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MINI-REVIEW

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Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*

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Abstract This paper is a review of strategies to introduce protein into the liquid medium of *Escherichia coli* K-12 industrial production cells. The cell design strategies are generally based on one of two general mechanisms. The first strategy involves a two-stage translocation using active transporters in the cytoplasmic membrane followed by passive transport through the outer membrane. Passive transport is achieved through either external or internal destabilization of the *E. coli* structural components. The latter can be achieved by transplantation of destabilizing components (lysis proteins) that work by permeabilization of the outer membrane from the interior of the cell, or by using cells carrying mutations of structural components. Passive transport can also be achieved by a chemical, mechanical, or enzymatic permeabilization directed from outside the cell. The second strategy is realized through transplantation of proteins capable of active transport over one or both of the membranes. This involves the transplantation of secretion mechanisms into the K-12 cell from pathogenic *E. coli* as well as from other species. The process design strategies are dependent on environmental conditions and must take into account changes in physical parameters, medium design, and influx of limiting carbon source in fed-batch cultivation.

Introduction

The overall goal in recombinant protein production is to simultaneously reach a high specific recombinant protein production rate (g/g,h), a high cell density, and a high product quality. Few strategies are however able to achieve more than one of these at a time and this is a considerable drawback. A high cell density can only be reached by fed-batch cultivation that is analogous to a feed profile, which leads to a continuously decreasing growth rate throughout the process. This profile can however be designed, as well as the point of induction. Both the specific protein productivity and the product quality are strictly dependent on these factors (Bylund et al. 2000; Curless et al. 1990; Hellmuth et al. 1994; Jensen and Carlsen 1989; Ryan et al. 1996; Sandén et al. 2002; Shin et al. 1998).

One strategy to avoid quality problems is to target the protein to different outer compartments of the host cell to give a more-favorable redox potential for proper folding, to avoid inclusion body formation and the majority of proteolytic proteins in the cytoplasm, and to achieve a primary purification. From the periplasm the protein can sometimes “leak” to the medium. This involves a selective passage through the outer membrane, to be distinguished from total cell lysis, which must generally be avoided due to the difficulty of reproducibility and control. Since several of the transport mechanisms are dependent on the physiological status of the cell, specifically the growth rate, both the expression system and the transport system must both be designed to work simultaneously and at their best under the same carbon and energy limitation. In this review, we shall consider the design strategies available for translocation to the medium in relation to industrial fed-batch conditions (Fig. 1).

Cell design for periplasmic transport followed by outer membrane passive transport

Transport to the periplasm

Transport signal recognition is mediated either through (1) the signal recognition particle system, which seems to direct hydrophobic segments and segments which are co-translationally synthesized to the membrane or (2) the recognition of a chaperone, usually SecB but also DnaK. Alkaline phosphatase, ribose-binding protein, and β-lactamase are examples where DnaK, DnaJ, and GrpE

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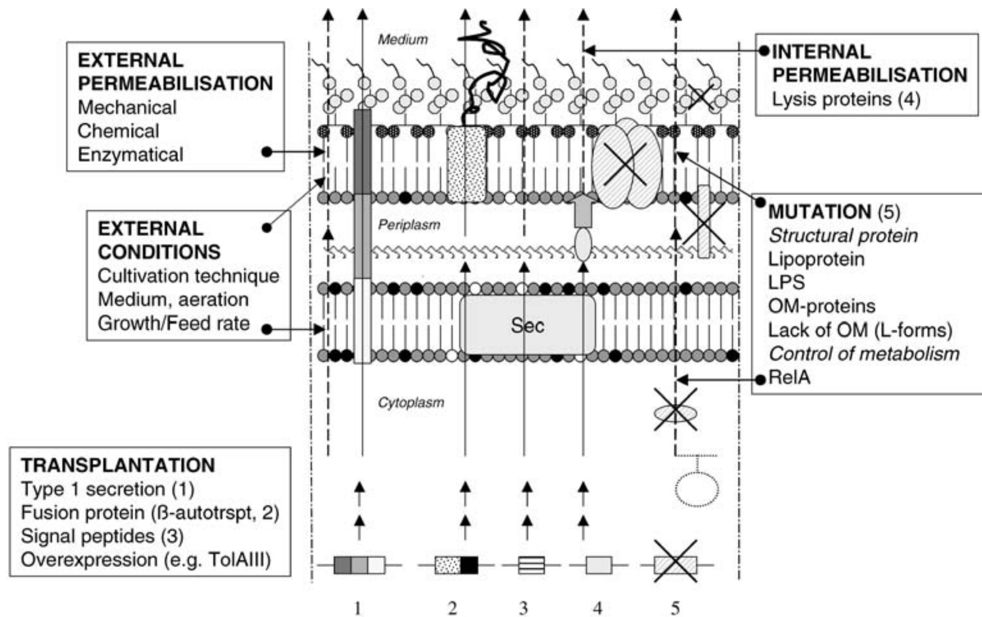

