Construction of an overproduction vector containing the novel *srp* (sterically repressed) promoter

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Abstract

We report the design, synthesis, and evaluation of a novel *Escherichia coli* promoter intended for use in overproduction of proteins that are deleterious to the host. In this sterically repressed promoter (*srp*), the *lac* operator site is positioned between the -10 and -35 elements, where it can interfere sterically with RNA polymerase and thereby prevent assembly of a poised transcriptional complex. An *srp*-containing phagemid, pKEN1, and a *tac*-containing phagemid, pHN1, which has been widely used in protein overproduction but is often unstable, are compared with respect to levels of uninduced and induced protein expression. The level of uninduced protein synthesis by the *srp* promoter in vivo is $\sim 50\%$ of that observed with *tac*, whereas the levels of induced protein synthesis with the 2 vectors are approximately equal. A remarkable increase in stability of overproduction and growth was observed when the toxic Ada protein was overproduced in pKEN1, demonstrating the potential utility of this vector in overproducing toxic proteins.

Keywords: overproduction; promoter leakage; transcriptional poisoning

Escherichia coli has been widely used for the expression of prokaryotic and eukaryotic genes, largely owing to the ease of introducing foreign DNA into bacteria on plasmid or phage vectors. Of particular value in the understanding of protein structure and function has been the availability of specialized overexpression vectors, which can afford large quantities of protein for biochemical, X-ray crystallographic, and/or NMR studies. Although such overexpression vectors have generally proven successful, they can sometimes fail when the overexpressed gene product is deleterious to the host cell (Brosius, 1984). In such cases, failure typically results from low-level constitutive expression of the gene product as a result of inadequate promoter repression; this can lead to instability of the plasmid in vivo, slow culture growth, and cell death (Rose & Shafferman, 1981; Caulcott et al., 1985).

The *lac* operon in *E. coli* has long provided the paradigm for negative gene regulation (Beckwith, 1987), hence components of its transcriptional control machinery have been widely utilized in protein overexpression systems. In the wild-type *lac* operon, the presence of the Lac repressor reduces expression $>1,300$ -fold in vivo; de-repression can be achieved with the inducer isopropyl- β -D-thiogalactopyranoside (IPTG) (Miller, 1978). Whereas it was previously thought that the Lac repressor down-regulates transcriptional initiation by blocking access of RNA

polymerase to the promoter, more recent evidence suggests that this is not the case, since the Lac repressor bound at the O_1 operator has been shown not to prevent the binding of RNA polymerase (Straney & Crothers, 1987; Lee & Goldfarb, 1991) (Fig. 1A, B). Moreover, RNA polymerase has actually been found to bind the repressor-bound promoter 100-fold tighter than the naked promoter. These results have been interpreted as suggesting that the dissociation of the Lac repressor is kinetically and thermodynamically coupled to the formation of an open transcriptional complex. In this model, RNA polymerase is "poised" to initiate *lac* message synthesis, and dissociation of the Lac repressor from its operator is rapidly followed by transcriptional initiation. Under conditions in which (1) the intracellular concentration of the Lac repressor is close to the concentration of operator sites, (2) the relative concentration of RNA polymerase is high, and (3) the promoter is strong, a single dissociation of Lac repressor from a poised transcriptional complex may result in a large number of transcription events before repression is reestablished. Conditions 1–3 are present inside *E. coli* cells carrying a plasmid-borne *lac*-controlled promoter—*lac*, *tac* (DeBoer et al., 1982, 1983; Amann et al., 1983), or *trc* (Amann et al., 1988)—and thus the problems of promoter leakage in such strains may be exacerbated by their access to poised transcriptional complexes. These considerations suggested that the efficiency of repression in *lac*-controlled overproducing strains may be improved by removal of transcriptional poisoning. The present experiments were undertaken in part to test that hypothesis.

