Technical Brief:

ACES™ Signal Sequence and YebF Expression Systems

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Introduction

E. coli is the host strain of choice for the production of recombinant proteins. Despite its long and successful history, several limitations remain when the desired protein cannot be expressed in a functional state. This is often due to the inherent properties of expressing heterologous proteins in the cytoplasm. One means of overcoming these impediments is to express the protein such that it is secreted into the periplasm or extracellular matrix, where correct folding is likely and proteolytic degradation is minimized. Periplasmic or extracellular protein export has been exploited for the production of a number of recombinant proteins.[1] The extracellular accumulation of a target protein provides several advantages. These include: [1] A simplified downstream purification scheme. *E. coli* does not naturally export a significant amount of proteins to the extracelluar matrix. Therefore, the contamination level of host cell proteins, as well as endotoxin and nucleic acids (contaminants that present considerable challenges to the purification of proteins destined for pharmaceutical use), are significantly reduced. [2] Enhanced biological activity. Protein export through the periplasmic space exposes proteins to a set of disulfide isomerases and foldases. These facilitate correct folding as well as provide an oxidizing environment which favors disulfide bridge formation. [3] Higher product stability and solubility. In addition to the presense of chaperones, there are fewer proteases in the periplasmic space and even fewer in the extracellular matrix. [4] The N-terminus is authentic to the native protein. During the export process the signal sequence is removed, yielding an exported polypeptide without extra N-terminal amino acids.

Protein secretion in *E. coli* and other Gram-negative bacteria is an extensively reviewed subject (see reference 2) and will be only briefly summarized here as it pertains to the export of recombinant proteins. The process of protein secretion is complex. In Gram-negative bacteria secreted proteins must cross two membranes employing one of five different translocation mechanisms. Each mechanism requires the involvement of several proteins. With regard to the use of secretory systems for the production of recombinant proteins, the type I and type II export systems have been most often used and excellent reviews have been published.[1,3]

The type I system is a single-step process where translocation bypasses the periplasmic space. The process requires five proteins,

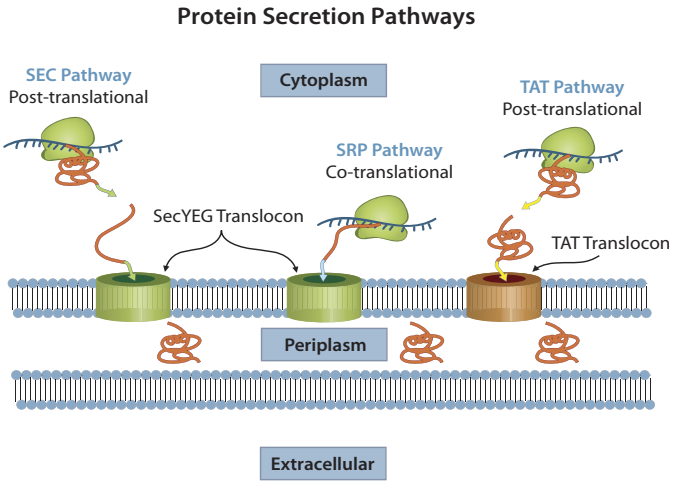


Figure 1. Diagram of the Type II secretion pathways of *E. coli.*

HlyA, HlyB, HlyD, an ABC transporter and TolC. The HlyB/D are inner membrane proteins bound to the ABC transporter. This transporter spans the two membranes and binds two TolC molecules on the outer membrane. The C-terminal portion of HlyA is sufficient to effect translocation through the HlyB/D-ABC-TolC complex and, when fused to a target protein, will direct secretion of the protein to this system. Disulfide bond formation occurs during export. There are three drawbacks to this system: [1] The size of the channel is limited, which restricts the size of the protein that can be translocated to about 200 amino acids,[4] [2] the HlyA sequence remains attached[5] and must be removed during purification and [3] co-expression of the translocation components are needed to increase capacity.

