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Strategies for Achieving High-Level Expression of Genes in *Escherichia coli*†

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INTRODUCTION

The choice of an expression system for the high-level production of recombinant proteins depends on many factors. These include cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications, and biological activity of the protein of interest, as well as regulatory issues in the production of therapeutic proteins (191, 254). In addition, the selection of a particular expression system requires a cost breakdown in terms of process, design, and other economic considerations. The relative merits of bacterial, yeast, insect, and mammalian expression systems have been examined in detail in an excellent review by Marino (362). In addition, Datar et al. (121) have analyzed the economic issues associated with protein production in bacterial and mammalian cells.

The many advantages of *Escherichia coli* have ensured that it remains a valuable organism for the high-level production of recombinant proteins (177a, 197, 254, 362, 406, 426, 510). However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism. This may be due to the unique and subtle structural features of the gene sequence, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, and the potential toxicity of the protein to the host. Fortunately, some empirical "rules" that can guide the design of expression systems and limit the unpredictability of this operation in *E. coli* have emerged. The major drawbacks of *E. coli* as an expression system include the inability to perform many of the posttranslational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, and the limited ability to facilitate extensive disulfide bond formation. On the other hand, many eukaryotic proteins retain their full biological activity in a nonglycosylated form and therefore can be produced in *E. coli* (see, e.g., references 170, 342, and 486). In addition, some progress has been made in the areas of extracellular secretion and disulfide bond formation, and these will be examined.

The objectives of this review are to integrate the extensive published literature on gene expression in *E. coli*, to focus on expression systems and experimental approaches useful for the overproduction of proteins, and to review recent progress in this field. Areas that have been covered in detail in recent reviews are included in abbreviated form in order to present their key conclusions and to serve as a source for further reading. As a matter of definition, the terms "periplasmic expression" and "extracellular secretion" will be used to refer to the targeting of protein to the periplasm and the culture medium, respectively, to avoid confusion.

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† This review is dedicated to the memory of William John Steele, an inspired scientist, a great man, mentor, and friend, who died on 8 December 1995. The world is a better place because of him.

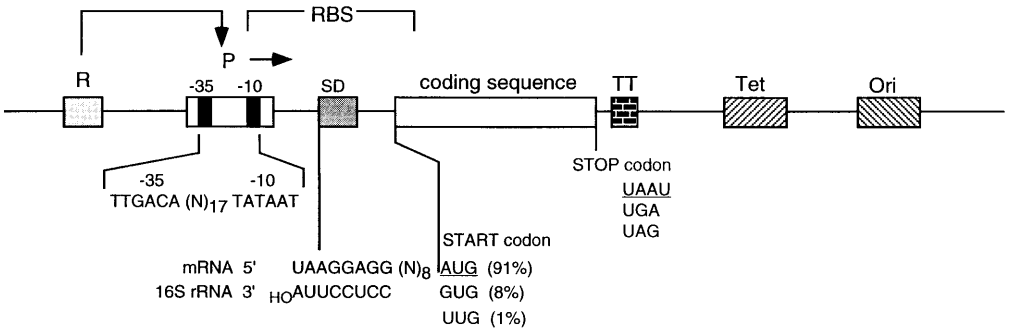

FIG. 1. Schematic presentation of the salient features and sequence elements of a prokaryotic expression vector. Shown as an example is the hybrid *tac* promoter (P) consisting of the −35 and −10 sequences, which are separated by a 17-base spacer. The arrow indicates the direction of transcription. The RBS consists of the SD sequence followed by an A+T-rich translational spacer that has an optimal length of approximately 8 bases. The SD sequence interacts with the 3′ end of the 16S rRNA during translational initiation, as shown. The three start codons are shown, along with the frequency of their usage in *E. coli*. Among the three stop codons, UAA followed by U is the most efficient translational termination sequence in *E. coli*. The repressor is encoded by a regulatory gene (R), which may be present on the vector itself or may be integrated in the host chromosome, and it modulates the activity of the promoter. The transcription terminator (TT) serves to stabilize the mRNA and the vector, as explained in the text. In addition, an antibiotic resistance gene, e.g., for tetracycline, facilitates phenotypic selection of the vector, and the origin of replication (Ori) determines the vector copy number. The various features are not drawn to scale.

CONFIGURATION OF EFFICIENT EXPRESSION VECTORS

The construction of an expression plasmid requires several elements whose configuration must be carefully considered to ensure the highest levels of protein synthesis (22, 64, 120, 142, 355, 538, 612). The essential architecture of an *E. coli* expression vector is shown in Fig. 1. The promoter is positioned approximately 10 to 100 bp upstream of the ribosome-binding site (RBS) and is under the control of a regulatory gene, which may be present on the vector itself or integrated in the host chromosome. Promoters of *E. coli* consist of a hexanucleotide sequence located approximately 35 bp upstream of the transcription initiation base (−35 region) separated by a short spacer from another hexanucleotide sequence (−10 region) (174, 232, 236, 344, 465). There are many promoters available for gene expression in *E. coli*, including those derived from gram-positive bacteria and bacteriophages (Table 1). A useful promoter exhibits several desirable features: it is strong, it has a low basal expression level (i.e., it is tightly regulated), it is easily transferable to other *E. coli* strains to facilitate testing of a large number of strains for protein yields, and its induction is simple and cost-effective (612).

Downstream of the promoter is the RBS, which spans a region of approximately 54 nucleotides bound by positions −35 (±2) and +19 to +22 of the mRNA coding sequence (269). The Shine-Dalgarno (SD) site (514, 515) interacts with the 3′ end of 16S rRNA during translation initiation (133, 532). The distance between the SD site and the start codon ranges from 5 to 13 bases (93), and the sequence of this region should eliminate the potential of secondary-structure formation in the mRNA transcript, which can reduce the efficiency of translation initiation (198, 229). Both 5′ and 3′ regions of the RBS exhibit a bias toward a high adenine content (140, 499, 502).

The transcription terminator is located downstream of the coding sequence and serves both as a signal to terminate transcription (465) and as a protective element composed of stem-loop structures, protecting the mRNA from exonucleolytic degradation and extending the mRNA half-life (35, 37, 147, 227, 249, 597).

In addition to the above elements that have a direct impact on the efficiency of gene expression, vectors contain a gene that confers antibiotic resistance on the host to aid in plasmid selection and propagation. Ampicillin is commonly used for this purpose; however, for the production of human therapeutic proteins, other antibiotic resistance markers are preferable to avoid the potential of human allergic reactions (42). Finally, the copy number of plasmids is determined by the origin of replication. In specific cases, the use of runaway replicons results in massive amplification of plasmid copy number concomitant with higher yields of plasmid-encoded protein (387, 415). In other cases, however, there appeared to be no advantage in using higher-copy-number plasmids over pBR322-based vectors (612). Furthermore, Vasquez et al. (572) reported that increasing the copy number of the plasmid decreased the production of trypsin in *E. coli* and Minas and Bailey (379) found that the presence of strong promoters on high-copy-number plasmids severely impaired cell viability.

TRANSCRIPTIONAL REGULATION

Promoters

A promoter for use in *E. coli* (Table 1) should have certain characteristics to render it suitable for high-level protein synthesis (207, 612). First, it must be strong, resulting in the accumulation of protein making up 10 to 30% or more of the total cellular protein.

Second, it should exhibit a minimal level of basal transcriptional activity. Large-scale gene expression preferably employs cell growth to high density and minimal promoter activity, followed by induction or derepression of the promoter. The tight regulation of a promoter is essential for the synthesis of proteins which may be detrimental to the host cell (see, e.g., references 68, 137, 544, 563, and 599). For example, the toxic rotavirus VP7 protein effectively kills cells and must be produced under tightly regulated conditions (592). However, in some cases, promoter stringency is inconsequential, because even the smallest amount of gene product drastically curtails bacterial survival because of its severe toxicity (615). For example, molecules that inactivate ribosomes or destroy the membrane potential would be lethal. Toxicity to the host is not restricted to foreign genes but may also result from the overexpression of certain native genes, such as the *traT* gene, which encodes an outer membrane lipoprotein (423), the *Eco*RI restriction endonuclease in the absence of the corresponding protective *Eco*RI modification methylase (423), and the *lon* gene (558). Furthermore, incompletely repressed expression systems can cause plasmid instability, a decrease in cell growth rate, and loss of recombinant protein production (40, 98, 374).

TABLE 1. Promoters used for the high-level expression of genes in *E. coli*

Promoter (source)	Regulation	Induction	Reference(s)
lac (*E. coli*)	*lacI*, *lacI*^{q}	IPTG	17, 18, 221, 460, 610
	lacI(Ts),[a] *lacI*^{q}(Ts)[a]	Thermal	234
	lacI(Ts)[b]	Thermal	604
trp (*E. coli*)		Trp starvation, indole acrylic acid	365, 470, 549, 612
lpp (*E. coli*)		IPTG, lactose[c]	128a, 142, 185, 275, 401
phoA (*E. coli*)	*phoB* (positive), *phoR* (negative)	Phosphate starvation	84, 274, 291, 306, 382, 562
recA (*E. coli*)	*lexA*	Nalidixic acid	145, 260, 428, 516
araBAD (*E. coli*)	araC	L-Arabinose	554
proU (*E. coli*)		Osmolarity	247
cst-1 (*E. coli*)		Glucose starvation	564
tetA (*E. coli*)		Tetracycline	125, 523
cadA (*E. coli*)	*cadR*	pH	102, 480, 561
nar (*E. coli*)	*fnr* (FNR, NARL)	Anaerobic conditions, nitrate ion	335
tac, hybrid (*E. coli*)	*lacI*, *lacI*^{q}	IPTG	7, 123, 471
	lacI[d]	Thermal	603
trc, hybrid (*E. coli*)	*lacI*, *lacI*^{q}	IPTG	65
	lacI(Ts),[a] *lacI*^{q}(Ts)[a]	Thermal	4, 9
lpp-lac, hybrid (*E. coli*)	*lacI*	IPTG	261, 263
P_{syn}, synthetic (*E. coli*)	*lacI*, *lacI*^{q}	IPTG	186
Starvation promoters (*E. coli*)			366
p_L (λ)	λ*cI*ts857	Thermal	43, 80, 129, 130, 240, 454
p_L-9G-50, mutant (λ)		Reduced temperature (<20°C)	187, 433
cspA (*E. coli*)		Reduced temperature (<20°C)	187, 206, 433, 551
p_R, p_L, tandem (λ)	λ*cI*ts857	Thermal	150, 493
T7 (T7)	λ*cI*ts857	Thermal	537, 548
T7-*lac* operator (T7)	*lacI*^{q}	IPTG	141, 190, 239
λp_L, p_{T7}, tandem (λ, T7)	λ*cI*ts857, *lacI*^{q}	Thermal, IPTG	375
T3-*lac* operator (T3)	*lacI*^{q}	IPTG	190, 605
T5-*lac* operator (T5)	*lacI*^{q}, *lacI*	IPTG	71, 390
T4 gene 32 (T4)		T4 infection	143, 210
nprM-lac operator (*Bacillus* spp.)	*lacI*^{q}	IPTG	605
VHb (*Vitreoscilla* spp.)		Oxygen, cAMP-CAP[e]	304, 305
Protein A (*Staphylococcus aureus*)			1,256, 349

