Mutations affecting the extreme C terminus of *Escherichia coli* haemolysin A reduce haemolytic activity by altering the folding of the toxin

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Escherichia coli haemolysin A (HlyA), an RTX toxin, is secreted probably as an unfolded intermediate, by the type I (ABC transporter-dependent) pathway, utilizing a C-terminal secretion signal. However, the mechanism of translocation and post-translocation folding is not understood. We identified a mutation (*hlyA99*) at the extreme C terminus, which is dominant in competition experiments, blocking secretion of the wild-type toxin co-expressed in the same cell. This suggests that unlike recessive mutations which affect recognition of the translocation machinery, the *hlyA99* mutation interferes with some later step in secretion. Indeed, the mutation reduced haemolytic activity of the toxin and the activity of β -lactamase when the latter was fused to a C-terminal 23 kDa fragment of HlyA carrying the *hlyA99* mutation. A second mutant (*hlyA Δ 6*), lacking the six C-terminal residues of HlyA, also showed reduced haemolytic activity and neither mutant protein regained normal haemolytic activity in *in vitro* unfolding/refolding experiments. Tryptophan fluorescence spectroscopy indicated differences in structure between the secreted forms of wild-type HlyA and the HlyA Δ 6 mutant. These results suggested that the mutations affected the correct folding of both HlyA and the β -lactamase fusion. Thus, we propose a dual function for the HlyA C terminus involving an important role in post-translocation folding as well as targeting HlyA for secretion.

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INTRODUCTION

Bacteria have developed a wide range of defence strategies to survive in hostile and competitive environments. Some of these strategies rely on the secretion of toxins that have various effects, including disruption of host membranes. The secretion of proteins by Gram-negative bacteria involves an extraordinarily wide variety of distinct mechanisms. The 107 kDa toxin haemolysin A (HlyA; a member of the RTX protein family) produced by invasive *Escherichia coli* strains, in particular in uropathogenic *E. coli*, is secreted via the type I (ABC) system (Holland *et al.*, 2005) (see Supplementary Fig. S1, available with the online version of this paper).

HlyA secretion (for recent reviews see Delepelaire, 2004; Holland *et al.*, 2005) is accomplished via a *trans*-envelope conduit or tunnel assembled in response to the presence of nascent HlyA (Balakrishnan *et al.*, 2001). This tunnel connects the cytoplasm of *E. coli* directly to the external surface and is composed of three proteins: HlyB, an ABC transporter, TolC, an outer-membrane protein with an extensive helical domain extending into the periplasm (Koronakis *et al.*, 2000), and HlyD, a so-called membrane fusion protein, which likely bridges HlyB and TolC to complete the tunnel. Secretion is initiated by the docking of the C-terminal secretion signal of HlyA to the HlyBD complex, which appears to involve direct interaction between the secretion signal and HlyB in competition with ATP (Benabdelhak *et al.*, 2003). HlyA is then transported directly to the cell surface through the HlyB:HlyD:TolC *trans*-envelope tunnel, where the restricted diameter of TolC (Andersen *et al.*, 2002) indicates that HlyA must be translocated as an unfolded polypeptide chain. Previous

Abbreviations: LB, Luria–Bertani; IB, inclusion body; SEC, size exclusion chromatography.

Four supplementary figures are available with the online version of this paper.

evidence suggests that HlyA molecules can then proceed to the external medium by two distinct mechanisms: directly to the medium as the free protein or following accumulation in a trypsin-accessible form on the cell surface (Pimenta *et al.*, 2005) with a final release step in association with outer membrane vesicles (Balsalobre *et al.*, 2006).

In type I secretion systems, the unprocessed targeting sequence, which is necessary and sufficient for secretion, is located at the C terminus of the protein. For HlyA, this has been subjected to extensive mutation and deletion studies and the targeting sequence has been shown to consist of a stretch of 50–60 aa at the extreme C terminus of the protein (Holland *et al.*, 2005). However, the targeting signal sequence is apparently not strongly conserved in either HlyA or other type I proteins. Nevertheless, while many individual residues can be substituted with little effect, some residues and some putative structural motifs distributed throughout the targeting sequence are required for full secretion activity. In particular, at least seven residues dispersed between positions –15 and –46 reduced secretion levels by 50 % or more when substituted. Moreover, the effects of these mutations were additive. Although overall the signal sequences for type I substrates are highly variable, some conservation at the extreme terminus is apparent when subfamilies of RTX proteins are compared. Thus, the last five C-terminal residues appear to constitute a relatively well-conserved motif in the haemolysin and leukotoxin subfamily which clearly differs, for example, from the C terminus of the RTX proteases (Holland *et al.*, 2003). This suggests that the extreme terminus may confer specificity on the secretion process at some level.

Calcium ions, bound to each RTX repeat, are required for the haemolytic activity of RTX proteins such as HlyA (Holland *et al.*, 2005), with calcium ions seemingly triggering refolding of the RTX proteins, either while they are still bound to the cell envelope or after release to the extracellular space. It is also assumed that intracellular refolding of RTX proteins is prevented due to the low cytosolic Ca^{2+} concentration (300 nM), despite extracellular Ca^{2+} concentrations of up to 10 mM (Jones *et al.*, 2002). Sanchez-Magraner *et al.* (2007) demonstrated that in the presence of Ca^{2+} , the β -roll structure of the RTX domain of HlyA is compacted and stabilized, and Chenal *et al.* (2009) recently described similar effects of Ca^{2+} on the otherwise disordered RTX domain of the adenylate cyclase toxin. These results and a further study (Herlax & Bakas, 2007) appear to point towards HlyA being a conformationally flexible molecule, whose properties are commensurate with secretion in a relatively unfolded state on the one hand and the requirement for radical conformational changes for insertion into membranes on the other. For HlyA or for other bacterial proteins secreted (in a presumably unfolded form) directly to the exterior, the mechanism of final folding on the cell surface remains an intriguing puzzle, with extracellular calcium playing an important role.

In a previous study, we identified a fusion protein, CIZ–HlyA (containing most of the LacZ protein), which blocked the Hly translocator. This enabled us to develop a secretion competition system involving co-expression of wild-type HlyA and the fusion protein to analyse the role of some important residues between positions –46 and –15 that affected secretion. Such mutations, when incorporated into the C-terminus of the fusion, were shown to be recessive, suggesting that the affected amino acids are involved in an early step of translocation, probably the recognition of the translocator (Holland *et al.*, 2003). Importantly, this system, in contrast with competition for secretion between wild-type and mutant HlyA, easily distinguishes the two forms of HlyA and gives an essentially null end point, unless secretion inhibition by the fusion is alleviated by a particular mutation. Here, we describe a mutant, *hlyA99*, which carries four substitutions in the last six residues of HlyA (Supplementary Fig. S2). This mutation displayed a dominant phenotype, since, when present in the CIZ–HlyA fusion, secretion of the wild-type HlyA is inhibited, indicating a defect in a later step in the translocation pathway. In fact, further studies described in this report indicate that the *hlyA99* mutation and another mutation, which removes the six C-terminal residues of HlyA (*hlyAdel6*), appear primarily to affect the folding and therefore the activity of the toxin, suggesting a novel role for the extreme C-terminal residues of HlyA.

