Expressing genes in different *Escherichia coli* compartments

Pierre Cornelis

Production of heterologous proteins or parts thereof in different extra-cytoplasmic compartments (in the periplasm, outer membrane or extracellularly) of *Escherichia coli* offers multiple applications, for example, in vaccine development, immobilised enzymes and bioremediation. Nowadays, not only surface display of short peptides, but also cell-surface anchoring or secretion of functional proteins is possible. Factors influencing folding, stability and export of extra-cytoplasmic proteins are also better understood.

Addresses
Laboratory of Microbial Interactions, Department of Immunology, Parasitology and Ultrastructure, Flanders Interuniversity Institute of Biotechnology, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint Genesius Rode, Belgium; e-mail: pcornel@vub.ac.be

Current Opinion in Biotechnology 2000, **11**:450–454

0958-1669/00/$ – see front matter

Abbreviations

GPI	glycosyl-phosphatidylinositol
Inp	*Pseudomonas syringae* ice-nucleation protein
OprI	*Pseudomonas aeruginosa* major lipoprotein

Introduction

The Gram-negative bacterium *Escherichia coli* remains the most versatile host for the production of heterologous proteins [1•]. Although for most applications it is desirable to achieve maximal production within the cytoplasm, targeting the protein to extracellular compartments may offer an interesting alternative, especially when cytoplasmic production results in toxicity or improper folding [1•]. Cell-surface display, using outer membrane proteins as carriers for epitopes, adhesins or metal-binding motifs has been the object of intense research during the past decade and has been reviewed recently [2,3]. In view of the attractive applications of surface display in different areas, including vaccine development, bioremediation and enzyme immobilisation, it is not surprising that intensive research in this domain has been ongoing during the past two years, leading to new and interesting developments. The intention of this review is to re-actualise the state of the art concerning the production of proteins in different Gram-negative bacterial compartments. Although Gram-positive bacteria can also be engineered to display proteins [3], this review will be limited to Gram-negative bacteria, in particular *E. coli*.

Soluble proteins: cytoplasm or periplasm?

The formation of disulfide bonds does not occur in the reducing environment of the cytoplasm because of the presence of thioredoxins. The paradigm of this reality is the enzyme alkaline phosphatase, which is active only in the periplasm [4]. It therefore became an accepted fact that proteins with disulfide bonds could only be properly folded in the periplasm. It is now evident, however, that multiple disulfide bonds can be formed in the *E. coli* cytoplasm of a *trxB* thioredoxin reductase mutant, especially when the periplasmic enzyme DsbC, which catalyses disulfide bond isomerisation, is engineered to be localised in the cytoplasm and expressed [5••,6].

Progress has also been made recently to enhance the recovery of active protein in the periplasm by growing the cells in the presence of NaCl, sorbitol and compatible solutes such as glycine betaine [7].

Surface display using outer-membrane structures

Porins

Figure 1 shows the different strategies that have been used to display both short peptides and large proteins on the surface of *E. coli*. The features of each system are summarised in Table 1.

Insertion of short amino-acid stretches can be achieved in extracellular loops of outer membrane proteins such as the maltoporin LamB [8–13], OmpS of *Vibrio cholerae* [14] or OmpC of *E. coli* [15•]. The applications of this technique range from the display of synthetic metal-binding motifs [8,12,15•], metallothioneins [9–11], or sequences responsible for cell adhesion [13,14].

The major drawback of the systems based on outer membrane porins is that the insertion must be in a permissive extracellular loop, and that the number of residues that can be inserted is rather limited (<60). One exception is the *V. cholerae* OmpS porin, where the fourth loop allows the insertion of up to 186 amino acid residues [14].