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Fig. 1. Schematic diagram of the *lac* (and *lac*-derived) promoters (A, B) and the *srp* promoter (C, D). Parts A and C show the physical organization of the promoters; B and D show the putative state of the promoters in vivo under noninduced conditions.

In this study, we have relocated the *lac* operator site within the promoter to a position between the -10 and -35 elements, in order to block access of RNA polymerase to the promoter (Brosius & Holy, 1984; Mulligan et al., 1985) (Fig. 1C, D). We show that Lac repressor indeed blocks the promoter in this construct and that blockage is associated with an increased stability of the construct expressing a toxic gene product.

Results

The objective of this study was to use mechanistic information about the functioning of the *lac* promoter to design a new *lac*-controlled promoter with improved efficiency of repression, which could be employed in overproduction of proteins having moderate toxicity. Existing overproduction systems based on control by the Lac repressor most often use *lac* UV5, *tac*, or *trc* promoters (DeBoer et al., 1982, 1983; Brosius et al., 1985; Szoke et al., 1987). Owing to the relative orientation of the *lac* operator with respect to the -10 and -35 elements in such promoters, they are capable of forming poised transcription complexes, which may contribute to inadequate promoter repression (Figs. 1B, 2B,C). In an attempt to circumvent this problem, we decided to relocate the *lac* operator to a position at which it would sterically block access of RNA polymerase to the promoter. After considering numerous possible placements of the *lac* operator site that would

in principle block access of RNA polymerase to a promoter, a location in the spacer region was chosen (Fig. 2D). This placement in the designed promoter allowed the maintenance of consensus -35 and -10 elements and the incorporation of an operator sequence that is closely related to the *E. coli lac O₁* operator and *lac* UV5 operators found in common cloning (M13 series, pUC) and overproduction (pKK223-3, pKK233-2) vectors. *lac O₁* is generally considered to be a 21-bp sequence (Sandler et al., 1983; Oehler et al., 1990), of which only 2 bp had to be changed in order to allow placement within the -35/spacer/-10 region. The necessary modifications at the ends of the *lac* operator involve positions that appear not to participate in protein-DNA contacts (Simons et al., 1984; Satorius et al., 1988; Wick & Matthews, 1991). Indeed, recent results have shown that a 14-mer fragment of the operator is sufficient for binding the repressor with high affinity (Karslake et al., 1992). Sequence-specific amino acid contacts to the operator site are believed to involve the 5'-GTGA and 5'-GTTA sequences in the right and left half-sites, respectively (Wick & Matthews, 1991; Karslake et al., 1992). The centers of these recognition sequences are 7 bp from the centers of the -10 and -35 sequences, and assuming 10 bp per turn of B-DNA, the centers of the operator half-sites are located on neither the front nor the back face of the duplex, relative to the -10 and -35 elements. As a tetramer of 38-kDa monomers (Beyreuther, 1978; Farabaugh, 1978; Pace et al., 1990), Lac repressor bound so proximally to the polymerase recognition sites is expected to prevent co-occupancy by RNA polymerase, as implied by DNase protection experiments (Schmitz & Galas, 1979). Although there are examples of proteins that bind the spacer and do not prevent RNA polymerase from binding (O'Halloran et al., 1989; Shewchuk et al., 1989), these systems probably rely on precise protein-protein and protein-DNA contacts that have been subjected to evolutionary optimization. We have termed the designed promoter *srp*, which derives from its being sterically repressed. The *srp* promoter was inserted by conventional techniques upstream from the *EcoR* I site of the phagemid pBS+. The opposing *lac* UV5 promoter on the distal end of the polylinker was then removed and replaced with the *rrnBT₁T₂* translational terminator (Gentz et al., 1981) (Fig. 3). The resulting expression vector is termed pKEN1.