METHODS

Bacterial strains and growth conditions. The *E. coli* strain NM522 [$\Delta(lac-proAB)$ *thi-1 supE hsdR17* ($r_k^- m_k^-$), (F', *proAB, lacI^q*, Z Δ M15)] was used for expression and secretion of HlyA and β lac–23kDaHlyA. Cells were grown in Luria broth (LB) at 37 °C or at 30 °C for competition experiments in order to limit expression from the thermo-inducible λ promoter (see below). CaCl_2 (10 mM) was added to LB when cultures were to be assayed for HlyA toxin activity. For translocation kinetics, SE5000 expressing *hlyCA* (pLG813) and *hlyBD* (pLG814) was grown in M63 minimal medium plus 1 mM CaCl_2 at 37 °C. *E. coli* BL21(DE3) [*ompT gal dcm lon hsdSB*($r_B^- m_B^-$) λ [DE3 (*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*)] was used for production of wild-type pro-HlyA (pSU2726) and pro-HlyA Del6 (pSU2726 del6) as inclusion bodies (IBs). Cultures were grown at 37 °C in 2 × YT broth (tryptone 16 g l⁻¹, yeast extract 10 g l⁻¹, NaCl, 5 g l⁻¹) and cells were harvested 3 h after induction. Strain CJ236 [*dut, ung, thi-1 relA*:pCJ105(Cm^r)] was used in order to produce uracil-rich single strand phage DNA for oligonucleotide mutagenesis. This strain was grown as indicated by the manufacturer (Bio-Rad). When required, antibiotics were supplied at a final concentration of 25 µg kanamycin ml⁻¹, 25 µg chloramphenicol ml⁻¹, 100 µg ampicillin ml⁻¹, 10 µg tetracycline ml⁻¹. IPTG (0.5 mM) was added to the culture to induce expression of hybrid proteins under the control of *lac* promoters.

Deletion of the terminal 18 nt of *hlyA* to generate *hlyAdel6*. The *hlyAdel6* mutation was constructed using PCR with pLG813 as template DNA and the following oligonucleotides: H3, 5'-GAGCTCAAATGCCAACAATAACC-3', hybridizing to the 5' end of *hlyA*; and KR1, 5'-AAATTAATAAAATTATATTGAGTTCGC-3', hybridizing to *hlyA* codons 1015–1018 (underlined) and to 3' non-coding sequence (stop codon is depicted in bold) thereby deleting

amino acid codons 1019–1024 (inclusive) but leaving the termination codon intact. Note that the four 5' nt of KR1 correspond to half of a blunt *SwaI* restriction site. The 3.1 kb PCR product was digested with *HindIII* and the 2.4 kb fragment cloned into *HindIII/SwaI*-digested pLG813. The *hlyAdel6* mutation was confirmed by sequencing. HlyA Del6 was constructed using ligase chain reaction and the following oligonucleotides: HlyAdelstop_for 5'-TATGGACGGAAGCTCAATAT-AATTGACAG-3', and HlyAdelstop_rev 5'-TGCTGATGCTGTCA-ATTATATTGAGTTC-3', which introduce a stop codon (bold) in pSU2726. Introduction of the stop codon was confirmed by sequencing.

Transfer of signal mutations into genes encoding β lac-23kDaHlyA and CIZ-HlyA. The point mutations F989P and (Δ 55) in the HlyA secretion signal were transferred from plasmid pLG816 containing these mutations into pLG811, which carries the CIZ-HlyA fusion (LacZ fused N-terminally to the 81 kDa C-terminal domain of HlyA), by *in vitro* recombination using the *BglII* and *NcoI* unique restriction sites present in both plasmids and localized at -86 and -850 bp from the 3' end of *hlyA*, respectively. In the case of the *hlyA99* mutation, the *Sall* and *NcoI* sites were used to transfer the DNA fragment from pLG813-*hlyA99* into pLG811. Transfer of *hlyA99* into the gene encoding β lac-23kDaHlyA was achieved by ligation of the 0.25 kb *BglII/SmaI* fragment of pLG813 carrying the mutation into pPSG51 cut by *BglII* and *HpaI*. The authenticity of the *in vitro* recombinant was confirmed by DNA sequencing.

Denaturation-renaturation of HlyA activity. This was carried out as described previously (Pimenta *et al.*, 2005).

Standard haemolytic assay and activity of HlyA determined by the titration method. These were carried out as described previously (Pimenta *et al.*, 2005). In the titration method, one haemolytic unit was defined as the amount of HlyA necessary to produce 50 % lysis of the erythrocytes. All results are expressed as averages of duplicate samples unless otherwise stated and are given per ml of culture supernatant.

Measurement of β -lactamase activity in culture supernatants. This was performed as described by Chervaux *et al.* (1995). Units of β -lactamase activity were proportional to the amount of fusion protein over at least a 20-fold range (0.05–1 μ g).

Preparation of protein samples from cells and supernatants. Cells were recovered from cultures by centrifugation at 12 000 g for 10 min and resuspended in SDS sample buffer. Protein-containing supernatants were obtained by two further successive centrifugations at 27 000 g each for 15 min and protein was precipitated by addition of TCA [final concentration 10 % (w/v)]. After 1 h on ice, proteins were pelleted by 30 min centrifugation at 16 000 g and resuspended in SDS sample buffer. Each sample was boiled for 10 min before loading on the gel.

Purification of wild-type and HlyAdel6. Pro-HlyA (lacking the internal acylation sites) and wild-type HlyA were purified from IBs. IBs were washed three times with buffer A (50 mM Tris/HCl, 1 mM DTT, 1 mM EDTA, 0.1 % NaN₃, 0.5 % Triton X-100, pH 7.0) and once with buffer B (50 mM Tris/HCl, 1 mM DTT, 0.1 % NaN₃, pH 7.0). After every washing step, the sample was centrifuged at 30 000 g, for 30 min at 4 °C. IBs were finally dissolved in 50 mM Tris/HCl, 8 M urea, pH 7.0. Purification was achieved by anion-exchange chromatography (DEAE fast flow Sepharose, GE Healthcare) with 50 mM Tris/HCl, 8 M urea, 0–300 mM NaCl, pH 7.0 and size exclusion chromatography (SEC; 50 mM Tris/HCl, 8 M urea, pH 7.0). Subsequent cation-exchange chromatography (SP Sepharose; GE Healthcare) and another SEC step resulted in pro-HlyA purified to homogeneity, which was suitable for fluorescence spectroscopy studies (Supplementary Fig. S3, available with the online version of this paper). During an early attempt to purify wild-type, acylated HlyA, we encountered problems with the hydrophobic

molecule sticking to the anion exchange column resin. Furthermore, the purified protein showed a strong tendency to form high molecular mass aggregates. We considered that this problem might be due to the acylation of pro-HlyA. Therefore, we decided to purify proteins (wild-type HlyA and HlyAdel6) for tryptophan fluorescence measurements, following expression in a strain lacking *hlyC* that normally activates HlyA via acylation of the two lysine residues. This procedure enabled us to purify successfully the two proteins without visible contamination, which is important for the fluorescence spectroscopy (Supplementary Fig. S3).