[a] *lacI* gene with single mutation, Gly-187 → Ser (72).
[b] *lacI* gene with three mutations, Ala-241 → Thr, Gly-265 → Asp, and Ser-300 → Asn (604).
[c] The constitutive *lpp* promoter (P_{lpp}) was converted into an inducible promoter by insertion of the *lac*UV5 promoter/operator region downstream of P_{lpp}. Thus, expression occurs only in the presence of a *lac* inducer (142).
[d] Wild-type *lacI* gene.
[e] cAMP-CAP, cyclic AMP-catabolite activator protein.

Lanzer and Bujard carried out extensive studies on the commonly used *lac*-based promoter-operator systems and demonstrated up to 70-fold differences in the level of repression when the operator was placed in different positions within the promoter sequence (328). Thus, when the 17-bp operator was placed between the −10 and −35 hexameric regions, a 50- to 70-fold-greater repression was caused than when the operator was placed either upstream of the −35 region or downstream of the −10 site (328).

A third important characteristic of a promoter is its inducibility in a simple and cost-effective manner. The most widely used promoters for large-scale protein production use thermal induction (λ p_L) or chemical inducers (*trp*) (Table 1). The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible hybrid promoters *tac* (123) or *trc* (65) are powerful and widely used for basic research. However, the use of IPTG for the large-scale production of human therapeutic proteins is undesirable because of its toxicity (159) and cost. These drawbacks of IPTG have until now precluded the use of the *tac* or *trc* promoter from the production of human therapeutic proteins and rendered the large-scale expression of proteins for basic research prohibitively expensive. The availability of a mutant *lacI*(Ts) gene that encodes a thermosensitive *lac* repressor (72) now permits the thermal induction of these promoters (4, 9, 234). In addition, the new vectors exhibit tight regulation of the *trc* promoter at 30°C (9). Two different *lac* repressor mutants that are thermosensitive (586, 604) as well as IPTG inducible (586) have recently been described. Although the wild-type *lacI* gene can be thermally induced (602, 603), this system is not tightly regulated and cannot be used in *lacI*^{q} strains, since a temperature shift does not override the tight repression caused by the overproduction of the *lac* repressor (603). Thus, this system is limited to the production of some proteins that are not detrimental to the host cell.

Cold-responsive promoters, although much less extensively studied than many of the other promoters included here, have been shown to facilitate efficient gene expression at reduced temperatures. The activity of the phage λ p_L promoter was highest at 20°C and declined as the temperature was raised (187). This cold response of the p_L promoter is positively regulated by the *E. coli* integration host factor, a sequence-specific, multifunctional protein that binds and bends DNA (164, 165, 188). The promoter of the major cold shock gene *cspA* (206, 551) was similarly demonstrated to be active at reduced temperatures (187). Molecular dissection of the *cspA* and p_L promoters led to the identification of specific DNA regions involved in the enhancement of transcription at lower temperatures; this has allowed the development of p_L derivatives that are highly active at temperatures below 20°C (433). The rationale behind the use of cold-responsive promoters for

gene expression is based on the proposition that the rate of protein folding will be only slightly affected at about 15 to 20°C, whereas the rates of transcription and translation, being biochemical reactions, will be substantially decreased. This, in turn, will provide sufficient time for protein refolding, yielding active proteins and avoiding the formation of inactive protein aggregates, i.e., inclusion bodies, without reducing the final yield of the target protein (433). It would be interesting to compare the transcriptional activities of other promoters derived from cold shock genes (288, 402).

Other promoters that have been characterized recently (Table 1) possess attractive features and should provide additional options for high-level gene expression systems. For example, the pH promoter (102, 561) is very strong: recombinant proteins are produced at levels of up to 40 to 50% of the total cellular protein (480). This expression level, however, will probably vary for different genes, because protein synthesis depends on translational efficiency as well as promoter strength.

E. coli promoters are usually considered in terms of a core region composed of the −10 and −35 hexameric sequences including a 15- to 19-bp spacer between the two hexamers (344). However, it has been proposed that elements outside the core region stimulate promoter activity (134). Many studies have demonstrated that sequences upstream of the core promoter increase the rate of transcription initiation in vivo (172, 213, 264, 290, 618). Gourse and colleagues have shown that a DNA sequence, the UP element, located upstream of the −35 region of the *E. coli* rRNA promoter *rrnB* P1, stimulates transcription by a factor of 30 in vitro and in vivo (290, 453, 468). The UP element functions as an independent promoter module because when it is fused to other promoters such as *lacUV5*, it stimulates transcription (453, 468). Upstream activation in *E. coli* and other organisms has been reviewed in detail (110). The ability of the UP element to act as a transcriptional enhancer when fused to heterologous promoters may be of general utility in high-level expression systems.

Although the extraordinary strength of the rRNA promoters P1 and P2 is well documented (173, 414), these promoters have not been exploited for the high-level production of proteins in *E. coli*, mainly because their regulation is more difficult. The in vivo synthesis of rRNA is subject to growth rate control (213), and P1 and P2 are active during periods of rapid cell growth and are downregulated when cells are in the stationary phase of growth. Therefore, the rRNA promoters would be continuously active or "leaky" during the preinduction phase. In vivo P2 is the weaker, less inducible promoter in rapidly growing cells. However, when uncoupled from P1, the P2 promoter shows increased activity (up to 70% of that of P1) and becomes sensitive to the stringent response, indicating that in its native tandem context, P2 is partially occluded (173, 289). Brosius and Holy (66) inserted the *lac* operator sequence downstream of the *rrnB* rRNA P2 promoter and achieved repression of P2 in strains harboring the *lacI*^{q} gene. Transcriptional activity was measured by the production of chloramphenicol acetyltransferase and by the expression of the 4.5S RNA. However, the P2 construction was only half as active as the *tac* promoter, and furthermore, when the *rrnB* P1 promoter was placed upstream of the P2 promoter, transcriptional repression was incomplete (66).

It is tempting to speculate that rRNA promoters could be tightly regulated by using the concept of inverted promoters (see the section on tightly regulated expression systems, below). Thus, a rRNA promoter could be cloned upstream of the gene of interest but in the opposite transcriptional direction. The use of λ integration sites and a regulated λ integrase would facilitate the inversion of the promoter for induction, and the presence of strong transcription terminators upstream of the highly active promoter would prevent destabilization of the vector during the preinduction phase.

Transcriptional Terminators

In prokaryotes, transcription termination is effected by two different types of mechanisms: Rho-dependent transcription termination depends on the hexameric protein rho, which causes the release of the nascent RNA transcript from the template. In contrast, rho-independent termination depends on signals encoded in the template, specifically, a region of dyad symmetry that encodes a hairpin or stem-loop structure in the nascent RNA and a second region that is rich in dA and dT and is located 4 to 9 bp distal to the dyadic sequence (83, 122, 439, 455, 456, 465, 594, 609). Although often overlooked in the construction of expression plasmids, efficient transcription terminators are indispensable elements of expression vectors, because they serve several important functions. Transcription through a promoter may inhibit its function, a phenomenon known as promoter occlusion (5). This interference can be prevented by the proper placement of a transcription terminator downstream of the coding sequence to prevent continued transcription through another promoter. Similarly, a transcription terminator placed upstream of the promoter that drives expression of the gene of interest minimizes background transcription (413). It is also known that transcription from strong promoters can destabilize plasmids as a result of overproduction of the ROP protein involved in the control of plasmid copy number as a result of transcriptional readthrough into the replication region (539). In addition, transcription terminators enhance mRNA stability (237, 404, 597) and can substantially increase the level of protein production (237, 572). Particularly effective are the two tandem transcription terminators T1 and T2, derived from the *rrnB* rRNA operon of *E. coli* (67), but many other sequences are also quite effective.

Transcriptional Antiterminators

In bacteria, many operons involved in amino acid biosynthesis contain transcriptional attenuators at the 5′ end of the first structural gene. The attenuators are regulated by the amino acid products of the particular operon. Thus, the availability of the cognate charged tRNA leads to the formation of a secondary structure in the nascent transcript followed by ribosome stalling. In the absence of the cognate charged tRNA, an antiterminator structure which prevents formation of the RNA hairpin in the terminator and prevents transcriptional termination is formed (325). The antiterminator element that enables RNA polymerase to override a rho-dependent terminator in the ribosomal RNA operons has been identified and is referred to as *boxA* (41, 341). Transcriptional antitermination is a remarkably complex process that involves many known and as yet unidentified host factors. This topic has been covered in great detail in two excellent recent reviews (110, 456). Here, we will briefly consider the use of antitermination elements that are useful in the expression of heterologous genes in *E. coli.*

One of the more powerful and widely used expression systems in *E. coli* makes use of the phage T7 late promoter (537, 548). The activity of this system depends on a transcription unit that supplies the T7 RNA polymerase, whose tight repression is essential to avoid leakiness of the T7 promoter. Several approaches have been used to regulate the expression of the T7 polymerase, and each has its own unique disadvantages (374). Mertens et al. (374) addressed this problem by constructing a reversibly attenuated T7 RNA polymerase expres-

sion cassette based on λ p_L regulation. Thus, the basal expression level of the T7 polymerase was attenuated by inserting three tandemly arranged transcription terminators between the promoter and the gene encoding the T7 polymerase. For induction, the phage λ-derived nut_L-dependent antitermination function was also incorporated to override the transcription block. Alternatively, an IPTG-inducible promoter was similarly used, allowing conditional reversion of attenuation upon induction (374).

