Toxic protein expression in *Escherichia coli* using a rhamnose-based tightly regulated and tunable promoter system

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The refinement of tightly regulated prokaryotic expression systems that permit functional expression of toxic recombinant proteins is a continually evolving process. Unfortunately, the current best promoter options are either tightly repressed and produce little protein, or produce substantial protein but lack the necessary repression to avoid mutations stimulated by leaky expression in the absence of inducer. In this report, we present three novel prokaryotic expression constructs that are tightly regulated by L-rhamnose and D-glucose. These expression vectors utilize the Escherichia coli *rhaT promoter and corresponding regulatory genes to provide titratable, high-level protein yield without compromising clone integrity. Together, these components may enable the stable cloning and functional expression of otherwise toxic proteins.*

INTRODUCTION

Recombinant protein expression in *Escherichia coli* is a commonly used technique to produce a wide array of functional proteins from a variety of organisms. The pLac system (1,2) is a popular expression vector for the production of nontoxic proteins. While this isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible system is capable of high-level protein expression, when toxic proteins are cloned into vectors of this class, low-level, untimely expression may stimulate a cascade of deleterious events ending in mutations that may affect target protein function, overproduction of target-directed proteases, or cell death. Therefore, tight expression control prior to target protein induction is critical for reproducible and functional expression of host-toxic proteins.

Several strategies have been described for reducing premature protein production. Some involve culture conditions: lower growth temperatures before and possibly during induction decrease the probability of a toxic event, and we have also observed that clones grown in stationary phase have an increased frequency of mutations in the toxic protein (data not shown). In addition, much attention has been focused on the development of tightly regulatable expression vectors. The pBAD (3) and T7 polymerase (4–7) systems have been popular vectors for the expression of toxic proteins. While the pBAD system, which relies on catabolite repression and positive induction, is tightly regulated, it produces a relatively small amount of recombinant protein. On the other hand, while T7 polymerase systems facilitate a higher level of expression than pBAD, they are prone to leaky expression. In the T7 system, recombinant protein expression is driven by promoter sequences recognized by T7 polymerase. The T7 polymerase gene is generally located on the bacterial chromosome under the control of a lactose-inducible bacterial promoter. Because these promoters are leaky in the absence of inducer, the transformation of T7-inducible plasmids bearing toxic protein sequences may result in selective pressure from immediate and untimely expression of the toxic protein.

We set out to design a protein expression system that combines tight regulation with high-level induction. To this end, we constructed an expression system based on the *E. coli rhaTRS* locus (Figure 1A). In the presence of L-rhamnose, RhaR activates transcription of *rhaR* and *rhaS*, resulting in an accumulation of RhaS. RhaS then acts as the L-rhamnose-dependent positive regulator of the *rhaT* promoter (8). The mechanism of expression and repression of the *rhaT* promoter has been studied extensively (8–11). This L-rhamnose-inducible promoter is also subject to catabolite repression (12), so although this promoter is capable of high-level recombinant protein expression in the presence of

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L-rhamnose, it is also tightly regulated in the absence of L-rhamnose by the addition of D-glucose (13–18). Given these properties, we elected to transfer the chromosomal sequence corresponding to the *rhaT* promoter and the *rhaR* and *rhaS* genes (for simplicity, referred to here as pRHA) to three different plasmid backgrounds and examined the suitability of this system for regulatable protein expression. The results presented here suggest that pRHA vectors represent a viable alternative *E. coli* expression system for the production of nontoxic proteins and an enabling technology for the functional production of otherwise toxic proteins.

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* strains used in this study were MG1655 (19), TOP10 [F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *Φ80lacZΔM15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^R) *endA1 nupG*], and BL21(DE3) [F-*ompT hsdS*$_B$ (r_B-m_B-) *gal dcm* (DE3)] (all from Invitrogen, Carlsbad, CA, USA).

Construction of pRHA-67, pRHA-113, pRHA-109, and pMPX-66

A portion of the rhamnose regulon containing the *rhaT* promoter and *rhaR* and *rhaS* regulatory genes (pRHA) was PCR-amplified from the MG1655 chromosome (Figure 1A). Three separate PCR amplifications of this region were performed. The reverse primer used in each reaction was the same and was tagged with *Hin*dIII (see Table 1 for all primer sequences). In each case, the forward primer was tagged with a restriction enzyme recognition site, a multicloning sequence, and an optimized Shine-Dalgarno sequence. However, the forward primer used in each reaction differed in the restriction enzyme site. For cloning into pUC18, *Kpn*I was used; for pBR322 lacking the *rop* gene, *Nde*I was employed; and for pBR322 containing the *rop* gene, the recognition site used was *Xho*I. All three pRHA PCR products (appropriately sized at 2074 bp) were gel-purified and blunt-end ligated into pCR-Blunt II-TOPO® (Invitrogen).

After sequence confirmation, each amplification product was removed from pCR-Blunt II-TOPO using the appropriate restriction endonucleases (described above) and ligated into the corresponding recipient vector (Figure 1B). First, pRHA was ligated into the high-copy plasmid pUC18, which had been previously digested with *Kpn*I and *Hin*dIII. This high-copy plasmid was designated pRHA-67. Second, pRHA was ligated into *Hin*dIII and *Nde*I digested pBR322 plasmid lacking the *rop* gene. This plasmid was designated pRHA-109 (medium copy). Third, pRHA was ligated into *Hin*dIII and *Sal*I (*Sal*I and *Xho*I are isoschizomers) digested pBR322 plasmid containing the *rop* gene. This plasmid was designated pRHA-113 (low copy). The Shine-Dalgarno sequence and multiple cloning site (MCS) of each plasmid were identical (Figure 1C). Each of the plasmids was transformed into chemically competent TOP10 cells for storage and analysis.