Trypsin treatment. *E. coli* NM522 carrying pPSG51 (wild-type or *hlyA99* signal sequence) and pLG814 or pLG339 (as a vector control minus *hlyBD*) were grown at 37 °C in LB plus antibiotics as described above. β lac-23kDaHlyA synthesis was induced by 0.5 M IPTG at OD₆₀₀=0.5 and growth was followed to OD₆₀₀=4. Supernatants were separated from cells by centrifugation at 4 °C (20 min at 3000 g in a Sorvall SA600 rotor). An aliquot (1 ml) of each supernatant was kept as a trypsin minus control; trypsin (bovine pancreatic, 100 μ g ml⁻¹ in 100 mM Tris/HCl, pH 8) was added to the remaining supernatant to a final concentration of 10 μ g ml⁻¹. A 1 ml aliquot was immediately taken and mixed with 30 μ g trypsin inhibitor ml⁻¹ (Sigma). Samples were taken at intervals and proteins were precipitated by 10 % TCA; 0.2 OD₆₀₀ units were loaded for SDS-PAGE (12 % acrylamide), before transfer to nitrocellulose filters. Proteins were then revealed using anti- β -lactamase or anti-haemolysin antibodies.

For treatment of whole cells, bacteria (3 OD₆₀₀ units) were suspended in MgCl₂ (10 mM), Tris/HCl (10 mM, pH 7.2) plus 50 μ g trypsin ml⁻¹ and incubated at 4 °C for 30 min; digestion was stopped by PMSF (5 mM) and excess trypsin inhibitor. Under these conditions, trypsin does not penetrate to the periplasm (Halegoua & Inouye, 1979).

Kinetic analysis of HlyA translocation. *E. coli* SE5000 (pLG814; pLG813 encoding either *hlyA* or *hlyA99*) was grown in M63 medium (1 mM CaCl₂) to OD₆₀₀=0.5; 7 ml samples were labelled with [³⁵S]methionine (20 μ Ci ml⁻¹=740 kBq ml⁻¹) for 30 s and incorporation was stopped by the addition of cold methionine (0.4 mg ml⁻¹). Samples (1 ml) were taken at different chase times and mixed with 0.9 ml ice-cold phosphate buffer (10 mM, pH 7.5, plus 50 μ g BSA ml⁻¹ as carrier, 3 mM sodium-azide and 50 μ M CCCP). Samples were centrifuged twice to give cell-free supernatants; protein was precipitated with 10 % TCA (w/v) and precipitates were suspended in 20 μ l sample buffer and analysed by SDS-PAGE (12 % acrylamide) and by fluorography.

Tryptophan fluorescence spectroscopy. Protein samples (wild-type, pro-HlyA and pro-HlyA Del6) were prepared by dilution from 50 mM Tris/HCl, 8 M urea (pH 7.0) into assay buffer containing 50 mM Tris/HCl, 20 mM Ca²⁺, 0.5–8 M urea (pH 7.0). Protein samples were incubated for between 24 and 120 h at 4 °C in assay buffer to reach equilibrium. Protein fluorescence was measured with a Fluorolog 3 instrument (HoribaJobinYvon) at 25 °C. Excitation wavelength was 295 nm and slits were set to 2 nm. Emission was recorded between 310 and 500 nm and slits were set to 5 nm. For subsequent analysis, the change in normalized fluorescence intensity at 329 nm was followed.

RESULTS

Isolation of the HlyA99 mutant and its initial characterization

An analysis of HlyA secretion using random hydroxylamine mutagenesis revealed a C-terminal mutation (*hlyA99*)

corresponding to four substitutions (all GC to AT transitions as expected for hydroxylamine mutagenesis) in the last six residues of the protein. This resulted in a sequence change from –TLTASA to –ILIVSV (Fig. 1a). This part of the normal signal sequence is particularly rich in hydroxylated amino acids and has been shown previously to be important but not essential for secretion (Holland *et al.*, 2003). Thus, deletion of the seven C-terminal residues was reported to allow secretion of HlyA protein to the medium of at least 30 % of wild-type levels (Koronakis *et al.*, 1989). However, no effect of such mutations on the activity of HlyA was examined. The *hlyA99* mutant in fact has a phenotype of low haemolytic activity on blood agar plates at 37 °C, giving colonies with small haloes (data not shown). Moreover, in liquid LB medium at 37 °C, levels of haemolytic activity were detected in the culture supernatant at approximately 10 % of that of the wild-type (Fig. 1b) (see also Table 1).

The *hlyA99* mutant protein is secreted relatively efficiently but haemolytic activity is reduced

In order to measure any secretion defect associated with the *hlyA99* mutation, the amount of HlyA protein in the supernatant of both mutant and wild-type cultures grown at 37 °C in LB medium was analysed by Western blotting with anti-HlyA antibodies (Fig. 1c) and by SDS-PAGE with

Coomassie-blue staining (Fig. 1d). Surprisingly, densitometric analysis (see Methods) indicated that the amount of protein secreted to the medium by the mutant was as high as 40–50 % of that obtained with wild-type haemolysin. Fig. 1(d) also indicates that the antibody detected some lower molecular mass bands in the culture supernatant; however, these are unlikely to be HlyA degradation products as they were also detected in the supernatant from cells not expressing HlyBD. The same relatively high level of secretion was obtained (data not shown) when the C-terminal 217 aa of HlyA (23 kDa) carrying the *hlyA99* mutation were expressed from the *lac* promoter of the M13mp18 phage (see Methods). The combination of all these results indicated that the mutant toxin was primarily defective in haemolytic activity.

To completely eliminate the possibility of other upstream mutations in *hlyA* being responsible for these properties, a 3' fragment of the mutant *hlyA99* gene, corresponding to the C-terminal 27 aa, was exchanged *in vitro* for the 3' end of an otherwise wild-type *hlyA* (see Methods). The reconstituted gene was then co-expressed in cells with *hlyBD* and again the secreted protein was found to have reduced activity. Thus, the level of secretion of the mutant protein was approximately 40 % of wild-type levels but the haemolytic activity corresponded to approximately 12 % of the wild-type level in the culture supernatant, giving an

Fig. 1. (a) C-terminal sequence of the mutant HlyA99 compared with the wild-type sequence. Double arrows indicate amino acid changes. (b) Haemolytic assay for the *hlyA99* mutant. *E. coli* NM522 containing the low copy number plasmids pLG814 (*hlyBD*) and pLG813 (*hlyCA*) carrying wild-type or mutant signal sequences. (c, d) Western blot analysis (c) and Coomassie-blue staining (d) of supernatant proteins from cultures (pLG814, *hlyBD*) also expressing *hlyC* and *hlyA* wild-type (lanes 1 and 4) or the *hlyA99* mutant (lanes 2 and 6) from pLG813; supernatants from cells lacking *hlyBD* are shown in lanes 3 and 5. Molecular mass markers and the position of HlyA are indicated.