The transcriptional antitermination region from the *E. coli rrnB* rRNA operon has been used in the expression vector pSE420, which utilizes the *trc* promoter (64). The rationale in this case was to facilitate transcription through areas of severe secondary structure, thus reducing the possibility of premature transcription termination by the host RNA polymerase. In this case, however, the presence of the *rrnB* antiterminator is apparently ineffective (64a).

Tightly Regulated Expression Systems

The advantages of tightly regulated promoters (see the section on promoters, above) have led to the design of many ingenious and highly repressible expression systems that are particularly useful for the expression of genes whose products are detrimental to host growth. The various approaches include the use of a "plating" method (544), the increase of the repressor-to-operator ratio (9, 391), induction by infection with mutant phage (68, 137), attenuation of promoter strength on high-copy-number vectors (587), the use of transcription terminators (374, 375, 413) in combination with antiterminators (374), the use of an inducible promoter within a copy-number-controllable plasmid (558), "cross-regulation" systems (97, 98), cotransformation of plasmids utilizing the SP6 RNA polymerase (473), and the use of antisense RNA complementary to the mRNA of the cloned gene (423). Finally, one elegant approach involves the principle of invertible promoters: the promoter, flanked by two λ integration sites, faces in the direction opposite that of the gene to be expressed and is inverted only by inducing site-specific genetic recombination mediated by the λ integrase (16, 21, 235, 441, 599).

The above systems have advantages as well as disadvantages, depending on their intended use. Thus, methods that rely on solid media cannot easily be used for large-scale expression. High-level repressor systems often cause a substantial decrease in protein yield (9, 531), thus necessitating optimization of the repressor-to-operator ratio (234). Induction mediated by λ phage adds further complexity to the system. The use of inverted promoter circuits involves complex vector constructions. Although most of the above systems have not yet been used for the high-level production of proteins on a large scale, they nevertheless provide important tools for the armamentarium of gene expression.

TRANSLATIONAL REGULATION

mRNA Translational Initiation

The extensive knowledge of the transcriptional process has allowed the use of prokaryotic promoters in cassette fashion, unaffected by the surrounding nucleotide context (232, 236, 317, 344). However, the determinants of protein synthesis initiation have been more difficult to decipher; this is not surprising, considering the complexity of this process (224, 579). It is now clear that the wide range of efficiencies in the translation of different mRNAs is predominantly due to the unique structural features at the 5′ end of each mRNA species. Thus, in contrast to the portable promoters, no universal sequence for the efficient initiation of translation has been devised. However, progress in this aspect of gene expression in *E. coli* has been strong, and general "guidelines" have emerged (131, 133, 196, 198, 218, 368, 369, 458, 579, 590).

The translational initiation region of most sequenced *E. coli* genes (91%) contains the initiation codon AUG. GUG is used by about 8% of the genes, and UUG is rarely used as a start site (1%) (218, 224, 535). In one case, AUU is used as the start codon for *infC* (75). This codon is required for the autogenous regulation of *infC*. The translational efficiency of the initiation codons in *E. coli* has been examined. AUG is the preferred codon by two- to threefold, and GUG is only slightly better than UUG (458, 573).

Shine and Dalgarno (514, 515) identified a sequence in the RBS of bacteriophage mRNAs and proposed that this region, subsequently called the Shine-Dalgarno (SD) site, interacts with the complementary 3′ end of 16S rRNA during translation initiation. This was confirmed by Steitz and Jakes (532). The spacing between the SD site and the initiating AUG codon can vary from 5 to 13 nucleotides, and it influences the efficiency of translational initiation (196). Extensive studies have been carried out to determine the optimal nucleotide sequence of the SD region, as well as the most effective spacing between the SD site and the start codon (28, 93, 131, 593). Ringquist et al. (458) examined the translational roles of the RBS and reached the following conclusions. (i) The SD sequence UAA GGAGG enables three- to sixfold-higher protein production than AAGGA for every spacing. (ii) For each SD sequence, there is an optimal although relatively broad spacing of 5 to 7 nucleotides for AAGGA and 4 to 8 nucleotides for UAAGG AGG. (iii) For each SD sequence, there is a minimum spacing required for translation; for AAGGA, this minimum spacing is 5 nucleotides, and for UAAGGAGG, it is 3 to 4 nucleotides. These spacings suggest that there is a precise physical relationship between the 3′ end of 16S rRNA and the anticodon of the fMet-$tRNA_f$ bound to the ribosomal P site (458).

The secondary structure at the translation initiation region of mRNA plays a crucial role in the efficiency of gene expression (132, 229, 233, 277, 295). It is believed that the occlusion of the SD region and/or the AUG codon by a stem-loop structure prevents accessibility to the 30S ribosomal subunits and inhibits translation (184, 451, 556). Several different strategies have been devised to minimize mRNA secondary structure. The enrichment of the RBS with adenine and thymidine residues enhanced the expression of certain genes (94, 412, 429). Similarly, the mutation of specific nucleotides upstream or downstream of the SD region suppressed the formation of mRNA secondary structure and enhanced translational efficiency (107, 223, 266, 336, 530, 583). Another approach takes advantage of the naturally occurring phenomenon of translational coupling in bacteria (506). The mechanism of translational coupling has been invoked to account for the coordinate expression of different proteins from polycistronic mRNAs. Thus, it was shown that the moderately strong *gal* promoter could direct the synthesis of galactokinase at very high levels when *galK* was translationally coupled to an upstream gene, suggesting that even a weak RBS may be highly efficient if it is accessible to ribosomes (506). Schümperli et al. (506) suggested that this regulatory mechanism might have important applications in biotechnology for the overproduction of proteins. Indeed, translational coupling has been widely used for the high-level expression of diverse genes (46, 359, 430, 438, 503, 504, 505, 552).

In addition to the binding of the SD region to the 16S rRNA, other interactions between mRNA and the ribosome are in-

volved during the initiation of translation. Cross-linking studies, for example, have shown that the ribosomal protein S1 is directly involved in recognition and binding of mRNA by the 30S ribosomal subunit (54). The structural and functional interactions of the many components of the prokaryotic translational initiation complex have been examined (160, 224, 321, 368, 369, 579).

Translational Enhancers

Sequences that markedly enhance the expression of heterologous genes in *E. coli* have been identified in both bacteria and phages. Olins et al. characterized a 9-base sequence from the T7 phage gene 10 leader (g10-L) that appears to act as a very efficient RBS. Compared with a consensus SD region, the g10-L sequence caused a 40- to 340-fold increase in the expression of several genes (425, 428). When placed upstream of a synthetic SD sequence, the g10-L sequence caused a 110-fold increase in the translational efficiency of the *lacZ* gene, estimated as the ratio of β-galactosidase activity to the level of *lacZ* mRNA (427). A model was proposed whereby this sequence functions to enhance translation by interacting with bases 458 to 466 of the 16S rRNA (427). An alternative explanation is that only mRNAs with a weak SD site are likely to benefit from the g10-L sequence and that this might be due to stabilization of the mRNA rather than to a specific interaction with the 16S rRNA (527). Others failed to observe a significant enhancement of protein production when using the g10-L sequence (9, 527). Sequences homologous to the T7 g10-L have also been identified in other bacteriophages (427).

Several other groups have identified U-rich sequences in the 5′ untranslated region (UTR) of mRNAs that act as enhancers of translation. McCarthy et al. (370) characterized a region in the *E. coli atpE* gene, immediately upstream of the SD site. This 30-base sequence was used to overexpress the human interleukin-2 and interferon beta genes (371, 493, 494). A U_8 sequence upstream of the SD site in the *rnd* mRNA encoding RNase D (620) was shown to be essential for efficient translation of this mRNA (622). Deletion of this region severely decreased translation without affecting the level of *rnd* mRNA or the transcriptional start site (621). Boni and coworkers demonstrated that the target for similar sequences is the S1 protein of the 30S ribosomal subunit (54, 565).

In a very interesting study, Sprengart et al. (527) demonstrated that sequences immediately downstream of the start codon play an important role during translation initiation. A specific region, termed the downstream box (DB), located between positions +15 to +26 of the T7 gene 0.3 coding region (526) or between positions +9 and +21 of the T7 gene 10 coding region (527) functions as a translational enhancer. The DB region is complementary to 16S rRNA nucleotides 1469 to 1483, termed the anti-downstream box (ADB). Deletion of the DB abolished translational activity (526). Conversely, optimization of the complementarity between the DB and the ADB sequences resulted in the highest level of expression of the *dhfr* fusion gene (527). Interestingly, the DB was not functional when shifted upstream of the initiation colon to the position of the SD sequence. The DB is present in a number of *E. coli* and bacteriophage genes (158, 242, 279, 326, 397, 442, 511).

These findings demonstrate convincingly that in addition to the SD site and the start codon, other sequences in the mRNA are important for efficient translation. Although the precise mechanisms of the observed effects are not always clear, the few studies cited above indicate that efforts to overexpress genes may benefit from the use of "translational enhancer" modules.

mRNA Stability

The process of mRNA degradation provides a major control point of gene expression in virtually all organisms (467). Although the concept of mRNA and its lability was established 35 years ago (60, 222, 283), the detailed understanding of the mechanisms of mRNA decay has presented a considerable challenge for a number of reasons (35). However, in spite of the many perplexing questions surrounding this important biological process, progress has been impressive (36, 147, 323, 407, 437). This section will consider specific determinants of mRNA stability that may have practical applications in the high-level expression of genes in *E. coli*.

