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Multi-Copy Genes that Enhance the Yield of Mammalian G Protein-Coupled Receptors in *Escherichia coli*

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Abstract

Low yields of recombinant expression represent a major barrier to the physical characterization of membrane proteins. Here, we have identified genes that globally enhance the production of properly folded G protein-coupled receptors (GPCRs) in *Escherichia coli.* Libraries of bacterial chromosomal fragments were screened using two separate systems that monitor: (i) elevated fluorescence conferred by enhanced expression of GPCR-GFP fusions and (ii) increased binding of fluorescent ligand in cells producing more active receptor. Three multi-copy hits were isolated by both methods: *nagD*, encoding the ribonucleotide phosphatase NagD; a fragment of *nlpD*, encoding a truncation of the predicted lipoprotein NlpD, and the three-gene cluster *ptsN-yhbJ-npr*, encoding three proteins of the nitrogen phosphotransferase system. Expression of these genes resulted in a 3- to 10-fold increase in the yields of different mammalian GPCRs. Our data is consistent with the hypothesis that the expression of these genes may serve to maintain the integrity of the bacterial periplasm and to provide a favorable environment for proper membrane protein folding, possibly by inducing a fine-tuned stress response and/or via modifying the composition of the bacterial cell envelope.

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Keywords

genetic engineering; G protein-coupled receptor; membrane protein; periplasmic expression followed by cytometric sorting (PECS); stress response

1. Introduction

Membrane proteins execute a large number of vital functions in living cells, such as maintenance of structure, transport of chemicals, signal transduction, energy production, and protection from harmful agents (Link and Georgiou, 2007). As might be expected from their central role in cellular physiology, approximately 50% of all drugs target membrane proteins (Hopkins and Groom, 2002). Despite their biological and pharmacological significance, however, the structure and function of membrane proteins is much less well understood compared to soluble proteins (Freigassner et al., 2009).

Structural and biochemical studies hinge on the availability of sufficient amounts of high-purity and correctly folded protein. Because membrane proteins are found in their natural cellular environments in quantities that are too low for biochemical analysis, they have to be prepared by expression in heterologous hosts (Link and Georgiou, 2007; Wagner et al., 2006). *Escherichia coli* have been by far the most successful host for the preparative expression of heterologous membrane proteins (Freigassner et al., 2009; Wagner et al., 2006). Unfortunately, many mammalian membrane proteins fail to form correctly in bacteria and instead accumulate as cytoplasmic inclusion bodies that are very difficult to refold (Link and Georgiou, 2007). Additionally, membrane protein overexpression often results in severe cell toxicity and low final biomass production, further limiting the yield (Wagner et al., 2007).

Recently, directed protein evolution has been applied in order to identify amino acid substitutions in prokaryotic and eukaryotic membrane proteins that lead to enhanced expression in *E. coli*. Following mutagenesis and screening, variants of certain hard-to-express membrane proteins have been isolated which can be produced in bacteria in more than an order of magnitude higher quantity compared to their parental sequences (Dodevski and Pluckthun, 2011; Molina et al., 2008; Sarkar et al., 2008).

Other groups been demonstrated that *E. coli* can be engineered genetically to produce significantly enhanced amounts of recombinant membrane proteins (Makino et al., 2011a). Chen *et al*. reported that co-expression of the chaperone/co-chaperone DnaK/DnaJ conferred a significant increase in the bacterial production of the magnesium transporter CorA, whereas co-expression of the membrane-bound protease FtsH and of the protein of unknown function YbaB was shown to enhance dramatically the accumulation of membrane-incorporated human G protein-coupled receptors (GPCRs) in *E. coli* (Chen et al., 2003; Link et al., 2008; Skretas and Georgiou, 2010). Additionally, *E. coli* variants carrying chromosomal lesions or transposon insertions that facilitate the expression of increased levels of membrane-integrated prokaryotic or eukaryotic membrane proteins have been reported (Massey-Gendel et al., 2009; Skretas and Georgiou, 2009). Finally, Baneyx and co-workers demonstrated that simultaneous deletion of *tig*, the gene encoding for the molecular chaperone trigger factor, and overexpression of *yidC*, the gene encoding for the membrane protein integrase YidC, results in a 3- to 7-fold increase in the accumulation of the *Haloterrigena turkmenica* deltarhodopsin, the *Natronobacterium pharonis* sensory rhodopsin II, and the *E. coli* sensory histidine kinase ZraS (Nannenga and Baneyx, 2011).

GPCRs constitute the largest eukaryotic membrane protein superfamily and are notoriously difficult to overexpress in prokaryotic as well as in eukaryotic hosts (Sarramegna et al.,

2003). Here, we describe the isolation of multi-copy genes that broadly enhance the yield of several type I GPCRs in *E. coli*. Libraries of genomic fragments were screened in parallel by two different fluorescence-activated cell sorting (FACS) assays that detected either improved expression of GPCR-GFP fusions or the binding of fluorescent ligand. Three different gene fragments conferring enhanced cell fluorescence were identified independently by both screens and shown to result in a markedly enhanced yield of membrane-integrated and properly folded receptor for several hard-to-express mammalian GPCRs. We hypothesize that these genes act by inducing a fine-tuned stress response that appears to be optimal for assisting protein integration into the *E. coli* membrane and/or via modifying of the composition of the bacterial cell envelope.

2. Materials and Methods

2.1 Strains and plasmids

The *E. coli* strain MC4100A (MC4100 *ara*+) (Santini et al., 2001) was utilized for all experiments. The expression vectors pBADSmRBla-NTR1 and pBADSmRBla-NTR1(D03) were constructed as follows: the protein sequences for a N-terminally truncated rat neurotensin receptor 1 (NTR1) (Tucker and Grisshammer, 1996) and the corresponding variant NTR1(D03) were retrieved from the GPCRDB website (http://www.gpcr.org/7tm/) and from Sarkar et al. (Sarkar et al., 2008), respectively. The genes encoding for these proteins were constructed by PCR-based oligonucleotide assembly using DNA primers which had been codon-optimized for expression in *E. coli* as described in previous work (Link et al., 2008). XbaI and PstI restriction sites were appended at the 5′ and 3′ termini of these genes, respectively. The gene encoding for TrxA was amplified from *E. coli* genomic DNA by gene-specific DNA primers that encoded PstI and HindIII restriction sites at the 5′ and 3′ termini of the gene, respectively. NTR1 or NTR1(D03) were then digested with XbaI-PstI, TrxA was digested with PstI-HindIII and the resulting gene fragments were used to construct pBADSmRBla-NTR1 and pBADSmRBla-NTR1(D03), respectively, by performing a double ligation into pBADSmRBlaBR2 (Skretas and Georgiou, 2010), from which the BR2 sequence had been excised by digesting with XbaI-HindIII. pBAD33BR2-GFP, pBAD33NKR1-GFP, and pBAD33CB1-GFP were constructed by subcloning BR2-GFP, NKR1-GFP, and CB1-GFP from pASKBR2-GFP, pASKNKR1-GFP, and pASKCB1-GFP (Link et al., 2008), respectively, into pBAD33 (Guzman et al., 1995) by using the restriction sites XbaI-HindIII. pBAD33CstA-GFP was constructed by amplification of *cstA* gene from *E. coli* genomic DNA and insertion into the XbaI-PstI sites of pBAD33BR2-GFP after removal of the BR2-encoding gene. The *E. coli* genomic library pTrcGL was constructed as follows: *E. coli* MC4100A chromosomal DNA was isolated by using the GenElute™ Bacterial Genomic DNA kit (Sigma). The extracted DNA was partially digested with FatI and separated with agarose gel electrophoresis. The DNA fragments with sizes from 0.6 to 2.5 and from 2.5 to 9 kbases were isolated and then ligated into the pTrc99a vector previously digested with NcoI to form a “short fragment library” and a “long fragment library”, respectively. The size of each library was ~10^6. The quality of each library was evaluated by performing DNA sequencing on ten randomly selected clones and it was found that each one contained a good representation of fragment sizes and cloned areas of the *E. coli* genome. The plasmids pTrcNagD, pTrcPcm, pTrcNlpD, pTrc(Pcm-NlpD), pTrc5′NlpD, pTrcNlpDΔ(349–380), pTrcPtsN, pTrcYhbJ, pTrcNpr, pTrc(PtsN-YhbJ), pTrc(YhbJ-Npr), and pTrc(PtsN-YhbJ-Npr) were constructed by PCR amplification of the corresponding genes from *E. coli* genomic DNA with sequence-specific DNA primers carrying XbaI and HindIII recognition sequences in their 5′ and 3′ ends, respectively, along with a ribosome-binding site. Gene sequence information was acquired from EcoCyc (www.ecocyc.org). The resulting PCR products were digested with XbaI and HindIII and ligated into similarly digested pTrc99a. For all constructed vectors, insertion of the gene with the correct sequence was verified with DNA sequencing. The plasmids pAU66-rpoH,

