence expression at various integration sites.

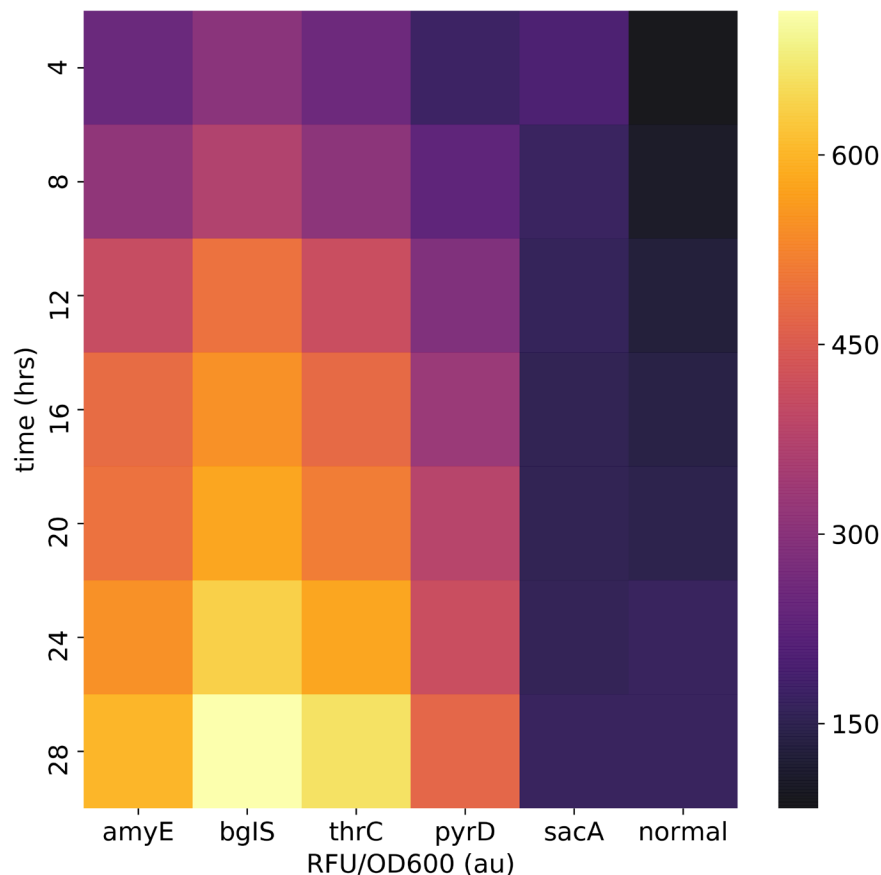


Figure 15. Plate reader fluorescence measurements in kinetic mode for constitutive promoters.

4.4.2 Tuning Expression Levels with an RBS Library

This *B. subtilis* toolkit was also used to implement and evaluate novel RBS libraries. Here, we tested three different sets of RBS pairings, coupling known promoter regions to existing RBS sequences, and RBS sequences designed using the RBS calculator.⁷⁰ The first set (Figure 16A) shows fluorescence intensities of the promoter-RBS pairings for PliaG-spoVG and PliaI-spoVG evaluated at different integration sites. Across all integration sites, both PliaG-spoVG and PliaI-spoVG had minimal expression over the wild-type control except for PliaG integrated at the *pyrD* site, which demonstrated significant fluorescence expression greater than the wild-type background fluorescence.

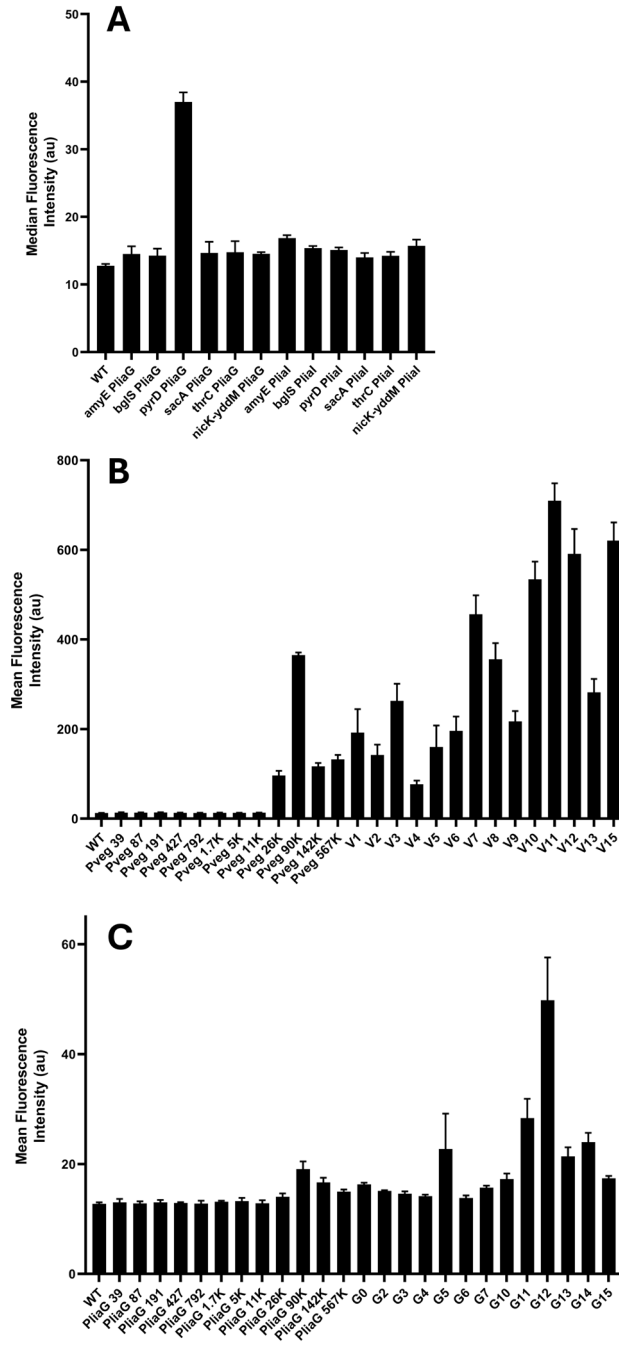


Figure 16. (A–C) Flow cytometry measurements for RBS library expressing mKate2 in stationary phase.

The second set of promoter-RBS combinations we tested (Figure 16B) shows fluorescence intensities of the Pveg promoter (a strong constitutive promoter in *B. subtilis*) paired with novelly engineered RBS sequences (39, 87, 191, 427, 792, 1.7K, 5K, 11K, 26K, 90K, 142K, and 567K) and previously defined RBS sequences (V1, V2, V3, V4, V5, V6, V7, V8, V9, V10, V11, V12, V13, and V15). The highest expressing combination was V11, with approximately 55-fold change in

fluorescence expression compared with the wild type. Interestingly, of the engineered RBS sequences, the 90K RBS exhibited significantly higher expression compared with its counterparts with higher predicted outputs, 142K and 567K.

The third set of promoter-RBS combinations (Figure 16C) echoes the same RBS library used in Figure 16B, yet it uses a weaker constitutive promoter, *PliaG*. From the flow cytometry tests, we observed significantly lower expression levels compared with the *Pveg* group. This was expected because *PliaG* is a weaker promoter than *Pveg*. The same RBS sequences, such as RBS11 and RBS12, that achieved higher fluorescence expression in the *Pveg* group, also generally appeared to have higher fluorescence expression when coupled to *PliaG*. The G11 and G12 showed approximately 2.2-fold and approximately 3.9-fold fluorescence expression higher than the wild-type control.

4.4.3 Use of *B. subtilis* Genetic Toolkit Parts for Manipulation of Melanin Synthesis

In demonstrating the utility of this framework of standardized tools, we cloned several variants of melanin synthesizing *B. subtilis* strains. In accordance with the workflow shown in Figure 2, we first cloned a tyrosinase gene derived from *Rhizobium etli*, denoted as *mela*, into an entry vector. This entry vector was then subsequently cloned into a TU vector and moved into two of the integration vectors: *amyE* spec and *bglS* spec, encoding *mela* expression through an interruption of amylase or β -glucanase activity. We also encoded the *mela* gene in the miniICE (yddM-nicK) backbone, which was subsequently integrated into a xylose-inducible conjugation strain JAB932.¹⁶ We then used this *mela*-encoded conjugation strain to introduce *mela* into undomesticated *Bacilli*. For the images shown in Figure 17, we show that all the genetically engineered *mela Bacillus* strains were able to synthesize melanin in minimal MNGE media supplemented with varying concentrations of tyrosine.

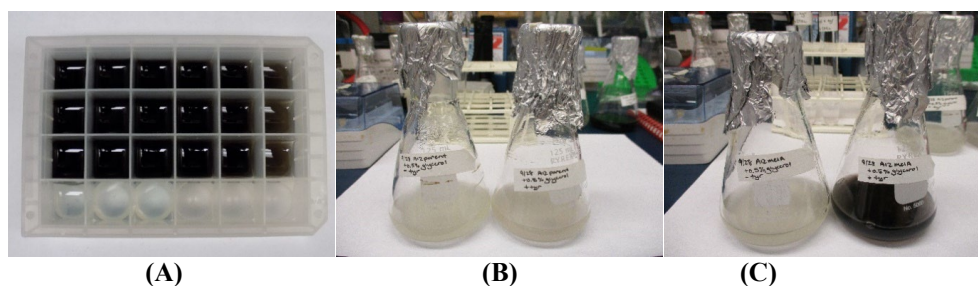


Figure 17. Melanin production in various *B. subtilis* strains. (A) Melanin production from *amyE* integration (top row), *bglS* integration (middle row), and miniICE integration strain (bottom row). (B) Negative controls for melanin production from an environmentally isolated *Bacillus* strain “A12.” Left flask is the A12 parent strain incubated in glycerol supplemented MNGE media. Right flask is the A12 parent strain incubated in glycerol supplemented MNGE with added tyrosine. (C) Melanin production from melanin miniICE transconjugant (an environmentally isolated *Bacillus* strain A12). Left flask is the A12 transconjugant strain incubated in glycerol supplemented MNGE media. Right flask is the A12 transconjugant strain incubated in glycerol supplemented MNGE with added tyrosine.

4.5 Secretion Tags and Spore Display Tags

We amassed a library of 100 secretion tags as Type 3a parts, 6 N-terminal spore display tags as Type 3a parts, and 6 C-terminal spore display tags as Type 3b parts. Although we have not completed validation of all these parts individually, they serve as a repository that can be used to rapidly build and screen tagged proteins for future biotechnology projects.

5. Conclusions

Standardized genetic part designs that are flexible, interchangeable, and amenable to automated liquid handling allow the creation of a toolkit from which researchers can explore, screen, and tune expression of biological products for biotechnology development. The implementation of standardized cloning workflows accelerates the Design–Build–Test–Learn cycle for biotechnology. In the last decade, the Army has been committed to biotechnology endeavors that standardized data management and handling, with an outlook toward data that can be mined or applied in ML for generations to come. Building a repository of genetic elements available in this standardized format provides agility for researchers to pivot rapidly to organisms, strains, or emerging systems biology engineering and biotechnology problems for the Army.