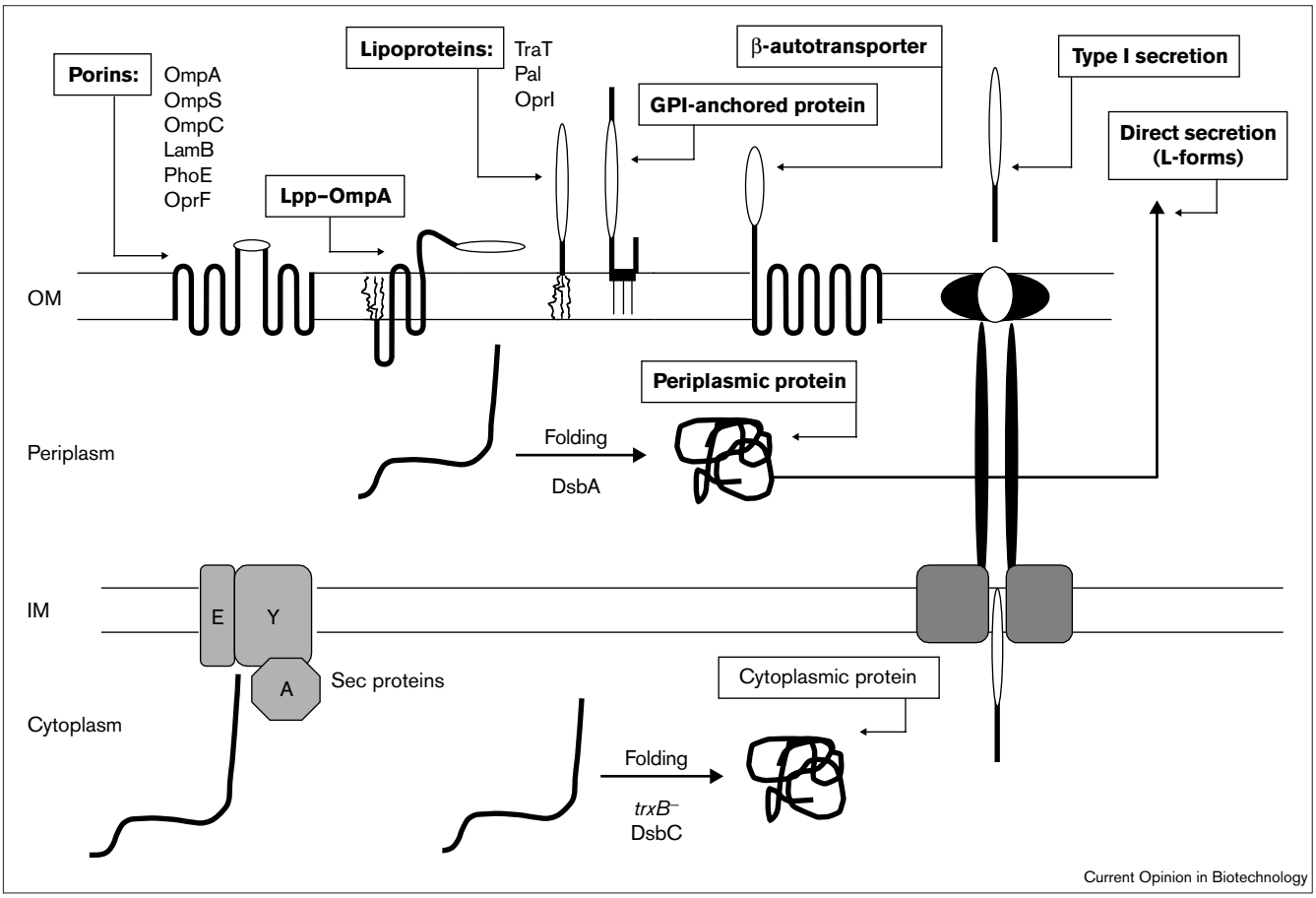
Fimbriae

Similarly, fimbriae and flagellin have been used to display short peptides [2,8]. Fimbriae displaying metal-binding motifs have been found to work very well for the sequestration of metals by recombinant *E. coli* cells [16,17••]. As for the porins, however, this system is limited by the size of the peptides that can be inserted (~15 residues).

