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Research review paper

Recombinant protein secretion in *Escherichia coli*

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Abstract

The secretory production of recombinant proteins by the Gram-negative bacterium *Escherichia coli* has several advantages over intracellular production as inclusion bodies. In most cases, targeting protein to the periplasmic space or to the culture medium facilitates downstream processing, folding, and in vivo stability, enabling the production of soluble and biologically active proteins at a reduced process cost.

This review presents several strategies that can be used for recombinant protein secretion in *E. coli* and discusses their advantages and limitations depending on the characteristics of the target protein to be produced.

Keywords: Recombinant proteins; *Escherichia coli*; Secretion; Periplasm; Type II

Contents

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1. Introduction

Most bacteria secrete proteins such as degradative enzymes, toxins, and other pathogenicity factors into the extracellular environment (Fernandez and Berenguer, 2000). In Gram-negative bacteria, secreted proteins have to cross the two membranes of the cell envelope, which differ substantially in both composition and function (Koebnik et al., 2000).

The type I, II, III, IV, and V secretion pathways are widespread among Gram-negative bacteria and their mechanisms differ significantly. Despite these differences, the systems have, in common, a need to recognise specifically their cognate substrates and promote secretion without compromising the barrier function of the cell envelope (Koster et al., 2000). This review discusses the type I and type II mechanisms that are used most commonly for recombinant protein secretion in *Escherichia coli* K-12 or B strains. The type III secretion pathway is characteristic of several pathogenic Gram-negative bacteria and has been reviewed by Cornelis and Van Gijsegem (2000). Type IV secretion comprises those pathways usually found in bacterial conjugation systems (Pallen et al., 2003) and has been reviewed by Christie (2001). The type V mechanism includes the autotransporter and the two-partner secretion systems (Pallen et al., 2003), and has been reviewed by Jacob-Dubuisson et al. (2001).

Finally, protein secretion to the culture medium may also occur by leakage of periplasmic contents, and thus is not always mediated by specific transport mechanisms as will be discussed in this review.

2. Recombinant protein secretion

Secretion of recombinant proteins to the culture medium or periplasm of *E. coli* has several advantages over intracellular production. These advantages include simplified downstream processing, enhanced biological activity, higher product stability and solubility, and N-terminal authenticity of the expressed peptide (Cornelis, 2000; Makrides, 1996; Mergulhão et al., 2004b).

As *E. coli* does not naturally secrete high amounts of proteins (Sandkvist and Bagdasarian, 1996), recovery of a recombinant gene product can be greatly simplified by a secretion strategy that minimises contamination from host proteins. Additionally, if the product is secreted to the culture medium cell disruption is not required for recovery and,

even in the case of periplasmic translocation, a simple osmotic shock or cell wall permeabilization can be used to obtain the product without the release of cytoplasmic protein contaminants (Mergulhão et al., 2004b; Shokri et al., 2003).

Biological activity is dependent on protein folding and, particularly if disulfide bonds must be formed, proper folding is unlikely in the reducing environment of the cytoplasm. Additionally, the correct pair bonding of cysteines contributes to the thermodynamic stability of the proteins (Kadokura et al., 2003; Maskos et al., 2003; Raina and Missiakas, 1997). The *E. coli* periplasm contains a series of enzymes such as disulfide-binding proteins (DsbA, DsbB, DsbC, and DsbD) and petidyl-prolyl isomerases (SurA, RotA, FklB, and FkpA) that promote the appropriate folding of thiol-containing proteins (Shokri et al., 2003).

Protein aggregation can result from chaperone limitation when gene expression is performed at nonphysiological levels (Hoffmann et al., 2004). In this situation, the intramolecular or intermolecular association of hydrophobic surfaces that are exposed prior to folding can cause the precipitation of folding intermediates (Carrio and Villaverde, 2002). Periplasmic or extracellular secretion can increase the solubility of a gene product as exemplified by the production of bacterial PNGaseF (Loo et al., 2002) and human granulocyte colony-stimulating factor (Jeong and Lee, 2001). Obtaining a soluble protein often constitutes a bottleneck in the production of proteins for structural studies or proteomics (Goulding and Jeanne Perry, 2003; Pedelacq et al., 2002; Yokoyama, 2003). The increased solubility of secreted protein may in part be due to dilution as the periplasm and the extracellular medium have a lower protein content than the cytoplasm (Makrides, 1996). Additionally, the cosecretion of molecular chaperones and medium supplementation with low molecular weight additives (such as L-arginine and glutathione) resulted in increased secretion and folding yields in the bacterial periplasm (Barth et al., 2000; Choi and Lee, 2004; Joly et al., 1998; Qiu et al., 1998; Schaffner et al., 2001; Winter et al., 2001).

Product secretion can provide a way to guarantee the N-terminal authenticity of the expressed polypeptide because it often involves the cleavage of a signal sequence (Mergulhão et al., 2000), thus avoiding the presence of an unwanted initial methionine on a protein that does not normally contain it. This extra methionine can reduce the biological activity and stability of the product (Liao et al., 2004) or even elicit an immunogenic response in the case of therapeutic proteins.

Protein secretion can increase the stability of cloned gene products. For instance it was shown that the half-life of recombinant proinsulin is increased 10-fold when the protein is secreted to the periplasmic space (Talmadge and Gilbert, 1982). Secretion was also useful in the production of penicillin amidase from *E. coli* as intracellular product degradation was a severe problem (Ignatova et al., 2003). The increased stability of gene products on the periplasm and in the culture medium probably results from the lower levels of *E. coli* proteases that can be found in these locations (Gottesman, 1996; Mergulhão et al., 2004b).

Protein secretion in *E. coli* is a complex process (Economou, 1999; Pugsley, 1993) and attempts to secrete recombinant proteins can face several problems. The most frequent are incomplete translocation across the inner membrane (Baneyx, 1999), insufficient capacity of the export machinery (Mergulhão and Monteiro, 2004; Mergulhão et al., 2004a; Rosenberg, 1998), and proteolytic degradation (Huang et al., 2001). Several factors can

influence the secretion of a recombinant protein in *E. coli*. It has been reported that protein size may influence secretion efficiency (Koster et al., 2000; Palacios et al., 2001) and that large cytoplasmic proteins may be physically impossible to translocate (Baneyx, 1999; Feilmeier et al., 2000). The amino acid composition of the leader peptide (Belin et al., 2004; Khokhlova and Nesmeyanova, 2004; Nakai and Kanehisa, 1991) and of the target protein (Kajava et al., 2000) is also important. There is an optimum rate of translation to achieve high-level secretion of heterologous proteins (Simmons and Yansura, 1996), and secretion may drop off severely at higher rates. This effect is probably a consequence of the limited secretion capacity of the *E. coli* transport machinery (Rosenberg, 1998). When this capacity is overwhelmed, the excess of expressed recombinant protein is likely to accumulate in inclusion bodies (Mergulhão and Monteiro, 2004; Mergulhão et al., 2004a). It is therefore important to optimise the expression level and one way to achieve this is by carefully balancing the promoter strength and gene copy number (Mergulhão et al., 2003a,b, 2004b).

The type I and type II secretion mechanisms are used by *E. coli* to secrete a number of native proteins. However, these systems have also been used widely for recombinant protein production. For this purpose, the choice between these two secretion mechanisms is dictated by the type of protein to be transported.

2.1. Type I secretion mechanism

Type I secretion systems transport proteins in one step across the two cellular membranes, without a periplasmic intermediate (Binet et al., 1997). *E. coli* normally uses this pathway for the secretion of high-molecular-weight toxins and exoenzymes (Fernandez and de Lorenzo, 2001). The type I secretion machinery is composed of two inner membrane proteins (HlyB and HlyD) that belong to the *A*TP *b*inding *c*assette (ABC) family of transporters, and an endogenous outer membrane protein, TolC (Fernandez and de Lorenzo, 2001; Gentschev et al., 2002; Koronakis, 2003). However, it has been reported that translocation can also be influenced by components of other secretion pathways including SecB (Sapriel et al., 2002, 2003). Although several type I transporters can been used for recombinant protein production, the *E. coli* α-haemolysin (HlyA) transporter is by far the most popular (Table 1). The C-terminal region of HlyA contains all the information required for efficient translocation and can therefore be used as a signal sequence for recombinant protein targeting. This system is very versatile, allowing the secretion of up to 5% of the total cell protein (Blight and Holland, 1994). In this pathway, the two ABC proteins HlyB and HlyD form a stable complex, which binds the recombinant protein bearing a C-terminal HlyA signal sequence and ATP in the cytoplasm (Fig. 1). A TolC trimer with a single hydrophilic pore (Andersen et al., 2002) binds the complex formed by HlyB, HlyD, and the recombinant protein, forming a channel connecting both cellular membranes. ATP hydrolysis by HlyB is required for protein transport through the channel but not for complex assembly (Thanabalu et al., 1998). After translocation TolC separates from the HlyB/HlyD complex, thus disconnecting the membranes.

Disulfide bond formation occurs during the passage of the polypeptide through the export conduit and is independent of inner membrane bound Dsb enzymes. The type I

Table 1
Examples of the secretion of recombinant proteins expressed as fusions to the HlyA signal sequence

Protein	Organism of origin	Promoter	References
β-Galactosidase	*Escherichia coli*	*lambda*	(Kenny et al., 1991)
β-gal-OmpF	*E. coli*	*lac*	(Mackman et al., 1987)
Alkaline phosphatase	*E. coli*	*lac*	(Gentschev et al., 1990)
Chloramphenicol acetyltransferase	*E. coli*	*cat*	(Kenny et al., 1991)
c-Type cytochromes	*E. coli*	*lac*	(Sanders et al., 2001)
Dihydrofolate reductase	*E. coli*	*tac*	(Nakano et al., 1992)
Eukaryotic proteins[a]	Various	*trc*	(Palacios et al., 2001)
IgA fragments	*Mus musculus*	*tac*	(Holland et al., 1990)
Interleukin-6	*Homo sapiens*	*lac*	(Li et al., 2002)
OmpF	*E. coli*	*lac*	(Holland et al., 1990)
Prochymosin	*Bos taurus*	*trp*	(Holland et al., 1990)
Prochymosin	*B. taurus*	*trp*	(Kenny et al., 1991)
ScFv antibodies	*H. sapiens*	*lac*	(Fernandez et al., 2000)

[a] Endochitinase (*Trichoderma harzianum*), green fluorescent protein (*Aequorea victoria*), human erythropoetin, and trout growth hormone.

pathway secretes proteins ranging from 50 to over 4000 amino acids, although the translocation channel can only accommodate globular proteins of up to 200 amino acids (Sapriel et al., 2003). The internal diameter of the channel is 3.5 nm and the length is 14 nm; these dimensions appear to be compatible with the secretion of partially folded molecules (Fernandez and de Lorenzo, 2001).

Although the type I secretion mechanism is capable of exporting the target protein to the culture medium, it has two significant drawbacks. Firstly, the secreted peptide remains attached to the signal sequence and therefore an additional cleavage step is required to obtain the intact native protein (Blight and Holland, 1994). Secondly, coexpression of the components of this system is often necessary to increase transport capacity. As in all coexpression systems, several proteins (i.e., host proteins, coexpressed transport components, and target protein) will compete for the *E. coli* native protein transport

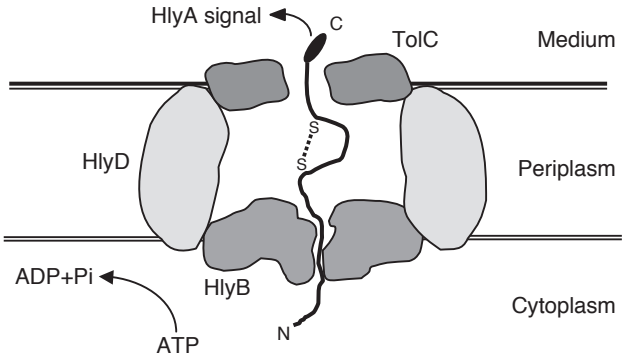

Fig. 1. Type I secretion mechanism, recombinant protein secretion through the α-haemolysin pathway of *E. coli*. The recombinant protein bearing a C-terminal HlyA signal peptide binds the HlyB/HlyD complex. ATP hydrolysis by HlyB is necessary for transport through the TolC channel during which disulfide bond formation occurs.

system and this can cause a severe production bottleneck. Very low protein translation rates must be used in order not to saturate the transport machinery (Shokri et al., 2003).