The type II secretion mechanism is a two-step process. Protein translocation through the inner membrane is accomplished by way of one of three pathways: SecB-dependent (SEC), signal recognition particle (SRP) or twin-arginine translocation (TAT) (Fig. 1). Most proteins in *E. coli* are exported to the periplasm via one of these pathways with the larger majority using SEC. Extracellular secretion by a type II mechanism is via the main terminal branch pathway, which involves a poorly defined complex of 12-16 proteins that are not expressed under normal laboratory conditions.[6] Therefore, most of the protein exported by the type II systems remains resident in the periplasm, which is also where most secreted recombinant proteins accumulate.

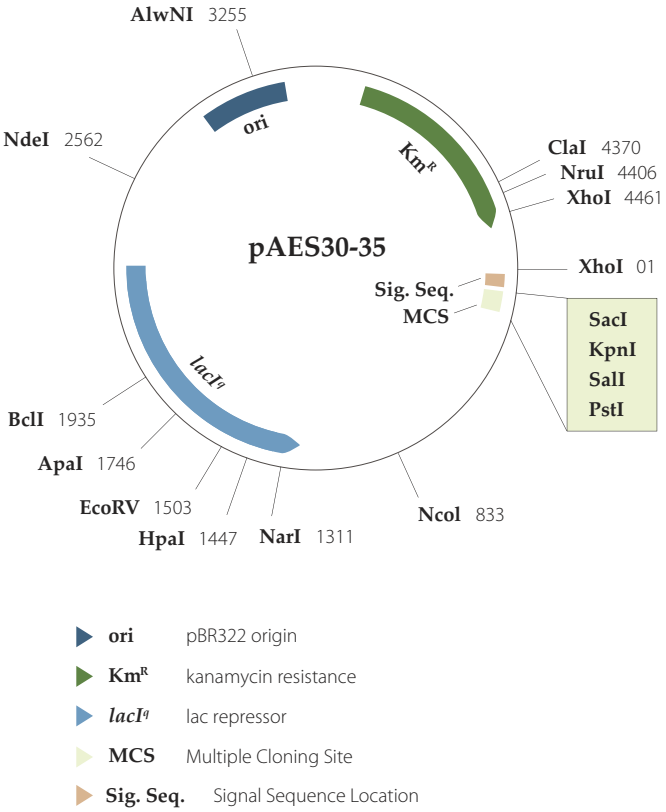
Each of the three pathways employs a different mechanism for translocation. Translocation in the SEC or TAT pathway is via a post-translational mechanism, whereas in the SRP pathway translocation is co-translational. Proteins translocated by the SEC pathway are unfolded prior to export and then refolded in the periplasm. In the TAT pathway, on the other hand, the proteins are translocated in a folded state. Not all proteins will be translocated equally well by any one export mechanism. In some cases a recombinant protein may not be exported at all by a particular pathway. For example, green fluorescent proteins, which cannot be completely unfolded due to covalent bond formation after folding,[7] cannot be exported via the SEC pathway, but can be exported via the TAT pathway.[8] The SRP pathway is the only pathway that can be used to export the DARPins, a class of soluble, fast folding proteins.[9] Thus, the challenge to utilizing protein secretion for the production of a heterologous protein in *E. coli* is the selection of the export pathway that is best suited for that particular protein. This requirement of matching the target protein to the appropriate export pathway to achieve translocation is the basis for Athena's ACES™ Signal Sequence Kit.

Export to the Periplasmic Space: ACES™ Signal Sequence Expression Vectors

Selecting the best export pathway is fundamentally a trial-and-error process. To facilitate the selection of the best export pathway for the secretion of a given recombinant protein, we have constructed a set of six expression vectors, each with a different signal sequence for export via the three type II pathways. The plasmid map for these six vectors is shown in Figure 2. ACES™ Signal Sequence Vectors are used to determine the optimal secretion pathway of a target protein.

Information about the primary or secondary structure of the target protein can be used to narrow down the choice of signal sequence(s) likely to successfully secrete the protein. For example, the SRP pathway is used by *E. coli* primarily for the targeting of inner membrane proteins (IMPs)[10] that belong to the helical bundle class (a highly diverse class regarding size, transmembrane segments and nature of periplasmic and cytoplasmic domains). IMPs are involved in key processes such as energy generation and conversion in the respiratory chain, cell division, signal transduction and transport processes.[11] Therefore, if the target protein resembles any of the IMPs, then it is most likely to be secreted efficiently using the SRP signal sequence. Likewise, if the target protein is known to have fast folding properties, or once folded, difficult to unfold, then the co-translational mechanism might be the best choice.