6. Future Outlook

The Army needs to dominate every environment in which it operates, from the urban to austere. The agility to rapidly pivot biotechnology toward the emergent

needs of the Army necessitates a repository of tools from which to build and a repository of data from which to learn. Standardized frameworks and processes in biology meet both of those needs. *B. subtilis* will continue to be an important chassis organism for biomanufacturing, supporting domestic supply chain resiliency and resource independence as well as environmental monitoring and sensing, supporting logistics and assured provisioning for the Army.

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Appendix. Sequence-Specific Information About the Toolkit

Table A-1. Guidelines for designing part overhangs.

Part type	Left flank	Right flank	Notes
1	GCAT <u>CGTCTC</u> ATC <u>GGTCTC</u> AC <u>CCT</u> GAATTCGCATCTAGA	AACGTGAGACCTGAGACGGCAT	
2a	GCATCGTCTCATC <u>GGTCTC</u> AAACG	GGAGTGAGACCTGAGACGGCAT	
2b	GCATCGTCTCATC <u>GGTCTC</u> AGGAGAAAA	TATGTGAGACCTGAGACGGCAT	
3	GCATCGTCTCATC <u>GGTCTC</u> ATATG	GGATCCTGAGACCTGAGACGGCAT	
3a	GCATCGTCTCATC <u>GGTCTC</u> ATATG	GGTCTTGAGACCTGAGACGGCAT	3, 3a: Leave out the start codon from the template, because the start codon is in the overhang.
3b	GCATCGTCTCATC <u>GGTCTC</u> ATTCT	GGATCCTGAGACCTGAGACGGCAT	3b: Leave out stop codon
4	GCATCGTCTCATC <u>GGTCTC</u> CAATCCTAACTCGAG	GCTGTGAGACCTGAGACGGCAT	
4a	GCATCGTCTCATC <u>GGTCTC</u> CAATCC	TAACTCGAGTGGCTGAGACCTGAGACGGCAT	
4b	GCATCGTCTCATC <u>GGTCTC</u> ATGGC	GCTGTGAGACCTGAGACGGCAT	
5	GCATCGTCTCATC <u>GGTCTC</u> AGCTG	ACTAGTGCACTGCACTGCAGTACATGAGACCTGAGACGGCAT	
6	GCATCGTCTCATC <u>GGTCTC</u> ATACA	GAGTTGAGACCTGAGACGGCAT	
7	GCATCGTCTCATC <u>GGTCTC</u> AGAGT	CCGATGAGACCTGAGACGGCAT	
8a	GCATCGTCTCATC <u>GGTCTC</u> ACCGAGCGGCCGC	GCGGCCGCCAATTGAGACCTGAGACGGCAT	
8b	GCATCGTCTCATC <u>GGTCTC</u> ACAAT	CCCTTGAGACCTGAGACGGCAT	

Yellow: BsmBI recognition site

Blue: BsaI recognition site

Underlined: BsmBI overhang

Red: BsaI overhang

Table A-2. Type 2 promoter library.

Name	Sequence
Pveg spoVG	AATTTTGTCAAAATAATTTTATTGACAACGTCTTATTAACGTTGATA TAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAA ATGTTACTAGAGAAAGGTGGTGAA
PlepA (native)	GAGTCAATGTATGAATGGATACGGGATATGAATCAATAAGTACGT GAAAGAGAAAAAGCAACCCAGATATGATAGGGAACCTTTCTCTTTCT TGTTTTACATTGAATCTTTACAATCCTATTGATATAATCTAAGCTAG TGTATTTTGC GTTTAATAGT
PlepA spoVG	GAGTCAATGTATGAATGGATACGGGATATGAATCAATAAGTACGT GAAAGAGAAAAAGCAACCCAGATATGATAGGGAACCTTTCTCTTTCT TGTTTTACATTGAATCTTTACAATCCTATTGATATAATCTAAGCTAG TGTATTTTGC GTTTAATAGTAAAGGTGGTGAACTA
PliaG spoVG	GTATCAATTCGCAAGCTTTTCCTTTATAATAAAGGTGGTGAACTA
PliaI spoVG	TACGACTCCGGTCTTATATAATCGTTTTGCATATCTTCCAACCTTGTA TAAGAAAAGGTGGTGAACTA
PcotYZ	GACAGCAACAAATACACTCGTAGCCATCCTAGTTATCACTCTTGTC CTCTAGGACCTAAAAGCAGAGCTAAAAACGCTCTGCTTTTTCTTAT TTTCCAAGCATATGATGAATATATAGACGTTACCCACACCAAGTG GGGCACGGGTACATATGTTGTTAAGGACTAAAGTCAAATACCCTAT AAT
Pxyl	CTAAAAAAATATTGAAAATACTGACGAGGTTATATAAGATGAAA ATAAGTTAGTTTGTTTAAACAACAACTAATA
Pspank-lacO- spoVG	CAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTGTGTAA TTGTGAGCGGATAACAATTAAGCTTTCGGCTGAAAGGTGGTGAACT AC

Table A-3. Type 2a promoter library.

Name	Sequence
Pveg	AATTTTGTCAAAATAATTTTATTGACAACGTCTTATTAACGTTGATATA ATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTT ACTAG
PliaG	GTATCAATTCGCAAGCTTTTCCTTTATAAT

Table A-4. Type 2b RBS library.

Name	Sequence
RBS_1.7K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACTAAC GAGGTTTCAA
RBS_5K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACAAA CGAGGCTTCAA
RBS_11K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACAAA CGAGGTTTCAA
RBS_26K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACTAAG GAGGCTTCAA
RBS_39	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACTAAC CAGGCTTCAA
RBS_87	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACTAAC CAGGTTTCAA
RBS_90K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACTAAG GAGGTTTCAA
RBS_142K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACAAA GGAGGCTTCAA
RBS_191	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACAAA CCAGGCTTCAA
RBS_427	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACAAA CCAGGTTTCAA
RBS_567K	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACAAA GGAGGTTTCAA
RBS_792	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGC CTCTACAAATAATTTTGTTTAATAACAAATAATTTTGTTTAAAGGCTAGACTAAC GAGGCTTCAA
R0	GGAGAAAAGATTAACTAATAAGGAGGACAAAC
R1	GGAGAAAAGCTCTTAAGGAGGATTTTAGA
R2	GGAGAAAAGGTGGGAAGGAGGACATTTCGAC
R3	GGAGAAAAGGTGGGAAGGAGGACATTTCGAC
R4	GGAGAAAATGACATGAAAGGAAGTATTTGAAA
R5	GGAGAAAAGGTGGGAAGGAGGTGATCCA
R6	GGAGAAAAGGTGGGAAGGAGGGGTTTCGAC
R7	GGAGAAAAGGTGGGAAGGAGGAAGTACT
R8	GGAGAAAAAATACCACGTAAAAAGGAGTAAATAT
R9	GGAGAAAAAATAATATCACCCAAAAGGAGAATTTTA
R10	GGAGAAAAAAGCTATAGCACACACAAAGGAGGGTTTTT
R11	GGAGAAAAAAGCTAATAGCACAGAGAAAAGGAGGGTTTTT
R12	GGAGAAAAAAGCTAAGAGCACACAGAAAAGGAGGGTTTTT
R13	GGAGAAAAAAGCTACGAGCACACAGAAAAGGAGGGTTTTT
R14	GGAGAAAAAAGCTACGAGCACACATAAAAGGAGGGTTTTT
R15	GGAGAAAAAAGCTAAGAGCACACAGAAAAGGAGGGTTTTT
R16	GGAGAAAAAAGCTAAAAATATAAGACACAAAAAAGGAGGGTTTTT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001.