To construct pMPX-66, the entire pBAD promoter region and *araC* regulatory gene from pBAD24 was moved into the same pUC18 plasmid backbone used for pRHA-67 (3,20). Primers were designed to replace the pBAD24 polylinker with that found in the pRHA expression vectors (depicted in Figure 1C). The sequences of the forward and reverse primers introduced *Hin*dIII and *Kpn*I sites, respectively. The resulting PCR fragment (1303 bp) was gel-purified and blunt-end ligated into a pCR-Blunt II-TOPO sequencing vector and, following sequence confirmation, ligated into pUC18 previously digested with *Hin*dIII and *Kpn*I. The resulting pMPX-66 plasmid was identical to pRHA-67 with the exception of the promoter and regulatory sequences.

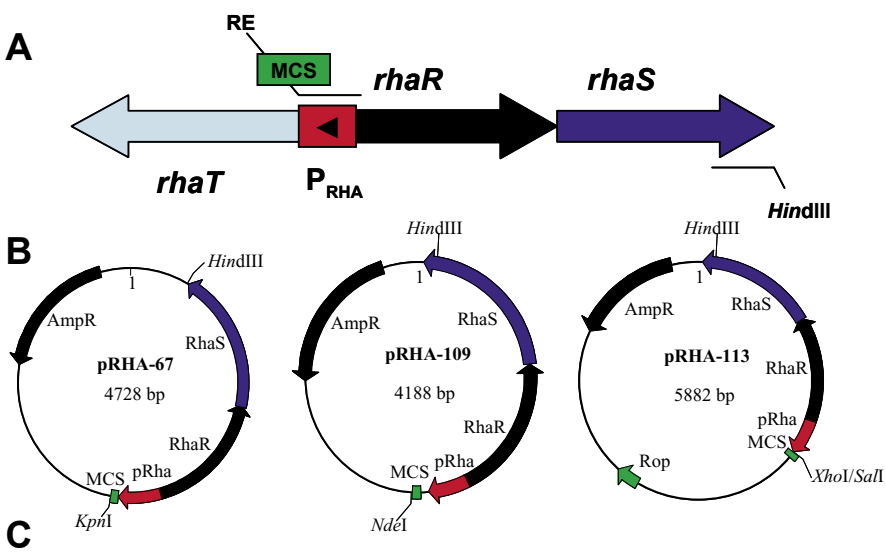


Figure 1. Cloning strategy, plasmid maps, and multiple cloning site descriptions of pRHA vectors. (A) Representation of the cloning strategy used to generate pRHA vectors from the *Escherichia coli* chromosome. The sense strand primer introduces a multiple cloning site (MCS) and the appropriate restriction enzyme recognition site (RE). (B) Plasmid maps of pRHA-67, pRHA-109, and pRHA-113 show relative sizes, positions, and respective directionalities of the pRHA components. Vector pMPX-66 is not shown. (C) MCS sequences and corresponding restriction sites are shown for plasmids pRHA-67, pRHA-109, and pRHA-113.

Cloning *tphoA*

ToxR-PhoA (TphoA) is a chimeric protein consisting of the transmembrane domain from *Vibrio cholera* ToxR tethered to bacterial alkaline phosphatase (BAP; PhoA) lacking the native signal sequence (21–28). The *phoA* gene (GenBank® accession no. U00096.2) lacking the signal sequence

Table 1. Oligonucleotide PCR Primers

Primer Name[a]	DNA Sequence
pRHA-F[b]	5′-**RE**GTACCCATACGCCCTTTCGGATGAGGGCGCGGGGATCCTCTAGAGTCGACAACCTCCTGAATTTCATTAC-GACC-3′
pRHA-R	5′-**AAGCTT**AATTAATCTTTCTGCGAATTGAGATGACGCC-3′
pBAD-F	5′-**AAGCTT**CAAGCCGTCAATTGTCTGATTCGTTACC-3′
pBAD-R	5′-**GGTACC**AATACGCCCTTTCGGATGAGGGCGCGGGGATCCTCTAGAGTCGACAACCTCCTGC-TAGCCCAAAAAAACGGGTATGG-3′
TphoA-F[b]	5′-**RE**CCGCGCTGCAGATGAACTTGGGGAATCGACTGTTTATTCTGATAGCGGTCTTACTTCCCCTCGCAGTAT-TACTGCTCATGCCTGTTCTGGAAAACCGGGCTGCTCAGGG-3′
TphoA-R[b]	5′-**RE**TTATTATTGTCATCGTCATCTTTATAATCTTTCAGCCCCAGAGCGGCTTTCATGG-3′
GFP-F	5′-**GTCGAC**ATGAGTAAAGGAGAAGAACTTTT-3′
GFP-R	5′-**TCTAGA**TTATTTGTATAGTTCATCC-3′
MalE-F[b]	5′-**RE**ATGAAAATAAAAACAGGTGCACGC-3′
MalE-NTR-R	5′-GGGCCCTGCCGTGTCGGATTCCGAGGTGTACGGCATCCCAGGTAAACGG-3′
MalE-NTR-F	5′-CCGTTTACCTGGGATGCCGTACACCTCGGAATCCGACACGGCAGGGCCC-3′
NTR-FLAG-R[b]	5′-**RE**TTATTATTTATCGTCATCTTTATAATCGTACAGGGTCTCCCGGGTGGCG-3′

[a]Primers are indicated as either forward (F) or reverse (R) orientation.
[b]See Materials and Methods for restriction endonuclease (RE) sites (shown as bold and underlined).

