Introduction

A difficult problem in engineering Gram-negative bacteria is the directed secretion of proteins to the extracellular environment (Wittrup, 2000; Harvey et al., 2004; Lee et al., 2006; Zhang et al., 2006). There are few secretion systems that are capable of exporting proteins through both the inner and outer membrane. This is an important tool for several applications in biotechnology. For example, the expression of some recombinant proteins at high titers can lead to the formation of inclusion bodies or retard cell growth (Sorensen and Mortensen, 2005). Secrecion is also critical when the function of nontarget proteins at high titers can lead to the formation of inclusion bodies or retard cell growth (Sorensen and Mortensen, 2005). Secretion is also critical when the function of nontarget proteins at high titers can lead to the formation of inclusion bodies or retard cell growth (Sorensen and Mortensen, 2005).

The T3SS is unique because it a well-characterized protein secretion system that translocates polypeptides through both the inner and outer membranes. This is in contrast to the Sec and Tat pathways, which deliver proteins to the periplasm (Georgiou and Segatori, 2005; Wickner and Schekman, 2005). Both types of signal peptides are demonstrated with up to 14% of expressed protein secreted. This work introduces new parts to control protein secretion in Gram-negative bacteria, which will be broadly applicable to problems in biotechnology.

The type III secretion system (T3SS) exports proteins from the cytoplasm, through both the inner and outer membranes, to the external environment. Here, a system is constructed to harness the T3SS encoded within Salmonella Pathogeneity Island 1 to export proteins of biotechnological interest. The system is composed of an operon containing the target protein fused to an N-terminal secretion tag and its cognate chaperone. Transcription is controlled by a genetic circuit that only turns on when the cell is actively secreting protein. The system is refined using a small human protein (DH domain) and demonstrated by exporting three silk monomers (ADF-1, -2, and -3), representative of different types of spider silk. Synthetic genes encoding silk monomers were designed to enhance genetic stability and codon usage, constructed by automated DNA synthesis, and cloned into the secretion control system. Secretion rates up to 1.8 mg l⁻¹ h⁻¹ are demonstrated with up to 14% of expressed protein secreted. This work introduces new parts to control protein secretion in Gram-negative bacteria, which will be broadly applicable to problems in biotechnology.

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Type II secretion can export proteins from the periplasm through the outer membrane; however, the secretion signal is difficult to identify and seems to be distributed throughout the protein, making heterologous protein secretion difficult (Pohlschroder et al., 2005). Remarkably, it has been shown that all of the genes associated with the Erwinia type II secretion system can be transferred to Escherichia coli and used to secrete Erwinia cellulases (Zhou et al., 1999). Alternatively, Gram-positive bacteria offer a single secretion event to the extracellular space and offer an attractive platform for secretion engineering.

In its natural context, the SPI-1 T3SS functions as a molecular syringe to inject effector proteins into mammalian host cells that facilitate invasion and pathogenesis (Altier, 2005). The SPI-1 T3SS forms a needle-like structure that crosses both the inner and outer membranes (Marlovits et al., 2006). A chaperone is required for secretion, as well as an N-terminal peptide tag that is not cleaved post secretion (Galan and Collmer, 1999). Secreted proteins can be folded in the cytoplasm and then are unfolded in an ATP-dependent reaction before secretion (Feldman et al., 2002; Lee and Schneewind, 2002; Akeda and Galan, 2005). It is expected based on needle dimensions that proteins must be at least partially unfolded to transit through the needle and would be required to re-fold outside the cell. The E. coli and Salmonella flagellum and Yersinia enterocolitica ysc T3SS have been shown to be able to export heterologous proteins (Russmann et al., 1998; Feldman et al., 2002; Lee and Schneewind, 2002; Majander et al., 2005; Chen et al., 2006; Konjuftca et al., 2006; Vegh et al., 2006). These systems have been used to inject foreign proteins and peptides into mammalian cells as a mechanism to confer immunity (Russmann et al., 1998; Boyd et al., 2000; Konjuftca et al., 2006).