In order to test the levels of uninduced and induced protein synthesis, we chose to insert the *E. coli ada* gene into pKEN1 (Lindahl et al., 1988). Evidence from this and other laboratories (B. Demple, pers. comm.) has indicated that constitutive overexpression of the Ada protein is deleterious to *E. coli*, which has caused difficulty in stable maintenance of Ada-overproducing cells (Myers et al., 1992). Although the factors governing the toxicity of Ada are not rigorously understood, a contributing factor is likely to be the high nonspecific affinity of the protein for DNA (F. Jackow & G.L. Verdine, unpubl. results), which may burden proper functioning of the genome. Given the high toxicity of Ada, inadequate promoter repression in uninduced cells would be expected to result in strong selection against maintenance of the overproducing phenotype; thus, decreased promoter leakage should result in increased stability. The Ada protein was therefore chosen as a demanding test of *srp* promoter function, specifically with regard to repressibility, inducibility, and plasmid stability.

We have previously reported construction of a *tac*-controlled overexpression vector, pHN1+ (Myers et al., 1992), which is

-35 17 bp -10

NNNNNNTTGACANNNNNNNNNNNNNNNNNNNTATAATNNNNNNNNNNNN
NNNNNNAACTGTNNNNNNNNNNNNNNNNNNATATTANNNNNNNTNNNNNN

-35 18 bp -10

CCAGGC**TTTACA**CTTTCTGCTCCGGCTCG**TATAAT**GTGTGG**AATTTGTGAGCCGATAACAATTT**CC
GGTCCG**AAATGT**GAAAGACGAAGGCCGAGC**ATATTA**CACACCT**TTAACA**CTCGCCTATTGT**TAAAG**

-35 16 bp -10 EcoRI

GAGCTGTTGACAATTAATCATCGGCTGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAACAGAATTC

CAGCACAACTGTTAATTAGTAGCCGAGCATATTACACACCTTAACACTCGCCTATTGTTAAAGGTGCTCTTGTCTTAAG

-35 17 bp -10 EcoR I

GCGCAATTGAC**CATTGTGAGCGGATAACAATATAA**TGTGTGGAAATTC
CCGCTTA**ACTGTAACACTTGCCTATTGTTATATTA**ACACACCTTAAG

$\longleftrightarrow$

lac O_{sm}

EcoR I Met
GAATTCTTAACCAGGAGCTGATT ATG
CTTAAGAATTGGTCCTCGACTAA TAC

Fig. 2. Promoter sequences described in the text. **A:** *E. coli* consensus promoter. **B:** *lacUV5* promoter/operator. **C:** *tac* promoter/operator as in the pHNI+ vector. **D:** *srf* promoter/operator as in the pKEN1 vector. **E:** The sequence from the *Eco*R I site to the start codon common to both pHNI-*ada* and pKEN1-*ada* constructs. Features indicated: large letters, RNA polymerase recognition sequences; bold letters, *lac* operators; pseudodyad elements indicated by arrows.

In order to test the relative stability of Ada overproduction in *E. coli* XA90(pKEN1-*ada*) (*srp* promoter) and XA90(pHN1-*ada*) (*tac* promoter), induction tests on cells transformed in parallel were carried out at 1 and 14 days after transformation. Between day 1 and 14, the cells were maintained on LB-Amp plates (Sambrook et al., 1989) at 4 °C. At day 1, 4 colonies of XA90(pKEN1-*ada*) and 2 colonies of XA90(pHN1-*ada*) were induced, all of which yielded successful Ada production. At day 14, 4 of 4 colonies of XA90(pKEN1-*ada*) were successfully induced; however, neither of the 2 XA90(pHN1-*ada*) clones could be induced, despite the fact that they were still resistant to ampicillin (data not shown). These results suggest that loss of over-