was PCR-cloned from *E. coli* strain MG1655. The forward primer was designed to introduce 90 nucleotides of the *V. cholera toxR* (GenBank accession no. M21249) transmembrane domain, and the reverse primer was designed to introduce a C-terminal FLAG® tag (29) for quantitation of full-length protein expression. The forward primer also added a *Sal*I site, and the reverse primer was tagged with an *Xba*I site for subcloning into pRHA-67, 109, 113, and pMPX-66. PCR of *phoA* with the *toxR* transmembrane domain (*tphoA*) yielded a 1401-bp product that was gel-purified and blunt-end ligated into pCR-Blunt II-TOPO. Following sequence confirmation, the *tphoA* insert was subcloned into vectors pRHA-67, pRHA-109, pRHA-113, and pMPX-66 using the described *Sal*I and *Xba*I sites. Cloning *tphoA* into pET11b (Novagen, Madison, WI, USA) was performed as described above, but substituting the *Nde*I and *Bam*HI sites for *Sal*I and *Xba*I, respectively.

Cloning *GFPM5*

GFPM5 (30), a fluorescence lifetime-stablized GFP mutant, was PCR-amplified from pGFPM5 and will be referred to as green fluorescent protein (GFP) in this work. The forward primer added a *Sal*I site while the reverse primer added an *Xba*I site. PCR of *GFP* yielded a 727-bp product that was gel-purified and blunt-end ligated into pCR-Blunt II-TOPO. Following sequence confirmation, positive *GFP* clones were digested with *Sal*I and *Xba*I, gel-purified, and subcloned into vectors pRHA-67, pRHA-109, pRHA-113, pUC19, and pMPX-66.

Cloning *malE::NTR*

Neurotensin receptor (NTR) is a seven-transmembrane domain G-protein coupled receptor (GPCR) cloned from rat (31). For expression of GPCR sequences in *E. coli*, it has been shown that including an N-terminal leader sequence of an *E. coli* exported protein (such as MalE) increases the membrane association of these complex proteins (31). To address this need, a *malE-NTR* fusion was cloned by designing PCR primers that added the MalE leader sequence (residues 1–370, GenBank accession no. AY605712) to the N terminus of the rat NTR (residues 43–424, GenBank accession no. P20789) as previously described (31) while adding *Sal*I and *Xba*I sites for subcloning. In addition to these restriction recognition sites, the reverse primer was designed to incorporate a C-terminal FLAG tag for quantitating full-length MalE::NTR production. Primary PCR of *malE-NTR* yielded a 2295-bp product that was gel-purified and blunt-end ligated into pCR-Blunt II-TOPO. Following sequence confirmation, positive clones were digested with *Sal*I and *Xba*I, gel-purified, and subcloned into vectors pMPX-66 and pRHA-67. Cloning *malE-NTR* into pET11b was performed as described above, but substituting the *Nde*I and *Bam*HI sites for *Sal*I and *Xba*I, respectively.

Growth and Expression Conditions for TphoA and GFP-Producing Strains

For expression of nontoxic TphoA and mildly toxic GFP in MG1655, a frozen glycerol stock of each strain was streaked on Luria-Bertani (LB) agar and grown overnight at 37°C. The following day, a single colony was selected and grown for 15 h in 3 mL LB broth containing ampicillin (100 µg/mL). Cultures were incubated in 13 × 100 mm polystyrene culture tubes at 37°C while rotating on a vertical-rotating carousel unless indicated otherwise. For maintenance purposes (growth in the absence of protein induction), 0.2% D-glucose was included in all 15 h and overnight incubations.

After the 15 h incubation, cultures were diluted 1/1000 into 3 mL

prewarmed LB broth containing ampicillin (100 µg/mL; LB-Amp) and grown to early exponential phase. Measuring absorbance at an A_{600} of 0.1, inducer was added at concentrations indicated (Figures 2–5). Growth was continued until the cell density reached an A_{600} of 1.0 (approximately 10^9 cells/mL). Induced cultures contained D-glucose where indicated (Figures 2–5).

Growth and Expression Conditions for *malE-NTR*-Containing Strains

For expression of toxic MalE-NTR, a frozen glycerol stock of each strain was streaked on LB agar and grown overnight at 30°C. The following day, a single colony was selected and grown for 15 h in 3 mL LB-Amp and D-glucose (0.2% w/v). Unless otherwise indicated, cultures were incubated in 13 × 100 mm polystyrene culture tubes at 30°C while rotating on a vertical-rotating carousel.

After the 15-h incubation, cultures of MG1655 cells containing pRHA-67::*malE-NTR*, pMPX-66::*malE-NTR*, or BL21(DE3) cells harboring pET11b::*malE-NTR* were diluted 1/1000 into 3 mL prewarmed LB-Amp and grown to early exponential phase. Cultures for induction were incubated slowly at 25°C to enable the efficient expression and membrane localization of MalE-NTR. At an A_{600} of 0.1, inducer was added to a final concentration of 100 µM, and growth was continued until the cell density reached an A_{600} of 1.0.

Western Blot Analysis

Western blot analysis was performed using standard techniques. Mouse monoclonal immunoglobulin G (IgG) directed toward PhoA (Sigma, St. Louis, MO, USA) or FLAG® tag (Sigma) were prepared as a 1:10,000 dilution in 10 mL phosphate-buffered saline (PBS) with 0.1% (v/v) Tween® 20 (PBST), containing 1% (w/v) bovine serum albumin (BSA). These solutions were used as primary antibody where indicated. Goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate was prepared at a 1:10,000 dilution in 10 mL PBST containing 1% BSA. This solution was used as secondary antibody for detection. For visualization, blots were developed using the Western Chemiluminescence Kit (Perkin-Elmer, Boston, MA, USA). Densitometry was performed using the Storm™ 860 densitometer (Molecular Dynamics; Amersham Biosciences, Piscataway, NJ, USA), and data were analyzed using ImageQuant™ 5.2 software (Amersham Biosciences).