It has been well-documented that the Sec(B) secretion pathway has been utilized to secrete the vast majority of recombinant proteins. Secretion via this pathway involves 9 different components: Trigger Factor, SecA, SecB, SecY, SecE, SecG, SecD, SecF and YajC. Despite its complexity, this pathway is the one most commonly used for secretion of native proteins in *E. coli*.[1] Ribosome-associated nascent chains of secreted proteins bind Trigger



pAES30	SRP	4,715 bp
AAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAGCGTTTAGCGCATCGGCG		
pAES31	**Sec**	**4,721 bp**
AAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCG		
pAES32	**Sec**	**4,721 bp**
AAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCCTGTGACAAAAGCG		
pAES33	**TAT**	**4,753 bp**
TCACTCAGTCGGCGTCAGTTCATTCAGGCATCGGGGATTGCACTTTGTGCAGGCGCTGTTCCACTGAAGGCCAGCGCAGCAGATCTACTAGT		
pAES34	**TAT**	**4,789 bp**
AACAATAACGATCTCTTTCAGGCATCACGTCGGCGTTTTCTGGCACAACTCGGCGGCTTAACCGTCGCCGGTATGCTGGGTCCGTCATTGTTAACGCCGCGACGTGCGACGGCAGCAGATCTACTAGT		
pAES35	**SRP**	**4,712 bp**
CGCGTACTGCTATTTTTACTTCTTTCCCTTTTCATGTTGCCGGCATTTTCG		

Figure 2. Vector map for the six signal sequence variants.

Factor, which is bound to ribosomes. This association is maintained until the pre-protein leaves the ribosome, thus preventing co-translational binding of nascent chains to SRP components. Proteins to be secreted are kept in a translocation-competent state by the chaperone SecB, which interacts with the mature region of the pre-protein to prevent premature folding.[10] SecY, E and G are components of the translocation channel between the bacterial inner and outer membranes. SecA is involved in the translocation of ATPase. SecD, F and YajC are accessory proteins that aid in

translocation into the periplasm, where proteins are folded into their final confirmation.

The TAT pathway is capable of transporting folded proteins across the inner membrane independently of ATP. This being the case, most of the proteins translocated by the TAT pathway are proteins that bind specific cofactors in the cytoplasm and are folded prior to export. The TAT pathway has been exploited to secrete antibody fragments, oxidoreductase, alkaline phosphatase and green fluorescent protein.[10] However, the TAT pathway is less efficient and slower than the Sec pathway. The largest protein known to be transported by the TAT system is the 142 kDa subcomplex of *E. coli* formate dehydrogenase.

To demonstrate that proteins are exported differentially, the genes encoding alkaline phosphatase (PhoA) from *E. coli* and a streptavidin-*Gaussia* luciferase hybrid protein (SA-Luc) were inserted downstream of the six signal sequences. Each of these proteins is a marker for secretion into the periplasm. PhoA is only enzymatically active when exported to the periplasm[12] and is non-functional if it accumulates in the cytoplasm. Likewise, we have shown that when SA-Luc (a heterologous semi-synthetic protein) is not exported, the protein forms inclusion bodies and does not exhibit biotin binding (streptavidin portion) or luminescent activity (luciferase portion).

