

N-tail translocation of mature β -lactamase across the *Escherichia coli* cytoplasmic membrane

Costas Mitsopoulos, Lida Hashemzadeh-Bonehi, Jenny K. Broome-Smith*

Biochemistry Group, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

Received 25 September 1997; revised version received 30 October 1997

Abstract Mature β -lactamase was attached to the N-terminus of human glycophorin C, an N-out membrane protein lacking a cleavable signal peptide (an N-tail membrane protein). When synthesised in *Escherichia coli* more than 30% of the intact mature β -lactamase-glycophorin C molecules assembled N-out, C-in into the cytoplasmic membrane. The N-tail translocated β -lactamase folded into an enzymatically active form, but it was more susceptible to proteolysis than the equivalent portion of β -lactamase-glycophorin C synthesised with an N-terminal signal peptide. Its translocation was virtually abolished when the N-out domain of glycophorin C was truncated or when the basic residues C-terminally flanking the glycophorin C membrane-spanning segment were replaced with neutral ones.

© 1997 Federation of European Biochemical Societies.

Key words: β -Lactamase; Protein translocation; Membrane protein; N-tail; Protein folding; *Escherichia coli*

1. Introduction

Proteins that are translocated across the bacterial cytoplasmic membrane or eukaryotic endoplasmic reticulum (ER) membrane are generally made with N-terminal cleavable signal peptides (SPs), which ensure that the mature portion enters the translocation channel and passes through the membrane with an overall N-out, C-in polarity. Most simple (singly spanning) membrane proteins with large extracytoplasmic domains are also made with SPs or uncleaved signal peptides that likewise facilitate the translocation of the more C-terminal portion (until, in the case of the N-out, C-in type I membrane proteins, the membrane-spanning segment [MSS] enters the channel) [1]. However, the type III membrane proteins have extracytoplasmic N-terminal domains but lack SPs. Translocation of their N-tails requires the MSS, and is compromised when positively charged residues are added to its N-terminal flank. This suggests that translocation of N-tails depends upon the MSS inserting with an N-out, C-in orientation into the membrane, and that the N-tail adopts an opposite-to-normal – N-in, C-out – polarity during its post-translational translocation across the membrane [2,3].

All known type III membrane proteins of *Escherichia coli*

have short (less than 20 amino acid) N-out domains, that might conceivably be driven through the lipid bilayer as a consequence of the oriented membrane insertion of the MSS. We demonstrated previously, however, that the human type III membrane protein, glycophorin C, which has a 58 amino acid N-tail, assembled with its native topology in *E. coli*, indicating that this bacterium has the capacity to translocate larger N-tails [4]. It was subsequently shown that the 100 amino acid N-tail of the polytopic ProW protein was efficiently localised to the *E. coli* periplasm in ProW truncates that contained the first three or more MSSs, and that in a truncate retaining only MSS1, with its C-terminal flank made basic, 65–70% of the N-tails were translocated [5,6]. Pf3-Lep derivatives were also shown to assemble with the type III topology in *E. coli*, but with decreasing efficiency as their N-tails were extended [7]. As N-tail translocation in ProW and Pf3-Lep derivatives was not detectably affected by treatments that impaired SecA or SecY, and bacterial N-tails contain few positively charged residues, it was proposed that N-tails are unable to use the Sec machinery for their translocation into the bacterial periplasm [5–7].

In contrast to the bacterial situation, membrane proteins with long N-tails are more common in eukaryotes and the mammalian Sec61 protein complex is known to be required for the insertion of glycophorin C into proteoliposomes [8]. It has also been found that destabilisation of the folding state rather than the size of the N-tail is crucial for type III membrane protein assembly in mammalian cells. Hence a short (29 amino acid) N-tail comprising a zinc-finger domain resisted translocation whereas an N-tail of 237 amino acids that was unable to fold properly into a compact three-dimensional structure was translocated [9].