Flow Cytometry Analysis

Cells (1×10^7) containing various expression constructs expressing GFP were analyzed by flow cytometry. Prior to analysis, the cells were washed twice in 1 mL of PBS containing 35 µg/mL chloramphenicol to remove nutrients and stop further protein production. After the final wash, cell pellets were resuspended in 1 mL PBS containing 35 µg/mL chloramphenicol and placed on ice until analysis using FACSAria® and FACSDiva® systems (BD Biosciences, San Jose, CA, USA).

Radioligand Binding Assays

Specific neurotensin ligand binding to NTR located in *E. coli* membranes was performed as previously described (32,33), with the exception that ^{125}I-neurotensin (^{125}I-NT) was employed. Membranes from 10^8 spheroplasted cells (33) expressing MalE-NTR were incubated 60 min with ^{125}I-NT. Specificity was determined by incubating membrane with ^{125}I-NT in the presence of nonradiolabeled NT, and with membranes lacking MalE-NTR. Following incubation, membrane reactions were introduced to 96-well polyvinylidene difluoride (PVDF) MultiScreen® opaque plates (Millipore, Bedford, MA, USA) for washing and detection. Prior to detection, membranes were washed 3 times with 100 µL PBS containing 15 mM $MgCl_2$ and 3 mM $CaCl_2$. Each wash solution was removed by vacuum filtration through the filter plate. Following the final wash, filters were dried and removed to scintillation vials for analysis. Human embryonic kidney (HEK 293) cell membranes (Perkin-Elmer) were used as a positive control in binding experiments. Control reactions were performed as described by the manufacturer.

RESULTS

Cloning pRHA Expression Constructs

The cloning strategy for construction of pRHA expression vectors is shown in Figure 1. The unique restriction sites found in the MCS are listed in order of proximity to the transcriptional start site of the *rhaT* promoter sequence (Figure 1C) (8). The Shine-Dalgarno sequence (Figure 1C, bold) was optimized from the native *rhaT* promoter to create the sequence 5′-AGGAGG-3′, where the last G is 9 bp upstream from A of the AUG start codon. Genes to be cloned should contain a PCR-inserted *Sal*I site immediately upstream of the AUG start codon. Therefore, following the insertion of the target gene into the pRHA construct, the start codon will be appropriately placed for translation (34).

The three plasmid constructs that were generated are shown in Figure 1B. Construct pRHA-67 was created in the pUC18 backbone, while pRHA-109 and pRHA-113 were created in a pBR322 backbone. The two pBR322-based constructs differ in the absence (pRHA-109) or presence (pRHA-113) of the *rop* gene, which acts to decrease copy number (35,36).

To determine the approximate copy number of each plasmid construct, plasmid DNA was prepared from 10^9 cells containing pRHA-67, pRHA-109, or pRHA-113. Prepared DNA was read on a spectrophotometer (Beckman Instruments 640 DU® Spectrophotometer; Beckman Coulter, Fullerton, CA, USA) at 260 nm. The pUC18-derived pRHA-67 was present at approximately 250 copies per cell. The pBR322-derived pRHA-109 (*rop* gene removed) was present at approximately 50 copies per cell. The pBR322-derived pRHA-113 (*rop* gene present) was present at approximately 7 copies per cell.

Characterization of pRHA Expression Vectors Using TphoA and GFP

To characterize the panel of rhamnose-inducible vectors, we analyzed the effects of inducer

concentration on both TphoA (Figure 2) and GFP (Figure 3) expression from pRHA-67, pRHA-109, and pRHA-113. The production level of nontoxic TphoA was found to be dependent upon both inducer concentration and plasmid copy number (Figure 2A). Moreover, as expected, the amount of nonspecific expression in the absence of inducer also increased with increased copy number. However, in the lowest copy-number plasmid, adding high levels of D-glucose (0.2%) appeared to completely repress this leaky expression through early and mid-exponential phases. To better characterize this catabolite repression event, cells containing pRHA-67::*tphoA* were grown in the presence of 0.1% and 0.2% D-glucose in the absence of inducer. From these cultures, cells taken at different points during exponential growth phase were analyzed for leaky TphoA production (Figure 2B). The results indicate that while cells ultimately consume added D-glucose to levels below those sufficient for catabolite repression, levels as high as 0.2% efficiently block leaky expression ($\leq A_{600}$ of 1.0). Interestingly, as shown in Figure 3B (and discussed below), the addition of inducer to cells growing in the presence of high D-glucose levels causes expression of cloned target proteins at levels equivalent to induced cells growing in the absence of D-glucose.

From these results, it appears that the level of protein production from the pRHA expression plasmids is titratable (in that the level of protein production per cell is proportional to the amount of inducer added). However, as previously shown for both the pBAD and pLac systems (20), it is possible that this apparent "rheostat" expression is a result of an all-or-none induction mechanism in a subpopulation of cells. To differentiate between these two possibilities, *GFP* was cloned into the three different pRHA constructs (pRHA-67, pRHA-109, and pRHA-113), expressed, and compared with GFP expressed from pMPX-66 and pUC19 (containing the pBAD and pLac regulatory sequences, respectively) using flow cytometry analysis (Figure 3). Consistent with published results, both pBAD and pLac appear to operate by an all-or-none induction mechanism (Figure 3A). Specifically, as inducer was decreased, GFP expression per cell remained constant, although in fewer total cells. However, as inducer was decreased further, GFP production stopped (≤100 µm for both arabinose and IPTG). In contrast, flow cytometry data for pRHA-67::*GFP* suggest that the pRHA expression system is titratable and operates as a "rheostat." Specifically, as inducer was decreased, although similar to pBAD and pUC19-containing cells in that fewer cells produced GFP, unlike these systems, the level of expressed GFP per cell decreased proportionally to the inducer concentration. This rheostat effect is also evident in the summary statistics for the flow cytometry analysis (Figure 3B) and is also observed in the medium- and low-copy pRHA constructs. Interestingly, the high-copy pRHA vector was able to produce similar amounts of GFP protein (Figure 3B) when inducer was added in the absence or presence of 0.2% D-glucose. The mechanism behind this observation is being explored. It should be noted that the addition of ≥100 µM IPTG was toxic (retarded growth) to cultures expressing GFP from pUC19.