A well-characterized regulatory network encoded within SPI-1 controls the dynamics of T3SS gene expression (Box 1) (Lucas and Lee, 2000). Environmental signals from two-component systems and global regulators control the expression of the HilC, HilD, and HilA transcription factors, which together form a commitment circuit for the expression of SPI-1 genes (Baja et al., 1996; Eichelberg and Galan, 1999; Lundberg et al., 1999; Kalir et al., 2001; Ellermeier et al., 2005). Within SPI-1, there is a genetic circuit that links the expression of effector proteins to the completion of functional needles (Darwin and Miller, 1999, 2000, 2001; Temme et al., 2008). The circuit consists of a transcription factor (InvF) that is only functional when bound to the SicA chaperone protein. Before the cell can secrete protein, the chaperone is sequestered by the SipB/C proteins. After functional needles are completed, SipB/C are secreted and SicA is free to bind InvF, thus turning on the circuit and gene expression from the sicA promoter (Darwin and Miller, 1999, 2000, 2001; Tucker and Galan, 2000).

The Salmonella SPI-1 T3SS has several properties that make it a good tool for the secretion of recombinant proteins. It is highly expressed under standard laboratory conditions (Luria-Bertani Broth at 37°C), with 10–100 needles per cell (Kubori et al., 1998). Under these conditions, effector proteins are secreted into the media in significant quantities without the need to co-culture with mammalian cells or expensive media components (Kubori and Galan, 2002). Finally, the N-terminal secretion tags, chaperone-binding domains (CBD), and chaperones have been identified (Fu and Galan, 1998; Hong and Miller, 1998; Bronstein et al., 2000; Tucker and Galan, 2000; Russmann et al., 2002; Zhang et al., 2002; Ehrbar et al., 2003; Lee and Galan, 2004; Wood et al., 2004; Karavolos et al., 2005; Higashiide and Zhou, 2006; Knodler et al., 2006). On the basis of

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**Box 1** A genetic circuit from the SPI-1 regulatory pathway is harnessed to control expression

![Box 1](https://wwwpartsregistryorg/)

| A | B |
|---|---|
| SPI-1 Commitment circuit | Secretion-activated circuit |
| Secretion control system (pCASP) | |

**Box 1** The regulatory network controlling T3SS self-assembly is shown (yellow background) (panel A). Within this network, an operon containing the chaperones, translocators, and effectors is controlled by a genetic circuit that becomes active once the T3SS is constructed and functional (green background, centered on invF and sicA). A secretion control system is constructed that contains all of the necessary parts for the T3SS to export heterologous proteins (orange background). The sicA promoter and ribosome-binding site drive the expression of the chaperone (SicP) and heterologous protein (green) fused to an N-terminal secretion signal (SptP). A TEV protease site is included after the tag such that it can be removed post secretion. The cryo-EM image of the T3SS is reproduced from reference (Marlovits et al., 2004). A map of the pCASP plasmid is shown (B). The secretion control system superpart is BBa_J64032 in the Registry of Standard Biological Parts (www.partsregistry.org) and the full pCASP sequence is available in Genbank (#EF179157).
In this earlier work, we have constructed a system that contains all of the necessary genetic parts to secrete heterologous proteins (Box 1). This system is demonstrated by using it to export a human protein and three spider silk monomers. Natural silks are abundant biomaterials that span a remarkable diversity of physical properties (Vollrath and Knight, 2001; Swanson et al., 2006). For example, the dragline silk of spider webs is extremely strong yet remains highly elastic. These materials have a number of uses including medical device implants, high strength fibers, advanced composites, and drug delivery systems (Lewis, 2006; Wang et al., 2006a, b; Hofmann et al., 2007; Lee et al., 2009). Spiders are not conducive to scalable agriculture, therefore requiring silk production to be done in a recombinant host (Lewis, 2006). Using a solution of natural or recombinant silk monomers, it has been shown that threads can be artificially spun, producing materials with properties approaching natural silks (Seidel et al., 2000; Lazaris et al., 2002). One of the key limitations in creating materials that match or exceed the natural properties is the lack of practical approaches for recombinant protein expression (Kluge et al., 2008).

In this paper, we use whole gene DNA synthesis to construct long, computationally designed DNA sequences that exactly match the wild-type amino-acid sequence for the known fragments of silk monomers (Figure 1). The amino-acid sequences of the synthetic spider silk genes are shown. Each silk sequence is labelled with a name and the gland in which it is produced. The repetitive regions used in the sequence entropy calculations are in red and green.