pAU66-ppiA, pAU66-ppiD, pAU66-degP, pAU66-ftsH, pAU66-lon, pAU66-ibpB, pAU66-bolA and pAU66-glnA used for stress response analysis were a kind gift from A. James Link (Princeton University).

2.2 Membrane protein overexpression

E. coli MC4100A cells freshly transformed with the appropriate expression vector, with and without a plasmid co-expressing an effector gene, were used for all protein production experiments. Single bacterial colonies were used to inoculate liquid LB cultures containing the appropriate combination of antibiotics (100 μg/mL spectinomycin, 40 μg/mL chloramphenicol, 100 μg/mL ampicillin, 50 μg/mL kanamycin). These cultures were used with a 1:100 dilution to inoculate fresh LB cultures with 0.1 mM IPTG, which were grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.5–0.7 with shaking. The temperature was then decreased to 25 °C and after a temperature equilibration period of 10–20 min, membrane protein expression was induced by the addition of 0.1% L-arabinose for 3–5 h.

2.3 Fluorescent ligand synthesis and labeling conditions

The fluorescent neurotensin analog BODIPY-Neurotensin(8–13) was prepared by coupling dipyrromethene boron difluoride (BODIPY) FL-X, SE (Invitrogen) to the minimal bioactive peptide sequence neurotensin (8–13) (AnaSpec) according to the manufacturer's instructions. The conjugated product was isolated by reverse-phase HPLC and purity was confirmed by mass spectrometry. For ligand labeling using periplasmic expression followed by cytometric sorting (PECS), cells overexpressing NTR1 or NTR1(D03) were harvested by centrifugation and resuspended in cold Tris-KCl buffer (50 mM Tris-HCl, pH 7.4, 150 mM KCl) (Sarkar et al., 2008) and 500 nM BODIPY-NT(8–13) followed by 1 h incubation at room temperature with shaking.

2.4 Flow Cytometry and FACS

Cell fluorescence (530/30 nm) was monitored using either a Becton-Dickinson FACSAria™ flow cytometer and analyzed with FACSDiva software or a Dako MoFlo™. For FACS screening, cells were initially gated on a side-scatter (SSC-H) versus forward-scatter (FSC-H) plot. ~10^5 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 LB as described above, and subjected to repeated rounds of PECS screening using FACS.

2.5 Membrane Isolation

Total membrane fractions were prepared from cells harvested from 500 mL LB cultures, resuspended in 10 mL of cold lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, 15% glycerol, 5 mM dithiothreitol) and lysed by double passing through a French press. Cell lysates were then centrifuged twice at 8,000 rpm for 20 min, the supernatant was collected and subjected to ultracentrifugation at 50,000 rpm for 1.5 hr at 4 °C. Total membranes corresponding to the pellet of the ultracentrifugation step were resuspended in 10 mL of cold lysis buffer and homogenized.

2.6 Western blot analysis

Western blotting was performed as described previously (Skretas and Georgiou, 2009) by using the following antibodies: mouse anti-GFP IgG (Clontech), goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma), and mouse anti-polyhistidine conjugated with horseradish peroxidase (Sigma).

2.7 Stress responsive promoter assay

MC4100A cells harboring pBADSmRNTR1(D03) and a pTrc99a vector encoding one of the isolated genes/gene clusters or empty vector were transformed with a plasmid encoding a stress responsive promoter-GFP fusion and plated on solid LB media containing the appropriate antibiotics. Single colonies were picked to inoculate liquid LB overnight cultures containing the same antibiotics. The following day, these cultures were used at a 1:100 dilution to inoculate fresh LB cultures containing 0.1 mM IPTG, and were grown aerobically at 37 °C to OD_{600} of 0.5–0.7. The temperature was then decreased to 25 °C, and membrane protein expression was induced by the addition of 0.1% L-arabinose. Cell fluorescence was monitored by flow cytometry at 0 and 5 h after arabinose addition.

3. Results

3.1 Screening for genes that enhance the expression of functional mammalian GPCRs in E. coli

The D03 mutant of the rat neurotensin receptor 1 (NTR1(D03)) has been previously engineered by directed evolution for improved expression in *E. coli* (Sarkar et al., 2008). To monitor the amount of active NTR1(D03) at the single-cell level, we used a variation of periplasmic expression followed by cytometric sorting (PECS) (Chen et al., 2001; Skretas and Georgiou, 2008) as described by Sarkar et al. (Sarkar et al., 2008). Briefly, cells are incubated in a high osmolarity buffer rendering the bacterial outer membrane permeable to fluorescently labeled NTR1 ligand, and highly fluorescent clones due to the production of increased amounts of active GPCR are isolated using FACS. Sorted cells are re-grown and subjected to additional rounds of screening with enrichment monitored by an increase in the mean fluorescence of the population.

The accumulation of the ligand-binding form of NTR1 in *E. coli* is markedly increased when it is expressed as an N- and C-terminal fusion to well folded partners (Tucker and Grisshammer, 1996). For this reason, we expressed NTR1(D03) as a fusion with TEM-1 β-lactamase (Bla) at the N terminus and thioredoxin (TrxA) at the C terminus under the control of the *araBAD* promoter (Figure 1A). Since most endogenous and heterologous membrane proteins are targeted to the inner membrane via the signal recognition particle (SRP) pathway, the SRP pathway-independent signal peptide of Bla (Beha et al., 2003) was replaced with the SRP-dependent DsbA signal peptide (Schierle et al., 2003) (Figure 1A). *E. coli* MC4100A cells (MC4100 *ara*+) (Santini et al., 2001) expressing Bla-NTR1(D03)-TrxA from the vector pBADSmRNTR1(D03) (Table 1) exhibited high levels of receptor-specific fluorescence when labeled with a dipyrromethene boron difluoride (BODIPY) fluorescent conjugate of the NTR1 agonist neurotensin(8–13) (BODIPY-NT(8–13)).

E. coli MC4100A (pBADSmRNTR1(D03)) cells were transformed with a library of genomic *E. coli* fragments with sizes between 0.6 and 9 kbp and inserted downstream of the *trc* promoter in pTrc99a. Approximately 10^7 bacterial colonies carrying both plasmids were collected, pooled, grown in liquid LB media and the expression of Bla-NTR1(D03)-TrxA was induced by the addition of 0.1% L-arabinose. After 3–5 hours at 25 °C, ~10^7 cells labeled with 500 nM BODIPY-NT(8–13) were screened and the population exhibiting the top 1–3% fluorescence was isolated using FACS. The isolated cells were re-grown and screened for 8 additional rounds, at which point the mean fluorescence of the population displayed a 2.7-fold increase relative to the library prior to sorting; no further increase in fluorescence was observed after additional rounds of sorting (Figure 1B). It is interesting to note that at that point there were two sub-populations present, one exhibiting high fluorescence, while the other lacked fluorescence (Figure 1B). The latter likely corresponds to cells which have undergone plasmid loss or which have acquired mutations that shut down gene expression and/or synthesis of the recombinant protein. Expression of NTR1 and

other GPCRs has been reported to be toxic to bacteria and causes the appearance of the non-fluorescent population (Link et al., 2008; Skretas and Georgiou, 2009; Wagner et al., 2007).

