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Expression of a Highly Toxic Protein, Bax, in *Escherichia coli* by Attachment of a Leader Peptide Derived from the GroES Cochaperone

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Abstract

Attempts to express a truncated form of murine Bax in the periplasm by using an expression vector that attached the OmpA signal sequence to the protein failed to alleviate this toxicity. In contrast, attachment of a peptide based on a portion of the *E. coli* cochaperone GroES reduced Bax's toxicity significantly and allowed good expression. The peptide, which was attached to the N-terminus, included the amino acid sequence of the mobile loop of GroES that has been demonstrated to interact with the chaperonin, GroEL. Under normal growth conditions, expression of this construct was still toxic, but generated a small amount of detectable recombinant Bax. However, when cells were grown in the presence of 2% ethanol, which stimulated overproduction of the molecular chaperones GroEL and DnaK, toxicity was reduced and good overexpression occurred. Two-dimensional gel electrophoresis analysis showed that approximately 15-fold more GroES-loop-Bax was produced under these conditions than under standard conditions and that GroEL and DnaK were elevated approximately 3-fold.

Keywords

Escherichia coli; chaperonin; protein expression; expression vector; GroES; GroEL; Bax; Bcl-2

Heterologous expression in various bacterial host strains or systems can allow the production of useable amounts of mammalian proteins that otherwise would be inaccessible. However, despite their simplicity and scalability, bacterial expression systems still have some limitations when applied to eukaryotic proteins. In some cases, the large-scale production of a protein is limited by toxicity of the overexpressed protein toward the host cell. Numerous strategies have been developed to allow successful production of heterologous proteins (1–13), but these methods are not universally successful. Here we describe another approach that allows expression of a highly toxic mammalian protein in *Escherichia coli*.

The mammalian gene *bax* is a member of the *bcl-2* family of apoptosis modulators (14–16). Although it is homologous to *bcl-2* and *bcl-X_L*, which inhibit apoptosis, *bax* has the opposite function and is an effector of cell death (16). Expression of *bax* has been reported to be highly toxic to *E. coli* (17). In an attempt to circumvent these difficulties, we evaluated alternative expression systems that attach a leader sequence to the expressed proteins. One of these systems, in widespread use, attaches the OmpA leader and targets the protein for excretion to the periplasm (11). We have also developed a new expression system that involves the GroEL/ES chaperonin system. *E. coli* possesses a number of molecular chaperone proteins, the best characterized of which are the GroEL/GroES chaperonins (18–25). These two protein complexes (the 14-subunit, double-ring GroEL and the 7-subunit single-ring GroES) interact to form an 870-kDa assembly that prevents protein aggregation and refolds denatured proteins (24–26). Crystallo-graphic and biochemical studies have shown that a specific loop of the GroES subunit binds to GroEL (24, 27, 28). Mutations in this loop interfere with the interaction of GroES and GroES-like proteins with GroEL (28).

The new expression system takes advantage of this interaction and consists of a new expression vector and the use of specific growth conditions during expression experiments. The expression vector generates a protein with an N-terminal leader that consists of the amino acid sequence of the GroEL-binding loop of GroES (S-loop) of *E. coli*. The growth conditions promote higher than normal expression of GroEL in the host. In the case of Bax, attachment of the loop leader sequence allowed strong overexpression of the resulting protein when cells were cultured in the presence of 2% ethanol. Despite this strong overexpression, the toxic effect of Bax on the host was reduced significantly. The data suggest that the expression system allowed chaperonin-mediated expression of a highly toxic protein in *E. coli*.

MATERIALS AND METHODS

Genes, strains, media, and growth conditions

The pSK-mBax plasmid containing the cDNA encoding murine *bax* was a generous gift from Dr. John C. Reed from the La Jolla Cancer Foundation. Murine *bcl-2* cDNA in the pMON vector (29) containing murine *bcl-2* cDNA was kindly provided by Dr. Timothy J. McDonnell at the University of Texas M. D. Anderson Cancer Center. *E. coli* strain JM83 was obtained from Dr. Deborah Hanson, Argonne National Laboratory, and strain JM105 was from Pharmacia (Table 1). For cloning, strains were cultured on LB or 2xYT medium (30) with ampicillin (100 µg/ml) where appropriate; for expression studies, strains were grown on LB medium with or without 2% ethanol added. The plasmid pASK40 was from Dr. Arne Skerra (11), pJF118EH was from Dr. Michael Bagdasarian (5). pTRC99a and pUC19 were from Pharmacia. Polymerase chain reaction (PCR) reagents were from Perkin–Elmer; isopropyl-β-D-thiogalactopyranoside (IPTG) was from U.S. Biochemicals; enzymes for molecular biology were from Promega (Madison, WI); all other chemicals were purchased from Sigma (St. Louis, MO).