Figure 1 Changes made to the Araneus spider silk DNA sequences. (A) The codon frequency is a measure of the abundance of codon sequences in the E. Coli genome relative to each amino acid. The average frequency is the mean of the codon frequencies across the entire sequence of the silk gene. The average codon frequency is shown for the spider (gray) and synthetic (black) genes. Very rare codons (<10 per gene, defined as frequencies <0.13) were entirely eliminated from the sequences. (B) The DNA sequence entropy of the repetitive units is shown for the wild-type spider (gray) and synthetic (black) genes. The repeat units for each silk monomer were manually aligned (Supplementary information) and the sequence entropy is calculated: \[S = -\frac{1}{N} \sum_{i=1}^{N} p_i \log p_i,\] where \(N\) is the length of the repeat unit and \(p_i\) is the probability that base \(i\) (A, T, G, C) occurs at position \(j\). The maximum of this function (when all four bases are equally represented at each position) is \(H = \log 4\). A lower sequence entropy indicates a higher degree of sequence identity between the repeat units. ADF-3 has extremely repetitive DNA sequences and this repetitiveness is effectively eliminated upon optimization. (C) The amino-acid sequences of the synthetic spider silk genes are shown. Each silk sequence is labelled with a name and the gland in which it is produced. The repetitive regions used in the sequence entropy calculations are in red and green.
extremely tough and elastic dragline, which anchors the web and is used as a lifeline for escape. Each of the synthetic genes is expressed and exported from the cell using the *Salmonella* SPI-1 T3SS.

**Results**

Three spider silk genes (ADF-1, -2 and -3) were computationally designed for expression in eubacteria and constructed using automated DNA synthesis and assembly. The wild-type DNA sequences of the spider silk genes contain rare codons (Supplementary information), which can result in truncated proteins and poor expression (Gustafsson *et al.*, 2004). The sequences also consist of repeated amino-acid units that correspond to repetitive underlying DNA sequences. The variation in the repeat amino-acid units is one of the key features that differentiate natural silk fibres (Figure 1C). These regions can be very large with over 100 nucleotides of exact identity, which is a potential target for homologous recombination, resulting in genetic instability. The degeneracy of the genetic code allows many alternative nucleotide sequences to encode the same protein. This enables synthetic genes to be designed that simultaneously redistribute the codon usage, while reducing the DNA repetitiveness (Figure 1A and B). Each re-designed gene was constructed using automated whole gene DNA synthesis (Supplementary information).

We constructed a genetic system to secrete heterologous proteins into the culture media (Box 1). This system consists of four genetic parts. A circuit is harnessed from the natural SPI-1 regulatory pathway that controls gene expression based on the capacity of the cell to export protein (Darwin and Miller, 1999, 2000, 2001; Temme *et al.*, 2008). The output of this circuit (PsicA) drives the transcription of an operon containing the heterologous protein fused to an N-terminal secretion tag and the associated chaperone. The human DH domain (Hussain *et al.*, 2001) was chosen as an initial target because it is small (24 kDa), easy to manipulate, and it expresses well in *Salmonella* without affecting cell growth. A protease cleavage site is included between the tag and exported protein so that the tag can be removed after secretion. All of these parts are combined onto a plasmid (Box 1) and are available in the Registry of Standard Biological Parts (http://www.partsregistry.org/). The part numbers (BBa_) are provided throughout the text.

Using the SicA:InvF circuit to control expression has two advantages. First, it restricts expression until the T3SS is built. This allows synthetic genes to be designed that simultaneously redistribute the codon usage, while reducing the DNA repetitiveness (Figure 1A and B). Each re-designed gene was constructed using automated whole gene DNA synthesis (Supplementary information).

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Using the SicA:InvF circuit to control expression has two advantages. First, it restricts expression until the T3SS is built and is functional (Darwin and Miller, 1999, 2000, 2001). This links expression to secretion capacity and can reduce the build-up of protein before secretion. The second advantage is that no exogenous inducer is required. The circuit can be maintained in the off state when cells are grown in low salt L-Broth (LB) and then turned on after the cells are induced by high salt LB (inducing medium) and the needle is constructed (Figure 2A) (Materials and methods) (Tartera and Metcalf, 1993). The sicA promoter has a low basal transcription rate and increases 200-fold in activity when induced. Additionally, the sicA promoter has no activity in *E. coli* under inducing conditions, which is a useful trait for cloning purposes (Figure 3A).

The sicA promoter turns on within 3 h after cells are shifted into inducing media (Figure 2A) (Temme *et al.*, 2008). At this point, secreted protein begins to accumulate in the media (Figure 2A). This can be clearly visualized in a Coomassie gel (Figure 3D) along with other proteins naturally secreted by *Salmonella*. There was no observable lysis or outer membrane shedding in the secretion assay, as determined using an antibody against the MalE periplasmic protein (Majander *et al.*, 2005).