2.2. Type II secretion mechanism

The general secretory pathway is a two-step process for the extracellular secretion of proteins mediated by periplasmic translocation (Koster et al., 2000). Three pathways can be used for secretion across the bacterial cytoplasmic membrane: the SecB-dependent pathway, the signal recognition particle (SRP), and the twin-arginine translocation (TAT) pathways. The second step (translocation across the outer membrane) involves specific protein machinery known as the secreton (see below).

2.2.1. Cytoplasmic membrane translocation

2.2.1.1. SecB-dependent pathway. The vast majority of secreted proteins uses the SecB-dependent pathway for translocation across the inner membrane. This pathway, whose constituents are listed in Table 2, is also the most commonly used for recombinant protein production (see Section 2.2.3). Ribosome-associated nascent chains of secreted proteins bind trigger factor (Fig. 2), which is bound to the ribosomes (Maier et al., 2003). This association is maintained until the preprotein leaves the ribosome, thus preventing cotranslational binding of the nascent chain to SRP components (Beck et al., 2000; Maier et al., 2003).

Table 2
Components of the SecB-dependent pathway

Protein	M_W (kDa)	Function	Reference
Trigger factor	48	Prevents binding to SRP components	(Hesterkamp et al., 1996; Patzelt et al., 2001)
SecA	102	Translocation ATPase	(Eser and Ehrmann, 2003; Wang et al., 2000)
SecB	16.6	Preprotein targeting, retards protein folding, modulates the activity of SecA	(Dekker et al., 2003; Driessen, 2001; Randall and Hardy, 2002; Ullers et al., 2004)
SecY	49	Component of the translocation channel, necessary for the high-affinity binding of SecA	(Shimokawa et al., 2003)
SecE	13.6	Component of the translocation channel	(Pugsley, 1993)
SecG	11.4	Facilitates SecA cycling through topology inversion, interacts with SecDF–YajC	(de Keyzer et al., 2003a; Mori and Ito, 2001)
SecD	67	Maintaining the proton-motive force, affects protein release from the channel	(de Gier and Luirink, 2001; Manting and Driessen, 2000)
SecF	35	Modulation of SecA activity	(Eichler, 2003)
YajC		Accessory protein, associates with SecF	(de Keyzer et al., 2003a; Nouwen and Driessen, 2002)

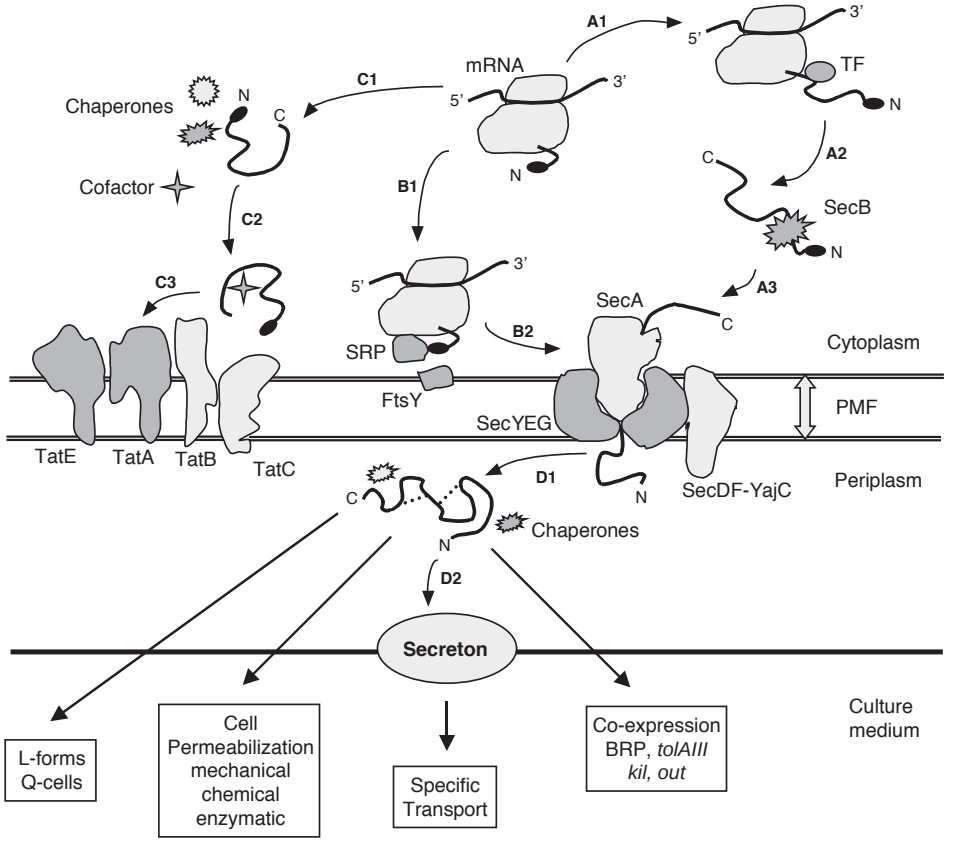

Fig. 2. Recombinant protein secretion by the type II mechanism and strategies for the extracellular release of recombinant proteins from the periplasm. On the SecB-dependent pathway, the protein emerges from the ribosome and binds to trigger factor (TF) (step A1). The protein is then recognised by SecB (step A2), which targets it to the membrane-bound SecA (step A3). At the translocation point, a group of proteins (Sec Y, SecE, and SecG) forms a translocation complex that threads the protein at the expense of ATP hydrolysis. At a later stage, the proton-motive force (PMF) can drive the translocation. On the SRP pathway, the nascent chain is recognised by SRP (step B1). The SRP–ribosome complex interacts with FtsY, thus releasing the nascent chain to the translocation site (step B2). On the TAT pathway, the protein is fully synthesized and folds in the cytoplasm where it can bind specific cofactors (step C1). The signal peptide is then recognised by TatC in the TatBC complex (step C2). Signal peptide binding promotes association of the complex with TatA oligomers at the expense of PMF. Protein translocation occurs through a channel formed by TatA and possibly TatE oligomers (step C3). Within the periplasm, the protein is folded and adopts tertiary or even quaternary structures (step D1). The protein is then transported by a secretion machinery named "secreton" which is composed by 12–16 proteins (step D2). Extracellular release of periplasmic proteins can also be achieved by several strategies like the use of leaky strains, cell membrane permeabilization, or coexpression of release proteins.

Secreted proteins targeted to the SecB-dependent pathway contain an amino-terminal signal peptide that functions as a targeting and recognition signal. These signal peptides are usually 18–30 amino acid residues long and are composed of a positively charged amino terminus (n-region), a central hydrophobic core (h-region), and a polar cleavage

region (c-region) (Choi and Lee, 2004; Fekkes and Driessen, 1999). The n-region is believed to be involved in targeting the preprotein to the translocase and binding to the negatively charged surface of the membrane lipid bilayer. Increasing the positive charge in this region has been shown to enhance translocation rates, probably by increasing the interaction of the preprotein with SecA (Fekkes and Driessen, 1999; Wang et al., 2000). The h-region varies in length from 7 to 15 amino acids. Translocation efficiency increases with the length and hydrophobicity of the h-region, and a minimum hydrophobicity is required for function (Wang et al., 2000).

Secreted proteins are kept in a translocation-competent state by the chaperone SecB (de Gier and Luirink, 2001), which interacts with the mature region of the preprotein to prevent premature folding (Khokhlova and Nesmeianova, 2003) and targets it to SecA (Fig. 2). In the presence of preprotein, SecB binds SecA (Fekkes et al., 1998; Woodbury et al., 2000), thus releasing the precursor protein that is transferred to SecA (Fekkes and Driessen, 1999). SecA binding to the preprotein is facilitated by the signal peptide, which it recognizes specifically (Kebir and Kendall, 2002; Miller et al., 1998). At this point SecA is bound to the SecY subunit of the SecYEG complex. Binding of ATP at one of the two ATP-binding sites on SecA causes the release of SecB from the membrane (van der Wolk et al., 1998). There is no consensus on how the Sec components form a functional translocon (Pugsley et al., 2004), and monomeric (Yahr and Wickner, 2000), dimeric (Breyton et al., 2002; Duong, 2003; Tziatzios et al., 2004), and oligomeric (Manting et al., 2000) translocons have been proposed. Binding of the preprotein to membrane-bound SecA results in the translocation of approximately 20 amino acids, and subsequent binding of ATP to SecA promotes SecA membrane insertion and translocation of an additional 15–20 amino acids. ATP hydrolysis releases the preprotein from SecA into the translocation channel (Driessen et al., 1998). ADP is then released and SecA deinserts from the membrane where it can be exchanged with cytosolic SecA. Multiple rounds of SecA insertion and deinsertion promote protein translocation through the channel (de Keyzer et al., 2003b; Economou, 1999). Proton-motive force (PMF) can complete translocation when the preprotein is halfway through the translocase, even in the absence of SecA (Nishiyama et al., 1999). The mechanism by which PMF drives translocation is unknown but it has been suggested that PMF assists in the initiation phase of protein translocation (Mori and Ito, 2003) and that it accelerates SecA membrane deinsertion (Nishiyama et al., 1999; van der Wolk et al., 1998).

It has been reported that the rate-limiting step for translocation is SecA release from the membrane (Manting and Driessen, 2000). Since the limited capacity of the *E. coli* transport system is one of the most serious drawbacks in the secretory production of recombinant proteins, optimising SecA deinsertion may be a useful strategy to extend the export capacity of the cells. Since SecA synthesis increases when export is blocked or saturated (Pugsley, 1993), overproduction of SecA could in principle enhance translocation by promoting the exchange between cytosolic and membrane-bound SecA (thus stimulating SecA release) or by promoting complex formation between SecA and SecB. However, SecA expression is down-regulated by binding of SecA to its own mRNA (Schmidt et al., 2001), a control mechanism not seen in other components of the SecB-dependent pathway (Pugsley, 1993), and one that might frustrate attempts to overexpress the protein.

The situation is further complicated because SecA translation is also regulated by the product of a cotranscribed upstream gene, *secM*, which acts as a monitor of *E. coli* secretion proficiency (Oliver et al., 1998; Sarker et al., 2000). When SecM secretion is limiting, ribosomes translating the *secM* ORF stall, exposing the *secA* ribosome binding site in *secM–secA* mRNA and stimulating *secA* translation (Butkus et al., 2003; Mori and Ito, 2001; Nakatogawa et al., 2004). However, when the cell has excess protein secretion capacity, translocation of SecM is efficient and *secA* translation is repressed. It has been reported (Sarker and Oliver, 2002) that certain signal peptide mutations in SecM can prevent its translocation, thereby rendering SecA translation constitutive. However it has also been shown that the expression of signal sequence-defective SecM is extremely toxic to the cell (Nakatogawa and Ito, 2002). In a final twist to this complex story, ectopic production of SecA from a *secA* gene in isolation from *secM* is less effective than expression from the wild-type *secM–secA* operon, possibly due to an inefficient membrane targeting of the SecA (Nakatogawa and Ito, 2004).