Table 1. Comparison of wild-type HlyA with variants carrying the *hlyA99* or *hlyAde16* mutation: effects on secretion and activity levels

Protein sample	Protein secreted to the medium (% of wild-type HlyA)*	Activity of culture supernatants (% of wild-type HlyA)*	Activity relative to specific activity (%)*	Haemolytic activity of culture supernatant per unit HlyA protein (% of wild-type HlyA)†	Haemolytic activity after renaturation by 6 M GnCl per unit HlyA protein (% of wild-type HlyA)†
HlyA (wild-type)	100	100	100	40.4 (100)	49.1 (100)
HlyA99	30	12	40	15.7 (39)	19.8 (40)
HlyA Del6	67	25	37	15.1 (37)	21.0 (43)

*Values given are averages of three independent experiments.

†Values given are averages of two independent experiments.

apparent reduction in specific activity of at least 2.5-fold (see Table 1).

The *hlyA99* mutation is dominant in competition experiments with wild-type HlyA

We showed previously that co-expression of wild-type HlyA and a large fusion protein composed of virtually the whole β -galactosidase protein fused upstream of the C-terminal 81 kDa of HlyA (CIZ–HlyA), in cells also expressing *hlyCBD* (Kenny *et al.*, 1994), leads to inhibition of wild-type HlyA secretion. The CIZ–HlyA fusion protein itself (encoded by pLG811) is secreted to the medium but only at very low levels, while large amounts of the fusion protein accumulate in the cell envelope in an *hlyBD*-dependent manner, presumably blocking the translocator. Importantly, we also showed that mutations located between residues –15 and –46 with respect to the HlyA C terminus that were introduced into the CIZ–HlyA signal sequence alleviated the inhibition of secretion of the co-expressed HlyA. These mutations could therefore be considered recessive.

In order to test the dominance or recessivity of *hlyA99* (substitution ILIVSV at the C terminus) in such a competition experiment, the HlyA99 mutation was introduced into the CIZ–HlyA secretion signal by *in vitro* recombination (see Methods). The CIZ–HlyA fusion itself has no haemolytic activity (data not shown), presumably

because it lacks most of the N-terminal hydrophobic region of HlyA. Therefore, in competition experiments, only the haemolytic activity of the co-expressed wild-type HlyA was detected in the culture medium. In cells expressing the CIZ–HlyA fusion, together with *hlyCBD* and wild-type *hlyA*, the colony halo size on blood agar was small or undetectable but increased as expected when mutations that reduce secretion of HlyA were introduced into the fusion (data not shown). However, when the *hlyA99* mutant secretion signal was introduced into the CIZ–HlyA molecule, no increase in halo size was observed. This result is in clear contrast with the recessive secretion signal mutations analysed previously, indicating that the HlyA mutation is dominant.

The competitive effect of CIZ–HlyA99 was then analysed in liquid culture by measuring the levels of haemolytic activity of co-expressed HlyA toxin released into the culture supernatant. Samples of supernatant and total cell proteins were taken at intervals from an exponentially growing culture and analysed for activity and by SDS-PAGE. Fig. 2(a) shows that on the basis of activity measurements, CIZ–HlyA99 inhibited the secretion of co-expressed HlyA as efficiently as a fusion carrying the wild-type signal. As expected, the presence of a non-sense mutation ($\Delta 55$), leading to the deletion of the 55 C-terminal amino acids of the CIZ–HlyA fusion, also alleviated this inhibition by CIZ–HlyA, which is in contrast with *hlyA99*. Moreover, another control, a point substitution at position –35 (Phe

Table 2. A β lac–HlyA fusion carrying the HlyA secretion signal mutation *hlyA99* has reduced specific activity

β -Lactamase activity was calculated for culture supernatants containing the β -lac-23kDaHlyA fusion protein carrying the wild-type secretion signal or the *hlyA99* mutant secretion signal, expressed from pLG813 and pPSG51, respectively. In order to compare apparent specific activities, the amount of secreted protein was determined by densitometry following SDS-PAGE analysis (see Fig. 5).

Protein sample	β -Lactamase activity per OD ₆₀₀ unit of culture supernatant ($\mu\text{mol min}^{-1}$ OD ₆₀₀ ⁻¹)	β -Lactamase specific activity in culture supernatant (% of wild-type β lac-23kDaHlyA)
Wild-type β lac-23kDaHlyA	0.227	100
<i>hlyA99</i> mutant β lac-23kDaHlyA	0.028	40

Fig. 2. Competition for secretion between wild-type HlyA toxin (pLG813) and CIZ-HlyA (pLG811) containing either a wild-type or a mutant secretion signal. All strains also express *hlyBD* from pLG814. (a) The growth of *E. coli* SE5000 in LB at 30 °C co-expressing wild-type HlyA and the 200 kDa fusion protein CIZ-HlyA, containing wild-type (□) or mutant signals: F989-P (◇), *hlyA99* (○) and Δ55 (△). Dotted lines indicate the haemolytic activity (corresponding to the amount of HlyA secreted in culture supernatant as described in Methods). (b) Western blot analysis of culture supernatants precipitated with TCA after harvesting at a time around the maximum for secretion in this medium (OD₆₀₀ ≈ 4). Each lane was loaded with 1 OD₆₀₀ equivalent. Lane 1, samples from the culture co-expressing wild-type HlyA and CIZ-HlyA; lane 2, CIZ-HlyA (F989-P); lane 3, CIZ-HlyA99; lane 4, CIZ-HlyAΔ55. Lane 5 corresponds to a sample from strain SE5000, which expresses wild-type toxin but not the CIZ-HlyA fusion. Molecular mass markers are indicated.

to Pro) which reduces secretion of HlyA when present in the CIZ-HlyA fusion (Chervaux & Holland, 1996), also alleviated the inhibition of HlyA secretion, as measured by haemolytic activity.