80 colonies were picked randomly after the fifth and ninth rounds of FACS sorting (40 from each round) and evaluated for BODIPY-NT(8–13) by FACS. 15 clones from each round exhibiting the highest FACS signals were selected and the identity of the expressed genomic fragment was determined by DNA sequencing. Three classes of different gene fragments were found in the clones isolated from the fifth round: the first class of gene fragments contained the *nagD* gene (*nagD*), the second the genes *pcm* and *nlpD* (*pcm-nlpD*), and the third containing the genes *hpf*, *ptsN*, *yhbJ*, and *npr* (*hpf-ptsN-yhbJ-npr*) (Table 2). In each class, the size of the fragment and the cloning junction varied indicating that they did not correspond to the same clone. The shortest fragments in each class are shown in Table 2. All 15 high-fluorescence clones isolated from the ninth round contained the *nagD* fragment. Co-expression of the genomic fragments in Table 2 resulted in a 2–3 fold increase in the binding of fluorescence ligand in permeabilized cells (Figure 1C). To further dissect the effects of each of *nagD*, *pcm* and *nlpD*, and *hpf*, *ptsN*, *yhbJ* and *npr* in the three genomic fragments isolated in the screen, each ORF and also truncations in the gene fragments were generated and their effect on BODIPY-NT(8–13) binding in cells co-expressing NTR1(D03) was determined (Table 2). The minimal fragments that conferred the increased fluorescence phenotype were: (i) the *nagD* gene, encoding for the ribonucleotide monophosphatase NagD; (ii) a deletion in *nlpD* (*nlpDΔ(349–380)*) encoding for a C-terminal truncation between amino acids 349 and 380 of the putative outer membrane lipoprotein NlpD; (iii) the entire *ptsN-yhbJ-npr* sequence, but not any individual ORF or combinations of ORFs encoded within the *hpf-ptsN-yhbJ-npr* fragment (Table 2 and Figure 2A). *ptsN-yhbJ-npr* encodes three of the components of the *E. coli* nitrogen phosphotransferase system. Western blotting experiments with isolated membrane fractions showed that co-expression of *nagD*, *nlpDΔ (349–380)*, and *ptsN-yhbJ-npr* results in a marked increase in the expression of membrane-incorporated NTR1(D03) and of wild-type NTR1 (Figure 2C).

3.2 A second screen for multi-copy genes that enhance the fluorescence of GPCR-GFP fusions identifies the same gene fragments

De Gier and co-workers have shown that the fluorescence of bacterial cells expressing GFP fusions to the C terminus of membrane proteins having this terminus facing the cytoplasm (C_{in}) correlates well with the amount of membrane-integrated protein expressed (Drew et al., 2001). Such fusions with GFP have been used extensively for monitoring the expression of membrane-integrated membrane proteins (Drew et al., 2006; Drew et al., 2001) and for genetic analyses (Link et al., 2008; Skretas and Georgiou, 2009). Whereas the direct ligand assay selects clones that display active GPCR, the fluorescence of cells expressing membrane protein-GFP fusions reflects the amount of non-aggregated protein; the latter may or may not be correctly folded or capable of ligand binding. The human bradykinin receptor 2 (BR2) and the neurokinin (substance P) receptor 1 (NKR1) are important targets for pharmaceutical discovery and their wild-type sequences express very poorly in *E. coli* (Dodevski and Pluckthun, 2011; Link et al., 2008). *E. coli* MC4100A cells carrying the plasmids pBAD33BR2-GFP or pBAD33NKR1-GFP expressing BR2-GFP and NKR1-GFP under the control of the *araBAD* promoter (Figure 3A and Table 1), respectively, were transformed with the *E. coli* genomic library described above. A library of 10^7 transformants was screened by FACS for 6–9 rounds with sorting of the top 1–3% most fluorescent cells at each round. The mean fluorescence of the BR2-GFP expressing clones after the last round was 3-fold higher than the fluorescence of cells expressing BR2-GFP alone, while the corresponding increase in fluorescence for the NKR1-GFP library was 8-fold (Figure 3B). 20 or more colonies were randomly selected from the last round, cells were grown in LB media and GFP fluorescence was evaluated by flow cytometry. Sequencing of the inserts

showed the presence of a variety of genes but, importantly, fragments containing *nagD*, *pcm-nlpD*, and *hpf-ptsN-yhbJ-npr* isolated above because of their ability to confer increased ligand binding to NTR1(D03) expressing cells, were also isolated here. These fragments mediated higher cell fluorescence in cells expressing BR2-GFP and, independently, NKR1-GFP. The minimal size fragments determined by the analysis described above, namely *nagD*, *nlpDΔ (349–380)* and *ptsN-yhbJ-npr* genes also confirmed high fluorescence with the GPCR-GFP fusions (Figure 3C) and were selected for further studies. It is possible that some of the gene fragments isolated via screening of genomic libraries using the GFP reporter screen, but not also in the ligand binding screen, may act to enhance the expression of membrane-integrated but inactive GPCR, in terms of ligand binding. The effect of these genes was not investigated further.

Flow cytometry and Western blot analyses were used to evaluate the effect of the isolated gene fragments on the yield of membrane-integrated polypeptide for a variety of proteins. Western blotting of membrane fractions from cells expressing BR2-GFP is shown in Figure 3D and reveals two bands with molecular mass close to the predicted one for BR2-GFP (~72 kDa). As it has been demonstrated previously by Poolman and co-workers (Geertsma et al., 2008), the higher molecular weight band corresponds to unfolded or misfolded GFP fusion, while the lower band comprises folded protein. The third and fourth bands with molecular mass <50 kDa that appears in the total membrane fractions of cells co-expressing *nagD*, *nlpDΔ (349–380)*, or *ptsN-yhbJ-npr* correspond most likely to degradation products. Based on these results, wild-type *E. coli* cells are able to produce very little membrane-embedded BR2 protein. Co-expression of *nagD*, *nlpDΔ (349–380)*, or *ptsN-yhbJ-npr*, however, results in accumulation of significantly enhanced amounts of membrane-integrated BR2 (Figure 3D). Similarly, the expression of other GPCRs, such the human central cannabinoid receptor (CB1), and of a homologous integral membrane protein, the putative *E. coli* peptide transporter CstA, was increased significantly, as indicated by the 3- to 10-fold enhancement in the fluorescence signal of cells expressing fusions of these membrane proteins with GFP with or without co-expression of the isolated genes (Figure 3C).