Construction of vectors for expressing S-loop-protein hybrids

Vectors designed to attach the GroEL-binding loop of GroES were constructed as follows. Oligonucleotides were synthesized (Yale Keck Laboratories) based on the published amino acid sequence of the *E. coli* GroES protein (31). Residues 16 through 33 comprise the conserved sequence EVETKSAGGIVLTGSAAA, which forms the loop known to participate in the interaction of GroES with GroEL (15, 27). A nucleotide sequence encoding this sequence and its complement were generated by the program Lasergene (DNASTar, Inc., Madison, WI) with the codon preferences for *E. coli*. Additional nucleotides encoding the overhang generated by an *EcoRI* digest of DNA were included at the 5' end of each to give the following: sense, 5'-

AATTATGGAAGTTGAAACCAAATGTGCTGGTGGTATCGTTCTGACCGGTTCTGCTGCTGCG-3'; antisense, 5'-

AATTCGCAGCAGCAGAACCGGTCAGAACGATACCACCAGCAGATTTGGTTTCAACCTTCCAT-3'. Hybridization of these two oligonucleotides generated the insert illustrated in Fig. 1. The insert was designed so that the reading frame of the leader sequence it generated bore the same relationship to the reading frame of the subsequent multicloning site that occurs in pASK40. This strategy allowed direct transfer of cloned genes from pASK40-based vectors to the S-loop vectors without alteration of the genes.

The insert was phosphorylated by using T4 polynucleotide kinase, purified with QiaEx resin (Qiagen), and ligated into the dephosphorylated *EcoRI* site of the vector pJF118EH (5). Transductants of strain JM105 were screened for the presence of an *AgeI* site, which is present in the insert but absent in pJF118EH. Positive transductants were screened for orientation of the insert; only one end regenerates an *EcoRI* site, and in the correct orientation that site is the first site of the multicloning site. *EcoRI*–*EcoRV* digests were analyzed on a 1.5% agarose gel for the presence of the 1260-bp fragment predicted for the correct orientation as opposed to the 1200-bp fragment predicted for the reverse orientation. The new vector was designated pATP004.

The multicloning site of pATP004 was replaced with the larger multicloning site of pUC19 by using the enzymes *EcoRI* and *HindIII*. Plasmids obtained from JM105 transductants were screened for the *KpnI* and *XbaI* sites which are present only in the pUC19 multicloning site. The resulting plasmid was designated pATP005.

Cloning genes into expression vectors

Nucleic acid sequences for murine *bax* and murine *bcl-2* found in GenBank entries L22472 (16) and M16506 (15) were used to design PCR primers that would (1) introduce an *EcoRI* site at the 5' end of the gene to allow cloning into the vector pASK40 in the correct reading frame. (2) terminate each gene prior to sequences encoding the terminal hydrophobic transmembrane segment, and (3) encode an additional 5 histidine residues at the 3' end of the gene to facilitate purification of expressed proteins (32). The protein that would be produced would include residues 4–169 of native Bax and 5–212 of Bcl-2. The PCR products were digested with *EcoRI* and *HindIII* and introduced into these sites in pASK40 by standard protocols (30). The *bax* gene and the gene encoding the *E. coli* malate

dehydrogenase, *mdh*, which had previously been cloned into pASK40 (33), were transferred into the *EcoRI*–*HindIII* site of pATP005.