Name	Sequence
ComX	GCATCGTCTCATCGGTCTCATATGCAAGATCTGATTAATTATTTTCTGAATTATCC GGAAGCACTGAAAAAACTGAAAAATAAAGAAGCATGCCTGATTGGCTTTGATGT TCAAGAAACAGAAACAATTATTAAGCATATAATGATTATTATCTGGGTTCTTGA GACCTGAGACGGCAT
LipA	GCATCGTCTCATCGGTCTCATATGAAAAAAGTACTTATGGCATTTCATTATTTGTTT ATCGCTGATTCTATCTGTTTTAGCCGCTCCGCCG TCTGGCGCCAAAGCCGTTCTTGAGACCTGAGACGGCAT
NprM	GCATCGTCTCATCGGTCTCATATGAAAAAGAAAAAACAGGCTTTAAAGGTATTAT TATCAGTTGGTATCCTTTCTTCATCATTTTGTCTTTGCACATACGAGCAGTGCCGGT TCTTGAGACCTGAGACGGCAT
YocH	GCATCGTCTCATCGGTCTCATATGAAAAAGACAATGATTACGTTTCAGTTTAGTTC TAATGTCACTGTTTCGGAGTGGCAAGTGGTGCATCAGCTGGTTCTTGAGACCTGAG ACGGCAT
YngK	GCATCGTCTCATCGGTCTCATATGTATATTAATAAATGTATAGGAAGCATCTTGT TTTTACTGCTGTTTTGTTTCGAGCGCGTTACCTGCTAAAGCAGGTTCTTGAGACCTG AGACGGCAT
WapA	GCATCGTCTCATCGGTCTCATATGAAAAAAGAAAGAGGGCGAACTTTAAAAGG TTCATTGCAGCATTTTTAGTGTTGGCTTAATGATTTTCATTAGTGCCAGCCGATGT ACTAGCAGGTTCTTGAGACCTGAGACGGCAT
AmyQ	GCATCGTCTCATCGGTCTCATATGATTCAAAAAAGAAAAAGAACAGTTTCATTTA GACTGGTTCTGATGTGCACACTGCTGTTTGTTCCTGCGGATTACAAAAACATC AGCAGGTTCTTGAGACCTGAGACGGCAT
SacB	GCATCGTCTCATCGGTCTCATATGAATATTAATAAATTTGCAAAACAAGCAACAG TTCTGACATTTACAACAGCACTGCTG GCAGGCGGCGCAACACAAGCATTTCAGGTTCTTGAGACCTGAGACGGCAT
AmyL	GCATCGTCTCATCGGTCTCATATGAAACAACAAAAAGACTGTATGCAAGACTG CTGACACTGCTGTTTGCACTGATTTT CTGCTGCCGCATTTCAGCAGCAGCAGCAGGTTCTTGAGACCTGAGACGGCAT
Csn	GCATCGTCTCATCGGTCTCATATGAAAATTTCAATGCAAAAAGCAGATTTTTGGA AAAAAGCAGCAATTTCACTGCTGGTT TTTACAATGTTTTTACACTGATGATGTCAGAAACAGTTTTTGCAGGTTCTTGAGA CCTGAGACGGCAT
AmyE	GCATCGTCTCATCGGTCTCATATGTTTGCAAAACGATTCAAAACCTCTTTACTGCC GTTATTCGCTGGATTTTTATTGCTGTTTCATTTGGTTCTGGCAGGACCGGCGGCTG CGAGTGCTGGTTCTTGAGACCTGAGACGGCAT
XynA	GCATCGTCTCATCGGTCTCATATGTTTAAATTTAAAAAAATTTTCTGGTTGGCCT GTCAGCAGCACTGATGTCAATTTCA CTGTTTTTCAGCAACAGCATCAGCAGGTTCTTGAGACCTGAGACGGCAT
YnfF	GCATCGTCTCATCGGTCTCATATGATTCCGAGAATTAATAAACAATTTGCGTTC TGCTGGTTTGCTTTACAATGCTGTCA GTTATGCTGGGCCCCGGGCGCAACAGAAGTTCTGGCAGGTTCTTGAGACCTGAGA CGGCAT
BglC	GCATCGTCTCATCGGTCTCATATGATGAGAAGAAGAAAAAGATCAGATATGAAA AGATCAATTTCAATTTTTATTACATGC CTGCTGATTACACTGCTGACAATGGGCGGCATGATTGCATCACCGGCATCAGCAG GTTCTTGAGACCTGAGACGGCAT
PelB	GCATCGTCTCATCGGTCTCATATGAAAAGACTGTGCCTGTGGTTTACAGTTTTTTC ACTGTTTCTGGTTCTGCTGCCGGGC AAAGCAGGTTCTTGAGACCTGAGACGGCAT
AprE	GCATCGTCTCATCGGTCTCATATGAGATCAAAAAAACTGTGGATTTCAGTCTGT TTGCACTGACACTGATTTTTACAATG GCATTTTCAAATATGTCAGTTCAAGCAGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
Bpr	GCATCGTCTCATCGGTCTCATATGAGGAAAAAAACGAAAAACAGACTCATCAGC TCTGTTTTAAGTACAGTTGTCATCAGTTCAGTCTGCTGTTTCCGGGAGCAGCCGGGG CAGGTTCTTGAGACCTGAGACGGCAT
Mpr	GCATCGTCTCATCGGTCTCATATGAAATTAGTTCCAAGATTCAGAAAAACAATGGT TCGCTTACTTAACGGTTTTGTGTTTGGCTTTGGCAGCAGCGGTTTCTTTTGGCGTA CCGGCAAAAG CGGGTTCTTGAGACCTGAGACGGCAT
TasA	GCATCGTCTCATCGGTCTCATATGGGCATGAAAAAAAAAACTGTCACTGGGCGTTG CATCAGCAGCACTGGGCCTGGCACTG GTTGGCGGCGGCACATGGGCAGGTTCTTGAGACCTGAGACGGCAT
LipA	GCATCGTCTCATCGGTCTCATATGAAATTTGTTAAAAAGAAGAATTATTGCACTGG TTACAATTCTGATGCTGTCAGTTACA TCACTGTTTGCACTGCAACCGTCAGCAAAAGCAGGTTCTTGAGACCTGAGACGGC AT
SpaS	GCATCGTCTCATCGGTCTCATATGTCAAAATTTGATGATTTTGATCTGGATGTTGT TAAAGTTTCAAAACAAGATTCAAAAATTACACCGCAAGGTTCTTGAGACCTGAG ACGGCAT
YwbN	GCATCGTCTCATCGGTCTCATATGTCAGATGAACAAAAAAAACCGGAACAAATT CATAGAAGAGATATTCTGAAATGGGGC GCAATGGCAGGCGCAGCAGTTGCAGGTTCTTGAGACCTGAGACGGCAT
YkuE	GCATCGTCTCATCGGTCTCATATGAAAAAAATGTCAAGAAGACAATTTCTGAAA GGCATGTTTGGCGCACTGGCAGCAGGC GCACTGACAGCAGGCGGCGGCTATGGCTATGCAGGTTCTTGAGACCTGAGACGG CAT
YuiC	GCATCGTCTCATCGGTCTCATATGAGAAAAAGTTATGACAATGTTTAGAAGATTTC TGATGACAGTTCTGTTTGTCTGGCA TTTATGACAACATTTTTTGCAGGTTCTTGAGACCTGAGACGGCAT
PhoD	GCATCGTCTCATCGGTCTCATATGGCATATGATTCAAGATTTGATGAATGGGTTG AAAAACTGAAAGAAGAATCATTCAA AATAATACATTTGATAGAAGAAAAATTATTCAAGGCGCAGGCAAAATTGCAGGC CTGTCA CTGGGCCTGACAATTGCACAATCAGTTGGCGCATTTGAAGTTAATGCAGGTTCTT GAGACCTGAGACGGCAT
protA	GCATCGTCTCATCGGTCTCATATGAAAAAAAAAAAAATTTTATTCAATTAGAAAAAC TGGGCGTTGGCATTGCATCAGTTACA CTGGGCACACTGCTGATTTCAAGGCGGCGTTACACCGGCAGCAAATGCAGGTTCTT GAGACCTGAGACGGCAT
ywmC	GCATCGTCTCATCGGTCTCATATGATGAAAAAAAGATTTTCACTGATTATGATGA CAGGCCTGCTGTTTGGCCTGACATACCG GCATTTGCAGGTTCTTGAGACCTGAGACGGCAT
dacB	GCATCGTCTCATCGGTCTCATATGCGCATTTTCAAAAAAGCAGTATTCGTGATCA TGATTTCTTTTCTTATTGCAACCGTAAATGTGAATACAGCACATGCTGGTTCTTGA GACCTGAGACGGCAT
yddT	GCATCGTCTCATCGGTCTCATATGAGAAAAAAAGAGTTATTACATGCGTTATGG CAGCATCACTGACACTGGGCTCACTG CTGCCGGCAGGCTATGCATCAGCAGGTTCTTGAGACCTGAGACGGCAT
yqoM	GCATCGTCTCATCGGTCTCATATGAAACTGAGAAAAGTTCTGACAGGCTCAGTTC TGTCATGGGCCTGCTGGTTTCAGCA TCACCGGCATTTGCAGGTTCTTGAGACCTGAGACGGCAT
yvcE	GCATCGTCTCATCGGTCTCATATGAGAAAAATCACTGATTACACTGGGCCTGGCAT CAGTTATTGGCACATCATATTCTG ATTCCGTTTACATCAAAAACAGCATCAGCAGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
elB	GCATCGTCTCATCGGTCTCATATGAAATATCTGCTGCCGACAGCAGCAGCAGGCC TGCTGCTGCTGGCAGCACAAACCGGCA ATGGCAGGTTCTTGAGACCTGAGACGGCAT
pel	GCATCGTCTCATCGGTCTCATATGAAAAAAGTTATGCTGGCAACAGCACTGTTTC TGGGCCTGACACCGGCAGGCGCAAAT GCAGGTTCTTGAGACCTGAGACGGCAT
nprE	GCATCGTCTCATCGGTCTCATATGGGTTTAGGTAAGAAATTGTCTGTTGCTGTGCG CTGCTTCGTTTATGAGTTTATCAATCAGCCTGCCAGGTGTTTCAGGCTGGTTCTTGA GACCTGAGACGGCAT
lytC	GCATCGTCTCATCGGTCTCATATGAGATCATATATTAAAGTTCTGACAATGTGCT TTCTGGGCCTGATTCTGTTTGTTCGG ACAGCACTGGCAGGTTCTTGAGACCTGAGACGGCAT
yhcR	GCATCGTCTCATCGGTCTCATATGCTGTCTGTCGAAATGATAAGCAGACAAAATC GTTGTCATTATGTGTATAAGGGAGGAAATATGATGAGGCGTATTCTGCATATTGT GTTGATCACGG CATTAATGTTCTTAAATGTAATGTACACGTTTGAAGCTGGTTCTTGAGACCTGAG ACGGCAT
PenP	GCATCGTCTCATCGGTCTCATATGAAGTTGAAAACCTAAAGCGTCAATAAAATTTCG GAATATGTGTTGGGCTTTTATGTTTAAGCATTACTGGTTTCACACCTTTTTTCAAC TCAACACATG CCGAAGCAGGTTCTTGAGACCTGAGACGGCAT
SacC	GCATCGTCTCATCGGTCTCATATGAAAAAAGACTGATTCAAGTTATGATTATGT TTACACTGCTGCTGACAATGGCATT TCAGCAGATGCAGGTTCTTGAGACCTGAGACGGCAT
YkwD	GCATCGTCTCATCGGTCTCATATGAAGAAAGCATTATTTTATCTGCTGCCGCTGC GGTTGGATTATTCACATTCCGGGGCGTACAGCAAGCATCAGCTGGTTCTTGAGAC CTGAGACGGCAT
Epr	GCATCGTCTCATCGGTCTCATATGAAAAACATGTCTTGCAAACCTGTTGTATCAG TCACTCTGTTTTTCAGTTTTCTCACCATAGGCCCTCTCGCTCATGCGGGTTCTTGA GACCTGAGACGGCAT
YncM	GCATCGTCTCATCGGTCTCATATGGCAAAACCGCTGTCAAAGGCGGCATTCTGG TTAAAAAAGTTCTGATTGCAGGCGCA GTTGGCACAGCAGTTCTGTTTGGCACACTGTCATCAGGCATTCCGGGCCTGCCGG CAGCA GATGCAGGTTCTTGAGACCTGAGACGGCAT
GlpQ	GCATCGTCTCATCGGTCTCATATGAGAAAAAATAGAATACTGGCCTTGTTTGTTT TGTCGCTGGGCTTACTCTATTTATGGTAACGCCAGTGTCGGCAGGTTCTTGAGA CCTGAGACGGCAT
YjfA	GCATCGTCTCATCGGTCTCATATGAAAAGACTGTTTATGAAGGCTTCATTGGTGT TATTCGCAGTAGTATTTGTTTTTGGCGTCAAAGGTGCACCCGCCAAGGCGGGTTC TTGAGACCTGAGACGGCAT
YopL	GCATCGTCTCATCGGTCTCATATGAAAAAAGTATTATGGCACTGGTTATTCTGG GCGCACTGGGCACATCATATATTTCA GCAGGTTCTTGAGACCTGAGACGGCAT
YoaW	GCATCGTCTCATCGGTCTCATATGATGAAAAAATGCTGATGCTGGCATTACAT TTCTGCTGGCACTGACAATTCATGTTGGC GAAGCATCAGCAGGTTCTTGAGACCTGAGACGGCAT
AspB	GCATCGTCTCATCGGTCTCATATGAAACTGGCAAAAAGAGTATCCGCATTAACAC CATCAACCACACTGGCAATCACAGCGAAAGCGGGTTCTTGAGACCTGAGACGGC AT
BglS	GCATCGTCTCATCGGTCTCATATGCCTTATCTGAAACGAGTGTTGCTGCTTCTTGT CACTGGATTGTTTATGAGTTTGTGTCAGTCACTGCTACTGCCTCAGCTGGTTCTT GAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