β -Lactamase is a globular protein of 263 amino acids, which normally requires a SP and the Sec machinery for its translocation into the bacterial periplasm [10]. We report here that when mature β -lactamase is attached to the N-terminus of glycophorin C, a proportion of the molecules assemble N-out, C-in fashion in the *E. coli* cytoplasmic membrane. Hence a very large (326 amino acid) signal peptide-less domain, comprised mainly of a polypeptide with a normal folding status and a normal content of positively charged residues, can undergo N-tail translocation in *E. coli*.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* K-12 strain used was NM522 [F' *lacI^q* Δ (*lacZ*)M15 *proA⁺B⁺supE thi* Δ (*lac-proAB*) Δ (*hsdMS-mcrB*)5]. pLHB28 and pLHB32 are chloramphenicol-resistant derivatives of the direct expression vector pYZ4 [11] encoding pre- β -lactamase-glycophorin C (SP-BlaM-gC) and mature β -lactamase-glycophorin C (BlaM-gC) respectively, under the control of the *lac* *uvr5* promoter. To construct

*Corresponding author. Fax: (44) (1273) 678433.
E-mail: j.k.broome-smith@sussex.ac.uk

Abbreviations: SP, signal peptide; MSS, membrane-spanning segment; BlaM-gC, mature β -lactamase-glycophorin C; SP-BlaM-gC, pre- β -lactamase-glycophorin C; MIC, minimum inhibitory concentration; CAT, chloramphenicol acetyltransferase; IPTG, isopropyl-1-thio- β -D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; ECL, enhanced chemiluminescence

pLHB28 the β -lactamase coding region of pLH15, which lacks a stop codon [12], was synthetically extended, incorporating a *SalI* site in the same frame as the *SalI* site spanning codons 2–4 of glycophorin C, and then the glycophorin C coding region, carried on a *SalI*-*SmaI* fragment, was fused to it. The region encoding the modified SP of pre- β -lactamase from pTG2 [13], extending from the *EcoRI* site spanning codons 2–4 and including the *BstEII* site at the end of the SP coding region, was then incorporated, and an *NcoI* site was introduced spanning the initiation codon. (The SP coding region of pLHB28 differs from that of pTG2 by the addition of an alanine codon adjacent to the initiation codon and the conversion of the third [isoleucine] codon to a glycine codon.) To generate pLHB32, which lacks the β -lactamase SP coding region, pLHB28 was digested with *NcoI* and *BstEII* and the protruding ends were made blunt by end-filling, and by partial end-filling with dGTP and end-trimming, respectively, and then ligated. To construct plasmids encoding mutant derivatives of SP-BlaM-gC and BlaM-gC, oligonucleotide-directed mutagenesis was performed on an M13 derivative containing the *SalI*-*SmaI* fragment that encodes glycophorin C, according to the method of Kunkel [14], and the mutant glycophorin C alleles were subcloned into pLHB28 and/or pLHB32 using available restriction sites. All fusion junctions and introduced mutations were verified by dideoxynucleotide sequencing.

2.2. Media and growth conditions

Strains were grown at 37°C in L-broth or L-agar, supplemented, where appropriate, with 30 μ g/ml chloramphenicol. To determine the MICs of ampicillin for individual cells of *E. coli* isolates synthesising β -lactamase-glycophorin C derivatives, the strains were grown for 5–7 h in L-broth, and then diluted 10³-fold. 5 μ l aliquots of the diluted cultures (containing 20–40 cells) were then spotted onto L-agar containing chloramphenicol, 1 mM isopropyl-1-thio- β -D-thio-

galactopyranoside (IPTG), and 0, 5, 10, 15, 20, 25, 50, 100, 150, 200, 300, 400, 500 or 600 μ g/ml ampicillin. The minimum inhibitory concentration (MIC) of ampicillin for cells individually exposed to the antibiotic (the single cell MIC of ampicillin) is defined as the lowest concentration that prevents 50% or more of the cells from forming colonies.

2.3. Assay of N-tail translocation

Cells of NM522 carrying pLHB28, pLHB32, or their derivatives, were grown in L-broth at 37°C to early log phase, induced to synthesise β -lactamase-glycophorin C derivatives by adding IPTG (50 μ M) and, after 2 h, were collected by centrifugation, washed and then resuspended in one-fifth of the culture volume of 0.3 M sucrose in 0.12 M Tris-HCl, pH 7.6. To convert the cells to spheroplasts, they were incubated on ice for 5 min with lysozyme (100 μ g/ml) and EDTA (4.5 mM). MgSO₄ (20 mM) was added to stabilise the spheroplasts, and they were then harvested by centrifugation. Aliquots of the resuspended spheroplasts were incubated for 5 min on ice with or without proteinase K (10 μ g/ml), and then phenylmethylsulphonyl fluoride (PMSF, 1 mM) was added. The spheroplast-associated and periplasmic proteins were then separated by centrifugation, TCA-precipitated, and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), enhanced chemiluminescence (ECL) Western blotting and, where appropriate, densitometry.