3.3 *nagD* and *nlpDΔ (349–380)*, but not *ptsN-yhbJ-npr*, induce selective changes in cellular stress responses

It has been recently suggested that the AAA+ membrane-bound protease FtsH, a factor whose overexpression increases significantly the accumulation of membrane-incorporated mammalian GPCRs in *E. coli* (Link et al., 2008), may be exerting its enhancing effects by activating specific heat shock and cell envelope stress response pathways (Xu and Link, 2009). Increased expression of stress-related genes, such as molecular chaperones and proteases, may be assisting the cell to cope with the production of these hard-to-express recombinant proteins (Xu and Link, 2009). To evaluate the impact of *nagD*, *nlpDΔ (349–380)* or *ptsN-yhbJ-npr* on bacterial stress responses, we utilized a set of transcriptional fusions (Zaslaver et al., 2006) of stress-responsive promoters with the gene encoding for the FACS-optimized GFP variant GFPmut2 (Cormack et al., 1996): P3*rpoH* (regulated by the extracytoplasmic sigma factor σ^E (Alba and Gross, 2004)); P*ppiA* and P*ppiD* (regulated by the extracytoplasmic stress response regulator CpxR (Raivio, 2005)); P*degP* (regulated by both σ^E and CpxR (Ruiz and Silhavy, 2005)); P*ftsH*, P*lon*, P*ibpB* (regulated by the heat shock factor σ^H (Zhao et al., 2005)); P*glnA* (regulated by the nitrogen limitation factor σ^N (Reitzer and Schneider, 2001)); and P*bolA* (transcribed by the general stress and stationary phase-associated factor σ^S (Weber et al., 2005)).

E. coli MC4100A cells carrying pBADSmRNTR1(D03) and either pTrc99a (empty vector), pTrcNagD, pTrcNlpDΔ(349–380), or pTrc(PtsN-YhbJ-Npr) were also transformed with plasmids carrying each of the promoter-GFP fusions described above. These cells were grown in liquid LB media in the presence of 0.1 mM IPTG so as to induce effector gene

expression, and cell fluorescence was measured by flow cytometry before and after 5 hours induction of GPCR overexpression by the addition of 0.1% L-arabinose. Overexpression of NTR1(D03) alone did not induce stress-responsive expression of GFP for the vast majority of the promoters tested, with the exception of a moderate increase in the case of the *ppiA* and *lon* promoters (Figure 4; compare bars corresponding to empty vector at t=0 and 5 h). This absence of stress response induction is consistent with what has been observed for a variety of integral membrane proteins in a very recently published study (Gubellini et al., 2011). Overexpression of NTR1(D03) together with *nlpDΔ(349–380)*, however, resulted in a significant increase in transcription from P3*rpoH*, P*ftsH*, and P*ppiD*, promoters (3.5-, 2.9-, and 2.7-fold, respectively) compared to the empty plasmid control and, to a lesser extent, from the P*ppiA*, P*bolA* and P*lon* promoters (2.3-, 2.3-, and 2.1-fold, respectively) (Figure 4). Expression of GFP from the P*ibpB*, P*degP* or P*glnA* promoters was only marginally higher compared to the empty vector control (1.9-, 1.8-, and 1.6-fold, respectively) (Figure 4 and data not shown). Similarly, co-expression of *nagD* resulted in a significant increase in transcription from P3*rpoH* (2.7-fold), a moderate increase from the P*ftsH* and P*ppiD* promoters (2.2- and 2.4-fold, respectively), and a marginal increase from the P*ppiA*, P*bolA* P*ibpB*, P*degP*, P*lon*, and P*glnA* promoters (1.7-, 1.7-, 1.9-, 1.5-, 1.4-, and 1.4-fold, respectively) compared to the empty vector control (Figure 4 and data not shown). The effect of *nlpDΔ(349–380)* on enhancing stress responsive gene expression was greater in all cases than that mediated by co-expression of *nagD*. In complete contrast to *nagD* and *nlpDΔ(349–380)*, expression of *ptsN-yhbJ-npr* did not confer any significant increase in GFP fluorescence from any promoter fusion tested (Figure 4 and data not shown). These results indicate that *nagD* and *nlpDΔ(349–380)* co-expression affects a variety of bacterial stress responses primarily relating to cell envelope stress and the cytoplasmic heat shock response. Although these responses are not induced at very high levels, they are significant and comparable to the activation levels of the extracytoplasmic responses under conditions where membrane protein production/assembly and/or folding are problematic in *E. coli*, such as depletion of the membrane protein integrase YidC and the presence of *secY* missense mutations that yield defective assembly of overexpressed model membrane proteins (Shimohata et al., 2007).

The extent to which a particular stress response regulon was induced was found to be strongly promoter-specific. For example, although expression of NTR1(D03) together with *nlpDΔ(349–380)* or *nagD* induced a high level of transcription from the σ^E-regulated P3*rpoH* promoter and from the Cpx-regulated P*ppiD* promoter, we observed no increase in GFP fluorescence from P*degP* which is positively regulated by both σ^E and CpxR (Ruiz and Silhavy, 2005) (Figure 4). In an analogous manner, a higher GFP fluorescence was observed from the σ^H-dependent promoters P*ftsH* compared to P*lon* and P*ibpB*. Induction of these stress responses appears to be primarily a consequence of the co-expression of *nagD* and *nlpDΔ(349–380)*, and not a side effect of the enhanced accumulation of the expressed GPCR, since (i) *ptsN-yhbJ-npr* co-expression fails to induce stress-responsive GFP expression, despite the fact that it is effective in enhancing the accumulation of membrane-bound GPCRs (Figures 2, 3, and 4) and (ii) overexpression of *nlpDΔ(349–380)* together with NTR1(D03) results in higher levels of stress response induction compared to *nagD* for all promoters, despite the fact that *nagD* co-expression results in larger accumulation of recombinant GPCR. Each of the cell envelope and heat shock responses regulate the expression of close to 100 individual genes, such as molecular chaperones, folding factors, and proteases, which assist the cell in coping with stress, such as that resulting from protein misfolding (Rhodius et al., 2006; Richter et al., 2010). Thus, it is possible that *nlpDΔ(349–380)* and *nagD* co-expression during GPCR production modulates the expression of a variety of stress-related genes in a way that it is “preparing” the cell to handle the production of hard-to-express proteins. Such a mechanism is also consistent with the beneficial effects

of the co-expression of the membrane protease FtsH on bacterial GPCR production (Xu and Link, 2009).

4. Discussion

In the present work, we searched for multi-copy bacterial genes and gene clusters which can act as enhancers of the accumulation of membrane-embedded and correctly folded mammalian GPCRs in *E. coli*. For this, we screened an *E. coli* genomic library using two different flow cytometric assays: (i) a PECS-based assay that monitors directly the binding of a fluorescently labeled ligand to active GPCR, and (ii) an assay that monitors the fluorescence of folded GPCR-GFP fusions. Using three model mammalian GPCRs (NTR1(D03), BR2, and NKR1) and the screens mentioned above, a common set of *E. coli* gene fragments were isolated in both screens: *nagD*, the gene encoding the ribonucleotide phosphatase NagD; the gene fragment *nlpDΔ(349–380)*, encoding a C-terminal truncation of the putative outer membrane lipoprotein NlpD, and a three-gene cluster *ptsN-yhbJ-npr*, encoding three proteins of the nitrogen phosphotransferase system. Co-expression of the isolated genes was found to result in a marked increase in the production of membrane-integrated and well folded protein for a variety of mammalian GPCRs: wild-type NTR1, NTR1(D03), BR2, CB1, and NKR1, as well as for a prokaryotic integral membrane protein, the putative *E. coli* peptide transporter CstA. Thus, the genes isolated here using two completely different expression screens and different model GPCRs, appear to broadly enhance the production of a variety of GPCRs and, possibly, of other membrane proteins.

All three identified genes/gene clusters are novel effectors of the accumulation of mammalian GPCRs in *E. coli*. It is interesting to note that, while in an earlier study we had shown that co-expression of the AAA+ membrane-bound protease FtsH enhances the yield of several GPCRs, no genomic fragments containing *ftsH* were identified by either one of our expression screens here. We note however, that *Fat*I, the restriction endonuclease which was used for the construction of the genomic DNA library, cleaves the *ftsH* gene at 13 sites and, thus, the presence of a clone with the intact gene in our library is highly unlikely.

