# Comprehensive Engineering of *Escherichia coli* for Enhanced Expression of IgG Antibodies

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## Abstract

The expression of IgG antibodies in *Escherichia coli* is of increasing interest for analytical and therapeutic applications. In this work, we describe a comprehensive and systematic approach to the development of a dicistronic expression system for enhanced IgG expression in *E. coli* encompassing: (i) random mutagenesis and high-throughput screening for the isolation of overexpressing strains using flow cytometry, and (ii) optimization of translation initiation via the screening of libraries of synonymous codons in the 5' region of the second cistron (heavy chain). The effects of different promoters and co-expression of molecular chaperones on full-length IgG production were also investigated. The optimized system resulted in reliable expression of fully assembled IgG at yields between 1 and 4 mg/liter of shake flask culture for different antibodies.

### Keywords

*Escherichia coli*; strain optimization; IgG expression; chemical mutagenesis; fluorescence-activated cell sorting (FACS); translational initiation region (TIR)

## 1. Introduction

Monoclonal antibodies occupy a prominent position in the drug market and account for over $40 billion in annual sales. There are currently more than 30 commercially available

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antibody-based therapeutics, including the anti-cancer drugs Herceptin® and Avastin® and the anti-inflammatory drugs Remicade® and Humira®. Currently, more than 280 antibody-based therapeutics are being evaluated in clinical trials (Strohl & Knight, 2009).

Monoclonal antibodies can be expressed either as full-length IgG molecules or as antibody fragments (scFv, Fab or F(ab)$_2$; (Andersen & Reilly, 2004, Humphreys, 2003)). Antibody fragments lack part of or the entire constant region (domains $C_H1$-$C_H3$ and $C_{\kappa,\lambda}$) and therefore fold more efficiently and can be produced at high yields in microbial hosts (Andersen & Reilly, 2004, Humphreys, 2003). Full-length antibodies, however, are often the preferred therapeutic antibody format due to the properties mediated by the $F_C$ domain, namely, significantly longer circulation half-life in mammals and antibody-dependent cell cytotoxicity and complement-dependent cell cytotoxicity (ADCC and CDC, respectively; (Mariani, 1990, Junghans, 1997)). Because the latter two properties depend on glycosylation and because of their relative complex disulfide bond pattern, full-length antibodies are typically expressed in mammalian cell lines, such as CHO or NSO cells (de la Cruz Edmonds *et al.*, 2006, Huang *et al.*, 2007, Liu *et al.*, 2008). However, the selection of stable cell lines appropriate for production is very time-consuming. Additionally, even though IgG yields in CHO cells can reach or exceed 10 g/L of culture, mammalian cell expression requires a long production cycle which in turn translates into much higher capital investment relative to microbial expression systems that benefit from much faster growth rates (Chartrain & Chu, 2008).

Early efforts to express full-length IgGs in bacteria failed, but more recently scientists at Genentech reported the expression of ~1 g/L of correctly folded IgG antibodies in *Escherichia coli*-fermented cultures (Simmons *et al.*, 2002, Reilly & Yansura, 2010). Bacterially expressed IgGs are aglycosylated and, therefore, do not display ADCC/CDC normally. However, aglycosylated antibody variants that exhibit unique abilities to engage cytotoxic innate immune cells have recently been engineered (Jung *et al.*, 2010). Antibodies lacking ADCC/CDC are in fact preferable in instances where the therapeutic function is receptor agonism or antagonism. Partly because of this reason and partly because of the lower capital investment and production costs, the bacterial expression of IgGs is attracting increasing interest and at least one *E. coli* expressed antibody is currently in advanced clinical development (http://www.gene.com/gene/pipeline/status/oncology/metmab/) (Martens *et al.*, 2006). Furthermore, full-length IgGs with tailored antigen-binding characteristics have been isolated from combinatorial libraries expressed in *E. coli* and screened by flow cytometry (Mazor *et al.*, 2007).

While significant yields of fully assembled IgG can be obtained by high-cell-density fermentation (Jung et al., 2010, Simmons et al., 2002), yields in shake flasks rarely exceed 1 mg of assembled full-length antibodies per liter of culture. Increasing the protein yield in shake flasks is particularly important in antibody discovery programs following combinatorial library screening where many variants have to be purified and examined individually for binding and biological function. Unlike large-scale expression which is more efficient when the heavy and light chains are expressed from different promoters on different plasmids (Simmons et al., 2002), in library screening experiments it is preferable that the heavy and light chains be expressed from a single promoter as a dicistronic operon (Mazor et al., 2007).

In this work, we describe a systematic program to enhance IgG yields from a dicistronic expression system in shake flasks by addressing all aspects relevant to protein synthesis and folding in bacteria, namely: (i) effect of promoter strength and signal peptide sequence for the transcription and membrane translocation, respectively, for both the light and heavy chains; (ii) host strain improvement by chemical mutagenesis and high-throughput screening

for enhanced expression; (iii) optimization of translation initiation for the second cistron of the dicistronic operon (heavy chain) via mutagenesis of the 5' region; and (iv) evaluation of the effects of molecular chaperones on IgG yield. In this manner, we were able to obtain up to 6-fold higher yields of full-length IgG compared to wild-type *E. coli* cells, and the engineered system to produce ~4 mg/L of properly assembled IgG in shake flask culture.

## 2. Materials and Methods

### 2.1 Reagents, Strains and Plasmids

All DNA modification enzymes were purchased from New England Biolabs. The *E. coli* strain JUDE-1 (DH10B (Invitrogen) harboring the 'F' factor derived from XL1-blue (Stratagene)) was utilized for all experiments unless otherwise mentioned. The construction of the IgG expression vectors pMAZ360-M18.1-IgG, pMAZ360-26.10-IgG and pMAZ360-YMF10-IgG has been described previously (Mazor et al., 2007). pMAZ360-9c8-IgG was generated by swapping the $V_H$ and $V_L$ of M18.1-IgG in pMAZ360-M18.1-IgG with those of 9c8-IgG by using the restriction sites *Nco*I/*Not*I and *Nhe*I/*Hind*III, respectively (Sean Carroll and GG, unpublished results). Molecular chaperones and other folding factors were expressed either from pBAD33-based plasmids (Guzman *et al.*, 1995) or from pAR3-based plasmids (Perez-Perez & Gutierrez, 1995). *dsbA*, *dsbC*, *fkpA*, *fkpA-dsbA*, and *tig* were expressed from pBAD-DsbA (laboratory stock), pBAD-DsbC (laboratory stock), pBAD-FkpA (Arredondo *et al.*, 2008), pBAD-FkpA-DsbA (Arredondo et al., 2008), and pBAD-Tig (Levy *et al.*, 2001), respectively. *dnaK/dnaJ*, *groEL/groES*, *HSPA5* (encoding for BiP), *secB*, and *secY/secE* were expressed from pAKJ (Perez-Perez *et al.*, 1995), pAG (Perez-Perez et al., 1995), pAR3-BiP (laboratory stock), pAB (Perez-Perez et al., 1995), and pAA4E (Perez-Perez et al., 1995), respectively. The gene encoding for Skp was amplified from *E. coli* genomic DNA with gene-specific DNA primers and was cloned into pBAD33 by using the restriction sites *Xba*I-*Hind*III. The *ppiB* gene encoding for mouse cyclophilin B (peptidylprolyl isomerase B) was synthesized by gene assembly PCR using codon-optimized oligonucleotides for *E. coli* expression designed with the assistance of the software DNAWorks (http://helixweb.nih.gov/dnaworks/). Following assembly, the gene was cloned into the available *Sac*I and *Xba*I site of pBAD33 fused with the *pelB* leader sequence to its N terminus.

