Appl Microbiol Biotechnol (2004) 64: 625–635
DOI 10.1007/s00253-004-1559-9

MINI-REVIEW

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Secretory and extracellular production of recombinant proteins using *Escherichia coli*

Received: 25 September 2003 / Revised: 25 December 2003 / Accepted: 30 December 2003 / Published online: 14 February 2004

Abstract *Escherichia coli* is one of the most widely used hosts for the production of recombinant proteins. However, there are often problems in recovering substantial yields of correctly folded proteins. One approach to solve these problems is to have recombinant proteins secreted into the periplasmic space or culture medium. The secretory production of recombinant proteins has several advantages, such as simplicity of purification, avoidance of protease attack and N-terminal Met extension, and a better chance of correct protein folding. In addition to the well-established Sec system, the twin-arginine translocation (TAT) system has recently been employed for the efficient secretion of folded proteins. Various strategies for the extracellular production of recombinant proteins have also been developed. For the secretory production of complex proteins, periplasmic chaperones and protease can be manipulated to improve the yields of secreted proteins. This review discusses recent advances in secretory and extracellular production of recombinant proteins using *E. coli*.

Introduction

Escherichia coli has been the "workhorse" for the production of recombinant proteins as it is the best-characterized host with many available expression systems (Lee 1996; Makrides 1996). However, *E. coli* cannot produce some proteins containing complex disulfide bonds, or mammalian proteins that require post-translational modification for activity. Nevertheless, many recombinant proteins have been successfully produced using *E. coli*. Overexpressed proteins are often produced in the form of inclusion bodies, from which biologically active proteins can only be recovered by complicated and costly denaturation and refolding processes. Furthermore, the final yields of these soluble refolded proteins are usually very low, due mainly to protein aggregation resulting from interactions between the hydrophobic regions of the proteins.

A variety of techniques have been developed to solve these problems, including the use of different promoters to regulate the level of expression, the use of different host strains, co-expression of chaperones, reduction of culture temperature, and secretion of proteins into the periplasm or culture medium. In this review, we discuss strategies for secretory and extracellular production of recombinant proteins using *E. coli*.

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Secretory production of recombinant proteins using *E. coli*

Secretory production of recombinant proteins provides several advantages compared to cytosolic production. For example, the N-terminal amino acid residue of the secreted product can be identical to the natural gene product after cleavage of the signal sequence by a specific signal peptidase. Also, there appears to be much less protease activity in the periplasmic space than in the cytoplasm. In addition, recombinant protein purification is simpler due to fewer contaminating proteins in the periplasm. Another advantage is that correct formation of disulfide bonds can be facilitated because the periplasmic space provides a more oxidative environment than the cytoplasm (Makrides 1996). Furthermore, it has recently been shown that secreted proteins can be applied in in-vivo activity assays as secretion gives the expressed protein (enzyme) greater

access to the substrate. Secreted proteins can also be used to screen protein libraries (Chen et al. 2001; Sroga and Dordick 2002).

In gram-negative bacteria, at least three different types of protein secretion systems have been described—type I, type II, and type III (Pugsley 1993), with type II being the most widely used. The type II system involves a two-step process in which a premature protein containing a signal sequence is exported to the periplasmic space using the Sec pathway and processed into a mature protein. Although heterologous proteins are often exported to the periplasm by the common Sec system, extracellular secretion of target proteins depends on the characteristics of signal sequences and proteins.

Generally, proteins found in the outer membrane or periplasmic space are synthesized in the cytoplasm as premature proteins. These premature proteins contain a short (15–30) specific amino acid sequence (signal sequence) that allows proteins to be exported outside the cytoplasm. A number of signal sequences have been used for efficient secretory production of recombinant proteins in *E. coli*, including PelB, OmpA, PhoA, endoxylanase, and StII. Although sequence diversity exists among these signal sequences, some common structural features have been identified. Table 1 shows the representative signal sequences that have been used for the secretory production of recombinant proteins in *E. coli*. Typical signal sequences are composed of a hydrophobic H-domain of about 10–20 amino acids that can be preceded by a short, positively charged N-domain of about two to ten amino acids (Fig. 1, Table 1). In general, signal sequences are rich in hydrophobic amino acids, such as alanine, valine, and leucine, a feature essential for secretion of the proteins into the periplasm of *E. coli* (Pugsley 1993). During transport of proteins out of the cytoplasm, the signal sequence is cleaved by signal peptidase to yield a mature protein product. The cleavage site (the C-domain) is usually less hydrophobic, contains a signal recognized by the signal peptidase, and conforms to the −3, 1 rule (Pugsley 1993)—that is, the residue at position −1 must have a small neutral side-chain, as is the case for alanine, glycine, and serine, and this also holds true for the residue at position −3. As can be seen from Table 1, alanine is most frequently found at the −1 and −3 positions, forming the so-called Ala-X-Ala box, which is recognized and cleaved by signal peptidase I. The secondary structure at the cleavage junction of preproteins also plays an important role in determining the cleavage site and protein processing (Pratap and Dikshit 1998). Thus, the selection of an optimal signal sequence is important for efficient secretory production of recombinant proteins. As can be seen from Table 2, the efficiency of protein secretion varies depending on the host strain, signal sequence, and the type of protein to be secreted. To date, there is no general rule in selecting a proper signal sequence for a given recombinant protein to guarantee its successful secretion. Several signal peptides, such as those listed in Table 1, must be examined in a trial-and-error type approach.