Lipoproteins

Outer membrane lipoproteins are anchored in the membrane only by virtue of their amino-terminal lipid tail modification, making them interesting candidates as amino-terminal fusion protein partners. Several lipoproteins from Gram-negative bacteria have been used to produce fusion proteins associated with the outer membrane. The first of such lipoprotein-based systems was the hybrid Braun's lipoprotein (Lpp)–OmpA protein comprising the signal peptide and eight amino-acids of the major Braun's lipoprotein of *E. coli* and amino acids 46–159 of the OmpA outer membrane protein [18]. This system has been used recently to display metallothionein at the *E. coli* surface, resulting in very low expression [10]. This system was also used to fuse an organophosphorus hydrolase

Figure 1



Schematic representation of the *E. coli* compartments (cytoplasm, periplasm and outer membrane [OM]), and the different systems used to localise proteins. For the surface displays, the passenger domain is shown in white. IM, inner membrane.

(OPH), resulting in surface displayed active OPH [19], and to display conformationally constrained peptides corresponding to a cystine knot of a squash-type protease inhibitor [20].

The peptidoglycan-associated lipoprotein (PAL) of *E. coli* has also been used as a fusion partner, but curiously, as a carboxy-terminal partner. The translocation through the inner membrane is achieved thanks to the presence of a pectin lyase (PelB) signal peptide [10,21].

The TraT lipoprotein of *E. coli*, encoded by the F plasmid, seems to be a very efficient fusion partner for the presentation of heterologous polypeptides at the bacterial surface [22•].

OprI, the 8 kDa major outer membrane lipoprotein of *Pseudomonas aeruginosa*, was found to be a versatile amino-terminal partner for the production of large fusion proteins that are, for the majority, cell-surface exposed [23,24]. The OprI expression vectors could also be used to surface display a foot and mouth disease virus epitope, not only in *E. coli* but also in *Salmonella* [24]. We also demonstrated that OprI-derived fusion proteins are highly immunogenic [23], allowing antigen presentation to cytotoxic T-lymphocytes in the context of class I molecules [25], and that they can skew the immune system towards a T-helper 1 response (Cote-Sierra *et al.*, personal communication).

Glycosyl-phosphatidylinositol anchored proteins

The observation that the ice nucleation protein (Inp) from *Pseudomonas syringae* is attached to the surface of the cell via a glycosyl-phosphatidylinositol (GPI) anchor was unexpected because only eukaryotic proteins were known to posses GPI anchors [26]. The Inp protein was used for the first time to display the *Zymomonas mobilis* levansucrase at *E. coli* surface [27] to produce an immobilised enzyme. Because the central region of the Inp is made of repeats, these can be easily replaced. The versatility of the Inp as a carrier for surface presentation of proteins or peptides was later demonstrated in number of applications, such as the production of cell-bound active carboxymethylcellulase [28,29•], presentation of HIV gp120 [30•], and hepatitis B virus surface antigen [31].

β-autotransporters

Another class of outer membrane-associated proteins, represented by the β-autotransporters, has a large amino-terminal

Table 1

Alternative ways to express, target and fold proteins in diverse compartments in *E. coli*.

Cellular compartment	Type of vector	Folding	Size of inserted peptides	Expression level	Applications	References
Cytoplasm	Classical expression vector	Yes, in a *trxB* mutant and DsbC in *trans*	Not relevant	High	Production of soluble proteins	[1•,2–4,5••,6]
Periplasm	Sec signal sequence	Yes	Not relevant	High	Production of soluble proteins	[1•,7]
Cell surface	Porins	Yes	Up to 150 residues	Fair	Epitopes, metal-binding motifs	[8–14,15•]
	Fimbriae	Yes	<50 residues	Fair	Epitopes, metal-binding motifs	[16,17••]
	Lipoproteins	Yes	Large polypeptides	Fair to high	Immobilised enzymes, metal-binding motifs, subunit vaccines	[18–21,22•,23–25]
	GPI anchor	Yes	Large polypeptides	Fair	Immobilised enzymes, metal-binding motifs, subunit vaccines	[27,28,29•,30•,31]
	β-autotransporters	Yes	Large polypeptides	Fair	Immobilised enzymes, metal-binding motifs, subunit vaccines	[32–34,35••,36]
Outside medium	Type I secretion	Not known	Large polypeptides	Fair	Subunit vaccines	[39–41]
	L-forms and vectors with signal sequence	Yes	Large polypeptides	Fair	Single-chain antibodies	[42–44]