In order to test whether the *srp* promoter in pKEN1-*ada* is actually more highly repressed than the *tac* promoter in pHN1-*ada*, freshly transformed XA90 clones of each phagemid were grown in parallel and divided into 2 portions at mid-log phase. One of the portions was induced with IPTG for 2 h, and the other was grown for the same time in the absence of IPTG. Whole cell lysates from the induced and uninduced samples were analyzed by SDS-PAGE; the results are presented in Figure 4. Upon induction, both strains synthesize approximately the same amounts of Ada protein (~40% of the total cell protein by scanning densitometry). The uninduced cells, on the other hand, expressed markedly different amounts of Ada: densitometric analysis of whole cell lysates from uninduced XA90(pKEN1-*ada*) and XA90(pHN1-*ada*) revealed that the former cells express roughly 50% the amount of Ada protein expressed by the latter (both uninduced). These results suggest that the 2 promoters possess roughly equivalent strengths under induced conditions, but differ in the levels of uninduced protein synthesis. Caution must be exercised in making universal conclusions about the relative overproduction efficiency of pHN1 and PKEN; since the 5'-mRNA leader sequences encoded by the *tac* and *srp* vectors are not identical, the vectors bearing these promoters may not perform identically when linked to genes other than *ada*.

Consistent with a lower level of uninduced Ada biosynthesis, XA90(pKEN1-*ada*) exhibits growth characteristics superior to XA90(pHN1-*ada*). Shown in Figure 5 are growth curves for uninduced cell cultures of pKEN1-*ada* (Fig. 5A) and pHN1-*ada* (Fig. 5B) in XA90. In these experiments, single colonies from plates (LB-Amp) of freshly transformed cells (<18 h after trans-

Fig. 3. Construction and circular plasmid map of pKEN1.

formation) were used to inoculate an overnight culture of LB-Amp. After ~12 h, the cells were diluted 100-fold into fresh LB-Amp and the cultures were maintained on a rotary shaker at 37 °C. Cell density of the fresh inoculation was monitored by optical density at 595 nm. At mid-log phase ($A_{595} = 0.6$), the cells were again diluted 100-fold with fresh LB-Amp broth and their cell density was recorded at frequent time intervals ($A_{595} = 0.006$ at $t = 0$ h). In the case of XA90(pKEN1-*ada*), individual clones gave rise to virtually superimposable growth curves; moreover, the overproducing clones grew at essentially the same rate as the vector control. Upon reinoculation and induction, all of

the XA90(pKEN1-*ada*) clones showed high-level induction of Ada (data not shown).

In contrast to the uniform growth rates seen with XA90(pKEN1) are the results of parallel cell growth in XA90(pHN1-*ada*) (Fig. 5B). In this case, of the 7 cultures for which growth curves were measured, only 2 grew (Fig. 5B, cultures 1 and 2) at a rate comparable to that observed for XA90(pKEN1-*ada*). Two other cultures grew at moderately reduced rates (cultures 3 and 4), and 3 barely grew at all (cultures 5–7). Only 2 cultures (1 and 2) produced measurable (by SDS-PAGE; not shown) amounts of Ada upon induction.

The impaired growth of pHN1-*ada* relative to pKEN1-*ada* is so severe that it can readily be observed on LB-Amp plates. Figure 6 shows a photograph of an LB-Amp plate streaked with overnight liquid (LB-Amp) cultures of the 2 strains. After several hours at 37 °C, XA90(pKEN1-*ada*) had formed dense streaks, while XA90(pHN1-*ada*) had hardly grown. After ~24 h at 37 °C, streaks of the 2 strains were indistinguishable; however, induction of the XA90(pHN1-*ada*) from colonies of that plate was not successful. A number of independent colonies gave the same result, ruling out random loss of the plasmid or the $F' lacI^{q1}$ allele as a contributing factor. Although the process giving rise to loss of overproduction is uncertain, it is clear that the loss is greatly accelerated in the pHN1 construct relative to the pKEN1 construct. Similar results have been obtained independently with pHN1 and pKEN1 constructs that overexpress the FKBP12, even when the cells were grown and stored in minimal media (P.K. Martin & S.L. Schreiber, pers. comm.).