CccA	GCATCGTCTCATCGGTCTCATATGAAATGGAACCCGCTTATTCCATTTTTGCTGAT CGCTGTTTTAGGAATTGGTCTAACTTTCTTTTTATCAGTAAAAGGAGGTTCTTGAG ACCTGAGACGGCAT
CitH	GCATCGTCTCATCGGTCTCATATGGGAAATACTCGTAAAAAAGTTTCTGTTATCG GAGCAGGTTTTACCGGAGCTACAACCTGCATTTTAAATCGCTCAAAAAGAGCTGGC AGACGTTGGTTCTTGAGACCTGAGACGGCAT
Csn	GCATCGTCTCATCGGTCTCATATGAAAATCAGTATGCAAAAAGCAGATTTTTGGA AAAAAGCAGCGATCTCATTACTTGTTTTCACCATGTTTTTACCCTGATGATGAGC GAAACGGTTT TTGCGGGTTCTTGAGACCTGAGACGGCAT
DacF	GCATCGTCTCATCGGTCTCATATGAAACGTCTTTTATCCACTTTGTTGATTGGTAT AATGCTGCTTACATTTGCACCGTCTGCATTTGCAGGTTCTTGAGACCTGAGACGG CAT
DltD	GCATCGTCTCATCGGTCTCATATGAAAAAGCGTTTTTTCGGTCCAATTATTTTGGC GTTTTATTCTATTCGCAGGCGCCATCGCAGGTTCTTGAGACCTGAGACGGCAT
FliL	GCATCGTCTCATCGGTCTCATATGAAGAAAAAGTTAATGATCATATTACTAATTA TTCTTATCGTAATTGGTGCTCTCGGGGCGGCGGCTGGTTCTTGAGACCTGAGACG GCAT
FliZ	GCATCGTCTCATCGGTCTCATATGAAAAAGAGTCAATATTTTATTGTTTTTATTG TTTTTTCGTTTTATTTCAGTGTACATCCGATTGCTGCTGCCGCGGCAGGTTCTTGAG ACCTGAGACGGCAT
LytB	GCATCGTCTCATCGGTCTCATATGAAATCTTGCAACAATTGATAGTGTGTTTAC TTGCTGCAATCTTATTATTGATTCCATCAGTTTCTTTTGCAGGTTCTTGAGACCTG AGACGGCAT
LytD	GCATCGTCTCATCGGTCTCATATGAAAAAGAGACTAATCGCACCTATGCTTCTAT CCGCCGCGTCCCTTGCCTTTTTGCATGTCTGGTTCTGCCAGGCAGGTTCTTGA GACCTGAGACGGCAT
LytR	GCATCGTCTCATCGGTCTCATATGAGAAACGAACGCAGAAAAAAGAAAAAACT TTATTACTGACAATTTTAAACGATAATCGGACTTCTTGACTCGGAAGTGGCGGCT ATGCATACTACT TATGGCATAAAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
Mdr	GCATCGTCTCATCGGTCTCATATGGACACAACAACAGCAAAACAGGCTTCCACC AAATTTGTGGTCTCGGTCTCCTGCTGGGCATCTTGATGTCTGCAATGGACAATA CGATTGTTGCCA CCGCGGGTTCTTGAGACCTGAGACGGCAT
PbpX	GCATCGTCTCATCGGTCTCATATGACAAGCCCAACCCGCAGAAAGAACTGCGAAA CGCAGACGGAGAAACTAAATAAAAGAGGAAACTGTTGTTTGGTCTTTTAGCA GTGATGGTTTGCA TTACGATTTGGAATGCTGGTTCTTGAGACCTGAGACGGCAT
Pel	GCATCGTCTCATCGGTCTCATATGAAAAAAGTGATGTTAGCTACGGCTTTGTTTT AGGATTGACTCCAGCTGGCGCGAACGCAGGTTCTTGAGACCTGAGACGGCAT
PelB	GCATCGTCTCATCGGTCTCATATGAAACGACTTTGTTTATGGTTACCGTCTTTAG CTTGTTTTTGGTTCTCTTGCCCTGAAAAAGCATTAGGTGGTTCTTGAGACCTGAGAC GGCAT
PhoA	GCATCGTCTCATCGGTCTCATATGAAAAAATGAGTTTGTTCAAAATATGAAAT CAAAACTTCTGCCAATCGCCGCTGTTTCTGTCCTTACAGCTGGAATCTTTGCCG AGCTGGTTCTTGAGACCTGAGACGGCAT
PhoB	GCATCGTCTCATCGGTCTCATATGAAAAAATCCCGAAGAAATTACTGCCTATCG CGTTTTTATCATCAATTGCGTTCAGCAGCTTAGCCAGCGGCAGTGTCCTGAAGC CAGCGCCGGTTCTTGAGACCTGAGACGGCAT
PhrA	GCATCGTCTCATCGGTCTCATATGAAATCTAAATGGATGTCAGGTTTGTGCTCG TTGCGGTCCGGTTCAGCTTACTCAGGTGATGGTTCATGCAGGTTCTTGAGACCT GAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
PhrC	GCATCGTCTCATCGGTCTCATATGAAATTGAAATCTAAGTTGTTTGTATTGTTT GGCCGCAGCCGCGATTTTTACAGCGGCTGGCGTTTCTGCTAATGCGGGTCTTGAG GACCTGAGACGGCAT
PhrF	GCATCGTCTCATCGGTCTCATATGAAATTGAAGTCTAAACTATTACTCTCTTGTCT GGCTCTAAGCACTGTGTTCGTGGCAACAACATTGCAGGTTCTTGAGACCTGAGA CGGCAT
PhrG	GCATCGTCTCATCGGTCTCATATGAAAAGATTCTGATTGGCGCAGGCGTCGCAG CGGTGATTTTATCAGGTTGGTTTATTGCGGGTTCTTGAGACCTGAGACGGCAT
PhrK	GCATCGTCTCATCGGTCTCATATGAAAAAACTTGTGCTTTGCGTATCTATTTTAGC TGTGATTTTAAGTGGAGTAGCTGGTTCTTGAGACCTGAGACGGCAT
RpmG	GCATCGTCTCATCGGTCTCATATGAGAAAAAAGATTACGTTAGCATGCAAGACAT GCGGAAACCGTAATTATACGACAATGAAGAGCTCTGCATCAGCGGGTCTTGAG ACCTGAGACGGCAT
SacB	GCATCGTCTCATCGGTCTCATATGAACATCAAAAAGTTTGCAAAACAAGCAACA GTATTAACCTTTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTTTGCGG GTTCTTGAGACCTGAGACGGCAT
SacC	GCATCGTCTCATCGGTCTCATATGAAAAGAGACTGATTCAAGTCATGATCATGT TCACCCTGCTGTTGACTATGGCATTTCGGCAGATGCAGGTTCTTGAGACCTGAG ACGGCAT
SleB	GCATCGTCTCATCGGTCTCATATGAAGTCCAAAGGATCGATTATGGCATGTCTCA TCCTTTTTTTCCTTTACAATAACGACGTTTATTAATACTGAAACGATCTCTGCCTTT TCGGGTTCTTGAGACCTGAGACGGCAT
SpoIID	GCATCGTCTCATCGGTCTCATATGAAACAATTCGCAATCACACTATCCGTAATAT GTGCATTGATTCTCTTGGTTCCCACACTCTTGGTTATACCATTCCAGCATAATAAG GAAGCGGGGGCCGGTTCTTGAGACCTGAGACGGCAT
SpoIIP	GCATCGTCTCATCGGTCTCATATGAGAAATAAACGCAGAAACAGACAGATTGTT GTTGCGGTAAATGGAGGGAAGCGGTAAAAGCCATCTTTCTATTATCGTAAGCC TTATCGTGATTT TTGTTCTATCCGGTGTGGGTTCTTGAGACCTGAGACGGCAT
SpoIIQ	GCATCGTCTCATCGGTCTCATATGAGAGAGGAAGAAAAGAAAACCTTCTCAAGTC AAAAAACTTCAGCAGTTTTTTCGTAACGCTGGGTGTTCCCTGCAATTTACTTAG TCAGTGCGGCCG TCATTTTAACAGCTGTCCTTGGTTCTTGAGACCTGAGACGGCAT
SpoIIR	GCATCGTCTCATCGGTCTCATATGAAAAAACAGTAATCATTTGTATATATATCT TTCTTTTATTATCCGGAGCGCTCGTAGGTTCTTGAGACCTGAGACGGCAT
TyrA	GCATCGTCTCATCGGTCTCATATGAATCAAATGAAAGATACAATATTGCTCGCCG GTCTCGGATTGATAGGCGGTTTCGATTGCCCTAGCCGGTTCTTGAGACCTGAGACG GCAT
Vpr	GCATCGTCTCATCGGTCTCATATGAAAAGGGGATCATTGCTTTCTGCTTGTA GTTTCGTCTTATTTTTGCGTTATCCACAGGCATTACGGGCGTTTCAGGCAGCTCCG GCTGGTTCTTGAGACCTGAGACGGCAT
YbbC	GCATCGTCTCATCGGTCTCATATGAGAAAAACAATATTCGCTTTTCTGACAGGGC TCATGATGTTTCGGAACGATAACCGCTGCCTCTGCTGGTTCTTGAGACCTGAGACG GCAT
YbbE	GCATCGTCTCATCGGTCTCATATGAAAACAAAGACACTGTTTCATATTCAAGTGCCA TTTTAACCTATCGATATTTGCGCCAAATGAAACCTTCGCAGGTTCTTGAGACCT GAGACGGCAT
YbbR	GCATCGTCTCATCGGTCTCATATGGATAAATTCTTAAACAACCGCTGGGCTGTGA AAATTATTGCTCTGCTTTTCGCGCTCTTGCTTTATGTGGCGGTTAACAGCGGTTCT TGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
YbdG	GCATCGTCTCATCGGTCTCATATGAAAACATTATGGAAAGTCCTCAAAATTGTTT TTGTCAGCTTGGCTGCTTTGGTTTTGCTTGTATCCGTCTCGGGTTCTTGAGACCTG AGACGGCAT
YbdN	GCATCGTCTCATCGGTCTCATATGGTGAAAAAATGGCTTATTCAATTCGCTGTTA TGCTTTCTGTCTTATCTACCTTTACGTATTCCGGCATCAGCTGGTTCTTGAGACCTG AGACGGCAT
YbfO	GCATCGTCTCATCGGTCTCATATGAAACGAATGATAGTGAGAATGACCCTGCCGC TGCTGATCGTTTGTCTGGCGTTTTCTTCTTTTTTCAGCCTCAGCCCGTGCTGGTTCTT GAGACCTGAGACGGCAT
YbxL	GCATCGTCTCATCGGTCTCATATGAAAAAATGGATATATGTTGTGCTTGTGCTGA GTATTGCAGGGATCGGCGGCTTCTCCGTCCACGCAGGTTCTTGAGACCTGAGACG GCAT
YbdK	GCATCGTCTCATCGGTCTCATATGAAACTTTTTAATCGGAAGGTCACCTTGGTTTC TCTTATCCTGATGGCTGTCTTTCAATTCTTCATGGCATTGATCATTAAACGGATTG TCATCAGTGGTTCTTGAGACCTGAGACGGCAT
YdhT	GCATCGTCTCATCGGTCTCATATGTTTAAGAAACATACGATCTCTTTGCTCATTAT ATTTTTACTTGCGTCTGCTGTTTTAGCAGGTTCTTGAGACCTGAGACGGCAT
YdjM	GCATCGTCTCATCGGTCTCATATGTTGAAGAAAGTCATTTTAGCCGCTTTTATCTT AGTAGGAAGTACTTTGGGAGCTTTTAGTTTTTCATCAGATGCCAGTGCGGGTTCT TGAGACCTGAGACGGCAT
YdjN	GCATCGTCTCATCGGTCTCATATGAAAAAAGAATCATATTATTATTAGCAGTGA TAATAGCGGCAGCTGCCGCGGGGTTGCGGGTTCTTGAGACCTGAGACGGCAT
YfhK	GCATCGTCTCATCGGTCTCATATGAAAAAGAAACAAGTAATGCTCGCTTTAACAG CTGCCGCAGGACTGGGTTTGACAGCACTTCATTCCGCTCCCGCAGCAAAAGCTGG TTCTTGAGACCTGAGACGGCAT
YfjS/Pd aA	GCATCGTCTCATCGGTCTCATATGAAGTGGATGTGTTCAATATGCTGCGCCGCCG TATTGCTTGCCGGAGGTGCAGCACAGGCGGGTTCTTGAGACCTGAGACGGCAT
YfkD	GCATCGTCTCATCGGTCTCATATGATGAAAAAGCTATTTCAATCCACACTTATTGT GTTGTTATTCTTTAGTTTTTTTCGGCGTTTCAGCCCATCCACGCGGGTTCTTGAGACC TGAGACGGCAT
YfkN	GCATCGTCTCATCGGTCTCATATGAGAATACAGAAAAGACGAACACACGTCGAA AACATTCTCCGTATTCTTTTGCCCCCAATTATGATACTTAGCCTAATCCTCCCAAC ACCACCCATT ATGCAGGTTCTTGAGACCTGAGACGGCAT
YhdC	GCATCGTCTCATCGGTCTCATATGAAATCCCTGCCGTATACCATCGCACTTCTTTT TTGCGGATTGATTATCGTGTCATGGCTGGTTCTTGAGACCTGAGACGGCAT
YhfM	GCATCGTCTCATCGGTCTCATATGAAAAAATAGTGGCAGCCATCGTGGTAATCG GTCTTGTTTATCGCATTTTTTTATCTTTACAGCCGATCAGGCGATGTGTATCAA TCGGTAGACG CGGGTTCTTGAGACCTGAGACGGCAT
YhjA	GCATCGTCTCATCGGTCTCATATGAAAAAAGCGGCGGCGGTTTTGCTGTCACTCG GTCTCGTTTTCGGTTTTTTCATACGGAGCTGGCCATGTGGCGGAAGCCGGTTCTTG AGACCTGAGACGGCAT
YjeN	GCATCGTCTCATCGGTCTCATATGAAAAAGAAACTAAAATTATACTTTCTCTCT TGGCAGCACTTATTGTTATATTGATAGTACTTCCAGTTCTATCTCCTGTTGTCTTT ACAGCTTCTT CGGGTTCTTGAGACCTGAGACGGCAT