Expression and analysis of proteins

Cultures containing plasmids were grown to $A_{600} = 0.2$ – 0.5 in LB medium containing 100 $\mu\text{g/ml}$ ampicillin and then induced with 0.1 mM IPTG. In most cases we continued to observe A_{600} after the introduction of IPTG. In certain experiments, 2% ethanol was included in the growth medium. Total cell proteins were solubilized by heating at 90°C for 5 min in the presence of 10% sodium dodecyl sulfate and 1 mM dithiothreitol. To prepare soluble, nondenatured proteins, cells were lysed with lysozyme (11). Expression of proteins was evaluated by gel electrophoresis (34) and, in the case of malate dehydrogenase, by enzymatic activity (35). The relative expression of proteins was estimated by quantitative analysis of extracts separated by two-dimensional gel electrophoresis (2DE) (36–39).

Two-dimensional gel electrophoresis

Cell pellets (7.85 OD₆₀₀ units) were lysed in 1 ml of a solution containing 9 M urea, 2% 2-mercaptoethanol, 4% Nonidet P-40, and 2% ampholytes (Bio-Rad; pH 8–10). The resulting homogenates were centrifuged for 5 min at 435,000g in a Beckman TL100 tabletop ultracentrifuge to remove particulates. The supernatants were then frozen at –70°C until electrophoresis. Isoelectric focusing in the first dimension was done essentially as described by Anderson and Anderson (38) with 50% pH 3–10 Biolyte, 25% pH 5–7 Biolyte, and 25% pH 5–7 Servalyte carrier ampholytes. After equilibration in sodium dodecyl sulfate buffer (34), the focused proteins were separated in the second dimension in slab gels containing a linear gradient of 10–17% polyacrylamide, essentially as described by O’Farrell (37) with modifications described by Anderson and Anderson (39). After electrophoresis, gels were fixed and stained in 0.125% (w/v) Coomassie Blue R250 in 2.5% phosphoric acid and 50% ethanol for approximately 24 h. The gels were then destained in 20% ethanol. Protein patterns were digitized by using an Eikonix 1412 CCD scanner interfaced with a VAXstation 4000-90. Image processing and generation of parameter lists, referred to as spot files, were as previously described (36). A master pattern was created by using a copy of a 2DE spot file from the “ethanol and induced” treatment group, so that the Bax protein was represented. Each of the patterns generated for the experiment, with four replicate patterns within each sample group, was matched to the master and then examined interactively for the appearance of new proteins, the loss of normally expressed proteins, and statistically significant quantitative differences between the control patterns and the “induced” or “ethanol and induced” patterns. The identity of the proteins was established by MALDI/MS of tryptic digests of spots excised from the replicate gels, performed by the Yale Keck Laboratory Protein Analytical Facility.

RESULTS

Expression of Bax and Bcl-2

Bax and Bcl-2 are members of the same family of apoptosis modulator proteins, but expression of Bax has been shown to be highly toxic to *E. coli* (17), whereas expression of Bcl-2 is not. We cloned genes encoding the cytoplasmic domains of both proteins into the

expression vector pASK40 to target the proteins for secretion into the periplasm. This expression vector generates recombinant proteins fused to the secretory signal sequence of the *E. coli* outer membrane protein, OmpA (11). Induction of the resulting expression vectors consistently gave moderate expression of OmpA-Bcl-2 but very poor expression of OmpA-Bax (Fig. 2A). The attachment of the OmpA leader did not alleviate the toxicity of Bax; induction resulted in an immediate decrease in the cell density in the culture, whereas growth of the culture expressing the OmpA-Bcl-2 hybrid was only slightly perturbed (Fig. 2B). These results indicate that even when targeted to the periplasm, Bax was highly toxic to the host cells. Because of the very low level of expression, we did not attempt to differentiate cytoplasmic versus periplasmic accumulation of the Bax.

Construction and expression of S-loop-Bax

A leader sequence encoding the GroEL-binding loop of GroES (S-loop, Fig. 1) was introduced into the expression vector pJF118EH to give vectors pATP004 and pATP005, as described under Materials and Methods. The presence of the leader in the new vector, pATP004, was confirmed by detection of an *Age*I site. Functionality of the vector was demonstrated by introducing the gene for the *E. coli* malate dehydrogenase. Induction of the resulting vector, pATP007, gave strong overexpression of a protein of the predicted molecular weight (35 kDa), as detected by protein gels stained with Coomassie. Enzyme activity assays showed that the recombinant enzyme was highly active, indicating that attachment of the S-loop did not interfere with proper folding and dimerization of the enzyme (40).