Each of the expression vectors carrying PhoA and SA-Luc fused to each of the six signal sequences was introduced into *E. coli* strain DH5α (*phoA* mutant) and expression of the respective protein measured. For PhoA, the cells were cultured on solid medium containing 1 mM IPTG to induce expression and 5-bromo-4-chloro-3-indol-phosphate, a substrate of PhoA, to measure enzyme activity and thus indicate a Pho^+ phenotype. When the PhoA protein was fused to the *phoA* (Sec), *sufI* (TAT) and *torA* (TAT) signal sequences (ss), Pho^+ cells were observed with the *phoA* signal sequence construct clearly yielding a positive result in contrast to a marginal positive signal for the *sufI* and *torA* signals. *ompA*ss (Sec), *dsbA*ss (SRP) and *torT*ss (SRP) did not yield Pho^+ cells indicating a lack of export. Therefore, for PhoA only the native signal sequence (Sec pathway) appeared to yield a high level of protein export. For SA-Luc, cultures were grown from single colonies to late exponential phase in Turbo Broth™ (AthenaES™) and expression induced by the addition of IPTG to a final concentration of 1 mM. After three hours post-induction, the cells were harvested and enzyme activity determined for pre- and post-induction samples. Figure 3 shows the relative increase in luciferase activity after induction. Luciferase activity was significantly higher when either the *sufI*ss (TAT) or *phoA*ss (Sec) was used, but significantly lower when the alternative TAT or Sec, or the two SRP signal sequences were used. Very low levels of luciferase were produced after induction when the protein was expressed from the parent pAES25 expression vector without a signal sequence (<800 RFU). These experiments demonstrated that the type of signal sequence used and, therefore, the protein export pathway to which the recombinant protein is directed, has

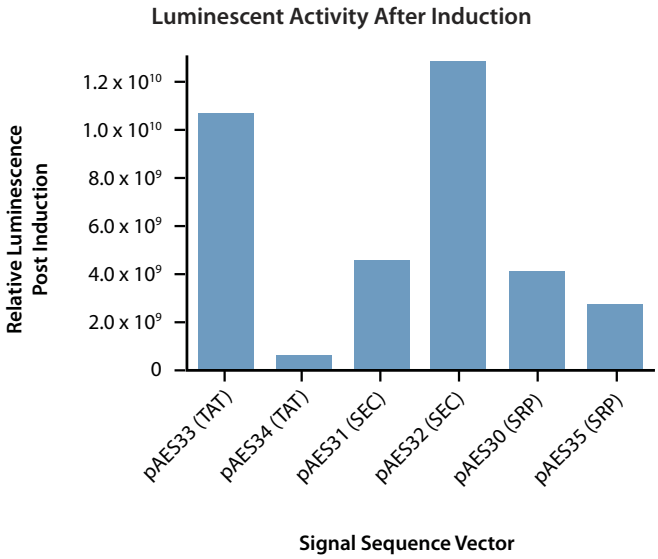


Figure 3. Luminescence was measured in whole cells. The parent plasmid, pAES25 with SA-Luc which does not have a signal sequence, gave 726 RLU post-induction.

a profound effect on the level of target protein that is accumulated.

Extracellular Export: ACES™ YebF Expression System

In spite of the inherent limitation of *E. coli* to export proteins to the culture medium, four basic techniques have been used to export recombinant proteins to the culture medium. One approach is to secrete the proteins to the periplasm and use chemical, biochemical, enzymatic or physical treatment to induce outer membrane leakage resulting in the release of the target protein to the medium. This approach is limited by the lack of export to the periplasm and loss of function after exposure to the treatments. The second approach is to use mutant strains of *E. coli* known as L-forms, which do not have an outer membrane.[13] In these strains, proteins that are exported through the cytoplasmic membrane end up in the culture medium because there is no periplasmic space. The drawback of these mutants is that they are growth impaired and prone to autolysis, which makes them difficult to employ in commercial-scale fermentations.[14] A third technique is to co-express lysis-promoting proteins such as Kil, TolAIII, or the *out* gene proteins with the recombinant target protein.[cited in 1] Finally, attempts have been made to exploit the type I and type II general secretory pathways of *E. coli* and other Gram-negative bacteria by fusing the export signals to the target protein and simultaneously co-expressing those proteins involved in a particular translocation process.[15,16,17] None of these approaches has been widely adopted and, in general, the production levels of the target proteins have been modest with a few noted exceptions.