3. Results

To determine whether glycophorin C could facilitate the translocation of N-terminally attached mature β -lactamase in *E. coli* we analysed the transmembrane disposition of

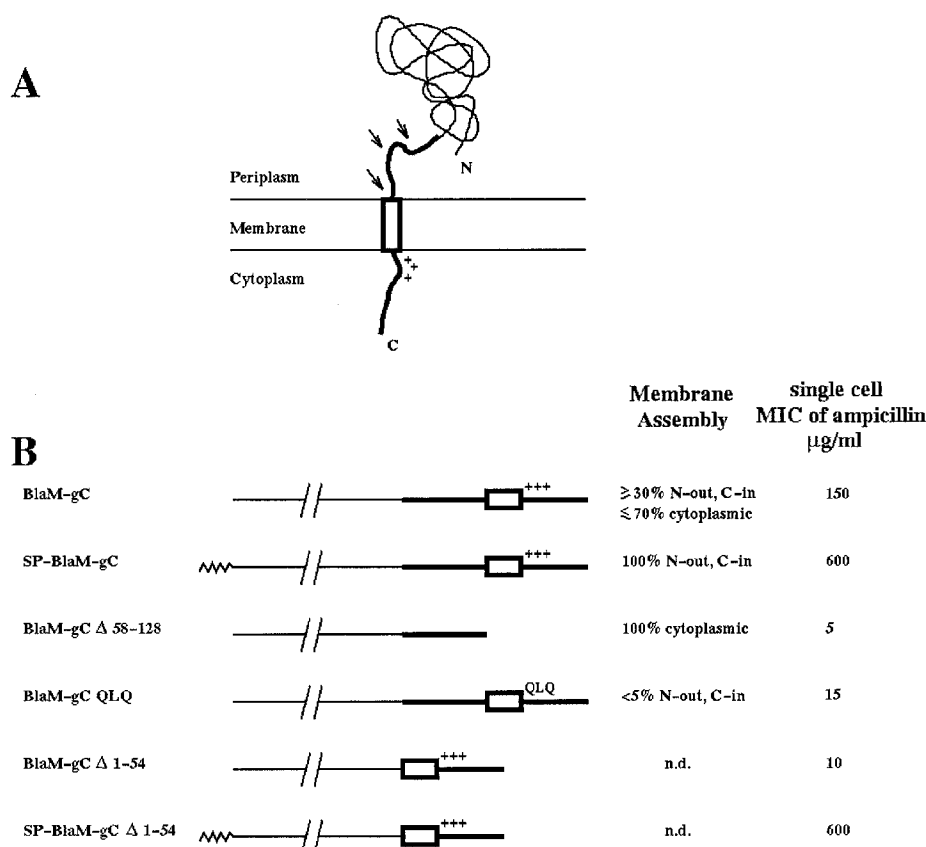


Fig. 1. A: Topology of β -lactamase-glycophorin C in the *E. coli* cytoplasmic membrane. The glycophorin C portion is shown in bold, and arrows indicate accessible proteinase K cleavage sites. The basic residues C-terminally flanking the MSS are indicated by + signs. B: Membrane assembly properties of the β -lactamase-glycophorin C derivatives analysed in this paper. Mutants are named according to the residues altered in the glycophorin C portion. The proportion of full-length BlaM-gC molecules that assembled into the membrane and the ability of BlaM-gC derivatives to protect producing cells from lysis by ampicillin are recorded. n.d. = not determined.

BlaM-gC and, for comparison, SP-BlaM-gC. In the latter case efficient translocation of the mature β -lactamase portion should be ensured by the provision of an N-terminal SP. Several mutants of BlaM-gC and SP-BlaM-gC were also analysed (details of the proteins used in this study are given in Fig. 1B).