Fig. 1 Summary of strategies for protein translocation during production of recombinant protein. The figure shows a cross-section of *Escherichia coli* K-12 membranes. *Sec*: Inner membrane secretory protein system. The phospholipid structure of the inner and outer membrane is shown in proportion to the amount of phosphatidylethanolamine (*gray*), phosphatidylglycerol (*black*) and cardiolipin (*white*). *Dashed arrows* indicate a passive leakage of protein or transport by unknown mechanisms while *solid arrows* indicate an active transport mechanism. *Gene boxes 1–5* indicate transport by: (1) type 1 secretion/ABC transport, (2) β-autotransport, (3) specific signal peptides (4) lysis proteins or (5) mutation resulting in either lack of structural elements such as lipoproteins, outer membrane proteins, parts of the lipopolysaccharide (LPS) structure or lack of outer membrane or mutation of control proteins leading to defective membrane structure

are alternatively used (Knoblauch et al. 1999; Wild et al. 1996). For some proteins it is recognized that no specific initial transporter is needed (de Gier et al. 1998). The translocation detection system recognizes an unfolded but not native preprotein, as shown by in vitro refolding experiments by Hendrick and Hartl (1993).

It is the positively charged N-terminal of the signal peptide which interacts with either SecA, i.e., the first soluble part of the protein translocon, or the acidic parts of the membrane. The latter lowers the energy barrier for membrane insertion of the hydrophobic α-helical part of the signal sequence (Briggs 1985; Nesmeyanova 1982). The insertion changes both the signal peptide (α-helix formation) and lipid organization (Keller et al. 1996; van Klompenburg and Kruijff 1998; de Vrije et al. 1988). If acidic phospholipids are the initial recognition factor, the polypeptide may have to move laterally in the membrane until it reaches the translocon. The realization of this will depend on the availability of acidic phospholipids and on the membrane fluidity, which is a function of the growth rate dynamics in the fed-batch process (Shokri et al. 2002).

The protein transport pathways converge at the Sec-translocon (Valent et al. 1998), which consist of SecA and two trimeric integral membrane domains, SecYEG and SecDFyajC, which together form a hydrophilic pore in the inner membrane (Doung and Wickner 1997; Driessen et al. 1998, 2001; Manting and Driessen 2000; Pugsley 1993; Valent et al. 1998). A recently identified protein, YidC, seems however competent to work with or without Sec interaction (van der Laan et al. 2001; Scotti et al. 1999). Once translocation is started, a specific stop-transfer hydrophobic segment determines whether translocation is interrupted and the protein diffuses into the lipid phase of the membrane (Doung and Wickner 1998; von Heijne 1997; Mothes et al. 1997). The latter process is strongly affected by the fluidity of the membrane and thus by the lipid composition and structure.

Translocation is driven by two energy sources, ATP and the proton motive force (Economou 2000; Driessen and Wickner 1991). SecA is bound to the membrane by both electrostatic and hydrophobic mechanisms and is stimulated by acidic phospholipids, secretory proteins, SecB, and SecE/SecY (Breukink et al. 1992; Hendrick and Wickner 1991; Ulbrandt et al. 1992). This seems very similar to the GTP-ase activity of FstY, the membrane receptor of the signal recognition particle, where membrane docking is supported by the presence of anionic phospholipids (de Leeuw et al. 2000).

It is not clear whether all proteins need all Sec components for transport. In the case of some proteins, the translocon was used mainly to gain increased transport specificity and efficiency. SecY-deficient strains can still transport the preproteins of OmpA, lipoprotein, and lambda receptor protein with high efficiency in vitro (Yang et al. 1997) and the M13 procoat protein appears not to require either SecA or SecY (de Gier et al. 1998). In vivo studies have, however, shown that SecY and SecE

are essential, whereas other subunits such as SecD and SecF are required only at low temperature (Danese and Silhavy 1998).

Once translocated to the periplasm, the signal sequence is cleaved off, a mechanism which seems to be dependent on several factors, including the structure of the membrane and the availability of the cleavage site. The first part of the product protein sequence is also known to affect the efficiency. This is important, since a frequent drawback in recombinant overproduction of proteins is clogging of the outer part of the inner membrane (Thomas et al. 1997).