### 2.2 IgG overexpression

*E. coli* cells were grown in modified NU medium (Mori, 1979) (4 g/L $KH_2PO_4$, 4 g/L $K_2HPO_4$, 7 g/L $Na_2HPO_4$, 1.2 g/L $NH_4Cl$, 1.2 g $(NH_4)_2SO_4$, 4 g/L yeast extract, 0.2 % $MgSO_4$, 0.5 % glycerol, 0.05 % glucose). Single bacterial colonies were used to inoculate liquid NU overnight cultures containing the appropriate antibiotics (100 µg/mL ampicillin with and without 40 µg/mL chloramphenicol). The following day, cultures were diluted 1:50 in 4 ml of fresh NU media and grown at 37 °C to an optical density at 600 nm ($OD_{600}$) of 0.7 with shaking. At that point, the temperature was decreased to 25 °C and after a temperature equilibration period of 5–20 min, protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for approximately 16 h. When molecular chaperones and other folding factors were co-expressed, 0.01% of L-arabinose was added to liquid medium prior to inoculation of the cells from the overnight culture.

### 2.3 Fluorescent Labeling and fluorescence-activated cell sorting (FACS)

Digoxigenin-dipyrromethene boron difluoride (digoxigenin-BODIPY) was purchased from Invitrogen. For labeling, cells were harvested by centrifugation and resuspended in cold 5X Tris-KCl buffer (250 mM Tris-HCl, pH 7.4, and 750 mM KCl) (Sarkar *et al.*, 2008) and 200 nM digoxigenin-BODIPY followed by 1 h incubation at room temperature with shaking.

Flow cytometry and sorting were carried out essentially as described previously (Skretas & Georgiou, 2009). Briefly, the cell fluorescence (530/30 nm) exhibited upon fluorescent antigen binding to IgG was monitored using a Becton-Dickinson FACSAria™ flow cytometer and analyzed with FACSDiva software. For FACS screening, cells were initially gated on a side-scatter (SSC-H) versus forward-scatter (FSC-H) plot. $2\times10^5$ clones corresponding to the top 1–3% fluorescent events were isolated, grown on LB agar plates with the appropriate antibiotics, harvested, and then grown in liquid NU media as described above, and subjected to repeated rounds of PECS screening using FACS.

## 2.4 SDS-PAGE and Western blotting

Cells were lysed by brief sonication cycles over ice and the lysate was clarified by centrifugation (12,000 X g for 20 min) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were normalized so that each lane contained an amount of total protein corresponding to the same number of cells as judged by $OD_{600}$ measurements. SDS–PAGE was performed on 4–20 or 12% gels under reducing (2.5% (v/v) 2-mercaptoethanol) and non-reducing conditions and gels were stained with GelCode Blue® (Pierce). For Western blot analyses, proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 16 h at 110 mA or 2 h at 350 mA. Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween-20 (PBST) for approximately 1 h at room temperature. Following a triple washing step with PBST, membranes were incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories) in PBST containing 0.5% non-fan dry milk at room temperature for approximately 1 h. After triple washing with PBST again, the probed proteins were visualized on X-ray film with SuperSignal West Pico chemiluminescent substrate (Pierce). The intensity of the band corresponding to fully assembled IgG was compared with serially diluted IgG1 standard (clinical grade trastuzumab antibody (anti-p185HER2, 4D5; kindly provided by Michael Rosenblum). Densitometry was carried out using a Quantity One™ (Biorad) densitometer.

## 2.5 Enzyme-linked immunosorbent assay (ELISA)

50 µl/well of 4 µg/ml solutions of digoxigenin-bovine serum albumin (BSA) (Invitrogen) were used to coat 96-well ELISA plates (Corning Incorporated) for 16 h at 4 °C with shaking followed by blocking with 50 µl/well of a 2% nonfat milk solution in PBS (PBSM) and shaking at room temperature for 2 h. Clarified cell lysates were diluted in PBSM so that each sample contained total protein corresponding to an equal number of cells as judged by $OD_{600}$. 50 µl of protein samples were added to the wells and allowed to bind for 1 h at room temperature followed by triple washing step with PBS + 0.5% Tween-20 (PBST). 50 µl of HRP-conjugated goat anti-human IgG (heavy and light chain) antibodies (Jackson ImmunoResearch Laboratories) diluted 1:4,000 into PBSM were then added and allowed to bind for 1 hour with shaking. After discarding the content of the wells, ELISA plates were visualized by the addition of 50 µl/well of Onestep™ Ultra TMB-ELISA (Thermo Scientific, Waltham, MA) and quenched with the addition of 50 µl/well of 2 N $H_2SO_4$.

## 2.6 Chemical Mutagenesis

Chemical mutagenesis was carried out essentially as described in previous work (Zhan *et al.*, 2004). Briefly, JUDE-1 cells harboring pMAZ360-26.10-IgG were grown in LB medium containing 100 µg/ml of ampicilin to an $OD_{600}$ of ~0.3. The cells contained in 5 ml of culture were collected by centrifugation at $2,800 \times g$ for 10 min, washed twice with cold citrate buffer (0.1M Sodium Citrate, pH=5.5) and resuspended in 1.9 ml of the same buffer, and treated with 50 µg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma) at

37°C for 15, 30 and 45 min. After MNNG treatment, the cells were mixed, washed twice with phosphate buffer (0.1 M $KH_2PO_4$, pH=7.0) and resuspended in 2 ml of the same buffer.