YjdB	GCATCGTCTCATCGGTCTCATATGAATTTCAAAAAAACGGTTGTTTCTGCTTTATC GATTTCTGCGTTAGCTCTGTGCGTCAGCGGGGTAGCTTCAGCTGGTTCTTGAGAC CTGAGACGGCAT
YjiA	GCATCGTCTCATCGGTCTCATATGGCAGCACAAACAGATTATAAAAAACAAGTT GTTGGCATTCTGCTGTCACTGGCATT GTTCTGTTTGTTTTTTCATTTTCAGAAGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
YkoJ	GCATCGTCTCATCGGTCTCATATGCTCAAGAAAAAATGGATGGTCGGTCTTTTAG CAGGGTGCCTAGCGGCCGAGGATTACAGCTACAACGCGTTTGCCGGTTCTTGAG ACCTGAGACGGCAT
YkvV	GCATCGTCTCATCGGTCTCATATGTTGACGAAGCGCTTGCTTACTATATACATTAT GTTATTAGGGTTGATTGCATGGTTTCCAGGTGCGGCACAAGCTGGTTCTTGAGAC CTGAGACGGCAT
YlaE	GCATCGTCTCATCGGTCTCATATGAAGAAAAACATTTGTAAAAAAGCTATGTTAA CGACTGCCGCAATGACCTCAGCTGCTCTATTAACGTTCCGGTCTGATGCAGCCAG CGCAGGTTCTTGAGACCTGAGACGGCAT
YlbL	GCATCGTCTCATCGGTCTCATATGCTACGTAAAAAACATTTTAGCTGGATGCTCG TCATCCTCATACTGATCGCCGTTTATCATTTATAAACTTCCATACTATATTACA AAACCGGGAG AAGCGGGTTCTTGAGACCTGAGACGGCAT
YlqB	GCATCGTCTCATCGGTCTCATATGAAAAAATCGGTTTATTGTTTATGTTATGTCT GGCAGCCCTGTTTACAATAGTTTCCCGGCACAGCAAGCAGACGCTGGTTCTTGA GACCTGAGACGGCAT
YlxF	GCATCGTCTCATCGGTCTCATATGTCCGGCAAAAAGAAAGAATCAGGTAAGTTCC GTTTCGGTTTTGCTTATCATTATCCTCCCGCTGATGTTTCTATTAATCGCAGGGGG ATTGTTCTTT GGGCTGCTGGTGGTTCTTGAGACCTGAGACGGCAT
YckD	GCATCGTCTCATCGGTCTCATATGAAAAGAATTACAATTAATATTATTACAATGT TTATTGCAGCAGCAGTTATTTCACTG ACAGGCACAGCAGAAGCAGGTTCTTGAGACCTGAGACGGCAT
YobB	GCATCGTCTCATCGGTCTCATATGAAAATTAGAAAAATTCTGCTGTCATCAGCAC TGTCATTTGGCATGCTGATTTTCAGCA GTTCCGGCACTGGCAGGTTCTTGAGACCTGAGACGGCAT
YurI	GCATCGTCTCATCGGTCTCATATGACAAAAAAGCATGGTTTCTGCCGCTGGTTT GCGTTCTGCTGATTTTCAGGCTGGCTG GCACCGGCAGCATCAGCATCAGCAGGTTCTTGAGACCTGAGACGGCAT
yvpA	GCATCGTCTCATCGGTCTCATATGAAAAAATTGTTTCAATTCTGTTTATGTTTGG CCTGGTTATGGGCTTTTCAAAATTT CAACCGTCAACAGTTTTTGCAGGTTCTTGAGACCTGAGACGGCAT
AbnA	GCATCGTCTCATCGGTCTCATATGAAAAAATGAAAAAATGAAAAAGATTCTG CATTTTTTCATCAGCAGCACTGGCAGCA GGCCTGATTTTTACATCAGCAGCACCGGCAGAAGCAGGTTCTTGAGACCTGAGA CGGCAT
YwjE	GCATCGTCTCATCGGTCTCATATGAAAGTTTTTATTGTTATTATGATTATTGTTGT TATTTTTTTTGCAGTGATTCTGCTG GATATTTTTATGGGCAGAGCAGGTTCTTGAGACCTGAGACGGCAT
YqgA	GCATCGTCTCATCGGTCTCATATGAAACAAGGCAAATTTTCAGTTTTTCTGATTCT GCTGCTGATGCTGACACTGGTTGTT GCACCGAAAGGCAAAGCAGAAGCAGGTTCTTGAGACCTGAGACGGCAT
lipB	GCATCGTCTCATCGGTCTCATATGAAAAAGTTCTGATGGCATTATTATTGCTT GTCACTGATTCTGTCACTTCTGGCA GCACCGCCGTCAGGCGCAAAGCAGGTTCTTGAGACCTGAGACGGCAT
YrvJ	GCATCGTCTCATCGGTCTCATATGAATAAAAAATATTTTGTCTGATTGTTTGCAT TATTTTTACATCAGCACTGTTTCCG ACATTTTCATCAGTTACAGCAGGTTCTTGAGACCTGAGACGGCAT
YbxI	GCATCGTCTCATCGGTCTCATATGAAAAATGGATTTATGTTGTTCTGGTTCTGTC AATTGCAGGCATTGGCGGCTTTTCA GTTTCATGCAGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
YolA	GCATCGTCTCATCGGTCTCATATGAAAAAAGAATTACATATTCAGTCTGCTGGCAC TGCTGGCAGTTGTTGCATTTGCATT ACAGATTCATCAAAAGCAAAAGCAGGTTCTTGAGACCTGAGACGGCAT
YqxI	GCATCGTCTCATCGGTCTCATATGTTTAAAAAACTGCTGCTGGCAACATCAGCAC TGACATTTTCACTGTCAGTGGTTCTG CCGCTGGATGGCCATGCAAAAGCAGGTTCTTGAGACCTGAGACGGCAT
NprB	GCATCGTCTCATCGGTCTCATATGAGAAATCTGACAAAAACATCACTGCTGCTGG CAGGCCTGTGCACAGCAGCACAATG GTTTTTGTACACATGCATCAGCAGGTTCTTGAGACCTGAGACGGCAT
YvbX	GCATCGTCTCATCGGTCTCATATGAAAAATGGCTGATTATTGCAGTTTCACTGG CAATTGCAATTGTTCTGTTTATGTAT ACAAAAGGCGAAGCAAAAGCAGGTTCTTGAGACCTGAGACGGCAT
YlxY	GCATCGTCTCATCGGTCTCATATGTATAAAAAATTGTTCCGTTTGCAGTTTTTCT GTTTCTGTTTTTGTTCATTTGAA ATGATGGAAAAATCCGCATGCACTGGATTATATTGGCGCAGGTTCTTGAGACCTGA GACGGCAT
YxiT	GCATCGTCTCATCGGTCTCATATGAAATGGAATAATATGCTGAAAGCAGCAGGC ATTGCAGTTCTGCTGTTTTCACTTTTT GCATATGCAGCACCGTCACTGAAAGCAGTTCAAGCAGGTTCTTGAGACCTGAGA CGGCAT
YkvT	GCATCGTCTCATCGGTCTCATATGACAACAAAATTTACAGCACTGGCAGTTTTTC TGCTGTGCTTTATGCCGGCAGCAAAA ATTGGTTCTTGAGACCTGAGACGGCAT
CotC	GCATCGTCTCATCGGTCTCATATGAAAAATAGACTGTTTATTCTGATTGCTTTTG CGTTATTTGCCTTTTCTGTCATTT GGCCAACCGTTTTTCCGTCATGATTCTGACAGTTCAAGCAGCAAAATCAGGTT CTTGAGACCTGAGACGGCAT
YolC	GCATCGTCTCATCGGTCTCATATGAAAAAAGACTGATTGGCTTTCTGGTTCTGG TTCCGGCACTGATTATGTCAGGCATT ACACTGATTGAAGCAGGTTCTTGAGACCTGAGACGGCAT
YqzG	GCATCGTCTCATCGGTCTCATATGATGATTAACAATGCGTTATTTGCCTGTCAGT GCTGGTTTTTGGCACAACAGCAGCA CATGCAGGTTCTTGAGACCTGAGACGGCAT
YndA	GCATCGTCTCATCGGTCTCATATGAGATTTACAAAAGTTGTTGGCTTTCTGTCAGT TCTGGGCCTGGCAGCAGTTTTTCCG CTGACAGCACAAGCAGGTTCTTGAGACCTGAGACGGCAT
YwfM	GCATCGTCTCATCGGTCTCATATGAAAGGCAATATTTATTCACTGTTTGTCTGAT TGCAGCATTTTTTTGGGGCACAACA GGCACAGTTCAAGCAGGTTCTTGAGACCTGAGACGGCAT
NucB	GCATCGTCTCATCGGTCTCATATGAAAAATGGATGGCAGGCCTGTTTCTGGCAG CAGCAGTTCTGCTGTGCCTGATGGTT CCGCAACAAATTCAAGGCGCATCATCAGGTTCTTGAGACCTGAGACGGCAT
YqxM	GCATCGTCTCATCGGTCTCATATGTTTAGACTGTTTCATAATCAACAAAAAGCAA AAACAAAAGTAAAGTTCTGCTGATT TTTCAACTGTCAGTTATTTTTTCACTGACAGCAGCAATTTGCCTGCAATTTTCAGA TGAT ACATCAGCAGGTTCTTGAGACCTGAGACGGCAT
YpuA	GCATCGTCTCATCGGTCTCATATGAAAAAATTTGGATTGGCATGCTGGCAGCAG CAGTTCTGCTGCTGATGGTTCCGAAA GTTTCACTGGCAGATGCAGGTTCTTGAGACCTGAGACGGCAT
YwmD	GCATCGTCTCATCGGTCTCATATGAAAAAACTGCTGGCAGCAGGCATTATTGGCC TGCTGACAGTTTCAATTGCATCACCG TCATTTGCAGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
YwtD	GCATCGTCTCATCGGTCTCATATGAATACACTGGCAAATTGGAAAAAATTTCTGC TGGTTGCAGTTATTATTTGCTTTCTG GTTCCGATTATGACAAAAGCAGAAATTGCAGAAGCAGGTTCTTGAGACCTGAGA CGGCAT
YdbK	GCATCGTCTCATCGGTCTCATATGAAACTGTTAATAGAAAAGTTACACTGGTTT CACTGATTCTGATGGCAGTTTTTCAA TTTTTTATGGCACTGATTATTAAGAATTGTTATTTTCAGGTTCTTGAGACCTGAG ACGGCAT
YwaD	GCATCGTCTCATCGGTCTCATATGAAAAAACTGCTGACAGTTATGACAATGGCAG TTCTGACAGCAGGCACACTGCTGCTG CCGGCACAATCAGTTACACGGCAGCACATGCAGGTTCTTGAGACCTGAGACGG CAT
YpjP	GCATCGTCTCATCGGTCTCATATGAAACTGTGGATGAGAAAAACACTGGTTGTTC TGTTTACAATTGTTACATTTGGCCTG GTTTACCGCCGGCAGCACTGATGGCAGGTTCTTGAGACCTGAGACGGCAT
LytF	GCATCGTCTCATCGGTCTCATATGAAAAAAACTGGCAGCAGGCCTGACAGCA TCAGCAATTGTTGGCACAACACTGGTT GTTACACCGGCAGAAGCAGGTTCTTGAGACCTGAGACGGCAT
WprA	GCATCGTCTCATCGGTCTCATATGAAAAGAAGAAAATTTTCATCAGTTGTTGCAG CAGTTCTGATTTTTGCACTGATTTTT TCACTGTTTTACCGGGCACAAAAGCAGCAGCAGGTTCTTGAGACCTGAGACGG CAT
PbpD	GCATCGTCTCATCGGTCTCATATGACAATGCTGAGAAAAATTATTGGCTGGATTC TGCTGCTGTGCATTATTCCGCTGTTT GCATTTACAGTTATTGCAGGTTCTTGAGACCTGAGACGGCAT
YjeM	GCATCGTCTCATCGGTCTCATATGAAAAAGAACTGCTGGCATCACTGGTTCTGT GCCTGTCACTGTCACCGCTGGTTTCA ACAAATGAAGTTTTTGCAGGTTCTTGAGACCTGAGACGGCAT
MotB	GCATCGTCTCATCGGTCTCATATGGCAAGAAAAAATGAAATGAAGAT GAACATGTTGATGAATCATGGCTGGTT CCGTATGCAGATATTCTGCACTGCTGCTGGCACTGTTTATTGTTCTGTATGCATC ATCAGGTTCTTGAGACCTGAGACGGCAT
LytE	GCATCGTCTCATCGGTCTCATATGAAAAACAAATTATTACAGCAACAACAGCA GTTGTTCTGGGCGCACTGTTTGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YknX	GCATCGTCTCATCGGTCTCATATGAAAAAGTTTGGATTGGCATTGGCATTGCAG TTATTGTTGCACTGTTTGTGGCATT AATATTTATAGATCAGCAGCACCGACATCAGGCTCAGCAGCTGGTTCTTGAGACC TGAGACGGCAT
YlxW	GCATCGTCTCATCGGTCTCATATGAGAGGCAAATCAGCAGTTCTGCTGTCACTGA TTATGCTGATTGCAGGCTTTCTGATT TCATTTTCATTTCAAATGACAAAAGAAAATAATAAATCAGCAGCAGCTGGTTCTT GAGACCTGAGACGGCAT
YnzA	GCATCGTCTCATCGGTCTCATATGAAACTGTCATTTACAAAATTCTGGTTATTCT GTTTGTGGCTTTCTGGTTTTTGGC CCGGATAAACTGCCGGCACTGGGCGCTGGTTCTTGAGACCTGAGACGGCAT
YobV	GCATCGTCTCATCGGTCTCATATGAAACTGGAAAGACTGCTGGCAATGGTTGTTC TGCTGATTTCAAAAAACAAGTTCAA GCAGCTGGTTCTTGAGACCTGAGACGGCAT
YodV	GCATCGTCTCATCGGTCTCATATGAAAGTTCCGAAAACAATGCTGCTGTCAACAG CAGCAGGCCTGCTGCTGCTCACTGACA GCAACATCAGTTTCAGCAGCTGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