A second, Sec-independent, mechanism for the targeting and recognition of inner membrane transport is mediated through the TatABCE translocation pathway (Sargent et al. 2001). This system seems to translocate protein in a folded conformation. It has been shown that translocation of the protein trimethylamine N-oxide reductase (TorA), which needs to be transported in a folded conformation by TAT, was highly dependent on the anionic phospholipids and on the lipid polymorphism (Mikhaleva et al. 1999). The distribution between TAT and Sec transport seems to depend on differences in the signal peptide, where the substrate for the TAT translocators is the specific twin arginine signal sequence (Cristóbal et al. 1999).

Transport to the outer membrane

From the periplasm in *E. coli* K-12 strains proteins can span the area between the membranes, between the membranes and the cell wall, or be directed to the outer membrane. Proteins of this character, such as flagella, outer membrane porines, and lipoproteins, have been used for the surface display of fusion peptides in *E. coli*, and more rarely to enhance leakage to the medium (Cornelis 2000; Francisco et al. 1992). However, Nagahari et al. (1985) reported the export of human ß-endorphin to the culture medium with the promoter, signal peptide and N-terminus of the OmpF protein. Since no other periplasmic protein was detected in the medium, the conclusion was that the protein was actively translocated over both membranes. The transport of proteins to the outer membrane is much less understood than transport over the inner membrane and no general mechanism or signal recognition system has been proposed. It is suggested that outer membrane-anchored proteins have membrane-directed signals, which in the case of lipoproteins are released by specific chaperone-like proteins such as the LolA-LolB system (Yokota et al. 1999).

In the periplasm, chaperones like the disulfide-binding proteins (DsbA, DsbC) and peptidyl-prolyl isomerases (SurA, RotA, FklB, FkpA) are important not only for correct folding of periplasmic proteins but also for outer membrane incorporation. SurA is important for outer membrane proteins, as is Skp, a 17-kDa molecular chaperone involved both in periplasmic protein folding and in the biogenesis of the outer membrane proteins such as OmpA, LamB, and PhoE. Skp insertion into lipid monolayers was strongly enhanced by acidic phospholipids, and Skp has also a strong affinity for lipopolysaccharides (LPS) and maps on the chromosome close to the genes of lipid A synthesis (de Cock et al. 1999). It is proposed that Skp plays an early role in biogenesis and binds proteins directly after translocation through the cytoplasmic membrane. Co-expression of this chaperone was shown to improve the solubility of single-chain antibody fragments in the periplasm (Bothmann and Plückthun 1998; Hayhurst and Harris 1999).

Laboratory strains of *E. coli* do not in general have the terminal branches for transport from the periplasm to the medium. However, it seems that cryptic genes coding for secretion and piliation are also present in *E. coli* K-12. This cryptic terminal secretion branch comprises 13–14 genes, which have been identified as outer membrane exporters of homology to the pullulanase translocon in *Klebsiella*. These proteins have been expressed from other promoters, since the native signal for induction of these genes under laboratory conditions is not yet known (Pugsley and Francetic 1998). An interesting feature is the possibility of using these proteins for secretion, especially since they are probably co-ordinated with the Sec system.

Passive transport over the outer membrane

Mechanical, chemical, and enzymatic permeabilization

A number of authors have suggested methods for partial breakage of the *E. coli* outer membrane and cell wall to achieve a selective disruption/permeabilization and thus to release the periplasmic proteins. These include chemical methods such as the use of polyethylene glycol, guanidine/Triton X-100, EDTA, magnesium, and calcium ions (Hettwer and Wang 1989; Kuboi et al. 1995; Naglac and Wang 1990), mechanical methods such as the use of ultrasonography (Feliu et al. 1997), and enzymatic methods. For a review of the subject, see Naglak and Wang (1990). Enzymatic methods include destabilization of the outer membrane by EDTA to render the cell more susceptible to lysozyme. This enzyme breaks the cell wall β-(1–4)-glycoside linkages from the inside and thus leads to the establishment of spheroplasts of *E. coli* (Bucke 1983; French et al. 1996; Neu and Heppel 1965; Witholt et al. 1976). This technique is however rarely used in large-scale production and not in integration with cultivation.

Permeabilization by import of lysis mechanisms

Permeabilization of the outer membrane can also take place from within the cell and is a common mechanism in pathogenic *E. coli*. This type I secretion system, which employs the Sec transport system in the first step, uses a mechanism based on the action of colicin lysis proteins, and the mechanism seems to resemble that employed by