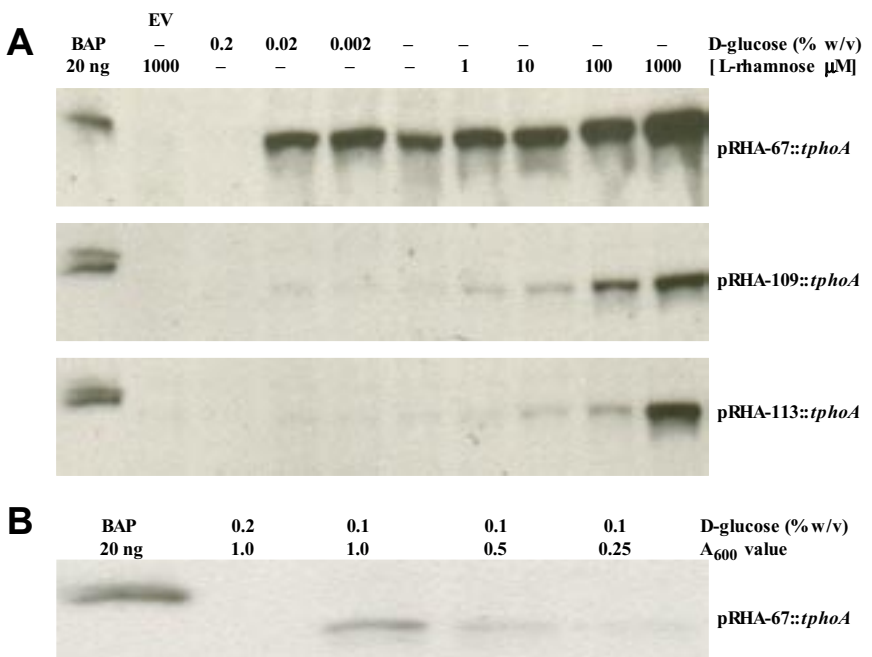
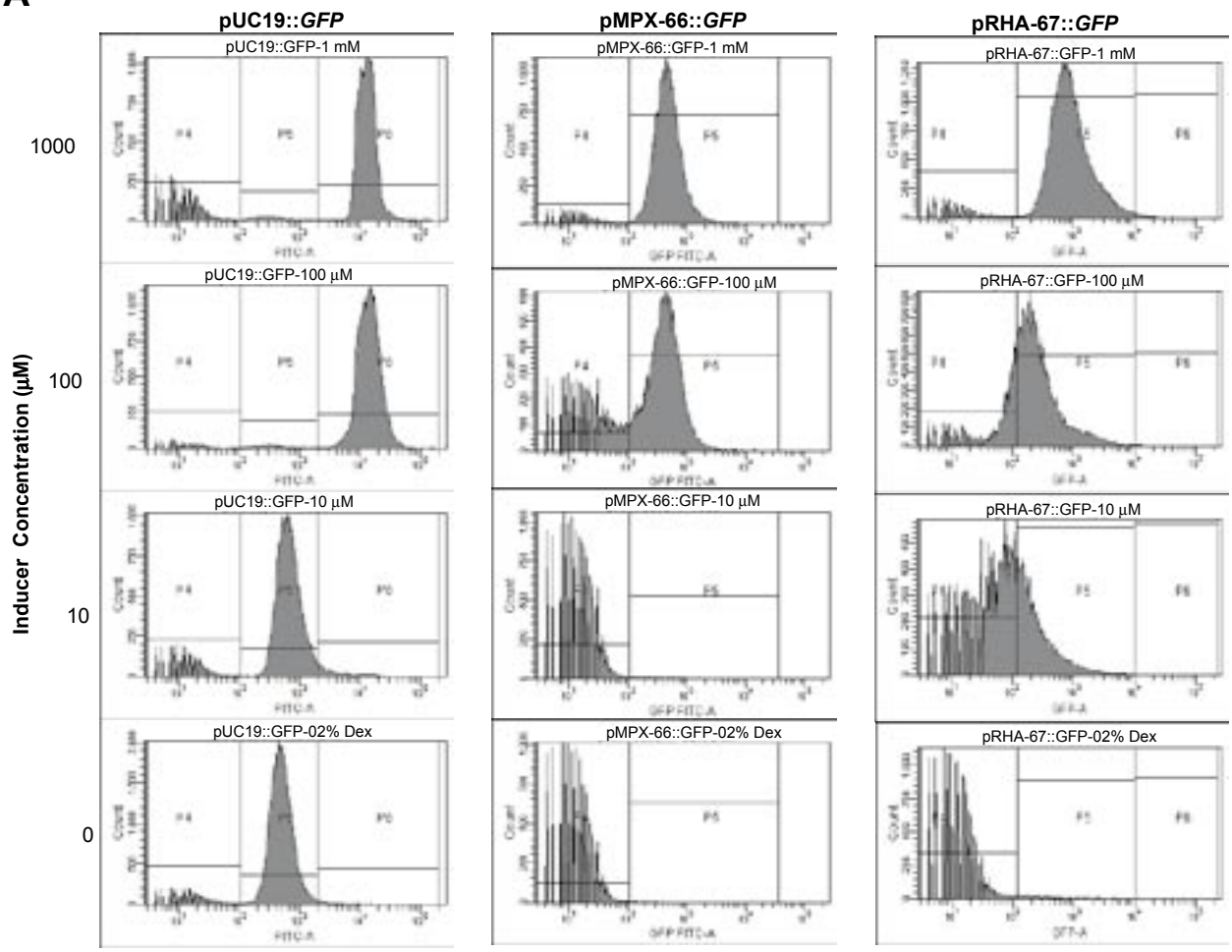