To gain potential insight into the mechanism by which the isolated genes/gene clusters aid the accumulation of membrane proteins, we tested how stress-responsive gene expression during GPCR production changes upon co-expression of the identified genes. These experiments revealed two distinct effects: (i) co-expression of the cluster *ptsN-yhbJ-npr* did not affect transcriptional activity from any promoter tested, whereas (ii) the co-expression of *nlpDΔ(349–380)* and *nagD* induced significantly a variety of bacterial stress responses upon GPCR overexpression, primarily cell envelope stress and the cytoplasmic heat shock response.

NagD is a member of one of the two *nag* operons, *nagABCD* and *nagE*, which include five genes responsible for *N*-acetylglucosamine (GlcNAc) utilization (Peri et al., 1990). GlcNAc is a key intermediate of uridine 5′-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc), a precursor in the biosynthesis of all amino sugar macromolecules including the peptidoglycan and the lipopolysaccharide (LPS) of *E. coli* (Barreteau et al., 2008; Bouhss et al., 2008; Ogata et al., 1999). In contrast to the products of the genes *nagABC* and *E* whose functions have been largely deciphered, the physiological role of the ribonucleotide phosphatase NagD is poorly characterized (Kuznetsova et al., 2006; Tremblay et al., 2006). We hypothesize, however, that NagD is enhancing GPCR production in an indirect manner by affecting GlcNAc metabolism and by changing to some extent the composition of the bacterial cell envelope. This could in turn be either (i) providing a more favorable environment for membrane protein folding, or/and (ii) inducing a level of periplasmic and cytoplasmic stress response which is beneficial for membrane protein production.

NlpD is an outer membrane lipoprotein (Ichikawa et al., 1994) that localizes at the bacterial septum during *E. coli* division. NlpD along with other LytM-domain proteins, such as EnvC, are thought to assist the separation of the shared cell wall material between the two daughter cells during cell division, possibly by activating the ability of the amidases AmiABC to hydrolyze the peptidoglycan (Uehara et al., 2010). Since the isolated NlpDΔ(349–380) fragment lacks the C-terminal LytM domain and the full-length NlpD protein failed to enhance GPCR production (Table 2), it seems unlikely that the cell division-mediating function of NlpD is responsible for the observed enhancement in membrane protein production. The cytotoxic effects of the high-level overexpression of full-length *nlpD* (Lange and Hengge-Aronis, 1994), however, could account for the failure of the non-truncated protein to enhance GPCR accumulation. Lipoproteins have very recently been identified as regulators of peptidoglycan-synthesizing enzymes, such as penicillin-binding proteins (Paradis-Bleau et al., 2010; Typas et al., 2010). Accordingly, NlpDΔ(349–380) co-expression could be enhancing membrane protein production by changing the composition of the bacterial periplasm, in a manner similar to the hypothesized effect of NagD. Although we have not investigated the folding of NlpDΔ(349–380) explicitly, another possibility is that the identified C-terminal truncation in NlpDΔ(349–380) causes the protein to misfold. The overexpression and/or misfolding of lipoproteins and other cell envelope proteins has been shown to activate the cell envelope stress response system (Alba and Gross, 2004; Raivio, 2005), an effect which has also been observed here with NlpDΔ(349–380). The induction of the periplasmic and cytoplasmic protein quality control systems is likely leading to enhanced expression of other proteins, such as FtsH, which promote the accumulation of membrane-embedded GPCRs (Link et al., 2008).

Surprisingly, we failed to detect upregulation of any of the stress promoters tested in cells co-expressing the gene cluster *ptsN-yhbJ-npr.* This cluster encodes two proteins, PtsN (or nitrophosphotransferase system enzyme IIA) and Npr, which are thought to be part of the bacterial nitrogen phosphotransferase system and which resemble components of the sugar phosphotransferase system (Pfluger-Grau and Gorke, 2010). Very interestingly, *ptsN* has been found to be a multi-copy suppressor of the essentiality of σ^E in *E. coli*, a factor which is required for maintaining the integrity of the periplasmic space in this organism (Hayden and Ades, 2008). *ptsN*, which is a σ^E-regulated gene itself, was found capable of lowering the basal activity of a variety of different bacterial cell envelope stress responses, such as the σ^E, Rcs, and Cpx pathways (Hayden and Ades, 2008). It must be mentioned that phosphorylation of PtsN is required for the ability of PtsN to suppress the lethality of the loss of σ^E activity and that PtsN phosphorylation is mediated by the function of Npr (Hayden and Ades, 2008; Pfluger-Grau and Gorke, 2010). The *ptsN-yhbJ-npr* cluster encodes also for YhbJ, a predicted ATPase with a poorly characterized physiological function. YhbJ has been reported to affect the synthesis of GlcN-6P, a key reaction in the biosynthesis of lipopolysaccharides and the peptidoglycan (Barreteau et al. 2008) by controlling the processing and stability of the small RNA regulator GlmZ (Kalamorz et al., 2007). Inactivation of *yhbJ* leads to overproduction of GlmS and increased synthesis of GlcN-6P. Very recently, *yhbJ* was identified as an important gene for isobutanol tolerance in *E. coli* (Atsumi et al., 2010) In that study, Liao and co-workers showed that genetic inactivation of *yhbJ* results in enhanced resistance to isobutanol and other higher-chain alcohols by a mechanism that presumably involves changes in the composition of the LPS of the outer membrane (Atsumi et al., 2010). GlcN-6P was proposed to play a central role in this process and, indeed, supplementation of a culture of a parental (*yhbJ*$^+$) *E. coli* strain with GlcNAc resulted in enhanced isobutanol tolerance (Atsumi et al., 2010).

Taken together, it appears that *nlpDΔ(349–380), nagD*, and *ptsN-yhbJ-npr* enhance the production of membrane-embedded and properly folded GPCRs not by interaction with these proteins directly (e.g. by assisting GPCR biogenesis, folding and/or assembly), but by

indirect effects. Based on the currently available knowledge about the physiological functions of the isolated effector genes, we hypothesize that a subtle balance between the induction of the periplasmic and cytoplasmic heat shock responses, and/or of mechanisms that help maintain the composition and integrity of the bacterial periplasmic space may be important for optimal membrane protein expression. According to our hypotheses, enhanced accumulation of membrane-embedded proteins could be occurring due to:

- **i.** Induction of stress responses, which may be up-regulating genes encoding for factors that assist membrane protein production, such as molecular chaperones, folding catalysts and proteases. For example, expression of *nlpDΔ(349–380)* or *nagD* up-regulates the promoter of *ftsH*, a gene which has been shown previously to enhance markedly the accumulation of well folded human GPCRs in the inner membrane of *E. coli* cells (Link et al., 2008). Although *ptsN-yhbJ-npr* did not induce gene expression from any of the stress-responsive gene promoters tested, its isolation as a factor that enhances GPCR accumulation is not inconsistent with this model, since phosphorylated PtsN (mediated by the function of Npr) has been found to be a multi-copy suppressor of the lethality caused by the loss of σ^E function in *E. coli* (Hayden and Ades, 2008). Thus, phosphorylated PtsN may have overlapping functions with σ^E and its overexpression may resemble a cellular status where *rpoE* is up-regulated.
- **ii.** Changes in the composition of the bacterial periplasm, thus providing a favorable environment for the folding of mammalian GPCRs, Indeed, the overexpression of recombinant membrane proteins has been shown to have severe consequences on the composition of the bacterial cell envelope (Wagner et al., 2007), and factors necessary for proper folding of these proteins may be limited. GlcNAc may be playing a key role in this process since two of the three isolated genomic fragments contain genes which are involved in glucosamine biosynthesis.

Mechanisms (i) and (ii) may be taking place interdependently, i.e. the induction of stress responses described in (i), for example, could be a direct result of altered periplasmic composition described in (ii). It is important to stress that the proposed mechanisms are only hypotheses at this point. The effects of stress response pathways and the consequences of altered cell wall biosynthesis on membrane protein production are not well understood presently and, therefore, additional work will be required in order to decipher the detailed role of the isolated genes.