Several different RNases participate in mRNA degradation in *E. coli*, including endonucleases (RNase E, RNase K, and RNase III) and 3′ exonucleases (RNase II and polynucleotide phosphorylase [PNPase]); no 5′ exonuclease has been identified to date in prokaryotes (35). mRNA degradation is not effected randomly by nonspecific endonucleolytic cleavage, since there is no inverse correlation between mRNA length and half-life (90). Two classes of protective elements are known to stabilize mRNAs in *E. coli*. One class consists of sequences in the 5′ UTRs of mRNAs (31), and the other class includes stem-loop structures from the 3′ UTRs and intercistronic regions (249). Some of these elements act as stabilizers when fused to heterologous mRNAs but only under restricted conditions. For example, the 5′ UTR of the bacteriophage T4 gene 32 increases the half-life of unstable mRNAs in *E. coli* but only in T4-infected cells (143, 210). The erythromycin resistance genes (*erm*) of gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* encode mRNAs that contain stabilizing elements in their 5′ UTRs. However, stabilization of mRNAs by the *ermC* and *ermA* 5′ UTRs is induced by an antibiotic that inhibits translation and causes ribosome stalling (32, 481, 482). Similarly, the stabilizing effect of the 5′ region of the bacteriophage λ p_L on λ p_L-*trp* transcripts requires λ infection (606).

In contrast to the above examples, the 5′ UTR of the *E. coli ompA* transcript prolongs the half-life of a number of heterologous mRNAs in *E. coli* under normal conditions of rapid cell growth (38, 96, 151, 152). Emory et al. showed that the presence of a stem-loop structure at or very near the extreme 5′ terminus of the *ompA* 5′ UTR is essential for its stabilizing effect. Furthermore, the half-life of a normally labile mRNA could be prolonged in *E. coli* by adding a hairpin structure at its 5′ terminus (152). It was proposed that *E. coli* mRNAs beginning with a long single-stranded segment are preferentially targeted by an RNase that interacts with the 5′ terminus before cleaving the mRNA at internal sites (152). It appears, therefore, that the addition of the *ompA* 5′ stabilizer to heterologous genes might enhance gene expression in *E. coli*. However, it is possible that mRNAs which contain internal RNA-processing sites are not protected by the presence of the 5′ stabilizer (31).

The other class of mRNA-protective elements consists of 3′ UTR sequences that can form stem-loop structures, thereby blocking exonucleolytic degradation of the transcript from the 3′ terminus (249). Wong and Chang (597, 598) identified such an element within the transcription terminator of the crystal protein gene of *Bacillus thuringiensis*. Fusion of this "positive retroregulator" to the 3′ termini of the penicillinase (*penP*) gene of *Bacillus licheniformis* and the human interleukin-2 cDNA increased the half-life of the mRNAs and enhanced the production of the corresponding polypeptides in both *B. subtilis* and *E. coli*. However, as in the case of some of the 5′ stabilizers, this 3′ retroregulator (597) is unlikely to act as a

universal mRNA stabilizer. For example, the replacement of the 3′-terminal hairpins of labile mRNAs with those from stable mRNAs did not enhance the expression of the labile transcripts (38, 89, 597). Furthermore, it has been suggested that gene expression might be enhanced by the use of host strains that are deficient in specific RNases, such as RNase II or PNPase. This, too, is unlikely to be an effective strategy, because the absence of RNase II or PNPase, as well as the overproduction of RNase II, had no effect on the average half-life of *E. coli* bulk mRNA (138, 139). Moreover, strains that were deficient in both RNase II and PNPase were inviable (138). These and other considerations led to the following conclusions: "It is unlikely that the disparate stabilities of most mRNAs that end in a stem-loop result from differential susceptibility of these terminal stem-loops to penetration by 3′ exonucleases," and, furthermore, "3′-exonucleolytic initiation of RNA decay probably is rare, except in the case of labile RNAs lacking a substantial 3′ hairpin and long-lived RNAs resistant to attack by all other types of ribonucleases" (35).

Translational Termination

The presence of a stop signal in the mRNA is an indispensable component of the translation termination process. In addition to the three termination codons, UAA, UGA, and UAG, this complex event involves specific interactions between the ribosome, mRNA, and several release factors at the site of termination (112, 553). In *E. coli*, RF-1 terminates translation at the UAG stop codon, RF-2 terminates translation at the UGA codon, and both RFs terminate translation at the UAA codon (507). An additional factor, RF-3, has recently been cloned (219, 377).

The design of expression vectors frequently includes the insertion of all three stop codons to prevent possible ribosome skipping. In *E. coli*, there is a preference for the UAA stop codon (508). A statistical analysis of more than 2,000 *E. coli* genes revealed local nonrandomness both in the stop codon and in the nucleotide immediately following the triplet (445, 553). The same workers tested the strengths of each of 12 possible tetranucleotide "stop signals" (UAAN, UGAN, UAGN) by an in vivo termination assay that measured termination efficiency by its direct competition with frameshifting. Termination efficiencies varied significantly depending on both the stop codon and the fourth nucleotide, ranging from 80% (UAAU) to 7% (UGAC). These findings indicate that the identity of the nucleotide immediately following the stop codon strongly influences the efficiency of translational termination in *E. coli* (445). Therefore, UAAU is the most efficient translational termination sequence in *E. coli*.

The sequence context at the 5′ end of the stop codon further influences the efficiency of termination. Thus, the charge and hydrophobicity properties of the penultimate (−2 location) C-terminal amino acid residue in the nascent peptide cause up to a 30-fold difference in UGA termination efficiency, whereas termination at UAG is less sensitive to the nature of the −2 amino acid residue (389). For the −1 location, α-helical, β-strand, and reverse-turn propensities are determining factors in UGA termination (48).

PROTEIN TARGETING

Cytoplasmic Expression

The formation of inclusion bodies remains a significant barrier to gene expression in the cytosol. Inclusion bodies do offer several advantages (Table 2). However, these are small consolation considering the arduous task of refolding the aggregated protein (469), the uncertainty of whether the refolded protein retained its biological activity, and the reduction in yield of the refolded and purified protein. To date, the precise physicochemical parameters that contribute to the formation of inclusion bodies remain unclear (322, 363, 381, 469, 495, 588). A statistical analysis of the composition of 81 proteins that do and do not form inclusion bodies in *E. coli* concluded that six parameters are correlated with inclusion body formation: charge average, turn-forming residue fraction, cysteine fraction, proline fraction, hydrophilicity, and total number of residues (591). The first two parameters are strongly correlated with inclusion body formation, while the last four parameters show a weak correlation. These findings were used to develop a model to predict the probability of inclusion body formation solely on the basis of the amino acid composition of a protein (591). This model was used to predict accurately the insolubility of the human T-cell receptor Vβ5.3 in *E. coli* (9).

Several experimental approaches have been used to minimize the formation of inclusion bodies and improve protein folding (496) (Table 2). These include the growth of bacterial cultures at lower temperatures (77, 495, 497, 517); the selection of different *E. coli* strains (302); the substitution of selected amino acid residues (118, 457); the coproduction of chaperones (8, 29, 52, 337, 613); the use of *E. coli* thioredoxin either as a fusion partner (330) or coproduced with the protein of interest (613); growth and induction of the cells under osmotic stress in the presence of sorbitol and glycyl betaine (49); addition of nonmetabolizable sugars to the growth medium (56); alteration of the pH of the culture medium (541); and the use of strains deficient in thioredoxin reductase (128, 447).

The reducing potential of the cytoplasmic redox state (156, 270) presents still another problem. Bacterial cytoplasmic proteins contain few cysteine residues and few disulfide bonds (156, 444). Most proteins that contain stable disulfide bonds are exported from the cytoplasm (559). Thus, mammalian proteins whose complex tertiary structure depends in part on disulfide bond formation may not be produced in their correct conformation in the bacterial cytoplasm (443). Bardwell et al. have proposed that the low frequency of disulfide bonds in cytoplasmic proteins may be due to the absence from the cytoplasm of a system for the formation of disulfide bonds, such as the DsbA and DsbB proteins (26, 27), and/or a mechanism that actively prevents the formation of disulfide bonds in the cytoplasm. Mutant *E. coli* strains that allow the formation of disulfide bonds in the cytoplasm were isolated (128). These mutations inactivate the *trxB* gene that encodes thioredoxin reductase (168) and contributes to the sulfhydryl reducing potential of the cytoplasm (258). Thioredoxin itself was unnecessary for disulfide bond formation (128). The precise sequence of events is not clearly understood, and the authors suggested that the cytoplasm may contain another thioredoxin-like protein that can be reduced by thioredoxin reductase; in the absence of thioredoxin reductase, the oxidized form of this unknown protein facilitates the formation of disulfide bonds in the cytoplasm (128). Other workers have recently used *E. coli* strains carrying null mutations in the *trxB* gene and observed significant amounts of functional disulfide-containing protein in the cytoplasm (447). These thioredoxin reductase-deficient strains should prove to be valuable tools for the production of complex proteins in *E. coli*.