Data shown in Fig. 2(b) confirm that CIZ-HlyA99 inhibited secretion of the co-expressed HlyA when immunoblotting was used to reveal the amount of each protein present in the culture supernatants. Thus, expression of CIZ-HlyA reduced the amount of HlyA protein found in the supernatant (Fig. 2b, lanes 1 and 5). In contrast, as expected, when mutation F989P (position -35) or the non-sense mutation (Δ55) was present in CIZ-HlyA, the competitive effect was alleviated and

significantly more HlyA protein was found in the supernatant (Fig. 2b, lanes 2 and 4). However, with *hlyA99* present in the CIZ-HlyA secretion signal, the amount of HlyA protein secreted and detected in the supernatant remained very low, while the amount of CIZ-HlyA itself present in the supernatants was reduced according to the particular secretion signal mutation introduced, as expected.

The data in Fig. 2 clearly show that when present in the CIZ-HlyA fusion, the *hlyA99* mutation is dominant, since it specifically inhibited secretion of co-expressed wild-type HlyA. Consequently, whilst the previously analysed mutations appeared to affect an early step in translocation, probably the initial recognition step, this *hlyA* mutation (Supplementary Fig. S2) must affect a later step in the transport pathway, resulting in a secreted protein with reduced activity.

Localization of cell-associated wild-type HlyA and HlyA99 probed by trypsin accessibility

Previous studies have indicated that production of *hlyA* mRNA is blocked when HlyA is not secreted, for example, in strains not expressing *hlyBD* (M. A. Blight and I. B. Holland, unpublished data). On the other hand, significant amounts of secreted HlyA remain associated with the cell envelope, accessible to extracellular trypsin, in strains expressing *hlyBD* (Pimenta *et al.*, 2005). When wild-type HlyA and HlyA99 were compared in the presence of HlyBD, high levels of the wild-type protein were found to be cell-associated, with apparently even higher levels detected in the case of the mutant HlyA99 protein (Fig. 3). When cells were treated with trypsin, the HlyA99 protein, like wild-type HlyA, was completely degraded by addition of trypsin to cells (Fig. 3) under conditions where the protease does not penetrate the cell

Fig. 3. Substantial amounts of wild-type HlyA and HlyA99 produced in the presence of *hlyBD* are secreted to the surface and are accessible to exogenous trypsin. Strain SE5000 expressing *hlyBD* (pLG814) and *hlyA* or *hlyA99* (from pLG813) was grown in LB medium at 37 °C. Total cells were harvested at OD₆₀₀=6 and the supernatant, containing Hly secreted to the medium, was removed by centrifugation and washing. The cells, resuspended in 10 mM Tris/HCL plus 10 mM MgCl₂ (see Methods), were either left untreated or treated with trypsin (50 µg ml⁻¹) as described in Methods. Total cell protein of cells of each strain (OD₆₀₀ equivalents) was then analysed by SDS-PAGE, followed by Western blotting against anti-HlyA antibody.

envelope (Halegoua & Inouye, 1979). In both wild-type and mutant, therefore, all cell-associated HlyA molecules have been secreted to the surface, are accessible to trypsin and apparently neither wild-type nor mutant HlyA accumulates in the cytoplasm. The control experiments (Supplementary Fig. S3) demonstrated that trypsin treatment has no detectable effect on total protein profiles from treated cells nor the low levels of cytoplasmic HlyA present in cells lacking HlyBD, indicating that trypsin does not penetrate into cells under these conditions. The results shown in Fig. 3 also suggest that the true secretion levels of HlyA99 were underestimated, with relatively more protein accumulating at the cell surface. Thus, these results indicated that the secretion of the HlyA99 mutant was relatively unimpaired. However, no conclusions about the kinetics of translocation can be drawn from these experiments.

Kinetics of translocation of wild-type HlyA and HlyA99

In order to compare the kinetics of translocation of wild-type HlyA and HlyA99 to the medium, cells expressing *hlyBD* and either mutant or wild-type *hlyA* were grown in M63 minimal medium at 37 °C, pulse-labelled with [³⁵S]methionine and chased with cold methionine (see Methods). As shown in Fig. 4, HlyA99 was secreted to the medium at levels about 40 % of that of the wild-type toxin, but the time-course of secretion was similar to that of the wild-type HlyA. In summary, these results indicated that the translocation of HlyA99 was not defective per se, although its ability to fold and leave the cell surface may be perturbed.

Fig. 4. Kinetics of translocation of wild-type HlyA (○) and HlyA99 (●). Strain SE5000 (pLG813, pLG814) was grown at 37 °C in M63 (+1 mM CaCl₂) to OD₆₀₀=0.5 and pulse-labelled with [³⁵S]methionine for 30 s and chased in cold medium; samples were analysed by SDS-PAGE and fluorography. X-ray films were scanned to quantify gel bands in arbitrary units (AU). SE5000 (pLG813) carried either wild-type *hlyA* or *hlyA99*.

The *hlyA99* mutation also affects the specific activity of β -lactamase in the chimeric β lac-23kDaHlyA protein

The HlyA99 mutation markedly affected the haemolytic activity of HlyA (Table 1). This is particularly evident in the case of HlyA Del6, where the mutation has relatively little effect on the levels of HlyA toxin secreted to the medium (see below). However, it has been generally assumed that the haemolytic domain of HlyA is delimited by the presence of the glycine-rich repeats located between residues 730 and 840. Consequently, it was surprising that mutations at the extreme C terminus of HlyA, within the approximately 50 residues thought to constitute the secretion signal, appeared to influence the activity of haemolysin molecules. We therefore considered the possibility that the secretion signal region has an additional function and that the effect of the mutations might indicate that the C-terminal region also plays an important role in the correct folding of HlyA molecules.

In an attempt to test the idea that the C terminus may be involved in folding of the protein, we analysed the effect of the *hlyA99* mutation on secretion and activity of a chimeric protein composed of *E. coli* TEM β -lactamase and a C-terminal part (23 kDa) of HlyA, which lacks the majority of the RTX repeats and the large N-terminal toxin domain. This fusion protein is only secreted in the presence of the translocator proteins HlyBD. Moreover, the secreted form of this fusion appeared to have the same specific activity as normal β -lactamase (Holland *et al.*, 2003). We introduced the cluster of four substitutions constituting the *hlyA99* mutation into the signal sequence of the β -lactamase-HlyA23 kDa fusion (β lac-23kDaHlyA) (see Methods). Secretion of the chimeric proteins, carrying either the wild-type or the mutant secretion signal, was followed by both measurement of β -lactamase activity in the medium and quantification of the amount of the corresponding protein in culture supernatants. Typical results are shown in Fig. 5a, b, where Coomassie-blue-stained and Western blot analyses of supernatant proteins are shown. In these experiments, cultures were grown to late exponential phase for maximum secretion levels. The amount of β lac-23kDaHlyA99 detected was usually between 30 and 50 % compared with the hybrid protein carrying a wild-type secretion signal. Both staining and immunodetection gave similar results. This level of secretion was close to the level of secretion of the HlyA toxin itself carrying the same mutation (see Fig. 1c, d).