domain exposed at the surface. They are anchored to the outer membrane via their carboxy-terminal domain consisting of β-barrels [32]. After autoproteolytic cleavage, their amino-terminal domain can be released into the culture medium. The prototype of β-autotransporters, the *Neisseria gonorrheae* IgA protease, has been engineered by replacing the amino-terminal domain by the polypeptide to be transported, as exemplified by the extracellular transport of cholera toxin B subunit [33,34]. In these reports, the authors suggested that the passenger domain could be transported only after the carboxy-terminal domain got inserted in the outer membrane, which required the amino-terminal passenger polypeptide to be maintained in an unfolded state in order to be secretion-competent [33,34]. Recent results, however, demonstrate that folding of the passenger domain (a single-chain Fv) in the periplasm does not hinder its transport, its exposure at the surface or its antigen-binding activity [35••]. Recently, another β-autotransporter, from *E. coli*, the adhesin-involved-in-diffuse-adherence (AIDA), was used to insert and transport small T-cell epitopes and the 11.6 kDa B subunit of the *E. coli* heat labile toxin (LTB) [36].

Secretion of heterologous proteins

The secretion of recombinant proteins, resulting in their liberation in the culture medium, is often desirable for easy recovery and purification. Of the four different secretion systems that have been described in Gram-negative bacteria (reviewed in [37]), only type I, the Sec-independent ATP-binding cassette (ABC)-transporter-mediated transport system, has been readily engineered to secrete passenger proteins. The prototype of type I secretion systems is the hemolysin transport system, which requires a short carboxy-terminal secretion signal, two translocators, HlyB and HlyD, and the outer membrane protein TolC [38]. The hemolysin secretion system can be engineered to export to the medium passenger polypeptides, providing that they are fused with the carboxy-terminal secretion signal of type I-secreted proteins, and that the genes for the secretion apparatus are also co-expressed [39–41].

An interesting alternative for secretion is the use of bacterial L-forms (mutants devoid of outer membrane and murein sacculus) in order to get products that normally are targeted to the periplasm directly into the medium [42–44]. In this system, polypeptides fused to a normal Sec-recognised amino-terminal signal sequence cross the cytoplasmic membrane via the Sec machinery and arrive in the extracellular space because of the absence of periplasm. Interestingly, correct folding of the proteins released into the medium was found to take place [43,44], probably because periplasmic enzymes such as DsbA are also released into the medium.

Conclusions

During the past few years, much progress has been made to improve the production of proteins in *E. coli*, not only in the cytoplasm, but also in other cellular compartments. It seems now that periplasmic expression should be limited to products that are toxic when present in the cytoplasm, as proteins with disulfide bridges can now be correctly folded in the cytoplasm by manipulating the thioredoxin pathway [5••,6].

Targeting proteins to the outer membrane is now achievable thanks to a range of different systems, including outer membrane porins, lipoproteins, GPI-anchored proteins, fimbriae, and autotransporters. These systems offer perspectives, among others, for the development of vaccines, immobilised enzymes, bioremediation, and metal bioabsorption.

The ultimate destination, the extracellular space, can now be reached by manipulating the type I Sec-independent systems, or by the use of L-forms in a Sec-dependent fashion. It would be interesting to investigate, in the future, if the L-forms can target outer membrane lipoproteins to the medium.