We have further carried out *in vitro* studies to evaluate if both Lac repressor and RNA polymerase are capable of binding the *srp* promoter simultaneously (data not shown). Although, as previously reported, a ternary complex was observed in gel retardation experiments with *tac* promoter/*lac* operator fragments (pHN1+), no such complex was detectable with the *srp* promoter/operator (pKEN1). This suggests that the *srp* promoter functions as designed *in vivo*.

Discussion

The overproduction of toxic proteins in *E. coli* is often hampered by slow cell growth and instability of the overproduction

Fig. 4. SDS-PAGE analysis of *ada* gene inserts in pHN1+ (*tac* promoter) and pKEN1 (*srp* promoter). I, induced cells; U, uninduced cells. Note the difference in the intensity of the 39-kDa Ada protein band in the uninduced lanes (shown by arrow). The gel on the right side contains a control lacking the *ada* gene.

Fig. 5. Growth curves of Ada-overproducing strains (uninduced liquid cultures). **A:** XA90(pKEN1-*ada*). **B:** XA90(pHN1-*ada*). Curves 1–7 represent the results for cultures grown from independent colonies.

vector. A predominant reason for these effects is ineffective repression of the strong promoters typically used in overproduction vectors. In *lac*-derived systems, the efficiency of repression is dependent on the affinity of Lac repressor for its operator as well as the concentration of Lac repressor within the cell. Furthermore, interactions between Lac repressor and RNA poly-

merase play a role in controlling the promoter and consequently affect the levels of uninduced protein synthesis (Lee & Goldfarb, 1991). This work attempts to exploit the knowledge of the interaction between Lac repressor and RNA polymerase to design and evaluate a novel promoter, which is intended to reduce significantly transcription in the uninduced state. The design of this promoter was inspired by the findings of Straney and Crothers (1987) that Lac repressor does not occlude RNA polymerase from binding the promoter and, moreover, that Lac repressor and RNA polymerase can co-occupy the promoter/operator region. More recent work by Lee and Goldfarb (1991) suggests that Lac repressor prevents RNA polymerase from proceeding beyond the initially formed transcription complex. By moving the operator from its native site to the region between the -10 and -35 sequences, we hoped to prevent the poised transcription complex from forming, by allowing Lac repressor to interfere sterically with access of RNA polymerase to the promoter. Such a system should reduce the levels of uninduced protein synthesis, since the polymerase and the repressor are competing for overlapping sites. Because promoter “leakage” presents a problem for the maintenance of toxic proteins in *lac*-derived vectors and can have deleterious effects on cell growth, we anticipated that such a promoter should be useful for protein overproduction. We have termed this new promoter *srp*, for sterically repressed, and have incorporated it into the phagemid vector pKEN1. Although all of the factors that govern the induced levels of protein expression in the pKEN1 vector are not yet elucidated, it is clear that the *srp*-containing vector, pKEN1, has a lower level of uninduced protein expression than the corresponding *tac*-controlled vector, pHN1+. A comparison between these vectors led to 3 observations: (1) Ada overproduction in XA90(pKEN1-*ada*) is significantly more stable than that in XA90(pHN1-*Ada*) when maintained on LB-Amp plates; (2) XA90(pKEN1-*ada*) has a lower level of noninduced expression than XA90(pHN1-*ada*); (3) pKEN1-*ada* grows faster and exhibits more uniform growth (from colony to colony) than pHN1-*ada* in both liquid and solid media; and (4) consistent with design, in vitro studies indicate that pKEN1 is unable to form a poised transcription complex.