Another promising strategy to increase *E. coli* translocation capacity uses *prl* (*pr*otein *l*ocalisation) mutations. These mutations map to the *secY* (*prlA*), *secE* (*prlG*), *secG* (*prlH*), and *secA* (*prlD*) genes (Manting and Driessen, 2000) and are thought to relax the protein-conducting channel, allowing the translocation of some proteins with defective signal sequences. One of the most effective mutations, *prlA4* (de Keyzer et al., 2002b; van der Wolk et al., 1998), has been shown to cause a 10-fold increase in translocation efficiency compared to wild type (de Keyzer et al., 2002a).

Although this pathway has been used extensively for recombinant protein production, it has one serious drawback. This system is not able to transport folded proteins and, since transport is largely posttranslational, the secretion of proteins that fold rapidly in the cytoplasm may not be possible. In these cases the protein should be targeted to the SRP or the TAT pathways.

2.2.1.2. SRP pathway. The *s*ignal *r*ecognition *p*article (SRP) pathway is used by *E. coli* primarily for the targeting of inner membrane proteins (Economou, 1999). This system has been exploited in the secretion of several recombinant proteins including Mtla–OmpA fusions (Neumann-Haefelin et al., 2000), MalF–LacZ fusions (Tian et al., 2000), maltose binding protein, chloramphenicol acetyl transferase (Lee and Bernstein, 2001; Peterson et al., 2003), and haemoglobin protease (Sijbrandi et al., 2003).

The system consists of several proteins and one RNA molecule (Table 3 and Fig. 2). SRP recognises its substrates by the presence of a hydrophobic signal sequence (hence the name signal recognition particle). The presence of an N-terminal signal sequence with a highly hydrophobic core, combined with a lack of a trigger factor binding site (Patzelt et al., 2001), results in cotranslational binding of the nascent chain to Ffh (Beck et al., 2000). For a productive interaction between the preprotein and Ffh, 4.5S RNA is required (Herskovits et al., 2000). It has been suggested (Fekkes and Driessen, 1999) that the interaction between SRP and the signal sequence is dependent on the hydrophobicity of the nascent chain since preproteins with more hydrophobic signal sequences are translocated with higher efficiency. It has been shown (Gu et al., 2003) that SRP binds the ribosome at a site that overlaps the binding site of trigger factor. A discriminating process has been proposed in which SRP and trigger factor alternate in transient binding to

Table 3
Components of the SRP pathway

Component	Localisation	Function	References
4.5S RNA	Cytoplasm	Binding of the nascent chain to Ffh	(Driessen et al., 2001; Peterson et al., 2003; Wild et al., 2004)
Ffh	Cytoplasm	Protein targeting to FtsY	(Driessen et al., 2001; Keenan et al., 2001; Wild et al., 2004)
FtsY	Cytoplasm and membrane	SRP receptor membrane targeting	(Drew et al., 2003; Eitan and Bibi, 2004; Koch et al., 2003)
SecAYEG			See Table 2
YidC	Inner membrane	Lateral diffusion of proteins. Present at about 3000 copies per cell	(Nouwen and Driessen, 2002; Serek et al., 2004; Urbanus et al., 2002; van der Laan et al., 2001)

the ribosome until a nascent peptide emerges. Depending on the characteristics of the nascent peptide, the binding of either SRP or trigger factor is stabilised, thus determining whether the peptide is targeted to the membrane via the SRP pathway, or post-translationally by the SecB pathway (Gu et al., 2003).

FtsY is found both in the cytoplasm and at the membrane (Herskovits et al., 2000), and can interact with ribosomal nascent chain–SRP complexes in the cytosol. Upon interaction with membrane lipids, the GTPase activities of FtsY and Ffh are stimulated, thus releasing the nascent chain to the translocation site (Nagai et al., 2003). This site may be the SecYEG translocon (Koch et al., 1999; Valent et al., 1998; Zito and Oliver, 2003), although it has been demonstrated that membrane insertion can occur independently of SecYEG (Cristobal et al., 1999b). Insertion of transmembrane segments can occur in the absence of SecA (Scotti et al., 1999) while translocation of large periplasmic loops is SecA-dependent (Neumann-Haefelin et al., 2000; Qi and Bernstein, 1999; Tian et al., 2000). The protein YidC was also identified as a translocase-associated component during insertion (Scotti et al., 2000). It has been proposed that this protein facilitates the diffusion of transmembrane segments into the lipid phase (van der Laan et al., 2001).

For recombinant protein production, SRP targeting can be achieved by engineering the hydrophobicity of the signal sequence (Bowers et al., 2003; de Gier et al., 1998; Peterson et al., 2003). This is advantageous if for instance the target protein folds too quickly in the cytoplasm, adopting a conformation incompatible with secretion by the SecB-dependent system (Lee and Bernstein, 2001; Schierle et al., 2003).

2.2.1.3. TAT pathway. Recently, a Sec-independent pathway was reported to be functional in *E. coli* (Santini et al., 1998; Sargent et al., 1998). This pathway has been termed the TAT (*t*win-*a*rginine *t*ranslocation) system because preproteins transported by it contain two consecutive and highly conserved arginine residues in their leader peptides.

The TAT pathway is capable of transporting folded proteins across the inner membrane (Stanley et al., 2000) independently of ATP (Yahr and Wickner, 2001) using the transmembrane PMF (de Leeuw et al., 2002). In most cases, the substrates of this pathway are proteins that bind specific cofactors in the cytoplasm and are folded prior to export (Bogsch et al., 1998; Santini et al., 1998). This system is related to the ΔpH-dependent protein import machinery of the plant chloroplast thylakoid membrane (Sargent et al.,

1999). The TAT pathway has been used in the secretion of several recombinant proteins including antibody fragments (De Lisa et al., 2003), glucose–fructose oxireductase (Blaudeck et al., 2001), ribose binding protein (Pradel et al., 2003), alkaline phosphatase (Masip et al., 2004), and green fluorescent protein (Barrett et al., 2003; De Lisa et al., 2002; Santini et al., 2001; Thomas et al., 2001).

The main components of this translocation system are summarised in Table 4 but their specific roles have not yet been firmly established. TatA has been proposed to form the transport channel (Palmer and Berks, 2003), although TatAB complexes have also been implicated in that function (Sargent et al., 2001). TatB and TatC are proposed to form a 1:1 complex that may provide the initial binding site for preprotein docking (Allen et al., 2002; de Leeuw et al., 2002; Schnell and Hebert, 2003). It has also been proposed that the signal sequence is recognised by TatC and then transferred to TatB (Alami et al., 2003). A mechanism for protein translocation by the TAT system (Fig. 2) was recently proposed by Palmer et al. (2004). In this study, the authors propose that the signal peptide is recognized by TatC, which is forming a complex with TatB. When signal peptide binding occurs, PMF promotes the association between the TatBC complex and TatA oligomers. The folded preprotein is then translocated by the TatA channel and the leader peptide is processed. Following translocation TatA dissociates from the TatBC complex (Palmer et al., 2004). It has also been reported that TatE can partially substitute TatA (Berks et al., 2000).

Like Sec signal peptides, TAT signal peptides are also composed of three regions: a positively charged region (n-region), a hydrophobic region (h-region), and a c-region that contains the cleavage site. The average size of these signal peptides is approximately 38 amino acids, which is 14 amino acids longer than the average Sec leader peptide. Most of this additional length is due to an extended n-region. TAT signal peptides bear the N-terminal consensus motif S/T-R-R-X-F-L-K, where X is highly variable (Blaudeck et al., 2001). Although the presence of both arginine residues is not an obligatory requirement for transport (Stanley et al., 2000), mutagenesis of one or both of these residues can affect membrane translocation (De Lisa et al., 2002; Ize et al., 2002). The h-region of TAT signal

Table 4
Components of the TAT pathway

Protein	Predicted size (kDa)	Characteristic	Reference
TatA	9.6 or 11.3[a]	60% Homologous to TatE; its expression is higher than other *tat* genes	(Gouffi et al., 2004; Porcelli et al., 2002; Sargent et al., 1998)
TatB	18.4[a]	Complexes with TatC and prevents its degradation	(Sargent et al., 1999)
TatC	28.9[a]	Likely to be a signal peptide binding component	(Allen et al., 2002; Behrendt et al., 2004; Buchanan et al., 2002)
TatD	29.5[a]	No effect on protein translocation; presents DNase activity	(Wexler et al., 2000)
TatE	6.9[b]	Can partially substitute TatA	(Berks et al., 2000)

[a] Two possible translation initiation sites exist for *tatA* (Sargent et al., 1998).
[b] DNA sequence retrieved from Genbank accession no. NP_308692. Molecular mass calculated by the program PROTPARAM (http://www.expasy.ch/tools/protparam.html).

peptides is usually less hydrophobic than that of Sec leader peptides. The c-region contains the cleavage site and shows a strong bias towards basic amino acid residues (Berks et al., 2000). It has not been established whether these signal peptides are cleaved by signal peptidase I or by some other protease (Oresnik et al., 2001).

It has been shown that transport via the TAT pathway is less efficient (De Lisa et al., 2004) and slower than the Sec pathway with transit half-times in the order of a few minutes (Santini et al., 1998; Sargent et al., 1998) instead of a few seconds (Berks et al., 2000). The largest protein known to be transported by a TAT system is the 142-kDa FdnGH subcomplex of *E. coli* formate dehydrogenase-N (Berks et al., 2000). Although recombinant protein targeting to the TAT pathway can be achieved simply by relying on wild-type levels of proteins produced by the *tatABCD* operon, it has been reported (Barrett et al., 2003; De Lisa et al., 2004) that this secretion mechanism is rapidly saturated, so for large-scale production, coexpression of the *tatABC* operon is necessary. As discussed above in the context of the type I secretion mechanism, the requirement for coexpression is a severe drawback for the application of the TAT pathway for production processes. Additionally, it has been suggested that the energy cost of translocation by this pathway may be excessive for high level secretion and this would explain its inherent low capacity (De Lisa et al., 2004). Despite these disadvantages, the TAT pathway is capable of transporting folded protein across the inner membrane, unlike the SecB or the SRP pathways (De Lisa et al., 2003).

2.2.2. Extracellular secretion

One advantage of extracellular secretion from *E. coli* is that because this bacterium does not normally secrete proteins into the culture medium (Hannig and Makrides, 1998; Pugsley et al., 1997), contamination of the product by host proteins can be minimized. This production strategy is also beneficial as proteolytic activity is greatly reduced in the culture medium (Gottesman, 1996). Proteins can reach the culture medium by nonspecific periplasmic leakage, by a type I mechanism, or by the second step of a type II mechanism.

Extracellular secretion by a type II mechanism constitutes the main terminal branch (MTB) of the general secretory pathway. This step is complex and requires 12–16 proteins that constitute the secreton (Lory, 1998; Pugsley et al., 1997; Sandkvist, 2001). Although the functions of individual secreton components are not known, some roles have been attributed by comparative analysis with other secretons that are highly conserved among Gram-negative bacteria (Nouwen et al., 1999, 2000; Possot et al., 2000; Sandkvist, 2001). When exiting peptides reach the periplasm, they are thought to adopt tertiary and even quaternary structures in order to be recognised by the MTB components. Although it is known that proteins have to adopt secretion-competent conformations to proceed further (Lory, 1998; Pugsley et al., 1997), no secretion signal on the folded proteins has been identified (Sandkvist, 2001).

E. coli contains a complete set of genes coding for MTB components (accounting for approximately 0.5% of the coding capacity of its genome) but they are not expressed under normal laboratory conditions (Francetic et al., 2000; Francetic and Pugsley, 1996; Pugsley and Francetic, 1998). Strategies for the extracellular secretion of recombinant proteins may include the overexpression of some secreton components or the optimisation of environmental conditions that allow their expression (Pugsley et al., 1997).