coliphage autolysis proteins. This mechanism relies on the internal permeabilization of the outer membrane in order to deliver the toxin to the medium. This occurs however without a cleavable amino terminal, i.e., by a mechanism similar to that of the hemolysin system. It has been shown that a specific sequence, the abovementioned release or lysis protein, can activate dormant phospholipase A when an appropriate promoter is used. The suggested mechanism is the hydrolysis of phosphatidylethanolamine (PE) to produce lysophosphatidylethanolamine (LPE), which in turn accumulates in the outer membrane, where it presumably makes the membrane permeable for proteins (van der Wal et al. 1995). This is the case for colicin A and for the colicin A lysis protein (Cal). However, leakage using the colicin E1 lysis protein (Kil) might not employ the same mechanism, since LPE was not detected in the outer membrane. When these proteins are used, OmpA, lipoprotein, LPS, and PE can be found in the medium and the lack of these compounds increases the leakage, as is seen in mutants. If the lysis proteins are overproduced, they easily lead to host cell lysis and death, and several central mechanisms are affected, leading to the inhibition of protein synthesis and loss of sugar uptake. However, the effect of BRP on the membrane may also be due to the signal peptide that is not degraded after processing and accumulates in the cytoplasmic membrane (van der Wal et al. 1998).

Examples of recombinant protein production using ColE1-lysis protein for medium leakage are penicillinase and the periplasmic marker alkaline phosphatase (Aono 1989; Kobayashi et al. 1986), β-glucanase (Miksch et al. 1997), and human growth hormone (Kato et al. 1987). An example of the use of the Cal-protein is the production of human growth hormone (Lloubés et al. 1993). The cloacin system (pCloDF13) has been used for the release of periplasmic ß-lactamase and a periplasmic chaperone (van der Wal et al. 1998), and for human growth hormone (Hsiung et al. 1989). Although several of these production systems are accompanied by cell death, the work of van der Wal et al. (1998) shows that protein engineering could be used to decrease and modulate the lethality of the protein. The internal lysis system builds on the recombinant production of a specific product protein with an appropriate inducible or constitutive promoter and with an appropriate signal peptide for periplasmic production. In some cases, production without signal peptide was used and the protein was still detected in the medium. The release or lysis factor, on the other hand, is co-expressed from a separate promoter. Several promoters have been suggested, in order to achieve a balance between stable production and cell lysis and death.

It is now known that the transmembrane proteins TolQRAB, Orf2, and PAL (*excC*) are part of the multiprotein complex distributed at contact sites between the inner and outer membrane. This system is used to import group A colicins, i.e., some colicins and cloacin, and by some filamentous phages to enter the cell (Lazdunski et al. 1998). Co-overexpression of TolAIII and a modified form of β-lactamase with the OmpA signal peptide resulted in the release of most periplasmic proteins into the extracellular medium in a manner that excluded periplasmic inclusion bodies, which were otherwise formed (Wan and Baneyx 1998). As with the lysis protein, cells were impaired by the TolAIII overexpression and their viability was reduced by three orders of magnitude.

Use of leaky mutants

Mutants of *E. coli* showing higher leakage properties can be formed from lack of structural elements, incomplete synthesis, or incomplete regulation of the synthesis of structural elements.

Several types of membrane or cell wall structural mutants have been described in the literature. The large number of mutants depends mainly on mutations of proteins such as lack of a murein lipoprotein (Lpp), proteins spanning the cytoplasmic to the outer membrane (TolA, TolB), lipoproteins such as the PAL lipoprotein associated with the cell wall (*excC*), outer membrane proteins (Omp, Env) or mutations in the structure of the LPS layer (Braun and Hanke 1974; Chatterjee et al. 1976; Fognini-Lefebvre et al. 1987; Hirota et al. 1977; Lazzaroni and Portalier 1981, 1982, 1992; Nikaido et al. 1977; Tamaki et al. 1971; Weigand and Rothfield 1976; Wu 1972). The LPS mutations vary in that they lack more or less of the polysaccharide structure, and this means that the capacity for binding proteins that are generally closely connected to this structure is reduced. Enhanced leakage, sensibility to dyes, detergents, and antibiotics, and a loss of phage-binding properties have been reported for several of these strains, specifically when they lack the heptose part of the structure and/or the phosphate diester bridges in the backbone. This effect was different in *Salmonella typhimurium*, depending on the growth rate and being more pronounced at higher dilution rates. Several of these strains are useless for production, since they are impaired in growth and lack the robustness required, especially in pharmaceutical production.

A drastic form of a structural mutation for production in *E. coli* is the so-called L-forms (Kujau et al. 1998). These are spherical cells derived by mutation and devoid of the periplasm and murein sacculus, which cannot be regenerated. These were used to produce antibody fragments in a complex medium. With these cells, the same specific productivity was reached as with intact *E. coli* cells for a specific product. This system is not however appropriate for industrial high cell density production, since it probably cannot be used without additional developments regarding stability and medium optimization.