The cytoplasmic expression of a gene without a leader requires the presence of an initiation codon, the most common one encoding methionine. Although this extraneous amino acid might have no adverse effect on the protein synthesized, there are specific cases in which the extra methionine has

TABLE 2. Relative merits of different compartments for gene expression in *E. coli* and strategies for the potential resolution of experimental problems

Compartment property	Strategy for resolution	Reference(s)
Cytoplasm		
Advantages		
Inclusion bodies: facile isolation of protein in high purity and concentration; target protein protected from proteases; desirable for production of proteins that, if active, are lethal to host cell		25, 99, 243, 294, 310, 469
Higher protein yields		
Simpler plasmid constructs		
Disadvantages		
Inclusion bodies: protein insolubility; refolding to regain protein activity; refolded protein may not regain its biological activity; reduction in final protein yield; increase in cost of goods	Lower growth temperature	495
	Cold shock promoters (lower temperature)	187, 206, 433
	Selection of different *E. coli* strains	302
	Amino acid substitutions	118, 282, 394, 457, 536
	Coexpression of molecular chaperones	119, 581, 613
	Fusion partners	330, 419, 591a
	Strains deficient in thioredoxin reductase	128, 447
	Sorbitol and glycyl betaine in culture medium	49
	Altered pH	541
	Sucrose, raffinose in growth medium	56
	Rich growth media	386
Reducing environment: does not facilitate disulfide bond formation	Strains deficient in thioredoxin reductase	128, 447
Authenticity: N-terminal methionine	Coexpression of methionine aminopeptidase	483, 513
Proteolysis	Protease-deficient strains	211
	Mutagenesis of protease cleavage sites	25, 243, 395
	Hydrophobicity engineering	394
	Fusion partners	59, 319, 393, 395, 567
	Fermentation conditions	24, 25, 100, 337
	Coexpression of phage T4 *pin* gene	519–521
	Coexpression of molecular chaperones	180, 489, 581
	Fusion of multiple copies of target gene	512
Purification is more complex (more protein types)	Affinity fusion partners (may require cleavage)	419
Periplasm		
Advantages		
Purification is simpler (fewer protein types)		416
Proteolysis is less extensive	Protease-deficient strains	372, 373
	Fusion partners	230
	Other approaches as above	
Improved disulfide bond formation/folding		
N-terminus authenticity		
Disadvantages		
Signal peptide does not always facilitate transport; protein export machinery overloaded?	Coexpression of signal peptidase I	570
	Co-overexpression of *prlF*	379
	Use of *prlF* mutant strains	525
	Coexpression of *prlA4* and *secE*	435
	Expression of *pspA*	311
	Coexpression of *sec* genes	581
	Fusion proteins	220
Reduced folding	Amino acid substitutions	314
	Coexpression of protein disulfide isomerase	268, 433a
Inclusion bodies may form	Coexpression of molecular chaperones	45
	Lower growth temperature	57, 58, 82
	Sucrose, raffinose in growth medium	56
Inner membrane		
To date not useful for high-level gene expression; may facilitate pharmacological studies, enzymatic activity studies, and other applications		220, 500

Continued on following page

TABLE 2—*Continued*

Compartment property	Strategy for resolution	Reference(s)
Extracellular medium		
Advantages		
Least level of proteolysis		309
Purification is simpler (fewest protein types)		
Improved protein folding		
N-terminus authenticity		
Disadvantages		
No secretion usually	Fusions to normally secreted proteins	303, 528
	Coexpression of *kil* for permeabilization	296, 309
	Fusion to *ompF* gene components	396
	Use of *ompA* signal sequence	316
	Use of protein A signal sequence	256
	Coexpression of bacteriocin release protein	261
	Use of glycine and bacteriocin release protein	617
	Glycine supplement in medium	10, 13
	Fusion partners	384
Protein diluted, more difficult to purify	Expanded-bed adsorption	231
	Concentration, affinity chromatography	
Cell surface		
To date not useful for high-level gene expression. May facilitate vaccine development, drug screening, biocatalysis, protein-protein interactions, and other applications		85, 111, 169, 178, 179, 253, 345, 352, 405

profound consequences. For example, the retention of the initiating methionine in RANTES, a member of the chemokine family of cytokines, completely abrogates the physiological activity of this molecule and confers potent antagonist properties to the methionylated RANTES (448). Similarly, an unnatural N-terminal methionine residue can alter the conformation of the human hemoglobin molecule (298). Moreover, it is possible that the presence of an extra amino acid will change the immunological properties of pharmaceutical proteins and create difficulties in the approval of a nonnative product for clinical use.

Bacterial translation is initiated by *N*-formylmethionine which is deformylated during synthesis (2) but not necessarily removed. The N-terminal methionine might be cleaved off by an endogenous methionine aminopeptidase (39) depending on the side chain length of the second amino acid residue (250). Thus, residues with small side chains such as Gly, Ala, Pro, Ser, Thr, Val, Cys, and, to a lesser degree, Asn, Asp, Leu, and Ile, facilitate the methionine aminopeptidase-catalyzed removal of the N-terminal methionine (250). One strategy that has been successfully used to remove the extra methionine residue from recombinant proteins in vivo is coexpression of the *E. coli* methionine aminopeptidase gene (483, 513). An alternative method for the in vitro generation of an authentic N terminus uses the exopeptidase dipeptidylaminopeptidase I. This enzyme removes dipeptides from the N terminus but cannot cleave peptide bonds containing a proline residue. Dalbøge et al. (117) produced human growth hormone containing an amino-terminal extension which was subsequently removed with dipeptidylaminopeptidase I to yield authentic growth hormone. This approach requires an amino-terminal extension that contains an even number of amino acid residues and is designed so that it enables the in vivo excision of the N-terminal methionine. In addition, the second or third amino acid residue in the target protein must be proline (117). A more elaborate method free of the above restrictions has been proposed to generate an authentic N terminus for any protein (117). The cotranslational amino-terminal processing in both prokaryotes and eukaryotes has been reviewed (301).

Protein degradation is more likely to occur in the cytoplasm of *E. coli* than in other compartments (550) because of the greater number of proteases located there (545, 546). This topic is examined in the section on protein degradation (below). Finally, another difficulty that affects cytosolic gene expression is the need to purify the target protein from the pool of the intracellular proteins. Calculations based on total DNA content predict that the *E. coli* chromosome may encode 3,000 to 4,000 genes (547), although not all of these are expressed under given growth conditions.

Periplasmic Expression

The periplasm offers several advantages for protein targeting. In contrast to the cytosolic compartment, the periplasm contains only 4% of the total cell protein (416) or approximately 100 proteins (450). The target protein is thus effectively concentrated, and its purification is considerably less onerous. The oxidizing environment of the periplasm facilitates the proper folding of proteins, and the cleaving in vivo of the signal peptide during translocation to the periplasm is more likely to yield the authentic N terminus of the target protein. Protein degradation in the periplasm is also less extensive (550).

The transport of a protein through the inner membrane to the periplasm normally requires a signal sequence (376, 490, 492, 575–577, 589). A wide variety of signal peptides have been used successfully in *E. coli* for protein translocation to the periplasm. These include prokaryotic signal sequences, such as the *E. coli* PhoA signal (127, 424), OmpA (127, 185, 205, 263, 339), OmpT (286), LamB and OmpF (255), β-lactamase (292, 574), enterotoxins ST-II, LT-A, LT-B (171, 388), protein A from *Staphylococcus aureus* (1, 256), endoglucanase from *B. subtilis* (348), PelB from *Erwinia carotovora* (44, 340), a degen-

erate PelB signal sequence (332), the murine RNase (498), and the human growth hormone signal (217).

However, protein transport to the bacterial periplasm is a particularly complex and incompletely understood process (449, 475, 492), and the presence of a signal peptide does not always ensure efficient protein translocation through the inner membrane. For example, whereas the bacterial production of human immunoglobulins has been quite successful (440, 522), the production of T-cell receptor variants in the periplasm has been considerably more difficult in spite of the structural similarities between these two families of molecules. Thus, in spite of the correct cleavage of the signal peptide, no T-cell receptor protein was detected in the periplasm (see, e.g., references 9 and 417). Correctly folded T-cell receptor fragments in the periplasm have been obtained by Wülfing and Plückthun (600), who induced the heat shock response at low temperature together with overexpression of DsbA. It was thought that this "shotgun approach" would induce a whole variety of chaperones, including yet undiscovered periplasmic ones (581). It is now clear that besides the signal peptide, other structural features in proteins are involved in membrane transport (55, 87, 108, 333, 346, 356, 466, 542).

Strategies for the improved translocation of proteins to the periplasm include the supply of components involved in protein transport and processing: the overproduction of the signal peptidase I (570), the use of *prlF* mutant strains (525), coexpression of the *prlA4* and *secE* genes (435), coexpression of the *prlF* gene (379), expression of the *pspA* gene (311), and downregulation (375), deletion (9), or nonuse (422) of the β-lactamase gene to avoid the possible overloading of transport mechanisms or competition for processing of signal peptides. The possibility of transport limitations is indicated in the study of Hsiung et al. (263), who observed the intracellular accumulation of a greater amount of human growth hormone precursor after IPTG induction, but no increase in the amount of translocated human growth hormone. In general, the mechanisms governing the translocation of proteins to the periplasm are not clearly understood yet.

Extracellular Secretion

The targeting of synthesized proteins for secretion to the culture medium presents significant advantages (Table 2). Unfortunately, *E. coli* normally secretes very few proteins and the manipulation of the various transport pathways to facilitate secretion of foreign proteins remains a formidable task (50). An understanding of the secretory pathways in *E. coli* is necessary to develop an appreciation of the difficulties involved in protein secretion. Pugsley (449) offers a detailed and excellent account of the secretory pathways in gram-negative bacteria, and Stader and Silhavy (528) examine heterologous protein secretion in a comprehensive and critical review. What follows is a brief summary of the main issues.

The methodological approaches to protein secretion in *E. coli* may be conveniently divided into two categories: (i) the exploitation of existing pathways for "truly" secreted proteins, as defined by rigorous criteria (528), and (ii) the use of signal sequences, fusion partners, permeabilizing proteins, nutrients, or other agents that effect protein secretion as a result of "leakage" or selective and limited permeability of the outer membrane. The first approach offers the advantage of specific secretion of the protein of interest and hence minimum contamination by nontarget proteins. Perhaps the best-known example is the hemolysin gene, which has been used for construction of secreted hybrid proteins (50, 257, 303, 357, 528). Secretion, however, is not a particularly efficient process. The second approach relies on the induction of limited leakage of the outer membrane to cause protein secretion (361, 422, 543). Examples are the use of the *pelB* leader (44), the *ompA* leader (316), the protein A leader (1, 256), the coexpression of bacteriocin release protein (261), the mitomycin-induced bacteriocin release protein along with the addition of glycine to the culture medium (617), and the coexpression of the *kil* gene for membrane permeabilization (296, 309). Some of these studies reported low or no extracellular activity of the cytoplasmic enzyme β-galactosidase, indicating that there was no appreciable cell lysis (13, 316, 617). In general, protein yields were modest.