Following synthesis of BlaM-gC or SP-BlaM-gC, the *E. coli* cells were converted to spheroplasts, and these were harvested and subjected to proteolysis, and the periplasmic and spheroplast-retained products were analysed by SDS-PAGE and Western blotting (see Fig. 2). In the case of SP-BlaM-gC the protease protection assay revealed that 100% of the molecules attained the N-out, C-in topology (Fig. 2A). Treatment with proteinase K, which is predicted to cleave several times within the N-tail of glycoporphin C (see Fig. 1A), yielded spheroplast-retained products that cross-reacted with antiserum to the C-terminal domain of glycoporphin C (products b and c, lane 2) but not with antiserum to β -lactamase (lane 6), and the smaller of these (product c) corresponded in size to glycoporphin C minus its N-tail. After its SP-mediated translocation β -lactamase, like many other periplasmic proteins, folds into an intrinsically protease-resistant form. Proteinase K treatment also generated a periplasmic product that cross-reacted with β -lactamase antiserum (product a, lane 8), but not with antiserum to the C-terminal domain of glycoporphin C, and corresponded in size to slightly C-terminally extended β -lactamase. As shown in Fig. 2B, probing with the glycoporphin C antibody revealed more than 30% of the full-length BlaM-gC molecules were cleaved by proteinase K to yield spheroplast-retained glycoporphin C minus its N-tail, indicating that they had acquired the N-out, C-in topology (compare lanes 1 and 2). In addition to the major band corresponding to full-length BlaM-gC, probing with β -lactamase antibody revealed many degradation products (lane 5). Proteinase K treatment resulted in the cleavage of about 30% of the full-length BlaM-gC molecules (compare lanes 5 and 6), and also generated the periplasmic C-terminally extended β -lactamase

product (lane 8). It was, however, present in lower amounts than expected (from the proportion of full-length BlaM-gC molecules that were cleaved), suggesting that it was unstable and was being degraded during the assay (see below). The rest of the BlaM-gC molecules and their degradation products were resistant to cleavage by exogenous proteinase K and fractionated with the pelleted spheroplasts, indicating that they remained in the cytoplasm. As the SP of pre- β -lactamase is known to retard the folding of the mature portion [15], but the C-terminally attached MSS of glycoporphin C is unlikely to be able to perform the same role, the β -lactamase portion of many molecules may fold rapidly in the cytoplasm, and prove translocation-incompetent, and eventually be degraded by cytoplasmic proteases. In both cases probing with antiserum to the cytoplasmic protein chloramphenicol acetyltransferase (CAT) revealed that virtually no CAT was present in the periplasmic fractions (Fig. 2, lanes 9–12). As CAT was resistant to degradation by the proteinase K (in Triton X-100-treated spheroplasts), its absence from the periplasmic fractions confirmed that the spheroplasts had remained intact during the protease protection assays. Hence we conclude that mature β -lactamase is translocated across the *E. coli* cytoplasmic membrane when it is attached to the N-terminus of glycoporphin C, but that the translocation of this very long (326 amino acid) N-tail is less efficient than that of unextended glycoporphin C N-tails [4], and is also less efficient than in the presence of a SP, in which cases all of the molecules assemble into the membrane with the N-out, C-in topology.

To compare their stabilities, the translocated β -lactamase-containing portions of BlaM-gC and SP-BlaM-gC (product a in Fig. 2A,B) were cleaved from the spheroplasts using proteinase K, as before, and the periplasmic fractions containing them were collected and incubated at 37°C for increasing times. As shown in Fig. 3, the half-life of the N-tail translocated β -lactamase-containing product was approximately 20 min, whereas that of the equivalent product derived from pre- β -lactamase-glycoporphin C exceeded several hours. When the periplasmic fractions were combined prior to incubation (in a 4:1 ratio of BlaM-gC-derived product:SP-BlaM-gC-derived product) the pattern of the degradation was unaltered (data not shown), confirming that the SP-translocated β -lactamase was indeed inherently less susceptible to proteolysis than the N-tail translocated β -lactamase. As these two polypeptides are predicted to differ, at most, by an additional N-terminal methionine (in the N-tail translocated product), it seems likely that the increased susceptibility of N-tail translocated β -lactamase to proteolysis is a consequence of its atypical mode of translocation.