Periplasmic leakage might be of some importance for extracelullar secretion (del Castillo et al., 2001; Rinas and Hoffmann, 2004) and may have several causes. During cell division, leakage of periplasmic contents can happen prior to the formation of individual outer membranes (Mergulhão et al., 2004a). The accumulation of recombinant protein in the periplasm may cause an osmotic pressure build-up, which can be the driving force for transport across the outer membrane (Hasenwinkle et al., 1997). Recombinant protein production can induce perturbations on the membrane (Pugsley et al., 1997), thus increasing its selective permeability (Slos et al., 1994), which may facilitate leakage. Periplasmic secretion may also induce cell lysis (Lee et al., 2001), resulting in the release of periplasmic content, particularly in older cultures.

Strategies employed to increase the permeability of the outer membrane have included mechanical (ultrasound), chemical (addition of magnesium, calcium, EDTA, glycine, and Triton X-100), and enzymatic (lysozyme) treatments (Choi and Lee, 2004; Shokri et al., 2003). Extracellular secretion can also be enhanced by variation of physical and chemical parameters (temperature, culture medium composition, pH, or aeration), or by taking advantage of the growth-coupled effects on membrane components (Rinas and Hoffmann, 2004; Shokri et al., 2003).

The choice of an appropriate strain can also dictate the success of a particular secretion strategy. The use of leaky strains (i.e., mutants that display disturbances in the synthesis of outer membrane components) has also been explored for recombinant protein secretion. The most drastic example is the use of bacterial L-forms (that lack periplasm and murein *sacculus*) in the production of penicillin G acylase (Gumpert and Hoischen, 1998), staphylokinase (Hoischen et al., 2002), miniantibodies (Kujau et al., 1998), and antibody fragments (Rippmann et al., 1998). However, the use of leaky mutants is usually not suitable for industrial production since these strains are growth-impaired and lack the necessary robustness for high-density fermentations (Ray et al., 2002).

The use of growth-arrested, metabolically active quiescent cells (Q-cells; Rowe and Summers, 1999) has proved successful for the extracellular secretion of antibody fragments (Mukherjee et al., 2004), although the mechanism of secretion is not known. Q-cells are generated by overexpression of a plasmid-encoded cell cycle regulator (Rcd) in an *hns205* mutant host. Nucleoid collapse in Q-cells results in global down-regulation of chromosomal gene expression but plasmid gene expression continues. Thus a recombinant protein can be expressed in an environment where resource competition for transcription, translation, and secretion is very much reduced. Mukherjee et al. (2004) reported that Q-cells in fed-batch culture secreted a biologically active antibody fragment into the culture medium 10 times faster than could be achieved in conventional culture.

Some coexpression strategies have been employed successfully to achieve culture medium release of recombinant proteins. The use of colicin E1 lysis protein (Kil) has been reported in the production of penicillin amidase (Ignatova et al., 2003), phytase (Kleist et al., 2003; Miksch et al., 2002), human C-reactive protein (Tanaka et al., 2002), interleukin-2 (Robbens et al., 1995), and β-glucanase (Miksch et al., 1997). Coexpression of the bacteriocin release protein (BRP) has been successfully used on the secretion of β-lactamase, chaperone FaeE, cloacin DF13 (van der Wal et al., 1995b,c, 1998), penicillin acylase (Lin et al., 2001), and alkaline protease (Fu et al., 2003). The *tolAIII* and *out* genes have also been coexpressed in the extracellular production of β-lactamase (Wan and

Table 5
Examples of secreted recombinant proteins

Protein	Signal	Promoter	Level	Location	Scale	Reference
β-Lactoglobulin	PelB	*T7*	33 ng/μg	Periplasm	(n/a)	(Chatel et al., 1999)
Antibodies	PelB	*arabinose*	700 mg/l	Medium	Fermenter, 14 l, OD 100	(Better et al., 1993)
Antifreeze peptide	OmpA	*tac*	16 mg/l	Medium	Shake flask, 200 ml, OD 2	(Tong et al., 2000)
bGPF	OmpA	*T7*	8 mg/l	Periplasm	Shake flask, vol and OD (n/a)	(Loo et al., 2002)
bPT inhibitor	SpA	*spA*	200 mg/l	Periplasm	Shake flask, 25 ml, OD (n/a)	(Nilsson and Abrahmsen, 1990)
CBD	Cex	*tac*	2.8 g/l	Periplasm	Fermenter, 1.5 l, OD 180	(Hasenwinkle et al., 1997)
Chitin deacetylase	Chitinase	*T7*	5.58 mg/l	Medium	Shake flask, 100 ml, OD 1.6	(Tokuyasu et al., 1999)
Cholera toxin B	OmpA	*arabinose*	1 g/l	Medium	Fermenter, 20 l, CDW 20 g/l	(Slos et al., 1994)
Cytochrome *P*450	PhoA	*phoA*	0.6 μM	Periplasm	Shake flask, 500 ml OD (n/a)	(Kaderbhai et al., 2001)
Growth hormone	OmpA	*lpp/lac*	28.4 mg/l	Periplasm	Shake flask, 1 l, OD 1.6	(Becker and Hsiung, 1986)
Growth hormone	DsbA	*lambda*	12 μg/ml	Periplasm	Shake flask, 100 ml, OD 3	(Soares et al., 2003)
hEGF	PhoA	*tac*	800 μg/l	Periplasm	Shake flask, vol and OD (n/a)	(Engler et al., 1988)
hGCSF	Exl	*trc*	3.2 g/l	Periplasm	Shake flask, 50 ml, OD (n/a)	(Jeong and Lee, 2001)
Hirudin	Asparaginase	*tac*	60 mg/l	Medium	Shake flask, 1 l, OD 6	(Tan et al., 2002)
Human leptin	Enx	*T7*	150 mg/l	Periplasm	Shake flask, 50 ml, OD (n/a)	(Jeong and Lee, 2000)
Human proinsulin	SpA	*spA*	28 μg/l	Medium	Shake flask, 100 ml, OD 1.5	(Mergulhão et al., 2001)
Human proinsulin	DsbA	*trc*	9.2 mg/g	Periplasm	Shake flask, 500 ml, OD 1	(Winter et al., 2001)
Human proinsulin	SpA	*spA*	1.2 mg/l	Periplasm	Shake flask, 25 ml, OD 2	(Mergulhão et al., 2000)
IGF-I	LamB	*arabinose*	2.5 g/l	Periplasm	Fermenter, 10 l, OD (n/a)	(Joly et al., 1998)
IGF-I	SpA	*spA*	30 mg/l	Periplasm	Shake flask, vol and OD (n/a)	(Samuelsson et al., 1996)
IGF-I	SpA	*spA*	10 μg/l	Periplasm	(n/a)	(Hammarberg et al., 1989)

Table 5 (*continued*)

Protein	Signal	Promoter	Level	Location	Scale	Reference
IGF-I	SpA	*spA*	30 mg/l	Medium	Fermenter, 10 l, OD (n/a)	(Moks et al., 1987)
IGF-I	SpA	*spA*	51 mg/l	Medium	Fermenter, 7 l, OD 25	(Abrahmsen et al., 1986)
IGF-I fragment	SpA	*spA*	5 mg/l	Medium	Shake flask, 1 l, OD (n/a)	(Lowenadler et al., 1987)
Immunotoxins	PelB	*T7*	0.6 g/l	Periplasm	Shake flask, 1 l, CDW 4 g/l	(Barth et al., 2000)
Leptin	OmpA	*lpp/lac*	4.5 mg/l	Periplasm	Shake flask, 500 ml, OD 1.5	(Guisez et al., 1998)
LFT	Seq X	*lac*	4 g/l	Medium	Fermenter, 5 l, OD 140	(Lee et al., 2001)
Miniproinsulin	SpA	*spA*	75 mg/l	Periplasm	(n/a)	(Kang and Yoon, 1994)
Mouse endostatin	PhoA	*phoA*	40 mg/l	Medium	Fermenter, 10 l, OD (n/a)	(Xu et al., 2002)
PhoA	PhoA	*phoA*	3400 U/mg	Periplasm	(n/a)	(Mikhaleva et al., 2001)
PhoA	Enx	*trc*	5.2 g/l	Periplasm	Fermenter, 6 l, OD 150	(Choi et al., 2000)
Phos D	PelB	*T7*	1.3 mg/l	Medium	Shake flask, 500 ml, OD 3	(Zambonelli et al., 2003)
Salmon calcitonin	OmpA	*tac/lac*	210 mg/l	Medium	Fermenter, 1 l, OD (n/a)	(Ray et al., 2002)
scFv antibody	PelB	*lambda*	160 mg/l	Medium	Fermenter, 4 l, OD 50	(Mukherjee et al., 2004)
scFV fragments	PelB	*T7*	19 μg/ml	Periplasm	Shake flask, vol and OD (n/a)	(Oelschlaeger et al., 2003)
scFV multimers	PelB	*lambda*	1 mg/l	Periplasm	Fermenter, 10 l, OD 14	(Bayly et al., 2002)
Staphylokinase	OmpA	*tac*	15 μg/ml	Periplasm	Shake flask, 250 ml, CDW 0.5 g/l	(Lee et al., 1998)
TPA	SthII	*arabinose*	180 μg/l	Periplasm	Fermenter, 15 l, OD 100	(Qiu et al., 1998)
TPA derivatives	OmpA	*lac*	29.6 μg/l	Medium	Shake flask, 100 ml, OD (n/a)	(Manosroi et al., 2001)
TPA variant	PelB	*T7*	4 ng/ml	Periplasm	Shake flask, vol and OD (n/a)	(Schaffner et al., 2001)

bGPF—bacterial glycoamidase PNGase F; bPT—bovine pancreatic trypsin; CBD—cellulose binding domain; CDW—cell dry weight; Cex—cellulase from *Cellulomonas fimi*; Enx—endoxylanase from *Bacillus* sp.; Exl—*Bacillus* sp. signal peptide; hEGF—human epidermal growth factor; hGCSF—human granulocyte colony-stimulating factor; IGF-I—insulin-like growth factor I; LFT—levan fructotransferase; lpp—lipoprotein promoter; OmpA—outer membrane protease A; PelB—pectate lysate from *Erwinia carotovora*; PhoA—alkaline phosphatase; Phos D—Phospholipase D; scFv—single chain variable fragment; Seq X—TMITNSSSV (Lac Z derived); Spa—protein A from *Staphylococcus aureus*; SthII—heat-stable enterotoxin II.

Note: Scale information is included when it is available from the cited reference. When it is not available, it is indicated by (n/a).

Baneyx, 1998) and endoglucanase (Zhou et al., 1999), respectively. Due to the potential deleterious effects of coexpression of these proteins on cell physiology (van der Wal et al., 1995a), they must be expressed from a tightly repressible promoter and induction must be independent of the product gene promoter.

2.2.3. How are recombinant proteins really transported?

Although studies cited here demonstrate that recombinant protein targeting to the TAT, SRP, or SecB-dependent pathways can be achieved with fruitful results, it is often impossible to guarantee that all molecules of the recombinant protein will be translocated by a single targeting pathway. Indeed it has been reported that the SRP and SecB-dependent pathways can be involved simultaneously in the targeting of a single protein (Froderberg et al., 2003), indicating some degree of overlap between these systems (Kim et al., 2001). In addition, competition between Sec- and TAT-dependent protein translocation has been suggested (Cristobal et al., 1999a) and it has also been reported that under Sec-deficient conditions, the export of Sec pathway substrates can be achieved by the TAT system (Pradel et al., 2003). This raises the intriguing possibility that when the maximum capacity of a specific translocation route has been reached (Mergulhão and Monteiro, 2004), protein translocation can be rescued by an alternative pathway as long as some compatibility requirements are met. The SecB-dependent pathway has been studied in far greater detail than the other two targeting pathways and it is therefore no surprise that most secretory recombinant protein production strategies use this system. Table 5 lists several examples of recombinant proteins that have been targeted to the SecB-dependent pathway. However, we cannot rule out the possibility that several translocation mechanisms operate simultaneously in these experiments. Furthermore, in some examples, the recombinant protein was found in the culture medium in significant amounts, which is most likely due to leakage of periplasmic contents as discussed above.