YojL	GCATCGTCTCATCGGTCTCATATGAAAAAAAAAATTGTTGCAGGCCTGGCAGTTT CAGCAGTTGTTGGCTCATCAATGGCA GCAGCACCAGGAGAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YomL	GCATCGTCTCATCGGTCTCATATGAGAAAAAAAAAGAGTTATTACATGCGTTATGG CAGCATCACTGACACTGGGCTCACTG CTGCCGGCAGGCTATGCAACAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YohH	GCATCGTCTCATCGGTCTCATATGAAAGATTTATTCTGGTTCTGTCATTTCTGTC AATTATTGTTGCATATCCGATTCAA ACAAATGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YpbG	GCATCGTCTCATCGGTCTCATATGAAACTGTCAGTTAAAATTGCAGGCGTTCTGA CAGTTGCAGCAGCAGCAATGACAGCAAAA ATGTATGCAACAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YpcP	GCATCGTCTCATCGGTCTCATATGAATAATAATAAACTGCTGCTGGTTGATGGCA TGGCACTGCTGTTTAGAGCATTTTTT GCAACAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YpmS	GCATCGTCTCATCGGTCTCATATGAATAAATGGAAAAGACTGTTTTTTATTCTGCT GGCAATTAATTTTTATTCTGGCAGCAGGC TTTGTTGCACTGGTTCTGCTGCCGGGCGAACAAGCACAAGTTGCTGGTTCTTGAG ACCTGAGACGGCAT
YpuD	GCATCGTCTCATCGGTCTCATATGGGCAGAATTAACAAAAATTACAATTCTGC TGGTTCTGCTGCTGCTGCTGGCAGGC GGCTATATGTATATTAATGATATTGAACTGAAAGATGTTCCGACAGCAATTGGCG CTGGTTCTTGAGACCTGAGACGGCAT
YqzC	GCATCGTCTCATCGGTCTCATATGACAAAAAGAGGCATTCAAGCATTTCAGGCG GCATTATTCTGGCAACAGCAGTTCTG GCAGCAGTTTTTTATCTGACAGATGAAGATCAAGCAGCAGCAGCTGGTTCTTGAG ACCTGAGACGGCAT
YraJ	GCATCGTCTCATCGGTCTCATATGACACTGACAAAAGTGAATGCTGTCAATGC TGACAGTTATGATTGCATCACTGTTT ATTTTTTCATCACAAGCACTGGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YuaB	GCATCGTCTCATCGGTCTCATATGAAAGAAAACTGCTGTCATCACTGGCAATTT CAGCACTGTCACTGGGCTGCTGGTT TCAGCACCAGCAGCATCATTTGCAGCAGAAGCTGGTTCTTGAGACCTGAGACGG CAT
YusW	GCATCGTCTCATCGGTCTCATATGCATCTGATTAGAGCAGCAGGCGCAGTTTGCC TGGCAGTTGTTCTGATTGCAGGCTGC AGATTTAATGAAGATCAACATCAAGCAGAAGGCGCTGGTTCTTGAGACCTGAGA CGGCAT
YvgO	GCATCGTCTCATCGGTCTCATATGAAAGAAATTAGAATTCCGATGACACTGGCAC TGGGCGCAGCACTGACAATTGCACCG CTGTCATTTGCATCAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YvgV	GCATCGTCTCATCGGTCTCATATGAAAAAAAAACAACAATCATCAGCAAAATTT GCAGTTATTCTGACAGTTGTTGTTGTT GTTCTGCTGGCAGCAATTGTTGCTGGTTCTTGAGACCTGAGACGGCAT
YvnB	GCATCGTCTCATCGGTCTCATATGAGAAAATATACAGTTATTGCATCAATTCTGC TGTCATTTCTGTCACTTCTGTCAAGC GGCGCTGGTTCTTGAGACCTGAGACGGCAT
YvpB	GCATCGTCTCATCGGTCTCATATGAAAACACTGAGAACACTGTGCGTTCTGATGA TTCTGTCAAGGCGTTATTTTTTTTGGC CTGAAAATTGATGCAGCTGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
Ywei	GCATCGTCTCATCGGTCTCATATGAAAAGACTGCTGGTTTCACTGAGAGTTTGGA TGGTTTTTCTGATGAATTGGGTAC CCGGATAGAAAAACAGCAAGAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YwdK	GCATCGTCTCATCGGTCTCATATGAAAGTTTTATTATTCTGGGCGCAATTAATGC ACTGCTGGCAGTTGGCCTGGGCGCA TTTGGCGCTGGTTCTTGAGACCTGAGACGGCAT
YweA	GCATCGTCTCATCGGTCTCATATGCTGAAAAGAACATCATTTGTTTCATCACTGTT TATTTTCATCAGCAGTTCTGCTGTCA ATTCTGCTGCCGTCAGGCCAAGCACATGCAGCTGGTTCTTGAGACCTGAGACGGC AT
YwgB	GCATCGTCTCATCGGTCTCATATGAAAATGAAATCAGGCATGGAACAAGCAGTTT CAGTTCTGCTGCTGCTGTCAAGACTG CCGGTTCAAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YwmB	GCATCGTCTCATCGGTCTCATATGAAAAAAAACAAGTTTCACATGCAATTATTA TTTCAGTTATGCTGTCAATTTGTTATT GCAGTTTTTTCATACAATTCATGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YwoF	GCATCGTCTCATCGGTCTCATATGAGAAAATGGTATTTTATTCTGCTGGCAGGCG TTCTGACATCAGTTATTCTGGCATT GTTTATGATAAAACAAAAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YwqC	GCATCGTCTCATCGGTCTCATATGGGCGAATCAACATCACTGAAAGAAATTCTGT CAACACTGACAAAAAGAATTCTGCTG ATTATGATTGTTACAGCAGCAGCAACAGCAGCTGGTTCTTGAGACCTGAGACGG CAT
YwqO	GCATCGTCTCATCGGTCTCATATGAAATTTCTGCTGTCAGTTATTGCAGGCCTGCT GATTCTGGCACTGTATCTGTTTTGG AAAGTTCAACCGCCGGTTTGGATTGCTGGTTCTTGAGACCTGAGACGGCAT
YwsB	GCATCGTCTCATCGGTCTCATATGAATAAACCGACAAAAGTGTTCACACTGG CACTGGCAGCAGGCATGACAGCAGCA GCAGCAGGCGGCGCAGGCACAATTCATGCAGCTGGTTCTTGAGACCTGAGACGG CAT
YwtC	GCATCGTCTCATCGGTCTCATATGAAATTTGTTAAAGCAATTTGGCCGTTTGTTC AGTTGCAATTGTTTTATGTTTATG TCAGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YwtF	GCATCGTCTCATCGGTCTCATATGGAAGAAAGATCACAAAGAAGAAAAAAAAAA AGAAAAGTGAAGAAATGGGTAAAGTT GTTGCAGGCCTGATGGCATTCTGGTTATTGCAGCAGGCTCAGTTGGCGCATATG CAGCTGGTTCTTGAGACCTGAGACGGCAT
YxaK	GCATCGTCTCATCGGTCTCATATGGTTAAATCATTTAGAATGAAAGCACTGATTG CAGGCGCAGCAGTTGCAGCAGCAGTT TCAGCAGGCGCAGTTTCAGATGTTCCGGCAGCAAAAGTTCTGCAACCGACAGCA GCATAT GCAGCTGGTTCTTGAGACCTGAGACGGCAT
YxiA	GCATCGTCTCATCGGTCTCATATGTTTAATAGACTGTTTAGAGTTTGCTTTCTGGC AGCACTGATTATGGCATTACACTG CCGAATTCAGTTTATGCAGCTGGTTCTTGAGACCTGAGACGGCAT
YybN	GCATCGTCTCATCGGTCTCATATGAATAAATTTCTGAAATCAAATTTTAGATTCT GCTGGCAGCAGCACTGGGCATTTCA CTGCTGGCATCATCAAAATTTATTAAAGCAGCTGGTTCTTGAGACCTGAGACGGC AT
YycP	GCATCGTCTCATCGGTCTCATATGAAAAAATGGATGATTACAATTGCAATGCTGA TTCTGGCAGGCATTGCACTGTTTGGT TTTATTTACCGCTGAAATCAGCTGGTTCTTGAGACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
CwlD	GCATCGTCTCATCGGTCTCATATGAGAAAAAACTGAAATGGCTGTCATTTCTGC TGGGCTTTATTATTCTGCTGTTTCTG TTTAAATATCAATTTTCAAATGCTGGTCTTGAGACCTGAGACGGCAT
MreC	GCATCGTCTCATCGGTCTCATATGCCGAATAAAAGACTGATGCTGCTGCTGCTGT GCATTATTATTCTGGTTGCAATGATT GGCTTTTCAGCTGGTTCCTTGAGACCTGAGACGGCAT
PbpB	GCATCGTCTCATCGGTCTCATATGATTCAAATGCCGAAAAAAATAAATTTATGA ATAGAGGCGCAGCAATTCTGTCAATT TGCTTTGCACTGTTTTTTTTTGTATTCTGGGCAGAATGGCAGCTGGTTCCTTGAGA CCTGAGACGGCAT
YocA	GCATCGTCTCATCGGTCTCATATGAAAAAAAAGAAAAGGCTGCTTTGCAGCA GCAGGCTTTATGATGATTTTTGTTTTT GTTATTGCAGCTGGTTCCTTGAGACCTGAGACGGCAT
YolI	GCATCGTCTCATCGGTCTCATATGAAAAATGGATTGTTCTGTTTCTGGTTCATG TGCAGCAGCAATTTCAATTTTTGTT TATGTTTCAACAGGCTCAGAAGCTGGTTCCTTGAGACCTGAGACGGCAT
YpmB	GCATCGTCTCATCGGTCTCATATGAGAAAAAAGCACTGATTTTTACAGTTATTT TTGGCATTATTTTTCTGGCAGTTCTG CTGGTTTCAGCATCAATTTATAAATCAGCAATGGCAGCTGGTTCCTTGAGACCTGA GACGGCAT
YqfZ	GCATCGTCTCATCGGTCTCATATGAAAAGACTGACACTGGTTTGCTCAATTGTTTT TATTCTGTTTATTCTGTTTTATGAT CTGAAAATTGGCACAATTCCGATTCAAGATCTGCCGGTTTATGAAGCATCAGCAG CTGGTTCCTTGAGACCTGAGACGGCAT
YrrL	GCATCGTCTCATCGGTCTCATATGTATATTAATCAACAAAAAAATCATTTTTTA ATAAAAAAGAATTATTCTGTCATCA ATTGTTGTTCTGTTTCTGATTATTGGCGGCGCATTTCTGGCTGGTTCCTTGAGACCT GAGACGGCAT
YrrR	GCATCGTCTCATCGGTCTCATATGAAAATTTCAAAAAGAATGAACTGGCAGTTA TTGCATTTCTGATTGTTTTTTTTCTG CTGCTGTGAGACTGGCAGAAATTGCTGGTTCCTTGAGACCTGAGACGGCAT
YrrS	GCATCGTCTCATCGGTCTCATATGTCAAATAATCAATCAAGATATGAAAATAGAG ATAAAAAGAAGAAAAGCAAATCTGGTT CTGAATATTCTGATTGCAATTGTTTCAATTCTGATTGTTGTTGTTGCAGCAGCTGG TTCTTGAGACCTGAGACGGCAT
YunA	GCATCGTCTCATCGGTCTCATATGATTACAGATATTTTTAAACCGGGCTGCAGAA AACTGTGCGTTTTTAATATGAAAGGC GATTATTTGTTAAAGTTCTGCTGTCAGCACTGCTGCTGCTGCTGTTGCAGCTGG TTCTTGAGACCTGAGACGGCAT
YunB	GCATCGTCTCATCGGTCTCATATGCCGAGATATAGAGGCCCGTTTAGAAAAAGA GGCCCGCTGCCGTTTAGATATGTTATG CTGCTGTCAGTTGTTTTTTTATTCTGTCAACAACAGTTTCACTGGCTGGTTCCTG AGACCTGAGACGGCAT
YveB	GCATCGTCTCATCGGTCTCATATGAATTATATTAAGCAGGCAAATGGCTGACAG TTTTTCTGACATTTCTGGGCATTCTG CTGTTTATTGATCTGGCTGGTTCCTTGAGACCTGAGACGGCAT
YyaB	GCATCGTCTCATCGGTCTCATATGGTTTATCAAACAAAAAGAGATGTTCCGGTTA CACTGATGATTGTTTTTCTGATTCTG CTGATTCAAGCAGATGCAGCTGGTTCCTTGAGACCTGAGACGGCAT
thnP	GCATCGTCTCATCGGTCTCATATGAAAAAATTATTAATTTCTGCTGCTGATTA ATCTGATTTCAAGGCACATTTATCCGATTACAACAGTTCATGCAGCTGGTTCCTGA GACCTGAGACGGCAT