One of the advantages of secretory protein production is that the authentic N-terminal amino acid sequence without the Met extension can be obtained after cleavage by the signal peptidase. However, this can be achieved only when the gene of interest is correctly fused to the cleavage site. Choi et al. (2000) reported on the use of a new signal sequence cloned from the *Bacillus* sp. endoxylanase gene (Jeong et al. 1998) for the secretory production of recombinant proteins. Within the signal peptidase cleavage site (A-S-A) of the endoxylanase signal sequence, there is a*Pst*I site that can be used for directly cloning the gene encoding the mature protein of interest. Upon cleavage by the signal peptidase, the mature protein can be produced without the need to change any amino acid sequence. Therefore, the endoxylanase signal sequence allows convenient cloning of genes encoding recombinant proteins for secretory production without changing either the sequence itself or the sequence of the mature protein. This is an important feature of the endoxylanase signal peptide as other signal sequences often cannot be used without changing the mature protein sequence. Using the endoxylanase signal sequence and the inducible *trc* promoter, *Bacillus* sp. endoxylanase and *E. coli* alkaline

Table 1 Representative signal sequences used for the secretory production of recombinant proteins in *Escherichia coli*. The signal sequence is composed of N-, H- and C-domains. The N-domains of signal sequences are shown in *bold* while the C-domains are *underlined*

Signal sequences	Amino acid sequences
PelB (pectate lyase B) from *Erwinia carotovora*	**MKY**LLPTAAAGLLLLAAQPAMA
OmpA (outer-membrane protein A)	**MKKT**AIAIAVALAGFATVAQA
StII (heat-stable enterotoxin 2)	**MKK**NIAFLLASMFVFSIATNAYA
Endoxylanase from *Bacillus* sp.	**MFKFKKK**FLVGLTAAFMSISMFSATASA
PhoA (alkaline phosphatase)	**MKQS**TIALALLPLLFTPVTKA
OmpF (outer-membrane protein F)	**MMKR**NILAVIVPALLVAGTANA
PhoE (outer-membrane pore protein E)	**MKKS**TLALVVMGIVASASVQA
MalE (maltose-binding protein)	**MKIKT**GARILALSALTTMMFSASALA
OmpC (outer-membrane protein C)	**MKVK**VLSLLVPALLVAGAANA
Lpp (murein lipoprotein)	**MKATK**LVLGAVILGSTLLAG
LamB (λ receptor protein)	**MMITLRK**LPLAVAVAAGVMSAQAMA
OmpT (protease VII)	**MRAK**LLGIVLTTPIAISSFA
LTB (heat-labile enterotoxin subunit B)	**MNKVK**CYVLFTALLSSLYAHG