Figure 2. Induction and repression of pRHA vectors. (A) Titration of TphoA expression from vectors pRHA-67::*tphoA*, pRHA-109::*tphoA*, and pRHA-113::*tphoA* in *Escherichia coli* MG1655 induced with varying amounts of L-rhamnose. Cultures were grown and induced. Each lane contains TphoA from 2.5×10^7 cells. MG1655 cells containing pRHA-67, pRHA-109, or pRHA-113 were used as empty vector (EV) controls. Primary antibody used was a mouse monoclonal antibody against PhoA. Purified bacterial alkaline phosphatase (BAP) was used as a positive control. (B) Loss of catabolite repression from pRHA-67::*tphoA* was tested by growing cultures as described in the absence of L-rhamnose and the presence of 0.1% (w/v) D-glucose. Equivalent numbers of cells were harvested at the indicated A_{600} values and analyzed by Western blot analysis. Cultures grown in 0.2% (w/v) D-glucose were used as a positive control for catabolite repression.

Comparative Expression Analysis

To compare levels of nontoxic protein expression between the pRHA system and other tightly regulated expression systems, TphoA was expressed from the pRHA vectors, pMPX-66 (pBAD regulation) and pET11b (T7 promoter regulation). The results indicate that pRHA-67::*tphoA* expresses approximately 2-fold more protein than pMPX-66::*tphoA* and equivalent amounts compared with the BL21(DE3)/pET11b system (Figure 4A). Moreover, these results show that repression is equivalent between pRHA-67 and pMPX-66, as no TphoA was detected in the presence of D-glucose and the absence of inducer. In contrast, the pET11b system showed considerable leakiness of TphoA expression (Figure 4A).

A



B

Inducer	pUC19::*GFP*	pMPX-66::*GFP*	pRHA-67::*GFP*	pRHA-109::*GFP*	pRHA-113::*GFP*
No D-glucose					
1000 µM	15,727/79.0%	542/88.7%	1172/88.6%	811/78.8%	659/82.3%
100 µM	15,222/78.1%	539/76.9%	520/71.1%	471/75.9%	256/82.7%
10 µM	0/0%	0/0%	340/30.5%	304/39.9%	168/33.4%
With D-glucose (0.2% w/v)					
1000 µM	15,644/11.1%	511/43.0%	1045/76.1%	270/79.0%	296/1.1%
100 µM	15,926/2.0%	528/11.2%	436/17.2%	240/32.3%	0/0%
10 µM	0/0%	0/0%	112/8.7%	0/0%	0/0%
D-glucose only (NI)	0/0%	0/0%	0/0%	0/0.1%	0/0%

Figure 3. Flow cytometry analysis of GFP expression. (A) Raw flow cytometry analysis data showing mean GFP fluorescence intensity peaks from MG1655 cells expressing GFP from pUC19::*GFP*, pMPX-66::*GFP*, and pRHA-67::*GFP* at the indicated inducer concentration (IPTG, arabinose, or L-rhamnose, respectively). Noninduced (NI) cultures were maintained in 0.2% (w/v) D-glucose. (B) Summarized results compare GFP expression from all expression constructs tested in the presence or absence of 0.2% (w/v) D-glucose. The ratios shown represent the mean fluorescence intensity per cell/percentage of cells expressing GFP. GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; FITC, fluorescein isothiocyanate.

Because pRHA-67 and pMPX-66 are equivalent in plasmid copy number, these results suggest that the pRHA expression system offers a viable alternative to the pBAD expression system by offering both tight regulation and high-level recombinant protein production. The pET11b vector has a lower copy number than both pRHA-67 and pMPX-66. Therefore, the amount of expression cannot be compared directly. However, the level of leaky expression in the presence of D-glucose and absence of inducer suggests that this system is not tightly regulated, and at comparable copy numbers to pRHA-67 would result in substantial toxicity when containing coding sequences for a host-toxic protein.

Expression Quantitation

Quantitation of recombinant TphoA expression was determined by densitometry based on a purified BAP standard curve of 100, 50, 25, and 12.5 ng. Extrapolating data from Figure 4B, 1 mM L-rhamnose induces 1×10^7 cells to produce 391.5 ng TphoA from pRHA-67::*tphoA*, 97.6 ng TphoA from pRHA-109::*tphoA*, and 61.4 ng TphoA from pRHA-113::*tphoA*. Under these conditions, pRHA-67::*tphoA* expresses approximately 40 mg TphoA/L or approximately 5.0×10^5 molecules TphoA per cell and >90% of TphoA is membrane-associated (data not shown).