One has also to look for new methods to improve the folding, and to decrease the degradation of the compartment-targeted protein. Expressing different periplasmic chaperones, such as Skp for outer membrane proteins [45••,46•,47] and LolA/LolB for outer membrane lipoproteins, can probably make this objective realisable [48•]. Another challenge is to minimise the protein degradation in the periplasm, which will be aided by a better understanding of the stress-related degradation pathway of misfolded proteins in the periplasm [49•].

Update

Since submission of this review, two important contributions in the domain of surface display were published. One concerns the display of the metal-binding metallothionein at the surface of the Gram-negative bacterium *Ralstonia eutropha* using the *Neisseria gonorrhoeae* IgA protease autotransporter [50••], while the second one describes the display of hepatitis B and C at the surface of *Salmonella typhi* using the *P. syringae* Inp [51].

Acknowledgements

We wish to thank Mark Lauwerijs and A Leitão for reading and comments, Yves Geunes and Marc Vauterin for solving some computer problems, and the journal staff for their help.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Baneyx F: **Recombinant protein expression in *Escherichia coli*.** *Curr Opin Biotechnol* 1999, **10**:411-421.
A recent and excellent review on factors that influence heterologous protein production in *E. coli*.

2. Georgiou G, Stathopoulos C, Daugherty P-S, Nayak A-R, Iverson B-L, Curtiss R III: **Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines.** *Nat Biotechnol* 1997, **15**:29-34.

3. Ståhl S, Uhlén M: **Bacterial surface display: trends and progress.** *Trends Biotechnol* 1997, **15**:185-192.

4. Åslund F, Beckwith J: **The thioredoxin superfamily: redundancy, specific and gray area genomics.** *J Bacteriol* 1999, **181**:1375-1379.

5. •• Bessette P-H, Åslund F, Beckwith J, Georgiou G: **Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm.** *Proc Natl Acad Sci USA* 1999, **96**:13703-13708.
Thioredoxin reductase mutants (*trxB*) can accumulate an active cytoplasmic form of the normally periplasmic alkaline phosphatase because the two cytoplasmic thioredoxins are oxidized and serve as catalysts for disulfide bond formation, resulting in higher yields of active enzyme compared to periplasmic expression. Even active tissue plasminogen activator (tPA) could be produced, provided that DsbC (a normally periplasmic protein disulfide isomerase) was produced in the cytoplasm.

6. Qiu J, Swartz J-R, Georgiou G: **Expression of active human tissue-type plasminogen activator in *Escherichia coli*.** *Appl Environ Microbiol* 1998, **64**:4891-4896.

7. Barth S, Huhn M, Matthey B, Klimka A, Galinski E-A, Engert A: **Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions.** *Appl Environ Microbiol* 2000, **66**:1572-1579.

8. Sousa C, Cebolla A, de Lorenzo V: **Enhanced metalloadsorption of bacterial cells displaying poly-His peptides.** *Nat Biotechnol* 1996, **14**:1017-1020.

9. Sousa C, Kotrba P, Ruml T, Cebolla A, de Lorenzo V: **Metalloadsorption of *Escherichia coli* cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB.** *J Bacteriol* 1998, **180**:2280-2284.

10. Valls M, González-Duarte R, Atrian S, de Lorenzo V: **Bioaccumulation of heavy metals with protein fusions of metallothionein to bacterial outer membrane proteins.** *Biochimie* 1998, **80**:855-861.

11. Kotrba P, Pospisil P, de Lorenzo V, Ruml T: **Enhanced metallosorption of *Escherichia coli* cells due to surface display of beta- and alpha-domains of mammalian metallothionein as a fusion to LamB protein.** *J Recept Signal Transduct Res* 1999, **19**:703-715.

12. Kotrba P, Dolecková L, de Lorenzo V, Ruml T: **Enhanced bioaccumulation of heavy metal ions by bacterial cells due to surface display of short metal binding peptides.** *Appl Environ Microbiol* 1999, **65**:1092-1098.

13. Pereira C-M, Favoreto S Jr, da Silveira J-F, Yoshida N, Castilho B-A: **Adhesion of *Escherichia coli* to HeLa cells mediated by *Trypanosoma cruzi* surface glycoprotein-derived peptides inserted in the outer membrane protein LamB.** *Infect Immun* 1999, **67**:4908-4911.