More recently, the fusion of the target protein to an *E. coli* protein that is transported to the culture medium, has been demon-

strated. In one example, the OmpF protein was used to export β-endorphin.[18,19] OmpF protein is an outer membrane porin that transports small hydrophilic molecules through the membrane. Despite the high level of production achieved using this system (0.33 g/L)[19] the size of the protein that could be exported appeared to be restricted to low mass polypeptides.[18] In addition, OmpF appears in the culture medium only from selected strains that, for unknown reasons, shed the OmpF protein.[19]

Another protein shown to support protein export to the culture medium is YebF.[20] YebF has an unknown function, but is an actual extracellular protein. It effectively transports both small and large prokaryotic and eukaryotic proteins to the extracellular medium in an active form.[20] In this study, the *E. coli* expression vector pMS119 (Ap^R, p*tac*),[21] was used to construct pYebFH_6/MS. This plasmid expresses wild-type YebF protein under the control of an IPTG-inducible promoter and has a C-terminal hexa-His affinity tag. Analysis of the subcellular localization of the YebFH_6 protein after induction showed that the protein accumulated in the culture medium. To demonstrate that YebF could facilitate the export of other proteins, C-terminal fusions were made by inserting the coding sequences for mature alkaline phosphatase (*E. coli phoA*), α-amylase (*Bacillus subtilis* X-23, *amy*) and human IL-2, between the C-terminal residue of YebF and the His tag. After induction all three proteins were found to accumulate in the culture medium, indicating that the YebF protein could effect extracellular transport of the fused protein. Importantly, cytoplasmic proteins did not leak into the medium. Therefore, YebF represents a potentially useful tool for facilitating the extracellular export of recombinant proteins.

We have expanded on the above work of Zhang *et al.*[20] by demonstrating that YebF export function works in several commonly used strains of *E. coli* for the expression of heterologous proteins including HB101, HMS174, BLR and TOP10. *E. coli* strains harboring pYebF-AmyH_6/MS ("YebF-Amy") or pYebF-PhoAH_6/T7 ("YebF-PhoA") were induced with 50 μM IPTG and samples were removed at 0, 3, 8 and 22 hours post-induction. Proteins that accumulated in whole cells and in the culture supernatant were analyzed by immunoblot and enzyme assay. For the immunoblot, the His-tagged proteins were detected using monoclonal anti-His tag antibody. The enzyme assays were performed using cell-free extracts and culture supernatants as described.[20]

Both fusion proteins appeared in the culture medium following induction (Fig. 4). The proteins exhibited a time-dependent increase in export level following induction with IPTG. Their appearance within the cells preceded their accumulation in the medium suggesting a rate-limiting process. The increase in enzyme activity for both fusion proteins paralleled the immunoblot (not shown). The immunoblot showed that the proteins may have undergone a processing event beyond the expected removal of the signaling peptide such that the amino-terminal portion of each YebF was removed (the antibody used was an anti-His tag which is a C-terminal epitope). The basis for this processing

Accumulation of YebF-Amy and YebF-PhoA

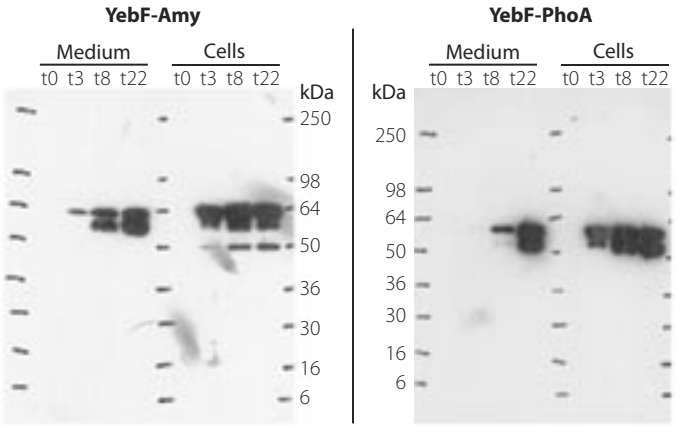


Figure 4. Immunoblot showing the accumulation of YebF-Amy and YebF-PhoA in the culture medium and intracellularly. Equal volumes of medium or whole cell extracts prepared in SDS-PAGE loading dye were loaded onto 4-20% acrylamide gradient gels. The separated proteins were electroblotted to nitrocellulose and reacted with anti-Penta His antibody.

event is unknown, but peptide sequence analysis of the purified proteins revealed the resulting N-termini to be identical.