The *bax* gene was transferred from the pASK40-based vector into pATP005 to generate pATP011. Under standard growth conditions, expression of the S-loop-Bax hybrid was still highly toxic to the host cells; cell densities dropped immediately upon induction of S-loop-Bax, just as had occurred with the OmpA-Bax protein. Expression of S-loop-Bax was further evaluated under culture conditions designed to enhance the expression of *E. coli* chaperones in the cell. The rationale for this approach was based on the assumption that folding of expressed S-loop proteins would be mediated through interaction with the chaperonin GroEL. Addition of ethanol is known to induce the expression of chaperones in *E. coli* (41), so we evaluated the growth of JM109 in LB medium supplemented with various concentrations of ethanol (Fig. 3). A compromise level of 2% ethanol (0.44 M) was chosen to stress the cells without seriously affecting their ability to produce proteins. At that concentration, the generation time increased from approximately 40 to 50 min, yet the biomass accumulation was approximately 80% of that for cultures lacking ethanol.

Induction of either the S-loop-Bax or the OmpA-leader-Bax protein, both in the presence and in the absence of ethanol, resulted in an abrupt decrease in the growth of the cultures (Fig. 4). However, in the presence of 2% ethanol, the culture expressing S-loop-Bax continued to grow, albeit much more slowly, whereas the cell density of the other cultures decreased immediately upon induction. At the end of the induction period (4 h), the cell density of the ethanol-containing culture was more than twice that of the culture lacking ethanol. The cultures were harvested and analyzed for protein expression (Fig. 5). A new protein of 23 kDa, the expected molecular weight of the S-loop-Bax hybrid, was clearly

evident only in the culture expressing S-loop-Bax in the presence of ethanol (arrow, lane 3). Possible low expression in the other induced cultures was obscured by host proteins in that region; very weak expression could not be ruled out in these experiments.

Quantitative two-dimensional gel electrophoresis

Separation of the induced proteins by two-dimensional gel electrophoresis allowed identification of proteins expressed preferentially under different conditions (Fig. 6). Four replicate gels of uninduced cultures and of induced cultures grown with or without ethanol were digitized and the abundance of the protein spots was analyzed statistically (36). Only four proteins were expressed at significantly higher levels in the induced, ethanol-treated culture than in the uninduced culture lacking ethanol. Those proteins were identified by MALDI/MS as DnaK, GroES, serine hydroxymethyl transferase, and S-loop-Bax (Table 2). The S-loop-Bax protein, which is seen in the lower left-hand quadrant of the gel, was not detected in uninduced cultures, but a faint spot was observed in that position in the extracts of the culture induced in the absence of ethanol (Fig. 6). Attributing that spot to S-loop-Bax allowed us to estimate that inclusion of ethanol resulted in a 14.7-fold increase in its production (Table 2). In combination with the higher cell density obtained in those cultures (Fig. 4), the net increase in yield per milliliter of culture was more than 30-fold. Induction in the absence of ethanol resulted in a slight decrease in GroES and DnaK. In the presence of ethanol, the abundances of these proteins increased approximately 3-fold relative to their amount in cultures induced in the absence of ethanol.

Cellular location of S-loop-Bax

In the experiments described above, cells were lysed under denaturing conditions. When cells were lysed under nondenaturing conditions and centrifuged, the S-loop-Bax protein was found primarily in the pellet (data not shown), indicating that most of the overexpressed hybrid was present as inclusion bodies. No attempt was made to solubilize the protein from this fraction.

DISCUSSION

Several obstacles to the expression of mammalian proteins in bacterial hosts diminish the utility of bacterial expression systems for large-scale heterologous protein production. These obstacles include codon usage, folding and assembly, cofactor incorporation, and posttranslational modification. Often heterologous proteins are poorly expressed or result in the formation of insoluble proteins that precipitate in inclusion bodies. Some heterologous proteins are highly toxic to the host cell. Toxicity may preclude obtaining good yields of the protein by reducing the cell density of the culture, limiting the amount of protein within the cell, or both.