Expression of Toxic Proteins

To compare toxic protein expression between the pRHA system and other tightly regulated expression systems, the GPCR chimera MalE-NTR was expressed from pRHA-67, pMPX-66 (pBAD regulation), and pET11b. The results show that the induction of MalE-NTR from the pBAD and pET systems was toxic, as indicated by inducer-based growth arrest (Figure 5A). In contrast, although culture doubling times were slightly longer in the presence of inducer, the expression of MalE-NTR from pRHA-67 was well tolerated. To measure the amount of full-length MalE-NTR, samples were analyzed by Western blot analysis to detect the presence of the carboxy-terminal FLAG tag (Figure 5B). These results indicate that pRHA-67 produced approximately 113 ng full-length MalE-NTR in 1×10^7 cells (approximately 1.0×10^3 MalE-NTR molecules per cell) compared with undetectable quantities from pMPX-66 and pET11b.

To demonstrate functional MalE-NTR production, the binding ^{125}I-NT ligand was measured by incubation with membranes from 1×10^8 cells (containing approximately 1×10^{11} full-length MalE-NTR proteins) in the presence and absence of unlabeled NT. The results indicate that the pRHA-67 expression system produces full-length and functional MalE-NTR capable of specific interaction with NT (Figure 5C). The average B_{max} was found to be approximately 20 fmol bound NT/1×10^8 MG1655 cells expressing MalE-NTR (1.2×10^{10} molecules NT bound to 1×10^{11} available full-length MalE-NTR proteins or 12% functional MalE-NTR proteins). The ability of NT to stimulate G-protein interactions with pRHA-67-expressed MalE-NTR was not tested. As a positive control, HEK 293 membranes were tested in parallel and exhibited a B_{max} of approximately 38 pmol bound NT/mg crude membrane. However, the number of NTR proteins in HEK 293 membranes was not disclosed by the manufacturer and no specific NTR antibody was available for quantitation, so no direct comparison can be made between these two membrane sources.

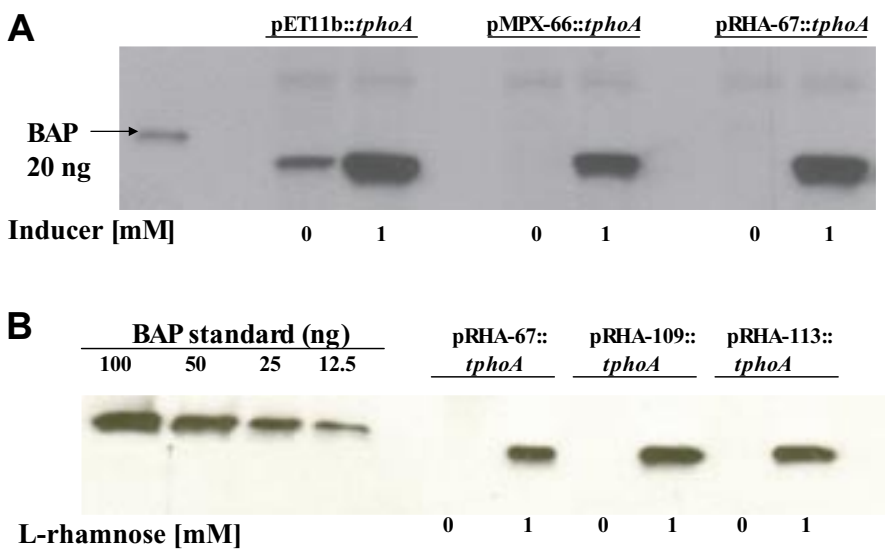


Figure 4. Comparative analysis of TphoA expression. (A) TphoA expression was compared between pET11b::*tphoA*, pMPX-66::*tphoA*, and pRHA-67::*tphoA*. Inducer was added at an A_{600} of 0.1. Noninduced cultures were maintained in 0.2% (w/v) D-glucose. Each lane contains TphoA from 5.0×10^7 cells. Western blot analysis was performed using mouse monoclonal anti-phoA as the primary antibody. Densitometry was performed as described. (B) TphoA expression from vectors pRHA-67::*tphoA*, pRHA-109::*tphoA*, and pRHA-113::*tphoA* was quantified by comparative densitometry to a BAP standard curve (100 to 12.5 ng). Protein expression was induced as described, and the numbers of cells loaded per well were 2.0×10^6, 1.0×10^7, and 1.5×10^7 for pRHA-67::*tphoA*, pRHA-109::*tphoA*, and pRHA-113::*tphoA*, respectively. Noninduced cultures were maintained in 0.2% (w/v) D-glucose. BAP, bacterial alkaline phosphatase.

DISCUSSION

The high-level expression of recombinant protein for structural studies or functional assays is of general interest. While many expression systems have been used for these applications, it is with limited exceptions that these systems are suitable for use with toxic proteins. The results presented here describe a new, tightly regulated bacterial expression system based on the *rhaTRS* locus of *E. coli*. This plasmid-based pRHA expression system is tightly regulated by the addition of D-glucose and capable of efficient protein production upon induction. As a result, these vectors are capable of expressing a wide variety of recombinant proteins, including

the stable cloning and expression of otherwise toxic proteins.

Presumably, the best system for the bacterial production of toxic proteins would be completely repressed until induced. Moreover, it would be capable of titrating protein production to meet the stringent expression criteria required by a variety of otherwise toxic recombinant proteins. Using the tightly regulated pRHA promoter and copy number-variant plasmid system developed here, we believe that we have met these criteria. First, these results show the pRHA expression system to be tightly regulated by catabolite repression and that expression can be turned on to high levels even in the presence of D-glucose. This feature allows for optimal repression during preinduction culture maintenance and high-level protein production upon induction. These results also show that the pRHA expression system induces protein production via a rheostat mechanism in the presence of subsaturating inducer concentrations.