β -Lactamase is a reliable phenotypic reporter of periplasmic localisation. Only if it is translocated to the periplasm does it render *E. coli* cells resistant to lysis by β -lactam antibiotics, such as ampicillin [16]. If it is produced in the cytoplasm individual cells remain unprotected against ampicillin-induced lysis and therefore fail to form colonies when they are exposed to as little as 2.5–5 μ g/ml ampicillin (the MICs required to prevent colony formation in non- β -lactamase-producing *E. coli* strains). Moreover, the level of ampicillin resistance conferred on individual *E. coli* cells by a fusion protein in which the β -lactamase portion is periplasmic increases as more β -lactamase molecules reach the periplasm [17]. Thus, as expected, not only SP-BlaM-gC but also BlaM-gC rendered *E. coli* cells

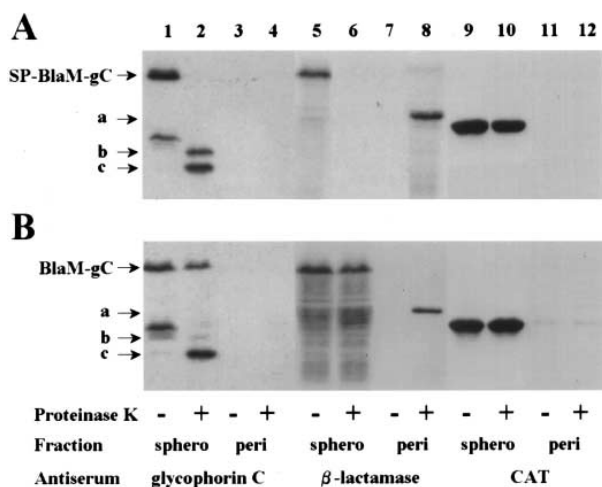


Fig. 2. Translocation of the N-tail of β -lactamase-glycoporphin C across the *E. coli* cytoplasmic membrane. A: Proteinase K accessibility in spheroplasts of β -lactamase-glycoporphin C made with a SP, and identification of spheroplast-bound and periplasmic products using antibody to the C-terminal domain of glycoporphin C, β -lactamase, and CAT. B: Proteinase K accessibility in spheroplasts of mature β -lactamase-glycoporphin C.