A possible explanation for poor expression of heterologous proteins in some cases is that the bacterial chaperone machinery becomes saturated and cannot provide sufficient assistance in folding of foreign proteins. Chaperones play a key role in folding and assembly of host proteins, but they also can refold proteins of mammalian origin *in vitro*. Recently, several reports have suggested that production and assembly of extrinsic proteins in *E. coli* can be

improved by overexpression of bacterial molecular chaperones (3, 4, 13). The *in vivo* mechanism for this apparent chaperone-mediated overproduction of soluble foreign proteins is not completely clear.

We have explored the possibility of modifying a protein substrate to obtain better interaction of foreign proteins with the bacterial chaperonin system. Expression of human Bax has been shown to be highly toxic to *E. coli*, resulting in cell death at concentrations as low as 0.01% of the total cellular protein (17). The region of the protein responsible for the toxicity has been determined to reside in residues 115–144 (42). We observed a similar severe toxicity caused by expression of residues 4–169 of murine Bax, even when the expressed protein was targeted for excretion to the periplasm by attachment of the OmpA leader. Overexpression of the host cell's chaperone systems by inclusion of ethanol in the growth medium failed to reduce the toxicity of OmpA-Bax. However, attachment of a leader sequence designed to increase the recombinant protein's affinity for the chaperonin, GroEL, successfully alleviated toxicity and allowed good expression and accumulation of Bax in *E. coli*. In the experiments described here, we provided a short (17 amino acid) leader sequence that could, in principle, increase affinity of newly expressed proteins for GroEL. The leader sequence was derived from the sequence of the *E. coli* GroES cochaperone mobile loop (S-loop) that is known to interact directly and specifically with GroEL chaperonin. The rationale of the approach was to target the newly expressed protein to the GroEL chaperonin. The strategy appears to work for Bax; the S-loop-Bax chimera was expressed at much higher levels than were other constructs and only when the molecular chaperones were moderately overproduced. The improved expression was not a general function of the presence of a leader sequence; the Bax chimera with the OmpA leader sequence was not effective in enhancing Bax expression even when chaperones were induced. In this example, the S-loop sequence was attached to the N-terminus of Bax. The use of an N-terminal leader was chosen because many proteins are expressed *in vivo* with such N-terminal extensions, although C-terminal extensions are also feasible. Also, as the N-terminus of the protein emerges first from the ribosome during protein synthesis, the leader sequence may facilitate initial steps in the folding process, possibly contributing to the reduction in toxicity in this manner by reducing the potential interaction of the toxic residues 115–144 with cellular components. The effect of C-terminal attachment of the S-loop remains to be determined.

Although our observations are indirect, it seems reasonable to propose that certain modifications to protein substrates that can enhance interaction with the chaperone machinery could be useful for production of some mammalian proteins in bacteria. We have shown that the addition of S-loop as a leader sequence has a beneficial effect on the expression of a highly toxic protein in *E. coli*. This new approach exploits the beneficial role of molecular chaperones in the expression of heterologous proteins in bacteria by altering the protein substrate. Broader application of this approach may allow access to many mammalian proteins that, thus far, have been too toxic to produce in bacteria. The approach can easily be combined with other expression tools, such as the addition of cleavable affinity tags that allow rapid purification of heterologous proteins. Proteins containing the S-loop extension may also fold more efficiently and be easier to purify because of higher expression levels and improved solubility. Furthermore, this or similar leader sequences

designed to increase the interaction of a protein with chaperonins and other molecular chaperones may also be useful for *in vitro* protein refolding. More tests are needed to explore these possibilities.

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M E V E T K S A G G I V L T G S A A A N S
 AATTATGGAGTGTGAACCAAAATCTGCTGTGTATGTGTCTGACCGGTTCGTGCTGCTGCG
 TACCTTCAACTTTGGTTTAGACGACACCATAGCAAGACTGGTCAAGACGACGACGCTTAA

FIG. 1.

The S-loop leader sequence and the double-stranded insert encoding the leader used to construct the expression vector pATP004. The top line indicates the amino acids encoded by the sense strand of the insert, starting at the initial methionine and including the residues required by the *EcoRI* site at the 3' end. Italicized residues are identical to the GroEL binding loop of GroES. The diagnostic *AgeI* site in the insert is underlined.

FIG. 2.