Accumulation of the fusion proteins in shake flask cultures was 20-50 mg/L; therefore, with a fully optimized fermentation process, production levels could reach well over 100 mg/L. We also observed that strains harboring expression vectors that produce YebF-Amy and YebF-PhoA exhibited a growth impaired phenotype when cultured on medium containing 1 mM but not at 50 μM IPTG. In contrast, a strain expressing a YebF-GFP fusion protein was fully inhibited by 50 μM IPTG. In addition, we subcloned the coding sequences of human GM-CSF and γ-interferon into pYebFH_6/MS and showed that the GM-CSF protein was exported, but that the interferon protein was not. As with the Amy and PhoA fusions, the GM-CSF construct exhibited a growth impaired phenotype when the cultures were induced with high levels of IPTG. These data, taken with the observation of residual target protein in the cell lysates (above and in reference 20), suggests a limitation which prevents full translocation of proteins that are over-expressed.

To determine if the export block of heterologous proteins fused to YebF can be alleviated by using alternative export pathways, we constructed a set of vectors where the wild-type signal sequence of YebF was replaced with alternative signal sequences. The DNA fragment encoding the wild-type *yeb*F, or the mature portion of *yebF,* was subcloned into plasmids pAES25[22] and pAES30 (*dsbA*ss) and pAES31 (*ompA*ss), respectively. To examine expression of YebF, each of the plasmids was introduced into *E. coli* strain TOP10. The resulting strains were then analyzed for their ability to express YebF and export the protein to the culture medium. The expression experiments were performed in 25 ml shake flask cultures at 30°C as described above. After 22 hours post-induction, the accumulation of YebF in the culture me-