3. Conclusions

The last decade has witnessed many developments in recombinant protein secretion by *E. coli*. Periplasmic secretion has been shown to be beneficial in the production of several recombinant proteins due to a higher stability of the gene product, correct folding, and facilitated downstream processing. A detailed elucidation of the Sec mechanism was crucial for these developments. On the other hand, the final stages of the SRP pathway have not been firmly established and our knowledge about the recently discovered TAT pathway is still in its infancy. These three targeting pathways can be used for the purpose of recombinant protein production and the choice among them is governed by the type of protein to be produced. The TAT pathway is the only one that can transport folded molecules from the cytoplasm, while the SRP pathway can be used for transport of proteins that fold too quickly and incorrectly in the cytoplasm. SecB is the best understood and most robust targeting pathway and is most widely used for recombinant protein production, although its utilization may sometimes be hampered by the limited capacity of the system. Recent developments on SecA regulation are bringing new insights on the tuning of this pathway aimed at increasing the secretion capacity of the cell.

Despite many attempts, recombinant protein targeting to the culture medium has proved very difficult and these systems have not been used widely on an industrial scale. Although the *E. coli* genome encodes the constituents of a secreton, these genes are not expressed under standard laboratory conditions. Research on the genetic and environmental conditions that promote the expression of these genes will contribute to a better understanding on how the *E. coli* secreton works, which may also bring benefits for recombinant protein production.

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References

Abrahmsen L, Moks T, Nilsson B, Uhlen M. Secretion of heterologous gene products to the culture medium of *Escherichia coli*. Nucleic Acids Res 1986;14:7487–500.

Alami M, Luke I, Deitermann S, Eisner G, Koch HG, Brunner J, et al. Differential interactions between a twin-arginine signal peptide and its translocase in *Escherichia coli*. Mol Cell 2003;12:937–46.

Allen SC, Barrett CM, Ray N, Robinson C. Essential cytoplasmic domains in the *Escherichia coli* TatC protein. J Biol Chem 2002;277:10362–6.

Andersen C, Koronakis E, Bokma E, Eswaran J, Humphreys D, Hughes C, et al. Transition to the open state of the TolC periplasmic tunnel entrance. Proc Natl Acad Sci U S A 2002;99:11103–8.

Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 1999;10:411–21.

Barrett CM, Ray N, Thomas JD, Robinson C, Bolhuis A. Quantitative export of a reporter protein, GFP, by the twin-arginine translocation pathway in *Escherichia coli*. Biochem Biophys Res Commun 2003; 304:279–84.

Barth S, Huhn M, Matthey B, Klimka A, Galinski EA, Engert A. Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. Appl Environ Microbiol 2000;66:1572–9.

Bayly AM, Kortt AA, Hudson PJ, Power BE. Large-scale bacterial fermentation and isolation of scFv multimers using a heat-inducible bacterial expression vector. J Immunol Methods 2002;262:217–27.

Beck K, Wu LF, Brunner J, Muller M. Discrimination between SRP- and SecA/SecB-dependent substrates involves selective recognition of nascent chains by SRP and trigger factor. EMBO J 2000;19:134–43.

Becker GW, Hsiung HM. Expression, secretion and folding of human growth hormone in *Escherichia coli* Purification and characterization. FEBS Lett 1986;204:145–50.

Behrendt J, Standar K, Lindenstrauss U, Bruser T. Topological studies on the twin-arginine translocase component TatC. FEMS Microbiol Lett 2004;234:303–8.

Belin D, Guzman L-M, Bost S, Konakova M, Silva F, Beckwith J. Functional activity of eukaryotic signal sequences in *Escherichia coli*: the ovalbumin family of serine protease inhibitors. J Mol Biol 2004; 335:437–53.

Berks BC, Sargent F, Palmer T. The Tat protein export pathway. Mol Microbiol 2000;35:260–74.

Better M, Bernhard SL, Lei SP, Fishwild DM, Lane JA, Carroll SF, et al. Potent anti-CD5 ricin A chain immunoconjugates from bacterially produced Fab′ and F(ab′)2. Proc Natl Acad Sci U S A 1993; 90: 457–461.

Binet R, Letoffe S, Ghigo JM, Delepelaire P, Wandersman C. Protein secretion by Gram-negative bacterial ABC exporters—a review. Gene 1997;192:7–11.

Blaudeck N, Sprenger GA, Freudl R, Wiegert T. Specificity of signal peptide recognition in tat-dependent bacterial protein translocation. J Bacteriol 2001;183:604–10.

Blight MA, Holland IB. Heterologous protein secretion and the versatile *Escherichia coli* haemolysin translocator. Trends Biotechnol 1994;12:450–5.

Bogsch EG, Sargent F, Stanley NR, Berks BC, Robinson C, Palmer T. An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. J Biol Chem 1998; 273:18003–6.

Bowers CW, Lau F, Silhavy TJ. Secretion of LamB–LacZ by the signal recognition particle pathway of *Escherichia coli*. J Bacteriol 2003;185:5697–705.

Breyton C, Haase W, Rapoport TA, Kuhlbrandt W, Collinson I. Three-dimensional structure of the bacterial protein–translocation complex SecYEG. Nature 2002;418:662–5.

Buchanan G, de Leeuw E, Stanley NR, Wexler M, Berks BC, Sargent F, et al. Functional complexity of the twin-arginine translocase TatC component revealed by site-directed mutagenesis. Mol Microbiol 2002; 43:1457–70.

Butkus ME, Prundeanu LB, Oliver DB. Translocon "pulling" of nascent SecM controls the duration of its translational pause and secretion-responsive secA regulation. J Bacteriol 2003;185:6719–22.

Carrio MM, Villaverde A. Construction and deconstruction of bacterial inclusion bodies. J Biotechnol 2002;96:3–12.

Chatel JM, Adel-Patient K, Creminon C, Wal JM. Expression of a lipocalin in prokaryote and eukaryote cells: quantification and structural characterization of recombinant bovine beta-lactoglobulin. Protein Expr Purif 1999;16:70–5.

Choi JH, Lee SY. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol 2004;64:625–35.

Choi JH, Jeong KJ, Kim SC, Lee SY. Efficient secretory production of alkaline phosphatase by high cell density culture of recombinant *Escherichia coli* using the *Bacillus* sp endoxylanase signal sequence. Appl Microbiol Biotechnol 2000;53:640–5.

Christie PJ. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. Mol Microbiol 2001;40:294–305.

Cornelis P. Expressing genes in different *Escherichia coli* compartments. Curr Opin Biotechnol 2000;11:450–4.

Cornelis GR, Van Gijsegem F. Assembly and function of type III secretory systems. Annu Rev Microbiol 2000;54:735–74.

Cristobal S, de Gier JW, Nielsen H, von Heijne G. Competition between Sec- and TAT-dependent protein translocation in *Escherichia coli*. EMBO J 1999;18:2982–90.

Cristobal S, Scotti P, Luirink J, von Heijne G, de Gier JW. The signal recognition particle-targeting pathway does not necessarily deliver proteins to the sec-translocase in *Escherichia coli*. J Biol Chem 1999; 274:20068–70.

de Gier JW, Luirink J. Biogenesis of inner membrane proteins in *Escherichia coli*. Mol Microbiol 2001; 40:314–22.

de Gier JW, Scotti PA, Saaf A, Valent QA, Kuhn A, Luirink J, et al. Differential use of the signal recognition particle translocase targeting pathway for inner membrane protein assembly in *Escherichia coli*. Proc Natl Acad Sci U S A 1998;95:14646–51.

de Keyzer J, Van Der Does C, Driessen AJ. Kinetic analysis of the translocation of fluorescent precursor proteins into *Escherichia coli* membrane vesicles. J Biol Chem 2002;277:46059–65.

de Keyzer J, van der Does C, Swaving J, Driessen AJ. The F286Y mutation of PrlA4 tempers the signal sequence suppressor phenotype by reducing the SecA binding affinity. FEBS Lett 2002;510:17–21.

de Keyzer J, van der Does C, Driessen AJ. The bacterial translocase: a dynamic protein channel complex. Cell Mol Life Sci 2003a;60:2034–52.

de Keyzer J, van der Does C, Kloosterman TG, Driessen AJ. Direct demonstration of ATP-dependent release of SecA from a translocating preprotein by surface plasmon resonance. J Biol Chem 2003b;278:29581–6.

Dekker C, de Kruijff B, Gros P. Crystal structure of SecB from *Escherichia coli*. J Struct Biol 2003; 144:313–9.

del Castillo FJ, Moreno F, del Castillo I. Secretion of the *Escherichia coli* K-12 SheA hemolysin is independent of its cytolytic activity. FEMS Microbiol Lett 2001;204:281–5.

de Leeuw E, Granjon T, Porcelli I, Alami M, Carr SB, Muller M, et al. Oligomeric properties and signal peptide binding by *Escherichia coli* Tat protein transport complexes. J Mol Biol 2002;322:1135–46.

De Lisa MP, Samuelson P, Palmer T, Georgiou G. Genetic analysis of the twin arginine translocator secretion pathway in bacteria. J Biol Chem 2002;277:29825–31.

De Lisa MP, Tullman D, Georgiou G. Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. Proc Natl Acad Sci U S A 2003;100:6115–20.

De Lisa MP, Lee P, Palmer T, Georgiou G. Phage shock protein PspA of *Escherichia coli* relieves saturation of protein export via the Tat pathway. J Bacteriol 2004;186:366–73.

Drew D, Froderberg L, Baars L, de Gier JW. Assembly and overexpression of membrane proteins in *Escherichia coli*. Biochim Biophys Acta 2003;1610:3–10.

Driessen AJ. SecB, a molecular chaperone with two faces. Trends Microbiol 2001;9:193–6.

Driessen AJ, Fekkes P, van der Wolk JP. The Sec system. Curr Opin Microbiol 1998;1:216–22.

Driessen AJ, Manting EH, van der Does C. The structural basis of protein targeting and translocation in bacteria. Nat Struct Biol 2001;8:492–8.

Duong F. Binding, activation and dissociation of the dimeric SecA ATPase at the dimeric SecYEG translocase. EMBO J 2003;22:4375–84.

Economou A. Following the leader: bacterial protein export through the Sec pathway. Trends Microbiol 1999;7:315–20.

Eichler J. Evolution of the prokaryotic protein translocation complex: a comparison of archaeal and bacterial versions of SecDF. Mol Phylogenet Evol 2003;27:504–9.

Eitan A, Bibi E. The core *Escherichia coli* signal recognition particle receptor contains only the N and G domains of FtsY. J Bacteriol 2004;186:2492–4.

Engler DA, Matsunami RK, Campion SR, Stringer CD, Stevens A, Niyogi SK. Cloning of authentic human epidermal growth factor as a bacterial secretory protein and its initial structure–function analysis by site-directed mutagenesis. J Biol Chem 1988;263:12384–90.

Eser M, Ehrmann M. SecA-dependent quality control of intracellular protein localization. Proc Natl Acad Sci U S A 2003;100:13231–4.

Feilmeier BJ, Iseminger G, Schroeder D, Webber H, Phillips GJ. Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. J Bacteriol 2000;182:4068–76.

Fekkes P, Driessen AJ. Protein targeting to the bacterial cytoplasmic membrane. Microbiol Mol Biol Rev 1999;63:161–73.