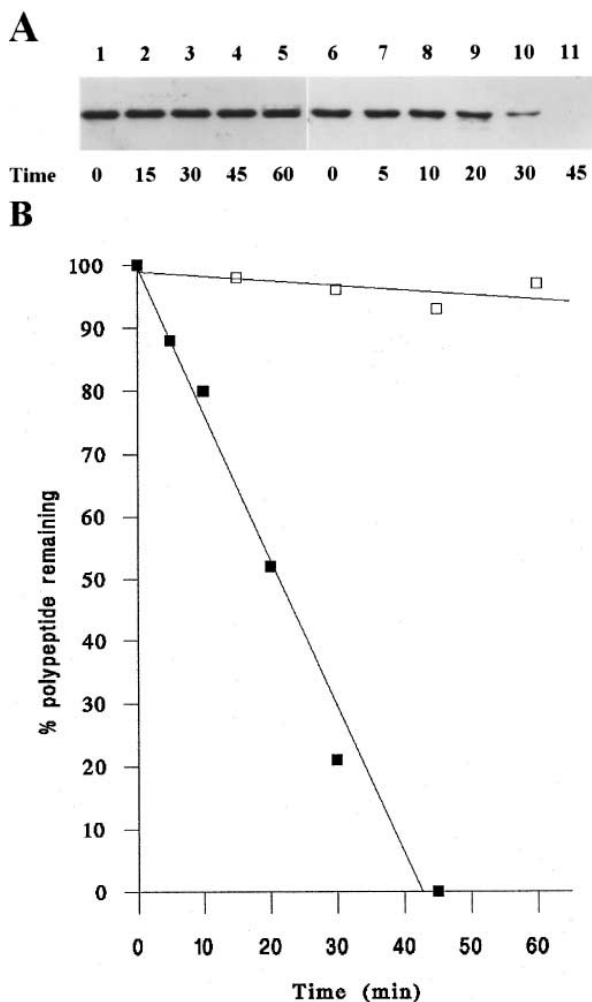


Fig. 3. Susceptibility of N-tail translocated β -lactamase to proteolysis. A: Spheroplasts containing β -lactamase-glycophorin C molecules that had been made with, or without, a SP were harvested and subjected to proteinase K treatment, and the periplasmic fractions were collected as for Fig. 2. These were immediately incubated at 37°C and aliquots were withdrawn at the times (in minutes) shown, TCA-precipitated and analysed by SDS-PAGE and Western blotting with antiserum to β -lactamase. B: The proportion of the N-terminal β -lactamase-containing product derived from BlaM-gC (■) and from SP-BlaM-gC (□) that remained after each time interval was determined densitometrically.

resistant to lysis by high levels of ampicillin (see Fig. 1B). The latter, however, conferred resistance to only one quarter of the concentration of ampicillin of the former, presumably because its β -lactamase portion is both less efficiently translocated and less stable than that of SP-BlaM-gC.

As shown in Fig. 1B, BlaM-gC Δ 58–128, which lacks the MSS and C-terminal domain of glycophorin C, was unable to confer any ampicillin resistance on *E. coli* cells, indicative of a cytoplasmic location. In confirmation of this, we found that in spheroplasts it remained fully resistant to cleavage by proteinase K (data not shown). These findings are compatible with the MSS being necessary for the N-tail translocation of β -lactamase. In the case of glycophorin C, replacing the three basic residues that C-terminally flank the MSS (arginine-82, arginine-86 and lysine-88) with neutral residues (glutamine, leucine, and glutamine, respectively) caused substantial topology inversion (L. Hashemzadeh-Bonehi, C. Mitsopoulos

and J.K. Broome-Smith, unpublished data). These same alterations dramatically reduced the ability of BlaM-gC to confer ampicillin resistance on *E. coli* cells (Fig. 1B), and protease protection assays on spheroplasts revealed that the N-tails of less than 5% of these charge-altered BlaM-gC molecules were translocated (data not shown). These findings imply that the correctly oriented (N-out, C-in) insertion of the MSS into the cytoplasmic membrane is crucial for optimal N-tail translocation of the attached β -lactamase. Finally we investigated the possibility that the N-tail of glycophorin C embodies features that facilitate the N-tail translocation of attached mature β -lactamase. As shown in Fig. 1B, a BlaM-gC derivative in which the residues comprising the N-out domain of glycophorin C were removed (BlaM-gC Δ 1–54) conferred only very low levels of ampicillin resistance on *E. coli* cells. When this polypeptide was supplied with a SP (SP-BlaM-gC Δ 1–54) it conferred the same level of ampicillin resistance on cells as SP-BlaM-gC, indicating that the removal of the N-out domain of glycophorin C did not affect the inherent activity of β -lactamase. These data strongly suggest, therefore, that most of the BlaM-gC molecules lacking the glycophorin C N-tail remain in the cytoplasm.

4. Discussion

We have shown that mature β -lactamase, a 263 amino acid enzyme which normally requires a SP for its translocation across the bacterial cytoplasmic membrane, and has a normal content of positively charged residues, can be translocated across the *E. coli* cytoplasmic membrane when it is attached to the N-tail of the human type III membrane protein, glycophorin C. In agreement with other studies we find that translocation of this N-tail requires the MSS and is strongly dependent upon it being able to adopt its N-out, C-in orientation in the membrane. A readily detectable proportion (more than 30% of the full-length BlaM-gC molecules) assembled with the N-out, C-in topology. This is a remarkable finding in view of the fact that considerably shorter N-tails composed of random neutral amino acids were very inefficiently translocated in *E. coli* [7], and that even a very small domain with a normal folding status resisted N-tail translocation in mammalian cells [9].

N-tail translocated BlaM proved much more susceptible to proteolysis than the equivalent portion of BlaM-gC molecules that had been synthesised with SPs. It is possible that the proteolytic susceptibility of N-tail translocated β -lactamase is a consequence of its arrival in the periplasm C-terminus first, rendering it unable to follow its normal folding pathway. Nevertheless it did attain an enzymatically active conformation, although we do not yet know whether its kinetic parameters are affected.