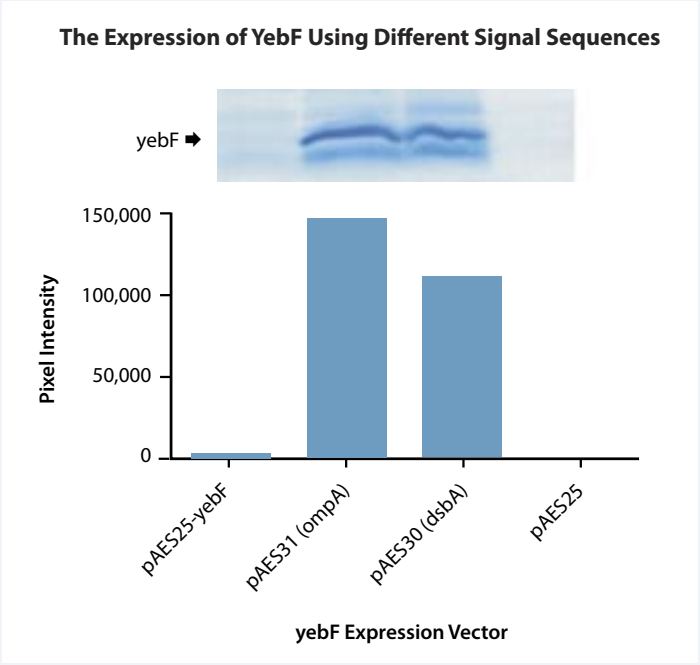


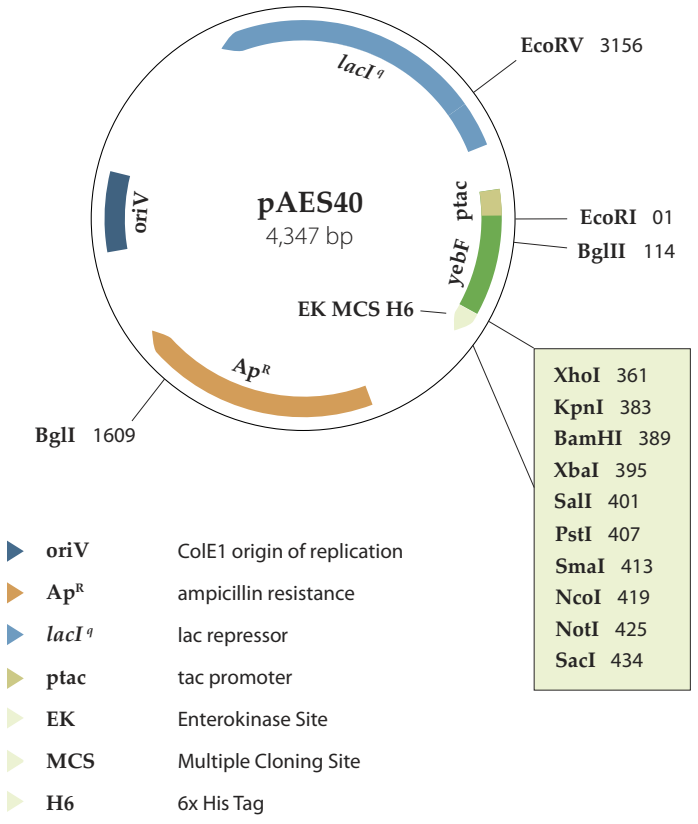
Figure 5. A portion of the stained gel corresponding to the location of YebF is shown above the graph. The x-axis labels correlate to the respective gel lane. The relative level of YebF accumulation was determined from the scanned gel image using TotalLab v2003.02 imaging software.

dium was determined by SDS-PAGE and immunoblot analyses. Figure 5 shows the relative level of YebF accumulation in the medium. This result showed that by exchanging the wild-type signal sequence of YebF for SEC (*ompA*)- or SRP (*dsbA*)-directed signal sequences, the level of YebF accumulation was increased by 46- and 35-fold, respectively. These data suggest that not only is YebF suitable for directing the translocation of recombinant proteins to the culture medium, but that by applying alternative signal sequences (which direct the proteins to the different export pathways) a significant increase in protein accumulation can be achieved.

To facilitate the use of YebF as a carrier protein for the extracellular production of recombinant proteins, we constructed the plasmid pAES40 (Fig. 6). The sequences extending from the *Xho*I to the *Hind*III sites of pYebFH$_6$/MS[20] were replaced with the sequences shown in the diagram. The C-terminal amino acids of YebF are shown in blue with the remainder of the sequence depicting the reading frame of YebF. An enterokinase proteolytic cleavage site was placed between the multiple cloning site (MCS) and the end of YebF to permit removal of the YebF sequences after export. A hexa-His sequence was placed at the end of the MCS to provide an affinity tag, if needed.

Conclusions

There have been many developments in recombinant protein secretion in *E. coli* over the past several years. Periplasmic secretion has been shown to be beneficial in the production of many recombinant proteins due to a higher stability of the gene product, correct folding, and facilitated downstream processing.



XhoI	EK Site			KpnI	BamHI	XbaI	SalI	PstI
CTC GAG	GAC GAT GAC GAT AAG			GGT ACC	GGA TCC	TCT AGA	GTC GAC	CTG CAG
SmaI	**NcoI**	**NotI**	**SacI**	**6xHis**				**Stop**
CCC GGG	CCA TGG	GCG GCC	GCA GAG CTC	CAC CAC CAC CAC CAC CAC				TAA

Blue - C-terminal amino acids of YebF
Orange - Enterokinase cleavage site

Figure 6. Plasmid map for the YebF export vector pAES40.

Each of the different secretory pathways has its advantages and drawbacks and, in the end, successful recombinant protein secretion will depend upon the individual protein to be produced.

From a commercial perspective, the export of target proteins to the culture medium has a benefit on several levels. In *E. coli* production systems there is a significantly lower level of cell contaminants, endotoxin, host cell proteins and nucleic acids, making purification easier thereby lowering production cost and processing duration. Further, it may be possible to produce proteins using this system that might otherwise not be expressed due to toxicity and folding errors. It is well known that for some mammalian proteins the reducing environment of the bacterial cytoplasm is not conducive to disulfide bond formation, whereas the oxidizing environment of the periplasm and culture medium are. It is for this reason that many researchers have attempted to secrete heterologous proteins. In addition, the system is adaptable to high throughput protein production and could be readily employed in automated systems.

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