The *relA* mutants are examples of mutations in the regulatory features of the phospholipid and fatty acid synthesis. The reduced guanosine tetraphosphate (ppGpp) formation in these mutants during stringent response could lead to a changed metabolism that in turn affects membrane flexibility. The influence of ppGpp both on

phospholipid and fatty acid synthesis is specifically related to the regulation of the distribution of the amounts of saturated and unsaturated fatty acids and the control of the proportions of PE to cardiolipin and phosphatidylglycerol (PG) (Magnuson et al. 1993; Merlie and Pizer 1973; Polakis et al. 1973; Seyfzadeh et al. 1993; Stein and Bloch 1976). Lower amounts of unsaturated than of saturated fatty acids were indeed found and also an increase in the cyclic fatty acids in a *relA* mutant (Gitter et al. 1995). It was shown that, in contrast to a *relA*$^+$ strain, the mutant releases proteins into the medium during amino acid limitation (Gitter and Riesenberg 1996; Gitter et al. 1995). Since many strains were recently discovered to carry the *relA* mutation, the product protein found in the medium was probably stimulated by the structural changes of this specific mutation. These mutants are however considered difficult to cultivate and to have a high degree of cell lysis accompanying the growth. Examples of *relA* mutants are CP-79, PA-1, JM 109, and MC 4100. From a database search of a common strain library (http//cgsc.biology.yale.edu), over 900 hits of *relA*-related mutants were found. There are many examples of the use of these strains in recombinant protein production, probably because they are generally more easy to transform due to the membrane defects.

Deletion mutants of phospholipid synthesis enzymes can be used to achieve cells with a drastically lower content of PE or PG (van Klompenburg and Kruijff 1998). The strategy of depleting the PE in favor of PG, which should enhance secretion, might be fruitful for enhanced translocation. The deletion of *pssA* leads to higher levels of PG, but it also leads to an accumulation of cardiolipin and phosphatidic acid. These mutations might, however, lack the stability required by a process organism.

The conclusion from the experiments with leaky mutants is that it is not clear whether it is possible to strictly influence the outer membrane with this technique. It is probable that both cell membranes are weakened and that the result is an undesired cell lysis.

Cell design by transplantation of active transport components of pathogenic *E. coli* and other Gram-negative strains

In pathogenic strains of *E. coli* several secretion mechanisms are available that are not present in K-12 strains, but which have a potential for use in traditional production strains. These include production systems for toxins, proteases, and filamentous phages. There are also mechanisms for display of bacterial appendages on the outer surface such as pili, flagella, fimbriae (curli), and crystalline S-layers. These systems have the potential for releasing product proteins, and some have been used for production (Blight et al. 1994; Cornelis 2000). The pathogenic *E. coli* strains include the following secretion systems: type I (e.g., hemolysin), type II (e.g., pullulanase/*Klebsiella*, out-system/*Erwinia*, pI/pIV/filamentous phages, type 4 pili, ß-autotransport/Gram-negative organisms and P and type 1 pili), type III (e.g., Yop production in *Yersinia*, flagellae), and type IV (e.g., pertussis toxin and DNA transfer) (Lory 1998; Stathopoulos et al. 2000). Type II secretion is, according to the terminology, the main terminal branch (MTB) of a general secretory pathway, where Sec transport is the first part. Many different proteins with different types of function, e.g., pilus formation and protein secretion, are secreted by this mechanism and share metabolic platforms (Sauvonnet et al. 2000; Stathopoulos et al. 2000).

ABC, type I secretion, is a mechanism that uses auxillary proteins of the inner and/or outer membrane to facilitate the single-stage transport to the medium without intermediates (Fath and Kolter 1993; Young and Holland 1999). The extracellular protein will thus contain the signal sequence that is not cleaved off. The role model for protein production is the hemolysin transport system (Blight and Holland 1994; Young and Holland 1999). Other ABC transport systems in *E. coli* include the systems for colicin V and microcin secretion (Young and Holland 1999). With the hemolysin transport system, protein is translocated in a protein channel (HlyB/HlyD/TolC), without a periplasmic intermediate, directly to the medium. This has been an attractive system, exemplified in recent reviews (Blight and Holland 1994; Cornelis 2000). Here it is the C-terminal signal peptide of HlyA (which does not contain the toxic properties of hemolysin), which is used for protein transport, but the genes for the secretion apparatus need to be co-expressed (Blight et al. 1994). As in all co-expression systems, this means that several proteins compete with *E. coli* native protein transport, and this makes transport a severe production bottleneck. This requires a very low protein translation rate in order not to overload the transport system. Few examples of production processes are thus available. However, the system is able to recognize a large number of proteins and thus has a high potential for the efficient production of many different proteins.