## 2.7 Construction of translational initiation region (TIR) libraries

A QuickChange® sire-directed mutagenesis kit (Stratagene) was used to introduce *Spe*I and *Stu*I restriction sites into the pMAZ360-26.10-IgG vector that flanks the *pelB* leader sequence of the $V_L$-$C_k$ gene to generate pMAZ360-26.10-TIR. The double stranded DNA libraries of the heavy and light chain genes containing the randomized TIRs were prepared by annealing the forward and reverse oligonucleotides shown in Supplementary Materials and Methods. The theoretical diversity of each library was $\sim3.7\times10^4$. For the construction of the light chain library, pMAZ360-26.10-TIR was digested with *Spe*I and *Stu*I and ligated with similarly digested light chain dsDNA fragments using T4 ligase. For the construction of the heavy chain library, pMAZ360-26.10-TIR was digested with *Nde*I and *Nhe*I and ligated with similarly digested heavy chain dsDNA fragments using T4 ligase. The generated plasmid libraries were transformed into JUDE-1 competent cells together by electroporation yielding more than $10^6$ clones. The quality of each library was assessed by sequencing 40–60 library members and it was found that all contain distinct nucleic acid sequences without insertions or deletions.

## 2.8 IgG purification

IgG antibodies were overexpressed in 500 ml cultures as described above and were purified from the growth medium supernatant and the soluble fraction of cell lysates using protein A sepharose (Thermo scientific) affinity chromatography.

# 3. Results

## 3.1 Development of a FACS-based assay for monitoring IgG expression at the single-cell level

We developed an assay for monitoring antibody expression at the single-cell level via flow cytometry by modifying a technique developed earlier by our group termed periplasmic expression followed by cytometric sorting (PECS) (Chen *et al.*, 2001). Briefly, *E. coli* cells expressing full-length IgGs in the periplasm are incubated in a high-osmolarity buffer that renders their outer membrane permeable to antigens (Fig. 1A). Addition of an excess of a fluorescently labeled small-molecule antigen allows the antigen to equilibrate into the periplasmic space and bind to the properly assembled IgG molecules. Thus, cell fluorescence is proportional to the number of functional, antigen-binding molecules in the periplasm. Clones containing mutations that increase IgG expression, display higher fluorescence and can be isolated by FACS. An important characteristic of PECS is that despite outer membrane permeabilization, the bacterial cells retain their viability and sorted cells can thus be immediately subjected to additional rounds of screening (Figure 1A).

Full-length and properly assembled IgGs have been expressed in *E. coli* from a dicistronic operon consisting of the light ($V_L$-$C_L$) and heavy ($V_H$-$C_H1$-$C_H2$-$C_H3$) chain genes fused with leader peptides that target both chains for periplasmic export under the control of an inducible promoter (Mazor et al., 2007). For expression optimization studies, we used as a model the IgG1 variant of the 26.10 scFv antibody which binds to the heart condition drug digoxin and its aglycone digoxigenin with high affinity (Chen *et al.*, 1999, Mazor et al., 2007). This antibody was selected as a model system because both digoxin and digoxigenin are small-molecule antigens appropriate for use as ligands in PECS (molecular weights 781 and 391 Da, respectively).

Three different *E. coli* strains were evaluated for IgG expression: JUDE-1 (DH10B (Invitrogen) harboring the 'F' factor derived from XL1-blue (Stratagene)), MC4100A (Santini *et al.*, 2001), and KS303 (MC1000 *lpp* 5508; (Strauch & Beckwith, 1988)). The latter strain contains an *lpp* deletion and therefore lacks the major outer membrane lipoprotein, a defect that confers a significant increase in the permeability of the outer membrane without eliciting cell lysis (Ni *et al.*, 2007). Each of these strains was transformed with the vector pMAZ360-26.10-IgG, which expresses the 26.10 IgG heavy and light chains as fusions with a *pelB* export leader under the control of the *lac* promoter as part of a dicistronic operon (Mazor et al., 2007). Cells were grown at 25 °C, IgG expression was induced with IPTG, cells were labeled with 200 nM digoxigenin-BODIPY in 5X Tris-KCl (a buffer that results in optimized outer membrane permeabilization (Sarkar et al., 2008)), and bacterial fluorescence was analyzed using flow cytometry. Highest levels of fluorescence compared to a negative control were observed in JUDE-1 (Figure 1B and data not shown) and therefore this strain was selected for further studies.

The effect of different promoters (*lac*, *trc*, *tet*, *araBAD, T7*) and leader peptides (the Sec pathway-dependent leader ss*pelB* (Manting & Driessen, 2000) or the signal recognition particle (SRP) pathway-dependent signal sequence ss*dsbA* (Schierle *et al.*, 2003)) on IgG yield were evaluated by flow cytometry and Western blotting (Figure 1B and data not shown). These experiments showed that a *lac* promoter with ss*pelB* and ss*dsbA* export of light and heavy chains respectively (*lac-pelB-dsbA* plasmid), gave the highest level of assembled IgG (Figure 1B). However, this combination of leader peptides and promoters had a significant effect on cell growth and resulted in low final cell yields (Figure 1C). These effects were alleviated when the ss*dsbA* was replaced with ss*pelB* (Figure 1C). Therefore, we elected to use ss*pelB* for the export of both the heavy and the light chains.

## 3.2 Random mutagenesis and screening for chromosomal lesions that result in enhanced IgG yield

JUDE-1 cells carrying the pMAZ360-26.10-IgG vector were subjected to random mutagenesis using the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as described in section 2.6. The resulting mutants were pooled to form the pre-sort library, propagated in liquid NU medium, and IgG expression was induced by the addition of IPTG as described in section 2.2. After labeling with 200 nM digoxingenin-BODIPY, ~$10^7$ cells were subjected to PECS screening using FACS. The cells exhibiting the top 1–3% fluorescence were collected in liquid medium, plated, propagated in liquid medium again, and subjected to additional rounds of PECS. The percent viability, (number of colony forming units divided by the number of sorted events measured FACS) of the chemically mutated cells in the utilized PECS buffer (5x Tris-KCl) was around 10%, whereas the parental strain JUDE-1 exhibited a viability of 30–40% under the same conditions. An increase in the average BODIPY fluorescence intensity was observed in every round until the fluorescent signal became saturated after the fifth round. At that point the mean fluorescence of the population was approximately 2.5-fold higher than the BODIPY fluorescence exhibited by the initial library (Figure 2A).

Three hundred single clones were selected at random from the fifth round population for further analysis. 26.10 IgG expression was evaluated first by ELISA of whole cell lysates using immobilized digoxigenin and then the seven clones with the highest ELISA signals, termed GS1–GS7, were further analyzed for expression of fully assembled IgG by Western blotting. All seven clones were found to yield enhanced production of properly assembled antibody compared to the parental *E. coli* strain JUDE-1 (Figure 2B and data not shown). As expected, curing of the pMAZ360-26.10-IgG plasmid resulted in loss of the ELISA signals, while retransformation with fresh pMAZ360-26.10-IgG vector restored the ELISA signal in 5/7 clones to the level of the original isolates (Figure 2C and data not shown).