However, when the β -lactamase activity in culture supernatants was measured, an eightfold reduction in activity levels of the chimeric protein carrying the *hlyA99* mutation, relative to the wild-type, was obtained, equivalent to a reduction in specific activity of 2.5-fold (40 %) (Table 2). When the stability of β lac-23kDaHlyA99, following secretion to the medium, was measured by its penicillinase activity, this remained essentially identical over 2 h to that of the fusion with a wild-type secretion signal (data not

Fig. 5. Analysis of the secretion efficiency of the β lac-23kDaHlyA fusion protein (encoded by pPSG51) carrying the *hlyA99* mutation. (a) Coomassie-blue-stained gel. Lanes 1 to 4, different OD₆₀₀ equivalents of samples containing the secreted β lac-23kDaHlyA with a wild-type secretion signal; lane 5, 1 OD₆₀₀ equivalent of supernatant from a culture expressing β lac-23kDaHlyA99; lane 6, 1 OD₆₀₀ supernatant equivalent from a culture expressing wild-type β lac-23kDaHlyA in a strain lacking *hlyBD*. (b) Western blot analysis of the same supernatant samples as in (a), using anti-HlyA antibody. The OD₆₀₀ loading equivalents are indicated. (c) Measurement of supernatant levels of secretion of β lac-23kDaHlyA, wild-type secretion signal or *hlyA99*. As in (a) and (b), supernatants from NM522 cultures expressing HlyBD and β lac-23kDaHlyA wild-type secretion signal or *hlyA99* were analysed by SDS-PAGE and Coomassie-blue staining. In order to compare the amounts of secreted proteins in each culture supernatant, serial dilutions were loaded on the same gel. OD₆₀₀ equivalents of wild-type culture supernatant are indicated. The position of the β lac-23kDaHlyA protein is indicated.

shown). These results therefore demonstrated that the *hlyA99* mutation has a similar debilitating effect on the specific activity of the attached β -lactamase as that found for the HlyA toxin itself. This result also clearly indicated that the *hlyA99* mutation does not simply affect the haemolytic activity per se, i.e. because the C terminus is directly required for toxin activity. Rather, this suggested that the mutation affects the correct folding of proteins in some way, for example, with the C terminus of HlyA normally acting as a folding nucleation centre as the polypeptide emerges from the HlyBD–TolC translocator.

Deletion of the six C-terminal residues mimics the effect of the *hlyA99* mutation

To examine further the role of the amino acids at the extreme C terminus of HlyA, we simply deleted the six C-terminal residues to generate HlyA Del6. The properties of this mutant were very similar to those of the *hlyA99* mutation. Removal of the six C-terminal residues had a modest effect on the level of secretion of the HlyA protein to the medium; this was approximately 70 % of the wild-type level (Table 1). However, as found with *hlyA99*, a major effect of the deletion of the C-terminal six residues was on the haemolytic activity in the culture medium, with a reduction in the apparent specific activity of close to threefold. Therefore, with both mutants, *hlyA99* and *hlyA Del6*, the results indicated that the extreme C terminus was in some way essential for activity.

The *hlyA99* and *hlyA Del6* mutations inhibit renaturation of HlyA to the haemolytic form following denaturation *in vitro*

Haemolysin inactivated *in vitro* by denaturation with urea or guanidine chloride (GnCl) can be renatured and therefore reactivated to the haemolytic form by rapid dilution. Moreover, we have shown that HlyA molecules with reduced activity due to mutations in HlyD can be restored to full activity following denaturation and then renaturation (Pimenta *et al.*, 2005). Wild-type haemolysin, HlyA99 and HlyA Del6 in culture supernatants were therefore concentrated by precipitation by ammonium sulphate, denatured by resuspending in 6 M GnCl and renatured by dilution into assay buffer (see Methods) to measure haemolytic activity.

Wild-type haemolysin regained slightly greater than 100 % activity after renaturation (Table 1), a result not unexpected due to the tendency of even wild-type HlyA to aggregate and lose activity in the growth medium (Goni & Ostolaza, 1998). Both mutant proteins (HlyA99 and HlyA Del6) showed a slight increase in activity after renaturation; however, their calculated specific activities remained at only 40 % relative to the wild-type protein. Therefore, *in vitro* these mutant proteins appear to display essentially the same reduced ability to refold to the active form as observed when they are initially secreted to the medium. This result rules out the possibility that initial misfolding is simply a consequence of perturbations

occurring during translocation onto the surface or prior to final release into the medium.

Protein folding of wild-type pro-HlyA and pro-HlyA Del6 monitored by fluorescence spectroscopy

To validate further our data that the extreme C terminus of HlyA plays a specific role in protein folding, we chose fluorescence spectroscopy to detect structural changes within the protein. For technical reasons, we used the pro-form of HlyA (see Methods), which, without acylation, is not able to lyse red blood cells (Holland *et al.*, 2003). Nevertheless, the non-acylated pro-HlyA was shown previously to bind and insert into liposome membranes as efficiently as the acylated form (Hyland *et al.*, 2001). Together, these results indicated that pro-HlyA and acylated HlyA molecules fold similarly. Thus, in light of the technical problems encountered during the purification of HlyA, we chose pro-HlyA as a suitable model protein to follow up our initial hypothesis that the C terminus of HlyA may provide a folding as well as a targeting function (see Supplementary Fig. S4).

When we compared the normalized tryptophan fluorescence of both the wild-type pro-HlyA and pro-HlyA Del6 proteins at high concentrations of urea (2–8 M), the results obtained were very similar (Fig. 6). In both cases, the observed constant decrease in fluorescence paralleling the increase in urea can be assigned to the unfolding of the HlyA protein. In support of this conclusion, lysis of red blood cells by HlyA treated with >3 M urea resulted in the

complete loss of lytic activity (data not shown). In contrast with the above results, at low concentrations of urea (in the range of 0.5–1 M) the wild-type protein and the HlyA mutant lacking the six C-terminal amino acid residues differed with respect to the effect on tryptophan fluorescence and therefore the structure of the pro-HlyA molecule. Thus, under these conditions the mutant protein appeared relatively more compact and more resistant to the significant conformational changes undergone by the wild-type protein. We therefore concluded that the fluorescence spectroscopy data support the hypothesis that the extreme C terminus of the HlyA molecule influences the structure/folding properties of HlyA and nicely complement our whole cell experiments that initially led us to the assumption of a dual role for the C terminus of HlyA.