14. Lång H, Mäki M, Rantakari A, Korhonen T-K: **Characterization of adhesive epitopes with the OmpS display system.** *Eur J Biochem* 2000, **267**:163-170.

15. • Xu Z, Lee S-Y: **Display of polyhistidine peptides on the *Escherichia coli* cell surface by using outer membrane protein C as an anchoring motif.** *Appl Environ Microbiol* 1999, **65**:5142-5147.
OmpC porin is the most abundant protein of *E. coli* and forms a trimer, each monomer having 16 transmembrane domains and eight external loops. Up to 84 amino acids could be inserted in loop 7. Cells displaying poly-His peptides attached to the OmpC porin could remove Cd^{2+} from solution.

16. Schembri M-A, Kjaergaard K, Klemm P: **Bioaccumulation of heavy metals by fimbrial designer adhesins.** *FEMS Microbiol Lett* 1999, **170**:363-371.

17. •• Kjaergaard K, Sørensen J, Schembri M-A, Klemm P: **Sequestration of zinc oxide by fimbrial designer chelators.** *Appl Environ Microbiol* 2000, **66**:10-14.
FimH, the receptor-recognizing element of type I fimbriae, was used to construct and display a library of peptides, followed by an enrichment in a gradient of ZnO-percoll. Clones binding ZnO were selected and the sequences of the corresponding peptides compared. This work shows that it is possible to use surface display to screen peptide libraries.

18. Francisco J-A, Earhart C-F, Georgiou G: **Transport and anchoring of beta-lactamase to the external surface of *Escherichia coli*.** *Proc Natl Acad Sci USA* 1992, **89**:2713-2717.

19. Richins R-D, Kaneva I, Mulchandani A, Chen W: **Biodegradation of organophosphorus pesticides by surface-exposed organophosphorus hydrolase.** *Nat Biotechnol* 1997, **15**:984-987.

20. Christmann A, Walter K, Wentzel A, Kratzmer R, Kolmar H: **The cystine knot of a squash-type protease inhibitor as a structural scaffold for *Escherichia coli* cell surface display of conformationally constrained peptides.** *Protein Eng* 1999, **12**:797-806.

21. Dhillon J-K, Drew P-D, Porter A-J-R: **Bacterial surface display of an antipollutant antibody fragment.** *Lett Appl Microbiol* 1999, **28**:350-354.

22. • Chang H-H, Sheu S-Y, Lo S-J: **Expression of foreign antigens on the surface of *Escherichia coli* by fusion to the outer membrane protein TraT.** *J Biomed Sci* 1999, **6**:64-70.
Fusions of the hepatitis B surface antigens comprising up to 98 amino acids or 72 residues from the snake venom protein rhodostomin could be

expressed and displayed at the cell surface. This article also mentions the immunostimulatory properties of TraT.

23. Cornelis P, Cote-Sierra J, Lim A Jr, Malur A, Tungpradabkul S, Tazka H, Leitão A, Martins C-V, di Perna C, Brys L *et al.*: **Development of new cloning vectors for the production of immunogenic outer membrane fusion proteins in *Escherichia coli*.** *Biotechnology* 1996, **14**:203-208.

24. Cote-Sierra J, Jongert E, Bredan A, Gautam D, Parkhouse M, Cornelis P, De Baetselier P, Revets H: **A new membrane-bound OprI lipoprotein expression vector. High production of heterologous fusion proteins in Gram(-) bacteria and the implications for oral vaccination.** *Gene* 1998, **221**:25-34.

25. Leitão A, Malur A, Cornelis P, Martins C-V: **Identification of a 25-aminoacid sequence from the major African swine fever virus structural protein VP72 recognised by porcine cytotoxic T lymphocytes using a lipoprotein based expression system.** *J Virol Methods* 1998, **75**:113-119.