FUSION PROTEINS

In recent years, there has been a remarkable increase in the sophistication and variety of fusion proteins used for biological research. The utility of fusion proteins spans an ever widening range of applications, and these have been examined in a series of comprehensive and excellent reviews (161, 331, 408, 409, 419, 487, 529, 566, 567). Table 3 includes most of the known fusion moieties. Other studies have addressed the design and engineering of excision sites, the sine qua non of fusion proteins, for the chemical or enzymatic cleavage and removal of fusion partners (78, 162, 163, 409, 419, 567). This section will briefly summarize the use of selected fusion systems that have a direct impact on high level production and, in some cases, secretion of target proteins.

Uhlén and colleagues developed a multifunctional fusion partner based on staphylococcal protein A or synthetic derivatives (Z) thereof. In addition to its utility as an affinity tag for purification (384, 411, 568), the protein A moiety acts as a solubilizing partner to improve folding (477, 478), and the presence of the protein A signal peptide causes the secretion of the gene product to the culture medium (1, 384, 385).

An alternative fusion partner is derived from streptococcal protein G (SPG), a bacterial cell wall protein that has separate binding regions for albumin within the amino-terminal domain and for immunoglobulin G within the carboxyl-terminal domain (157). A minimal albumin-binding domain consisting of 46 amino acid residues derived from SPG (411) was used as an affinity tag for the purification of cDNA-encoded proteins (329). Furthermore, the combination of both protein A and SPG domains (148, 418) formed a tripartite fusion protein, thus providing an additional purification option and further protecting the target protein from proteolytic degradation (230, 393). An interesting and potentially important application of the SPG albumin-binding domain is its ability to stabilize short-lived proteins in the peripheral circulation of mammals, an effect mediated by the binding of the SPG domain to serum albumin, a protein with a long half-life. Studies have demonstrated that the SPG-derived fusion partners enhanced the half-life of human soluble CD4 in mice (420) and reduced the clearance of human soluble complement receptor type 1 in rats (360).

A more elaborate affinity system that uses seven different affinity tags was recently constructed (410). This multipartite system allows the use of a wide variety of conditions for both the binding and elution steps and provides a useful tool for the production, detection, and purification of recombinant proteins.

The linkage of thioredoxin to target proteins dramatically increases the solubility of fusion proteins produced in the *E. coli* cytoplasm and prevents the formation of inclusion bodies (330, 591a). Similarly, the thioredoxin homolog DsbA (26, 27, 216) has been used as a fusion partner to direct the transport of proteins to the periplasm (109).

TABLE 3. Fusion partners and their applications[a]

Fusion partner	Ligand/matrix	Purification conditions
Flag peptide	Anti-Flag monoclonal antibodies, M1, M2	Low calcium, EDTA, glycine
His_6	Ni^{2+}-nitrilotriacetic acid	Imidazole
Glutathione-*S*-transferase	Glutathione-Sepharose	Reduced glutathione
Staphylococcal protein A	Immunoglobulin G-Sepharose	Low pH, IgG-affinity ligand
Streptococcal protein G	Albumin	Low pH, albumin-affinity ligand
Calmodulin	Organic ligands, peptide ligands, DEAE-Sephadex	Low calcium
Thioredoxin	ThioBond resin	Ion exchange
β-Galactosidase	TPEG[b]-Sepharose	Borate
Ubiquitin		
Chloramphenicol acetyltransferase	Chloramphenicol-Sepharose	Chloramphenicol
S-peptide (RNase A, residues 1–20)	S-protein (RNase A, residues 21–124)	Denaturing or nondenaturing conditions
Myosin heavy chain		Differential solubility in low/high salt
DsbA		
Biotin subunit (in vivo biotinylation)		
Avidin	Biotin	Denaturation (urea, heat)
Streptavidin	Biotin	Denaturation (urea, heat)
Strep-tag	Streptavidin	2-Iminobiotin, diaminobiotin
c-myc	Anti-*myc* antibody	
Dihydrofolate reductase	Methotrexate-agarose	Folate buffer
CKS[c]		
Polyarginine	S-Sepharose	NaCl
Polycysteine	Thiopropyl-Sepharose	Dithiothreitol
Polyphenylalanine	Phenyl-Superose	Ethylene glycol
lac repressor	*lac* operator	Lactose analog, DNase, restriction endonuclease
T4 gp55		
Growth hormone N terminus		
Maltose-binding protein	Amylose resin	Maltose
Galactose-binding protein	Galactose-Sepharose	Galactose
Cyclomaltodextrin glucanotransferase	α-Cyclodextrin-agarose	α-Cyclodextrin
Cellulose-binding domain	Cellulose	Water
Hemolysin A, *E. coli*		
λ *c*II protein		
TrpE or TrpLE		
Protein kinase site(s)		
$(AlaTrpTrpPro)_n$		Aqueous two-phase extraction
HAI[d] epitope		
BTag (VP7 protein region of bluetongue virus)	Anti-BTag antibodies	
Green fluorescent protein		

The fusion of genes to the ubiquitin sequence increased the yield of proteins from undetectable to 20% of the total cellular protein (76, 595). Similar results have been obtained by many other workers (reference 319 and references therein). The remarkable increase in protein yield was thought to be due to protection of the target protein from proteolysis, improved folding, and efficient mRNA translation (76). Ubiquitin or the ubiquitin metabolic pathway is absent in prokaryotic organisms. To remove the ubiquitin moiety from fusion proteins, Baker et al. (19) coexpressed the ubiquitin-specific protease Ubp2 in *E. coli*, thus effecting the cotranslational cleavage of ubiquitin from the fusion protein.

MOLECULAR CHAPERONES

It is now well established that the efficient posttranslational folding of proteins, the assembly of polypeptides into oligomeric structures, and the localization of proteins are mediated by specialized proteins termed molecular chaperones (33, 69, 104, 149, 183, 189, 246, 350, 364, 601). The demonstration that efficient production and assembly of prokaryotic ribulose bisphosphate carboxylase in *E. coli* require both GroES and GroEL proteins (208) led to an increasing interest in the use of molecular chaperones for high-level gene expression in *E. coli* (106). The experimental results from the use of chaperones, however, have been inconsistent, and thus far the effects of chaperone coproduction on gene expression in *E. coli* appear to be protein specific (581). For example, although the GroESL plasmids have been disseminated to more that 400 workers, only half of those who used them reported an improvement in gene expression (350a). This is consistent with recent observations that whereas the coproduction of thioredoxin in *E. coli* caused a dramatic increase in the solubility of eight vertebrate proteins, the coproduction of the GroESL chaperones increased the solubilities of only four of those proteins (613). It is also unclear whether the in vivo levels of different chaperone species are limiting under conditions of gene overexpression. For example, Knappik et al. (312) examined the effect of folding catalysts on the production of antibody fragments in the periplasm. Whereas the presence of the disulfide-forming protein DsbA was absolutely required in vivo, its overexpression did not increase the yield of antibody fragments. Wall and Plückthun (581) and Georgiou and Valax (180) revisited the assumptions and expectations behind the

TABLE 3—*Continued*

Detection	Applications	References
Antibody	Purification, detection	63, 259, 313, 446, 540
Antibody	Purification, detection	251, 252, 578, 619
Biochemical assay, antibody	Expression, purification, detection	101, 167, 225, 226, 462, 524
	Expression, purification, detection	411, 477, 568
	Expression, purification, detection	148, 230, 329, 418
Antibody, fluorescent calmodulin ligand	Purification, detection	403
Antibody	Expression, purification	330, 351, 591a
Biochemical assay, antibody	Expression, purification, detection	181, 182, 192, 278, 472, 518, 569
Antibody	Expression	19, 76, 319, 595
	Purification	144, 166, 315, 459
Biochemical assay	Purification, detection	307, 308
	Purification	596
	Expression, purification	109
Labeled biotin	Detection, purification	114, 608
Antibody	Purification, detection	6
	Purification, detection, assay systems	484, 485
	Detection, purification	410, 501
Antibody	Purification, detection	392, 584, 585
	Purification	281
	Expression	146
	Purification, refolding	61, 487, 488, 533, 534
	Purification	436
	Purification	436
	Purification, screening peptide libraries	115, 177, 347, 354, 491
	Expression	215
	Expression	176, 271, 365, 380
	Purification	34, 136, 358
	Purification	555
	Purification	244
	Purification, enzyme immobilization	431, 432
	Secretion into culture medium	303, 357, 528
		398–400
	Expression	267, 611
	In vitro phosphorylation, purification	88, 300
	Purification	318
	Purification, reverse epitope tagging[e]	557
Antibody	Detection, purification	582
UV light	Detection	81,113, 241

[a] In addition to their utility in purification and detection, specific fusion peptides may confer advantages to the target protein during expression, such as increased solubility, protection from proteolysis, improved folding, increased yield, and secretion. These advantages are denoted as Expression in the Applications column. The engineering of specific protease sites in many fusion proteins facilitates the cleavage and removal of the fusion partner(s).

[b] TPEG, *p*-aminophenyl-β-D-thiogalactoside.

[c] CKS, CTP:CMP-3-deoxy-D-manno-octulosonate cytidyltransferase.

[d] HAI, influenza virus hemagglutinin.

[e] Reverse epitope tagging refers to tagging of the chromosomal rather than the plasmid-encoded protein, to avoid the need to remove the fusion partner.

use of chaperones for gene expression and provided detailed and rigorous assessments. This section is a distillation of the take-home lessons.

Normally, protein folding proceeds toward a thermodynamically stable end product (434, 476). Proteins that are drastically destabilized will probably fold incorrectly, even in the presence of chaperones. Thus, the truncation of polypeptides, the production of single domains from multisubunit protein complexes, the lack of formation of disulfide bonds which ordinarily contribute to protein structure (320, 559), or the absence of posttranslational modifications such as glycosylation (116) may make it impossible to attain thermodynamic stability. Moreover, it is now clear that different types of chaperones normally act in concert (69, 327). Therefore, the overproduction of a single chaperone may be ineffective. For example, the overproduction of DnaK alone resulted in plasmid instability which was alleviated by the coproduction of DnaJ (52). Similarly, the coexpression of three chaperone genes in *E. coli* increased the solubility of several kinases (79). In some cases, it may be necessary to coexpress chaperones cloned from the same source as the target protein (105). Still another variable to consider is growth temperature. For example, GroES-GroEL coexpression increased the production of β-galactosidase at 30 but not 37 or 42°C, whereas DnaK and DnaJ were effective at all temperatures tested (180). Finally, the overexpression of chaperones can lead to phenotypic changes, such as cell filamentation, that can be detrimental to cell viability and protein production (52).