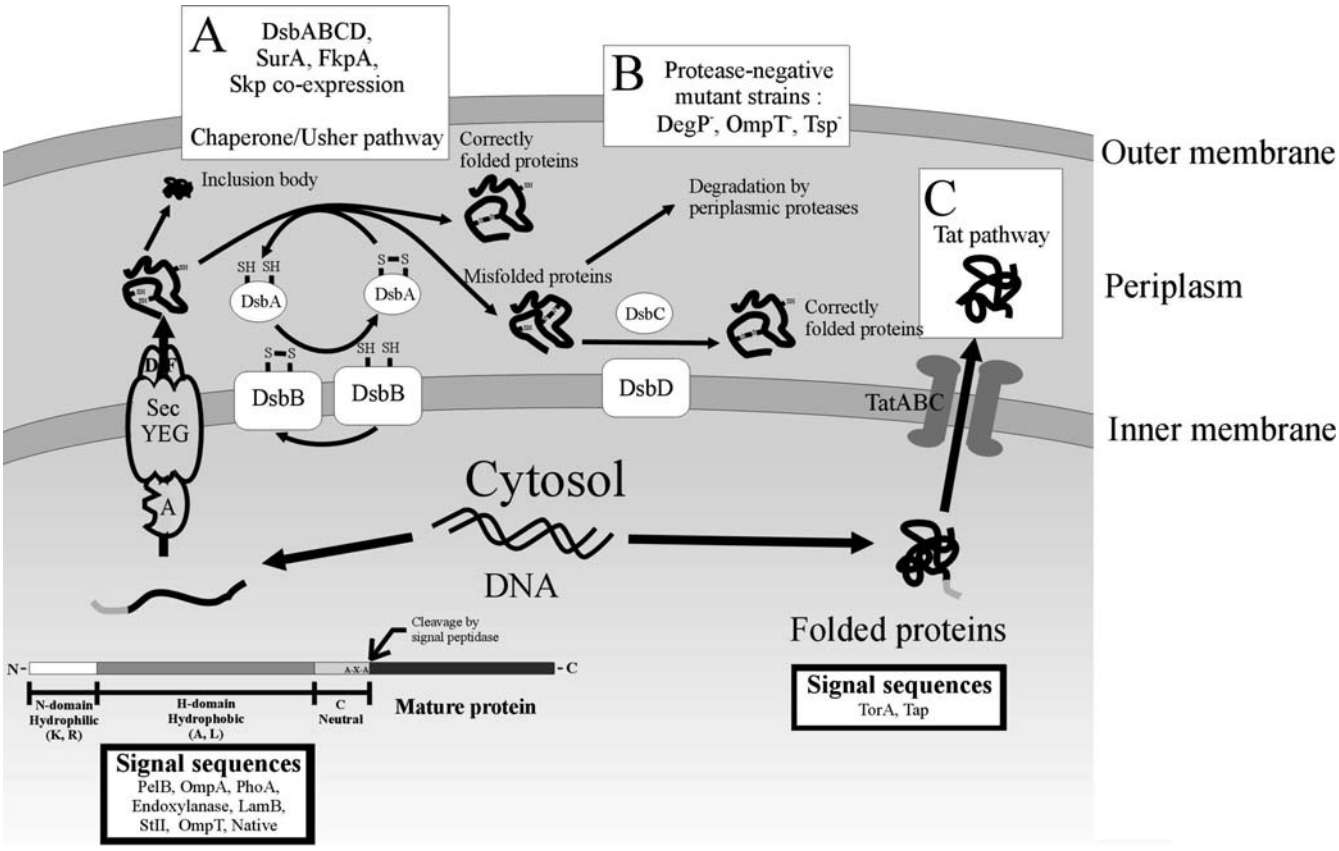

Fig. 1A–C Strategies for the secretory production of recombinant proteins in *Escherichia coli.* The Sec system, the twin-arginine translocation (TAT) system, and the strategies for enhancing secretory protein production using periplasmic chaperones and protease-negative mutants are shown.**A** The co-expression of periplasmic chaperones, such as disulfide-bond formation (Dsb) family proteins, SurA, FkpA, and Skp, can improve the efficiencies of secretory production and protein folding (Arie et al. 2001; Bothmann and Pluckthun 1998, 2000; Jeong and Lee 2000; Kurokawa et al. 2001; Lazar and Kolter 1996; Qiu et al. 1998; Wulfing and Rappouoli 1997; Zavialov et al. 2001).**B** Protease-negative mutant strains can improve secretory production of recombinant proteins by reducing proteolysis (Park et al. 1999; Wulfing and Rappuoli 1997).**C** A novel TAT system can directly secrete the folded proteins (Angelini et al. 2001; Barrett et al. 2003; Santini et al. 2001; Thomas et al. 2001)

phosphatase could be secreted into the *E. coli* periplasm. Furthermore, human leptin and granulocyte-colony stimulating factor (G-CSF) were also efficiently secreted into the *E. coli* periplasm using this sequence (Jeong and Lee 2000, 2001; Yim et al. 2001).

More recently, a novel twin-arginine translocation (TAT) system was discovered. The TAT system is Sec independent and is capable of secreting folded proteins by employing a particular signal peptide containing a twin-arginine sequence. Green fluorescent protein (GFP) fused to the twin-arginine signal peptide of trimethylamine-*N*-oxide (TMAO) reductase (TorA), and alkaline phosphatase (Tap) from *Thermus thermophilus,*which also uses the TAT system, were successfully secreted into the periplasm of *E. coli* (Angelini et al. 2001; Barrett et al. 2003; Santini et al. 2001; Thomas et al. 2001). Since the TAT system allows secretion of proteins already folded in the *E. coli* cytosol, it may have advantages over the Sec system, particularly for those proteins that may be folded before they can reach the Sec machinery or that contain complex disulfide bonds.