A second round of chemical mutagenesis and screening was performed by pooling GS1–GS5 clones carrying the pMAZ360-26.10-IgG plasmid, followed by exposure to MNNG. After seven rounds of FACS screening as above, the fluorescent signal saturated at a level nearly 10-fold higher than that of the parental JUDE-1 cells (Figure 2A). Seven clones, termed TM1–TM7, from the seventh round of sorting were randomly selected for further study and shown to exhibit approximately 5-fold higher cell fluorescence, which was dependent on the presence of the pMAZ360-26.10-IgG plasmid. Among TM1–TM7, six clones regained fluorescence upon retransformation with fresh vector (TM1–TM6). Western blot analysis revealed that TM1–TM6 produce 10–20 times more fully assembled IgG compared to the parental JUDE-1 cells (Figure 2B) and this was accompanied by markedly higher ELISA signals (Figure 2C). For comparison, bacterial clones isolated after the first round of mutagenesis, resulted in approximately 3-fold enhancement in the yield of 26.10 IgG (Figure 2B). However, with the exception of TM4, the other *E. coli* mutant strains isolated after the second round of mutagenesis grew more slowly and reached a lower final cell culture density after overnight growth compared to wild-type JUDE-1 (Figure 2D). For this reason, TM4 was selected for further studies.

It is interesting to note that the increased yield of fully assembled IgG in the TM mutants was accompanied by an appreciable increase in the production of the heavy and light chains (Figure 2B) indicating that the selected chromosomal lesions in these mutants affected protein synthesis and/or export and stability in the periplasm.

### 3.3 Isolation of heavy chain TIR variants

The IgH, IgL dicistronic operon in pMAZ360-26.10-IgG results in accumulation of an excess of light chain (first cistron gene product) over heavy chain (Figure 2B and data not shown). Swapping the order of the light and heavy chain ORFs of the IgG operon of the pMAZ360 vector did not improve IgG assembly (data not shown). We reasoned that a more balanced ratio of heavy:light chain synthesis may facilitate proper assembly. In *E. coli*, the protein synthesis rate is often critically dependent on the rate of translation initiation which in turn is influenced by the propensity of the 5' end of the mRNA around the Shine-Dalgarno sequence to form stable secondary structure that occludes ribosome binding. Even single nucleotide changes in the translation initiation region, which extends from the 5' end of the RBS to about 20 nucleotides downstream of the start codon, can affect the efficiency of translational initiation (Kudla *et al.*, 2009, Simmons & Yansura, 1996). Several previous studies have demonstrated that the TIR region can be engineered to enhance heterologous protein expression and secretion (Simmons & Yansura, 1996, Bandmann & Nygren, 2007, Ahn *et al.*, 2008). Similarly, optimized TIR sequences have been shown to be important for the expression of full-length IgGs in a system where the IgH and IgL were expressed from monocistronic operons (Simmons et al., 2002).

In order to select for a TIR sequence that confers a more favorable ratio of IgH:IgL, degenerate oligonucleotides were used to generate two libraries of pMAZ360-26.10-IgG plasmids carrying silent mutations in the TIR of both the heavy chain or light chain genes. The theoretical diversity for each of the heavy and light chain libraries was $3.7 \times 10^4$ (Figure 3A). First, we attempted to transform the constructed library into the evolved TM4 strain. The acquired mutations in TM4, however, had a dramatically decreasing effect on the transformation efficiency of the strain. Thus, we focused on wild-type JUDE-1 cells. Approximately $10^6$ colonies of *E. coli* JUDE-1 cells transformed with the TIR libraries in pMAZ360-26.10-IgG were pooled and subjected to successive rounds of PECS screening for enhanced binding to digoxigenin-BODIPY using FACS. The fluorescence signal was saturated after the sixth round of sorting (Figure 3A), at which point 90 colonies were picked randomly, grown in microtiter-well plates and the expression of functional antibody protein in whole-cell lysates was determined by ELISA. Interestingly, the TIR region in 15

clones showing the highest ELISA signals were found to contain mutations only in the heavy chain TIR. 13/15 clones had a common nucleotide sequence (Figure 3B). Western blotting and ELISA analysis of IgG expressed in JUDE-1 cells using the consensus TIR sequence revealed a 2- to 3-fold improvement in IgG assembly (Figure 3C) leading to a proportional increase in antigen binding as detected by ELISA (Figure 3D).

The thermodynamic stability of the RNA encompassing the TIR sequence, i.e. a 60 nucleotide-long region that included the ribosome-binding site and 30 nucleotides downstream of the ATG initiation codon was calculated using two different algorithms, Mfold and RBS calculator. Mfold (Zuker, 2003), predicts mRNA secondary structure, whereas the RBS calculator (Salis *et al.*, 2009) predicts relative translational initiation rates by using a free energy model to calculate the total change in Gibbs free energy upon binding of the 30S ribosome complex to a ribosome binding site on the mRNA. Consistent with the experimental results, Mfold predicted that the selected mutant sequences had a lower ΔG of folding relative to the parental gene (Figure 3E). Similarly, the RBS calculator program predicted that the selected TIR sequence should result in 10-fold higher translational initiation rate relative to that of the TIR sequence contained in the original vector (35370 vs. 3730 of arbitrary units, respectively).

### 3.4 Effect of Molecular Chaperone and Cysteine Oxidoreductase Co-expression

Co-expression of molecular chaperones has been shown in a number of studies to assist the production and folding of several recombinant proteins, including antibody fragments (Levy et al., 2001, Lin *et al.*, 2008, Mavrangelos *et al.*, 2001, Zhang *et al.*, 2002, Jurado *et al.*, 2002). The yield of the 26.10 IgG expressed in the TM4 mutant strain in cells with and without co-expression of various chaperones and enzymes known to influence heterologous protein expression, was evaluated. Specifically, the effect of co-expression of the periplasmic chaperones Skp and FkpA, the cytoplasmic molecular chaperones/co-chaperones DnaK/DnaJ, GroEL/GroES and the peptidyl-prolyl cis/trans isomerase trigger factor, the human endoplasmic reticulum molecular chaperone BiP, the mouse ER peptidyl-prolyl cis-trans isomerase Cyclophilin B (Feige *et al.*, 2009), the periplasmic disulfide oxidoreductases and isomerases DsbA and DsbC (Zhang et al., 2002), and an engineered FkpA-DsbA chimera (Arredondo et al., 2008) was investigated. Furthermore, we tested the effect of co-expressing the components of the Sec secretory pathway SecB and SecE/SecY which mediate export into the periplasm. We found that co-expression of DsbA, DsbC, BiP, and SecE/SecY individually resulted in 2- to 3-fold increased yield of fully assembled 26.10 IgG (Table 1). The most significant effect was observed in cells co-expressing DsbA, which increased the yield of assembled, antigen-binding antibody by more than 3-fold (Table 1). This result is consistent with very recent findings that DsbA overexpression enhances the yield of properly folded IgG in the periplasmic space of *E. coli* cells (Reilly & Yansura, 2010).