Expression of Bcl-2 and Bax with the OmpA leader. (A) Lanes 1 and 2, OmpA-Bcl, uninduced and induced 4 h, respectively; lanes 3 and 4, OmpA-Bax, uninduced and induced 4 h, respectively. The arrow indicates the expressed OmpA-Bcl protein (calculated molecular weight 26.9 kDa). MW markers are aprotinin (6.5 kDa), lysozyme (14 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), glutamate dehydrogenase (55 kDa), bovine serum albumin (66 kDa), fructose-6-phosphate kinase (84 kDa), phosphorylase B (97 kDa), β -galactosidase (97 kDa), and myosin (205 kDa). (B) Growth of cells containing (●) OmpA-Bcl-2 vector and (○) OmpA-Bax vector during induction experiment. IPTG was added when the cultures' OD₆₀₀ reached 0.5.

FIG. 3. Growth of *E. coli* JM105 in the presence of increasing amount of ethanol in LB medium: no ethanol (◆) and 1 (■), 2 (▲), 3 (×), and 4% (+) ethanol.

FIG. 4.

Growth of transformants expressing Bax with different leader sequences. Solid symbols represent the S-loop leader, open symbols, the OmpA leader. Uninduced cultures (■, □) were grown without ethanol. Induced cultures (0.1 mM IPTG was added at the arrow) were grown either with (●, ○) or without (◆, ◇) 2% ethanol.

FIG. 5.

Expression of S-loop-Bax and OmpA-Bax in the presence and absence of ethanol. Lanes 1–3 display extracts of cells containing the S-loop-Bax plasmid and lanes 4–6 cells containing the OmpA-Bax plasmid. For lanes 1, 2, 4, and 5, cells were grown without ethanol and either not induced (lanes 1 and 4) or induced 4 h with 0.1 mM IPTG (lanes 2 and 5). For lanes 3 and 6, cells were grown in the presence of 2% ethanol and induced 4 h with 0.1 mM IPTG. The arrow indicates the expressed S-loop-Bax protein (calculated molecular weight 21.4 kDa). MW markers are lysozyme (14 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), glutamate dehydrogenase (55 kDa), bovine serum albumin (66 kDa), fructose-6-phosphate kinase (84 kDa), phosphorylase B (97 kDa), β -galactosidase (97 kDa), and myosin (205 kDa).

FIG. 6.

Two-dimensional gel electrophoresis of extracts prepared from cultures containing the S-loop-Bax expression vector. (A) Uninduced cells (corresponding to symbol ■ of Fig. 4); (B) induced cells grown in the absence of ethanol (◆ of Fig. 4); (C) induced cells grown in the presence of ethanol (● of Fig. 4). Cultures were induced 4 h with 0.1 mM IPTG. Proteins are 1, Dnak; 2, GroEL; 3, serine hydroxymethyl transferase; 4, S loop-Bax.

TABLE 1

Plasmids Used in Studies

Plasmid	Components	Source
pASK40	<i>bla. lacIq</i> , P/Olac, <i>t lpp</i> , <i>fl-IG</i> , <i>ompA</i>	(11)
pJF118EH	<i>bla</i> , <i>lacIq</i> , P <i>tac</i>	(5)
pATP004	GroES loop encoding leader in pJF118EH	This study
pATP005	Multicloning site of pUC19 substituted for that of JF118EH in pATP004	This study
pMDH1	<i>E. coli mdh</i> in pASK40	(33)
pATP007	<i>E. coli mdh</i> in pATP004	This study
pBCLR19.3	Murine <i>bcl-2</i> in <i>EcoRI/HindIII</i> sites of pASK40	This study
pBAX001	Murine <i>bax</i> in <i>EcoRI/SalI</i> sites of pASK40	This study
pATP011	Murine <i>bax</i> from pBAX001 in pATP005	This study

TABLE 2

Qualitative Analysis of Protein Expression

Protein	Condition	Average density	Relative amount
DnaK	Control	17,300	1
	+IPTG	11,400	0.66
	+IPTG, +EtOH	36,100	2.09
GroEL	Control	22,200	1
	+IPTG	15,700	0.71
	+IPTG, +EtOH	43,800	1.98
Serine hydroxymethyl transferase	Control	24,500	1
	+IPTG	13,900	0.57
	+IPTG, +EtOH	40,400	1.65
S-loop-Bax	Control	0	—
	+IPTG	3,100	1
	+IPTG, +EtOH	45,500	14.7