26. Kozloff L-M, Turner M-A, Arellano F: **Formation of bacterial membrane ice-nucleating lipoglycoprotein complexes.** *J Bacteriol* 1991, **173**:6528-6536.

27. Jung H-C, Lebeault J-M, Pan J-G: **Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*.** *Nat Biotechnol* 1998, **16**:576-580.

28. Jung H-C, Park J-H, Park S-H, Lebeault J-M, Pan J-G: **Expression of *Bacillus subtilis* carboxymethylcellulase on the surface of *Escherichia coli* using *Pseudomonas syringae* ice nucleation protein.** *Enzyme Microb Technol* 1998, **22**:348-354.

29. • Kim Y-S, Jung H-C, Pan J-G: **Bacterial cell surface display of an enzyme library for selective screening of improved cellulase variants.** *Appl Environ Microbiol* 2000, **66**:788-793.
This is the follow-up of the previous article [28] where the authors take advantage of the complete surface-exposure of the recombinant carboxymethylcellulase to obtain, by DNA shuffling, mutants with improved growth capacity on CM-cellulose. Analysis of the mutants showed that the substitutions occurred in the catalytic site of the enzyme.

30. • Kwak I-D, Yoo S-K, Kim E-J: **Cell surface display of human immunodeficiency virus type 1 gp120 on *Escherichia coli* by using ice nucleation protein.** *Clin Diagn Lab Immunol* 1999, **6**:499-503.
This article shows that there is no apparent limitation to the size of the fusion protein that can be produced using Inp as a GPI-anchored fusion partner.

31. Kim E-J, Yoo S-K: **Cell surface display of hepatitis B virus surface antigen by using *Pseudomonas syringae* ice nucleation protein.** *Lett Appl Microbiol* 1999, **29**:292-297.

32. Henderson I-R, Navarro-Garcia F, Nataro J-P: **The great escape: structure and function of the autotransporter proteins.** *Trends Microbiol* 1998, **6**:370-378.

33. Klauser T, Pohlner J, Meyer T-F: **Extracellular transport of cholera toxin B subunit using *Neisseria* IgA protease β–domain: conformation-dependent outer membrane translocation.** *EMBO J* 1990, **9**:1991-1999.

34. Klauser T, Pohlner J, Meyer T-F: **Selective extracellular release of cholera toxin B subunit by *Escherichia coli*: dissection of *Neisseria* Igaβ–mediated outer membrane transport.** *EMBO J* 1992, **11**:2327-2335.

35. •• Veiga E, de Lorenzo V, Fernández L-A: **Probing secretion and translocation of a β-autotransporter using a reporter single-chain Fv as cognate passenger domain.** *Mol Microbiol* 1999, **33**:1232-1243.
This article demonstrates that a single chain antibody passenger domain needs to be folded in the periplasm in order to be active after translocation. In a *dsbA* mutant, the scFv is translocated more efficiently but is unable to bind its antigen. This means that disulfide bridges need to be formed for an active fusion partner to be translocated. This result raises the question of how a folded protein can be translocated by an autotransporter.

36. Konieczny M-P-J, Suhr M, Noll A, Autentrieth I-B, Schmidt M-A: **Cell surface presentation of recombinant (poly-) peptides including functional T-cell epitopes by the AIDA autotransporter system.** *FEMS Immun Med Microbiol* 2000, **27**:321-332.

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

38. Koronakis V, Stanley P, Koronakis E, Hughes C: **The HlyB/HlyD-dependent secretion of toxins by Gram-negative bacteria.** *FEMS Microbiol Immunol* 1992, **105**:45-54.

39. Tzschaschel B-D, Guzman C-A, Timmis K-N, de Lorenzo V: **An *Escherichia coli* hemolysin transport system-based vector for the export of polypeptides: export of Shiga-like toxin IieB subunit by *Salmonella typhimurium aroA*.** *Nat Biotechnol* 1996, **14**:765-769.