Transport systems from other organisms for all types of protein localization have also been expressed in *E. coli* with various degrees of success, of which most belong to the type II secretion system (Blight et al. 1994). This includes the β-autotransporters, proteins that in their sequence also include all information for transport and that are present in *E. coli* and several other Gram-negative organisms (Hederson 1998). This simple system consists of one protein part that is anchored to the outer membrane by the C-terminal domain and an N-terminal domain that has the potential to be cleaved off and released into the outer medium. Examples of the use of this transport system for production include the use of *Neisseria gonorrheae* IgA protease for the release of cholera toxin B subunit (CtxB) and single-chain antibody fragment (scFv) into the medium (Klauser et al. 1992). The presentation of peptides, functional T-cell epitopes, and enterotoxin (ETB) with the *E. coli* AIDA ß-autotransporter resulted, however, in surface display of the peptides, since this system does not seem to have the

necessary autoproteolytic capacity (Konieczny et al. 2000).

The question of whether or not proteins are folded or whether they even need to be folded before outer membrane translocation is still open. While the CtxB protein depends on transport in an unfolded state, ScFv fragments were to some extent transported with intact disulfide bridges (Klauser et al. 1992; Veiga et al. 1999). Some of the systems for outer membrane translocation have a proven transport of proteins in a folded conformation. Examples are the disulfide bond formation for pullulanase/*Klebsiella*, (Pugsley 1992) and colicin V/*E. coli* (Young and Holland 1999). Also in the case of the one-stage protein transport process of filamentous phages by the pIV homo-multimer, the protein is assembled before transport (Kazmierczak et al. 1994). However, other proteins of type II secretion, such as the β-autotransporter, need to be unfolded (Klauser et al. 1992). If folding is needed, the question arises as to how such a large pore could function without disruption of the cell integrity. This might be solved by the hypothesis of Kazmierczak et al. (1994) that relies on pIV and PulD being able to induce the *psp* operon (phage-shock response), also induced by osmotic shock, heat, and ethanol treatment, which might lead to occasional and temporary opening of parts of the channel.

An important question is to solve the stress-related interactions in the cell to achieve both high productivity and high-quality protein. In the periplasm, a heat shock-induced system is present that closely resembles and probably interacts with the cytoplasmic *rpoH* system. The *rpoE* gene product, σ^E/σ^{24}, induces this response. There is also a close interaction with the Cpx signaling pathway that is used to control stress response, pilus biogenesis, and the regulation of expression (Danese and Silhavy 1998; Hung et al. 2001; Missiakas and Raina 1997). Most of the excreted proteins have their own set of translocators, ATP-ases, and chaperones that may be protein specific and cannot be exchanged, and this further complicates the situation (Lory 1998; Stathopoulus et al. 2000).

Effects of signal peptide and/or unknown reasons of leakage

Signal peptides from *E. coli* and other sources of several outer membrane proteins that are transported by the general secretion pathway have been used to achieve periplasmic or surface expression with various degrees of success. However, signal peptides seem to play an important role in protein export to the outer medium. This has been described in some cases where no other leakage/transport strategy was employed, but where an alternative signal was used. The signal peptide characteristics are to some extent specific and deviate from the general structure in that the N-terminal can be much larger. This signal peptide is generally imported from other organisms or from pathogenic *E. coli* to the K-12 protein production strains. At least the larger part of this sequence is at some time cleaved off, since most products have their proposed activity and their correct size. The question is thus how this transfer is recognized, how it is fuelled, and how the signal sequence can assist in the process if it is already cleaved off as is described for exported proteins?

The surface-anchored staphylococci Protein A, which has a specifically extended N-terminal part of the signal sequence, was believed to enhance medium transport of insulin-like growth factor I in *E. coli* (Abrahamsén et al. 1986; Moks et al. 1987) and constitutes an example of this category of secretion. Specific interest is naturally given to organisms that have well-developed mechanisms for medium transport such as *Bacillus.* These signals have been used on several occasions and some systems resulted either in an enhanced periplasmic production or in leakage. The use of the signal sequence from a *Bacillus* endoxylanase for the production of human leptin in *E. coli* was reported to enhance periplasmic production, which was not achieved with OmpA or PelB signals, but it did not result in outer membrane secretion (Jeong and Lee 2000). The extracellular production of cyclodextrin glycosyltransferase (CGTase) by the native *Bacillus* sp E1 signal peptide without cell lysis was however successful and was shown to depend strongly on the source, in contrast to other bacilli CGTases (Yong et al. 1996). Secretion was also achieved with a serine protease, where the signal sequence also acted as a folding mediator and to gain secretion (Peek et al. 1993). Successful secretion has also been achieved when heterologous proteins were produced with the import of the native production protein signal in *E. coli*. Examples include the production of bovine RNase with the original eukaryotic signal sequence (Schein et al. 1992) and an antigen from *Mycobacterium bovis* (MPB70) with its native signal, where it was suggested that secretion was enhanced by hydrophobic interaction between the outer membrane and the hydrophobic MPB70 protein (Hewinson and Russel 1993). Although the exact mechanism has not been proven in these cases, secondary effects of blocking of the secretory system where the foreign protein can be a reason for the secretion cannot be excluded. This leads to an inhibition of important membrane component transport, proteins and lipids, which impairs the membrane function. This is seen when the signal peptide of the bacteriocin release protein gets stuck in the inner membrane (van der Wal et al. 1995), but it is also a well-known effect seen by researchers studying the translocation mechanisms by blocking the secretor proteins and/or using proteins that are not functional. An example is the concomitant mutation in SecA and the overproduction of LamB-lacZ, which blocks the transporters and thus leads to accumulation of membrane vesicles in the cytoplasm, causes changes in the LPS structure and resulting in lower amounts of cardiolipin and the unsaturated fatty acid C18:1 (Thommassen et al. 1989). In production, Georgiou and Shuler (1988) observed a lower level of outer membrane proteins and