Our results demonstrate that *E. coli* can translocate N-tails that are not depleted of positively charged residues. N-tail translocation of the 149-residue, highly basic, staphylococcal nuclease has also been reported in *E. coli* although its efficiency was not assessed [18]. Two naturally occurring mammalian type III proteins with exceptionally long (> 200 amino acid) N-tails that are not depleted for basic residues have also been described [19,20]. As well as having a low content of basic residues, no part of the glycophorin C N-tail has a high propensity for α -helix formation. These features may be important in enabling the translocation of the glycophorin

C N-tail to proceed efficiently, hence favouring the translocation of a substantial proportion of the attached nascent β -lactamase molecules before they have a chance to fold up, into translocation-incompetent structures, in the cytoplasm.

Although treatments that impaired SecA and SecY function did not detectably inhibit the translocation of ProW and Pf3-Lep N-tails [5–7], it is possible that these polypeptides had a low requirement for the Sec component tested because they were relatively short and depleted for basic residues. It remains unknown, therefore, whether type III proteins can use the *E. coli* translocase. As its SP-mediated translocation is demonstrably Sec-dependent, the N-tail of BlaM-gC should constitute a good substrate for testing the Sec dependence of N-tail translocation.

Acknowledgements: This work was funded by the Wellcome Trust and by the UK BBSRC.

References

- [1] von Heijne, G. and Manoil, C. (1990) *Protein Eng.* 4, 109–112.
- [2] Dalbey, R.E., Kuhn, A. and von Heijne, G. (1995) *Trends Cell Biol.* 5, 380–383.
- [3] Spiess, M. (1995) *FEBS Lett.* 369, 76–79.
- [4] Hennessey, E.S., Hashemzadeh-Bonehi, L., Hunt, L.A. and Broome-Smith, J.K. (1993) *FEBS Lett.* 331, 159–161.
- [5] Whitley, P., Zander, T., Ehrmann, M., Haardt, M., Bremer, E. and von Heijne, G. (1994) *EMBO J.* 13, 4653–4661.
- [6] Whitley, P., Gafvelin, G. and von Heijne, G. (1995) *J. Biol. Chem.* 270, 29831–29835.
- [7] Cao, G. and Dalbey, R.E. (1994) *EMBO J.* 13, 4662–4669.
- [8] Oliver, J., Jungnickel, B., Görlich, D., Rapoport, T. and High, S. (1995) *FEBS Lett.* 362, 126–130.
- [9] Denzer, A.J., Nabholz, C.E. and Spiess, M. (1995) *EMBO J.* 14, 6311–6317.
- [10] Broome-Smith, J.K., Tadayyon, M. and Zhang, Y. (1990) *Mol. Microbiol.* 4, 1637–1644.
- [11] Zhang, Y. and Broome-Smith, J.K. (1990) *Gene* 96, 51–57.
- [12] Chervaux, C., Sauvonnet, N., Le Clainche, A., Kenny, B., Hunt, L.A., Broome-Smith, J.K. and Holland, I.B. (1995) *Mol. Gen. Genet.* 249, 237–245.
- [13] Kadonaga, J.T., Gautier, A.E., Sraus, D.R., Charles, A.D., Edge, M.D. and Knowles, J.R. (1984) *J. Biol. Chem.* 259, 2149–2154.
- [14] Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 488–492.
- [15] Laminet, A.A. and Plückthun, A. (1989) *EMBO J.* 8, 1469–1477.
- [16] Broome-Smith, J.K. and Spratt, B.G. (1986) *Gene* 49, 341–349.
- [17] Broome-Smith, J.K., Bowler, L.D. and Spratt, B.G. (1989) *Mol. Microbiol.* 3, 1813–1817.
- [18] Kim, H., Paul, S., Gennity, J. and Inouye, M. (1994) *Mol. Microbiol.* 11, 819–831.
- [19] Wen, D., Peles, E., Cupples, R., Suggs, S.V., Bacus, S.S., Luo, Y., Trail, G., Hu, S., Silbiger, S.M., Levy, R.B., Koski, R.A., Lu, H.S. and Yarden, Y. (1992) *Cell* 69, 559–572.
- [20] Leung-Hagesteijn, C., Spence, A.M., Stern, B.D., Zhou, Y., Su, M.-W., Hedgecock, E.M. and Culotti, J.G. (1992) *Cell* 71, 289–299.