Despite many successful examples, such as those described above, secretory production of heterologous proteins in*E. coli* remains problematic. Obstacles include: (1) incomplete processing of signal sequences, (2) variable secretion efficiency depending on the characteristics of the proteins, (3) low or undetectable amounts of recombinant protein secretion, (4) formation of inclusion bodies in the cytosol and periplasm when using strong promoters, and (5) incorrect formation of disulfide bonds (Chung et al. 1998; Jeong and Lee 2000; Lucic et al. 1998; Pritchard et al. 1997; Wong et al. 2003). The first three problems have been solved using a trial-and-error type approach; different promoters, signal sequences, and host strains were examined under various culture conditions (e.g. temperature). The third problem might be due to periplasmic proteolysis rather than poor secretion machinery. In this case, a host strain deficient in periplasmic proteases can be used (see below). The fourth and fifth problems have been solved by manipulating periplasmic chaperones, as shown below. In addition, the use of the TAT system may be a good alternative to solve some of these problems.

Table 2 Representative secretory production of recombinant proteins in *E. coli*

Model proteins	Signal sequences	Hosts	Characteristics	References
Staphylokinase (sak)	OmpA	*E. coli* JM109	15 μg/ml	Lee et al. 1998
Human leptin	Endoxylanase	*E. coli* BL21(DE3)	41% of total proteins, DsbA coexpression	Jeong and Lee 2000
Staphylokinase (sak)	Native, PelB, LamB, MelE, OmpA	*E. coli*	140 IU/ml (periplasm), 530 IU/ml (extracellular)	Pratap and Dikshit 1998
Green fluorescent protein (GFP)	TorA (TMAO reductase)	*E. coli* MC4100	TAT pathway	Barrett et al. 2003; Santini et al. 2001; Thomas et al. 2001
Human interleukin-2 receptor α-chain (hIL-2Rα)	StII	*E. coli* DH5α		Dracheva et al. 1995
Arthrobacter levan fructotransferase	LacZ derived secretion motif	*E. coli* JM109	2 g/l	Lee et al. 2001
Mutated heat-labile enterotoxin	Native	*E. coli* KS476 (*degP*)	DsbA coexpression, DegP knock-out strain	Wulfing and Rappouli 1997
Thermoalkaliphilic lipase	Native, OmpA	*E. coli* JM105	660000 U/ml	Rua et al. 1998
Cholera toxin, and B subunit	Heat-labile enterotoxin LTIIb	*E. coli* TX1	190 mg/ml	Jobling et al. 1997
Alkaline phosphatase	Endoxylanase	*E. coli* HB101	5.2 g/l	Choi et al. 2000
Human interleukin-1β-caf1 fusion protein	Caf1	*E. coli* JM105	Chaperone/usher pathway	Zavialov et al. 2001
Mouse endostatin	PhoA	*E. coli* DH5α	40 mg/l	Xu et al. 2002
Subtilisin E	Gene III	*E. coli* TOP10	Cell-based kinetic assay	Sroga and Dordick 2002
Penicillin G acylase	Native (IB)	*E. coli* DH5α		Sriubolmas et al. 1997
Human granulocyte colony-stimulating factor (GCSF)	Endoxylanase	*E. coli* BL21(DE3)	22% of total proteins	Jeong and Lee 2001; Yim et al. 2001
Protein A-β-lactamase	Protein A	*E. coli* HM114	112500 U/l	Park et al. 1999
Hirudin III	L-Asparaginase II	*E. coli* JM105	60 mg/l	Tan et al. 2002
Protein A-PhoA fusion protein	PelB	*E. coli* BL21(DE3)		Chowdhury et al. 1994
Penicillin acylase (PAC)	Native	*E. coli* HB101	DegP coexpression	Lin et al. 2001a
Human cytochrome P4501A1	PhoA	*E coli* TB1	4500 nmol/l	Kaderbhai et al. 2000
Manduca diuresin	OmpA	*E. coli* JM101	Proteolysis	Wong et al. 2003
scFv antibody	OmpA	*E. coli* JM109	Bacteria L form	Rippmann et al. 1998
Pullulanase (PulA)	Naïve	*E. coli* K12	dsbA effect	Pugsley et al. 2001
Human proinsulin	SpA	*E. coli* AF1000	4.6 mg/l,*uspA* and *uspB* promoter	Mergulhao et al. 2003
20-kDa human growth hormone	*npr* (neutral protease gene), ompA	*E. coli* W3110	76 mg/l, glutathione reductase coexpression	Uchida et al. 1997
Human grannulocyte-colony stimulating factor (hGCSF)	PelB	*E. coli* BL21(DE3)	Not secreted	Chung et al. 1998
Human, mouse leptin	OmpA	*E. coli* MC1061	1.46 mg/ml	Guisez et al. 1998
Peptide:*N*-glycosidase F (PNGase F)	OmpA	*E. coli* BL21(DE3)	8 mg/l	Loo et al. 2002

Table 2 (continued)