40. Spreng S, Dietrich G, Niewiesk S, ter Meulen V, Gentschev I, Goebel W: **Novel bacterial systems for the delivery of recombinant protein or DNA.** *FEMS Immunol Med Microbiol* 2000, **27**:299-304.

41 Spreng S, Dietrich G, Goebel W, Gentschev I: **The *Escherichia coli* haemolysin secretion apparatus: a potential universal antigen delivery system in Gram-negative bacterial vaccine carriers.** *Mol Microbiol* 1999, **31**:1596-1598.

42. 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**:506-509.

43. Rippman J-F, Klein M, Hoischen C, Brocks B, Rettig W-J, Gumpert J, Pfizenmaier K, Mattes R, Moosmayer D: **Prokaryotic 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**:4862-4869.

44. Kujau M, 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 *Escherichia coli* producer strain.** *Appl Microbiol Biotechnol* 1998, **49**:51-58.

45. •• Schäfer U, Beck K, Müller M: **Skp, a molecular chaperone of gram negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins.** *J Biol Chem* 1999, **274**:24567-24574.
This work demonstrates that Skp binds to the outer membrane protein OmpA immediately after its emergence from the plasma membrane and is needed for its release from the membrane. Although a *skp*-null mutant is viable, when this mutation is combined with a deletion in the gene *degP*, encoding a periplasmic protease needed for the degradation of misfolded outer membrane proteins, the resulting mutant was not viable at 37°C.

46. • De Cock H, Schäfer U, Potgeter M, Demel R, Müller M, Tommassen J: **Affinity of the periplasmic chaperone Skp of *Escherichia coli* for phospholipids, lipopolysaccharides and non-native outer membrane proteins. Role of Skp in the biogenesis of outer membrane protein.** *Eur J Biochem* 1999, **259**:96-103.
The authors demonstrate that Skp associates with phosholipids and non-native outer membrane proteins, suggesting that Skp plays an early chaperone role for outer membrane proteins.

47. Bothmann H, Plückthun A: **Selection for a periplasmic factor improving phage display and functional periplasmic expression.** *Nat Biotechnol* 1998, **16**:376-380.

48. • Yokota N, Kuroda T, Matsuyama S, Tokuda H: **Characterization of the LolA-LolB system as the general lipoprotein localization mechanism of *Escherichia coli*.** *J Biol Chem* 1999, **274**:30995-30999.
The most recent article on the LolA (periplasmic chaperone) and LolB (outer membrane chaperone) proteins that are necessary for the outer membrane insertion of outer membrane lipoproteins such as Lpp, Pal and others. The authors demonstrate also that overexpression of LolB can greatly increase the outer membrane localisation of lipoproteins.

49. • Raivio T-L, Silhavy T-J: **The σ^E and Cpx regulatory pathways: overlapping but distinct envelope stress responses.** *Curr Opin Microbiol* 1999, **2**:159-165.
The σ^E pathway, in particular, senses the presence of misfolded proteins in the periplasm, resulting in the induction of the periplasmic protease DegP and the peptidyl-prolyl isomerase FkpA.

50. •• Valls M, Atrian S, de Lorenzo V, Fernández L-A: **Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for immobilization of heavy metals in soil.** *Nat Biotechnol* 2000, **18**:661-665.
A metallothionein gene was displayed at the surface of *E. coli* using the *Neisseria gonnorrhoeae* IgA protease autotransporter and binding of Cd^{2+} was demonstrated. The same construct was later inserted via a mini-transposon in the chromosome of the soil Gram-negative, metal-resistant bacterium *Ralstonia eutropha*. This recombinant *R. eutropha*, when inoculated in Cd^{2+} contaminated soil, was found to dramatically improve the growth of *Nicotiana bentamiana*, indicating that surface display on bacteria able to survive in soil can be used for metal binding and detoxification.

51. Lee J-S, Shin K-S, Pan J-G, Kim C-J: **Surface-displayed viral antigens on *Salmonella* carrier vaccine.** *Nat Biotechnol* 2000, **18**:645-648.