Table A-5. Type 3a secretion tag library. All sequences are in the 3a template designed for direct Golden Gate domestication into YTK001 (continued).

Name	Sequence
phrC-ext	GCATCGTCTCATCGGTCTCATATGAAACTGAAATCAAAACTGTTTGTATTGTCCT GGCAGCAGCAGCAATTTTACAGCA GCAGGCGTTTCAGCAAATGCAGAAGCACTGGATTTTCATGTTACAGCTGGTTCTT GAGACCTGAGACGGCAT
phrC-CSF	GCATCGTCTCATCGGTCTCATATGAAACTGAAATCAAAACTGTTTGTATTGTCCT GGCAGCAGCAGCAATTTTACAGCA GCAGGCGTTTCAGCAAATGCAGAAGCACTGGATTTTCATGTTACAGAAAGAGGC ATGACAGGATCCTGAGACCTGAGACGGCAT

Table A-6. Type 3 parts.

Name	Sequence
mKate2	GCATCGTCTCATCGGTCTCATATGGTTAGTGAGCTCATTAAAGA AAACATGCACATGAACTCTACATGGAAGGAACCGTCAACAAC CATCATTTTAAATGTACCTCCGAGGGGGAAGGTAAGCCTTATGA AGGTACTCAAACGATGCGTATCAAGGCGGTCTGAAGGAGGCCCA CTCCCTTTTTCGTTTCGACATCCTGGCTACCAGCTTCATGTATGGT TCTAAGACGTTTATAAACCATAACGCAGGGCATTCTGATTTTTTC AAACAATCCTTCCCGGAGGGTTTTACTTGGGAGCGTGTTACCAC ATACGAAGACGGAGGGGTGCTCACTGCAACACAAGATACGTCA TTGCAAGATGGCTGTCTGATTTACAATGTTAAGATACGGGGAGT AAATTTTCCGTCTAACGGTCCTGTAATGCAAAAAAAAAACGTTGG GTTGGGAAGCGAGTACGGAAACGTTATATCCTGCAGACGGGGG ATTAGAGGGTCGTGCCGACATGGCGTTGAAGTTGGTAGGAGGC GGGCACTTGATCTGCAACTTAAAGACCACTTATCGGTCAAAGAA GCCAGCTAAGAACTTAAAGATGCCTGGGGTCTATTACGTCGATC GTCGCCTTGAGCGCATCAAAGAAGCGGACAAAGAACATATGT AGAGCAACACGAAGTCGCCGTTGCCCGCTACTGCGACCTTCCAT CAAACTGGGCCATCGGTGAATCCTGAGACCTGAGACGGCAT
itag-mKate2	GCATCGTCTCATCGGTCTCATATGGATTCAATAGAAAAGGTAAG CGAATTTGCCACCATGGTTAGTGAGCTCATTAAAGAAAACATGC ACATGAACTCTACATGGAAGGAACCGTCAACAACCATCATTTT AAATGTACCTCCGAGGGGGAAGGTAAGCCTTATGAAGGTACTC AAACGATGCGTATCAAGGCGGTCTGAAGGAGGCCCACTCCCTTTT GCGTTTCGACATCCTGGCTACCAGCTTCATGTATGGTTCTAAGAC GTTTATAAACCATAACGCAGGGCATTCTGATTTTTTCAAACAATC CTTCCCGGAGGGTTTTACTTGGGAGCGTGTTACCACATACGAAG ACGGAGGGGTGCTCACTGCAACACAAGATACGTCATTGCAAGA TGGCTGTCTGATTTACAATGTTAAGATACGGGGAGTAAATTTTC CGTCTAACGGTCCTGTAATGCAAAAAAAAAACGTTGGGTTGGGAA GCGAGTACGGAAACGTTATATCCTGCAGACGGGGGATTAGAGG GTCGTGCCGACATGGCGTTGAAGTTGGTAGGAGGCGGGCACTTG ATCTGCAACTTAAAGACCACTTATCGGTCAAAGAAGCCAGCTAA GAACTTAAAGATGCCTGGGGTCTATTACGTCGATCGTCGCCTTG AGCGCATCAAAGAAGCGGACAAAGAAACATATGTAGAGCAACA CGAAGTCGCCGTTGCCCGCTACTGCGACCTTCCATCAAACTGG GCCATCGGTGAATCCTGAGACCTGAGACGGCAT