### 3.5 Optimized expression of various IgG antibodies

The 26.10 IgG was expressed in the TM4 mutant strain carrying the pMAZ360-26.10-IgG vector with and without the selected, optimized IgH TIR sequence and in the presence or absence of DsbA co-expression (Figure 4A). Unfortunately, the effects of the optimized TIR sequence and DsbA co-expression in the TM4 mutant were not additive (Figure 4A). Thus, for subsequent studies we elected to employ TM4 with the engineered TIR to avoid the need for having a second plasmid for DsbA expression.

We compared the yield of fully assembled IgG in *E. coli* TM4 from plasmids expressing heavy and light chains as a dicistronic operon transcribed from P*lac* and containing the engineered TIR, with that obtained using the parental strain JUDE-1 and unmodified TIR.

Three different IgG antibodies were used: the anti-digoxin 26.10 antibody, the 9c8 nanomolar affinity anti-complement factor C1S antibody (Sai Reddy and GG, unpublished results), and M18.1, a humanized antibody that binds to the *Bacillus anthracis* Protective Antigen (PA) with high affinity; (Mazor et al., 2007). All three antibodies are human IgG1 variants with a C kappa light chain and variable regions that originated from different murein germ lines. IgG overexpression was carried out in 100 mL shake flask cultures. The accumulation of fully assembled IgG in cell lysates and in the extracellular fluid was determined by Western blotting and the yield was estimated by densitometric analysis of the band intensity and comparison to serial dilutions of a commercially available IgG1 (Herceptin®) as standard (Figure 4B). As observed also in previous work where full-length IgG antibodies expressed using a dicistronic system had been purified to near homogeneity (Mazor et al., 2007), wild-type *E. coli* cells accumulated 0.1–1.3 mg of fully assembled IgGs per liter of shake flask culture. Use of the optimized expression system, however, resulted in a 6-fold increase for 9c8-IgG, 4-fold increase in the yield of 26.10 IgG and a 2.5-fold increase for M18.1 IgG, yielding up to 4 mg/L of full-length antibody. This generally observed expression-enhancing effect occurs partly due to the beneficial mutations acquired in TM4 and partly due to the use of the evolved TIR, as the expression of different IgG antibodies in TM4 cells but without the use of the optimized TIR sequence resulted in smaller increases in the production of fully assembled IgG (Figure 4C). Highly purified 26.10-IgG antibody expressed with the the optimized expression system could be obtained from cell lysates by protein A affinity chromatography (Figure 4D).

## 4. Discussion

In eukaryotic cells, antibody biogenesis is critically dependent on interactions of the newly secreted polypeptides in the ER with a network of molecular chaperones and with protein disulfide bond isomerases (Melnick & Argon, 1995, Vanhove *et al.*, 2001, Elkabetz *et al.*, 2005). Recapitulating IgG assembly *in vitro* has been challenging. Evidence from biophysical studies suggests that the rate limiting step in the assembly of the $F_{AB}$ domain of IgGs is the folding of the $C_H$ chain which is normally disordered and folds upon association with the $C_L$ (Feige et al., 2009). Very recently, protocols for the efficient refolding of IgG from *E. coli* inclusion bodies have been developed (Hakim & Benhar, 2009). However, the expression of soluble and correctly assembled IgG antibodies is usually preferable for routine studies in antibody discovery and for large-scale preparative purposes. Achieving high-level IgG biogenesis in bacteria which lack the mammalian molecular chaperone network requires a fine balance between protein synthesis, secretion and folding. The formation of disulfide bonds within each of the immunoglobulin domains in IgG and in the hinge region joining the two heavy chains are essential for the assembly of the native tertiary structure. Although a variety of engineered *E. coli* strains that enable disulfide bond formation in the cytoplasm are now available (Faulkner *et al.*, 2008), expression of correctly assembled full-length IgG in bacteria having an oxidizing cytoplasm has not been demonstrated. Therefore, full-length antibody production in bacteria requires export into the oxidative environment of the periplasmic space, as well as slow growth, slow protein synthesis rates and balanced ratio of heavy/light chain production that favor proper chain association (Levy et al., 2001, Mazor et al., 2007, Simmons et al., 2002).

In this work, we report the systematic optimization of IgG antibody expression in bacteria from a dicistronic operon by examining transcription, translation, *in vivo* protein folding and in parallel with genetic approaches. The light and heavy chains were placed in a dicistronic operon transcribed from an inducible promoter, because: (i) a dicistronic operon is desirable for the recovery of antibody DNA during the screening of IgGs from libraries (Mazor et al. 2007, 2010); (ii) monocistronic expression of heavy and light chain genes fused the *pelB* signal peptide and transcribed from the *lac* promoter resulted in lower IgG yields relative to