Two recent reports have shown that the coexpression of the human (268) or rat (433a) protein disulfide isomerase (PDI) with the target gene enhances the yield of correctly folded protein in the *E. coli* periplasm. Disulfide bond formation in

TABLE 4. Low-usage codons in *E. coli*[a]

Codon(s)	Amino acid
AGA, AGG, CGA, CGG	Arg
UGU, UGC	Cys
GGA, GGG	Gly
AUA	Ile
CUA, CUC	Leu
CCC, CCU, CCA	Pro
UCA, AGU,UCG, UCC	Ser
ACA	Thr

[a] The reported frequency of codon usage varies depending on the author (based on references 293, 580, and 623).

the *E. coli* periplasm is facilitated by a group of proteins that maintain the correct redox potential (26). It is thought that DsbA, a soluble periplasmic protein, directly catalyzes disulfide bond formation in proteins whereas DsbB, an inner membrane protein, is involved in the reoxidation of DsbA (227a). Eukaryotic PDI was capable of complementing the phenotypes of *dsbA* null mutants (268, 433a), but its function was virtually abolished in *dsbB* mutants (433a). In addition, the ability of PDI to enhance the yield of target proteins was increased in the presence of exogenously added glutathione (268, 433a). These observations suggest that PDI depends on the presence of bacterial redox proteins for its reoxidation. The coexpression of rat PDI has also been reported to enhance the correct folding of tissue plasminogen activator (433a).

Protein misfolding can be attributed to the intracellular concentration of aggregation-prone intermediates. Thus, although the subject of this review is the maximization of protein synthesis, reducing the rate of protein synthesis should disfavor protein misfolding. Indeed, the use of weaker promoters or conditions of partial induction from stronger promoters can result in larger amounts of soluble protein (180, 253). Kadokura et al. (291) showed that the ability of *E. coli* mutants to secrete a large amount of alkaline phosphatase into the periplasm was due to a lower synthetic rate of the *phoA* gene product.

CODON USAGE

Genes in both prokaryotes and eukaryotes show a nonrandom usage of synonymous codons (214, 228, 272, 509, 623). The systematic analysis of codon usage patterns in *E. coli* led to the following observations (124). (i) There is a bias for one or two codons for almost all degenerate codon families. (ii) Certain codons are most frequently used by all different genes irrespective of the abundance of the protein; for example, CCG is the preferred triplet encoding proline. (iii) Highly expressed genes exhibit a greater degree of codon bias than do poorly expressed ones. (iv) The frequency of use of synonymous codons usually reflects the abundance of their cognate tRNAs. These observations imply that heterologous genes enriched with codons that are rarely used by *E. coli* (Table 4) may not be expressed efficiently in *E. coli*.

The minor arginine $tRNA^{Arg\ (AGG/AGA)}$ has been shown to be a limiting factor in the bacterial expression of several mammalian genes (62), because the codons AGA and AGG are infrequently used in *E. coli* (91, 95, 214). The coexpression of the *argU* (*dnaY*) gene that codes for $tRNA^{Arg\ (AGG/AGA)}$ (175, 343) resulted in high-level production of the target protein (62). The production of β-galactosidase decreased when AGG codons were inserted before the 10th codon from the initiation codon of the *lacZ* gene (92). Similarly, Goldman et al. (204) reported that translational inhibition of a test mRNA was much stronger in both arginine and leucine cases when the consecutive low-usage codons were located near the 5′ end of the mRNA. Ivanov et al. (280) reported that tandem AGG triplets caused a substantial inhibition of gene expression independent of their localization in mRNA. These workers attributed the inhibitory effect to a competition of the tandem AGGAGG codons with the natural SD sequence. Other studies showed that protein production levels could be increased either by substitution of high-usage codons for low-usage ones (see, e.g., references 3, 70, 135, 145, 248, 262, 383, 452) or by coexpression of the "rare" tRNA gene (62, 126). The expression of the ICP4 gene from herpes simplex virus was shown to be inefficient because of the presence of an almost continuous stretch of 19 serine residues (73). The efficiency of ICP4 synthesis was not improved by silent mutations in this serine-rich region, supplementation of the growth medium with serine, overexpression of seryl-tRNA synthetase, or expression of $tRNA^{Ser5}$. The level of gene expression was inversely proportional to the number of serine codons in this region (73). Although this is certainly an extreme case, it is indicative of the adverse effects of long stretches of similar codons on translational efficiency.

In contrast, other workers reported very efficient expression of genes that contained low-usage codons (see, e.g., references 154, 265, 334, 464, and 616). Similarly, in the case of the human T-cell receptor Vβ5.3 gene that contains 4% AGA/AGG codons, expansion of the intracellular pool of $tRNA^{Arg\ (AGG/AGA)}$ did not significantly increase the amount of Vβ5.3 detected in the cells (9).

The evolutionary significance of codon usage patterns, as well as mechanistic explanations for the effects of codon usage, has been advanced by many workers (74, 92, 124, 155, 204, 245, 276, 293, 463, 474). To date, however, it has not been possible to formulate general and unambiguous "rules" to predict whether the content of low-usage codons in a specific gene might adversely affect the efficiency of its expression in *E. coli*. The experimental results may be confounded by several variables, such as positional effects, the clustering or interspersion of the rarely used codons, the secondary structure of the mRNA, and other effects (204, 293). Nevertheless, from a practical point of view, it is clear that the codon context of specific genes can have adverse effects on both the quantity and quality of protein levels. Usually, this problem can be rectified by the alteration of the codons in question, or by the coexpression of the cognate tRNA genes.

PROTEIN DEGRADATION

Proteolysis is a selective, highly regulated process that plays an important role in cellular physiology (200, 203, 378). *E. coli* contains a large number of proteases that are localized in the cytoplasm, the periplasm, and the inner and outer membranes (25, 199, 201, 212, 367). These proteolytic enzymes participate in a host of metabolic activities, including the selective removal of abnormal proteins (201, 212). Protein damage or alteration may result from a variety of conditions, such as incomplete polypeptides, mutations caused by amino acid substitutions, excessive synthesis of subunits from multimeric complexes, posttranslational damage through oxidation or free-radical attack, and genetic engineering (201). Such abnormal proteins are efficiently removed by the bacterial proteolytic machine. To date, the mechanisms of protein degradation are incompletely understood, and it is unlikely that all proteolytic pathways or enzymes operating in *E. coli* have been identified yet. For example, a new protease associated with the outer membrane

was recently discovered (297) and a fascinating new mechanism for the degradation of abnormal proteins in *E. coli* has just been uncovered (299). Nevertheless, the intense scientific interest in this area has generated new tools and strategies for minimizing the degradation of heterologous proteins in *E. coli*.

Although the precise structural features that impart lability to proteins are not known, some determinants of protein instability have been elucidated. In a series of systematic studies, Varshavsky and colleagues formulated the "N-end rule" that relates the metabolic stability of a protein to its amino-terminal residue (14, 15, 209, 560, 571). Thus, in *E. coli*, N-terminal Arg, Lys, Leu, Phe, Tyr, and Trp conferred 2-min half-lives on a test protein, whereas all the other amino acids except proline conferred more than 10-h half-lives on the same protein (560). As discussed above (see the section on cytoplasmic expression), amino acids with small side chains in the second position of the polypeptide facilitate the methionine aminopeptidase catalyzed removal of the N-terminal methionine (250). Therefore, these studies suggest that Leu in the second position would probably be exposed by the removal of the methionine residue and would destabilize the protein.

The second determinant of protein instability is a specific internal lysine residue located near the amino terminus (14, 15, 86). This residue is the acceptor of a multiubiquitin chain that facilitates protein degradation by a ubiquitin-dependent protease in eukaryotes. Interestingly, in a multisubunit protein, the two determinants can be located on different subunits and still target the protein for processing (287).

Another correlation between amino acid content and protein instability is presented in the PEST hypothesis (461). On the basis of statistical analysis of eukaryotic proteins that have short half-lives, it was proposed that proteins are destabilized by regions enriched in Pro, Glu, Ser, and Thr, flanked by certain amino acid residues. Phosphorylation of these PEST domains leads to increased calcium binding, which in turn facilitates the destruction of the protein by calcium-dependent proteases. It was suggested that PEST-rich proteins may be produced efficiently in *E. coli*, which apparently lacks the PEST proteolytic system (461).

Strategies for minimizing proteolysis of recombinant proteins in *E. coli* have been reviewed in detail (25, 153, 395) and are summarized in Table 2. These include protein targeting to the periplasm (550) or the culture medium (230), the use of protease-deficient host strains (211), growth of the host cells at low temperature (100), construction of N- and/or C-terminal fusion proteins (59, 230, 319, 393), tandem fusion of multiple copies of the target gene (512), coexpression of molecular chaperones (489, 581), coexpression of the T4 *pin* gene (519–521), replacement of specific amino acid residues to eliminate protease cleavage sites (243), modification of the hydrophobicity of the target protein (394), and optimization of fermentation conditions (24, 338).