an increase in the outer membrane sensitivity to detergents, which indicates changes in the LPS structure, accompanied by the overproduction of recombinant *β*-lactamase. The reason for the blocking of the transport channel of the signal peptide is probably different in different cases, and may be due either to the nature of the signal peptide, the inefficiency of the signal peptidase, or to the demand of translocation capacity arising from the rate of protein synthesis. The translocated protein or at least the initial parts of the protein closest to the cleavage site also affects the cleavage efficiency.

There are also cases in the literature of extracellular protein recovery, without cell lysis, where no specific strategy was employed to achieve the product protein in the medium (Horn et al. 1996). The mechanism for such translocation over the outer membrane is rarely described, but seems to be related to the nature of the protein. This translocation or "leakage" was often described in terms of homologous but overexpressed *E. coli* proteins like *β*-lactamase (Shorki et al. 2002) and alkaline phosphatase (Nesmeyanova et al. 1997). In these cases, Sec transport (or partly used Sec transport) results in a short-lived folding intermediate, followed by outer membrane translocation. It is suggested that alkaline phosphatase translocates through the outer membrane either by a mechanism similar to that of colicin transport or through membrane vesiculation (Nemeyanova et al. 1997). It has furthermore been shown that ß-lactamase from *Bacillus licheniformis* can be transported to the medium even without the signal peptide (Frate et al. 2000).

Process design based on varying the environmental conditions

Changes in physical conditions

Variation of physical parameters such as temperature, pH, or aeration has been suggested as a means of influencing the partitioning between protein inside and outside the cell, since structural changes are a function of these parameters (Arneborg et al. 1993; McGarrity and Armstrong 1981). Temperature has a major effect on all physical and chemical parameters, including translocation. A reduction in temperature reduces the transport (DiRienzo and Inouye 1979), since the membrane goes from a liquid-crystalline bilayer to a more-ordered gel phase (van Klompenburg and de Kruijff 1998). Thus, temperature is difficult to use specifically for leakage enhancement. Another important parameter influencing the outer membrane is the partial pressure of carbon dioxide, which is used for food preservation because of its effect on membrane fluidity. Since an increase in aeration also reduces the amount of carbon dioxide, the membrane structural changes might be secondary to the changed carbon dioxide partial pressure. Well-oxygenated conditions are known to increase the amount of cyclic fatty acids (Knivett and Cullen 1965) and this increases the rigidity of the membrane.

Medium effects

The growth medium also has a large impact on the function of the membrane, and cultivation in rich and minimal media was seen to give very different results after ultrasound treatment of cells. Although it was fairly easy to recover the periplasmic fraction in rich medium, a pure salt medium gave total cell lysis (Larsson, unpublished results). Addition of yeast extract and amino acids such as glycine and histidine to the growth medium produced a dose-dependent effect on the periplasmic release (Harrison et al. 1997; Kaderbhai et al. 1997). It is known that the presence of amino acids can affect the membrane structure, and methionine starvation was shown to cause a drop in the amount of cyclopropane fatty acids (Jungkind and Wood 1974). The production and secretion of human growth hormone using the lysis protein strategy was less in a minimal medium than in a medium supplemented with casamino acids (Hsiung et al. 1989). The addition of salts such as sodium chloride was shown to influence the outer membrane structure in the stationary phase of batch grown cells (McGarrity and Armstrong 1975). The content of divalent cations was also important for the transport of compounds over the membrane (Nikaido and Vaara 1996; Witholt et al. 1976). It is believed that these ions, such as magnesium and calcium, block the protein pores of the outer membrane. This was shown to reduce the transport of lysozyme and other molecules transported in this way. There is also an effect on the LPS stability, since this structure has binding sites with a high affinity for calcium ions.