the dicistronic system used here (data not shown). It should be noted, however, that a monocistronic expression system that capitalized on a proprietary PhoA promoter system and the StII signal peptide has been used for preparative production (Simmons et al., 2002). The dicistronic operon for the expression of the heavy and light chains was cloned into the previously described pMAZ360 vector (Mazor et al., 2007), but it could alternatively be also incorporated into the *E. coli* chromosome. A modification of a high-throughput technique (PECS) developed earlier for the isolation of antibodies with desired antigen specificity (Chen et al., 2001), was employed for the monitoring of antibody expression in *E. coli* at the single-cell level and for the screening of mutant libraries. Two rounds of chemical mutagenesis, each followed by several rounds of FACS sorting, led to the isolation of mutant bacteria that displayed a marked increase in the production of fully assembled IgG, as monitored both by ELISA and Western blotting. Mutant strains were shown to accumulate higher levels of heavy and light polypeptides indicating that the chromosomal lesions affect protein synthesis in a manner that favorably affects the formation of fully assembled antibodies. It should be noted that increased protein synthesis does not necessarily translate into higher IgG yields, as evident by the fact that expression from stronger promoters (T7, *trc*) had a detrimental effect on the IgG yield (data not shown). Consequently, in the mutant strain TM4, mutations in the chromosome must have had a subtle effect on the rate of transcription or translation that in turn was favorable for protein folding in the periplasm. However, we can not rule out the possibility that the acquired mutations affect the degradation or translocation of the antibody heavy and light chain in a manner that favors their accumulation. Because this strain was obtained following two rounds of chemical mutagenesis we anticipate that it will contain multiple mutations whose individual effects on protein expression will require extensive additional analyses. The genetic lesions that result in enhanced full-length IgG production in TM4 can be identified by standard mutation mapping techniques (Zhan et al., 2004) coupled with the flow cytometric screening assay described here, or by whole genome sequencing (MacLean *et al.*, 2009, Srivatsan *et al.*, 2008). Combinatorial library screening was also used to isolate optimized TIR sequences that enhanced the translation of the second cistron (heavy chain) and thus resulted in a more favorable heavy:light chain ratio in the periplasm. The requirement for a balanced expression of heavy and light chains for the production of correctly assembled antibodies or $F_{AB}$ fragments has also been observed in earlier studies (Humphreys, 2003, Simmons et al., 2002). Interestingly, combinatorial screening yielded TIR sequences that were predicted to display less stable RNA secondary structure and enhance translation initiation. This genetic verification of model predictions suggests that computational tools, namely Mfold, and more importantly, the RBS calculator are reliable tools for improving translation. To our knowledge, this work is the first attempt to engineer full-length IgG expression by generating *E. coli* mutant libraries and employing high-throughput screening to isolate the desired overexpression strains.

Finally, as part of this study we also examined the effects of co-expression of several molecular chaperones and enzymes that are known to assist protein folding. It was hypothesized that co-expression of cytoplasmic chaperones may assist in the secretion of the heavy and light chains into the periplasm by preventing the cytoplasmic aggregation of the pre-protein, containing the signal peptide (Berges *et al.*, 1996). However, no significant effect on IgG yield was observed in this case. At the same time, co-expression of the translocon components SecY/E resulted in a reproducible 2-fold enhancement in the amount of fully assembled IgG, suggesting that the capacity of the secretory apparatus may be limiting. Similar to these results, Perez-Perez et al. in earlier studies showed that co-expression of SecY/E enhanced the secretion of human interleukin-6 in *E. coli* by 1.2 to 10.8 fold (Perez-Perez *et al.*, 1996, Perez-Perez *et al.*, 1994). A similar enhancement in the production of full-length IgG was observed upon overexpression of the eukaryotic chaperone BiP. BiP has been found previously to promote antibody folding together with

protein disulfide isomerase (PDI) *in vitro* (Mayer *et al.*, 2000). In a cell-free IgG production system utilizing *E. coli* lysate, however, BiP was found ineffective in increasing the yields of fully assembled IgG (Frey *et al.*, 2008). A more significant increase in IgG yield was obtained by co-expressing the periplasmic oxidoreductase DsbA or the disulfide bond isomerase DsbC. The beneficial effect of co-expression of cysteine oxidoreductases on the folding of multi-disulfide-bond proteins including $F_{AB}$ antibody fragments is well established (Georgiou & Segatori, 2005, Levy et al., 2001). Consistent with these observations, full-length IgG could be produced with an *in vitro* transcription-translation system containing *E. coli* lysate only in the presence of a disulfide bond isomerase/ oxidoreductase, PDI or DsbC, while the redox potential of the bacterial lysate was found to be critical for the yields of intact IgG antibody (Frey et al., 2008).

While the co-expression of molecular chaperones and enzymes that assist folding was found to enhance IgG yields in TM4, the introduction of the engineered TIR did not show a further synergistic effect. A likely explanation for this result is that the simultaneous expression of three secreted polypeptides, i.e. the heavy and light chains as well as the chaperone/foldase saturates the export machinery. However other explanations, such as a decrease in the level of transcription from the *araBAD* promoter in TM4, for example, cannot be ruled out. Regardless of the mechanism, this finding underscores the need to balance a diverse array of physiological processes in the course of engineering cells for enhanced heterologous protein expression.

In summary, the above studies: (i) highlight the utility of high-throughput screening for strain and vector optimization for protein expression; (ii) demonstrate a systematic approach for addressing various biological processes that can impact transcription, protein synthesis, secretion and folding, and importantly (iii) led to the development of an expression system that resulted in reproducibly 3- to 6-fold higher expression of fully assembled IgG for three antibodies comprising different germline V genes and complementarity determining regions (CDRs). The engineered bacterial expression system is thus likely to be useful for the rapid expression of diverse antibodies generated in antibody discovery programs. Such evolved systems could be analyzed and optimized further, e.g. by metabolic analysis and engineering (Boghigian *et al.*, 2010), to generate specialized antibody-production strains. Finally, we note that the systematic approach applied to the optimization of antibody expression described here could serve as a model for enhancing the expression of other secreted heterologous proteins in bacteria.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Abbreviations

| | |
|---|---|
| **IgG** | immunoglobulin G |
| **TIR** | translational initiation region |
| **PECS** | periplasmic expression followed by cytometric sorting |
| **FACS** | fluorescence-activated cell sorting |
| **MNNG** | N-methyl-N'-nitro-N-nitrosoguanidine |
| **IPTG** | isopropyl-β-D-thiogalactopyranoside |
| **PA** | *Bacillus anthracis* Protective Antigen |

| **ELISA** | enzyme-linked immunosorbent assay |
| **SDS-PAGE** | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **BODIPY** | dipyrromethene boron difluoride |
| **BSA** | bovine serum albumin |
| **PVDF** | polyvinylidene fluoride |
| **SRP** | signal recognition particle |
| **OD$_{600}$** | optical density at 600 nm |
| **LB** | Luria-Bertani |
| **PBS** | phosphate-buffered saline |
| **HRP** | horseradish peroxidase |
| **ORF** | open reading frame |
| **PDI** | protein disulfide isomerase |
| **CDR** | complementarity determining regions |