There are multiple chaperones that target proteins to the SPI-1 T3SS and they interact with different N-terminal tags (Table I). A secretion assay was performed for the known tag/chaperone pairs to compare the amount of protein that is exported to the supernatant. This was repeated for each protein in this study to identify the optimal combination (Figure 2B; Supplementary information S2) (Materials and methods) (Collazo and Galan, 1996). The SpIP/SicP combination was found to yield the largest amount of secreted protein for DH, ADF-2 (Figure 2B), and ADF-3 (Supplementary information S2). For ADF-1, the optimum pair was SopB/SigE.

The SpIP tag consists of a 15 amino-acid signal sequence and 152 amino-acid CBD (Lee and Galan, 2004). The SptP CBD interacts with SicP chaperone dimers, which direct the SptP-tagged protein to the SPI-1 T3SS (Stebbins and Galan, 2001; Akeda and Galan, 2005). It has been shown that the signal sequence directs the protein generally to T3SSs and flagella. The CBD causes the protein to be directed specifically to the SPI-1 T3SS (Lee and Galan, 2004). The T3SS secretion signal is not self-cleaving. A tobacco etch virus (TEV) protease site is added after the tag so that it can be removed after export.

Variants of the pCASP plasmid were constructed by removing the tag and chaperone parts and tested for the loss of secretion. When expressed without the N-terminal SptP tag, there is a dramatic decline in the amount of DH protein that appears in the supernatant (Figure 3A). The deletion of the tag from the silk monomers results in 100-fold less protein in the supernatant (Table II). Secretion is also reduced when the SicP chaperone is only expressed at wild-type levels and not co-transcribed on the plasmid (-SicP). We tested the effect of expressing the chaperone in conjunction with a (-) tag protein in the pCASP system. The construct was run using the secretion assay and analyzed by western blot (Figure 3B).

Secretion was also tested for *Salmonella* strains where components of the SPI-1 T3SS and flagellum have been knocked out (Figure 3C). The sicA promoter differs in activity for these knockouts, so a plasmid was constructed where the chaperone and tagged protein are under the control of an IPTG-inducible promoter. There was little effect when the flagella master regulators (AflF/DC) are knocked out. However, when critical structural components of the SPI-1 T3SS are knocked out (AflgH/JK/ABC), secretion is eliminated. These results were confirmed using a set of earlier constructed knockout strains (ΔinvA and ΔflfGHI) (Supplementary information) (Lee and Galan, 2004). Together, these data indicate that the observed protein secretion is SPI-1 dependent.

Each synthetic silk gene was ligated into the pCASP secretion plasmid with the optimal tag/chaperone pair and
plasmid under the control of the chaperone pair is shown. All known SPI-1 N-terminal tag and chaperone pairs are tested for the secretion of ADF-2 (Table I). Each pair was cloned into the pCASP lysate samples is shown. The same comparison was made for each remaining silk protein (Supplementary information).

superimposed on the graph indicating the activation of the transcription of an operon containing the protein to be secreted, which is fused to the SptP N-terminal secretion signal. The SicP chaperone is also encoded, which binds and directs the heterologous protein to the SPI-1 T3SS.

The yields of secreted protein using type III secretion (Table II) are as good or better than other methods of extracellular protein production (Choi and Lee, 2004). Earlier reported values range from 0.5 to 10 mg l\(^{-1}\) h\(^{-1}\) in shake flasks using a range of protein export and recovery methods (Lucic et al, 1998; Tong et al, 2000; Fu et al, 2003). Unlike the Sec and Tat pathways, the T3SS translocates proteins through both membranes to the extracellular environment.

Natural Yersinia, Shigella, and Salmonella effector proteins have been observed to accumulate up to grams per liter in the growth media. The secretion rate of the SPI-1 T3SS has been measured in individual cells to be 7–60 proteins per cell per second when secreting a natural effector (Enninga et al, 2005; Schlumberger et al, 2005). This corresponds to an approximate theoretical yield of 10 mg l\(^{-1}\) h\(^{-1}\) OD for a protein the size of ADF-2. Our system is able to achieve ~10% of this yield for the spider silk ADF-2 in a shake flask culture.