For purposes of comparison in the present study, we have altered the minimum number of parameters in our design of the *srp* promoter. However, there are a number of ways in which *srp*-based vectors might be improved. Studies on the pseudo-operators (*lac* O_2 and *lac* O_3) have shown that they have lower intrinsic binding affinities than *lac* O_1 for the Lac repressor; however, *lac* O_2 and *lac* O_3 appear to directly interact in a co-operative fashion with repressor bound at *lac* O_1 (Oehler et al., 1990). Work from a number of laboratories suggests that repression in the wild-type *lac* operon involves looping of DNA, where a single repressor tetramer binds to 2 operators (Gralla, 1989; Oehler et al., 1990). Inactivation of both *lac* O_2 and *lac* O_3 was demonstrated to decrease repression ~ 70 -fold (Oehler et al., 1990). In light of these data, it is noteworthy that the *lac* promoter derivatives borne on common cloning and overproduction vectors lack one or both of the 2 distant operator sites, and thus these promoters are inherently less tightly controlled than the wild-type *lac* promoter. Thus, inclusion of an upstream operator site in pKEN1 may enhance the level of repression. Furthermore, repression via the *srp* promoter may possibly be improved by replacing *lac* O_{srp} with a higher-affinity operator site. From artificial *lac* operator sequences found in yeast,

Fig. 6. Growth of Ada-overproducing strains on an LB-Amp agar plate. *tac* refers to XA90(pHN1-*ada*), and *srp* refers to XA90(pKEN1-*ada*).

chicken, and mouse, Simons et al. (1984) created a symmetrical 14-bp *lac* operator sequence (5'-TGTGAGCGCTCAC-3') that binds *lac* operator DNA with 8-fold greater affinity than *lac* O₁.

Vectors containing the *srp* promoter have been used to overproduce a number of proteins and have performed particularly well when the protein was toxic to the cell and therefore could not be maintained in conventional vectors (P.K. Martin & S.L. Schreiber, pers. comm.). The *srp*-based strategy complements an overproduction strategy based on a coupled T7 RNA polymerase/promoter system (Tabor & Richardson, 1985; Studier et al., 1990; Sodeoka et al., 1993). The T7-based vectors have the advantage of exclusively producing a protein under the control of the T7 promoter, by inhibiting the *E. coli* RNA polymerase with rifampicin. However, the T7 RNA polymerase gene, borne on an additional plasmid or on the genome, needs to be tightly repressed or, alternatively, induction can be achieved upon infection by a bacteriophage that provides the T7 RNA polymerase. An alternate strategy, already in use in commercial vectors, is to increase the intracellular levels of Lac repressor by placement of its coding sequence (*lacI*) on the overproduction vector. Our design, undertaken as a means of further understanding *lac*-controlled systems and employing knowledge gained from the interaction of RNA polymerase with its promoter as well as with the Lac repressor, has allowed the rational design of a novel repression system with altered protein-protein and protein-DNA interactions.

Materials and methods

Media, chemicals, and enzymes

Ampicillin (sodium salt; Sigma) was dissolved in distilled, deionized water (ddw) at a concentration of 2.5 mg/mL (250× solution) and passed through an Acrodisc (Gelman Scientific) 25-μm filter. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were obtained from Bachem, Inc. (Torrance, California). IPTG was dissolved in ddw at a concentration of 23.8 mg/mL and passed through an Acrodisc 25-μm filter. A 25-mg/mL solution of X-gal in *N,N*-dimethylformamide (Aldrich) was freshly made before each use. Sodium dodecyl sulfate, *N,N'*-methylene-bisacrylamide, and acrylamide were purchased from Bio-Rad. Urea was purchased from Bethesda Research Laboratories (BRL). Agarose (BRL) and Seaplaque LMP agarose (FMC Bioproducts, Rockland, Maine) were used with TBE (10× stock for 1 L: 108 g Tris base, 55 g boric acid, 9.3 g Na₂EDTA, pH 8.3) as the buffer. DNA was dissolved in water or 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Phenol (BRL) was washed and saturated with 1× TE. Radiochemicals used in sequencing were purchased from Amersham Corporation. *Eco*R I, *Hind* III, *Sal* I, and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals (BMB). *Afl* II was obtained from Anglian Biotech Limited. *Ssp* I was obtained from New England Biolabs (NEB). *Pvu* II, *Bam* HI, and T4 polynucleotide kinase were from BRL.