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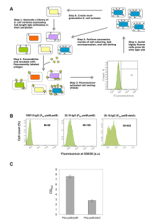
**Figure 1. Single-cell Fluorescent Detection of IgG Expression**
**(A)** General scheme of *E. coli* mutagenesis and isolation of clones displaying increased IgG expression using periplasmic expression with cytometric screening (PECS). **(B)** Fluorescence histograms of *E. coli* JUDE-1 cells over-expressing either 26.10 IgG or YMF10 IgG (anti-PA antibody (Mazor et al., 2007)); negative control) with different combinations of leader peptides for heavy and light chain periplasmic export. Statistics correspond to a population of 10,000 cells. M: mean fluorescence intensity. **(C)** Growth of *E. coli* JUDE-1 cells over-expressing 26.10 IgG antibody with different leader peptides for heavy and light chain periplasmic export. $OD_{600}$: optical density at 600 nm, $P_{lac}$-*pelB-pelB*: *lac* promoter with *pelB* leader peptide for light and heavy chain periplasmic export, $P_{lac}$-*pelB-dsbA*: *lac* promoter with *pelB* leader peptide for periplasmic export of light chain and *dsbA* leader peptide for periplasmic export of heavy chain.
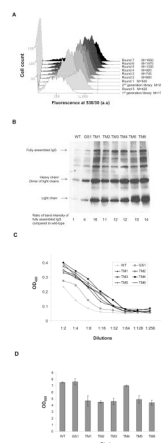
**Figure 2. Screening of Chemically Mutated *E. coli* for IgG Expression**
**(A)** FACS screening of mutated JUDE-1 cells expressing 26.10 IgG labeled with digoxigenin-BODIPY. M: mean fluorescence intensity. **(B)** Western blotting of 26.10 IgG expressed in isolated clones after two rounds of mutagenesis and screening. The increase in the yield of fully assembled IgG relative to wild-type (WT) JUDE-1 (lower panel) was determined by scanning densitometry. **(C)** ELISA assays of IgG level in cell lysates. $OD_{420}$: optical density at 420 nm. **(D)** Growth of isolated mutant clones after 16 h of 26.10-IgG overexpression. Experiments were carried out in triplicate and the error bars correspond to one standard deviation from the mean values. In (B) and (C) protein samples were normalized by $OD_{600}$ so as to contain total protein that corresponds to the same number of bacterial cells. $OD_{600}$: optical density at 600 nm.

E

## Original TIR sequence
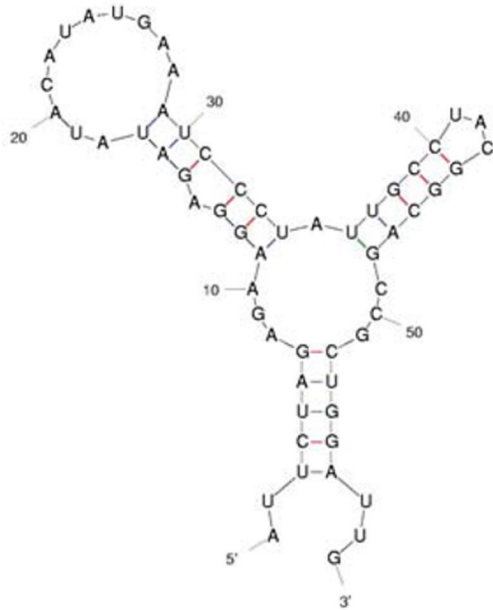


$\Delta G^{\circ}= -11.1$ kcal/mol

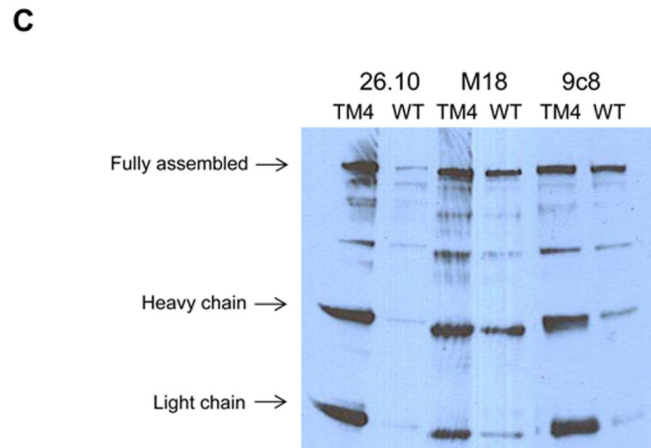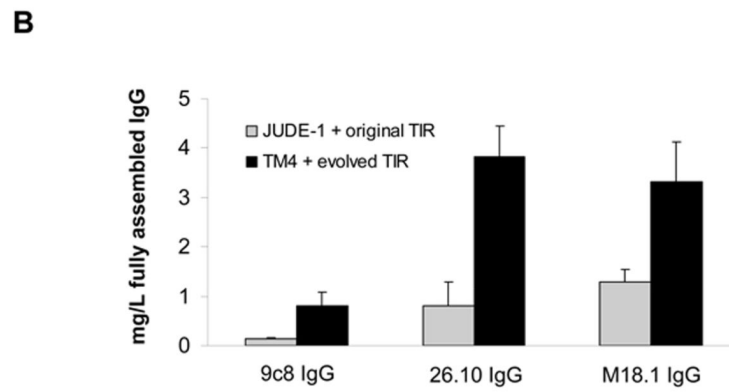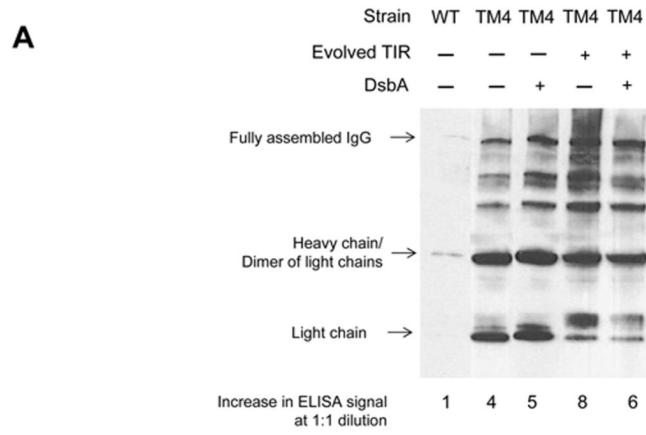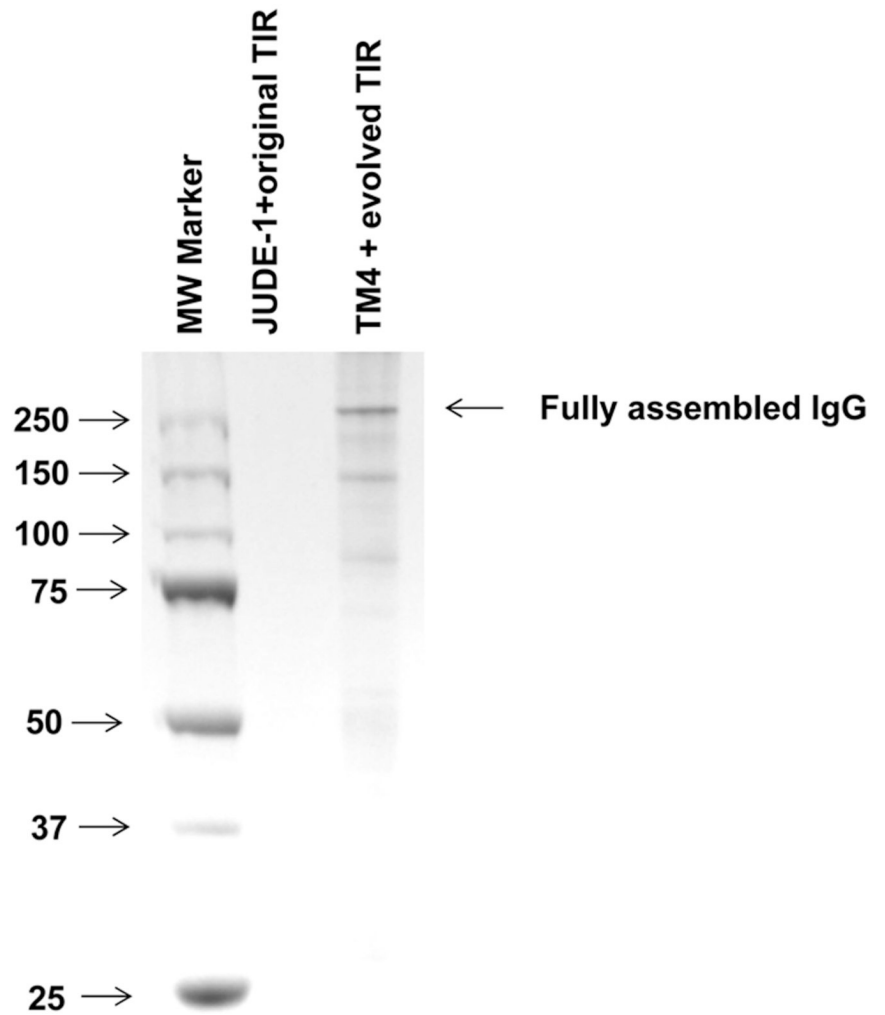## Evolved TIR sequence
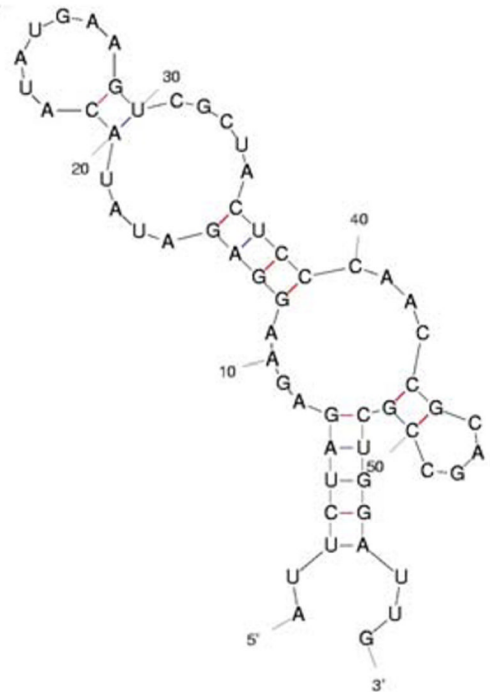