The results reported here serve to underscore the great promise of genetic approaches towards the engineering of specialized microbial “cell factories” for the production of recombinant membrane proteins, similarly to what has been achieved for a variety of soluble proteins and small molecules (Ajikumar et al., 2010; Atsumi et al., 2008; Makino et al., 2011a; Makino et al., 2011b; Nicolaou et al., 2010; Pfeifer et al., 2001; Souza et al., 2011). Facile bacterial expression systems for GPCR production will alleviate a major bottleneck in the study of this large protein class, aid our understanding of the limiting steps in recombinant membrane protein expression, and finally provide unanticipated insights in membrane protein biogenesis and stress responses in bacteria..

Acknowledgments

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Abbreviations

PECS	periplasmic expression followed by cytometric sorting
GPCR	G protein-coupled receptor
FACS	fluorescence-activated cell sorting
GFP	Green fluorescent protein
NTR1	rat neurotensin receptor 1
NTR1(D03)	NTR1 D03 variant
BR2	human bradykinin receptor 2
NKR1	human neurokinin (substance P) receptor 1
CB1	human central cannabinoid receptor
TrxA	thioredoxin A
BODIPY	dipyrromethene boron difluoride
GlcNAc	N-acetylglucosamine
UDP-GlcNAc	uridine 5′-diphospho-N-acetyl-Dglucosamine
LPS	lipopolysaccharide
GlcN-6P	glucosamine-6-phosphate
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Highlights

- Two expression screens were used to isolate genes that enhance bacterial GPCR production
- Three multi-copy enhancer genes were isolated using both methods
- Co-expression of these genes enhanced membrane protein production by 3- to 10-fold
- These genes may be serving to maintain the integrity of the bacterial periplasm

Fig. 1A

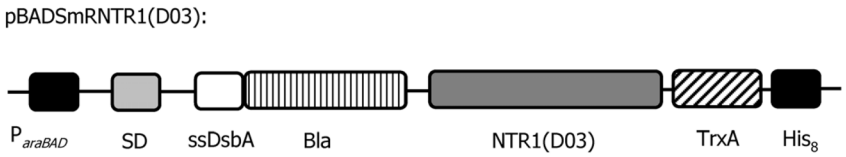


Fig. 1B

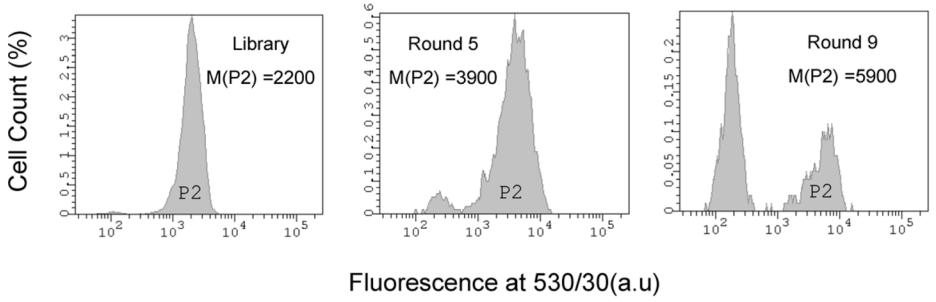


Fig. 1C

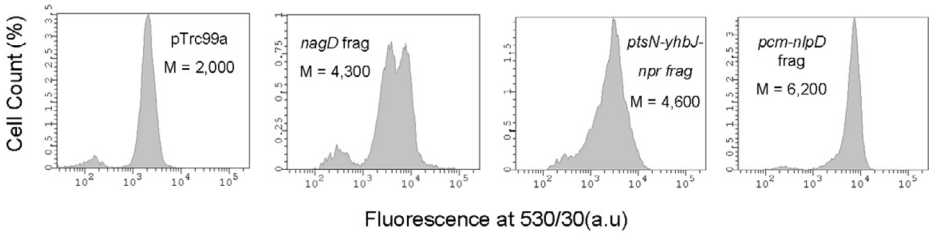


Figure 1.

(A) Schematic of the pBADSmRNTR1(D03) and pBADSmRNTR1 constructs used in this study to express NTR1(D03) and wild-type NTR1, respectively. The gene encoding for wild-type NTR1 or NTR1(D03) is flanked by the SRP-recognized DsbA signal sequence (ssDsbA) and a 3′ gene encoding thioredoxin (TrxA) tagged with an octahistidine sequence (His_8). Expression of this gene fusion was placed under the control of the *araBAD* promoter. SD: Shine-Dalgarno sequence. **(B)** PECS screening of MC4100A cells carrying pBADSmRNTR1(D03) and pTrcGL for enhanced NTR1(D03) production using BODIPY-NT(8–13). After five rounds of FACS sorting, a two-fold increase in the average fluorescence (M) of the high-fluorescence population (P2) was observed, while a three-fold increase in BODIPY-NT(8–13) fluorescence was observed at saturation after nine rounds. **(C)** Comparison of the BODIPY-NT(8–13) fluorescence of wild-type MC4100A cells carrying pBADSmRNTR1(D03) and of MC4100A cells co-expressing the isolated genomic

fragments *nagD*, *pcm-nlpD*, and *hpf-ptsN-yhbJ-npr*. In all panels shown above, NTR1(D03) was expressed at 25 °C for approximately 3–5 h. Fluorescence histograms correspond to a population of 10,000 cells. a.u: arbitrary units.

Fig. 2A

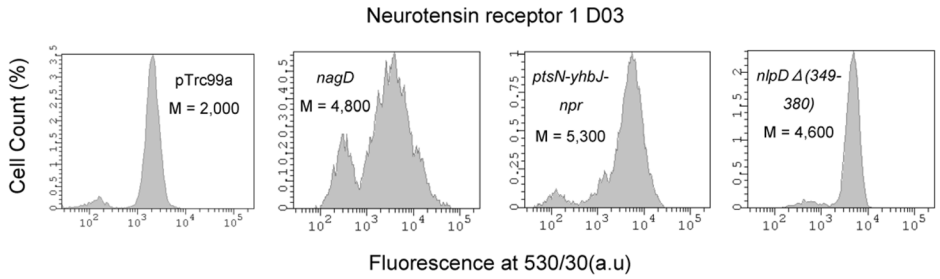


Fig. 2B

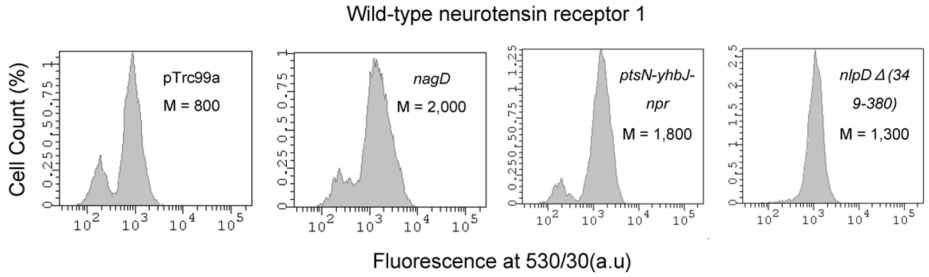


Fig. 2C

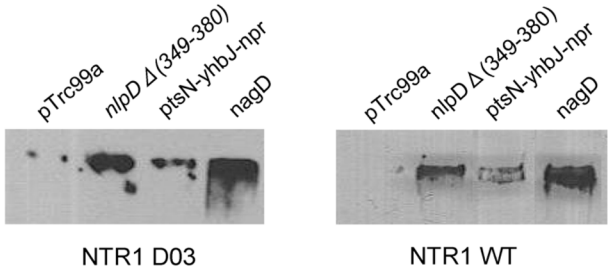


Figure 2.