Model proteins	Signal sequences	Hosts	Characteristics	References
Thermus thermophilus alkaline phosphatase (Tap)	Native	*E. coli* CC118	TAT pathway	Angelini et al. 2001
βhCG (chorionic gonadotropin)	LTB (heat labile enterotoxin)	*E. coli* DH5α		Pillai et al. 1996
Human proinsulin	DabA fusion	*E. coli* RB791	9.2 mg/g, Arg and ethanol addition	Winter et al. 2000
Alkaline phosphatase	Degenerated PelB	*E. coli* W3110		Le Calvez et al. 1996
Tn *phoA*	SpaA fusion at different site	*E. coli* CC202		Holt and Raju 2000
Human nerve growth factor β	OmpT	*E. coli* JM109	Dsb protein coexpression	Kurokawa et al. 2001
Human tissue plasminogen activator	StII	*E. coli* SF110	Dsb protein coexpression	Qiu et al. 1998

Enhancement of secretion efficiency by manipulating periplasmic chaperones

Many proteins contain disulfide bonds that need to be correctly formed to be functional. Some proteins containing disulfide bonds can be produced in active forms in the periplasm or extracellular medium using the secretion system. However, production of large and complex recombinant proteins in the *E. coli* periplasm can be limited by low secretion levels and folding problems, leading to periplasmic inclusion bodies. Many periplasmic chaperones have been characterized, and subsequently used for the efficient secretion of recombinant proteins. These proteins assist in correct folding of secreted proteins and prevent the formation of periplasmic inclusion bodies. Strategies for enhancing secretion efficiency of recombinant proteins in *E. coli* are presented in Fig. 1.

Large and complex proteins from mammalian cells frequently contain disulfide bonds which contribute to their stability and, in many cases, are essential for their catalytic activities. However, the *E. coli* cytosol is a rather reduced environment, and thus disulfide bonds are not normally formed. The enzymes that catalyze disulfide bond formation play key roles in folding many secreted proteins. For example, the Dsb (disulfide-bond formation) family of proteins catalyzes both the formation of new disulfide bonds and the rearrangement of existing ones. Dsb proteins contain one or more highly conserved thioredoxin-like motifs (C-X-X-C) which are important for disulfide oxidoreductase activity. DsbA and DsbB are oxidoreductases that allow the formation of disulfide bonds (Fig. 1). Subsequent rearrangement of the newly formed disulfide bonds is sometimes necessary since they can be formed among incorrectly paired cysteines, trapping substrate proteins in a misfolded conformation. Normally, misfolded proteins in the periplasm do not readily accumulate, being rapidly degraded by DegP protease. Disulfide-bond rearrangement is catalyzed by two periplasmic disulfide bond isomerases, DsbC and DsbD (Fig. 1). Recent studies revealed that the overexpression of Dsb proteins increased secretion efficiency, folding, and the solubility of recombinant proteins in the periplasmic space (Jeong and Lee 2000; Kurokawa et al. 2001; Qiu et al. 1998; Wulfing and Rappouoli 1997).

SurA, FkpA, and Skp are another set of folding catalysts and chaperones in the periplasmic space (Bothmann and Pluckthun 2000; Missiakas et al. 1996). SurA, which was identified in *E. coli* during starvation survival, shares sequence similarity with parvulin, a cytoplasmic peptidyl prolyl isomerase (PPI) in *E. coli.* Lazar and Kolter (1996) demonstrated that SurA assists in periplasmic folding of three outer-membrane proteins (OmpA, OmpF, and LamB) and of some other secreted proteins. SurA promotes folding of several otherwise unstable proteins (e.g. Protein A-β-lactamase hybrid protein) and proteins prone to aggregation. FkpA, which is a heat-shock periplasmic peptidylprolyl *cis*/*trans* isomerase, was shown to suppress the formation of modified maltose-binding protein (MalE31) inclusion bodies and to enhance

periplasmic expression and folding efficiency of functional antibody fragments in *E. coli* (Arie et al. 2001; Bothmann and Pluckthun 2000). Skp is also a periplasmic chaperone and it appears to improve phage-antibody display and periplasmic expression (Bothmann and Pluckthun 1998).

Sometimes, efficient protein secretion is hindered due to the degradation of target proteins by cell-envelope proteases, such as DegP, OmpT, protease III, and Tsp. Strategies to minimize proteolysis include reducing cultivation temperatures and using protease-negative mutant strains (Park et al. 1999; Wulfing and Rappuoli 1997).

Zavialov et al. (2001) demonstrated another novel approach using the chaperone/usher pathway for the secretion of human interleukin-1β and F1 antigen (Caf1) fusion protein into the periplasmic space. In this system, the Caf1M chaperone assisted periplasmic folding and enhanced chimeric protein solubilization.