Plasmids and bacterial strains

pBS+ phagemid and *E. coli* XL1-Blue (genotype: *endA1*, *hsdR17* (r_k−, m_k+), *supE44*, *thi*, 1−, *recA1*, *gyrA96*, *relA1*,

lac−, [F', *proAB*, *lacI*^qΔM15, Tn10 (tet^r)] were purchased from Stratagene Cloning Systems (La Jolla, California). *E. coli* XA90 was a gift of M. Ptashne (phenotype: Δ*lac pro* XIII, *ara*, *nalA*, *argE* (am), *thi*−, *rif*^r, [F' *lacI*^{q1}, *lacZY*⁺, *proAB*⁺]).

Insertion of an *srp* promoter cassette into pBS+

Complementary 34-mer oligonucleotides, SRP I and SRP II (Fig. 3), were designed to install the *srp* promoter when annealed and inserted into the *Eco*R I site of the phagemid vector pBS+ (Stratagene). pBS+ was chosen because it has several useful features as a cloning vehicle: (1) multiple cloning site, the unique restriction sites of which will be fully maintained upon insertion of the *srp* cassette; (2) high copy number, as conferred by its pUC origin of replication; (3) *bla* gene encoding β-lactamase, which permits simple plasmid selection in the presence of ampicillin; and (4) bacteriophage f1 origin of replication, which allows packaging and extrusion of the phagemid DNA upon superinfection with helper phage.

Insertion of the *rrnBT*₁*T*₂ transcriptional terminator

As mentioned previously, it is desirable to incorporate an efficient, rho-independent transcription terminator into an overexpression vector. The *rrnBT*₁*T*₂ was chosen for the present study because this tandem terminator has been used successfully in other expression systems (Amann & Brosius, 1985). A 593-bp *Hind* III–*Ssp* I fragment of pKK223-3 (Pharmacia) containing *rrnBT*₁*T*₂ was generated. The 593-bp *Hind* III/*Ssp* I fragment containing *rrnBT*₁*T*₂ was ligated into *Hind* III/*Pvu* II-cut pBS+*srp*1 and the ligation mixture transformed into XL1-Blue cells. Miniplasmid preparations from individual transformants were digested to verify the presence of the desired construct (pKEN1). The integrity of the promoter/operator/polylinker/terminator region of pKEN1 was fully verified by dideoxy sequencing.

Introduction of the *ada* gene into pKEN1

The *ada* gene, including translational initiation elements, was obtained as a 1,259-bp *Eco*R I/*Hind* III restriction fragment from the Ada-overproducing vector pMT69 (Myers et al., 1992) and was ligated into *Eco*R I/*Hind* III-cut pKEN1. The resulting construct was transferred into *E. coli* XA90, which contains the F' *lacI*^{q1} allele and consequently has a higher level of constitutive Lac repressor expression.

Induction and SDS-PAGE analysis of protein-overproducing constructs

A single colony was used to inoculate 1 mL of LB-Amp medium (Sambrook et al., 1989), which was incubated at 37 °C overnight on a rotary shaker. A 100-μL aliquot of this was used to inoculate 5 mL of LB-Amp broth in a 50-mL Corning tube. The tube was shaken at 37 °C until the cells had reached the mid-logarithmic growth stage (*A*₅₅₀ = 0.5). Separate 1-mL aliquots were transferred either to tubes containing 10 μL 100 mM IPTG (induced cells) or to an empty tube (uninduced cells). After being shaken for 2 h at 37 °C, 1 mL was removed from each tube and microcentrifuged. The cell pellet was resuspended in 1× SDS-PAGE buffer (Sambrook et al., 1989) and incubated in a boil-

ing water bath for 3 min. A 5- μ L aliquot was then analyzed by 20% SDS-PAGE.

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