(A–B) Comparison of the BODIPY-NT(8–13) fluorescence of MC4100A cells carrying pBADSmRNTR1(D03) (A) and pBADSmRNTR1 (B) without (pTrc99a) or with co-expression of the isolated genes/gene clusters *nagD*, *nlpDΔ(349–380)*, and *ptsN-yhbJ-npr*. Fluorescence histograms correspond to a population of 10,000 cells. M: mean value; a.u: arbitrary units. **(C)** Western blots on isolated total membrane fractions of MC4100A cells expressing NTR1(D03) from pBADSmRNTR1(D03) (left) and NTR1 from pBADSmRNTR1 (right) without and with co-expression of the isolated genes/gene clusters. Blots were probed with an anti-polyhistidine antibody. The amount of total proteins loaded on each pair of lanes corresponds to an equal number of cells. No additional (degradation) bands were visible. In all panels shown above. NTR1 and NTR1(D03) were expressed at 25

°C for approximately 3–5 h. Fluorescence histograms correspond to a population of 10,000 cells. a.u: arbitrary units.

Fig. 3A

Fig. 3B

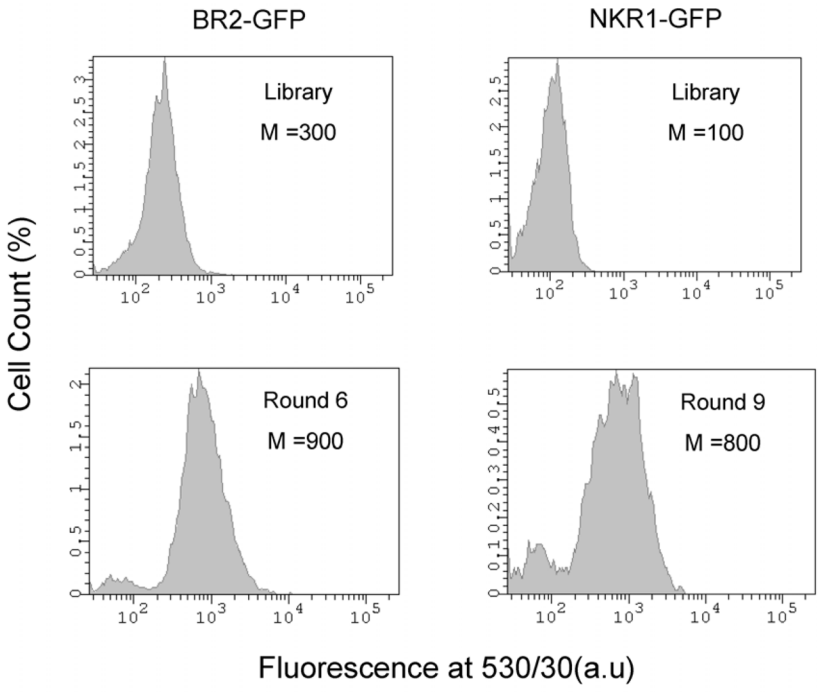


Fig. 3C

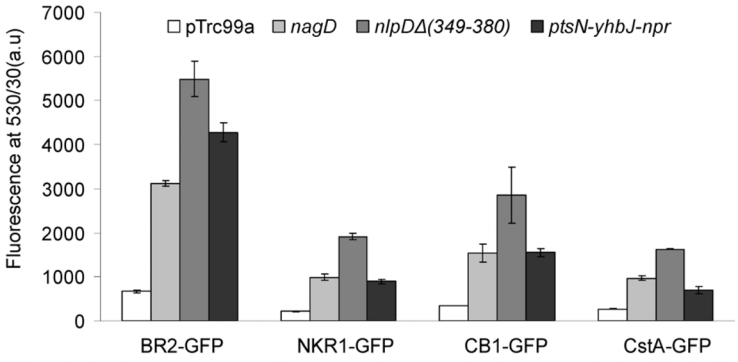


Fig. 3D

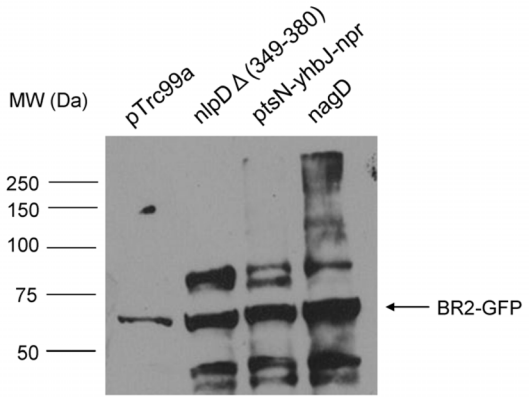


Figure 3.

(A) The pBAD33MP-GFP constructs used in this study to express membrane proteins with C-terminally fused GFPmut2 (Cormack et al., 1996). MP-GFP expression was placed under the control of the *araBAD* promoter. No additional signal sequence was added to the membrane protein for membrane targeting. All membrane proteins which were studied using the MP-GFP system have their C terminus located in the cytoplasm, and thus folding of GFP is expected to take place in the cytoplasmic space of *E. coli*. SD: Shine-Dalgarno sequence; TEV: tobacco etch virus cleavage site. **(B)** FACS screening of an *E. coli* genomic library for enhanced accumulation of BR2-GFP (left) and NKR1-GFP (right). A three-fold increase in BR2-GFP fluorescence was observed after six rounds of sorting, while an eight-fold increase in NKR1-GFP fluorescence was observed after nine rounds of FACS. **(C)** Comparison of the fluorescence of MC4100A cells expressing BR2-GFP, NKR1-GFP, CB1-GFP, or CstA-GFP without (pTrc99a, empty plasmid) and with co-expression of *nagD*, *nlpDΔ(349–380)*, and *ptsN-yhbJ-npr*. Fluorescence histograms correspond to a population of 10,000 cells. a.u:

arbitrary units. Error bars correspond to one standard deviation from the mean value of three replica experiments. **(D)** Comparison of the levels of BR2-GFP production in wild-type MC4100A cells (pTrc99a, empty plasmid) and MC4100A cells co-expressing *nagD*, *nlpDΔ(349–380)*, and *ptsN-yhbJ-npr* by Western blotting using an anti-GFP antibody. Extracts of an equal number of cells were loaded in each lane. In (B) and (C), MP-GFP fusions were expressed at 25 °C for approximately 5 h.