Extracellular production of recombinant proteins

Extracellular production of recombinant proteins has several advantages over secretion into the periplasm (Shokri et al. 2003). Extracellular production does not require outer-membrane disruption to recover target proteins, and, therefore, it avoids intracellular proteolysis by periplasmic proteases and allows continuous production of recombinant proteins. Based on these advantages, various strategies have been developed in *E. coli* for the extracellular production of recombinant proteins (Fig. 2, Table 3).

A number of methods have been applied to promote extracellular secretion of recombinant proteins from *E. coli*. These include the use of biochemicals, physical methods (osmotic shock, freezing and thawing), lysozyme treatment, and chloroform shock. However, these methods can be applied only after harvesting cells. *E. coli* normally does not secrete proteins extracellularly except for a few classes of proteins such as toxins and hemolysin. Secreted proteins can leak from the periplasmic space into the

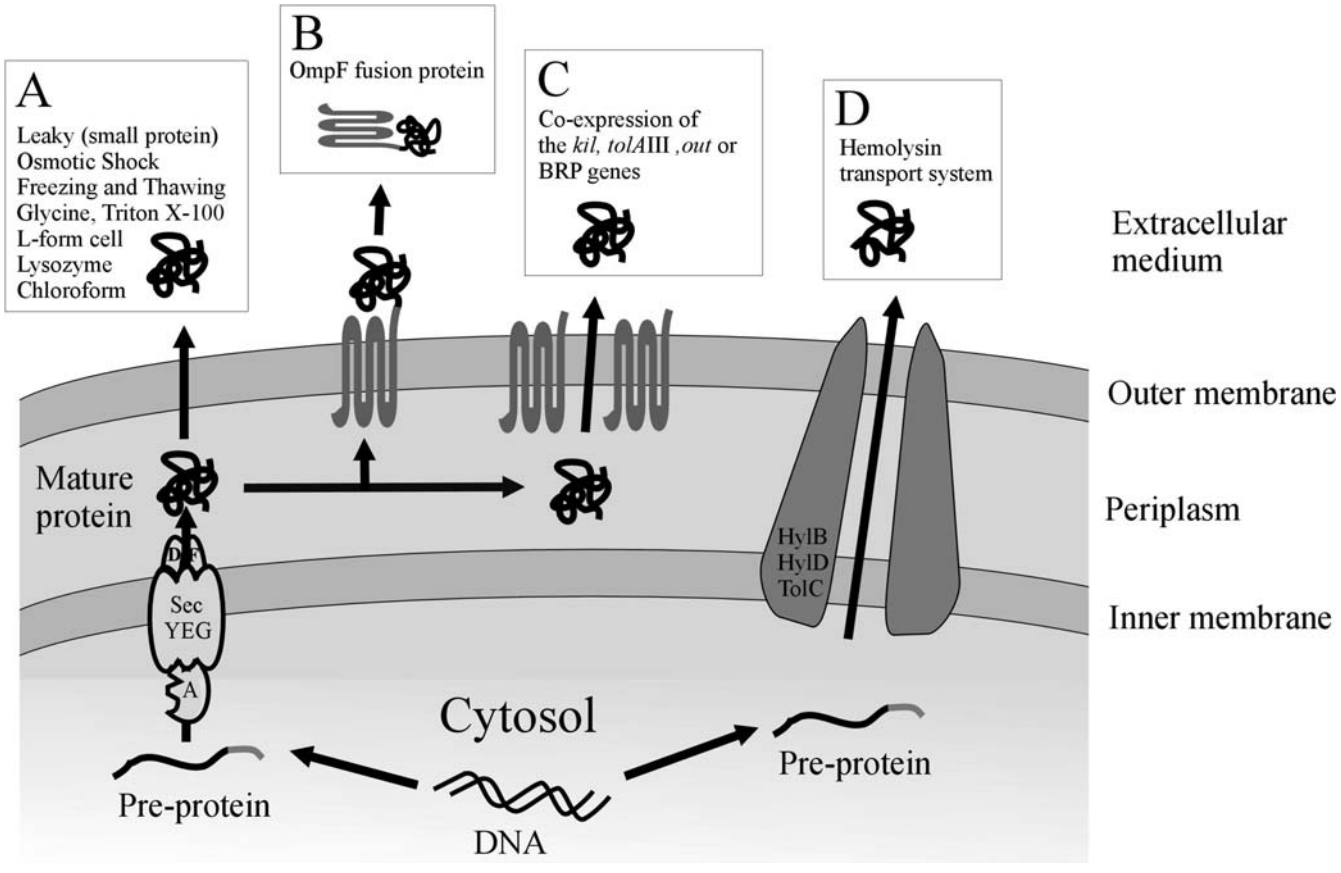

Fig. 2A–D Strategies for the extracellular production of recombinant proteins by *E. coli*. **A** Recombinant proteins can be excreted into the culture medium by treating cells with various agents or by using L-form cells (Jang et al. 1999; Kaderbhai et al. 1997; Kujau et al. 1998; Yang et al. 1998).**B** Recombinant protein fused to outer-membrane protein F (OmpF) of *E. coli* can be excreted into the culture medium (Jeong and Lee 2002; Nagahari et al. 1985). **C** Proteins secreted into the *E. coli* periplasm can also be released into the culture medium by co-expression of *kil*, *out* genes, the gene encoding the third topological domain of the transmembrane protein TolA (TolAIII), or the bacteriocin-release protein gene (Fu et al. 2003; Kleist et al. 2003; Lin et al. 2001b; Miksch et al. 1997; 2002; Robbens et al. 1995; van der Wal et al. 1995, 1998; Wan and Baneyx 1998; Zhou et al. 1999).**D** The target protein fused to the C-terminal hemolysin secretion signal can be directly excreted into the culture medium through the hemolysin transport system (Fernandez et al. 2000; Li et al. 2002)

Table 3 Representative extracellular production of recombinant proteins in *E. coli*

Model proteins	Signal sequences	Hosts	Characteristics	References
Levansucrase, β-lactamase	Native	*E. coli* DH5α	5% of total proteins, glycine supplement	Jang et al. 1999
Winter flounder antifreeze	OmpA	*E. coli* JM105	16 mg/l	Tong et al. 2000
Human β-endorphin	OmpF hybrid	*E. coli* BL21(DE3)	Hybrid proteins in medium	Jeong and Lee 2002
Exoglucanase	OmpA	*E. coli* JM101	143 U/ml	Lam et al. 1997
Human epidermal growth factor (hEGF)	OmpA	*E. coli* JM101	325 mg/l	Sivakesava et al. 1999
Taq I restriction endonuclease	MBP hybrid	*E. coli* XL1-Blue	30000 U/l	Toksoy et al. 2002