DISCUSSION

Secretion of proteins carrying a C-terminal secretion signal via the type I pathway are, in all probability, translocated in an unfolded form across the cell envelope (see Discussion in the paper by Pimenta *et al.*, 2005). Consistent with this idea, different lines of evidence indicate that type I polypeptides do actually undergo some folding on the cell surface. First, previous studies have implicated lipopolysaccharide as a requirement for formation of haemolytically active HlyA (Bauer & Welch, 1997). Second, mutations in either TolC (Vakharia *et al.*, 2001) or HlyD (Pimenta *et al.*, 2005) also result in secreted HlyA with reduced haemolytic activity. This seems likely to result from perturbed translocation through the translocator, conceivably leading to uncoordinated misfolding as the protein emerges on the surface, since *in vitro* denaturation and renaturation of HlyA secreted from these mutants largely restored haemolytic activity and therefore normal folding. Strikingly, such a restoration or rescue of the normal folding pathway was not obtained with the HlyA mutants studied here. Finally, an important contributor to folding of HlyA on the surface is most likely the binding of calcium ions to the RTX repeats, as first suggested by Baumann *et al.* (1993) based on the crystal structure of the type I protein alkaline protease from *Pseudomonas aeruginosa*. In reality, the extremely low levels of free Ca^{2+} present in the *E. coli* cytoplasm (less than 1 μM), which is equivalent to around 100 ions (Jones *et al.*, 2002), most probably precludes or severely limits any folding in the cytoplasm based on this mechanism.

Residues close to the C terminus of HlyA were previously shown to be important for secretion of the toxin to the medium, since progressive deletion of from between 7 and 27 residues from the C terminus resulted in secretion of HlyA to the medium at levels of between 30 % and less than 0.5 %, respectively, compared with the wild-type toxin (see Holland *et al.*, 2003). The *hlyA99* mutation involves four substitutions in the last six C-terminal residues, including the terminal Ala to Val (see Fig. 1a), with the most significant changes probably being substitutions of small

Fig. 6. Protein fluorescence spectroscopy of wild-type pro-HlyA (○) and pro-HlyA Del6 (●). Protein samples were purified to homogeneity and prepared and analysed as described in Methods. A characteristic change in fluorescence emission was observed at 329 nm and normalized fluorescence was plotted against urea concentration. For low urea concentrations (0.5–1 M) a significant difference in protein fluorescence was detected, which can be assigned to a difference in protein structure. Values given are averages of three independent experiments and different equilibration times (24–120 h).

hydroxylated Thr residues (positions -4 and -6) for larger Ile residues. In unpublished studies, we showed that the terminal Ala could be replaced by Pro or Gln without any detectable effect on secretion. However, compatible with some specific function, the immediate upstream region is relatively well conserved (L-Hx-Hx-Hx-A, where Hx is hydroxylated or Ala) in HlyA-like RTX toxins. Importantly, previous studies showed that type I signal sequence mutations, which mostly reduce secretion levels to well below 50 % and map to positions -15 to -46 with respect to the C terminus, were recessive, i.e. they alleviated competitive inhibition of secretion of wild-type HlyA by the co-expressed CIZ-HlyA fusion. Thus, such mutations apparently affected an early step in secretion of HlyA, probably recognition of the translocator (Holland *et al.*, 2003). In contrast, the *hlyA99* mutation, when present in CIZ-HlyA, was shown to be dominant in competition experiments, indicating that the mutation primarily affects a later step which is after initiation of translocation of HlyA (Fig. 2).

The amount of HlyA99 protein detected as being secreted to the medium was reduced compared with the wild-type, with final levels varying from 30 to 50 % in different experiments. However, as we show in this study (see also Pimenta *et al.*, 2005), we note that secretion efficiency can be routinely underestimated, since significant amounts of wild-type HlyA are translocated to the outer cell surface but are not released. This is in line with recent findings that in addition to direct extrusion to the medium, HlyA can subsequently be released from the surface in the form of outer membrane vesicles (Balsalobre *et al.*, 2006). Interestingly, even larger amounts were apparently found on the surface with the HlyA99 mutant compared with the wild-type (Fig. 5a), consistent with the altered folding of the mutant protein.

Further evidence that translocation is modestly affected by the HlyA99 mutation was shown by pulse-chase experiments which showed that the time-course for translocation of the mutant is similar to that of wild-type HlyA. Importantly, the *hlyAdel6* deletion mutation, which like *hlyA99* has a major effect on the activity of HlyA, showed only a minimal secretion defect with close to 70 % of wild-type levels in the medium. We conclude, therefore, that the effect of these C-terminal mutations is primarily to reduce the activity of secreted molecules rather than their translocation efficiency.

Three possible explanations for the reduced haemolytic activity of the HlyA mutants can be envisaged: (i) if the extreme C terminus of HlyA is specifically required for modification by HlyC; (ii) if the extreme C terminus is directly implicated in lysis of erythrocytes; or (iii) if the C terminus has a specific role in refolding of secreted molecules. A previous study (Stanley *et al.*, 1994) has shown that the two specific sites for HlyC-dependent acylation are located 288 and 414 residues, respectively, upstream of the C-terminal secretion signal. Involvement

of the C terminus in the acylation reaction seems extremely unlikely, since HlyA lacking the N-terminal part containing the proposed amphipathic helices, or deletion of the C-terminal part, which contains the secretion signal, were still acylated normally *in vitro* (Stanley *et al.*, 1994). Despite many detailed genetic and biochemical studies of the properties of HlyA and its interaction with membranes, no evidence has been presented in the literature to indicate that the C terminus of HlyA is directly involved in haemolytic activity. In contrast, we suggest that several findings in this study point to a role for the extreme C terminus in protein folding. Thus, the *cis*-dominant effect of the *hlyA99* mutation on the activity of β -lactamase fused to the C terminus of HlyA is most easily explained in this way. Moreover, the HlyA mutant proteins analysed in this study were essentially refractory to reactivation following denaturation in urea, consistent with inherently defective molecules, which cannot refold correctly *in vitro*. This is in complete contrast with the successful restoration of activity to inactive HlyA secreted from HlyD or TolC mutants (Pimenta *et al.*, 2005; Vakharia *et al.*, 2001). This result, combined with the relatively normal kinetics of translocation of HlyA99, appears to rule out effects on folding of these HlyA mutants incurred through perturbed movement through the translocator.

Importantly, tryptophan fluorescence spectroscopy studies to monitor protein folding in the presence of urea clearly revealed significant differences between the wild-type pro-HlyA and pro-HlyA Del6 mutants at low urea concentrations (Fig. 6). The wild-type protein in fact appeared more susceptible to structural changes under these conditions, suggesting that the mutant may have a relatively more stable or compact conformation; this may be less compatible with insertion of the HlyA molecule into membranes. The reduced activity of the deletion mutant may be particularly instructive since this implies a positive, active role in the normal refolding for the C terminus rather than a disruptive effect on folding created by the multiple mutations in HlyA99. The results of this experiment nevertheless point towards a crucial involvement of the extreme C terminus of HlyA in the folding of the toxin, acting in concert with other folding mechanisms, as the polypeptide emerges on the cell surface from the translocator.