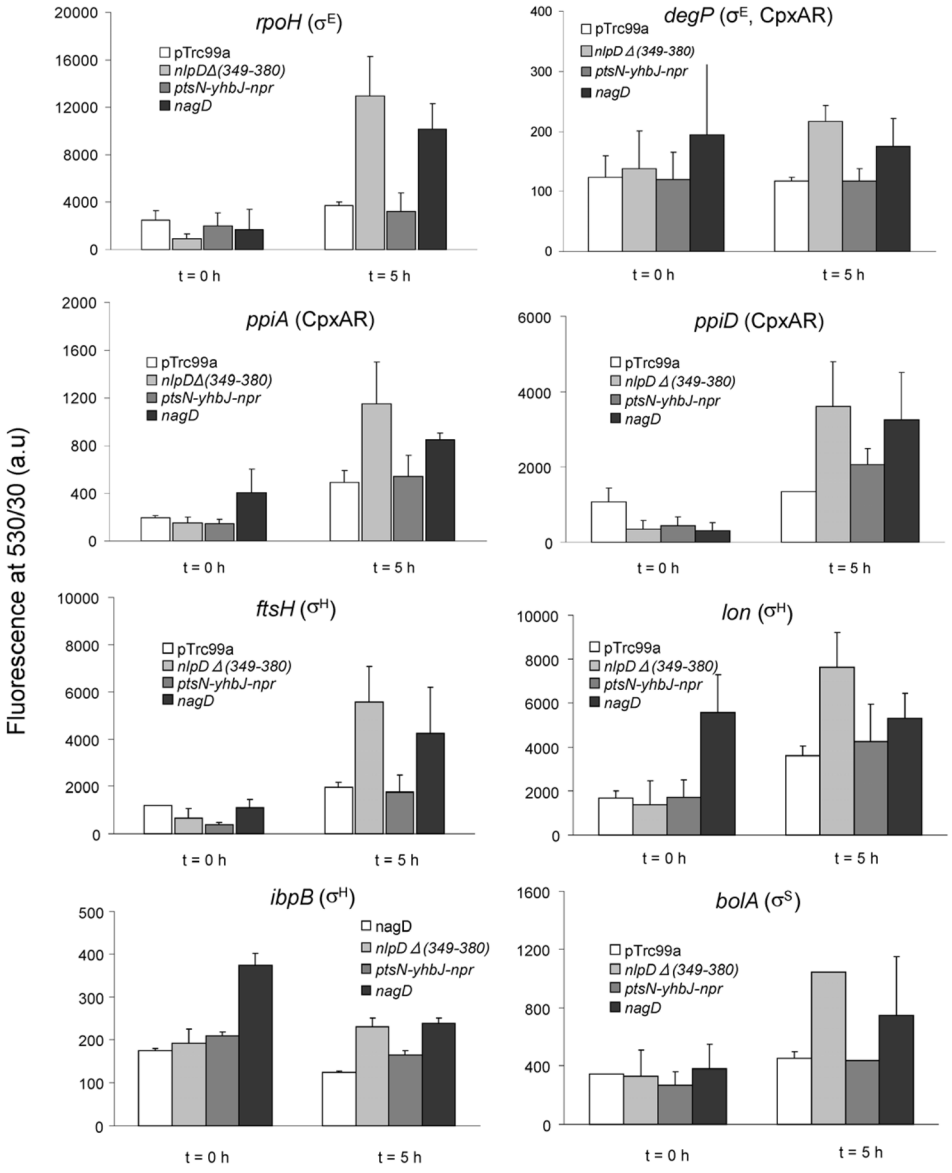


Figure 4.

Comparison of *gfpmut2* expression from different stress-responsive *E. coli* promoters before (t=0 h) and after NTR1(D03) overexpression (t=5 h) in wild-type MC4100A cells (pTrc99a, empty plasmid) and MC4100A cells co-expressing *nagD*, *nlpDΔ(349–380)*, and *ptsN-yhbJ-npr* by flow cytometry. Fluorescence measurements correspond to a population of 10,000 cells and the error bars to one standard deviation from the mean value of three independent experiments a.u: arbitrary units.

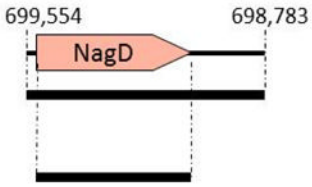
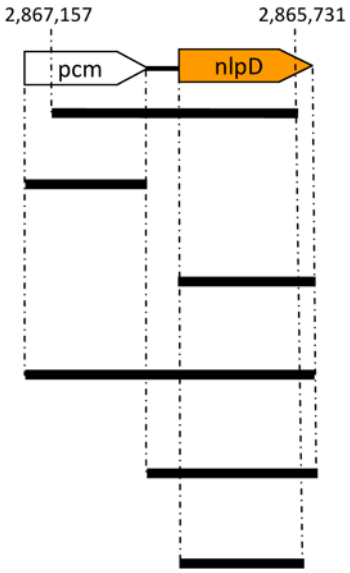
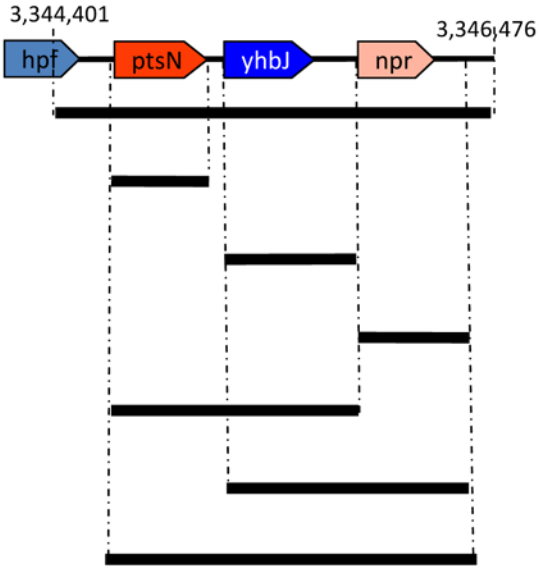
Table 1

Plasmids used in this work

Plasmid	Protein expressed	Marker	Origin of replication	Source
pBADSmRNTR1(D03)	ssDsbA-Bla-NTR1(D03)-TrxA-His_8	SmR	ACYC	This work
pBADSmRNTR1	ssDsbA-Bla-NTR1-TrxA-His_8	SmR	ACYC	This work
pBAD33BR2-GFP	FLAG-BR2-GFP-TEV-His_8	CmR	ACYC	This work
pBAD33NKR1-GFP	FLAG-NKR1-TEV-GFP-His_8	CmR	ACYC	This work
pBAD33CB1-GFP	FLAG-CB1-TEV-GFP-His_8	CmR	ACYC	This work
pBAD33CstA-GFP	FLAG-CstA-TEV-GFP-His_8	CmR	ACYC	This work
pTrc99a	-	AmpR	ColE1	GE Healthcare
pTrcGL	*E. coli* genomic library	AmpR	ColE1	This work
pTrcNagD	NagD	AmpR	ColE1	This work
pTrcPcm	Pcm	AmpR	ColE1	This work
pTrcNlpD	NlpD	AmpR	ColE1	This work
pTrc(Pcm-NlpD)	Pcm-NlpD	AmpR	ColE1	This work
pTrc5′NlpD	5′ non-coding region-NlpD	AmpR	ColE1	This work
pTrcNlpDΔ(349–380)	NlpDΔ(349–380)	AmpR	ColE1	This work
pTrcPtsN	PtsN	AmpR	ColE1	This work
pTrcYhbJ	YhbJ	AmpR	ColE1	This work
pTrcNpr	Npr	AmpR	ColE1	This work
pTrc(PtsN-YhbJ)	PtsN-YhbJ	AmpR	ColE1	This work
pTrc(YhbJ-Npr)	YhbJ-Npr	AmpR	ColE1	This work
pTrc(PtsN-YhbJ-Npr)	PtsN-YhbJ-Npr	AmpR	ColE1	This work
pAU66-P3rpoH	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PppiA	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PppiD	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PdegP	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PftsH	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-Plon	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PibpB	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PbolA	GFP	KanR	SC101	(Zaslaver *et al*, 2006)
pAU66-PglnA	GFP	KanR	SC101	(Zaslaver *et al*, 2006)

Table 2

Fluorescence of MC4100A cells expressing Bla-NTR1(D03)-TrxA with co-expression of the indicated genes/gene clusters, labeled with BODIPY-NT(8–13) and measured using flow cytometry.

Clone	Genomic location	BODIPY-NT(8–13) fluorescence
***nagD* fragment**		4,560 ± 250
nagD		4,520 ± 250
***pcm-nlpD* fragment**		5,780 ± 670
pcm		2,430 ± 70
nlpD		2,190 ± 170
pcm-nlpD		2,360 ± 410
5′ non-coding region-*nlpD*		2,540 ± 490
nlpDΔ(349–380)		4,550 ± 20
***hpf-ptsN-yhbJnpr* fragment**		3,990 ± 376
ptsN		1,766 ± 412
yhbJ		2,310 ± 397
npr		1,586 ± 21
ptsN-yhbJ		1,006 ± 106
yhbJ-npr		1,251 ± 61
ptsN-yhbJ-npr		4,289 ± 724