Pectate lyase	PelB	*E. coli* BL21(DE3)	2200 U/ml	Matsumoto et al. 2002
Fungal ribotoxin α-sarcin	OmpA	*E. coli* BL21(DE3)	3 μg/ml	Rathore et al. 1997
Fimbrial molecular chaperone FaeE	Native	*E. coli* C600	21 mg/l, bacteriocin-release protein	van der Wal et al. 1995
Fimbrial molecular chaperone FaeE	Native	*E. coli* C600	Modified bacteriocin-release protein	van der Wal et al. 1998
Penicillin acylase	Native	*E. coli* HB101, MDΔP7	Bacteriocin-release protein	Lin et al. 2001b
β-Glucanase	Native	*E. coli* JM109	150 U/ml,*kil* coexpression	Miksch et al. 1997
sFV/TGF-α	OmpA,	*E. coli* K802	170-fold increased, glycine, Triton X-100	Yang et al. 1998
scFv Antibody		*E. coli* HB2151	Hemolysin system	Fernandez et al. 2000
Phytase	Native	*E. coli* BL21(DE3)	50 U/ml, *kil* coexpressioin	Miksch et al. 2002
Phytase	Native	*E. coli* BL21(DE3)	120 U/ml, *kil* coexpressioin, HCDC	Kleist et al. 2003
Erwinia chrysanthemi endoglucanase	Native	*E. coli* B	4–6% of total proteins, *out* coexpression	Zhou et al. 1999
Mini-antibodies McPC603scFvDhlx	OmpA	*E. coli* RV308	10 mg/l, bacterial L-form	Kujau et al. 1998
Insulin-like growth factor binding protein-2 (IGFBP-2)	PelB	*E. coli* BL21	Gene III product fusion	Lucic et al. 1998
Murine interleukin-2 (mIL2)	OmpA	*E. coli* K12 RP1ΔM15	16 mg/l, *kil* gene	Robbens et al. 1995
Heat stable alkaline protease	Native	*E. coli* XL1-Blue	1500 U/ml, bacteriocin-release protein	Fu et al. 2003
TEM-β-lactamase	OmpA	*E. coli* BL21(DE3)	TolAIII coexpression	Wan et al. 1998
Human C-reactive protein (CRP)	PhoA (ALP)	*E. coli* JM109	*kil*coexpression	Tanaka et al. 2002
Human interleukin 6	HlyA	*E. coli* XL1-Blue	70.4 ng/ml, hemolysin system	Li et al. 2002

culture medium possibly due to an increased permeability of the cell membrane during a lengthy incubation period. Small proteins secreted into the periplasm are frequently released into the culture medium (Tong et al. 2000). In general, movement of recombinant proteins from the periplasm to the culture medium is the result of compromising the integrity of the outer membrane. However, care must be exercised during such recombinant protein production so as not to compromise cellular integrity, which often causes cell death. Interestingly, glycine or Triton X-100 supplemented to the medium retarded formation of inclusion bodies in the periplasm and increased the extracellular production efficiency of recombinant proteins (Jang et al. 1999; Kaderbhai et al. 1997; Yang et al. 1998). Glycine has been found to induce morphological changes, such as an enlarged spheroidal morphology in *E. coli*, as it is incorporated into peptidoglycan. Glycine supplementation may slightly disrupt peptidoglycan cross-linkages and cell membrane integrity. Yang et al. (1998) reported that adding 2% (w/v) glycine dramatically increased extracellular production of sFV/TNF-α and β-glucosidase.