Interestingly, Chenal *et al.* (2009) and Sotomayor Perez *et al.* (2010) recently showed that millimolar concentrations of Ca^{2+} ions *in vitro* promote the compaction of the normally disordered RTX region of a type I protein, together with increased formation of stable secondary and tertiary structures. This is consistent with the findings of Pimenta *et al.* (2005) who showed that the amount of HlyA secreted and the activity of HlyA increased in line with the extracellular Ca^{2+} concentration over a millimolar range. Chenal *et al.* (2009) indeed proposed that Ca^{2+} -dependent folding of the RTX region of emerging HlyA proteins could contribute a nucleus for folding of the more proximal regions of the protein. Intriguingly, the geometry of the

HlyA molecule – with conceivably at least two distinct types of folding pathway triggers, positioned towards the C terminus of the protein – the RTX Ca^{2+} repeats and the C-terminal end itself, suggest that the C terminus may lead the way out of the translocator. Finally, we conclude that the role of the 50–60 amino acid HlyA secretion signal region may not only be limited to targeting HlyA to the translocator but also be involved in promoting the correct folding of the toxin.

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REFERENCES

- Andersen, C., Koronakis, E., Bokma, E., Eswaran, J., Humphreys, D., Hughes, C. & Koronakis, V. (2002). Transition to the open state of the TolC periplasmic tunnel entrance. *Proc Natl Acad Sci U S A* **99**, 11103–11108.
- Balakrishnan, L., Hughes, C. & Koronakis, V. (2001). Substrate-triggered recruitment of the TolC channel-tunnel during type I export of hemolysin by *Escherichia coli*. *J Mol Biol* **313**, 501–510.
- Balsalobre, C., Silvan, J. M., Berglund, S., Mizunoe, Y., Uhlin, B. E. & Wai, S. N. (2006). Release of the type I secreted alpha-haemolysin via outer membrane vesicles from *Escherichia coli*. *Mol Microbiol* **59**, 99–112.
- Bauer, M. E. & Welch, R. A. (1997). Pleiotropic effects of a mutation in *rfaC* on *Escherichia coli* hemolysin. *Infect Immun* **65**, 2218–2224.
- Baumann, U., Wu, S., Flaherty, K. M. & McKay, D. B. (1993). Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. *EMBO J* **12**, 3357–3364.
- Benabdelhak, H., Kiontke, S., Horn, C., Ernst, R., Blight, M. A., Holland, I. B. & Schmitt, L. (2003). A specific interaction between the NBD of the ABC-transporter HlyB and a C-terminal fragment of its transport substrate hemolysin A. *J Mol Biol* **327**, 1169–1179.
- Chenal, A., Guijarro, J. I., Raynal, B., Delepierre, M. & Ladant, D. (2009). RTX calcium binding motifs are intrinsically disordered in the absence of calcium: implication for protein secretion. *J Biol Chem* **284**, 1781–1789.
- Chervaux, C. & Holland, I. B. (1996). Random and directed mutagenesis to elucidate the functional importance of helix II and F-989 in the C-terminal secretion signal of *Escherichia coli* hemolysin. *J Bacteriol* **178**, 1232–1236.
- Chervaux, C., Sauvonnnet, N., Le Clainche, A., Kenny, B., Hung, A. L., Broome-Smith, J. K. & Holland, I. B. (1995). Secretion of active beta-lactamase to the medium mediated by the *Escherichia coli* hemolysin transport pathway. *Mol Gen Genet* **249**, 237–245.
- Delepierre, P. (2004). Type I secretion in Gram-negative bacteria. *Biochim Biophys Acta* **1694**, 149–161.
- Goni, F. M. & Ostolaza, H. (1998). *E. coli* alpha-hemolysin: a membrane-active protein toxin. *Braz J Med Biol Res* **31**, 1019–1034.
- Halegoua, S. & Inouye, M. (1979). Translocation and assembly of outer membrane proteins of *Escherichia coli*. Selective accumulation of precursors and novel assembly intermediates caused by phenethyl alcohol. *J Mol Biol* **130**, 39–61.
- Herlax, V. & Bakas, L. (2007). Fatty acids covalently bound to alpha-hemolysin of *Escherichia coli* are involved in the molten globule conformation: implication of disordered regions in binding promiscuity. *Biochemistry* **46**, 5177–5184.
- Holland, I. B., Benabdelhak, H., Young, J., de Lima Pimenta, A., Schmitt, L. & Blight, M. A. (2003). Bacterial ABC transporters involved in protein translocation. In *ABC Proteins: from Bacteria to Man*, pp. 209–241. Edited by I. B. Holland, S. P. C. Cole, K. Kuchler & C. F. Higgins. London: Academic Press.
- Holland, I. B., Schmitt, L. & Young, J. (2005). Type I protein secretion in bacteria, the ABC-transporter dependent pathway. *Mol Membr Biol* **22**, 29–39.
- Hyland, C., Vuillard, L., Hughes, C. & Koronakis, V. (2001). Membrane interaction of *Escherichia coli* hemolysin: flotation and insertion-dependent labeling by phospholipid vesicles. *J Bacteriol* **183**, 5364–5370.
- Jones, H. E., Holland, I. B. & Campbell, A. K. (2002). Direct measurement of free Ca^{2+} shows different regulation of Ca^{2+} between the periplasm and the cytosol of *Escherichia coli*. *Cell Calcium* **32**, 183–192.
- Kenny, B., Chervaux, C. & Holland, I. B. (1994). Evidence that residues –15 to –46 of the haemolysin secretion signal are involved in early steps in secretion, leading to recognition of the translocator. *Mol Microbiol* **11**, 99–109.
- Koronakis, V., Koronakis, E. & Hughes, C. (1989). Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *EMBO J* **8**, 595–605.
- Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. & Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**, 914–919.
- Pimenta, A. L., Racher, K., Jamieson, L., Blight, M. A. & Holland, I. B. (2005). Mutations in HlyD, part of the type I translocator for hemolysin secretion, affect the folding of the secreted toxin. *J Bacteriol* **187**, 7471–7480.
- Sanchez-Magraner, L., Viguera, A. R., Garcia-Pacios, M., Garcillan, M. P., Arrondo, J. L., de la Cruz, F., Goni, F. M. & Ostolaza, H. (2007). The calcium-binding C-terminal domain of *Escherichia coli* alpha-hemolysin is a major determinant in the surface-active properties of the protein. *J Biol Chem* **282**, 11827–11835.
- Sotomayor Perez, A. C., Karst, J. C., Davi, M., Guijarro, J. I., Ladant, D. & Chenal, A. (2010). Characterization of the regions involved in the calcium-induced folding of the intrinsically disordered RTX motifs from the *Bordetella pertussis* adenylate cyclase toxin. *J Mol Biol* **397**, 534–549.
- Stanley, P., Packman, L. C., Koronakis, V. & Hughes, C. (1994). Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science* **266**, 1992–1996.
- Vakharia, H., German, G. J. & Misra, R. (2001). Isolation and characterization of *Escherichia coli* *tolC* mutants defective in secreting enzymatically active alpha-hemolysin. *J Bacteriol* **183**, 6908–6916.

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