Table A-7. Type 3b parts.

Name	Sequence
<i>amyE</i>	GCATCGTCTCATCGGTCTCATTCTATGTTTGCAAAACGATTCAAAACCTCTT TACTGCCGTTATTCGCTGGATTTTATGCTGTTTCATTGGTTCTGGCAGG ACCGGCGGCTGCGAGTGCTGAAACGGCGAACAAATCGAATGAGCTTACAG CACCGTCGATCAAAAGCGGAACCATTTCTTCATGCATGGAATTGGTCGTTCA ATACGTTAAAAACACAATATGAAGGATATTCATGATGCAGGATATACAGCC ATTCAGACATCTCCGATTAACCAAGTAAAGGAAGGGAATCAAGGAGATAA AAGCATGTGGAAGTGGTACTGGCTGTATCAGCCGACATCGTATCAAAATGG CAACCGTTACTTAGGTACTGAACAAGAATTTAAAGAAATGTGTGCAGCCG CTGAAGAATATGGCATAAAGGTCATTGTTGACGCGGTTCATCAATCATACCA CCAGTGATTATGCCGCGATTTCCAATGAGGTTAAGAGTATTCCAAACTGGA CACATGGAAACACACAAATTA AAAA ACTGGTCTGATCGATGGGATGTCACG CAGAATTCATTGCTCGGGCTGTATGACTGGAATACACAAAATACACAAGT ACAGTCCTATCTGAAACGGTTCTTAGACAGGGCATTGAATGACGGGGCAG ACGGTTTTTCGATTTGATGCCGCCAAACATATAGAGCTTCCAGATGATGGCA GTTACGGCAGTCAATTTTGCCGAATATCACAAATACATCTGCAGAGTTCC AATACGGAGAAATCCTGCAGGATAGTGCCTCCAGAGATGCTGCATATGCG AATTATATGGATGTGACAGCGTCTAACTATGGGCATTCCATAAGGTCCGCT TTAAAGAATCGTAATCTGGGCGTGTGCAATATCTCCCACTATGCATCTGAT GTGTCTGCGGACAAGCTAGTGACATGGGTAGAGTCGCATGATACGTATGC CAATGATGATGAAGAGTCGACATGGATGAGCGATGATGATATCCGTTTAG GCTGGGCGGTGATAGCTTCTCGTTCAGGCAGTACGCCTCTTTTCTTTTCCAG ACCTGAGGGAGGCGGAAATGGTGTGAGGTTCCCGGGGAAAAGCCAAATAG GCGATCGCGGGAGTGCTTTATTTGAAGATCAGGCTATCACTGCGGTCAATA GATTTCACAATGTGATGGCTGGACAGCCTGAGGAACTCTCGAACCCGAAT GGAAACAACCAGATATTTATGAATCAGCGCGGCTCACATGGCGTTGTGCTG GCAAATGCAGGTTTCATCCTCTGTCTCTATCAATACGGCAACAAAATTGCCT GATGGCAGGTATGACAATAAAGCTGGAGCGGGTTCATTTCAAGTGAACGA TGGTAAACTGACAGGCACGATCAATGCCAGGTCTGTAGCTGTGCTTTATCC TGATGATATTGCAAAAGCGCCTCATGTTTTCTTGAGAATTACAAAACAGG TGTAACACATTCTTTCAATGATCAACTGACGATTACCTTGCGTGCAGATGC GAATACAACAAAAGCCGTTTATCAAATCAATAATGGACCAGAAACGGCGT TTAAGGATGGAGATCAATTCACAATCGGAAAAGGAGATCCATTTGGCAA ACATACACCATCATGTTAAAAGGAACGAACAGTGATGGTGTAAACGAGGAC CGAGAAATACAGTTTTGTTAAAAGAGATCCAGCGTCGGCCAAAACCATCG GCTATCAAAATCCGAATCATTGGAGCCAGGTAAATGCTTATATCTATAAAC ATGATGGGAGCCGAGTAATTGAATTGACCGGATCTTGGCCTGGA AAACCA ATGACTAAAAATGCAGACGGAATTTACACGCTGACGCTGCCTGCGGACAC GGATACAACCAACGCAAAAGTGATTTTTAATAATGGCAGCGCCCAAGTGC CCGGTCAGAATCAGCCTGGCTTTGATTACGTGCTAAATGGTTTATATAATG ACTCGGGCTTAAGCGGTTCTCTTCCCCATTGAGGATCCTGAGACCTGAGAC GGCAT

Table A-7. Type 3b parts (continued).

Name	Sequence
<i>glS</i>	GCATCGTCTCATCGGTCTCATTCTATGCCTTATCTGAAACGAGTGTTGCTGC TTCTTGTCACCTGGATTGTTTATGAGTTTGTTTGACGTCACCTGCTACTGCCTC AGCTCAAACAGGTGGATCGTTTTTTGACCCTTTTAACGGCTATAACTCCGG TTTTTGGCAAAAAGCAGATGGTTATTCGAATGGAAATATGTTCAACTGCAC GTGGCGGGCTAATAACGTATCAATGACGTCATTGGGTGAAATGCGTTTAGC GCTAACAAGCCCAGCTTATAACAAGTTTGACTGCGGGGAAAACCGTTCTGT TCAAACATATGGCTATGGACTTTATGAAGTCAGAATGAAACCAGCTAAAA ACACAGGGATCGTTTCATCGTTCTTCACTTACACAGGTCCAACAGATGGAA CTCCTTGGGATGAGATTGATATCGAATTTTTAGGAAAAGACACAACGAAG GTTCAATTTAACTATTATACAAATGGTGCAGGAAACCATGAGAAGATTGTT GATCTCGGGTTTGATGCAGCCAATGCCTATCATACGTATGCATTGATTGG CAGCCAACTCTATTAAATGGTATGTCGACGGGCAATTAACATACTGCA ACAAACCAAAATCCGACAACACCTGGAAAGATCATGATGAAGTTGTGGAA TGGCACGGGTGTCGATGAATGGCTTGGCTCCTACAATGGTGTAATCCGCT ATACGCTCATTATGACTGGGTGCGCTATACAAAAAATAAGGATCCTGAG ACCTGAGACGGCAT
<i>mjDOW</i>	GCATCGTCTCATCGGTCTCATTCTATGAAGGGAACCTACTACATCAACCAC GGTGACCCATTGATGTACTTGAAGAAGCACATCAAGTTGAGACAATTCTTG GAAGGTTGGCAAGAAAACGTTGTTATCGAAAAGCCAAAGTCTATCTTGAT CATCAGTGACACTGGGACACCAACGTTCCAACCGTTAACTTCGTTGAACA CTGTGACACCATCCACGACTTCGACGACTACCCAGACCCATTGTACCAAAT CCAATACCGCGCTCCAGGTGCTCCAACTTGGCTAAGAAGGTTGAAGAATT GCTTAAGGAATCTGGTATGGAATGTGAAATCGACACCAAGAGAGGTTTGG ACCACGCTGCTTGGTTCCCATTTGATGTTTCAATGTACCCAGAAGCTAACATCC CAATCTGTGAATTGTCTGTTCAACCATCTAAGGACGGTATCCACCACTACA ACGTTGGTAAAGCTTTGTCTCCATTGTTGCAACAAGGTGTTTTGATCATCG GTTCTGGTGGAACCGTTCAACCATCTGACGACACCCACACTGTCCAAACG GTGTTGCTCCATGGGCTATCGAGTTTCGACAACCTGGTTGGAGGACGCTTTC TCTCGGGTAGATACGAGGACGTTAACAACCTTCAAGAAGTTGGCTCCAACT GGGAAATCTCTACCCAGGTCAAGAACACTTGTACCCATTGCACGTTGCTT TGGGTGCTGCTGGTAAGAACCCAAAGACCCAATTGATCCACCGGTCTTGGG CTGCTAACGGTGTTCGGTTACTCTACCTACAACCTTACCCCCAACCCCA AAAGACCGACGGATCCTAAGGATCCTGAGACCTGAGACGGCAT

List of Symbols, Abbreviations, and Acronyms

AI	Artificial Intelligence
ARL	Army Research Laboratory
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
bp	Base Pairs
CDS	Coding Sequence
CFU	Colony-Forming Unit
DEVCOM	U.S. Army Combat Capabilities Development Command
DNA	Deoxyribonucleic Acid
DO	Dropout
DoW	Department of War
<i>E. coli</i>	<i>Escherichia coli</i>
FA	Final Assembly
FACS	Fluorescence-Activated Cell Sorting
FSC	Forward Scatter
GC	Guanine Cytosine
gRNA	Guide Ribonucleic Acid
ICE	Integrative and Conjugative Element
LB	Luria–Bertani
MIC	Minimum Inhibitory Concentration
ML	Machine Learning
PCR	Polymerase Chain Reaction
RBS	Ribosome Binding Site
sfGFP	Super Folder Green Fluorescent Protein
SP	Signal Peptide
SRP	Signal Recognition Particle
SSC	Side Scatter
TU	Transcriptional Unit
YTK	Yeast Toolkit

1 DEFENSE TECHNICAL
(PDF) INFORMATION CTR
DTIC OCA

1 DEVCOM ARL
(PDF) FCDD RLB CB
TECH LIB