Although the variety of approaches for protein stabilization attests to the ingenuity of the investigators, the usefulness of some of the above methods may be limited, depending on the intended use of the recombinant protein. Thus, for example, the presence of fusion moieties on the target protein may interfere with functional or structural properties (51) or therapeutic applications of the product. The engineering of enzymatic or chemical cleavage sites for the subsequent removal of the fusion partners is a complex process that involves numerous considerations: the accessibility of the cleavage sites to enzyme digestion; the purity, specificity, and cost of the commercially available enzymes; the authenticity of the N or C termini upon enzymatic digestion; the possible modification of the target protein upon chemical treatment, and so forth (see, e.g., references 78, 162, 419, and 567). For the large-scale production of fusion proteins, some of these difficulties are amplified. Similarly, the fusion of multiple copies of the target gene to create multidomain polypeptides (512) requires the subsequent conversion to monomeric protein units by cyanogen bromide cleavage. In this case, the target protein must not contain internal methionine residues and must be able to withstand harsh reaction conditions. Moreover, a limited extend of amino acid side chain modification may occur, and the toxicity of cyanogen bromide presents a significant issue for large-scale cleavage reactions. Similarly, the rational modification of a protein sequence requires extensive structural information which may not be available. Molecular chaperones have been used successfully to stabilize specific proteins (395), but this approach remains a hit-or-miss affair (581).

The cytoplasm of *E. coli* contains a greater number of proteases than does the periplasm (545, 546). Therefore, proteins located in the periplasm are less likely to be degraded. For example, proinsulin localized to the periplasm was 10-fold more stable that when produced in the cytoplasm (550). However, proteolytic activity in the periplasm is substantial (367). Secretion into the culture medium would provide a better alternative in terms of protein stability. Unfortunately, the technology for secretion of proteins from *E. coli* into the culture medium is still in its infancy (528) (see the section on extracellular secretion, above). A major catalyst of protein degradation in bacteria is the induction of heat shock proteins in response to a variety of stress conditions, such as the thermal induction of gene expression or the accumulation of abnormal or heterologous proteins in the cytoplasm (194). Under these conditions, the production of the *lon* gene product, protease La (195), and other proteases is enhanced. This problem is minimized by the use of host strains deficient in the *rpoH* (*htpR*) locus (201, 211, 421). The *rpoH* gene encodes the RNA polymerase σ^{32} subunit, which regulates several proteolytic activities in *E. coli* (20, 193). Hosts that carry the *rpoH* mutation have been patented (202) and have been demonstrated to dramatically increase the production of foreign proteins in *E. coli* (see, e.g., references 4, 9, 47, 70, and 373). Strain SG21173 (211), which is deficient in proteases La and Clp and the *rpoH* locus, is particularly effective in protein production (9). A large number of protease-deficient hosts exists (see, e.g., references 23 and 211), including some that are deficient in all known protease loci that affect the stability of secreted proteins (372).

Before leaving this section, it is worth repeating a caveat on the use of protease-deficient strains (581): proteolysis may be an effect rather than a cause of folding problems, serving as a disposal system to remove misfolded and aggregated material (238). Therefore, it is possible that the absence of proteases will result in increased toxicity to the host as a result of the accumulation of abnormal proteins.

FERMENTATION CONDITIONS

Protein production in *E. coli* can be increased significantly through the use of high-cell-density culture systems, which can be classified into three groups: batch, fed batch, and continuous. These methods can achieve cell concentrations in excess of 100 g (dry cell weight)/liter and can provide cost-effective production of recombinant proteins. Detailed reviews of large-scale fermentation systems have been published (338, 607, 614). The composition of the cell growth medium must be carefully formulated and monitored, because it may have significant metabolic effects on both the cells and protein production. For example, the translation of different mRNAs is differentially affected by temperature as well as changes in the

culture medium (reference 284 and references therein). Nutrient composition and fermentation variables such as temperature, pH and other parameters can affect proteolytic activity, secretion, and production levels (24, 25, 153, 324, 338, 614). Specific manipulations of the culture medium have been shown to enhance protein release into the medium. Thus, supplementation of the growth medium with glycine enhances the release of periplasmic proteins into the medium without causing significant cell lysis (10, 13). Similarly, growth of cells under osmotic stress in the presence of sorbitol and glycyl betaine causes more than a 400-fold increase in the production of soluble, active protein (49).

High-cell-density culture systems suffer from several drawbacks, including limited availability of dissolved oxygen at high cell density, carbon dioxide levels which can decrease growth rates and stimulate acetate formation, reduction in the mixing efficiency of the fermentor, and heat generation. The techniques that are used to minimize such problems have been examined in detail (338). A major challenge in the production of recombinant protein at high cell density is the accumulation of acetate, a lipophilic agent that is detrimental to cell growth (285, 338, 353). A number of strategies have been developed to reduce acetate formation in high-cell-density cultures, but these suffer from several drawbacks (338). This problem was recently resolved through the metabolic engineering of *E. coli* (11, 12, 479). The *alsS* gene from *B. subtilis* encoding the enzyme acetolactate synthase was introduced into *E. coli* cells. This enzyme catalyzes the conversion of pyruvate to nonacidic and less toxic byproducts. The reduction in acetate accumulation caused a significant improvement in the production of recombinant protein (12, 479). Mutant strains of *E. coli* that are deficient in other enzymes have also been developed and shown to produce less acetate and higher levels of human recombinant proteins (30, 103, 273).

CONCLUSIONS AND FUTURE DIRECTIONS

An efficient prokaryotic expression vector should contain a strong and tightly regulated promoter, an SD site that is positioned approximately 9 bp 5′ to the translation initiation codon and is complementary to the 3′ end of 16S rRNA, and an efficient transcription terminator positioned 3′ to the gene coding sequence. In addition, the vectors require an origin of replication, a selection marker, and a gene that facilitates the stringent regulation of promoter activity. This regulatory element may be integrated either in the vector itself or in the host chromosome. Other elements that may be beneficial include transcriptional and translational "enhancers," as well as "minicistrons" in translationally coupled systems. These may be gene specific; therefore, their utility must be tested case by case. The translational initiation region of a gene must be free of secondary structures that may occlude the initiation codon and/or block ribosome binding. UAAU is the most efficient translation termination sequence in *E. coli*.

There are many different prokaryotic vectors that allow the tight regulation of gene expression. The experimental approaches to achieve tight regulation of promoter activity range from the simple repositioning of the operator in *lac*-based systems to the construction of elaborate "cross-regulation" systems. These vectors are efficient, and each system has its own niche in prokaryotic gene expression. The demonstrated effectiveness of a thermosensitive *lac* repressor now allows the thermal regulation of *lac*-based promoters in lieu of using IPTG.

To date, there is no generally applicable strategy to prevent the degradation of a wide variety of mRNA species in *E. coli*. Although certain 5′ and 3′ stem-loop structures have been shown to block mRNA degradation, these seem to stabilize only specific mRNAs, under restricted conditions. One exception appears to be the 5′ UTR of the *E. coli ompA* transcript, which prolongs the half-life of a number of heterologous mRNAs in *E. coli*. The use of strains deficient in specific RNases has been ineffective for enhanced gene expression.

Each of the four "compartments" for targeted protein production, i.e., the cytoplasm, periplasm, inner and outer membranes, and growth medium, offers advantages and disadvantages for gene expression, depending on the experimental objectives. The formation of inclusion bodies can be minimized by a variety of techniques, but it remains a significant barrier to high-level protein production in the cytoplasm. To date, the effectiveness of molecular chaperones has been protein specific. It is possible that this is due to conditions that prevent the formation of a thermodynamically stable end product, such as the production of severely truncated proteins or single domains from multisubunit protein complexes, lack of formation of disulfide bonds, suboptimal growth conditions, absence of posttranslational modifications, and the normally concerted action of multiple types of chaperones in vivo. Nevertheless, molecular chaperones have been used very successfully for the enhanced production of specific proteins.

The wide variety of existing fusion partners have utility in the production, detection, and purification of recombinant proteins. Specific fusion moieties can increase the folding, solubility, resistance to proteolysis, and secretion of recombinant proteins into the growth medium.

Protein misfolding, attributed to the intracellular concentration of aggregation-prone intermediates, may be minimized by a combination of experimental approaches: replacement of amino acid residues that cause aggregation, coexpression of molecular chaperones and foldases, reduction of the rate of protein synthesis, the use of solubilizing fusion partners, and the careful optimization of growth conditions.

Codon usage can have adverse effects on the synthesis and yield of recombinant proteins. However, the mere presence of "rare" codons in a gene does not necessarily dictate poor translation of that gene. Currently, we do not know all the rules that link codon usage and translation of a transcript. The lack of consistent results in the published literature on codon usage may be due to several variables, such as positional effects, the clustering or interspersion of the rare codons, secondary structure of the mRNA, and other effects. Positional effects appear to play an important role in protein synthesis. Thus, the presence of rare codons near the 5′ end of a transcript probably affects translational efficiency. This problem may be rectified by the alteration of the culprit codons, and/or the coexpression of the cognate tRNA genes.

Much progress has been made in the elucidation of specific determinants of protein degradation in *E. coli*. Effective approaches for the minimization of proteolysis in *E. coli* include the targeting of protein to the periplasm or the culture medium, the use of protease-deficient host strains, the construction of fusion proteins, the coexpression of molecular chaperones, the coexpression of the T4 *pin* gene, the elimination of protease cleavage sites through genetic engineering, and the optimization of fermentation conditions. Host strains that are deficient in the *rpoH* (*htpR*) locus are among the best, particularly for thermally induced expression systems.

Future challenges in the use of *E. coli* for gene expression will involve the following factors. The first is the achievement of enhanced yields of correctly folded proteins by manipulating the molecular chaperone machinery of the cell. Perhaps this might be done by the coexpression of multiple chaperone-

encoding genes or by methods that activate a large battery of chaperone molecules in the cell.

The second is the realization of a "true" and robust secretion mechanism for the efficient release of protein into the culture medium. There are several available systems that facilitate secretion of recombinant proteins into the culture medium. Some of these are based on the use of signal peptides, fusion partners, and permeabilizing agents that cause disruption and limited leakage of the outer membrane. Other efforts are directed at pirating existing secretion pathways that promise greater specificity of secretion. Work in this area will necessitate an improved understanding of the various secretion pathways in *E. coli*.

The third is the endowment of the prokaryotic cell with the ability to perform some of the posttranslational modifications found in eukaryotic proteins, such as glycosylation. This might be done by the engineering of eukaryotic glycosylating enzymes into the *E. coli* chromosome.

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