Another method of extracellular protein production involves fusing the product to a carrier protein that is normally secreted into the medium (e.g. hemolysin), or to a protein expressed on the outer membrane (e.g. OmpF). For example, human β-endorphin could be secreted into the culture medium when fused to OmpF (Jeong and Lee 2002; Nagahari et al. 1985). Recently, a method of releasing active scFv antibody and human interleukin-6 into the culture medium using the hemolysin secretion pathway was reported (Fernandez et al. 2000; Li et al. 2002). The hemolysin transport system (Hly) is a type-I secretory apparatus that forms a protein channel between the inner and outer membranes o f*E. coli*. Hemolysin toxin (HlyA) is secreted by direct passage of the HlyA polypeptide from the cytoplasm to the extracellular medium using the hemolysin transport system (Fig. 2). For extracellular production using the hemolysin secretion pathway, the target protein is fused to the C-terminal hemolysin secretion signal. The Hly system appears to be an attractive candidate for the extracellular production of recombinant proteins.

Proteins secreted into the *E. coli* periplasm can also be released into the culture medium by co-expression of *kil* (Kleist et al. 2003; Miksch et al. 1997; 2002; Robbens et al. 1995) or the gene coding for the third topological domain of the transmembrane protein TolA (TolAIII) (Wan and Baneyx 1998). Zhou et al. (1999) reported extracellular production of the *Erwinia chrysanthemi*endoglucanase by employing the *out* genes from *E. chrysanthemi* EC16, which are responsible for the efficient extracellular secretion of pectic enzymes. Co-expression of the *out* genes increased production of active endoglucanase and released enzymes equivalent to over half of the total activity into the extracellular medium.

Another approach to the extracellular production of target proteins uses L-form cells, wall-less, or wall-deficient cells (Kujau et al. 1998; Rippmann et al. 1998). Recently, Kujau et al. (1998) demonstrated that L-form *E. coli* cells were capable of secreting into the culture medium a recombinant antibody fragment (single-chain phosphorylcholine-binding scFv from human McPC603) fused to the OmpA signal sequence under the control of the *lac* promoter. A correctly folded and dimerized mini-antibody was secreted directly, and remained stable in the culture medium.

Bacteriocin release protein (BRP) can also be used in the extracellular production of recombinant proteins in *E. coli*. BRP is a 28-amino-acid lipoprotein that activates detergent-resistant phospholipase A, resulting in the formation of permeable zones in the cell envelope through which proteins can pass into the culture medium (Fu et al. 2003; Lin et al. 2001b; van der Wal et al. 1995). However, co-expression of the BRP gene can damage the cell envelope and cause release of other cellular proteins. Recently, van der Wal et al. (1998) reported that a modified BRP gene (Lpp-BRP) could be used for the extracellular production of K88 fimbrial molecular chaperone FaeE without growth inhibition, lysis, or contaminating proteins.

Conclusions

Recent advances in our understanding of the protein secretory machinery and mechanism in *E. coli* have led to the development of various strategies to enhance secretory production of recombinant proteins. However, despite the successful development of various recombinant protein secretion systems, several problems remain to be solved. First, many large and complex proteins of eukaryotic origin are not efficiently secreted. Second, without trial-and-error, it is somewhat difficult to select a proper host-vector system and a signal sequence for the secretion of a desired protein. Third, the high-cell-density culture techniques for the secretory production of recombinant proteins are less well developed than those for cytosolic production. The first and second problems will be solved as our understanding of protein secretion pathways, folding mechanisms, periplasmic chaperone function, and signal sequences advances further. An obvious alternative solution to the first problem is simply not to adhere to the *E. coli* expression system, and instead use other organisms, including mammalian cells, as is currently practiced for a number of mammalian proteins. The third problem is that less research has been devoted to secretory production than to cytosolic expression, and therefore, can be solved by more research into the former. *E. coli* has been successfully used for both industrial- and laboratory-scale cytosolic production of recombinant proteins. Similar success with secretory and extracellular production of recombinant proteins using *E. coli* will likely follow.

Acknowledgements This review was supported by the Korean Systems Biology Research Grant (M10309020000-03B5002-00000) from the Ministry of Science and Technology. Support from IBM through the IBM-SUR program is greatly appreciated.

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