Fekkes P, de Wit JG, van der Wolk JP, Kimsey HH, Kumamoto CA, Driessen AJ. Preprotein transfer to the *Escherichia coli* translocase requires the co-operative binding of SecB and the signal sequence to SecA. Mol Microbiol 1998;29:1179–90.

Fernandez LA, Berenguer J. Secretion and assembly of regular surface structures in Gram-negative bacteria. FEMS Microbiol Rev 2000;24:21–44.

Fernandez LA, de Lorenzo V. Formation of disulphide bonds during secretion of proteins through the periplasmic-independent type I pathway. Mol Microbiol 2001;40:332–46.

Fernandez LA, Sola I, Enjuanes L, de Lorenzo V. Specific secretion of active single-chain Fv antibodies into the supernatants of *Escherichia coli* cultures by use of the hemolysin system. Appl Environ Microbiol 2000;66:5024–9.

Francetic O, Pugsley AP. The cryptic general secretory pathway (gsp) operon of *Escherichia coli* K-12 encodes functional proteins. J Bacteriol 1996;178:3544–9.

Francetic O, Belin D, Badaut C, Pugsley AP. Expression of the endogenous type II secretion pathway in *Escherichia coli* leads to chitinase secretion. EMBO J 2000;19:6697–703.

Froderberg L, Houben E, Samuelson JC, Chen M, Park SK, Phillips GJ, et al. Versatility of inner membrane protein biogenesis in *Escherichia coli*. Mol Microbiol 2003;47:1015–27.

Fu Z, Hamid SB, Razak CN, Basri M, Salleh AB, Rahman RN. Secretory expression in *Escherichia coli* and single-step purification of a heat-stable alkaline protease. Protein Expr Purif 2003;28:63–8.

Gentschev I, Hess J, Goebel W. Change in the cellular localization of alkaline phosphatase by alteration of its carboxy-terminal sequence. Mol Gen Genet 1990;222:211–6.

Gentschev I, Dietrich G, Goebel W. The *E coli* alpha-hemolysin secretion system and its use in vaccine development. Trends Microbiol 2002;10:39–45.

Gottesman S. Proteases and their targets in *Escherichia coli*. Annu Rev Genet 1996;30:465–506.
Gouffi K, Gerard F, Santini CL, Wu LF. Dual topology of the *Escherichia coli* TatA protein. J Biol Chem 2004;279:11608–15.
Goulding CW, Jeanne Perry L. Protein production in *Escherichia coli* for structural studies by X-ray crystallography. J Struct Biol 2003;142:133–43.
Gu S-Q, Peske F, Wieden H-J, Rodnina MV, Wintermeyer W. The signal recognition particle binds to protein L23 at the peptide exit of the *Escherichia coli* ribosome. RNA 2003;9:566–73.
Guisez Y, Fache I, Campfield LA, Smith FJ, Farid A, Plaetinck G, et al. Efficient secretion of biologically active recombinant OB protein (leptin) in *Escherichia coli*, purification from the periplasm and characterization. Protein Expr Purif 1998;12:249–58.
Gumpert J, Hoischen C. Use of cell wall-less bacteria (L-forms) for efficient expression and secretion of heterologous gene products. Curr Opin Biotechnol 1998;9:506–9.
Hammarberg B, Nygren PA, Holmgren E, Elmblad A, Tally M, Hellman U, et al. Dual affinity fusion approach and its use to express recombinant human insulin-like growth factor II. Proc Natl Acad Sci U S A 1989;86:4367–71.
Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in *Escherichia coli*. Trends Biotechnol 1998;16:54–60.
Hasenwinkle D, Jervis E, Kops O, Liu C, Lesnicki G, Haynes C, et al. Very high-level production and export in *Escherichia coli* of a cellulose binding domain for use in a generic secretion–affinity fusion system. Biotechnol Bioeng 1997;55:854–63.
Herskovits AA, Bochkareva ES, Bibi E. New prospects in studying the bacterial signal recognition particle pathway. Mol Microbiol 2000;38:927–39.
Hesterkamp T, Hauser S, Lutcke H, Bukau B. *Escherichia coli* trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. Proc Natl Acad Sci U S A 1996;93:4437–41.
Hoffmann F, van den Heuvel J, Zidek N, Rinas U. Minimizing inclusion body formation during recombinant protein production in *Escherichia coli* at bench and pilot plant scale. Enzyme Microb Technol 2004;34:235–41.
Hoischen C, Fritsche C, Gumpert J, Westermann M, Gura K, Fahnert B. Novel bacterial membrane surface display system using cell wall-less L-forms of *Proteus mirabilis* and *Escherichia coli*. Appl Environ Microbiol 2002;68:525–31.
Holland IB, Kenny B, Steipe B, Pluckthun A. Secretion of heterologous proteins in *Escherichia coli*. Methods Enzymol 1990;182:132–43.
Huang HC, Sherman MY, Kandror O, Goldberg AL. The molecular chaperone DnaJ is required for the degradation of a soluble abnormal protein in *Escherichia coli*. J Biol Chem 2001;276:3920–8.
Ignatova Z, Mahsunah A, Georgieva M, Kasche V. Improvement of posttranslational bottlenecks in the production of penicillin amidase in recombinant *Escherichia coli* strains. Appl Environ Microbiol 2003;69:1237–45.
Ize B, Gerard F, Zhang M, Chanal A, Voulhoux R, Palmer T, et al. In vivo dissection of the Tat translocation pathway in *Escherichia coli*. J Mol Biol 2002;317:327–35.
Jacob-Dubuisson F, Locht C, Antoine R. Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. Mol Microbiol 2001;40:306–13.
Jeong KJ, Lee SY. Secretory production of human leptin in *Escherichia coli*. Biotechnol Bioeng 2000; 67:398–407.
Jeong KJ, Lee SY. Secretory production of human granulocyte colony-stimulating factor in *Escherichia coli*. Protein Expr Purif 2001;23:311–8.
Joly JC, Leung WS, Swartz JR. Overexpression of *Escherichia coli* oxidoreductases increases recombinant insulin-like growth factor-I accumulation. Proc Natl Acad Sci U S A 1998;95:2773–7.
Kaderbhai MA, Ugochukwu CC, Kelly SL, Lamb DC. Export of cytochrome *P*450 105D1 to the periplasmic space of *Escherichia coli*. Appl Environ Microbiol 2001;67:2136–8.
Kadokura H, Katzen F, Beckwith J. Protein disulfide bond formation in prokaryotes. Annu Rev Biochem 2003;72:111–35.
Kajava AV, Zolov SN, Kalinin AE, Nesmeyanova MA. The net charge of the first 18 residues of the mature sequence affects protein translocation across the cytoplasmic membrane of Gram-negative bacteria. J Bacteriol 2000;182:2163–9.

Kang Y, Yoon JW. Effect of modification of connecting peptide of proinsulin on its export. J Biotechnol 1994;36:45–54.

Kebir MO, Kendall DA. SecA specificity for different signal peptides. Biochemistry 2002;41:5573–80.

Keenan RJ, Freymann DM, Stroud RM, Walter P. The signal recognition particle. Annu Rev Biochem 2001;70:755–75.

Kenny B, Haigh R, Holland IB. Analysis of the haemolysin transport process through the secretion from *Escherichia coli* of PCM, CAT or beta-galactosidase fused to the Hly C-terminal signal domain. Mol Microbiol 1991;5:2557–68.

Khokhlova OV, Nesmeianova MA. Interaction of SecB and SecA with the N-terminal region of mature alkaline phosphatase on its secretion in *Escherichia coli*. Mol Biol (Mosk) 2003;37:712–8.

Khokhlova OV, Nesmeyanova MA. Interdependent effects of the charge of the N-terminal region of the signal peptide, SecA, and SecB on secretion of alkaline phosphatase in *Escherichia coli*. Mol Biol (Mosk) 2004;38:239–46.

Kim J, Rusch S, Luirink J, Kendall DA. Is Ffh required for export of secretory proteins? FEBS Lett 2001;505:245–8.

Kleist S, Miksch G, Hitzmann B, Arndt M, Friehs K, Flaschel E. Optimization of the extracellular production of a bacterial phytase with *Escherichia coli* by using different fed-batch fermentation strategies. Appl Microbiol Biotechnol 2003;61:456–62.

Koch HG, Hengelage T, Neumann-Haefelin C, Mac Farlane J, Hoffschulte HK, Schimz KL, et al. In vitro studies with purified components reveal signal recognition particle (SRP) and SecA/SecB as constituents of two independent protein-targeting pathways of *Escherichia coli*. Mol Biol Cell 1999;10:2163–73.

Koch HG, Moser M, Muller M. Signal recognition particle-dependent protein targeting, universal to all kingdoms of life. Rev Physiol, Biochem Pharmacol 2003;146:55–94.

Koebnik R, Locher KP, Van Gelder P. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol Microbiol 2000;37:239–53.

Koronakis V. TolC—the bacterial exit duct for proteins and drugs. FEBS Lett 2003;555:66–71.

Koster M, Bitter W, Tommassen J. Protein secretion mechanisms in Gram-negative bacteria. Int J Med Microbiol 2000;290:325–31.

Kujau MJ, Hoischen C, Riesenberg D, Gumpert J. Expression and secretion of functional miniantibodies McPC603scFvDhlx in cell-wall-less L-form strains of *Proteus mirabilis* and *Escherichia coli*: a comparison of the synthesis capacities of L-form strains with an *E coli* producer strain. Appl Microbiol Biotechnol 1998;49:51–8.

Lee HC, Bernstein HD. The targeting pathway of *Escherichia coli* presecretory and integral membrane proteins is specified by the hydrophobicity of the targeting signal. Proc Natl Acad Sci U S A 2001;98:3471–6.

Lee S, Kim I, Kim D, Bae K, Byun S. High level secretion of recombinant staphylokinase into periplasm of *Escherichia coli*. Biotechnol Lett 1998;20:113–6.

Lee J, Saraswat V, Koh I, Song KB, Park YH, Rhee SK. Secretory production of *Arthrobacter levan* fructotransferase from recombinant *Escherichia coli*. FEMS Microbiol Lett 2001;195:127–32.

Li Y, Chen CX, von Specht BU, Hahn HP. Cloning and hemolysin-mediated secretory expression of a codon-optimized synthetic human interleukin-6 gene in *Escherichia coli*. Protein Expr Purif 2002;25:437–47.

Liao YD, Jeng JC, Wang CF, Wang SC, Chang ST. Removal of N-terminal methionine from recombinant proteins by engineered *E coli* methionine aminopeptidase. Protein Sci 2004;13:1802–10.

Lin W-J, Huang S-W, Chou CP. High-level extracellular production of penicillin acylase by genetic engineering of *Escherichia coli*. J Chem Technol Biotechnol 2001;76:1030–7.

Loo T, Patchett ML, Norris GE, Lott JS. Using secretion to solve a solubility problem: high-yield expression in *Escherichia coli* and purification of the bacterial glycoamidase PNGase F. Protein Expr Purif 2002; 24:90–8.

Lory S. Secretion of proteins and assembly of bacterial surface organelles: shared pathways of extracellular protein targeting. Curr Opin Microbiol 1998;1:27–35.

Lowenadler B, Jansson B, Paleus S, Holmgren E, Nilsson B, Moks T, et al. A gene fusion system for generating antibodies against short peptides. Gene 1987;58:87–97.

Mackman N, Baker K, Gray L, Haigh R, Nicaud JM, Holland IB. Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. EMBO J 1987;6:2835–41.

Maier R, Eckert B, Scholz C, Lilie H, Schmid FX. Interaction of trigger factor with the ribosome. J Mol Biol 2003;326:585–92.

Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev 1996;60:512–38.

Manosroi J, Tayapiwatana C, Gotz F, Werner RG, Manosroi A. Secretion of active recombinant human tissue plasminogen activator derivatives in *Escherichia coli*. Appl Environ Microbiol 2001;67:2657–64.

Manting EH, Driessen AJ. *Escherichia coli* translocase: the unravelling of a molecular machine. Mol Microbiol 2000;37:226–38.

Manting EH, van Der Does C, Remigy H, Engel A, Driessen AJ. SecYEG assembles into a tetramer to form the active protein translocation channel. EMBO J 2000;19:852–61.

Masip L, Pan JL, Haldar S, Penner-Hahn JE, De Lisa MP, Georgiou G, et al. An engineered pathway for the formation of protein disulfide bonds. Science 2004;303:1185–9.

Maskos K, Huber-Wunderlich M, Glockshuber R. DsbA and DsbC-catalyzed oxidative folding of proteins with complex disulfide bridge patterns in vitro and in vivo. J Mol Biol 2003;325:495–513.

Mergulhão F, Monteiro G. Secretion capacity limitations of the Sec pathway in *Escherichia coli*. J Microb Biotechnol 2004;14:128–33.

Mergulhão F, Monteiro G, Kelly A, Taipa M, Cabral J. Recombinant human proinsulin: a new approach in gene assembly and protein expression. J Microb Biotechnol 2000;10:690–3.

Mergulhão FJ, Monteiro GA, Cabral JM, Taipa MA. A quantitative ELISA for monitoring the secretion of ZZ-fusion proteins using SpA domain as immunodetection reporter system. Mol Biotechnol 2001; 19:239–44.

Mergulhão F, Monteiro G, Larsson G, Sandem A, Farewell A, Nystrom T, et al. Medium and copy number effects on the secretion of human proinsulin in *Escherichia coli* using the universal stress promoters *uspA* and *uspB*. Appl Microbiol Biotechnol 2003a;61:495–501.

Mergulhão FJM, Monteiro GA, Larsson G, Bostrom M, Farewell A, Nystrom T, et al. Evaluation of inducible promoters on the secretion of a ZZ-Proinsulin fusion protein. Biotechnol Appl Biochem 2003b; 38:87–93.

Mergulhão FJ, Taipa MA, Cabral JM, Monteiro GA. Evaluation of bottlenecks in proinsulin secretion by *Escherichia coli*. J Biotechnol 2004a;109:31–43.

Mergulhão FJM, Monteiro GA, Cabral JMS, Taipa MA. Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*. J Microb Biotechnol 2004b;14:1–14.

Mikhaleva NI, Golovastov VV, Zolov SN, Bogdanov MV, Dowhan W, Nesmeyanova MA. Depletion of phosphatidylethanolamine affects secretion of *Escherichia coli* alkaline phosphatase and its transcriptional expression. FEBS Lett 2001;493:85–90.

Miksch G, Neitzel R, Fiedler E, Friehs K, Flaschel E. Extracellular production of a hybrid beta-glucanase from *Bacillus* by *Escherichia coli* under different cultivation conditions in shaking cultures and bioreactors. Appl Microbiol Biotechnol 1997;47:120–6.

Miksch G, Kleist S, Friehs K, Flaschel E. Overexpression of the phytase from *Escherichia coli* and its extracellular production in bioreactors. Appl Microbiol Biotechnol 2002;59:685–94.

Miller A, Wang L, Kendall DA. Synthetic signal peptides specifically recognize SecA and stimulate ATPase activity in the absence of preprotein. J Biol Chem 1998;273:11409–12.

Moks T, Abrahmsen L, Holmgren E, Bilich M, Olsson A, Uhlen M, et al. Expression of human insulin-like growth factor I in bacteria: use of optimized gene fusion vectors to facilitate protein purification. Biochemistry 1987;26:5239–44.

Mori H, Ito K. The Sec protein–translocation pathway. Trends Microbiol 2001;9:494–500.

Mori H, Ito K. Biochemical characterization of a mutationally altered protein translocase: proton motive force stimulation of the initiation phase of translocation. J Bacteriol 2003;185:405–12.

Mukherjee KJ, Rowe DCD, Watkins NA, Summers DK. Studies of single-chain antibody expression in quiescent *Escherichia coli*. Appl Environ Micobiol 2004;70:3005–12.

Nagai K, Oubridge C, Kuglstatter A, Menichelli E, Isel C, Jovine L. Structure, function and evolution of the signal recognition particle. EMBO J 2003;22:3479–85.

Nakai K, Kanehisa M. Expert system for predicting protein localization sites in Gram-negative bacteria. Proteins 1991;11:95–110.

Nakano H, Kawakami Y, Nishimura H. Secretion of genetically-engineered dihydrofolate reductase from *Escherichia coli* using an *E coli* alpha-hemolysin membrane translocation system. Appl Microbiol Biotechnol 1992;37:765–71.

Nakatogawa H, Ito K. The ribosomal exit tunnel functions as a discriminating gate. Cell 2002;108:629–36.

Nakatogawa H, Ito K. Intraribosomal regulation of expression and fate of proteins. ChemBioChem 2004;5:48–51.

Nakatogawa H, Murakami A, Ito K. Control of SecA and SecM translation by protein secretion. Curr Opin Microbiol 2004;7:145–50.

Neumann-Haefelin C, Schafer U, Muller M, Koch HG. SRP-dependent co-translational targeting and SecA-dependent translocation analyzed as individual steps in the export of a bacterial protein. EMBO J 2000;19:6419–26.

Nilsson B, Abrahmsen L. Fusions to staphylococcal protein A. Methods Enzymol 1990;185:144–61.

Nishiyama K, Fukuda A, Morita K, Tokuda H. Membrane deinsertion of SecA underlying proton motive force-dependent stimulation of protein translocation. EMBO J 1999;18:1049–58.

Nouwen N, Driessen AJ. SecDFyajC forms a heterotetrameric complex with YidC. Mol Microbiol 2002;44:1397–405.

Nouwen N, Ranson N, Saibil H, Wolpensinger B, Engel A, Ghazi A, et al. Secretin PulD: association with pilot PulS, structure, and ion-conducting channel formation. Proc Natl Acad Sci U S A 1999;96:8173–7.

Nouwen N, Stahlberg H, Pugsley AP, Engel A. Domain structure of secretin PulD revealed by limited proteolysis and electron microscopy. EMBO J 2000;19:2229–36.

Oelschlaeger P, Lange S, Schmitt J, Siemann M, Reuss M, Schmid RD. Identification of factors impeding the production of a single-chain antibody fragment in *Escherichia coli* by comparing in vivo and in vitro expression. Appl Microbiol Biotechnol 2003;61:123–32.

Oliver D, Norman J, Sarker S. Regulation of *Escherichia coli* secA by cellular protein secretion proficiency requires an intact gene X signal sequence and an active translocon. J Bacteriol 1998;180:5240–2.

Oresnik IJ, Ladner CL, Turner RJ. Identification of a twin-arginine leader-binding protein. Mol Microbiol 2001;40:323–31.

Palacios JL, Zaror I, Martinez P, Uribe F, Opazo P, Socias T, et al. Subset of hybrid eukaryotic proteins is exported by the type I secretion system of *Erwinia chrysanthemi*. J Bacteriol 2001;183:1346–58.

Pallen MJ, Chaudhuri RR, Henderson IR. Genomic analysis of secretion systems. Curr Opin Microbiol 2003;6:519–27.

Palmer T, Berks BC. Moving folded proteins across the bacterial cell membrane. Microbiology 2003;149:547–56.

Palmer T, Sargent F, Berks BC. Light traffic: photo-crosslinking a novel transport system. Trends Biochem Sci 2004;29:55–7.

Patzelt H, Rudiger S, Brehmer D, Kramer G, Vorderwulbecke S, Schaffitzel E, et al. Binding specificity of *Escherichia coli* trigger factor. Proc Natl Acad Sci U S A 2001;98:14244–9.

Pedelacq JD, Piltch E, Liong EC, Berendzen J, Kim CY, Rho BS, et al. Engineering soluble proteins for structural genomics. Nat Biotechnol 2002;20:927–32.

Peterson JH, Woolhead CA, Bernstein HD. Basic amino acids in a distinct subset of signal peptides promote interaction with the signal recognition particle. J Biol Chem 2003;278:46155–62.

Porcelli I, de Leeuw E, Wallis R, van den Brink-van der Laan E, de Kruijff B, Wallace BA, et al. Characterization and membrane assembly of the TatA component of the *Escherichia coli* twin-arginine protein transport system. Biochemistry 2002;41:13690–7.

Possot OM, Vignon G, Bomchil N, Ebel F, Pugsley AP. Multiple interactions between pullulanase secreton components involved in stabilization and cytoplasmic membrane association of PulE. J Bacteriol 2000;182:2142–52.

Pradel N, Santini CL, Ye CY, Fevat L, Gerard F, Alami M, et al. Influence of tat mutations on the ribose-binding protein translocation in *Escherichia coli*. Biochem Biophys Res Commun 2003;306:786–91.

Pugsley AP. The complete general secretory pathway in Gram-negative bacteria. Microbiol Rev 1993;57:50–108.

Pugsley AP, Francetic O. Protein secretion in *Escherichia coli* K-12: dead or alive? Cell Mol Life Sci 1998;54:347–52.

Pugsley AP, Francetic O, Possot OM, Sauvonnet N, Hardie K. Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria—a review. Gene 1997;192:13–9.

Pugsley AP, Francetic O, Driessen AJ, de Lorenzo V. Getting out: protein traffic in prokaryotes. Mol Microbiol 2004;52:3–11.

Qi HY, Bernstein HD. SecA is required for the insertion of inner membrane proteins targeted by the *Escherichia coli* signal recognition particle. J Biol Chem 1999;274:8993–7.

Qiu J, Swartz JR, Georgiou G. Expression of active human tissue-type plasminogen activator in *Escherichia coli*. Appl Environ Microbiol 1998;64:4891–6.

Raina S, Missiakas D. Making and breaking disulfide bonds. Annu Rev Microbiol 1997;51:179–202.

Randall LL, Hardy SJ. SecB, one small chaperone in the complex milieu of the cell. Cell Mol Life Sci 2002;59:1617–23.

Ray MV, Meenan CP, Consalvo AP, Smith CA, Parton DP, Sturmer AM, et al. Production of salmon calcitonin by direct expression of a glycine-extended precursor in *Escherichia coli*. Protein Expr Purif 2002;26:249–59.

Rinas U, Hoffmann F. Selective leakage of host-cell proteins during high-cell-density cultivation of recombinant and non-recombinant *Escherichia coli*. Biotechnol Prog 2004;20:679–87.

Rippmann JF, Klein M, Hoischen C, Brocks B, Rettig WJ, Gumpert J, et al. Procaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of *Proteus mirabilis* leads to active product and overcomes the limitations of periplasmic expression in *Escherichia coli*. Appl Environ Microbiol 1998;64:4862–9.

Robbens J, Raeymaekers A, Steidler L, Fiers W, Remaut E. Production of soluble and active recombinant murine interleukin-2 in *Escherichia coli*: high level expression, Kil-induced release, and purification. Protein Expr Purif 1995;6:481–6.

Rosenberg HF. Isolation of recombinant secretory proteins by limited induction and quantitative harvest. BioTechniques 1998;24:188–91.

Rowe DC, Summers DK. The quiescent-cell expression system for protein synthesis in *Escherichia coli*. Appl Environ Microbiol 1999;65:2710–5.

Samuelsson E, Jonasson P, Viklund F, Nilsson B, Uhlen M. Affinity-assisted in vivo folding of a secreted human peptide hormone in *Escherichia coli*. Nat Biotechnol 1996;14:751–5.