Whole gene DNA synthesis is a useful tool to make large-scale changes to genes. In this work, three spider silk genes were computationally designed for expression in eubacteria and constructed using automated DNA synthesis and assembly. Only the amino-acid sequence information is required for gene construction, rather than physical genetic material from the source organism. The degeneracy of the genetic code allows many alternative nucleotide sequences to encode the same protein. This enables synthetic genes to be designed that simultaneously match the recombinant host’s codon usage, while reducing the DNA repetitiveness. As of their highly repetitive amino-acid sequences, silk monomers represent one of the most challenging targets of automated DNA synthesis and assembly. The three synthetic ADF genes built here...
represent all of the known sequence information for these silks, but they are still fragments of the complete silk genes. The ability to construct the wild-type genes from information alone will make it possible to further explore—and modify—the amazing diversity of natural materials. More broadly, declining automated synthesis costs make it possible to rapidly construct large libraries of proteins, enzymes, or pathways from many diverse organisms using only information retrieved from sequence databases (Bayer et al., 2009). This use of synthetic metagenomics could be particularly applicable to areas of material space such as spider silks, when cDNA is difficult to obtain from the natural source and when isolated is unstable in recombinant hosts. Concurrently, it enables the large-scale modification of the sequences for expression in a recombinant host. This approach will revolutionize how natural diversity is explored when engineering cells.

**Table 1** SPI-1 N-terminal tags and chaperones

| Tag | Length $^a$ | Chaperone | Part numbers $^b$ | References |
|-----|------------|-----------|------------------|------------|
| SipA | 169 | InvB | BBa_J64035 | Bronstein et al. (2000) |
| SipC | 167 | SicA | BBa_J64040 | Tucker and Galan (2000) |
| SopE2 | 96 | InvB | BBa_J64037 | Higashide and Zhou (2006) |
| SopE2 | 105 | InvB | BBa_J64038 | Ehrhar et al. (2008) and Karavolos et al. (2005) |
| SptP | 167 | SicP | BBa_J64008 | Fu and Galan (1998) |
| SopB | 168 | SigE | BBa_J64041 | Hong and Miller (1998) and Knodler et al. (2006) |
| SopD | 40 | none | BBa_J64042 | Wood et al. (2004) and Zhang et al. (2002) |

$^a$Number of N-terminal amino acids included in the tag.
$^b$The part number from the Registry of Standard Biological Parts (http://www.partsregistry.org/). These numbers correspond to superparts that include the chaperone, ribosome-binding site, and the N-terminal tag.

The ability to construct the wild-type genes from information alone will make it possible to further explore—and modify—the amazing diversity of natural materials. More broadly, declining automated synthesis costs make it possible to rapidly construct large libraries of proteins, enzymes, or pathways from many diverse organisms using only information retrieved from sequence databases (Bayer et al., 2009). This use of synthetic metagenomics could be particularly applicable to areas of material space such as spider silks, when cDNA is difficult to obtain from the natural source and when isolated is unstable in recombinant hosts. Concurrently, it enables the large-scale modification of the sequences for expression in a recombinant host. This approach will revolutionize how natural diversity is explored when engineering cells.
Table II  Yields of expressed and secreted silk (nmol-1 h-1)\textsuperscript{a}

|        | + Tag\textsuperscript{b} | - Tag\textsuperscript{c} |
|--------|--------------------------|--------------------------|
| Mass (kDa) | Super\textsuperscript{d} | Lysate | Super\textsuperscript{d} | Lysate |
| ADF-1   | 30.8 | 2.9 ± 1.0 | 31 ± 5 | 0.01 ± 0.006 | 34 ± 5 |
| ADF-2   | 25.0 | 70 ± 15 | 410 ± 80 | 0.4 ± 0.1 | 200 ± 30 |
| ADF-3   | 56.2 | 6.9 ± 0.4 | 90 ± 21 | 0.02 ± 0.006 | 68 ± 22 |

\textsuperscript{a}The reported numbers are the mean of four (−tag ADF-2) independent measurements and the error is 1 s.d.

\textsuperscript{b}Rates are shown when the silk monomer is fused to the N-terminal secretion tag (+ tag) and when expressed without the tag (−tag).

\textsuperscript{c}Rates for the secreted protein collected in the supernatant (super) and nonsecreted protein (lysate) are shown.

\textsuperscript{d}When expressed without the tag, the protein is only detectible in the supernatant upon 30 × concentration (Materials and methods).

Materials and methods
See Supplementary information for detailed Materials and methods.

Supplementary information
Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

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