Recently, a new strain appropriate for T7 polymerase-responsive vectors in which the pBAD promoter drives production of T7 RNA polymerase has been described (37). This strain, BL21-AI, is reported to provide improved maintenance and expression of toxic genes. Although not tested in this study, the results presented here suggest that the BL21-AI/T7 promoter system may behave similarly to the pRHA vectors in both maintenance and expression of host-toxic proteins. However, the direct comparative performance of these systems remains to be tested.

In addition to the unique regulatory properties of the pRHA system, the results presented here indicate that by varying the plasmid copy number, an additional regulatory control is available to fine-tune the expression of fastidious and/or toxic recombinant proteins. For example, a lower copy number pRHA vector may be employed to further balance inducer concentration by adjusting the number of gene copies being induced. Therefore, this panel of pRHA expression plasmids may be useful as a multicondition screening tool to quickly identify maintenance and expression conditions required for uncharacterized proteins. Finally, these results indicate that the pRHA expression system accommodates full-length and functional expression of recombinant proteins found to be toxic using other tightly regulated systems. Taken together, these results suggest the pRHA expression system may be an enabling technology for cloning and expression of a wide variety of recombinant proteins, including those found to be toxic by other methods.

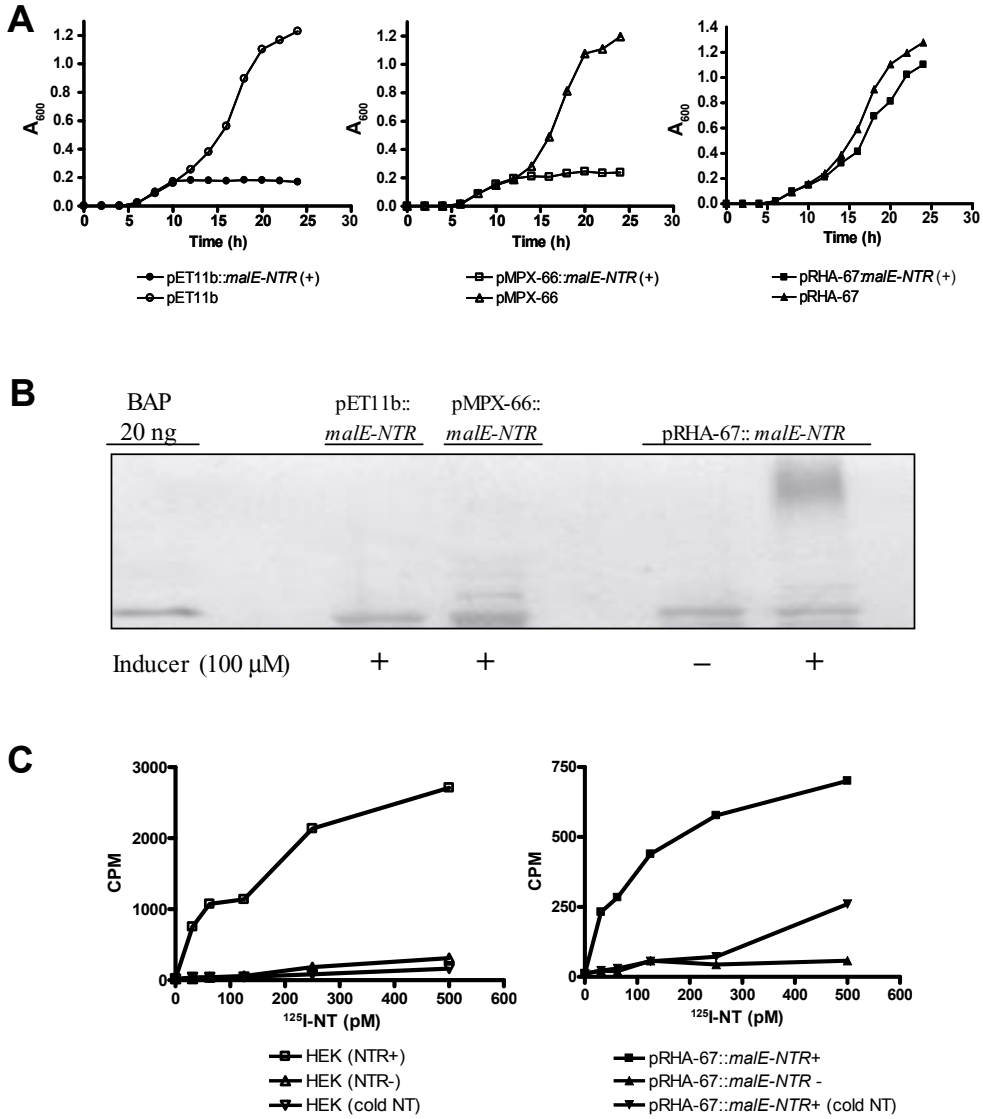


Figure 5. Expression and functional analysis of MalE-NTR production. (A) The expression of MalE-NTR is well tolerated by MG1655 harboring pRHA-67::*malE-NTR* in comparison to MG1655 harboring pMPX-66::*malE-NTR* or BL21(DE3) cells harboring pET11b::*malE-NTR*. Culture growth was monitored by A_{600} taken every 2 h. (B) Full-length expression of MalE-NTR with the C-terminal FLAG tag is detectable from MG1655/pRHA-67::*malE-NTR*. Cultures were grown and induced to produce MalE-NTR as described. Western blot analysis was performed using a mouse monoclonal antibody against FLAG tag and qualitated by comparison to a FLAG-tagged BAP positive control. (C) pRHA-67::*malE-NTR* produces functional MalE-NTR capable of specific binding to neurotrophin. Excess nonlabeled neurotrophin was used to determine specificity. BAP, bacterial alkaline phosphatase; CPM, counts per minute; ^{125}I-NT, ^{125}I-neurotensin; HEK, human embryonic kidney.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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