$\Delta G^{\circ}= -5.2$ kcal/mol

**Figure 3. Screening of a Library for Optimized Translational Initiation Region (TIR) Sequences**
**(A)** Construction of TIR libraries for light and heavy chain genes and screening by FACS.
M: mean fluorescence intensity, ss*pelB*: *pelB* signaling sequence **(B)** Nucleotide sequences
of the heavy chain TIRs of the isolated clones. **(C)** Western blotting of JUDE-1 cells
carrying the pMAZ360-26.10-IgG vector either with the original or the selected heavy chain
TIR sequences under non-reducing (left) and reducing (right) conditions. All lanes were
loaded with total protein corresponding to the same number of cells as judged by $OD_{600}$. **(D)**

ELISA determination of antigen-binding activity in cell lysates from mutant clones. Experiments were carried out in triplicate and the error bars correspond to one standard deviation from the mean values. **(E)** Prediction of mRNA secondary structure for the original and the evolved heavy chain TIR sequences using the Mfold software (Zuker, 2003). $\Delta G^o$: Gibbs free energy of unfolding.

**A**

Strain | WT | TM4 | TM4 | TM4 | TM4

Evolved TIR | — | — | — | + | +

DsbA | — | — | + | — | +

Fully assembled IgG →

Heavy chain/
Dimer of light chains →

Light chain →

Increase in ELISA signal
at 1:1 dilution | 1 | 4 | 5 | 8 | 6

**B**



**C**

**Figure 4. Yield of Various Fully Assembled IgGs in Shake Flask Cultures**
**(A)** Yield of assembled 26.10 IgG in different combinations of strain, heavy chain TIR sequence, and DsbA co-expression determined by Western blotting and ELISA (bottom). WT: *E. coli* JUDE-1 cells. **(B)** Production of full-length antibody for different IgG variants expressed either in JUDE-1 cells carrying the original pMAZ360 vector or in TM4 carrying the pMAZ360 vector containing the engineered heavy chain TIR sequence. Experiments were carried out in triplicate and the error bars correspond to one standard deviation from the mean values. **(C)** Western blot analysis of the production of full-length antibody for different IgG variants expressed either in JUDE-1 or TM4 cells carrying pMAZ360 vector with the original heavy chain TIR sequence. All lanes were loaded with total protein corresponding to the same number of cells as judged by $OD_{600}$. **(D)** SDS-PAGE analysis of 26.10 IgG following protein A affinity chromatography of cell lysates from either JUDE-1 cells carrying the pMAZ360-26.10-IgG vector with the original heavy chain TIR sequence or TM4 cells carrying the pMAZ360-26.10-IgG vector with the engineered heavy chain TIR sequence. MW: molecular mass in kDa.

**Table 1**

Effect of co-expressing molecular chaperones and other folding factors on the expression and of fully assembled 26.10-IgG in TM4 cells.

| Co-expressed folding factor | Expression of full-length 26.10-IgG (in arbitrary units) |
|---|---|
| No factor | 100 |
| Trigger factor | $57 \pm 5$ |
| DnaK/DnaJ | $136 \pm 21$ |
| GroEL/GroES | $144 \pm 12$ |
| Skp | $120 \pm 14$ |
| BiP | $198 \pm 10$ |
| SecB | $68 \pm 7$ |
| SecY/SecE | $206 \pm 3$ |
| DsbA | $319 \pm 27$ |
| DsbC | $236 \pm 13$ |
| Cyclophilin B | $73 \pm 2$ |
| FkpA | $73 \pm 2$ |
| FkpA-DsbC | $83 \pm 5$ |