Sanders C, Wethkamp N, Lill H. Transport of cytochrome c derivatives by the bacterial Tat protein translocation system. Mol Microbiol 2001;41:241–6.

Sandkvist M. Biology of type II secretion. Mol Microbiol 2001;40:271–83.

Sandkvist M, Bagdasarian M. Secretion of recombinant proteins by Gram-negative bacteria. Curr Opin Biotechnol 1996;7:505–11.

Santini CL, Ize B, Chanal A, Muller M, Giordano G, Wu LF. A novel sec-independent periplasmic protein translocation pathway in *Escherichia coli*. EMBO J 1998;17:101–12.

Santini CL, Bernadac A, Zhang M, Chanal A, Ize B, Blanco C, et al. Translocation of jellyfish green fluorescent protein via the Tat system of *Escherichia coli* and change of its periplasmic localization in response to osmotic up-shock. J Biol Chem 2001;276:8159–64.

Sapriel G, Wandersman C, Delepelaire P. The N terminus of the HasA protein and the SecB chaperone cooperate in the efficient targeting and secretion of HasA via the ATP-binding cassette transporter. J Biol Chem 2002;277:6726–32.

Sapriel G, Wandersman C, Delepelaire P. The SecB chaperone is bifunctional in *Serratia marcescens*: SecB is involved in the Sec pathway and required for HasA secretion by the ABC transporter. J Bacteriol 2003;185:80–8.

Sargent F, Bogsch EG, Stanley NR, Wexler M, Robinson C, Berks BC, et al. Overlapping functions of components of a bacterial Sec-independent protein export pathway. EMBO J 1998;17:3640–50.

Sargent F, Stanley NR, Berks BC, Palmer T. Sec-independent protein translocation in *Escherichia coli* A distinct and pivotal role for the TatB protein. J Biol Chem 1999;274:36073–82.

Sargent F, Gohlke U, De Leeuw E, Stanley NR, Palmer T, Saibil HR, et al. Purified components of the *Escherichia coli* Tat protein transport system form a double-layered ring structure. Eur J Biochem 2001;268:3361–7.

Sarker S, Oliver D. Critical regions of secM that control its translation and secretion and promote secretion-specific secA regulation. J Bacteriol 2002;184:2360–9.

Sarker S, Rudd KE, Oliver D. Revised translation start site for secM defines an atypical signal peptide that regulates *Escherichia coli* secA expression. J Bacteriol 2000;182:5592–5.

Schaffner J, Winter J, Rudolph R, Schwarz E. Cosecretion of chaperones and low-molecular-size medium additives increases the yield of recombinant disulfide-bridged proteins. Appl Environ Microbiol 2001;67:3994–4000.

Schierle CF, Berkmen M, Huber D, Kumamoto C, Boyd D, Beckwith J. The DsbA signal sequence directs efficient, cotranslational export of passenger proteins to the *Escherichia coli* periplasm via the signal recognition particle pathway. J Bacteriol 2003;185:5706–13.

Schmidt MO, Brosh Jr RM, Oliver DB. *Escherichia coli* SecA helicase activity is not required in vivo for efficient protein translocation or autogenous regulation. J Biol Chem 2001;276:37076–85.

Schnell DJ, Hebert DN. Protein translocons: multifunctional mediators of protein translocation across membranes. Cell 2003;112:491–505.

Scotti PA, Valent QA, Manting EH, Urbanus ML, Driessen AJ, Oudega B, et al. SecA is not required for signal recognition particle-mediated targeting and initial membrane insertion of a nascent inner membrane protein. J Biol Chem 1999;274:29883–8.

Scotti PA, Urbanus ML, Brunner J, de Gier JW, von Heijne G, van der Does C, et al. YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. EMBO J 2000;19:542–9.

Serek J, Bauer-Manz G, Struhalla G, Van Den Berg L, Kiefer D, Dalbey R, et al. *Escherichia coli* YidC is a membrane insertase for Sec-independent proteins. EMBO J 2004;23:294–301.

Shimokawa N, Mori H, Ito K. Importance of transmembrane segments in *Escherichia coli* SecY. Mol Genet Genomics 2003;269:180–7.

Shokri A, Sandén AM, Larsson G. Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*. Appl Microbiol Biotechnol 2003;60:654–64.

Sijbrandi R, Urbanus ML, ten Hagen-Jongman CM, Bernstein HD, Oudega B, Otto BR, et al. Signal recognition particle (SRP)-mediated targeting and Sec-dependent translocation of an extracellular *Escherichia coli* protein. J Biol Chem 2003;278:4654–9.

Simmons LC, Yansura DG. Translational level is a critical factor for the secretion of heterologous proteins in *Escherichia coli*. Nat Biotechnol 1996;14:629–34.

Slos P, Speck D, Accart N, Kolbe HV, Schubnel D, Bouchon B, et al. Recombinant cholera toxin B subunit in *Escherichia coli*: high-level secretion, purification, and characterization. Protein Expr Purif 1994; 5:518–26.

Soares CRJ, Gomide FIC, Ueda EKM, Bartolini P. Periplasmic expression of human growth hormone via plasmid vectors containing the lambda pL promoter: use of HPLC for product quantification. Protein Eng 2003;16:1131–8.

Stanley NR, Palmer T, Berks BC. The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. J Biol Chem 2000;275:11591–6.

Talmadge K, Gilbert W. Cellular location affects protein stability in *Escherichia coli*. Proc Natl Acad Sci U S A 1982;79:1830–3.

Tan S, Wu W, Liu J, Kong Y, Pu Y, Yuan R. Efficient expression and secretion of recombinant hirudin III in *E coli* using the L-asparaginase II signal sequence. Protein Expr Purif 2002;25:430–6.

Tanaka T, Horio T, Matuo Y. Secretory production of recombinant human C-reactive protein in *Escherichia coli*, capable of binding with phosphorylcholine, and its characterization. Biochem Biophys Res Commun 2002;295:163–6.

Thanabalu T, Koronakis E, Hughes C, Koronakis V. Substrate-induced assembly of a contiguous channel for protein export from *E coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. EMBO J 1998;17:6487–96.

Thomas JD, Daniel RA, Errington J, Robinson C. Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. Mol Microbiol 2001;39:47–53.

Tian H, Boyd D, Beckwith J. A mutant hunt for defects in membrane protein assembly yields mutations affecting the bacterial signal recognition particle and Sec machinery. Proc Natl Acad Sci U S A 2000;97:4730–5.

Tokuyasu K, Kaneko S, Hayashi K, Mori Y. Production of a recombinant chitin deacetylase in the culture medium of *Escherichia coli* cells. FEBS Lett 1999;458:23–6.

Tong L, Lin Q, Wong WK, Ali A, Lim D, Sung WL, et al. Extracellular expression, purification, and characterization of a winter flounder antifreeze polypeptide from *Escherichia coli*. Protein Expr Purif 2000;18:175–81.

Tziatzios C, Schubert D, Lotz M, Gundogan D, Betz H, Schagger H, et al. The bacterial protein-translocation complex: SecYEG dimers associate with one or two SecA molecules. J Mol Biol 2004;340:513–24.

Ullers RS, Luirink J, Harms N, Schwager F, Georgopoulos C, Genevaux P. SecB is a bona fide generalized chaperone in V. Proc Natl Acad Sci U S A 2004;101:7583–8.

Urbanus ML, Froderberg L, Drew D, Bjork P, de Gier JW, Brunner J, et al. Targeting, insertion, and localization of *Escherichia coli* YidC. J Biol Chem 2002;277:12718–23.

Valent QA, Scotti PA, High S, de Gier JW, von Heijne G, Lentzen G, et al. The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon. EMBO J 1998;17:2504–12.

van der Laan M, Houben EN, Nouwen N, Luirink J, Driessen AJ. Reconstitution of Sec-dependent membrane protein insertion: nascent FtsQ interacts with YidC in a SecYEG-dependent manner. EMBO Rep 2001;2:519–23.

van der Wal FJ, Luirink J, Oudega B. Bacteriocin release proteins: mode of action, structure, and biotechnological application. FEMS Microbiol Rev 1995a;17:381–99.

van der Wal FJ, ten Hagen CM, Oudega B, Luirink J. The stable bacteriocin release protein signal peptide, expressed as a separate entity, functions in the release of cloacin DF13. FEMS Microbiol Lett 1995b;131:173–7.

van der Wal FJ, ten Hagen-Jongman CM, Oudega B, Luirink J. Optimization of bacteriocin-release-protein-induced protein release by *Escherichia coli*: extracellular production of the periplasmic molecular chaperone FaeE. Appl Microbiol Biotechnol 1995c;44:459–65.

van der Wal FJ, Koningstein G, ten Hagen CM, Oudega B, Luirink J. Optimization of bacteriocin release protein (BRP)-mediated protein release by *Escherichia coli*: random mutagenesis of the pCloDF13-derived BRP gene to uncouple lethality and quasi-lysis from protein release. Appl Environ Microbiol 1998;64:392–8.

van der Wolk JP, Fekkes P, Boorsma A, Huie JL, Silhavy TJ, Driessen AJ. PrlA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA–SecY interaction during the initiation of translocation. EMBO J 1998;17:3631–9.

Wan EW, Baneyx F. TolAIII co-overexpression facilitates the recovery of periplasmic recombinant proteins into the growth medium of *Escherichia coli*. Protein Expr Purif 1998;14:13–22.

Wang L, Miller A, Kendall DA. Signal peptide determinants of SecA binding and stimulation of ATPase activity. J Biol Chem 2000;275:10154–9.

Wexler M, Sargent F, Jack RL, Stanley NR, Bogsch EG, Robinson C, et al. TatD is a cytoplasmic protein with DNase activity No requirement for TatD family proteins in sec-independent protein export. J Biol Chem 2000;275:16717–22.

Wild K, Rosendal KR, Sinning I. A structural step into the SRP cycle. Mol Microbiol 2004;53:357–63.

Winter J, Neubauer P, Glockshuber R, Rudolph R. Increased production of human proinsulin in the periplasmic space of *Escherichia coli* by fusion to DsbA. J Biotechnol 2001;84:175–85.

Woodbury RL, Topping TB, Diamond DL, Suciu D, Kumamoto CA, Hardy SJ, et al. Complexes between protein export chaperone SecB and SecA Evidence for separate sites on SecA providing binding energy and regulatory interactions. J Biol Chem 2000;275:24191–8.

Xu R, Du P, Fan JJ, Zhang Q, Li TP, Gan RB. High-level expression and secretion of recombinant mouse endostatin by *Escherichia coli*. Protein Expr Purif 2002;24:453–9.

Yahr TL, Wickner WT. Evaluating the oligomeric state of SecYEG in preprotein translocase. EMBO J 2000;19:4393–401.

Yahr TL, Wickner WT. Functional reconstitution of bacterial Tat translocation in vitro. EMBO J 2001;20:2472–9.

Yokoyama S. Protein expression systems for structural genomics and proteomics. Curr Opin Chem Biol 2003;7:39–43.

Zambonelli C, Morandi P, Vanoni MA, Tedeschi G, Servi S, Curti B, et al. Cloning and expression in *Escherichia coli* of the gene encoding *Streptomyces* PMF PLD, a phospholipase D with high transphosphatidylation activity. Enzyme Microb Technol 2003;33:676–88.

Zhou S, Yomano LP, Saleh AZ, Davis FC, Aldrich HC, Ingram LO. Enhancement of expression and apparent secretion of *Erwinia chrysanthemi* endoglucanase (encoded by celZ) in *Escherichia coli* B. Appl Environ Microbiol 1999;65:2439–45.

Zito CR, Oliver D. Two-stage binding of SecA to the bacterial translocon regulates ribosome–translocon interaction. J Biol Chem 2003;278:40640–6.