Acidic/alkaline media are known to increase/decrease the amount of cyclic fatty acids, respectively (Knivett and Cullen 1965), but the mechanisms are not known. In the case of *E. coli*, this might lead to an effect during acetic acid formation, which is strictly growth rate dependent.

In an attempt to directly influence the membrane phospholipid structure during cultivation, Gitter and Riesenberg (1996) fed various combinations of phospholipids in combination with various fatty acids to *E. coli* cells, including a set of heterologous origin. Dependent on the combination, a variation in the leakage of the model protein was achieved. This was shown to be very dependent on the presence of an intact stringent response mechanism, by studies of a stringent and relaxed *relA* strain pair. However, the uptake of phospholipids is rather poor in *E. coli*, and this strategy is probably not useful for enhancing secretion.

Growth-coupled effects on membrane components

Membrane protein translocators seem to be dependent on environmental conditions. Both SecA, SecB, SecE, SecY and trigger factors are dependent on the carbon source used for growth. The amount of SecB is dependent on cAMP-CRP (Seoh and Tai 1997). Low growth rates on glucose where catabolite repression is relieved might thus enhance SecB-dependent transport.

The major findings indicate that the membrane becomes more rigid when the stationary phase is approached and the growth rate declines. The total amount of phospholipids does not vary with growth rate, but the amount of specific phospholipids does. A reduction in PE towards lower growth rates is accompanied by an increase in phosphatidylserine, which is generally not present at other growth rates (Shokri et al. 2002). The increased rigidity is also due to accumulation of cyclic fatty acids in all phospholipids and an increased amount of cardiolipin at the expense of PG (Cronan 1968; Cronan and Rock 1996; Knivett and Cullen 1965; Shokri et al. 2002). It should be recognized that this generally constitutes the point where the induction of protein production takes place and it is thus not suited for efficient translocation.

The transition to increased rigidity, which was earlier shown to take place first in the stationary phase, starts at low/intermediate growth rates, as seen from continuous cultivation data (Arneborg et al. 1993; Shokri et al. 2002). Here there is a shift to higher amounts of PG, but also a very distinct accumulation of unsaturated fatty acids specifically in PG (Shokri et al. 2002). This is also the point of lowest concentration of cyclic fatty acids and constitutes the point of highest protein export to the medium (20% of the specifically produced protein).

It is known that there is a strong coupling between protein translocation and the structure and acidity of the membrane lipids. This coupling was shown for both membranes, but relates specifically to the amounts of acidic phospholipids and unsaturated fatty acids of the inner membrane. The role of PG in the establishment of membrane topology (van Klompenburg et al. 1997) is well known. It has been reported many times that increased amounts of PG facilitate protein translocation (van Klompenburg and de Kruijff 1998; Nesmeyanova 1982; Shokri et al. 2002; Thommassen et al. 1989), and conversely that the efficiency of the translocators and the signal peptidase is severely reduced when PG is decreased (de Vrije et al. 1988). Low contents of both cardiolipin and PG severely impair protein translocation, whereas a lack of cardiolipin alone does not (van Klompenburg and de Kruijff 1998; Thommassen et al. 1989). However, PE may also play a role in protein transport. This lipid adopts a non-bilayer hexagonal H_{II} phase conformation in model membranes, specifically when larger amounts of divalent cations are present (Cullis et al. 1980, de Kruijff et al. 1985). This specific structure is also important for viability and for protein translocation, probably because of the membrane packing pressure effects that could be imposed by this lipid in vivo. Translocation of alkaline phosphatase also seems to be impaired in a *pssA* mutant unable to produce PE (Mikhaleva et al. 2001). The conclusion of van der Does et al. (2000) is that SecA activity is dependent on the anionic phospholipids, whereas PE stimulates the non-bilayer formation, which in turn stimulates the activity of the translocases SecYEG. It also seems that PE plays a role in the functionality of certain proteins, e.g., the lactose permease, and that PE is important for the uptake of several amino acids as well as sugar transport (Dowhan 1997). This could be compared with the effects seen when an internal permeabilization, due to the expression of lysis proteins, changes the PE status and causes leakage.

Conclusion

For the production of recombinant protein, *E. coli* still remains a very attractive alternative because of its simplicity, safety, and the known properties of this organism, which exceed those of all other production systems used. However, an often-cited drawback has been a lack of possibilities for secretion. Research has shown that this is not correct and that several interesting techniques are available. It can generally be concluded that cultivation under dynamically changing glucose-limiting conditions can alter both membrane structure and regulation completely. In order to improve future production, more research is needed to study membranes under the limitations of industrial processes.

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